DEFINING TURTLE DIVERSITY

PROCEEDINGS OF A WORKSHOP ON GENETICS, ETHICS, AND TAXONOMY OF FRESHWATER TURTLES AND TORTOISES

CAMBRIDGE, MASSACHUSETTS, 8–12 AUGUST 2005

EDITED BY

H. BRADLEY SHAFFER, NANCY N. FITZSIMMONS,
ARTHUR GEORGES, AND ANDERS G.J. RHODIN

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Contributions in Turtle and Tortoise Research

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COVER ILLUSTRATIONS

***Front Cover*:** Radiated tortoise, *Astrochelys radiata (*previously in *Geochelone*) (Testudinidae), in the wild at Cap Ste. Marie Special Reserve, southern tip of Madagascar. Photo by ANDERS G.J. RHODIN.

***Frontispiece*:** Mating radiated tortoises, *Astrochelys radiata (*previously in *Geochelone*) (Testudinidae), in captivity at Berenty Nature Reserve, southern Madagascar. Photo by ANDERS G.J. RHODIN.

***Back Cover (Upper)*:** Big-headed Amazon River turtle, *Peltocephalus dumerilianus* (Podocnemididae), in captivity at Villavicencio, Colombia. Photo by RUSSELL A. MITTERMEIER.

***Back Cover (Lower)*:** Musk turtle, *Sternotherus odoratus* (Kinosternidae), in the wild on the South Branch of the Potomac River, West Virginia, USA, showing particularly striking facial markings. Photo by PETER PAUL VAN DIJK.

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PREFACE

H. Bradley Shaffer, Arthur Georges,
Phillip Q. Spinks, and Nancy N. FitzSimmons
Workshop Organizing Committee

Genetics. Conservation. Genomics. Systematics. Ethics. And of course, turtles.

In 2004, when one of us (HBS) had the privilege to conduct sabbatical research at the University of Canberra, we posed the simple question: where has genetics-based research in turtles been, and where is it going in the next decade? We knew a few important pieces of the answer, and thought we could see glimmers of directions to others. With over 40% of the world's turtle fauna IUCN Red-Listed (http://www.iucnredlist.org/), it was clear that conservation and management was no longer the purview of those who work on marine turtles and giant tortoises. Rather, the entire community of turtle researchers was aware of the issues, and virtually all turtle biologists were anxious to work toward effective international management. It was also clear that the systematics community, which relies so heavily on genetic data to recognize species and higher taxa, had made some amazing strides forward, including one of the most effective mergers of the professional and "amateur" biological communities for any group of organisms. However, these and other successes also raised a series of truly thorny issues that the community needed to tackle. How can evolutionary geneticists contribute to conservation in the most meaningful, and most efficient ways possible? When non-traditional material, like turtles in private, living collections, are used to name new species, how does one voucher those species for others to study? If museum specimens, including tissue specimens, are the foundation of research ranging from systematics to conservation to evolutionary biology, what are the "best ethical practices" when most turtle species are long-lived and threatened in the wild? In the rush of enthusiasm to bring new genetic tools to bear on traditional problems in turtle systematics, how should we best cope with the taxonomic instability that now exists, where it seems like half of the generic names we used 10 years ago have been replaced (is it *Clemmys marmorata, Actinemys marmorata,* or *Emys marmorata*)? For that matter, what are the current names in use, and why do we care so much about them anyway?

It was clear to us that the time was ripe for a meeting that brought together those members of the research community who dealt with genetics and turtles to discuss these and other issues, and try to bring both clarity and guidance to the next decade of work. This decade will be a critical one for turtle conservation—according to the IUCN 2007 Red List, 140 of the 212 species that have been evaluated are in the highest categories of endangerment (Extinct, Extinct in the Wild, Critically Endangered, Endangered, or Vulnerable); depending on how one does the calculations, that implies that somewhere between 66% (140/212) and 44% (140/319) of the world's currently recognized ca. 319[7] species of chelonians are in very serious trouble. Genetics can help with issues as diverse as maximizing breeding strategies in captive assurance colonies, to identifying cryptic diversity, to clarifying the phylogenetic prioritization of key taxa.

We reasoned that individuals working in isolation tend to be fragmented in their approaches, whereas group consensus and collaborative efforts can lead to the most efficient use of limited human and financial resources. We also reasoned that it was critically important to include young researchers at the start of their careers, 'seasoned' professionals (others may refer to us with a different label), and individuals from a variety of research, teaching, government and non-government organizations, including a few leaders from groups who do not do genetics, but who contribute to the overall genetics research program through their taxonomic or conservation-oriented work. Our goal was to bring together researchers who use genes to learn about turtles, get them in a room for a week, and produce a series of papers that summarized our collective thoughts on some of the critical issues in turtle genetics, the current state of the science, and important future directions. We brought these turtle genetics experts together also with a small group of turtle-focused individuals who use primarily non-genetic methods to study and conserve turtles. The interactions and exchanges of professional opinions and perspectives benefited both groups and helped our collective vision to grow. The papers in this monograph represent the results of that week of brainstorming, followed by months of careful writing, additional outside input and participants, peer-review and editing, and finally, publication in this comprehensive volume.

It takes several elements to make a meeting of 40-plus opinionated, passionate researchers work well. The proper venue is critical, and Jim Hanken literally and figuratively opened the doors of the Museum of Com-

parative Zoology (Harvard University) to our group. In hosting the meeting, Jim provided the ideal meeting place—the MCZ is steeped in the best traditions of excellence in turtle systematics and evolutionary biology, and has state-of-the-art meeting space that allowed us to meet, break-out, have internet access, and drink excellent espresso day and night. Having a local host that looks out for the group can transform a meeting from a chore to a pleasure. Anders Rhodin opened his home to a rowdy group of genetic cheloniophiles, and made sure that everyone was comfortable and able to work to their fullest capacity. It also takes money. The NSF provided the primary support for our meeting, with critical additional funding from the MCZ (Jim Hanken), Chelonian Research Foundation (Anders Rhodin), and Conservation International (Russ Mittermeier). And of course, it takes a group of people who are willing to give up a week of their time to meet, brainstorm, argue, and produce a final result. That was the group effort, and it produced what we believe is, at least in part, a blueprint for the next decade.

Postscript: On 31 October 2006, it was announced that the first full turtle genome project (for the painted turtle, *Chrysemys picta*) is moving forward (http://www.genome.gov/11007951, http://www.genome.gov/10002154); the project is now slated for early 2008. The genetics and genomics world continue to move at a decidedly non-turtle pace. Our hope is that the papers in the volume will provide ideas and directions for how to use these amazing genetic resources to study, understand, and save the turtles of the world.

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Defining Turtle Diversity: Proceedings of a Workshop on Genetics, Ethics, and Taxonomy of Freshwater Turtles and Tortoises

EXECUTIVE SUMMARY

H. Bradley Shaffer, Nancy N. FitzSimmons, Arthur Georges, and Anders G.J. Rhodin

Genetic data, in combination with strong field studies, form one of the cornerstones of evolutionary, conservation, and population biology. Turtles are particularly well suited to benefit from the insights that genetics can bring to important management issues. With only 313 currently recognized extant species worldwide, turtles are a manageable group from a phylogenetic perspective, such that conservation and management biologists are able to identify species complexes that will benefit from additional genetic analyses. Additionally, field studies of threatened and endangered turtles have identified conservation and management questions needing genetic answers. Given the severe survival threats facing the world's turtle and tortoise fauna, it is clear that the research and conservation communities, more than ever before, need to work together to help identify, manage, and renew the world's depleted turtle populations.

Freshwater turtles and tortoises have received considerable attention from the genetics research community, and several important trends came out of the comprehensive literature analyses by FitzSimmons and Hart, and Engstrom et al. First, the vast majority of work to date has been based on mitochondrial (mt) DNA, with input from the nuclear genome coming from population level analyses of microsatellite data and (in the older literature) allozymes. Given the recent work from several groups demonstrating that mtDNA can be strongly affected by hybridization, introgression, and incomplete lineage sorting, the genetics community needs to continue efforts to develop new nuclear DNA tools that will allow testing of phylogenetic, phylogeographic, and population genetic hypotheses with independent datasets. In addition, taxonomic and geographical biases exist in the areas of concentration of genetic research on turtles. In particular, North American turtles have received most of the research attention to date. While this focus is related to the high concentration of the genetics research community in North America, most of the critically important conservation problems are for non-North American taxa. The call by FitzSimmons and Hart for renewed research attention in other regions of the world is essential, both for filling in basic gaps in our knowledge of turtle genetics and to address the most crucial conservation needs faced by worldwide turtle and tortoise populations.

McGaugh et al. point out the stunning new tools that are becoming available for genetic studies, ranging from individual paternity analysis to deep phylogenetics to identifying genes associated with unique phenotypes. Many of these newest tools are just being applied to turtles, and the possibilities for asking and answering new questions are truly astonishing. As new genomic-level resources for turtles become available, it should become both easier and less expensive to achieve these new research goals. The availability of the first full genomic sequence for a turtle (*Chrysemys picta*, scheduled for delivery in 2008) will be a huge boost for this research agenda.

The Turtle Taxonomy Working Group (TTWG) focused on the important issues surrounding the scientific names that we apply to turtles, what they should represent, and their value in scientific communication and conservation biology; in essence, how do we recognize and define turtle diversity? Particularly as molecular genetic data have been applied to phylogenetic problems, the names that we apply to monophyletic groups (clades) at the genus level or higher have become quite unstable in recent years. While many of these taxonomic changes may be positive, too much change can lead to instability that is at odds with effective communication and conservation legislation. The TTWG recommended a set of "Guidelines for Best Scientific Practices for Revising Taxonomy" that could serve to stabilize taxonomy by recognizing the role that scientific nomenclature plays in biology, and the ways that we can use names to effectively communicate critical biological knowledge.

As we have continued to discover and investigate more of the world's turtle populations, and applied increasingly refined morphologic and genetic characters and criteria for recognizing and documenting chelonian diversity, the number of distinct turtle taxa have grown dramatically. The Turtle Taxonomy Working Group, in a separate chapter, documents this diversity by providing a complete, up-to-date list of all currently recognized extant or recently extinct turtle species and subspecies (consisting of 319 species and 146 additional subspecies, for 465 total turtle taxa), including a list of over 100 issues in turtle taxonomy that have either undergone recent taxonomic change or are in dispute or in need of some type of resolution. Of the currently recognized modern turtle taxa, 6 species plus 3 additional subspecies (9 total taxa) have gone extinct since 1500 AD, leaving us currently with 313 living turtle species, 143 additional living subspecies, and 456 living turtle taxa.

Assembling a complete phylogenetic tree for all species of turtles is a critical goal, and Iverson et al. summarize the literature to date on turtle phylogeny. They then go on to construct a set of supertrees that stand as the best composite hypotheses on turtle phylogeny assembled to date. Progress in the last 20 years on turtle phylogenetics has been incredible, and we can anticipate that in another decade turtles will emerge as one of the most completely understood clades of vertebrates. Major accomplishments include progress on the "backbone tree" of turtles, and phylogenetic relationships within Trionychidae, Geoemydidae, and Testudinidae. Given both the high species diversity and conservation concerns with these three families, this progress is important and timely. As Iverson et al. emphasize, much of this progress relies on mtDNA, and an infusion of nuclear DNA data and analyses will represent the next major step forward in assembling a true Tree of Life for turtles.

Conservation genetics is a theme that runs throughout this monograph, and is particularly emphasized by the Turtle Conservation Genetics Working Group. Key areas where population genetics approaches have been effective for turtle conservation include the identification of management units and cryptic lineages, understanding gene flow among natural populations, and forensic research on the exploitation of endangered taxa. One of the most important outcomes of this work has been in strengthening the working relationship between research biologists, agency and management scientists, and the captive breeding communities.

Syed et al. consider a related way in which the genetics and conservation communities work together for common goals through captive breeding and assurance colonies. Both *in-situ* and *ex-situ* captive management programs have provided a valuable hedge against extinction in the form of strong captive breeding programs, and some of the world's most endangered turtles (ranging from the Madagascar ploughshare tortoise, *Astrochelys yniphora*, to the critically endangered Australian western swamp turtle, *Pseudemydura umbrina*) are now being propagated and repatriated into the wild. Genetics plays a key role by identifying cryptic lineages that require independent management, as well as guidance in avoiding close inbreeding and resultant inbreeding depression. All of these approaches to conservation effectively use genetic technologies to help conserve turtles, and they represent a rich history of sharing resources and material across a variety of partners that has benefited turtle conservation as a discipline.

Another theme, and one that is often overlooked in academic treatises, is an explicit focus on ethical considerations in research programs. Two groups consider ethics from rather different vantage points in this volume. Burke et al. take a broad view of legal considerations—given the sometimes conflicting and confusing laws surrounding domestic and international research, how can individual researchers move forward with their work and respect the critical laws that govern and manage endangered species? The answers are not always simple, but they are important and need to be addressed by anyone who works on natural populations. A related theme explored by Lehn et al. is the ethics surrounding vouchering—that is, collecting representative material for long-term storage in standard specimen-based museums. Traditional voucher specimens constitute the physical record by which we often judge historical changes in species' ranges, and they are an absolute necessity for work in systematics and taxonomic descriptions requiring type specimens. However, as turtles become increasingly rare in the wild, euthanizing specimens as vouchers is often in direct conflict with the stated goals of conservation and management. These are difficult issues, and both Burke et al. and Lehn et al. consider the material already available in traditional museum collections, alternative media such as digital photographs or tissue samples, and investigator responsibilities in considering how traditional and non-traditional specimen acquisition should proceed.

Finally, one must consider the issue of data distribution in an Internet-driven world. The turtle community currently has several useful Internet resources, including the EmySystem site, and Kiester and Bock discuss how such a website can be developed as an improved portal to important data, advances, and issues related to conservation and organismal biology. They identify and discuss three key challenges: continuous content update, quality assurance and control, and synthesis and integration. All of these are issues that must be dealt with, and Kiester and Bock offer a concrete proposal for a website that would serve the needs of the turtle research and conservation communities into the future.

We have clearly made enormous strides in the last two decades in the use of genetics to further our understanding of evolutionary history, current demography, and conservation biology of the world's turtle and tortoise fauna. Major challenges still remain, particularly if we are to prevent the further loss to extinction of the relatively few species of turtles currently living on earth. However, we are coming to understand the species and lineages that require the most urgent conservation, and we can see clear, demonstrable progress in populations of taxa ranging from giant Galapagos tortoises to diminutive swamp turtles. Genetics has played a key role in some of these successes, and will continue to do so in the future, particularly as genomic resources become increasingly available.

The stakeholders in the international turtle conservation community include individuals and institutions from the diverse research and conservation communities of academics, non-governmental organizations, governmental agencies, international organizations and regulatory bodies, philanthropic foundations, zoos and aquaria, and private herpetoculturists. Collaboration between these various stakeholders who work with or care for turtles is the key to current and future progress, and the turtle community has been a leader in establishing and fostering such collaborative efforts. We are proud of these efforts, and continue to encourage them as we focus on expanding and accelerating progress for turtle conservation worldwide.

Defining Turtle Diversity: Proceedings of a Workshop on Genetics, Ethics, and Taxonomy of Freshwater Turtles and Tortoises
H. Bradley Shaffer, Nancy N. FitzSimmons, Arthur Georges, and Anders G.J. Rhodin, Eds.
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Genetic Studies of Freshwater Turtles and Tortoises: A Review of the Past 70 Years

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ABSTRACT. – Powerful molecular techniques have been developed over many decades for resolving genetic relationships, population genetic structure, patterns of gene flow, mating systems, and the amount of genetic diversity in animals. Genetic studies of turtles were among the earliest and the rapid application of new genetic tools and analytical techniques is still apparent in the literature on turtles. At present, of the 198 freshwater turtles and tortoises that are listed as not extinct by the IUCN Red List, 69 species worldwide are listed as endangered or critically endangered, and an additional 56 species are listed as vulnerable. Of the ca. 300 species of freshwater turtles and tortoises in the world, ca. 42% are considered to be facing a high risk of extinction, and there is a need to focus intense conservation attention on these species. This includes a need to (i) assess our current state of knowledge regarding the application of genetics to studies of freshwater turtles and tortoises and (ii) determine future research directions. Here, we review all available published studies for the past 70 years that were written in English and used genetic markers (e.g., karyotypes, allozymes, DNA loci) to better understand the biology of freshwater turtles and tortoises. We review the types of studies conducted in relation to the species studied and quantify the countries where the studies were performed. We track the changing use of different genetic markers through time and report on studies focused on aspects of molecular evolution within turtle genomes. We address the usefulness of particular genetic markers to answer phylogenetic questions and present data comparing population genetic structure and mating systems across species. We draw specific attention to whether authors have considered issues of turtle conservation in their research or provided new insights that have been translated into recommendations for conservation management.

KEY WORDS. – Reptilia; Testudines; conservation; turtle; tortoise; molecular; phylogeny; phylogeography; population genetics; mating system; hybrid; DNA; mtDNA; microsatellite

Since the advent of techniques to view condensed chromosomes in the early 1900s, researchers began studying turtle genomes (Oguma, 1936, 1937; Risley, 1936) to quantify genetic variation among and within species and to reconstruct the relationships among taxa. In the decades that followed, more powerful and finely discriminating techniques and genetic markers were developed that are now used to investigate (i) genetic relationships among taxa (phylogeny), (ii) genetic diversity and structure and patterns of gene flow among populations, (iii) mating systems and the extent of multiple paternity, (iv) histories of captive lineages, (v) origins of forensic specimens, and (vi) aspects of molecular evolution within taxa (e.g., Bull et al., 1974; Carr and Bickham, 1981; Bickham et al., 1985; Avise et al., 1992). Typically the early work with a new genetic approach was mostly theoretical, with the assessment of the utility of each technique and tool following later. For example, a debate over the usefulness of molecular markers versus morphological data in determining phylogeny was raised (regarding turtles, see Seidel and Lucchino, 1981; Shaffer et al., 1997; Iverson, 1998; McLuckie et al., 1999), which instigated analytical approaches for dealing with diverse data sets (Pupko et al., 2002; Wortley and Scotland, 2006b). In contrast, the application of molecular markers to studies of mating systems was rapidly adopted as early genetic studies demonstrated that actual paternity did not always correspond to expectations from observed matings (Galbraith, 1993; Galbraith et al., 1993; Valenzuela, 2000; Pearse and Avise, 2001; Pearse et al., 2002; Roques et al., 2004; Johnston et al., 2006). In recent years, new techniques that allow faster throughput have expanded the range of species that can be studied with genetic techniques, as well as the types of questions that can be addressed using this technology (e.g., Zhang and Hewitt, 2003; Parham et al., 2006a, 2006b; McGaugh et al., 2007). Declining costs, greater global communication, and web-based availability of DNA sequences and primers (e.g., GenBank) have facilitated collaborations among researchers and increased sample sizes, making genetic studies more amenable to rigorous statistical analyses (e.g., Holmes, 2003). In addition to these developments, increasingly sophisticated software packages to process and analyze data are becoming available to an ever-

expanding user community (e.g., Felsenstein, 2004; Pearse and Crandall, 2004). These conceptual and technological advances have increased the role of genetics in studies of wildlife, including turtles, and we contend that they could play a larger role in advancing turtle conservation (see Turtle Conservation Genetics Working Group, 2007, McGaugh et al., 2007).

Within the conservation community, turtles are considered to be in a crisis situation, brought about by human activities (reviewed in van Dijk et al., 2000; Klemens, 2000; Turtle Conservation Fund, 2002). Currently, out of 200 species of freshwater turtles and tortoises listed by the Worldwide Conservation Union (IUCN) in their Red List (IUCN, 2006), 24 are listed as Critically Endangered, 45 as Endangered, 56 as Vulnerable, 2 as Extinct in the Wild, and 9 as Extinct. However, about 100 species of freshwater turtles are not listed by IUCN in the Red List, either because they are more common or have not yet been evaluated for listing. This means that at least about 42% of freshwater turtles and tortoises are considered to be facing a high risk of extinction, and are in need of urgent conservation action. Immediate attention is also needed for an additional 11 species that are listed as Data Deficient.

Several causes of turtle declines exist worldwide, including illegal harvest, poorly regulated legal harvests, habitat loss and ensuing fragmentation, disruption of ecosystems, incidental catch and drowning in fishing gear, and other unintentional take such as road mortality or predation by domestic and feral animals (Mitchell and Klemens, 2000; Thorbjarnarson et al., 2000; van Dijk et al., 2000; Turtle Conservation Fund, 2002). Turtles have been prized as pets or killed for commercial products and although some of this trade is met by commercial farms, illegal harvest from the wild occurs on a broad scale in many areas (Thorbjarnarson et al., 2000). Recently, the Asian turtle crisis has brought to light the devastating declines in turtle abundance in that region of the world. Asian turtle trade numbers are astounding: 15,500 metric tons annually, 10.3 million turtles annually, about 28,000 turtles per day (van Dijk et al., 2000; Turtle Conservation Fund, 2002). These turtles are used as pets, for religious purposes, as food, and in traditional Chinese medicine, among other things. Similarly, Schlaepfer et al. (2005) documented the impact of trade in amphibians and reptiles in and out of the United States, and expressed explicit concern for 19 species of turtles they expected were particularly vulnerable due to trade activities.

Increasingly, researchers are using molecular genetic techniques to answer resource management questions involving the maintenance of genetic diversity, delineation of appropriate units for management, mating systems, and the genetic history of individuals. Broader insights have been gained through studying multiple species at both local and landscape levels to determine how past processes have shaped existing genetic patterns. Such approaches have been used with freshwater turtles and tortoises because this group of animals represent model species for uncovering landscape history (Walker and Avise, 1998): they date back over 200 million years to the Triassic era, they display extreme longevity (generation time), high philopatry, and relatively low dispersal rates. The application of conservation genetics to study threatened and endangered turtles will certainly expand during the coming decades with the advent of further technological and conceptual developments. The increasing use and future potential of powerful molecular genetic techniques for the study of freshwater turtles and tortoises warrants a thorough and critical evaluation of the progress in this rapidly evolving research field and an assessment of our current state of knowledge (see also McGaugh et al., 2007).

Here, we characterize the body of English-language literature on freshwater turtle and tortoise genetics that has been published over the last 70 years (1936-2006). We categorize the types of papers written and document the species included, the genetic markers used, and the geographic focus of the studies. We consider the usefulness of particular genetic markers and the thoroughness of the papers and summarize aspects of the data obtained from each paper. We draw specific attention to whether authors have explicitly considered issues of turtle conservation in their research or provided new insights that have been translated into recommendations for conservation management.

In this comprehensive review, we: (1) describe which of the many available molecular genetic techniques have been useful singly and, more recently, in combination, for definitively resolving important issues of taxonomy and uncovering ecological, evolutionary, and conservation-relevant aspects of turtle biology; (2) present an analysis of experimental and research design for all papers collected that includes a thorough evaluation of hypotheses tested and trends in techniques used to date; (3) draw specific attention to turtles as a model system for certain types of studies and to the important role that genetic studies have in turtle conservation; and (4) discuss how turtle conservation efforts will be enhanced with genetic knowledge and suggest areas for future research and directions. An underlying aim of this review is to better inform those working within the field and to increase their ability to make comparative assessments. We hope to advance the field by pointing out the strengths and limitations, in general terms, of the previous work. We look to uncover any general principles that emerge concerning the genetic structure of turtle populations. And finally, we seek to encourage an increased application of genetic tools to address questions of conservation concern.

METHODS

We selected publications for this review from several sources. First, we conducted online searchers using the Web of Science, Google Scholar, Biological Abstracts, and the Science Citation Index using the keywords ‘turtle and genetic’, ‘tortoise and genetic’, ‘turtle and mtDNA’, ‘tortoise and mtDNA’, ‘turtle and microsatellite’ and ‘tortoise and

microsatellite'. We also conducted both online and hardcopy searches using the same keywords in several journals and monitored other selected journals for new papers from 2003–06. We performed the last web search using Web of Science on 1 Oct 2006. We selected records from the 1930s through September 2006 to cover the time period from the early karyotype studies of turtle chromosomes until the present. The resulting list of publications was reduced to reviewable works according to six main criteria: (1) the paper was published in English in a peer-reviewed journal; (2) the primary focus on the paper was on the use of genetics to better understand turtles or their DNA; (3) the paper was attainable in hardcopy or pdf; (4) the work was not in the form of an unpublished thesis; (5) the paper contained new data that the authors generated (not just reviews); and (6) the paper represented more than a brief published abstract. Articles from the authors' personal libraries were included when they matched the above criteria, as were articles obtained through email requests to a selection of turtle geneticists worldwide, including all participants in the workshop. This included some manuscripts that were 'in press' at the time of the review. We examined the references of each paper for new articles to review and continued this process until no further publications emerged. A small number of papers (n = 10) proved difficult to obtain but a more limited set of data was obtained from their abstracts.

We tracked each study's purpose and methodology, and summarized the data presented and types of analyses performed. Data were collected on the country of origin of the first author, the continental region and countries of origin of the turtles that composed the focus of each study, the taxonomic level of focus, and the name of each turtle species studied, including all those included in phylogenetic analyses. Species names within the text of this review mostly represent the names used within each paper, with reference to names currently recorded by the Turtle Taxonomy Working Group (2007b). For each study, we noted the year, journal, types of genetic markers used, and total sample sizes. We grouped papers by their predominant focus into seven categories: Phylogeny, Taxonomy, Molecular Evolution, Population Genetics, Phylogeography, Techniques, and Mating Systems. In our initial overview of all papers, each paper was assigned to only one category based on its predominant focus. However, in the more detailed review of each category, relevant data from a single paper were often reviewed in more than one category. For example, if a mating study also provided data on allele frequencies for the population, then the relevant data were considered under Mating Systems as well as in Population Genetics. For all the categories, if particular data of interest were not readily available within the paper, but could be calculated, then we calculated them. Descriptions of each category and the data collected are given below.

Phylogeny. — To be considered as a phylogenetic study, the paper had to depict a phylogeny in some form. Papers that were mostly focused on understanding the geographic context of intra-species phylogenies were instead categorized in Phylogeography. However, all Phylogeography papers that presented phylogenetic data had those data included in the relevant phylogeny analyses. Data were collected on the number of taxa used, total sample size, the name of each species, and where appropriate, the names of species considered as an outgroup (OG) based on the authors' choice of OG species in constructing phylogenies. We collected data on bootstrap values for nodes that grouped species versus nodes grouping higher-level taxa to produce an index of effectiveness of the genetic markers used in producing phylogenetic trees. A single tree was selected from each paper for each marker used (or from a combined dataset if provided), with preference given for trees that used a maximum parsimony analysis because of the prevalence of this approach in the literature. Percent bootstrap support for each node was tallied as being either 100%, 90-99%, 80-89%, 70-79%, or <70% and categorized as a node representing individuals of the same species, or a node comprising multiple species. In general, bootstrap values of >90% are typically considered highly significant, values of 70-89% marginally significant, and <70% is considered limited evidence of monophyly within a clade (Hillis and Bull, 1993). Data were also collected on whether the paper included an analysis of morphological data, or compared results to morphological characters, and whether the analyses included a combined genetic and morphological dataset. For each genetic marker, data were collected on the mean and range in genetic divergence within species, genus, and family levels. We noted whether a paper discussed the rate of evolution for the markers used, and in doing so, if they used published rates of divergence, or presented new calculations to estimate time frames of divergence. Notes were made on what the authors emphasized as new findings and if recommendations for taxonomic revisions were made.

Taxonomy. — This category included studies that were focused on systematics and species identification. Some papers were self-described as phylogenetic, but did not include phylogenies and so were included here instead. Taxonomic papers were diverse, and included those designed to address questions of species identification and relationships to sister taxa, the origins of captive individuals, and history of hybrids. As in the Phylogeny section, notes were made on new findings and recommendations for taxonomic revisions.

Molecular Evolution. — These studies focused on molecular processes within the DNA of turtles, but not necessarily on turtle populations or turtles themselves. Early papers included investigations into chromosome size and structure and expression of allozymes in different tissues, whereas more recent papers concerned the evolution of specific genes and chromosomes and environmental damage to chromosomes. Although these studies often only used one or a few species, they were categorized by taxon level based on how the results were interpreted (i.e., often considered to represent all turtles). For the majority of Molecular

Evolution papers, the geographic location of the study and origin of the species used was usually noted as 'not applicable'.

Population Genetics. — These studies focused primarily on a single species and were concerned with issues of genetic diversity, population genetic structure and gene flow, and in limited cases, forensics. Data were collected on the number of populations studied, mean and range in sample sizes per population, number of loci used, mean and range in the number of alleles per population and per locus, and mean and range in the observed heterozygosity per population and per locus. Data on genetic structure were collected as the mean F_{st} value (a measure of genetic structure among populations that ranges from 0–1) for each type of genetic marker used. These values were categorized by the distance between populations as 0–49 km, 50–99 km, 100–499 km, 500–1000 km and >1000 km. For several analyses, these data had to be derived from the papers. For studies that used microsatellites, we noted whether assignment tests (e.g., Piry et al., 2004) were used to estimate rates of migration or identify migrants, and if so, what percentage of self-assignment to populations occurred. Results were considered relative to habitat and categorized as river, lake, semi-aquatic, or terrestrial.

Phylogeography. — In this category, papers focused on the distribution of genetic lineages within a species in relation to geography. Most included phylogenetic trees depicting the species' lineages, some included broader phylogenetic trees to show the placement of the species within its genus or family. In addition to the collection of phylogenetic data, we noted the number of populations studied, mean and range in sample sizes per population, number of mtDNA haplotypes and nDNA alleles found, geographic scale of the study, and whether the authors tested for and found an isolation by distance (IBD) effect. We also noted whether these studies set out to test a specific hypothesis about the distribution of genetic diversity and if comparative analyses were made across species.

Techniques. — Papers in this category described new techniques that had a specific focus on genetic studies of freshwater turtles and tortoises and those that reported on new genetic markers in these species. Some of these papers included genetic data obtained from testing new techniques, and these data were collected as described in the relevant categories.

Mating Systems. — These studies used nuclear markers to investigate the mating system of particular species through analyses of the extent of multiple paternity within clutches. Data were collected on the number of clutches studied, mean clutch size, range and mean number of offspring sampled per clutch, number of females studied, mean and range in the number of clutches sampled per female, number of loci used, probability of detecting multiple paternity, percent multiple paternity observed, the overall percent of offspring fathered by dominant males, and mean and range in the number of fathers involved in multiply-sired clutches. We also considered whether evidence of sperm storage was given, whether there was any difference in hatching success between single- and multiple-sired clutches, and the inferred mutation rate.

Conservation Analysis. — In addition to the collection of data outlined above, a primary aim of the review was to determine the extent to which the published genetic literature was concerned with turtle conservation, and produced work of conservation relevance. For this aim, we categorized papers by the extent to which conservation issues were reported in the introduction and then revisited within the discussion. Comments were made that ranged from 'none' or 'one sentence' to 'focus of the paper', which were translated into categories of 'low' or 'high' conservation concern. Recently, several papers have been concerned with issues of hybridization, particularly as it relates to proper species identification and the focus of conservation efforts. Because of this, we noted whether hybridization was considered as a possible explanation of results or if it was the focus of the paper. We also determined whether the species studied were included in the IUCN Red List (IUCN, 2006) and if so what their status was. This allowed us to compare the extent to which genetic studies have been directed at species of special conservation concern.

RESULTS AND DISCUSSION

Impact and Scope. — The number of turtle genetic papers published in peer-reviewed journals has increased

Table 1. Journals containing at least 2% (n = 3 or more) of reviewed papers (n = 262) that were published on freshwater turtle and tortoise genetics from 1936–2006.

	Journal	#	%
1	Copeia	21	8.1
2	Molecular Phylogenetics and Evolution	18	6.9
3	Molecular Ecology	12	4.6
4	Herpetologica	11	4.2
5	Molecular Ecology Notes	10	3.8
6	Chelonian Conservation and Biology	8	3.1
7	Comparative Biochemistry and Physiology (B)	8	3.1
8	Conservation Genetics	9	3.5
9	Biological Conservation	6	2.3
10	Canadian Journal of Zoology	6	2.3
11	Journal of Herpetology	6	2.3
12	Science	6	2.3
13	Evolution	5	1.9
14	Molecular Biology and Evolution	5	1.9
15	Zoologica Scripta	5	1.9
16	Conservation Biology	4	1.5
17	Cytologia	4	1.5
18	Gene	4	1.5
19	Genetica	4	1.5
20	Journal of Heredity	4	1.5
21	Cytogenetics and Cell Genetics	3	1.2
22	Ecotoxicology	3	1.2
23	Gene	3	1.2
24	Hamadryad	3	1.2
25	Proc. National Academy of Sciences, USA	3	1.2
26	Systematic Biology	3	1.2
27	Texas Journal of Science	3	1.2
	Total	177	66.0

Table 2. Summary of the number and percentage of papers using mitochondrial (mtDNA) markers and/or nuclear (nDNA) markers in studies of freshwater turtles and tortoises across categories of reviewed papers.

Paper Category	No. papers	using mtDNA markers No.	%	using nDNA markers No.	%	using mtDNA & nDNA markers No.	%
Phylogeny	66	44	66.7	31	47.0	12	18.2
Taxonomy	56	8	14.3	54	96.4	3	5.4
Molecular Evolution	41	6	14.6	38	92.7	3	7.3
Population Genetics	39	10	25.6	34	87.2	6	15.4
Phylogeography	31	29	93.5	4	12.9	4	12.9
Techniques	16	1	6.3	13	81.3	0	0.0
Mating Systems	13	0	0.0	13	100.0	0	0.0
Total (or Average)	262	98	(Avg 31.6)	187	(Avg 73.9)	28	(Avg 8.4)

steadily since 1930s when genetic tools were first used to investigate genetic diversity in turtles (Risley, 1936; Oguma, 1936, 1937). Our literature search produced 262 papers that were published in a variety of peer-reviewed journals (Table 1). In total, 88 journals are represented in this review, and an additional six other sources in books or special editions were added for a total of 94 sources. Overall, 27 journals contained 66.0% of the papers (n = 177) in this review. Noteworthy is the fact that papers on freshwater turtles and tortoise genetics have appeared six times in the journal *Science* but never in *Nature*. Using the Journal Citation Reports found on the ISI Web of Knowledge website (http://portal.isiknowledge.com), we found the impact factor for 53 of the journals, representing 205 papers. For these papers, the impact factor ranged from 0.22 – 31.6, with an average score of 3.5. Additionally, 19 papers were published in journals with an impact factor greater than 5.0, including the journals *Molecular Biology and Evolution; Proceedings of the National Academy of Sciences, USA; Systematic Biology; Science;* and *European Journal of Biochemistry*.

The number of papers published between 1936–2006 on freshwater turtle and tortoise genetics was not equally distributed among the seven described categories (Table 2). Papers that focused on Phylogeny (25.2%) and Taxonomy (21.4%) dominated the literature for several decades, followed by papers on Molecular Evolution (15.6%). Molecular Evolution papers date back to 1936, and Taxonomy papers have the second longest history of publication (Fig. 1). Fewer papers focused on Population Genetics (14.9%), Phylogeography (11.8%), Techniques (6.1%), and Mating Systems (5.0%), with most being published within the last decade (Table 2 and Fig. 1). The biggest surge in publications has been a recent increase in the number of Phylogeny papers (Fig. 1).

To couch the reviewed papers in a geographic framework, we tracked the country of first authors by category of paper (Fig. 2). Across all categories, USA first authors dominated (175 papers, 66.8%), followed by Japanese (17 papers, 6.5%) and German authors (14 papers, 5.3%) and authors from 15 other countries (56 papers, 21.4%). To further understand global coverage of freshwater turtle and tortoise genetics studies, we tracked the geographic region of each study by category (Fig. 3). The majority of studies focused on freshwater turtles and tortoises living in North

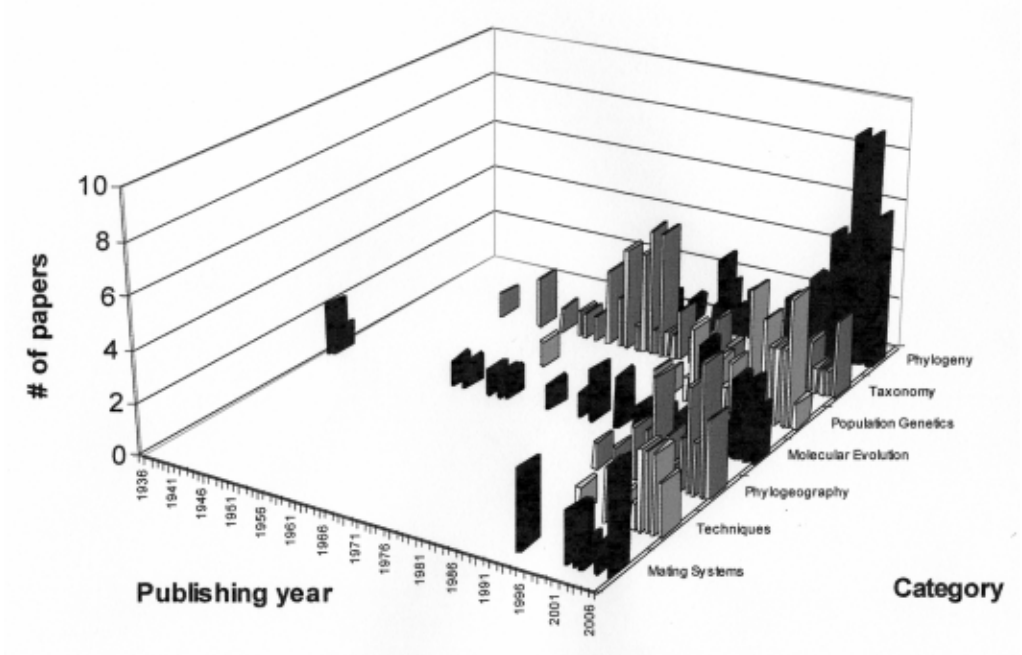

Figure 1. Number of reviewed papers published each year from 1936–2006 (n = 262) in each of the seven defined categories.

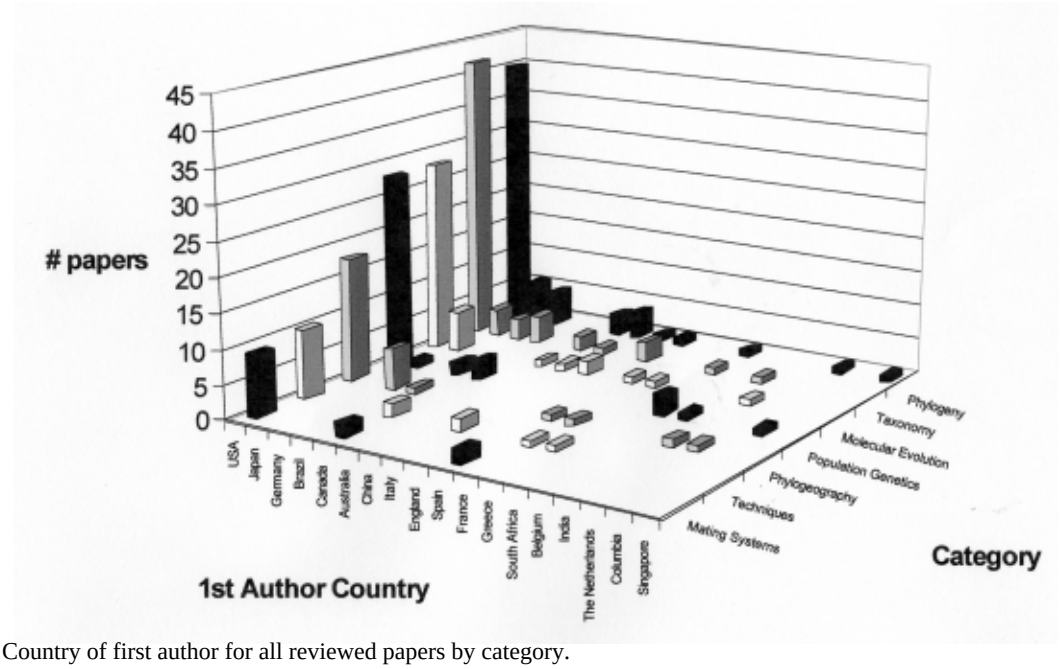

Figure 2. Country of first author for all reviewed papers by category.

America (95 papers, 36.3%), including the USA and Canada as well as a few broader studies that also included Central or South America. Many other studies included samples from around the globe (34 papers, 13.0%), followed by studies of turtles in South America (31 papers, 11.8%), Asia (29 papers, 11.1%), Europe (24 papers, 9.2%), and Africa (15 papers, 5.7%). A limited number of studies collected samples at several locations in the Southern Hemisphere (7 papers, 2.7%), or specifically in Australia (6 papers, 2.3%), or in the Neotropics (1 paper, 0.4%). Several studies (n = 20, 7.6%), particularly those focused on Molecular Evolution or Techniques, could not be meaningfully assigned to a geographic region.

Genetic Markers. — Various mitochondrial DNA (mtDNA) and nuclear markers were utilized across all categories of papers (Table 2). Overall, mtDNA markers were used in 32.8% of the papers included in this review. Mitochondrial DNA markers were most frequently used in Phylogeography (100%) and Phylogeny (69.8%) papers, but were underrepresented in Techniques (6.3%) and Mating System (0%) papers. A higher diversity of nuclear markers was prevalent across all categories of

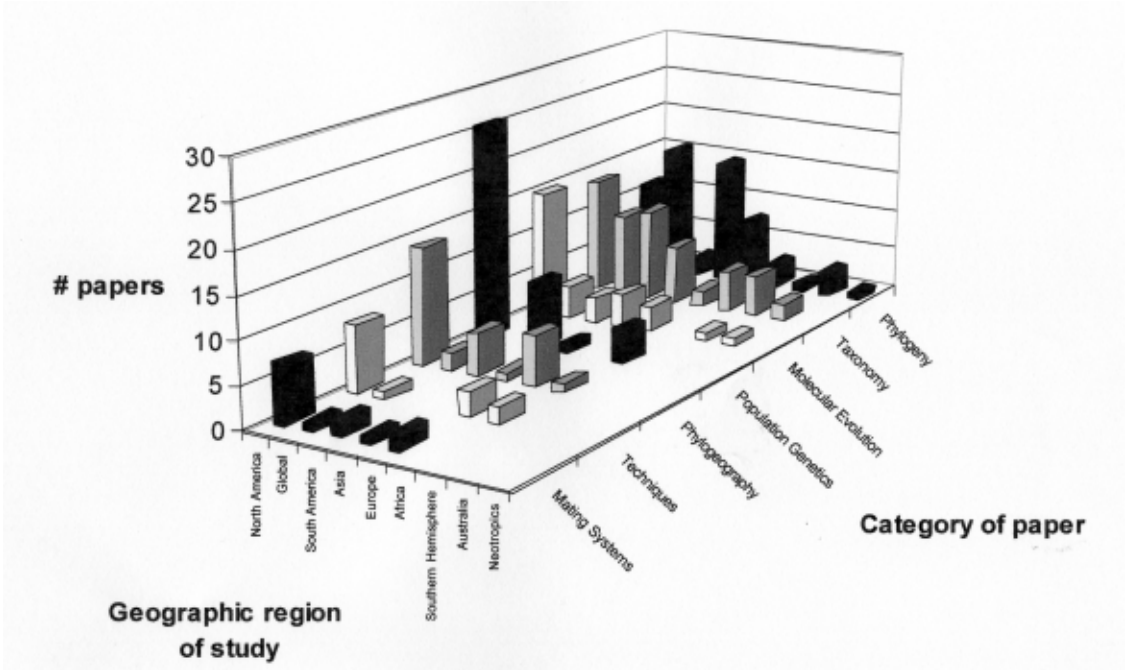

Figure 3. Category of paper published versus the geographic region of study. Note that North America includes USA and Canada as well as papers covering the Americas; South America includes South Pacific, Galápagos, Mexico, and Central America; Asia includes China, Southeast Asia, India, and Indo-Asia; Europe includes Europe, Mediterranean, and Eurasia; Africa includes North African countries and the Western Indian Ocean areas, including Madagascar; Southern Hemisphere includes broad phylogenetic or taxonomic studies across the region; and Neotropics includes Mexico, Nicaragua, El Salvador, and Honduras.

Table 3. Summary of the types of mitochondrial DNA (mtDNA) markers used across categories of reviewed papers.

	Category								
	Phylogeny	Taxonomy	Molecular Evolution	Population Genetics	Phylo-geography	Techniques	Mating Systems	Total No.	%
Total no. papers per category	66	56	41	39	31	16	13	262	
Mitochondrial DNA marker:									
cyt B	24	6	3	2	10			45	**17.2**
control region and D-loop	9	1	1	3	13	1		28	**10.7**
12sRNA	13		1		3			17	**6.5**
tRNA	10	3		1	2			16	**6.1**
ND4	12	1		1	1			15	**5.7**
16sRNA	10				2			12	**4.6**
mtDNA-RFLP			1		6			7	2.7
COI	4	1			1			6	2.3
complete mtDNA seq	1		1	2				4	1.5
PCR-RFLP	1			2	1			4	1.5
ND5		1		2				3	1.1
ND6		1		2				3	1.1
ND2				1				1	0.4
a.a. sequence of mtDNA proteins	1							1	0.4
ND1	1							1	0.4
ND3					1			1	0.4
restriction site	1							1	0.4
protein coding genes			1					1	0.4
total DNA CsCl			1					1	0.4

Note: **Boldface** values highlight the five mtDNA markers used in >3.0% of the papers (n = 262) included in this review.

papers and these markers were used in 73.2% of the reviewed papers (Table 2). Nuclear DNA markers had a high level (> 80%) of use in Mating Systems, Molecular Evolution, Taxonomy, Population Genetics, and Techniques papers. These markers were used in nearly half of all Phylogeny papers, but were used relatively infrequently (13.8%) in Phylogeography papers. Relatively few papers (8.4%) overall reported on the combined use of mtDNA and nuclear DNA markers. Authors of Phylogeny papers used both nuclear and mtDNA markers to the greatest extent (18.2%) followed by papers in Population Genetics (15.4%) and Phylogeography (12.9%) (Table 2).

Cytochrome *b* (cyt*b*) was first used in turtle studies in 1994 to address the systematics of *Graptemys* (Lamb et al., 1994) and became the predominant mtDNA marker across all categories, appearing in 17.2% of the papers. Second to cyt*b* was the control region, initially used in a phylogeographic study (Walker et al., 1995), and found in 11.1% of reviewed papers (Table 3). Sequencing of the complete mtDNA was first used to study molecular evolution and turtle affinities in 1998 (Zardoya and Meyer, 1998a) and later, sequencing of the complete mtDNA control region was first used to construct a phylogeny of *Kinosternon flavescens* (Serb et al., 2001). Compared to the mtDNA markers used, a broader diver-

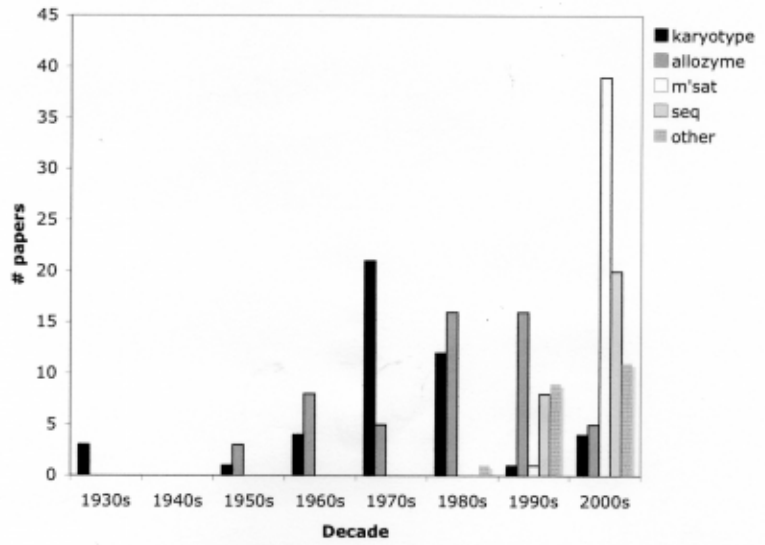

Figure 4. Change through time in the number of reviewed papers using different classes of nuclear markers; categorized as karyotypes (all chromosome studies except those using flow cytometry), allozymes (includes blood proteins), nuclear microsatellites (m'sats), all nDNA loci that were sequenced (seq) and other (includes RAPDs, ISSR-PCR, flow cytometry).

Table 4. Summary of the types of nuclear markers or techniques used across categories of reviewed papers.

	Category							
	Phylogeny	Taxonomy	Molecular Evolution	Population Genetics	Phylo-geography	Techniques	Mating Systems	Total No. **(%)**
Total no. papers per category	66	56	41	39	31	16	13	**262**
Nuclear marker/technique:								
allozymes/protein variants (hemoglobin, serum albumin, transferrin, isoelectric focusing)	11	21	6	13	1	2	54	**20.6**
karyotype	7	28	10				45	**17.2**
microsatellites	1	1	15	3	12	8	40	**15.3**
flow cytometry, chromosomes, micronuclei, telomeres	1		10				11	**4.2**
R35	4	1	1		1		7	2.7
genomic fingerprinting with ISSR-PCR	2	3					5	1.9
poIIII/Sine retroposon/LINE	1		3				4	1.5
RAPDs		1		3			4	1.5
RAG-1/RAG-2	2		1				3	1.1
fingerprint						2	2	0.8
myoglobin	1	1					2	0.8
Minisatellite				2			2	0.8
alpha enolase	1						1	0.4
aromatase			1				1	0.4
Introns			1				1	0.4
Sf1 gene expression			1				1	0.4
LDH-A and LDH-B	1						1	0.4
genomic hybridization			1				1	0.4
WT1 and Sox9			1				1	0.4
ZFY and Sox			1				1	0.4
total DNA-CsCl gradients			1				1	0.4
U17 snoRNA			1				1	0.4
DPLA	1						1	0.4
GAG	1						1	0.4
GAPDH					1		1	0.4
blood proteins		1					1	0.4
prion protein						1	1	0.4
chromosomes	1						1	0.4
FSHβ			1				1	0.4

Note: **Boldface** values highlight nuclear markers used in >3.0% of the papers in this review.

sity of nuclear markers was found in the studies, with a major shift in the type of markers used over the years of the study (Fig. 4). Allozyme/protein variants and karyotypes predominated in the early literature, with 20.7% and 17.2% of papers, respectively, using those markers. Microsatellites were first used to study turtle populations when Sites et al. (1999) first published on this technique in Amazonian river turtles (*Podocnemis expansa*) in 1999. These markers have come to dominate, with use in 15.3% of papers, mostly in the categories of Population Genetics and Mating Studies. More recently, nuclear introns were first sequenced in turtles to build a phylogeny of side-necked turtles in 1998 using c-mos in addition to mtDNA sequences (Georges et al., 1998).

As expected, the use of markers varied across the categories of papers (Tables 3 and 4). Phylogeny papers relied most strongly on cyt*b* (38.1%) and also often used 12sRNA, ND4, tRNAs, 16sRNA, and a variety of allozyme/protein variants (>14% each). Taxonomy papers predominantly used karyotypes (45.9%) and allozyme/protein variants (34.4%), particularly in earlier (i.e., before 1995) papers. Cyt*b* was the most frequently used (9.9%) mtDNA marker in the Taxonomy category. Molecular Evolution papers focused on nuclear DNA, particularly at the level of chromosomes, using detailed techniques to determine karyotypes and investigate chromosome damage, including the use of flow cytometry. Population Genetic papers also relied heavily on nuclear markers, initially using allozyme/protein variants (33.3%), which have been largely supplanted by microsatellites (38.5%). Phylogeography papers preferred sequencing the control region (48.3%) and cyt*b* (34.5%), but began with the use of mtDNA-restriction fragment length polymorphisms (RFLPs) in 1989 (Lamb et al., 1989). Techniques papers mostly involved the recent development of microsatellite markers, which accounted for 75% of those papers. Not surprisingly, Mating Systems papers only employed nuclear markers, originally by using allozymes (Scribner et al., 1993) and DNA fingerprinting (Galbraith, 1993), but since 2000 (Valenzuela, 2000), only microsatellite data have been reported.

Phylogeny. — Papers discussed in this section include the 66 papers that were placed in the Phylogeny category and an additional 40 papers that also included phylogenetic trees as a part of their results. This does not include papers that only presented the relationship among haplotypes using parsimony network analysis. Of these 105 papers, 61.9%

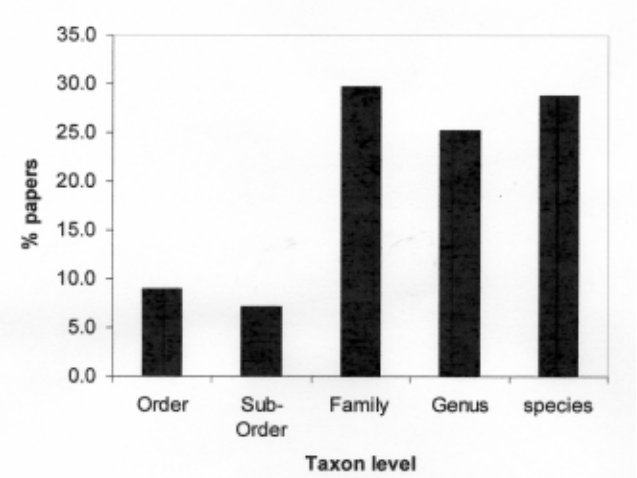

Figure 5. Taxon level of focus for reviewed papers in the Phylogeny category.

had been categorized under Phylogeny, 17.1% as Phylogeography, 7.6% as Population Genetics, 7.6% on Taxonomy, and 5.7% as Molecular Evolution. The primary focus of these papers was within a family (30.5%), genus (25.7%), or species (26.7%), and fewer papers dealt with questions at the level of order (9.5%) or sub-order (7.6%) (Fig. 5).

Among the phylogenetic data, there was wide diversity in the types of phylogenetic analyses and trees presented and the amount of data provided. To address the scope of phylogenetic trees, 96 datasets that provided the relevant data were selected. Only 15.6% of phylogenetic trees were focused on a single species; the remaining trees averaged 15 species per tree, with a range from 2–70. Outgroup (OG) taxa were used to root the trees in 67.7% of these papers, although several of the remaining 31 trees could have benefited from their inclusion. For those papers using OGs, the number of OGs ranged from 1–9, with 38.8% (n = 25) using only a single OG. The most comprehensive paper (in terms of the number of taxa used) sequenced 70 taxa plus 9 OGs for cyt*b* and 12S ribosomal mtDNA and all 23 genera of the Geoemydidae for the nuclear intron R35 (Spinks et al., 2004). Among the papers that used sequence data, variable numbers of basepairs were sequenced, from around 350 bp, up to the entire mtDNA control region, which provided up to 16.2–19.4 kilobases of data (Parham et al., 2006a, 2006b) . These larger datasets were used to study the phylogeny of several species of Mediterranean tortoises and to determine the relationships among *Platysternon*, *Kinosternon*, and *Chelydra* (Parham et al., 2006a,b). Most phylogenetic analyses used only a single specimen per taxon, and we observed a broad range in terms of the information provided about the specimens. Several papers that used sequence data only provided GenBank accession numbers for the sequences, but no information about sample location or any identifiers for the original specimen. A minority of all papers gave comprehensive information about location, museum catalogue numbers if appropriate, or other voucher identifiers for the specimens. For a discussion of this important subject, see Lehn et al. (2007).

Estimates of sequence divergence were provided in 56 papers. To compare divergence estimates across genetic markers, the average divergence values at different taxonomic levels (i.e., within species, within genera, within families, across families) were used where available. For papers that only reported the range in divergence estimates, the mid-point value of the range was used. Variable divergence values were reported in the literature, even within the same category of marker type and taxonomic level (Fig. 6). As expected, in all categories there was significantly less divergence observed at nuclear markers as compared to mtDNA markers (p = 0.45; 1-tailed, paired t-test). Substantial levels (>5%) of

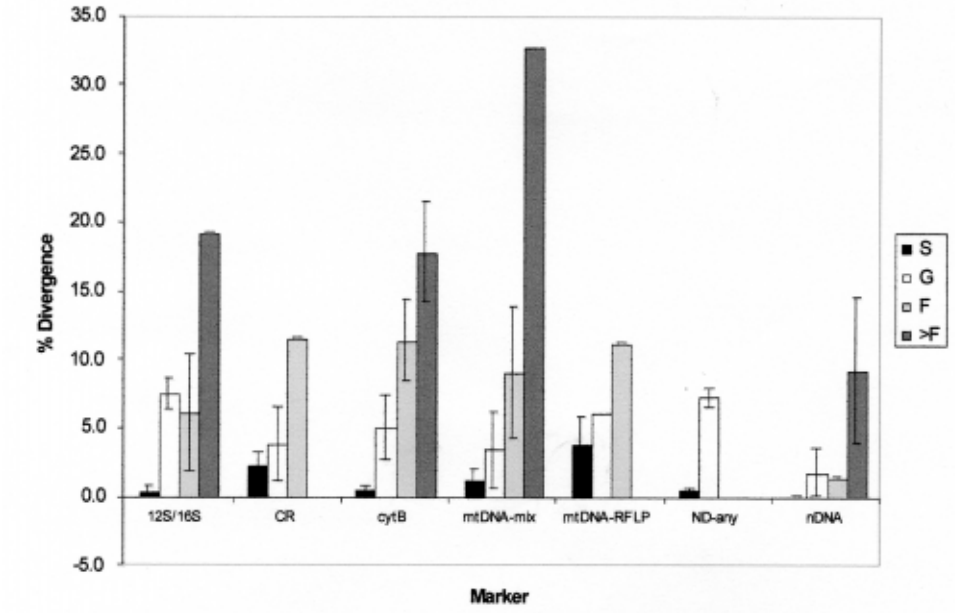

Figure 6. Average percent sequence divergence showing S.D. from papers with phylogenetic data; categorized as within species (S), within genus (G), within family (F), and across families (> F). Markers include mtDNA 12S/16S, control region (CR), cytochrome*b* (cyt*b)*, a combination of mtDNA genes (mtDNA-mix), mtDNA-RFLP, ND-any (ND1, ND2, ND3, ND4, ND5, ND6), and nDNA.

divergence were only seen in nDNA when comparing between families or higher taxonomic groupings. Significant differences in divergence were observed among the different taxonomic levels (p = 0.0004, ANOVA). Within species, the highest average divergence was observed in mtDNA-RFLP studies, followed by the control region. Within genera, the 12s/16s data and the NADH dehydrogenase genes (ND-all) provided the highest average divergence, with large standard deviations for most other markers. Divergence averaged around 10% within families for most markers, and 20% or higher above the family level. The highest value came from a combined use of cyt*b* and 12s data to compare genetic distances among the five major cryptodire lineages (Shaffer et al., 1997).

Whether or not turtle DNA evolves at a slow pace is still an unresolved topic within the literature. Across all 262 papers reviewed, 52 discussed rates of molecular evolution, ranging from broad-scale changes in chromosome number and structure (e.g., Bickham, 1981; Bickham and Rogers, 1985; Carr et al., 1986; Muhlmann-Diaz et al., 2001), to estimates of sequence divergence and microsatellite mutation rates (e.g., Lamb et al., 1994; Valenzuela, 2000). Most authors relied on previously published estimates, particularly the slow rate of mtDNA divergence (0.25%/Myr) first presented by Avise et al. (1992). Since then, only four papers have derived rates of mtDNA sequence divergence by calibrating against fossil dates (Lamb et al., 1994; Lenk et al., 1999), the formation of the Grand Canyon (Osentoski and Lamb, 1995), or the emergence of the Galápagos Islands (Beheregaray et al., 2004). Whereas the rates based on fossils indicated a slow rate of sequence divergence (0.3–0.46%/Myr), those based on geologic dates estimated faster rates (1.6–3.4%/Myr), at least for the control region. Several papers speculated on the rate of change based upon their data, and of those, six papers supported a faster rate and 20 papers supported a slower rate. Six papers discussed the possibility of mutation rate heterogeneity among lineages, and four of these tested whether the sequences evolved in a clock-like manner. Within the literature there was support for clock-like mutation among species of *Geochelone* (now *Chelonoidis nigra* and *C. chilensis*) and *Pyxis* in Madagascar (Caccone et al., 1999a) and within subspecies of *Testudo hermanni* (Fritz et al., 2006a). But at higher taxonomic levels, variation in mutation rates seemed to occur across lineages (Fujita et al., 2004; Near et al., 2005; Fritz et al., 2006a; Parham et al., 2006b). In terms of evolution at microsatellite loci, inferred mutation rates were within the range reported for other species, and they included some relatively high (10^{-2}–10^{-3}) values (Valenzuela, 2000; Pearse et al., 2002, 2006b; Roques et al., 2004).

Several authors have noted the importance of considering morphological data when analyzing phylogeny (e.g., Wortley and Scotland, 2006a,b), yet few papers provided the data to do so. While 60.9% (64 of 105) of papers discussed or mentioned aspects of comparative morphology, only 8.6% (9 papers) presented an analysis of morphological data. Of these papers, five included phylogenetic analyses of combined genetic and morphological data, and four presented comparative phylogenetic analyses of both separate and combined data sets. All of these papers demonstrated improved resolution using the combined phylogeny (see Shaffer et al., 1997; Iverson, 1998; Stephens and Wiens, 2003; Engstrom et al., 2004). Burke et al. (1996) suggested a need to include additional data from various sources, including fossil morphology and ecological traits, as well as genetic data. However, Shaffer et al. (1997) found that addition of fossil data to phylogenetic analyses did not lead to greater resolution, but instead produced greater uncertainty for the nodes surrounding the fossil taxa. For a comprehensive list of papers that include phylogenetic trees based on either genetics or morphology, see Iverson et al. (2007).

Increasingly, phylogenetic papers on turtles are providing analyses of both mtDNA and nuclear datasets, particularly as awareness increases about the limitations of mtDNA data (Bazin et al., 2006, but see Mulligan et al., 2006). Papers varied in whether they presented each data set independently, as well as in a combined tree, and the extent to which they tested the appropriateness of the model used to combine their data (see Pupko et al., 2002). To investigate the effectiveness of mtDNA, nDNA, and combined mtDNA/nDNA datasets to produce robust phylogenetic trees, we conducted an analysis of bootstrap values using a single tree for each category of genetic marker contained in a paper. Data were taken from 77 trees, which indicated overall that 37.5% of nodes within species had bootstrap values of > 90% in comparison to 44.1% for nodes at a taxonomic level higher than species. Nodes with < 70% bootstrap support were common, ranging from 48.8% within species to 37.1% at higher order nodes. However, the lower values were often associated with nodes that were less important to the specific research questions. Of the 77 trees, 64 used only mtDNA, including three that used complete control region, six that used only nDNA, and seven that combined mtDNA and nDNA datasets. ANOVA tests indicated significant differences in bootstrap values that were based upon these different classes of markers, both for clades within species (p = 0.002) and clades at higher taxonomic levels (p = 0.021). A summary of bootstrap values indicated that the complete control region provided the most robust phylogenies, particularly at the level within species (Fig. 7). For nodes within species, the combined data sets performed the next best, while for nodes at higher levels, the nDNA data performed somewhat better than mtDNA, which was somewhat better than the combined datasets. Particularly for the nodes at higher than species level, these results depended upon the specific choice of markers and the scope of the study, and for two of the combined datasets, these included morphological data.

It is clear from this review that there is a need to critically evaluate the effectiveness of different markers, but to do so requires that more authors present individual trees

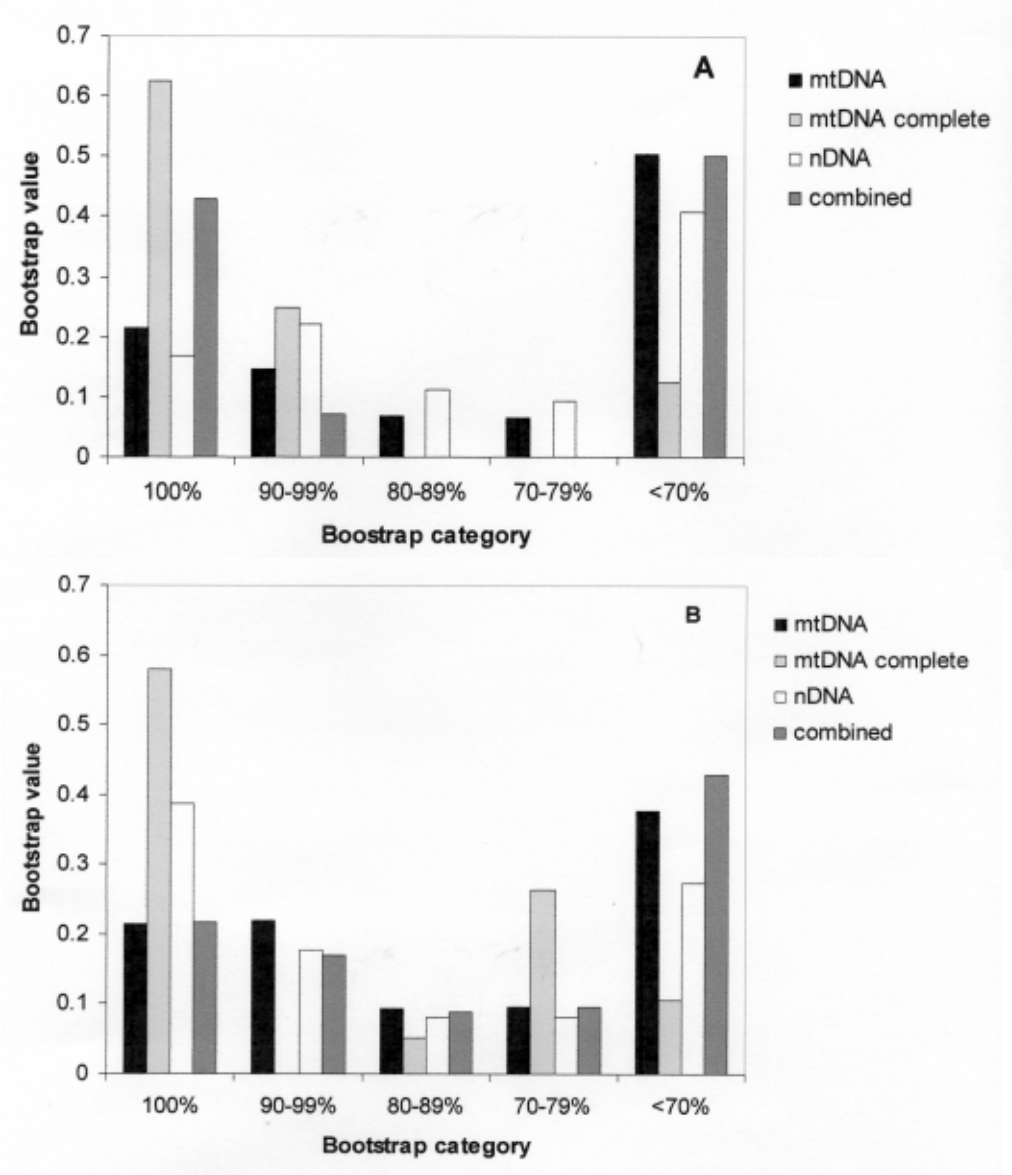

Figure 7. Percentage of nodes in phylogenetic trees that grouped genetic lineages of either **(A)** the same species, or **(B)** different species or higher taxonomic groupings, and had bootstrap values of either <70%, 70-79%, 80-89%, 90-99%, or 100%.

for data sets both individually and combined. In an attempt to do this by combining all available trees to produce a composite phylogenetic tree for all turtles, Iverson et al. (2007) found several gaps in need of resolution. These authors noted, in particular, the need to use a multilocus approach and to expand the geographic range of sampling within a species, including type specimens.

To assess the impact of phylogenetic studies on taxonomic issues, 97 papers were categorized as either (1) supporting the current (at that time) taxonomy, (2) suggesting that a change was needed, but either not specifying the change, or recommending that further data were needed, or (3) proposing a taxonomic change. Of the 97 papers, 29.9% of papers supported the current taxonomy, 38.1% provided evidence that changes were needed, and 32.0% proposed specific taxonomic changes. It is apparent that the taxonomy of turtles is still a work in progress (see Turtle Taxonomy Working Group, 2007a,b) and based on the small number of papers overall that included morphologic data, the greatest emphasis has shifted to using genetic data. An increased use of larger fragments of DNA, including sequencing of the complete control region, or entire mtDNA and an array of nuclear genes is warranted, as is a greater focus on morphology.

Taxonomy and Systematics. — Papers included in this section were all categorized into the initial Taxonomy grouping. Included here were papers that also considered phylogeny, but did not include a phylogenetic tree. We found 56 papers that were focused on taxonomic questions at levels within species (16.1%), genera (25.0%), family/subfamily (41.1%), or a higher level of taxonomy (17.9%). Compared to the papers analyzed in the Phylogeny section, we found a decreased emphasis on species and an increased emphasis on family/subfamily taxonomy in this category.

Many of these papers were exploratory and provided the first genetic analysis of the taxonomy under study. The lack of phylogenetic trees within these papers corresponded to a shift in the types of genetic markers used, with only 7.3% of papers sequencing mtDNA compared to 37.0% using allozymes or blood proteins, 50.0% using karyotypes, and 5.6% using other nuclear markers. Surprisingly, to an even greater extent than in the phylogenetic papers, little emphasis was placed on morphology. Only 32.9% of papers mentioned or discussed morphology and of these, only three papers (Vogt and McCoy, 1980; Seidel and Lucchino, 1981; Gerlach and Canning, 1998) included an analysis of morphological data. However, the lack of morphologic evidence or phylogenetic analysis did not preclude Taxonomic papers from making contributions to taxonomy, because 29.2% of papers indicated that changes were likely needed but required further work, and 12.5% (11 papers) proposed taxonomic revisions. Given the lack of a phylogenetic analysis, it is somewhat surprising how reliable the recommended taxonomic changes have been. Relative to the taxonomy presented by the Turtle Taxonomy Group (2007b), 19 of 20 recommendations are currently recognized as the current taxonomy.

Within this monograph, the Turtle Taxonomy Working Group (2007a) makes a plea for systematists to be both thorough and cautious in making taxonomic revisions. Among both the Phylogeny and Taxonomy papers we analyzed, 51 papers (35.2%) suggested that taxonomic change was likely needed, and 37 of them (25.5%) proposed taxonomic changes. Contrary to the recommendations of the Taxonomy Working Group (2007a) very few of these papers described the specific criteria used to govern their recommendations. For a discussion of turtle taxonomic methods and guidelines, and insights into issues of turtle taxonomy, see the Turtle Taxonomy Working Group (2007a).

Molecular Evolution. — For the past 70 years, turtles have been an important clade for the study of molecular evolution. As genetic techniques have advanced, studies of turtles have kept pace with the testing of new techniques. Of the 41 papers categorized as being primarily focused on Molecular Evolution, the first of these were karyotype studies in 1936 on the sex chromosomes of the soft-shelled turtle *Amyda japonica* (now *Pelodiscus sinensis*) (Oguma, 1936) and musk turtle *Sternotherus odoratus* (Risley, 1936). This sparked interest in looking for sex chromosomes in a variety of species (e.g., Sites et al., 1979; Carr and Bickham, 1981) and eventually to recent investigations of gene expression and chromosome structure in turtles with temperature-dependent versus genetic sex determination (Murdoch and Wibbels, 2003; Valenzuela et al., 2006; Ezaz et al., 2006). Changes to chromosomes have also been used to assess the impacts of radiation and pollution at contaminated sites (Bickham et al., 1988; Lamb et al., 1991; Swartz et al., 2003) and changes in telomere length (Girondot and Garcia, 1998). Early allozyme studies in turtles provided several insights into the functioning of proteins, including studies of serum proteins (Cohen and Stickler, 1958) and hemoglobin anti-adaptation (Manwell and Schlesinger, 1966). More recently, turtles have been the focus of studies of transposons (Endoh et al., 1990; Ohshima et al., 1996; Kajikawa et al., 1997), developmental genes (Spotila et al., 1994, 1998), and small nucleolar RNA proteins (Cervelli et al., 2003). Recently, the construction of a BAC library for *Chrysemys picta* has led to investigations of the structure of turtle genomes (see McGaugh et al., 2007).

Population Genetics. — This section included 39 papers categorized under Population Genetics and additional data from papers on Mating Systems, Phylogeny, Phylogeography, Taxonomy, and Techniques, providing 57 total papers for analysis. This collection of papers included data on 39 species and 24 genera, with most papers (n = 30) only studying a single species. The most frequently sampled genera were *Geochelone (*now *Astrochelys* or *Chelonoidis*) and *Gopherus*, with 10 papers each. The most well represented species were *Geochelone* (now *Chelonoidis*) *nigra* with six papers, *Gopherus agassizii*, *Trachemys scripta*, and *Emydoidea blandingii* with five papers each, *Podocnemis expansa* with four papers, and *Gopherus polyphemus* with three papers.

Within the population genetic data, there was a large range in sample size and completeness of sampling design. Total sample size per study ranged from 12 to 453, with a mean of 126 (±108). Surprisingly, half the studies had sample sizes of < 80. The number of populations (or locations) reported on ranged from one (14% of papers) to 19, with a mean of 6.5 (± 5.6). Mean sample size per population (or location) ranged from 1.2 to 145.0, with an overall mean value of 36 (± 38). Across all studies, 39% had sample sizes of < 20 per population. Although these smaller samples sizes were appropriate for Techniques papers that provided introductory data on microsatellite diversity, in several papers low sample sizes were not adequate for addressing the broad geographic or the fine-scale questions posed.

For the most part, papers reporting population genetic data used a similar number of loci regardless of whether allozymes or microsatellite markers were employed (Fig. 8).

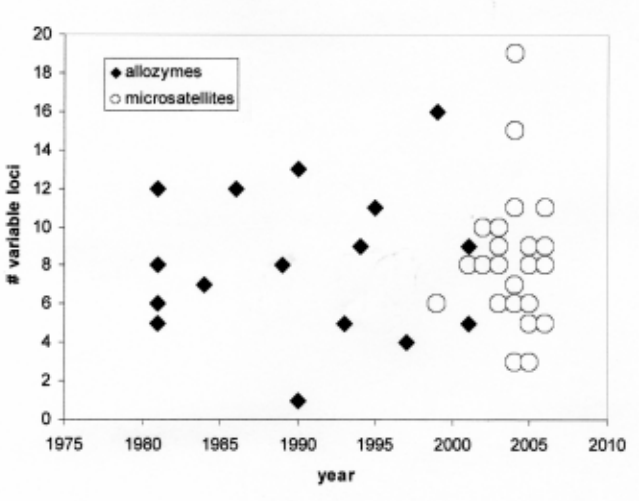

Figure 8. Scatter plot showing the number of variable loci over time for both allozymes and microsatellites for all Population Genetics papers.

The average number of loci was 8.3 (range 1–16) for allozymes and 7.9, respectively (range 3–19) for microsatellites. Of the 33 papers that reported microsatellite data, 24% used 10 or more loci, and there was no positive correlation between the number of loci used and the year of the study since their first use in *Podocnemis expansa* (Sites et al., 1999).

Genetic Diversity and Population History. — Comparing values of observed heterozygosity across species is problematic for allozyme studies because the value reported typically includes in the estimate a variable number of non-polymorphic loci, which were also tested when conducting the research. In these studies, the total number of loci ranged from 1 to 23, with an average of 17 loci tested. In several studies, heterozygosity values were not given per population, rather only an overall per-locus value. For allozymes, the reported average observed heterozygosity values ranged from 0.00 to 0.88 per population, with an overall average of 0.16. In contrast, the average observed heterozygosity per population using microsatellite loci was much higher at 0.64, but with a similar range of 0.34 to 0.88.

Genetic diversity as measured by the number of alleles was low for allozymes, with an average of 2.0 and a range of only 1.1 to 3.2 alleles per locus. Microsatellite loci have proven to be highly variable in turtles, with an average value of 9.3 and range of 2.7 to 21 alleles per locus. Within populations, the number of microsatellite alleles ranged from 1.5 to 18.0, with an average of 8.2 alleles. The number of mtDNA haplotypes found across studies ranged widely from a single haplotype in giant tortoises sampled across the Indian Ocean (Palkovacs et al., 2003) to 83 in *Emys* (formerly *Clemmys*, now also *Actinemys*) *marmorata* sampled across its range (Spinks and Shaffer, 2005); with an average of 14.9 haplotypes in all studies.

Few studies looked directly at the question of whether reduced habitat size, or increased isolation, led to changes in genetic diversity. One exception was a comparative study of painted turtles (*Chrysemys picta*) and spotted turtles (*Clemmys guttata*) living in small isolated ponds versus those in large connected ponds (Parker and Whiteman, 1993). That study uncovered reduced genetic diversity in spotted turtles in small ponds, but found no effect on painted turtles. Tests for evidence of past bottlenecks and population expansion that were based upon equilibrium expectations of heterozygosity were presented in nine studies, beginning in 2002 with a study indicating past bottlenecks in three populations of the geometric tortoise (*Psammobates geometricus*) (Cunningham et al., 2002). Six other studies indicated past bottlenecks in at least some populations across a diversity of habitats, including rugged islands (Beheregaray et al., 2003a; 2003b), deserts (Edwards et al., 2004a), woodlands (Schwartz and Karl, 2005), sand prairies (Kuo and Janzen, 2004), and large river systems (Pearse et al., 2006a). In contrast, no evidence of bottlenecks was found in populations of the angulate tortoise (*Chersina angulata*) of coastal South Africa (Lesia et al., 2003) or in Blanding's turtle *(Emydoidea blandingii)* populations found in lakes and rivers (Mockford et al., 2005) of Nova Scotia.

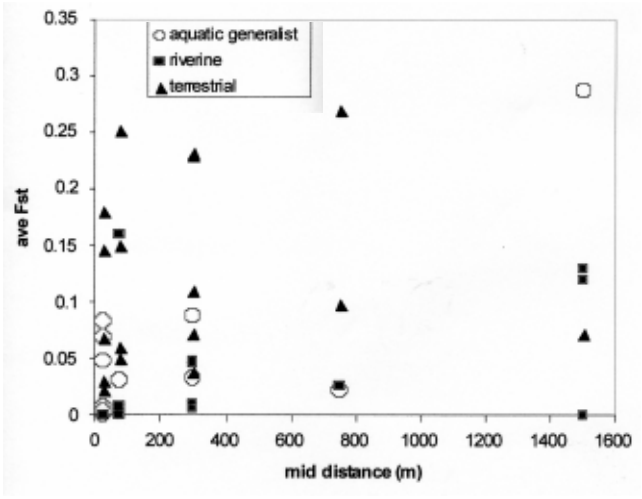

Figure 9. Scatter plot of F_{st} values versus mid distance (m), by habitat types for all reviewed papers providing population genetics data.

Genetic Structure and Gene Flow. — Surprisingly, we found no positive relationship between genetic structure and distance across studies (Fig. 9). Average pairwise F_{st} values were determined for five distance intervals and tested for variation in a single factor ANOVA. Mean F_{st} values for each category ranged from 0.06 (1–49 km) to 0.14 (1000+ km), but the differences across categories were not significant ($p = 0.45$). When these data were categorized by habitat type, there was a general trend toward increasing F_{st} values with increasing distance within categories. Notable exceptions to this were high F_{st} values at short distances for Galápagos tortoise populations of different islands (Ciofi et al., 2002) and low F_{st} values at a large distance for Indian star tortoises (*Geochelone elegans*) (Gaur et al., 2006). The lack of correlation between genetic structure and geographic distance across studies means that at present, generalizations and extrapolations from one species to another cannot be made with much confidence.

Few papers provided data that allowed for a comparison of genetic structure between mtDNA versus microsatellite markers. In the seven papers where data were available, we detected a strong positive correlation ($r^2 = 0.70$; $p = 0.011$). If these values are translated into estimates of gene flow (Nm, the number of migrants per generation; Slatkin, 1987), the average Nm value calculated from the mtDNA data was 0.43 and that from microsatellite data was 2.6. Given an expected 2x greater gene flow for nuclear versus mtDNA genes, on average there was 3.1x greater gene flow at nuclear genes than expected, perhaps reflecting a tendency toward male-biased gene flow in turtles. However, any such conclusions are tempered by problems associated in comparing genetic markers that vary in the nature and rate of mutations.

Patterns of gene flow as explained by an isolation-by-distance (IBD) effect was discussed or tested in 12 papers.

Of these, five papers stated that IBD likely occurred, but did not test for it, and one paper tested for IBD but did not find it in *Podocnemis* (Bock et al., 2001). The remaining six papers tested for IBD using Mantel tests or spatial autocorrelation tests and found that it occurred (Scribner et al., 1986; Edwards et al., 2004a; Freedburg et al., 2005; Hauswaldt and Glen, 2005; Spinks and Shaffer, 2005) in a variety of habitats. Correlation coefficients (r^2) ranged from 0.022 (Freedburg et al., 2005) to 0.79 (Hauswaldt and Glen, 2005). Distances over which IBD was found ranged widely. Scribner et al. (1986) used spatial autocorrelation to find IBD operating on a scale of 1.5–3.5 km among different aquatic habitats in *Trachemys scripta*, and Freedberg et al. (2005) used Mantel tests to find IBD among nesting *Graptemys kohnii* (now *pseudogeographica*) females along a 4 km lakeside beach. At the other extreme, IBD was found for distances up to 3000 km among estuarine populations of *Malaclemys terrapin* (Hauswaldt and Glen, 2005) and up to 4900 km in river populations of *Podocnemis expansa* (Pearse et al., 2006a). In terrestrial habitats, IBD was found in *Gopherus agassizii* among populations separated by distances up to 190 km (Edwards et al., 2004a).

Genotyping. — Other powerful new approaches used the genotypes of individuals to assign them to the most likely population of origin based on the *a priori* designation of populations. These included assignment tests in GENECLASS-2 (Piry et al., 2004), and WHICHRUN (Banks and Eichert, 2000), and modelling approaches to define populations based on genotypes or allele frequencies as in STRUCTURE (Pritchard et al., 2000) or BAPS (Corander et al., 2004); for a review see Pearse and Crandall (2004). These types of tests have been used in 39% of the microsatellite studies that provided population genetic data, with a range of results. The lowest levels (30%) of assignment of individuals to the populations from which they were sampled were found in desert tortoise (*Gopherus agassizii*) populations located within a 200 km radius (Edwards et al., 2004a). In contrast, values of >90% were found in at least some populations of Galápagos tortoises (*Chelonoidis nigra*) (Ciofi et al., 2002; Russello et al., 2006), gopher tortoises (*Gopherus polyphemus*) (Schwartz and Karl, 2005), wood turtles (*Glyptemys insculpta*) (Tessier et al., 2005), and Amazon river turtles *Podocnemis expansa* (Pearse et al., 2006a). Important new applications of assignment tests included the assignment to populations of origin for confiscated turtles, as done recently for Indian star tortoises to inform repatriation efforts (Gaur et al., 2006) and for captive Galápagos tortoises to better manage captive breeding and repatriation programs (Burns et al., 2003; Milinkovitch et al., 2004).

Phylogeography. — Analyses of phylogeographic data included 31 papers categorized in Phylogeography and data from 15 additional papers that were categorized as primarily Phylogenetic or Population Genetics papers. Although most of these papers aimed to better understand the historic processes leading to the observed phylogeographic patterns, less than half (46.7%) stated a specific hypothesis about the processes they were investigating, so it was often not clear whether the studies were designed effectively. All papers relied on mtDNA markers including mtDNA-RFLP, PCR-RFLP, and sequencing approaches. Of these, 22 papers used control region data, 19 used cyt*b*, 22 included data from more than one mtDNA locus, and 11 papers compared their results to nDNA analyses.

We observed a large range in the scope of the studies in terms of sampling effort and geographic coverage. Total sample sizes ranged from 14 to 802, with an average of 102 samples across all studies. Notable sampling efforts included comprehensive studies of Galápagos tortoises (n = 802; Beheregaray et al., 2004) and the European pond turtles (*Emys orbicularis*) (n = 423; Lenk et al., 1999) across their ranges. Across all studies, samples were collected from an average of 19 localities (range of 2 to 117) with an average of 17 samples collected per site (range of 1.3 to 67). Study sites ranged from a local focus of under 100 km for Galápagos tortoises on Isabela Island (Beheregaray et al., 2003a) to extensive studies across more than 4000 km for *Chrysemys picta* in North America (Starkey et al., 2003), *Emys orbicularis* in Europe (Fritz et al., 2004), *Geochelone sulcata* in sub-Saharan Africa (Livoreil and van der Kuyl, 2005), and *Testudo graeca* in North Africa and the Middle East (van der Kuyl et al., 2005). However, across the majority of studies, there was at best only limited sampling effort, both in terms of numbers of individuals sampled per site and the geographic range and focus.

Although a strong case has been made for taking a comparative phylogeographic approach, this has not been the case for studies of freshwater turtles and tortoises. This is somewhat surprising given that it was through early comparative studies of turtles that Walker and Avise (1998) first demonstrated the value of phylogeographic studies. Avise (2000) recommended comparative approaches that include the use of multiple genetic markers, multiple species, and multiple types of data to investigate patterns of concordance. However, across the 46 papers analyzed, only three papers studied more than one turtle species and less than half the papers (47.8%) used multiple genetic markers. Of the three papers that analyzed multiple species, two of these were the seminal phylogeographic studies, which used 14 and 2 species each (Walker and Avise, 1998 and Walker et al., 1998a, respectively), the remaining paper compared three species of turtles (Weisrock and Jansen, 2000). Within the discussion sections of Phylogeographic papers, there has been some improvement over time, with an additional six papers mentioning phylogeographic patterns in other turtles or other species (i.e., fish). To date, little has been accomplished in phylogeographic studies regarding the analysis of nuclear datasets in conjunction with mtDNA data, although nine studies made comparisons to allozyme or microsatellite data. Recently, Spinks and Shaffer (2005) demonstrated the potential for problems with nuclear markers in their study of *Emys* (= *Actinemys*) *marmorata* across its range. In their paper, sequence data from GAPDH and the intron R35 were so lacking in variation that the authors dropped them from

the analysis. However, Guiking et al. (2002) found that genomic fingerprinting via ISSR-PCR provided new insights into the history of *Cyclemys* in comparison to sequence data from cyt*b*, adding promise for this approach.

Walker and Avise (1998) argued that turtle species are particularly useful for comparative phylogeographic studies to uncover historic landscape processes due to their fidelity to sites, reduced dispersal, and longevity, which can prolong the historic footprint. However, to date, few such comparative studies have been completed. Thus, there is a need to increase the number of species studied across a region and to increase the number of genetic markers used to address these questions. Through better understanding of phylogeographic patterns across species, we can more fully understand how turtles are responding to landscape change across time and relate this to present conservation concerns. There is also a need to be more explicit regarding the testing of specific hypotheses when conducting phylogeographic and species delimitation studies and to analyze multiple genes when doing so.

Techniques. — Of the 16 papers classed under the Techniques category, 75% reported on the development of new microsatellite markers. Interestingly, the first paper to publish on turtle microsatellites reported on a microsatellite motif found within the control region of the African side-necked turtle, *Pelomedusa subrufa* (Zardoya and Meyer, 1998b). This region has been confirmed in other turtles (e.g., Serb et al., 2001; Pearse et al., 2006a) and its potential usefulness as a genetic marker has been considered (Zardoya and Meyer, 1998b). However, mtDNA microsatellites found in other species were shown to be fraught with problems due to heteroplasy within samples (Lundt et al., 1998). The trend to publish microsatellite primer notes began with primers for the Blanding's turtle (Osentoski et al., 2002), followed by 9 papers published in *Molecular Ecology Notes* and one in *Conservation Genetics* (King and Julian, 2004). The remaining techniques papers reported on sample-collection protocols. This included one paper that compared a non-invasive sampling technique of using skin scrapings, in comparison to blood and scutes, in which skin scrapings, but not scutes, provided reliable PCR products (Tessier and Lapointe, 2003). In a summary techniques paper, a case was made for the continued use of allozymes, with Buth and Rainboth (1998) providing detailed information on the optimized conditions for running 73 different allozyme loci in turtles. Many new techniques are being explored and new primers continue to be developed; within this monograph, see McGaugh et al. (2007) for a review of new genetic approaches and see Engstrom (2007) for information on turtle primers for microsatellites and sequencing.

Mating Systems. — Of the 13 papers on Mating Systems, two were review papers (Galbraith, 1993; Pearse and Avise, 2001), and the rest provided data on 10 species from seven genera. Most studies (n = 11) surveyed wild populations, a few of which were part of long-term studies with marked individuals. Two studies focused on captive populations, in which mating opportunities were controlled (Palmer et al., 1998; Johnson et al., 2006). Initially, the technique of DNA fingerprinting (Galbraith et al., 1993) and allozymes (Scribner et al., 1993) were used to study mating systems. However, the current trend is to use microsatellites exclusively due to the larger number of alleles per locus. The primary focus of most of the Mating Systems papers has been to understand the extent of multiple paternity (MP) and the role of sperm storage, with limited attention focused on implications for conservation. Whereas 35.7% of papers in this category mentioned conservation or indicated the threatened or endangered status of the species, it is only recently that these papers have discussed the conservation implications of the mating system of the species studied (e.g., Moon et al., 2006; Pearse et al., 2006b; Roques et al., 2006).

Multiple paternity of clutches was found in all studies in this category and most analyses suggested that it involved two or sometimes more males (Valenzuela, 2000; Pearse et al., 2006b) (Table 5). Sperm storage was apparent in all studies that examined multiple clutches of females, both within and between seasons. However, in many cases the results needed to be tempered by the limitations of small

Table 5. Mating system studies over time in freshwater turtles and tortoises; FP = fingerprint, allzy = allozyme, m'sat = microsatellite.

Species	marker	# loci	# females	# clutches mean	# offspring	# offspring range	% MP range	# fathers	% offspring of 1st father	Reference
Chelydra serpentina	FP	2	3	3	12.5	12 to 13	66%	1 to 2	-	Galbraith et al. 1993, Galbraith 1993
Chrysemys picta	m'sat	2-3	32	113	5.5	1 to 13	12%	1 to 2	79%[b]	Pearse et al., 2001b
Chrysemys picta	m'sat	2-3	227	227	5.6	1 to 14	11%[a]	1 to 2	~85%	Pearse et al., 2002
Clemmys insculpta	FP	n/a	n/a	6	-	-	33%	-	-	Galbraith, 1993
Emys orbicularis	m'sat	6	11	20	6.9	5 to 10	10%	2	-	Roques et al., 2006
Gopherus agassizii	allzy	3	12	12	5.6	5 to 8	50%	1 to 2	-	Palmer et al., 1998
Gopherus polyphemus	m'sat	9	7	7	7.6	4 to 11	29%	2?	57-80%	Moon et al., 2006
Podocnemis expansa	m'sat	8	2	2	32.5	16 to 46	100%	2 to 3+	37-67%	Valenzuela, 2000
Podocnemis expansa	m'sat	7	32[d]	32	21.4[c]	9 to 76	9%	1 to 2+	-	Pearse et al., 2006a
Testudo graeca	m'sat	3	8	15	3.1	1 to 5	20%	-	-	Roques et al., 2004
Testudo horsfieldii	m'sat	5	4	11	2.7	2 to 3	27%	1 to 2	50%	Johnston et al., 2006

[a]if the %MP is calculated only for clutches with >6 hatchling sampled (n = 12 clutches), the value increases to 33% (Pearse et al., 2002)
[b]for MP with previous years sperm
[c]based on 28 clutches, the remaining 4 clutches averaged 71.8 sampled
[d]females not available for genotyping

clutches, and small sample sizes, except for *Podocnemis*, which lay larger clutches (Table 5). Two studies were able to demonstrate that multiple paternity sometimes results from sperm storage from previous seasons. In captive desert tortoises, *Gopherus agassizii*, multiple paternity had occurred due to previous mating in the wild (Palmer et al., 1998). In a wild population of *Chrysemys picta*, studied over a four-year period, sperm storage from previous years was a contributor to multiple paternity (Pearse et al., 2001b). It should be noted, however, that determining the existence of multiple paternity relies on being able to identify mutations and mis-scored alleles. Unfortunately, few studies addressed quality-control issues, and some studies relied on a low number of loci (i.e., 2–3). This makes the determination of multiple paternity versus mutations a statistical exercise, sometimes with broad confidence intervals. Mutation rates were estimated for some of the studies and ranged from 8.9 x 10^{-4} to 2.7 x 10^{-2} (Valenzuela, 2000; Pearse et al., 2002, 2006b; Roques et al., 2004), indicating that mutations are likely to be encountered in mating studies of turtles. Of these studies, only Roques et al. (2004) and Pearse et al. (2002) were able to confirm the presence of some mutations by observing size changes to the mother's alleles in her offspring.

Several important questions remain regarding the synergy between turtle mating systems and individual fitness in turtles. Some studies have attempted to address the question of whether hatching success increases with multiple paternity, but with limited and conflicting outcomes. In a large study of *Chrysemys picta*, Pearse et al. (2002) found evidence in support of a positive correlation, and Roques et al. (2006) found increased hatching success in the second clutches of female *Emys orbicularis* if these were fathered by a second male. However, in a wild population of *Gopherus polyphemus* there was some evidence of reduced hatch success in 2 of 7 clutches showing multiple paternity (Moon et al., 2006). Few studies have aimed to evaluate male fitness correlates, even though all document variable mating success by males in multiply-sired clutches, including variation in male success across multiple clutches. Additionally, some males were found to have mated with more than one female in studies of wild populations of *Chrysemys picta* (Pearse et al., 2001b, 2002) and *Emys orbicularis* (Moon et al., 2006). In *Emys orbicularis*, Moon et al. (2006) found evidence of a negative relationship between female size and rates of multiple paternity and a positive relationship between male length and mating success, though sample sizes were small.

Two novel applications of mating systems to understand wild populations were presented, both involving *Chrysemys picta*. In a unique approach to understanding the dynamics of mating systems, Scribner et al. (1993) analyzed hatchlings (and juveniles) from particular nesting areas, rather than from specific nests, to find that the genetic structure of cohorts varied across time and space within a marsh. This information was applied to an understanding of the long-term dynamics of the population. In contrast, analysis of clutch paternity was used in a novel way to test a mark-recapture technique, in which captures and recaptures were determined through an analysis of clutch paternity, using the clutches of different females each year (Pearse et al., 2001a). In this way, nine males were recaptured out of 133 clutches and estimates were made of the size of the local male population and percent of breeders, though confidence intervals were large.

Mating system studies in turtles have documented that multiple paternity and sperm storage are common in turtles (see also Pearse and Avise, 2001), but the application of this knowledge to issues of conservation and captive breeding programs in turtles needs exploration (for a review, see Syed et al., 2007). In general, there is a need for more rigorously addressing theoretical questions and increasing the scope of mating system studies, including a better understanding of sperm competition and long-term fitness. Future studies should follow the lead of those studies that sampled the mothers (and potential fathers where possible), used larger sample sizes and analyzed more loci, and applied their results to the ecology and conservation of turtles. With relevance to mating systems and for conservation purposes, we need to know the extent to which hybridization is natural, and if so, how to incorporate that information into conservation programs (Parham et al., 2001; Stuart and Parham, 2004; Spinks and Shaffer, 2005, 2006). This can be a particularly difficult issue if not well explained to policy makers, given the potential confusion over a species' status in conservation legislation.

Hybrids. — Issues of hybridization in natural populations and confusion over the naming of species that were later shown to be unnatural hybrids from captive bred populations were the topics of 36 (13.7%) of the reviewed papers (Table 6). Of these papers, 26 studied natural popu-

Table 6. Analysis of reviewed papers by category that discussed hybrids or issues of hybridization.

Category	No. papers/ category	No. mentioned hybrids	No. focused on hybrids	Total no. dealing with hybrids	% of category	% of all papers
Phylogeny	66	8	5	13	19.7	5.0
Taxonomy	56	6	6	12	21.4	4.6
Molecular Evolution	41	2	0	2	4.9	1.9
Population Genetics	39	3	1	4	10.3	1.5
Phylogeography	31	5	0	5	16.1	1.9
Techniques	16	0	0	0	0.0	0.0
Mating Systems	13	0	0	0	0.0	0.0
Total (or Average)	262	24	12	36	(Avg 10.3)	14.9

lations, seven focused on captive turtles, and the remaining three tested hybrid individuals of unknown origin. Evidence of hybridization between species or subspecies was presented in 18 of the papers, with four papers relying only on mtDNA, seven using nDNA, and the remaining seven papers using both types of markers. Since the 1950s, several papers used genetic techniques to confirm the presence of natural hybrids in wild populations (e.g., Zweig and Crenshaw, 1957; Crenshaw, 1965b; Seidel and Atkins, 1987; Georges et al., 2002) or to conclude that past hybridization must have occurred to produce observed phylogenetic relationships (e.g., Spinks et al., 2004; Spinks and Shaffer, 2006).

We noted a concern in the literature that rates of hybridization in the wild may increase due to anthropogenic effects of habitat alteration, reductions in population size, and transport of individuals. Crenshaw (1965b) recognized these problems in hybrid populations of *Pseudemys rubiventris* and *P. floridana* (now *concinna*), in which large variation in population sizes between the two species was seen along hybrid zones, and in one locality a man-made lake had provided the opportunity for hybridization. Particularly for turtles, which have a long history as pets or as a food source, intentional hybridization through captive breeding and human transport are likely scenarios in many areas. Several cases were documented of mistaken identity of described species that genetic studies later revealed to be hybrids (e.g., Parham et al., 2001; Shi et al., 2005; Stuart and Parham, 2006), and others have verified the ability of freshwater turtles to hybridize (e.g., Yasukawa et al., 1992; Schilde et al., 2004; Buskirk et al., 2005). There is also genetic evidence that turtles have likely been transported by people into new habitats (Sato and Ota, 1999; Álavarez et al., 2000; Fritz et al., 2004), which may lead to hybridization. Surprisingly, no thorough investigation of turtles in a hybrid zone has yet been completed to determine the specifics of the mating patterns and the geographic extent of introgression; this is clearly an area for future research.

Conservation. — Across all reviewed papers, 32.8% of papers (n = 86) made some mention of conservation and of these, 25.6% (n = 67) provided conservation recommendations (Table 7). However, of the 67 papers providing conservation recommendations, most of these comments were of limited extent. Only 31 papers (11.9% of total) could be considered as papers focused on conservation, based on criteria that they (1) mentioned conservation in their introduction, (2) provided recommendations, and (3) also had the word 'conservation' in their title or the paper was published in a conservation or environmental journal (*Animal Conservation*, *Biological Conservation*, *Chelonian Conservation and Biology*, *Conservation Biology*, *Conservation Genetics*, or *Ecotoxicology*). Under this more restricted definition, Population Genetics papers dominated the conservation literature with 15 papers, followed by Phylogeography (7 papers) and Phylogeny (5 papers). There were only two papers on Mating Systems that focused on conservation and these discussed issues of effective population size and management implications (Pearse et al., 2006b; Roques et al., 2006). No Techniques papers were focused on conservation, since most papers reported on the development of microsatellite makers to address conservation issues. All of the conservation-focused papers were all relatively recent (i.e., since 1994); the first were population genetics papers on gopher tortoises (Morafka et al., 1994) and *Clemmys* (= *Actinemys*) *marmorata* (Gray, 1994).

Within their respective categories, Population Genetics papers had the highest percentage of papers focused on conservation, followed by Phylogeography (38.5 and 24.1%, respectively). However, in both categories, the papers were fairly limited in geographic scope. Most studies were conducted in North America, six papers reported on populations in South America, two studies were performed in Africa (Cunningham et al., 2002; Livoreil and van der Kuyl, 2005), and one study each focused on turtles from Madagascar (Leuteritz et al., 2005), India (Gaur et al., 2006), and China (Stuart and Parham, 2006). Included here were important forensics papers that demonstrated the presence of illegally harvested turtles at meat markets in the southeastern USA (Roman and Bowen, 2000), and on the identification of regional origins for confiscated Indian star tortoises for purposes of repatriation (Gaur et al., 2006).

Given the need to have a clear taxonomy based on a resolved phylogeny, and because of the strong arguments that have been put forward about this need (in this monograph, see Iverson et al., 2007; Lehn et al., 2007; Syed et al., 2007), there was a surprising lack of focus on issues of conservation within papers in both the Phylogeny (7.9% of papers) and Taxonomy (1.6% of papers) categories. However, these papers had a broader scope, from the USA to South America, China and SE Asia, and they were focused

Table 7. Conservation analysis of reviewed papers by category; Cons. = conservation, rec. = recommendations.

Category	No. papers/ category	Cons. mentioned in introduction	Cons. rec. provided	Cons. mentioned anywhere	% Total Cons.	% Total Cons. across all papers
Population Genetics	39	31	30	31	79.5	11.9
Phylogeny	66	17	11	17	27.0	6.5
Phylogeography	31	12	13	15	51.7	5.7
Techniques	16	9	2	9	56.3	3.4
Taxonomy	56	5	5	7	11.5	2.7
Mating Systems	13	5	4	5	38.5	1.9
Molecular Evolution	41	1	2	2	5.0	0.8
Total (or Average)	262	80	67	86	(Avg 38.5)	33.0

on determining the status of species of high conservation concern and determining unique lineages in need of conservation (i.e., Parham et al., 2001, 2004; Engstrom et al., 2002; Russello et al., 2006; Spinks and Shaffer, 2006; Stuart and Parham, 2006).

We constructed a summary list of all species that were included in the reviewed papers to show (1) the number of times each species was covered by the different categories of papers, and (2) their status if listed on the IUCN Red List (IUCN, 2006). This resulted in 1675 records (Appendix 1), which allowed an assessment of whether efforts at a species level correlated with conservation needs. By far, the greatest number of records were for species in the Testudinoidea (44.8%) and Emydidae (23.1%), followed by the Chelidae (10.0%) and Kinosternidae (9.4%); the remaining families represented < 5% of the records. These records represent only a small percentage of species that are likely to need conservation attention. If we define 'species of concern' as those that are IUCN-listed (IUCN, 2006) as Extinct in the Wild, Critically Endangered, Endangered, or Vulnerable, they were represented across all families by 43.6% of the records and by the four families as follows: Testudinoidea 51.2%, Emydidae 27.1%, Chelidae 34.2%, and Kinosternidae 13.6%. Conversely, 85.2% of 'species of concern' were included to some degree in the data of the reviewed papers. Overall, the majority (84%) of studies on species of concern were from Phylogeny (67.8%) and Taxonomy (16.3%) papers, and it appears that many of these records arise from including these species in multi-species phylogenies, without necessarily being a focus of the paper. Furthermore, only 6.9% of the records of 'species of concern' were associated with Population Genetics or Phylogeography papers. Iverson et al. (2007) stated that the genera most in need of phylogenetic resolution are *Pelusios*, *Podocnemis*, *Testudo*, *Kinosternon*, *Batrachemys*, *Elseya*, *Trachemys*, *Graptemys*, and *Pseudemys*; within which there are currently 25 'species of concern'. The conclusion of those authors reflects the need for a more focused persistence in many cases, as there are already 289 records of those genera in Phylogenetic and Taxonomy papers; representing 17.3% of all records, yet major questions remain.

It was apparent in our review that conservation themes were not pervasive across all categories of papers, even in recent years. However, whereas previously it was acceptable to routinely sacrifice turtles to conduct the research, this is now an infrequent occurrence. This change in attitude may largely be due to changing techniques that no longer require specific tissues, as well as changing attitudes about acceptable sampling protocols and ethics (see Burke et al., 2007). Within a conservation context, there is a need for improved vouchering of specimens, including properly referenced tissue collections obtained through ethical decision making (see Lehn et al., 2007). We hope that this review highlights the worldwide need for turtle conservation research using molecular genetic techniques. For reviews on the potential scope of conservation genetics for turtles and on the need for genetic data in captive breeding and reintroduction programs, see Turtle Conservation Genetics Working Group (2007) and Syed et al. (2007) in this monograph.

CONCLUSIONS AND FUTURE DIRECTIONS

Genetic studies of turtles have been among the earliest genetic studies, and the rapid application of new genetic tools and analytical techniques is apparent within the literature on turtles. This review represents the first attempt to assemble and examine a substantial number of published studies on freshwater turtle and tortoise genetics. Turtle geneticists began using molecular genetic techniques in the past primarily to study turtle systematics and the structure of chromosomes. Since these initial studies, the techniques and markers have improved, and we are now able to provide greater resolution to phylogenetic analyses and to discover aspects of turtle biology that previously were recoverable only after several years of ecological sampling (see Turtle Conservation Genetics Working Group, 2007; McGaugh et al., 2007). It is now possible to estimate levels of gene flow using a diversity of markers, to estimate migration rates and identify potential migrants, and to determine whether there is sex-biased gene flow. Markers can be used to quantify the relatedness between individuals within a population or sampling site, and population boundaries can be defined to inform management scale and strategies. For example, knowing how connected different subunits of a population are helps to resolve the spatial scale necessary for effective conservation.

Our review revealed that (1) studies conducted on freshwater turtles and tortoises in North America dominate the literature; (2) Phylogenetic and Taxonomic studies represent the top two categories of papers published on these species, though Molecular Evolution and Population Genetics papers are increasingly represented in recent publications; (3) the majority of studies use either mitochondrial or nuclear markers, not a combination of the two classes of markers; (4) few papers are focused on issues of conservation, and these are mostly limited to Population Genetics and Phylogeographic studies; and (5) though progress has been made, there is a need for a broader and more rigorous study of this imperiled group of vertebrates. Thus, there are strong needs for (1) the international community to support researchers outside of North America in conducting turtle genetics research; (2) collaborations to resolve the remaining Phylogenetic and Taxonomic questions; (3) development of reliable markers, particularly new nDNA markers, for use in multilocus approaches; (4) greater emphasis on the application of genetics to conservation issues in turtles; and (5) increased sample sizes, geographic scope, and analytical rigor to produce more meaningful results.

Future Conservation Efforts. — We did not find a strong emphasis on conservation issues in the literature we reviewed, or on informing mangers or policy. Unfortunately, this lack of emphasis is also pervasive across conservation biology journals. In a review of recent (i.e., since

2001) papers published in three top conservation biology journals, Fazey et al. (2005) found that only 37% and 20% of papers were highly relevant to management or policy, respectively. Additionally, only 13% of papers evaluated conservation actions, and these were typically related to restoration, translocations, or species recovery efforts. However, even if the numbers were higher, Pullin et al. (2004) found that managers do not usually use scientific papers when developing management plans. This indicates that even if more papers took a conservation focus, it may not be enough of an effort, thus, there is a growing need to communicate our science to policy makers.

Making use of high-profile IUCN lists should be done to prioritize species to be targeted for future studies with high relevance to policy or management. One important consideration that is not apparent in this review is the extent to which turtle geneticists have provided their results to managers and policy makers in the form of technical reports and presentations. Many researchers have their work funded by management agencies and conservation organizations, and these results do not always get published in readily accessible journals. An evaluation of this unseen level of communication, and the degree to which positive change has been implemented due to knowledge of genetic data, would be of great use.

Despite the fact that freshwater turtles and tortoises have been the focus of recent and intense conservation attention (e.g., because of the current Asian Turtle Crisis; van Dijk et al., 2000), there are still a relatively small number of published molecular genetic studies for the most Critically Endangered or Data Deficient turtle taxa. The necessary role that conservation genetic studies have in turtle conservation is apparent and suggests areas needing future research. As well, the results of our review are pertinent to researchers designing new studies. For example, of particular interest to new researchers may be the information on where studies have *not* been conducted (Fig. 3), new or under-utilized mtDNA and nuclear DNA markers (Tables 3 and 4, respectively), and the lack of studies on, for example, mating systems in hybrid zones.

In closing, to reiterate; at least 42% of freshwater turtles and tortoises are considered to be facing a high risk of extinction, and there is a need to focus intense conservation attention on these species. Taxonomic controversy must be resolved with policy makers to ensure the best interpretation is integrated into legislation (see Lehn et al., 2007). For example, the taxonomy proposed by the Turtle Taxonomy Working Group (2007b), represents 17 changes to the names of 'species of concern' since their listing on the IUCN Red List (IUCN, 2006). It will not be sufficient, however, to focus on taxonomy; there are also strong needs for phylogeographic and population genetic studies and to some extent, mating system studies, particularly for species that may be in need of relocation or captive breeding (see Syed et al., 2007). The Turtle Conservation Fund (TCF) in 2003 produced a list of the world's top 25 most endangered turtles (see <http://www.conservation.org/xp/news/press_releases/2003/051503.xml>). For these turtles, only nine papers reported on their phylogeography or population genetics; eight of these were on the Galápagos tortoise, and one focused on the geometric tortoise of South Africa (Cunningham et al., 2002). There is obviously a great deal more to be done. A primary goal of this review has been to elucidate where the gaps in our knowledge exist, particularly regarding conservation needs, in the hopes of inspiring both new and current turtle researchers to conduct appropriate studies and to disseminate their results to those who need the information. The time to act is upon us.

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Appendix 1. All species for which genetic data was provided in the reviewed papers, including IUCN status and the number of times the species was included in each category of reviewed papers. Also included are all freshwater turtles and tortoises listed by the IUCN 2006 Red List as Extinct in the Wild (EW), Critically Endangered (CR), Endangered (EN), Vulnerable (VU), or Data Deficient (DD) for which genetic data were provided. Category abbreviations are: Mating, Mating Systems; ME, Molecular Evolution, P'geny, Phylogeny; P'geo, Phylogeography; PG, Population Genetics; Tax, Taxonomy; Tech, Technology. Note: The values represent any inclusion of data about the particular species, and do not imply that the studies were focused on that species.
[1] previous names follow Turtle Taxonomy Working Group (2007b), previous names provided on subsequent lines
[2] IUCN abbreviations are: EX, extinct; EW, extinct in wild; CR, critically endangered; EN, endangered; VU, vulnerable, LR, low risk; n/a, not on the IUCN Red List (IUCN 2006). *listed on IUCN Red List (IUCN 2006) under previous name.

Species[1]	IUCN Status[2]	Mating	ME	P'geny	P'geo	PG	Tax	Tech	Total
Cryptodira									
Chelydridae									
Chelydra serpentina	n/a	2	6	12	2		4	1	27
Macrochelys temminckii	VU (1994)		2	6	1	2	2		13
Dermatemydidae									
Dermatemys mawii	CR (2001)		2	7			2		11
Kinosternidae/Kinosterninae									
Kinosternon acutum	LR (1994)			2					2
Kinosternon alamosae	n/a			2					2
Kinosternon angustipons	VU (1994)								**0**
Kinosternon bauri	n/a		2	6	3		4	1	16
Kinosternon chimalhuaca	n/a			1					1
Kinosternon dunni	VU (1994)			1					1
Kinosternon flavescens	n/a		3	7			3		13
Kinosternon herrerai	n/a			2					2
Kinosternon hirtipes	n/a		1	5			1		7
Kinosternon integrum	n/a		1	2					3
Kinosternon leucostomum	n/a						2		2
incl. *K. spurrelli*	-								
Kinosternon oaxacae	LR (1994)			4					4
Kinosternon scorpioides	n/a		4	5			7		16
incl. *K. cruentatum*	-								
Kinosternon sonoriense	VU (1994)		4						4
Kinosternon subrubrum	n/a		3	5	2		4		14
Sternotherus carinatus	n/a			5			3		8
Sternotherus depressus	VU (1994)			4	1		1		6
Sternotherus minor	n/a		2	5	2		3		12
incl. *Kinosternon minor*	-								
Sternotherus odoratus	n/a		2	13	2		4		21
Staurotypinae									
Claudius angustatus	LR (1994)		2	2					4
Staurotypus salvinii	LR (1994)		4	2			2		8
Staurotypus triporcatus	LR (1994)		1	9			1		11
Emydidae									
Deirochelyinae									
Chrysemys picta	n/a	3	7	9	1	3	7	1	31
Deirochelys reticularia	n/a		2	4	1		4		11
Graptemys babouri	LR (1994)			3			2		5
Graptemys caglei	VU (1994)			3					3
Graptemys ernsti	LR (1994)			3					3
Graptemys flavimaculata	EN (1994)			3			2		5
Graptemys geographica	n/a		1	5	2		2		10
Graptemys gibbonsi	LR (1994)			3					3
Graptemys nigrinoda	LR (1994)			3			1		4
Graptemys oculifera	EN (1994)			3			1		4
Graptemys ouachitensis	n/a			2	1		1		4
Graptemys psuedogeographica	n/a		4	7	1	1	7		20
Graptemys pulchra	n/a			3			1		4
Graptemys sabinensis	n/a		1	1					2
Graptemys versa	LR (1994)			3			1		4
Malaclemys terrapin	LR (1994)		2	5	1	1	4	1	14
Pseudemys alabamensis	EN (1994)						2		2
Pseudemys concinna	n/a		2	1	1	1	10		16
incl. *P. mobilensis*	-								
Pseudemys gorzugi	LR (1994)						1		1
Pseudemys nelsoni	n/a						1		1
Pseudemys rubriventris	LR (1994)					2	1	1	4
Pseudemys texana	n/a						2		2
Trachemys adiutrix	EN (1994)								**0**
Trachemys callirostris	n/a						1		1
Trachemys decorata	VU (1994)			1			4		5
incl. *Chrysemys decorata*	-								
Trachemys deucssata	n/a						3		3
Trachemys dorbigni	n/a						1		1

Trachemys emolli	n/a						1		1
Trachemys gaigeae	VU (1994)						1		1
Tracehemys ornata	n/a		1				1		2
Trachemys scripta	LR (1994)		16	17	2	4	11	1	51
incl. *Chrysemys scripta*	-								
incl. *Pseudemys scripta*	-								
Trachemys stejnegeri	LR (1994)			1			5		6
Trachemys terrapen	VU (1994)			1			5		6
incl. *Chrysemys terrapen*	-								
incl. *Ttrachemys felis*	-								
Trachemys venusta	n/a						1		1
Trachemys yaquia	n/a						1		1
Emydinae									
Actinemys marmorata	*		2	12	2	1	6		23
incl. *Emys marmorata*	-								
incl. *Clemmys marmorata*	VU (1994)								
Clemmys guttata	VU (1994)		4	6		1	6		17
Emydoidea blandingii	LR (1994)		4	7		3	3	2	19
incl. *Emys blandingii*	-								
Emys orbicularis	LR (1994)	1	3	14	4	5			27
Emys trinacris	n/a			1					1
Glyptemys insculpta	VU (1994)	1	1	7		2	5	2	18
incl. *Clemmys insculpta*	-								
Glyptemys muhlenbergii	*			6			3		9
incl. *Clemmys muhlenbergii*	EN (1994)								
Terrapene carolina	LR (1994)		5	7			5		17
Terrapene coahuila	EN (1994)		1	5			2		8
Terrapene nelsoni	DD (1994)			4					4
Terrapene ornata	LR (1994)		2	7		1	2		12
Platysternidae									
Platysternon megacephalum	EN (1994)			10					10
Testudinoidae (Testuguria)/Bataguridae (Geoemydidae)									
Batagur baska	CR (1994)			2					2
Callagur borneoensis	CR (1994)		1	6					7
Chinemys nigricans	EN (1994)			2					2
incl. *Chinemys kwangtungensis*	-								
Cuora amboinensis	VU (1994)		3	15			3		21
Cuora aurocapitata	CR (1994)			6					6
Cuora flavomarginata	EN (1994)			11			1		12
Cuora galbinifrons	CR (1994)			13			2		15
Cuora mccordi	CR (1994)			5					5
Cuora mouhotii	*		1	11			2		14
incl. *Pyxidea mouhotii*	EN (1994)								
Cuora pani	CR (1994)			6					6
Cuora picturata	n/a			7			1		8
(Cuora serrata) hybrid	n/a			4			1		5
Cuora trifasciata	CR (1994)			10			3		13
Cuora yunnanensis	EX (1994)			2					2
Cuora zhoui	CR (1994)			4					4
Cyclemys atripons	n/a			1			1		2
Cyclemys dentata	LR (1994)			6			5		11
Cyclemys oldhamii	n/a						1		1
Cyclemys pulchristriata	n/a						1		1
Cyclemys shanensis	n/a			2			2		4
Geoclemys hamiltoni	VU (1994)			2			1		3
Geoemyda japonica	EN (1994)			4					4
Geoemyda spengleri	EN (1994)			6					6
Hardella thurjii	VU (1994)			3					3
Heosemys annandalei	*		1	7					8
incl. *Hieremys annandalii*	EN (1994)								
Heosemys depressa	CR (1994)			3					3
Heosemys grandis	VU (1994)			8			1		9
Heosemys spinosa	EN (1994)		3	8			1		12
Kachuga dhongoka	EN (1994)			3					3
Kachuga kachuga	CR (1994)								**0**
*Kachuga trivittata***	EN (1994)						2		2
Leucocephalon yuwonoi	CR (1994)			4					4
Malayemys subtrijuga	VU (1994)		3	12			2		17
Mauremys annamensis	CR (1994)			6					6
Mauremys caspica	n/a		3	9	1	1	5		19
(Mauremys iversoni) hybrid	DD (1994)			4			1		5
Mauremys japonica	LR (1994)			9			1		10
Mauremys leprosa	n/a			4	2		1		7
(Mauremys megalocephala)	n/a			2					2

Chinemys megalocephala; hybrid	-							
Mauremys mutica	EN (1994)		14			5		19
Mauremys nigricans	*		6			1		7
Chinemys nigricans	EN (1994)							
(Mauremys pritchardi) hybrid	DD (1994)		1			1		2
Mauremys reevesii	*	3	24		1	4	2	34
Chinemys reevesii	EN (1994)							
Chinemys megalocephala	EN (1994)							
Mauremys rivulata	n/a		4	2		1	1	8
Mauremys sinensis	*	2	8			2		12
Ocadia sinensis	EN (1994)							
Melanochelys tricarinata	VU (1994)							**0**
Melanochelys trijuga	LR (1994)		7					7
Morenia ocellata	VU (1994)		3			1		4
Morenia petersi	VU (1994)							**0**
Notochelys platynota	VU (1994)		8					8
(Ocadia glyphistoma) hybrid	DD (1994)		1			1		2
(Ocadia philippeni) hybrid	DD (1994)		1			1		2
Orlitia borneensis	EN (1994)	2	8					10
Pangshura smithii	*					4		4
Kachuga smithii	LR (1994)							
Pangshura tecta	*	1	2			4		7
Kachuga tecta	LR (1994)							
Pangshura sylhetensis	*							**0**
Kachuga sylhetensis	EN (1994)							
Pangshura tentoria	*		2					2
Kachuga tentoria	LR (1994)							
Rhinoclemmys annulata	LR (1994)		2					2
Rhinoclemmys areolata	n/a	2	4			1		7
Rhinoclemmys diademata	n/a		2			1		3
Rhinoclemmys funerea	LR (1994)		6			1		7
Rhinoclemmys melanosterna	n/a		2					2
Rhinoclemmys nasuta	LR (1994)		1					1
Rhinoclemmys pulcherrima	n/a	1	5			2		8
Rhinoclemmys punctularia	n/a	1	5			3		9
Rhinoclemmys rubida	VU (1994)							**0**
Rhinoclemmys sp.	n/a		5					5
Sacalia bealei	EN (1994)	1	10			3		14
(Sacalia pseudocellata) hybrid	DD (1994)					1		1
Sacalia quadriocellata	EN (1994)		5		1	1		7
Siebenrockiella crassicollis	VU (1994)	3	7			3		13
Siebenrockiella leytensis	*		2					2
Heosemys leytensis	CR (1994)							
Vijayachelys silvatica	*							**0**
Geoemyda silvatica	EN (1994)							
Testudinidae								
Aldabrachelys arnoldi *Dipsochelys arnoldi*	n/a		3			2		5
Aldabrachelys daudini	n/a					1		1
Aldabrachelys grandidieri	n/a		1			2		3
Aldabrachelys hololissa	n/a					5		5
Astrochelys radiata *Geochelone radiata*	VU (1994)		4	2			1	7
Astrochelys yniphora *Geochelone yniphora*	EN (1994)		4	1				5
Chelonoidis carbonaria *Geochelone carbonaria*	n/a	2	5	2		4		13
Chelonoidis chilensis *Geochelone chilensis*	* VU (1994)	1	3	2		1		7
Chelonoidis denticulata *Geochelone denticulata*	* VU (1994)	2	7	2		4		15
Chelonoidis nigra *Geochelone nigra* incl. *Geochelone elephantopus*	* VU (1994)	1	5	5	3	1		15
Chelonoidis petersi *Geochelone petersi*	n/a -					1		1
Chersina angulata	n/a		1	1				2
Cylindraspis borbonica extinct	n/a					1		1
Cylindraspis indica *Testudo indica* incl. *Testudo graii*	n/a		1			2		3

extinct									
Cylindraspis inepta	n/a			1			1		2
extinct									
Cylindraspis peltastes	n/a			1			3		4
extinct									
Cylindraspis triserrata	n/a			1					1
extinct									
Cylindraspis vosmaeri	n/a			1			2		3
extinct									
Dipsochelys dussumieri	*			5			5		10
Aldabrachelys dussumieri									
incl. *Aldabrachelys gigantea*	-								
incl. *Aldabrachelys gouffei*	-								
incl. *Aldabrachelys ponderosa*	-								
incl. *Aldabrachelys sumeirei*	-								
incl. *Geochelone gigantea*	VU (1994)								
Eurotestudo hermanni	*		1	4	1		3	1	10
Testudo hermanni	LR (1994)								
Geochelone elegans	LR (1994)		1	2	1		1		5
Geochelone elephantopus	VU (1994)			1			2		3
Geochelone platynota	CR (1994)			1					1
Geochelone sulcata	VU (1994)			3			1		4
Gopherus agassizii	VU (1994)	1	3	4	4	6	2	2	22
Gopherus berlandieri	LR (1994)		2	2	1	1	2	1	9
Gopherus flavomarginatus	VU (1994)			1	1	1		1	4
Gopherus polyphemus	VU (1994)	1	3	6	3	2	1	1	17
Homopus aerolatus	n/a						1		1
Homopus boulengeri	n/a			1					1
Homopus signatus	n/a			1					1
Indotestudo elongata	EN (1994)		2	7			1		10
Indotestudo forstenii	EN (1994)			5					5
Indotestudo travancorica	VU (1994)			4					4
Kiniyxs erosa	DD (1994)			1					1
Kiniyxs homeana	n/a		1	1			1		3
Malacochersus tormieri	VU (1994)			5					5
Manouria emys	EN (1994)			6					6
Manouria impressa	VU (1994)			2					2
Psammobates geometricus	EN (1994)					1			1
Psammobates tentorius	n/a			1					1
Pyxis arachnoides	VU (1994)			3					3
Pyxis planicauda	EN (1994)			3					3
Stigmochelys pardalis	n/a		1	14	2		1		18
Testudo gigantea	n/a		1						1
Testudo graeca	VU (1994)	1		6	2		1		10
Testudo kleinmanni	CR (2001)			4					4
Testudo marginata	LR (1994)			6			1	2	9
incl. *T. weissingeri*	-								
Testudo horsfieldii	VU (1994)	1		7	1				9
Agrionemys horsfieldii	-								
Carettochelyidae									
Carettochelys insculpta	VU (1994)			8			1		9
Trionychidae/Cyclanorbinae									
Cyclanorbis elegans	LR (1994)			1					1
Cyclanorbis senegalensis	LR (1994)			2					2
Cycloderma aubryi	n/a			1					1
Cycloderma frenatum	n/a			1					1
Lissemys punctata	LR (1994)		1	6			2		9
Lissemys scutata	DD (1994)								**0**
Trionychinae									
Amyda cartilaginea	VU (1994)			1					1
Apalone ferox	n/a		3	3	1		1		8
Apalone mutica	n/a		1	1	1		1		4
Apalone spinifera	*		3	5	1		1		1
Apalone ater	CR (1994)								0
Aspideretes gangeticus	VU (1994)			1					1
Aspideretes hurum	VU (1994)			1					1
Aspideretes leithii	VU (1994)			1					1
Aspideretes nigricans	EW (2001)			1					1
Chitra chitra	CR (1994)			3					3
Chitra indica	EN (1994)			3					3
Chitra vandijki	n/a			3					3
Dogania subplana	LR (1994)						3		3
Nilssonia formosa	EN (1994)			1					1
Palea steindachneri	EN (1994)			2					2
Pelochelys bibroni	VU (1994)			3					3

Pelochelys cantorii	EN (1994)			3					3
Pelodiscus sinensis	VU (1994)		4	8		1	1		14
incl. *Amyda japonica*	-								
Rafetus euphraticus	EN (1994)			1					1
Rafetus swinhoei	CR (1994)								**0**
Trionx triunguis	n/a			1	1				2
Pleurodira									
Chelidae									
Acanthochelys macrocephala	LR (1994)			1			1		2
Acanthochelys pallidipectoris	VU (1994)			3			1		4
Platemys pallidipectoris									
Acanthochelys radiolata	LR (1994)			1					1
Acanthochelys spixii	LR (1994)		1	1					2
Platemys spixii	-								
Chelodina burrungandjii	n/a			1					1
Chelodina expansa	n/a			3			1		4
Chelodina longicollis	n/a		2	11			3		16
Chelodina mccordi	CR (1994)			1					1
Chelodina novaeguineae	LR (1994)			3					3
Chelodina oblonga	LR (1994)			5			1		6
Chelodina parkeri	VU (1994)			1					1
Chelodina pritchardi	EN (1994)			1					1
Chelodina reimanni	LR (1994)			1					1
Chelodina rugosa	*			7			1		8
incl. *C. siebenrocki*	LR (1994)								
Chelodina steindachneri	n/a			2			1		3
Chelus fimbriata	n/a		1	10			3		14
Elseya bellii	EN (1994)								**0**
Elseya branderhorstii	VU (1994)								**0**
Elseya dentata	n/a			6			1		7
Elseya georgesi	DD (1994)			1					1
Elseya latisternum	n/a			10			1		11
Elseya novaeguineae	LR (1994)			2					2
Elseya purvisi	DD (1994)			1					1
Elusor macrurus	EN (1994)			5					5
Emydura macquarii	*			6			3		9
incl. *E. krefftii*									
incl. *E. signata*	LR (1994)								
Emydura subglobosa	LR (1994)			3					3
Emydura victoriae	n/a			2			1		3
Hydromedusa maximiliani	VU (1994)				1	1			2
Hydromedusa tectifera	n/a			3			1		4
Mesoclemmys dahli	*						1		1
Phrynops dahli	CR (1994)								
Mesoclemmys gibba	n/a			7			3		10
Mesoclemmys zuliae	*								**0**
Phrynops zuliae	VU (1994)								
Rhinemys hogei	*								**0**
Phrynops hogei	EN (1994)								
Phrynops geoffroanus	n/a			3			1		4
Phrynops hilarii	n/a						1		1
Phrynops rufipes	n/a			1			1		2
Platemys platycephala	n/a		3	4			3		10
Pseudemydura umbrina	CR (1994)			4					4
Rheodytes leukops	VU (1994)			4			1		5
Pelomedusidae									
Pelomedusa subrufa	n/a		2	9			4	1	16
Pelusios broadleyi	VU (1994)								**0**
Pelusios castaneus	n/a			1			1		2
Pelusios niger	n/a						1		1
Pelusios sinuatus	n/a			3			2		5
Pelusios subniger	LR (1994)		1	1			3		5
Pelusios upembae	DD (1994)								**0**
Pelusios williamsi	n/a		1	4					5
Podocnemidae (Podocnemididae)									
Erymnochelys madagascariensis	EN (1994)			2			3	1	6
Peltocephalus dumerilianus	VU (1994)			2			3		5
Podocnemis erythrocephala	VU (1994)						1		1
incl. *Podocnemis cayennensis*	-								
Podocnemis expansa	LR (1994)	2	1	5	4		4		16
Podocnemis lewyana	EN (1994)						3		3
Podocnemis sextuberculata	VU (1994)						3		3
Podocnemis unifilis	VU (1994)		1	1		1	7		10
Podocnemis vogli	n/a						3		3
TOTAL		13	170	977	74	47	370	24	1675

Defining Turtle Diversity: Proceedings of a Workshop on Genetics, Ethics, and Taxonomy of Freshwater Turtles and Tortoises
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From Molecules to Organisms: Research Applications of Modern Genetic Tools for Turtle Biology and Conservation

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ABSTRACT. – Molecular methods are a powerful complement to traditional field practices in illuminating the evolution and ecology of turtles. We illustrate how standard approaches such as DNA sequencing and microsatellites have, and will continue, to shed light on numerous aspects of turtle biology. We also forecast the impact of selected technologies such as amplified fragment length polymorphisms (AFLPs), small interspersed nuclear elements (SINEs), single nucleotide polymorphisms (SNPs), bacterial artificial chromosome libraries (BACs), and gene expression techniques. These tools continue to help clarify the demography, population genetics, phylogeography, and phylogenetics of turtles, and hold great potential to elucidate developmental and life history questions in this group. This additional insight, allowed by molecular methods, may ultimately aid in the preservation of turtles by honing conservation and management efforts.

KEY WORDS. – turtles, small interspersed nuclear elements (SINEs), single nucleotide polymorphisms (SNPs), bacterial artificial chromosome libraries (BACs), gene expression, microsatellites, mitochondrial DNA, amplified length polymorphisms (AFLPs)

Molecular genetic techniques have allowed invaluable insight while complementing traditional field and morphological studies fundamental to ecological and evolutionary questions. Especially welcomed by the turtle community are non-invasive methods that have been a great tool in elucidating demographics (Pearse et al., 2001), mating systems (Pearse et al., 2002), and **phylogenetic** and **phylogeographic** (see glossary for highlighted words) relationships (Spinks et al., 2004; Spinks and Shaffer, 2005; Krenz et al., 2005; Parham et al., 2006b) in these long-lived, wide-ranging, and often highly endangered taxa. In addition to the well-established and widely used genetic methods, emerging techniques will allow studies of genome-wide variation and gene expression, thereby accessing some important questions in turtle biology. Implementing such technologies has the potential to revolutionize our ability to address ecological and evolutionary questions in turtles, including adaptation, longevity, and sex determining mechanisms, and this information will ultimately be useful in conservation efforts.

This review is intended to highlight the capabilities and limitations of traditional and emerging molecular techniques while emphasizing their utility in studies of conservation, evolution, and ecology of turtles. We show how standard approaches such as DNA sequencing and **microsatellite** analysis have, and will continue, to shed light on numerous aspects of turtle biology (see Fig. 1), and we also forecast the impact of a selected few new techniques such as **bacterial artificial chromosome** libraries and **microarrays**.

MOLECULAR MARKERS

Mitochondrial Genes and Genomes

Mitochondria are small organelles found in the cytoplasm of eukaryotic cells that possess their own **genomes** that encode products crucial to cellular adenosine triphosphate (ATP) production. The typical vertebrate mitochondrial (mt) genome is a circular, haploid genome (ca. 16,500 base pairs) that contains 37 **genes** (Boore, 1999). Because the mt genome is usually transmitted maternally, and generally lacks recombination, it is inherited as a single **locus** (Avise, 2004). These features, along with a relatively high mutation rate, make sequences from the **mtDNA** locus ideal for many kinds of evolutionary studies (Fig. 1).

Bowen et al. (1989) and Lamb et al. (1989) were the first workers to apply mtDNA data to chelonian questions, using variation in mtDNA to assess phylogeographic structure in *Chelonia mydas* and *Gopherus agassizii*, respectively. The first complete mt

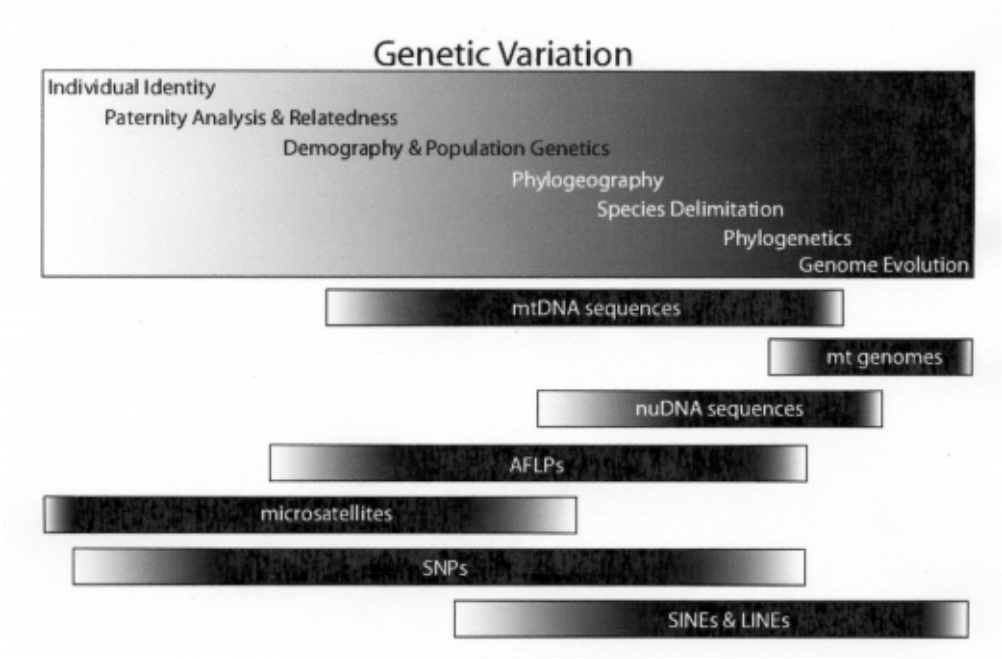

Figure 1. Diagram representing the continuum of genetic variation exhibited in biological systems, from the smallest amount of molecular differences (light) distinguishing conspecific individuals to the greatest amount of genetic divergence (dark) seen between phylogenetically distant taxa. Particular fields of inquiry within ecology and evolutionary biology typically deal with only a subset of this gradient of genetic variation and therefore only certain markers will be appropriate for such fields. The text elaborates on technical and logistical aspects of each tool's utility within this context. Technological advances may enable each class of molecular markers to span beyond the boundaries illustrated here, which show where markers are well-suited (dark) or of limited utility (light).

genome sequenced from a turtle (*Pelomedusa subrufa*) was used to assess the phylogenetic position of turtles relative to other amniotes (Zardoya and Meyer, 1998), while the first study to use mt genome data exclusively in turtles examined the phylogenetic relationships of a small group of Old World tortoises including *Testudo*, *Indotestudo*, and *Malacochersus* (Parham et al., 2006b).

Applications of mtDNA

Demography and Population Genetics. — Mitochondrial DNA has been widely used to study processes that determine the geographic distribution of genetic diversity within and among populations. Early comparisons of population genetic structure in mtDNA and nuclear markers performed in *Chelonia mydas* were landmark studies in demonstrating how sex-biased gene flow in turtles could be inferred from such data (Karl et al., 1992, FitzSimmons et al., 1997a,b). Beyond elucidating the current spatial distribution of genetic variation, mtDNA has been used in other vertebrate systems to examine change in genetic diversity and population structure through time. Because of its high copy number, mtDNA remains the most probable source of genetic population signature from ancient specimens.

Phylogeography. — Since mtDNA is haploid, maternally inherited, and possesses a rapid mutation rate, it should track recent population splitting events with higher fidelity than a single nuclear marker under many biologically plausible scenarios (Moore, 1995; Hickerson and Cunningham, 2005). Thus, mtDNA gene regions have been the most widely used molecular markers to reconstruct population histories and assess phylogeographic structure in turtle species (Fig. 2; e.g., Starkey et al., 2003; Spinks and Shaffer, 2005).

Species Identification and Forensics. — Because multiple copies of the mitochondrion exist in each cell, mtDNA analysis can be particularly useful in identifying the taxonomic or geographic origin of otherwise unidentifiable or poor quality samples (e.g., cooked meat, egg shells, carapace, blood smears, feces). For example, Hsieh et al. (2006) sequenced sections of cytochrome *b* to identify *Kachuga tecta* from poorly stored shells, helping the Council of Agriculture in Taiwan positively document violations of CITES regulations. In another case, Roman and Bowen (2000) used mtDNA to assess whether turtle meat in southeastern U.S. markets was harvested from legitimate sources (e.g., unprotected species). This study showed that even alligator meat was being sold as turtle and led the authors to coin the name "mock turtle syndrome" (Roman and Bowen, 2000).

Mitochondrial DNA can be used in conjunction with other datasets, including either morphological or nuclear molecular markers, to identify hybrid individuals. This approach has recently shown that numerous specimens purported to represent rare and endangered turtle species were actually hybrid individuals from the pet trade (Parham et al., 2001).

Phylogeny. — As mentioned above, mtDNA is particularly amenable to genealogical reconstruction and several features of mt genomes suggest that entire mt genomes are especially well suited for chelonian phylogenetics. First, be-

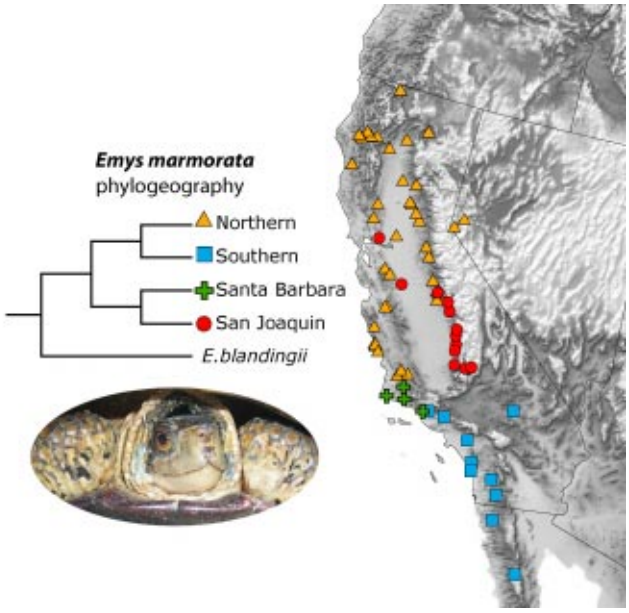

Figure 2. Phylogeography of *Emys* (or *Actinemys*) *marmorata* in western North America (Spinks and Shaffer, 2005). Employing both mtDNA and nuDNA markers and integrating phylogenetic and population genetic analyses in *marmorata* highlights the value of phylogeographies in assessing the evolutionary and biogeographic history of turtle taxa. The phylogeographic approach is also useful in revealing spatial patterns of genetic diversity and setting management priorities. Relationships between the four major mtDNA lineages their geographic distribution follow Spinks and Shaffer (2005). Photo by James Parham.

cause the order and content of genes is highly conserved within mitochondria (Boore, 1999), with no **introns** and spacer DNA, these genomes are easy to align and provide thousands of homologous characters for phylogenetic analyses (Fig. 3). Such large datasets are often necessary to resolve uncertain or incorrect relationships recovered from smaller DNA fragment data (Cummings et al., 1995; Zardoya and Meyer, 1996). For example, small mtDNA and nuclear (nu) DNA fragments initially suggested that turtles are nested within the Diapsida, rather than sister to all other reptiles (Hedges, 1994; Platz and Conlon, 1997). However, the placement of turtles within the Diapsida varied and support for any particular hypothesis was weak. Subsequently, complete mt genome data firmly placed turtles sister to archosaurs (Zardoya and Meyer, 1998; Kumazawa and Nishida, 1999), an arrangement later corroborated by multiple nuclear loci (Hedges and Poling, 1999). Second, the mt genome is composed of loci with vastly different rates of evolution, from hypervariable third positions in cytochrome *b*, to nearly immutable stems in 16S r**RNA**. Thus, mitogenomic datasets should provide resolution at various phylogenetic levels. Third, mt genome features and gene rearrangements have been shown to be valuable phylogenetic characters. Because gene rearrangements are typically rare, and generally considered irreversible, such characters are assumed to be virtually **homoplasy** free (Boore et al., 1995; Boore, 1999).

Genome Evolution. — Complete mt genome sequences may provide a better understanding of how genomes evolve. Because mt genomes are so small, aspects of evolution that are difficult to study in the nuclear genome may be tractable in the mt genome (Boore, 1999). A number of obvious questions include whether certain gene rearrangements or **duplications** occur more commonly than others, whether rates of rearrangements or duplications correlate with rates of sequence evolution, and whether novel mitochondrial features correspond to particular physiological or life history attributes (Boore, 1999). For instance, through sequencing the mt genome of *Platysternon* a partial mt genome duplication was inferred (Parham et al., 2006a). Further, the hypothesized loss of supernumerary genes excluded the duplicated control region, a characteristic that is relatively unique in metazoans and sets *Platysternon* apart from most other extant species of turtles (Parham et al., 2006a).

Marker Development. — Comparing levels of variation across entire mt genomes for a few focal taxa allows researchers to identify the most appropriate mtDNA markers for their research. Additionally, obtaining mt genome data for the major turtle clades would facilitate the quick development of other mtDNA markers in related taxa.

Data Collection and Analyses. — Total DNA is generally isolated by one of a number of conventional DNA extraction methods (Maniatis et al., 1982), often from tissue samples taken nondestructively in the field. Targeted mtDNA gene regions can then be amplified via **PCR** (Saiki et al., 1988) using a wide array of **primers** known to work in turtles (Engstrom et al., 2007). Long PCR can be employed to amplify large portions of the mt

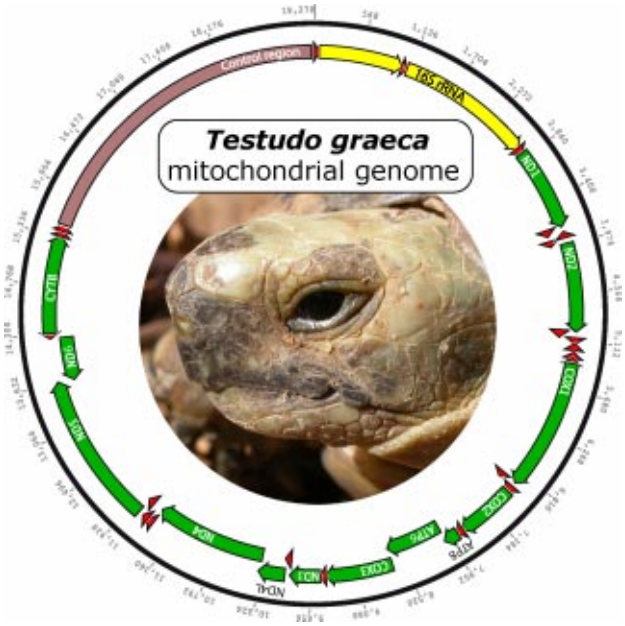

Figure 3. Diagram of the complete mitochondrial genome of *Testudo graeca* (GenBank NC 007692; Parham et al., 2006a). The mt genome of *T. graeca* is representative of turtles and most other vertebrates in overall size, gene content, and gene order. This circular, haploid genome is less than 20 kb with a single control region (brown), two rRNA genes (yellow), thirteen protein coding genes (green) and 22 tRNA genes (red). The mt genome is drawn to scale; arrows indicate the direction of transcription of loci (5' to 3'). The utility of specific loci and the primers used to capture those markers are reported by Engstrom et al. (2007). Photo by James Parham.

genome, or rolling circle amplification (RCA; Dean et al., 2001; Hawkins et al., 2002) can be used to generate entire mt genomes. These amplified products are then used in sequencing reactions that label the four DNA nucleotides (Sanger et al., 1977) and run on an automated machine that reads the labeled nucleotides.

Obtaining sequences from mtDNA gene regions is relatively inexpensive and efficient compared to the cost and time involved in collecting equivalent data from other classes of markers with similar properties and applications. Furthermore, primers that readily amplify many mtDNA regions in turtles are common (Engstrom et al., 2007), and rapid screening of variation in small mtDNA regions for large numbers of individuals is now possible (Avise, 2004; DeSalle and Amato, 2004).

However, collecting entire mt genome data is non-trivial, and the most efficient way to gather these data may be in collaboration with genome centers that have perfected the rapid and efficient acquisition of whole mt genomes (e.g., Joint Genome Institute, Lawrence Berkeley National Laboratory).

Limitations. — Because the haploid mt genome does not recombine, and is uniparentally inherited, all the genes in the mt genome effectively represent a single, linked locus. Thus, analyses based on multiple mt genes or entire mt genomes only represent single-locus estimates of **demography**, population history, or phylogeny. Likewise, inferences made from mtDNA to delimit species or reconstruct population or species histories should be made judiciously. Mitochondrial DNA phylogenies represent the branching history of mitochondria (**gene tree**) and may not track organismal history (**species tree**) flawlessly (reviewed in Avise, 2004), and thus should be corroborated by other evidence (Morando et al., 2004; Avila et al., 2006).

Phylogeographic studies of single species or closely related taxa focus on how evolutionary processes operate in natural populations (Avise, 2000), but the abundance of these studies in the literature belie the difficulties inherent in reconstructing complex demographic histories. The possible influences of past migration, divergence in isolation or with gene flow, and population bottlenecks or expansions, are difficult to disentangle (Knowles, 2004). Furthermore, introgression, incomplete lineage sorting, and natural selection may confound phylogeographic studies (Funk and Omland, 2003). As a consequence, mtDNA phylogeographic analyses have become increasingly sophisticated to accommodate these limitations (Ballard and Whitlock, 2004; Templeton, 2004; Hickerson and Cunningham, 2005).

For deeper phylogenetic questions the rapid rate of mtDNA evolution may lead to homoplasy between deep clades, possibly misleading even mitogenomic estimates of phylogeny (Curole and Kocher, 1999). However, some mtDNA and mt genome data collected in turtles (e.g., Feldman and Parham, 2002; Parham et al., 2006b) do not appear to have suffered from **saturated** data, and newer-mixed model methods of analysis (Yang, 1996) may accommodate and correct for at least some mutational history that can mislead phylogenetic inference (Engstrom et al., 2004; Brandley et al., 2005). Conversely, gene duplications and rearrangements that should be useful for deep level questions in mt genome data (Boore, 1999) may be rare or **autapomorphic**. Parham et al. (2006b) examined both sequence variation and mitogenomic features among major chelonian clades and found that gene rearrangements and duplications were restricted to a single taxon, and thus were phylogenetically uninformative.

Finally, nuclear sequences of mitochondrial origin (**numts**) are relatively common among metazoan taxa (Zhang and Hewitt, 1996; Bensasson et al., 2001) and can seriously mislead any genetic analysis if these nuclear copies of mtDNA are mistaken for authentic mtDNA (Zhang and Hewitt, 1996). Nuclear **pseudogenes** of mtDNA have been reported in turtles (Stuart and Parham, 2004; Spinks and Shaffer, 2007), and may be relatively common.

Future Applications. — We suggest several directions for the future use of mtDNA gene regions and mt genomes in chelonian biology. Most likely, these directions will include a combination of both mitochondrial and nuclear data to address a range of conservation and evolutionary questions. For example, maternally inherited mtDNA and paternally inherited nuDNA markers (Y or W linked loci in taxa with genotypic sex determination) could be used in combination to estimate sex-specific gene flow or other demographic parameters and assess population genetic structure. Already mtDNA and single copy nuDNA sequences have been used in concert to tackle phylogenetic and phylogeographic questions (Krenz et al., 2005; Spinks and Shaffer, 2005; Parham et al., 2006a). Mitogenomic data, in particular, might be combined with nuclear sequences to build a robust chelonian phylogeny that could provide the backbone for any comparative turtle study. Rapidly evolving mtDNA sequences can also be used in combination with **Mendelian** markers, such as microsatellites and single nucleotide polymorphisms (**SNPs**; Morin et al., 2004) for studies of metapopulation structure and conservation genetics (Pearse et al., 2006a). Because mitochondria play an essential role in cellular metabolism, investigations of the molecular evolution of the mt genome may convey metabolic and respiratory adaptations in turtles (e.g., Doiron et al., 2002). Lastly, we anticipate the expanded use of mtDNA, and molecular markers in general, to address broader ecological and evolutionary questions in turtles (Stephens and Wiens, 2003, 2004), and the extension of these findings into conservation biology.

Nuclear Markers: Sequences, Microsatellites, and AFLPs

In contrast to the mitochondrial genome, the nuclear genome contains a huge number of coding and non-coding regions (introns and intergenic spacers) that are subject to different mutation mechanisms and rates (Li, 1997). Thus the nuclear genome offers a virtually unlimited set of potential markers that are informative across the entire range of phylogenetic divergence and can be applied to a wider array

of questions relative to mtDNA data, including studies of adaptive radiation, life histories, hybridization, species delimitation, and phylogenetic inference (including estimates of divergence times [Near et al., 2005]; Fig. 1 summary; Avise 2004; but see Zhang and Hewitt, 2003, for a description of all technologies as well as an in-depth implementation guide).

Nuclear Gene Regions

Applications of Nuclear Gene Data

Phylogeography. — Karl et al. (1992) first used nuclear markers (**restriction digests** of anonymous loci) to estimate global population structure of the marine turtle *Chelonia mydas*, but few subsequent nuclear-sequence based phylogeographic studies have been published on freshwater turtles (FitzSimmons et al., this volume). Phylogeographic studies of Galapagos tortoises *Geochelone* (Caccone et al., 2004) and the western pond turtle *Emys [= Actinemys] marmorata* (Spinks and Shaffer, 2005) have met with limited success because of extremely low variation of nuclear relative to mitochondrial gene regions. This may be a general limitation of most nuclear gene regions accessible by conventional technologies (Zhang and Hewitt, 2003), but newer methods of screening for large numbers of anonymous nuclear loci will likely offer multiple unlinked high resolution markers for future phylogeographic studies (see Jennings and Edwards, 2005, for a recent example in birds).

Species Delimitation. — Allozymes have been used for species delimitation in turtles (e.g., Georges et al., 2002), but the use of nuclear DNA sequence for this purpose is not as prevalent in vertebrates as is the use of mitochondrial markers. Nuclear ribosomal DNA (e.g., internal transcribed spacer [ITS] DNA) has been used for studies of species classifications in algae and nematodes (LaJeunesse, 2001; Chilton, 2004), and primers are available for ITS in turtles although it is not known if this marker would provide an appropriate amount of variability for species delimitation in Testudines (Engstrom et al., 2007).

Phylogeny. — Combining nuclear gene regions can resolve the Testudines phylogenetic history, which has long terminal branches that may result in ambiguous placement of some taxa (Bergsten, 2005). In fact, multiple nuclear genes have been informative about the placement of turtles within Amniota (Hedges and Poling, 1999; Iwabe et al., 2005), single loci have been useful for resolving relationships within Testudines (Fujita et al., 2004), and a combination of nuclear and mtDNA indicated the separation between Platysternidae and Chelydridae (Krenz et al., 2005)

Data Collection and Analyses. — While data are collected using the same protocols as those used for mtDNA gene regions (conventional extraction from field preserved tissue samples; conventional PCR followed by automated sequencing of product, albeit **cloning** of the product is sometimes needed before sequencing), the efficiency of collecting sequence data is usually more difficult because primers are often borrowed from published sequences developed for other vertebrate groups, and must then be optimized. For example, the nuclear gene *glyceraldehydes-3 phosphate dehydrogenase* (*GADPH*) used by Spinks and Shaffer (2005) was amplified with primers originally developed for birds (Friesen et al., 1997).

Furthermore, duplicated regions can cause problems for phylogenetic and other analyses if one is unknowingly comparing **paralogs** and not **orthologs** (Li, 1997). Therefore, for every nuclear marker developed, a Southern hybridization should be performed to confirm single-copy status as Fujita et al. (2004) did when introducing the nuclear intron R35 as a phylogenetic tool in turtles. Lastly, heterozygosity is more prevalent in nuclear regions and generally requires cloning to resolve.

Limitations. — While nuclear sequences offer many advantages, there are multiple processes operating with greater frequency than in mtDNA and these may confound both data collection and various types of analyses. Additional efforts may be needed to evaluate possible influences of recombination, **codon** bias, duplicated genes, rate variation across characters or taxa, compositional bias, and heterozygosity (Maddison, 1997; Posada and Crandall, 2002; Harris, 2003), and to resolve gene tree – species tree discordance (Edwards and Beerli, 2000; Hudson and Turelli, 2003).

Future Directions. — Data analyses are improving as increasingly refined methods become available for mixed-model analyses (Yang, 1996) of multi-gene data sets for phylogenetic inference (Pagel and Meade, 2004), delimiting species (Sites and Marshall, 2003), and phylogeographic analyses (Templeton, 2004). In addition, steps to improve the alignment process of multigene data sets over a large number of taxa have been taken. At shallower levels of divergence, network methods will become more sophisticated (Cassens et al., 2003, 2005), as will demographic modeling under more biologically plausible scenarios (Hickerson and Cunningham, 2005; Jennings and Edwards, 2005).

Lastly, many conservative vertebrate nuclear gene primers will become applicable for turtle studies, as a result of the National Science Foundation's "Assembling the Tree of Life" (ATOL) initiative (Crandall and Buhay, 2004). Of the 22 projects supported by the ATOL project, five focus exclusively on vertebrates (including birds, archosaurs, amphibians, squamate reptiles, and cypriniform fishes; see: http://ucjeps.berkeley.edu/bryolab/ATOL/?page=projects), and other eukaryote projects are also likely to discover at least some highly conserved regions that can be employed in turtle studies.

Microsatellites

Microsatellite markers, or simple-sequence repeat (SSR) loci, are hyper-variable, iterated 1-6 bp motifs that have been detected in virtually all organismal genomes (Ellegren, 2000; Li et al., 2002). SSR markers constitute a subset of codominant Mendelian loci that are usually assumed to be selectively **neutral** and randomly distributed across **eu-**

chromatic genomes, although these assumptions are not always met (Li et al., 2002). **Alleles** originate by a number of non-conventional mutation mechanisms, which alter the number of repeat units in the alleles segregating at a given locus, and are easily distinguishable based on the length of a PCR product amplified with primers flanking the SSR region. The ease of screening polymorphisms, along with the typically high variability (up to 50 alleles per locus in a population; DeWoody and Avise, 2000), has made SSRs the markers of choice for a wide array of analyses (Avise, 2004; see Bennett, 2000, for in-depth technical review).

Applications of Microsatellites

Paternity Analysis and Relatedness. — Microsatellites are frequently used to estimate individual fitness and some components of breeding structure, in the context of single vs. multiple paternity, and the related phenomenon of sperm storage (both relevant issues in freshwater turtles; see Pearse and Avise, 2001; Pearse et al., 2002, 2006b; for examples).

Demography, Population Genetics, and Phylogeography. — Microsatellites have been utilized to estimate population genetic and phylogeographic structure, especially with regard to the identification of genetic 'breaks' – **Evolutionarily Significant Unit (ESU) or Management Unit (MU)** boundaries – an issue of crucial importance in the design of conservation strategies for endangered species (reviews in Fraser and Bernatchez, 2001; Frankham et al., 2002; Moritz, 2002; DeSalle and Amato, 2004; see Pearse et al., 2006a, for a turtle example). Similarly, microsatellites have been recently used to: (1) evaluate the genetic consequences of recent population bottlenecks (Waldick et al., 2002; Kuo and Janzen, 2004), (2) estimate population sizes and between-deme migration rates (Nichols and Freeman, 2004), (3) estimate natal dispersal (Berry et al., 2004), (4) detect hybridization (see Burns et al., 2003, for an example in turtles), and (5) provide identification in wildlife forensics (Avise, 2004).

In a recent study, Fritz et al. (2005) used microsatellite repeat motifs as primers to amplify ISSRs (inter-simple sequence repeats). By using the repeat motif as a primer, these authors were able to amplify a suite of bands particular to different *Testudo* species. This DNA "fingerprinting" method, in conjunction with mitochondrial DNA, was then used to reject the uniqueness of *Testudo weissingeri* (Fritz et al. 2005).

Data Collection and Analyses. — Microsatellite loci are typically isolated via enrichment probes, which requires less time than previous methods of clone screening (see Fischer and Bachmann, 1998). Once markers are developed, DNA is typically amplified using fluorescently labeled primers, following basic PCR protocols (Sites et al., 1999; Valenzuela, 2000). Amplification reactions are analyzed by electrophoresis, and alleles are scored based on the length of fragments (**electromorphs**). High-throughput genotyping can be achieved by using different fluorescent dyes to label loci with non-overlapping allele sizes in a single automated run or in a single PCR reaction (both terms are referred to as "multiplexing").

Limitations. — Although they are widely utilized, microsatellites have well-characterized limitations as well. From a theoretical perspective, Estoup et al. (2002) reviewed the relationship between SSR mutation models and homoplasy of alleles and showed that basic assumptions about mutational mechanisms are often not met in real data sets. In addition, although SSR loci are generally assumed to be neutral, evidence implicates their influence in clearly non-neutral processes such as genetic disorders (Li et al., 2002), and Vasemägi et al. (2005) found nine microsatellites linked to **Expressed Sequence Tags (EST)** that deviated significantly from neutral expectation. There is also selection against repeat motifs that would produce **frame shifts** in coding regions (e.g., di- and tetra-nucleotide repeats; Metzgar et al., 2000). The nonrandom distribution of SSR loci in the genome further suggests that assumptions of neutral evolution are not always accurate.

Operationally, using primers from related species can affect results by leading to alleles that are shorter, and less variable due to differential amplification (i.e., **ascertainment bias**; Hutter et al., 1998; Amos et al., 2003), or that do not amplify at all (so-called "null" alleles; Zenger et al., 2003). The strength of these effects is directly proportional to the genetic distance from the species for which the loci were originally isolated (Shepherd et al., 2002; Wright et al., 2004).

Future Directions. — Recent studies showed that electromorph (fragment length) data alone tended to underestimate population divergence (Balloux et al., 2000; Fisher et al., 2000). By sequencing microsatellite alleles one can infer mutational processes directly, by checking for consistency in repeat motif for each population sampled (see Engstrom et al., 2007). Electromorph data accompanied by sequence information can paint a more accurate picture of population differentiation (Colson and Goldstein, 1999).

In addition, a variety of approaches have been developed that are appropriate for the evaluation of population genetic structure in non-equilibrium conditions, which are the most likely demographic scenarios for declining species (see reviews by Pearse and Crandall, 2004; Manel et al., 2005). Further, recent empirical studies have shown the advantages of using multiple complementary analytical methods, including equilibrium and non-equilibrium methods, to detect different signals in genetic datasets (e.g., Lemaire et al., 2005; Pearse et al., 2006a). Lastly, combining Mendelian markers and mtDNA sequences can result in powerful inferences about demographic and meta-population structure and histories (FitzSimmons et al., 1997b; Pearse et al., 2006a).

Amplified Fragment Length Polymorphism (AFLP)

The amplified fragment length polymorphism (**AFLP**) method (Vos et al., 1995) is a relatively new technique for generating genome-wide estimates of genetic variation. The AFLP method combines two older molecular techniques (RFLP and RAPD) to quickly and inexpensively produce

numerous, variably sized DNA fragments. Profiles of these anonymous DNA fragments represent multilocus **genotypes** that can be used to answer questions at a wide range of biological scales. For example, these DNA profiles can be used to create distance matrices for phylogenetic reconstruction (Koopman, 2005), estimate population structure (e.g., Mock et al., 2002) or as DNA fingerprints to assess parentage (Mueller and Wolfenbarger, 1999).

The AFLP method has seen little use in animal systems (Bensch and Akesson, 2005) and has not been applied in any chelonian studies, but shows great promise. In the absence of a well-characterized genome, the AFLP method can provide a useful assessment of genome-wide variation in turtles. While there are some limitations inherent to AFLP data, the low cost and ease of use indicate that AFLPs could become valuable markers in a wide range of turtle ecological and evolutionary studies.

Applications of AFLPs

Demography and Population Genetics. — Population genetic studies of animal populations currently emphasize the use of microsatellite or mtDNA sequence data to provide estimates of population structure, gene flow, historical bottlenecks and other population parameters. While rapid rates of evolution in both microsatellites and mtDNA provide investigators with a workable pool of genetic variation to analyze, in most systems, these markers offer a limited view of overall genetic variation in the genome. Furthermore, microsatellite development can be a time consuming and expensive endeavor that generally yields less than 20 usable loci (Zane et al., 2002). AFLPs, on the other hand, can quickly and inexpensively provide a more complete view of genome-wide variation for estimates of population level processes (Bensch and Akesson, 2005). Although AFLP data cannot be scored for more than two alleles at any locus (1/0), or used to detect **heterozygotes**, as they are dominant, rather than co-dominant markers, the shear number of polymorphic AFLP loci can be as powerful as a several variable microsatellite loci in providing highly resolved genotypes (Gerber et al., 2000). Thus, AFLP data may be a useful molecular tool for tackling demographic questions.

Phylogenetics, Phylogeography, and Species Delimitation. — AFLP data can be used to reconstruct the branching history of populations and taxa. Phylogeographic surveys using AFLPs, in particular, could quickly identify cryptic lineages that may represent important management units or cryptic species and could identify regions of hybridization and backcrossing (Miller, 2000). AFLP data can be used directly in the character-based method of maximum parsimony, or compressed into distance matrices to be analyzed with clustering methods for phylogeographic and phylogenetic analysis (Koopman, 2005). However, adequate resolution of many phylogenetic questions may require hundreds or even thousands of AFLP loci (Albertson et al., 1999).

AFLP data could also be used in conjunction with other markers to delimit species when such datasets show concordant geographic boundaries exhibited by distinct populations, similar and separate evolutionary histories, or any other number of empirical situations (reviewed in Sites and Marshall, 2003, 2004).

Adaptive Variation. — The AFLP method may be useful in helping ecologists and evolutionary biologists explore the relationship between genotype and phenotype in chelonian systems. Specifically, researchers may find sets of AFLP loci that are correlated with particular phenotypes of interest. Furthermore, researchers can identify loci that are under selection by comparing the observed distribution of genetic variation at AFLP loci with expectations based on neutral processes (Wilding et al., 2001; Campbell and Bernatchez, 2004).

Data Collection and Analyses. — Following standard DNA extraction/isolation (Maniatis et al., 1982), genomic DNA is cut with two restriction enzymes (Vos et al., 1995) creating hundreds of thousands of DNA fragments. To reduce the number of DNA fragments to a more manageable amount, two rounds of PCR (Saiki et al., 1988) are used to selectively amplify a small portion of the DNA fragments originally cut by the restriction enzymes (Vos et al., 1995). This final pool of amplified DNA fragments can be fluorescently labeled and read on any standard fragment analysis machine (e.g. ABI 3100).

Raw AFLP data consist of a number of DNA fragments of varying lengths. Each fragment is assumed to represent a unique locus in the genome. Individuals that possess a specific fragment have one allele (1), while those that lack the same fragment have the alternative allele (0). Thus, with AFLP data, heterozygotes cannot be distinguished from **homozygotes**, and each locus is assumed to be diallelic in this **dominant** marker system.

Once all the presence/absence data have been collected, any number of analyses can be conducted, though some assumptions regarding Hardy-Weinberg equilibrium may be required to calculate heterozygosity for certain population genetic measures (Bensch and Akesson, 2005).

Limitations. — The chief limitation of AFLP data is that they are not **codominant**. Furthermore, each AFLP locus contains relatively little information (presence or absence of an allele). Thus codominant markers, especially those with high allelic diversity such as microsatellites, actually contain far greater resolving power per locus than AFLPs. To compensate for this deficiency in information content per locus, an AFLP data set must contain many more loci than most other marker systems (Bensch and Akesson, 2005).

Because AFLPs are dominant markers, Hardy-Weinberg equilibrium must be assumed in order to estimate population genetic parameters. Thus AFLPs cannot be used to independently test for violations of Hardy-Weinberg equilibrium in population genetic surveys (Bensch and Akesson, 2005).

Another potential problem of AFLP data is the anonymous nature of loci. Each DNA fragment is assumed to represent a unique locus. Yet, size homoplasy has occurred among smaller DNA fragments (Vekemans et al., 2002) and could seriously confound analyses of genetic diversity

(Vekemans et al., 2002) and phylogenetic reconstruction (Koopman, 2005).

Feasibility. — The quick set-up time involved in collecting AFLP data (often less than a week) and low cost of processing samples make AFLP the most inexpensive and efficient method of assessing genome-wide variation. The AFLP technique can be used without any prior knowledge of a turtle's genome to provide genotypes for a large number samples at a sizeable number of loci. Furthermore, the genetic profiles are highly reproducible across different laboratories. The protocols and equipment required to collect AFLP data should be found in any reasonably equipped molecular genetic laboratory. Moreover, the laboratory procedures have been further streamlined and standardized by a number of commercially manufactured kits.

Regardless of the cost and ease of data collection, AFLP data are not a panacea. Depending on the question and the system, other markers that do not suffer from the same major limitations of AFLP data may be more appropriate (e.g., microsatellites, DNA sequences).

Future Directions. — Future applications in which AFLP are likely to be used include further refinement of our understanding of the genome and its expression into the phenotype. For example, applications include gene mapping such as in **QTL studies** (though crosses are required) and in the discovery of SNPs for chelonian studies (every informative AFLP potentially contains an informative SNP). Another very interesting application of AFLPs is in the study of gene expression. Instead of using whole genomic DNA as the original template for the procedure, **cDNA** generated from expressed mRNA can be used. Using AFLP on cDNA allows researchers to generate global gene expression profiles that may be associated with a particular phenotype, developmental stage, or tissue type of interest (Bachem et al., 1996, 1998).

The AFLP method has not yet been used by turtle biologists, yet the technique can easily be applied to any number of ecological and evolutionary questions. AFLP data should be used judiciously in providing complementary datasets for the estimation of demographic and population genetic parameters (better addressed with microsatellites and SNPs), and in the reconstruction of phylogeographic and phylogenetic histories (better addressed with mtDNA and nuDNA sequence data), but may be ideal in delimiting species (Fig. 1). Further, sex specific AFLPs can be used indicate the heterogametic sex in species with cryptic sex chromosomes (Griffiths and Orr, 1999). Regardless, the low cost and ease of use suggest that the AFLP method shows great potential as a powerful molecular tool for turtle biologists.

MARKERS ON THE HORIZON

Short and Long Interspersed Nuclear Elements (SINEs and LINEs)

An exciting and relatively new set of molecular markers are **SINEs and LINEs** – repetitive elements with no obvious function that are dispersed randomly throughout the genomes of most eukaryotes (reviewed by Weiner et al., 1986; Shedlock and Okada, 2000; Shedlock et al., 2004).

LINEs (long interspersed nuclear elements) are **transposons** that contain some of the basic machinery of a retrovirus, including a gene for reverse transcriptase (RTase), but do not have the ability to cross-infect cells or individuals. LINE length is variable, but most typically spans a 1-7 kb (Kidwell, 2002). LINEs maintain their integrity within the genome, functioning as self-replicating elements that proliferate randomly by a copy-and-paste process involving an RNA intermediary. Those that lose that function progressively lose their identity through mutation, but are replaced elsewhere within the genome by the continued proliferation of functional elements within the same family. Thus families of functional LINEs reside within the genome, their relationship to each other determined by sequence **homology**. Such families may be longstanding, spanning much or all of the vertebrate radiation, for example. Relatively few LINEs are functional at any one time and the frequency of their propagation is governed by the intranuclear and intragenomic environment (Weiner, 2002).

SINEs (short interspersed nuclear elements) are also transposable but are much shorter elements (70-500 bp), lack a gene for RTase, and rely on a functional corresponding LINE to provide the RTase to support their proliferation (Kajikawa and Okada, 2002). SINEs too form families that are maintained by the balanced processes of gain through replication of functional elements (requiring a functional RTase recognition site) and loss through random mutation. SINEs have attracted particular attention because of their manageable size and because they usually are represented by $>10^4$ copies per SINE type per vertebrate genome (Kazazian and Moran, 1998; Shedlock et al., 2004).

Applications of SINEs and LINEs to Chelonian Biology

Demography and Population Genetics. — Where a SINE family is still actively proliferating, their utility extends beyond phylogenetics into population biology (Batzer et al., 1996). For example, insertion or lack of insertion of the *Alu* element for 100 loci provided sufficient polymorphism to estimate diversity among and within human populations (Watkins et al., 2003). Sampling of many SINE loci, which are dispersed across the genome, enabled inferences regarding the genetic distance to ancestral states and population subdivision with very little sampling error. In fact, resampling methods regard 50 loci to be sufficient for future studies (Watkins et al., 2003).

Species Delimitation and Phylogenetics. — Other applications where an unambiguous marker is of value may be found in species identification for forensics where the SINEs are fixed at the level of species. In addition, SINEs have been successful at identifying the close relationship between humans and chimps and discovering previously undetected radiations in cichlid species of the east African rift lakes (Shedlock et al., 2004).

For turtle biologists, SINEs are significant because their first application to reptiles is a study of the phylogenetic relationships among geoemydid turtles (Fig. 4; Sasaki et al., 2004). SINEs and LINEs yield phylogenetic information at three levels. The first is at the sequence level, providing information on the phylogeny of the element, and thus the species (or clade) that carries it, in the same way as for any nuclear marker. The second is at the level of the presence or absence of representatives of SINE or LINE families in the entire genome, from which we can infer their origin in a common ancestor to the exclusion of other taxa of interest. The third level involves their use as positional markers, where an individual SINE element at a particular locus can be identified by developing primers for its unique flanking region and scored as present or absent.

It is as positional markers that SINEs and LINEs come into their own as phylogenetic markers. They have a suite of remarkable properties straight out of the notebook of the pioneer of phylogenetic systematics, William Hennig (1966):

(a) They are discrete and recognizable DNA elements that proliferate through the nuclear genome by a copy-and-paste mechanism, rather than the cut-and-paste mechanisms of DNA transposons, so the history of their proliferation can be uncovered using traditional approaches to phylogenetic reconstruction using sequence data. This said, it is the presence or absence of the SINE or LINE at a specific location that is the novel character, and the sequence data internal to the marker is secondary to this.

(b) They insert into the genome essentially at random (though there is a slight bias in favor of AT rich regions) so the probability of homoplasy arising through a second insertion at the same site is remote. This assertion has been supported by an intensive study of the *Alu* SINE of primates (Roy-Engel, 2002). In any case, such an insertion does not overwrite the first and so if a duplicate insertion were to occur it would most likely be easily detected when the element and flanking region are sequenced, unless substantial deterioration has occurred (e.g., Ray et al., 2005).

(c) SINE or LINE insertion at a particular locus is considered irreversible, because flanking regions are created upon insertion and provide a signature of the insertion even in the unlikely event that the element "jumps" out of the previous spot.

(d) Absence of a SINE or LINE is accompanied by a robust positive control, so that there are three possibilities – amplification product contains a SINE or LINE, amplification product does not contain the SINE or LINE, no amplification because of mutation at the primer site. An absence of a SINE or LINE is an absence, provided there is successful amplification.

(e) The marker has clear homology across taxa and the polarities of the character states are unambiguous (i.e., the absence of the SINE or LINE and flanking regions at a specific location in the genome is unambiguously the ancestral state, and presence is unambiguously the derived state).

Once found, a SINE or LINE inserted at a specific location is a nuclear marker that is essentially free of homoplasy, which can occur only through introgression of a SINE element following interspecific hybridization or through gene-tree/species-tree disparity (Hillis, 1990; Miyamoto, 1999). Phylogenetic characters with these attributes potentially offer a treasure trove for systematic biology (Shedlock and Okada, 2000).

Data Collection and Analyses. — The human genome contains nearly 1.5 million SINEs (Shedlock et al., 2004). This abundance in genomes makes isolating and characterizing new SINEs relatively easy given the large playing field. Main approaches for SINE isolation include screening a genomic library with a probe which is designed for a particular SINE family of interest or sequencing of large chunks of the genome and using this information to predict the presence of SINEs

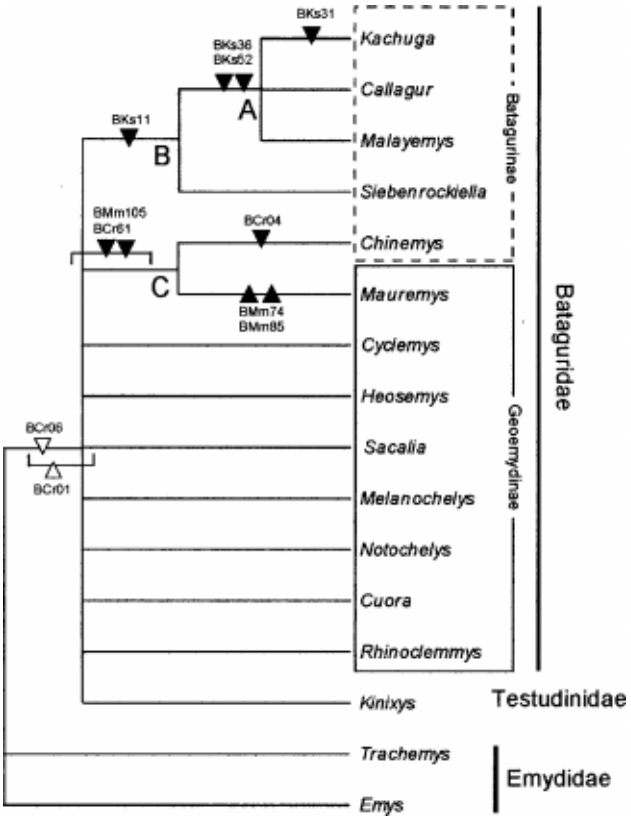

Figure 4. Phylogenetic relationships revealed from among the 16 species of Testudinoidea by the application of the SINE approach (after Sasaki et al., 2004). Arrowheads denote the insertion of tortoise polIII class SINEs. SINE insertions into loci BCr01 and BCr06 reveal a close relationships between Bataguridae (= Geoemydidae) and Testudinidae to the exclusion of the Emydidae. SINE insertions at loci BKs36 and BKs52 indicate that *Kachuga (= Pangshura) smithii*, *Callagur (= Batagur) borneoensis*, and *Malayemys subtrijuga* form a monophyletic group (clade A) within the *Batagur* complex. An insertion at BKs11 locus suggests monophyly of *Siebenrockiella* with the above three species (clade B). A close relationship between *Chinemys reevesii* (recently changed to *Mauremys reevesii*; Feldman and Parham, 2004; Spinks et al., 2004) and *Mauremys mutica kami* is suggested by SINE insertions at loci BCr61 and BMm105 (clade C).

computationally (Shedlock et al., 2004). Recent approaches also design a primer identical to the conserved polymerase III promoter and use PCR or genomic screening to isolate the new SINE (Shedlock et al., 2004; Borodulina and Kramerov, 2005). Additional information on the characterization of new SINEs and the use of SINEs in systematics is briefly summarized by Shedlock et al. (2004).

Limitations. — Limitations on the utility of SINES derive from the limited life of a particular retroelement as an identifiable and recoverable sequence in the genome, or the limited life of the flanking sequence that enables homology of the positional element to be established. Once inserted, the actual SINE and its flanking regions deteriorate over time through mutation to the point that they are not detectable. This aspect diminishes the utility of the technique beyond 50-150 million years (Shedlock and Okada, 2000; Shedlock et al., 2004).

A second limitation is that unlike sequence data, one cannot expect SINEs to provide information across all nodes of a phylogeny. This was evident in the turtle study (see Fig. 4; Sasaki et al., 2004) where despite considerable effort, solid information was obtained on only four nodes in the cryptodire phylogeny. This situation will improve as options for screening SINEs improve, such as when genomic information on target taxa increases, leading to greater numbers of loci. There may also be novel approaches on the horizon for targeting specific phylogenetic hypotheses at the time of screening for informative SINEs (e.g., screening after selected subtractive hybridization).

A third limitation is that these positional markers, informative as they may be for resolving tree topology, cannot be used for determining branch lengths or dating divergences. For this we must rely upon comparisons of the actual DNA sequences of the SINEs or LINEs or comparisons of sequence data from the flanking regions (Del Pozzo and Guardiola, 1990; Shedlock and Okada, 2000).

Future Directions. — Overall, the future of SINEs for resolving important questions in turtle phylogeny looks bright. Their abundance in the genome provides the opportunity to address the second limitation by identifying a very great number of SINE markers, so that resolution will ultimately be obtained across most or all of the important nodes in the turtle phylogeny, within the 50 million year window. This development will be greatly assisted by improved knowledge of the turtle genome, either through the development of selected BAC libraries (see below) and ultimately, one hopes, a turtle genome project. In the meantime, novel approaches to focusing attention on particular problematic nodes may be possible by combining subtractive hybridization with screening.

Single Nucleotide Polymorphisms (SNPs)

Single nucleotide polymorphisms (SNPs) are rapidly becoming valuable genetic markers because they are the most common source of variation among individuals – a SNP occurs on average every 300-500 bases in the human genome (Zhao et al., 2003). SNPs are generated by point mutations in the genome when one nucleotide is replaced by another (i.e., substitution). This definition is often broadened to include single-base **indels** where an extra base is inserted or deleted during the replication of DNA. In principle, SNPs can have as many as four alternative allelic states (i.e., adenine, guanidine, cytosine, or thiamine), but because of the rarity of the mutations (i.e., 10^{-8} to10^{-9} mutations per generation per site), tri or tetra-allelic states are virtually non-existent within related taxa. As a result, SNPs are often referred to as bi-allelic markers (Vignal et al., 2002).

SNPs are informative genetic markers for population, conservation, and evolutionary genetic studies when the least abundant allele reaches a frequency of 1% or greater in the population; a threshold that eliminates sites that are variable because of infrequent sequencing errors (Kwok and Gu, 1999; Wakeley et al., 2001; De La Vega et al., 2002). These traits of ubiquitous variation and high utility have recently been harnessed and applied to studies of evolutionary genetics, population genetics, hybridization, and wildlife forensics, and show great promise in chelonian studies (Bensch et al., 2002; Stickney et al., 2002; Belfiore et al., 2003; Aitken et al., 2004; Seddon et al., 2005).

Applications of SNPs

Paternity and Relatedness. — The typically bi-allelic character of SNPs creates a requirement for many more loci to be genotyped for parentage and relatedness studies compared to multi-allelic markers. It is estimated that 60 maximally informative SNPs would be required to provide the same level of paternity exclusion and estimates of relatedness as 14 microsatellite loci with an average allelic diversity of 9.5 (Krawczak, 1999). This number jumps to 100 when SNPs are only 20-30% heterozygous, a level closer to actual diversity (Krawczak, 1999; Glaubitz et al., 2003) However, once the SNP assays are developed, they could potentially produce better quality data and be more cost effective and efficient than microsatellites.

Demography, Population Genetics, and Phylogeography. — Similar to paternity studies, a larger number of SNP versus microsatellite loci are required for estimates of genetic diversity, gene flow, effective population size, and other population parameters (Morin et al., 2004). The extra effort required in isolating loci is offset by the better resolution obtained from SNPs with fewer assumptions compared to microsatellites (Brumfield et al., 2003). Estimates of population parameters such as $\mathbf{F_{ST}}$ are likely to be more accurate with SNPs than with microsatellites and AFLP because (i) their mutational mechanisms are relatively well characterized, (ii) they may be less subject to homoplasy, (iii) they potentially have a reduced interlocus sampling variance as a consequence of the large number of loci available for analysis, and (iv) they have less within-population variation which guards against artificially low F_{ST} estimates (Kalinowski, 2002; Nicholson et al., 2002; Brumfield et al., 2003).

Species Delimitation. — In the identification of cryptic species and hybridization, the application of SNPs has been extremely successful using a relatively small numbers of markers. For example, Belfiore et al. (2003) developed three SNPs that were 90% effective in discriminating among four species of Eurasian vole (*Microtus)*, a success rate higher than at the nuclear *p*53 locus (DeWoody, 1999). Further, a study of willow warblers used a single SNP to distinguish two subspecies that could not be differentiated using mitochondrial or microsatellite markers (Bensch et al., 2002). SNPs, owing to their codominance, are also effective in the detection of hybridization and introgression (see Saetre et al., 2001, for a more extensive description).

Wildlife Forensics. — SNPs will have immense utility in wildlife forensics, especially when poaching evidence consists of samples that may yield degraded DNA, such as a fragment of carapace or meat from a market (Sarkar and Kashyap, 2003). SNPs can be genotyped from degraded DNA more efficiently than any other nuclear marker due to the small size of the DNA fragment being amplified, and diagnostic SNPs can be found at all taxonomic levels. For example, a SNP assay has been developed in the Chinook salmon which can identify the country of origin of the fish and thereby aid in the regulation of the trade (see Smith et al., 2005).

Evolutionary Genetics. — SNPs are useful in the detection of adaptive variation and in drawing inferences on population demographic history. Signatures of natural selection in populations have been detected with comprehensive SNP maps (Akey et al., 2002; Nielsen, 2005). The abundance of SNPs in the genome and their potential for rapid genotyping makes them ideal markers to map Quantitative Trait Loci (QTL). QTL studies seek to identify the loci responsible for phenotypic traits, and can thereby shed light on how continuous traits are inherited in populations and the influence of evolutionary processes on these traits (Slate et al., 2002; Weinig and Schmitt, 2004; Slate, 2005).

Data Collection and Analyses. — SNP discovery (ascertainment) is successful through BACs and other previously sequenced information (Marth et al., 2001; Saetre et al., 2001; Bensch et al., 2002; Primmer et al., 2002; Belfiore et al., 2003; Brugmans et al., 2003; Nicod and Largiader, 2003). Alternative strategies include the reduced representation shotgun approach (RRS) in which DNA from many individuals are mixed together and subjected to restriction enzyme digestion. The resultant fragments are incorporated into plasmids. This plasmid library is then sequenced, and overlapping sequences are screened for SNP polymorphisms (Altshuler et al., 2000). More recent approaches identify the SNPs causing a polymorphism in the allelic states of an AFLP marker (i.e., present and absent states) and convert these into SNP markers (Nicod and Largiader, 2003; similar to the approach in Fitzpatrick and Shaffer, 2004). SNPs may be discovered in restriction enzyme recognition sites, the primer annealing sites or within an AFLP fragment itself (Bensch et al., 2002; Brugmans et al., 2003). These techniques appear to be promising for the discovery of large numbers of SNP loci in non-model organisms.

A diverse array of methods is available for genotyping SNPs (reviewed extensively by Kwok, 2001). Well established methods such as PCR-RFLP and PCR-SSCP can be used to cost effectively genotype SNPs using standard laboratory equipment (Doi et al., 2004). High throughput can be achieved using newer methods such as primer extension (Li et al., 1999), hybridization (Howell et al., 1999), and invasive cleavage assays (Lyamichev et al., 2000). For rapid genotyping, these techniques can be modified to be used in microarray platforms (Dalma-Weiszhausz and Murphy, 2002; Heller, 2002; Jenkins and Gibson, 2002). In addition, a rapid form of sequencing by DNA synthesis, pyrosequencing, which produces light upon the incorporation of the correct nucleotide, can be advantageous over hybridization applications (Ronaghi, 2001).

Limitations. — Discovery of SNPs without ascertainment bias has been a major limitation to their use. Biases can be reduced by the selection of a large panel of individuals for screening and inclusion of loci that display lower levels of variability (Nielsen, 2000; Schlötterer and Harr, 2002). Statistical analyses to correct for biases in SNP data are also available, such as maximum likelihood models (Kuhner et al. 2000; Nielsen, 2000).

Future Directions. — SNPs are emerging as markers with the potential for wide ranging applications in chelonian biology. For some applications, only a few SNP loci are required, such as for species diagnostics and identifying the geographic origins of individuals; applications which will be particularly useful in wildlife forensics to monitor trade of turtle populations worldwide. Furthermore, an exciting application of SNPs will be to study adaptive evolution in turtles to gain insights on how phenotypic traits are inherited and how they might respond to changes in environmental conditions. However, ascertainment bias remains a major hurdle that must be overcome before SNPs can be reliably used in population and evolutionary studies.

GENOMICS AND GENE EXPRESSION

Comparative Genomics: BACs

The comparative genomics of vertebrates is still in its infancy, with only a single avian genome sequenced thus far and no non-avian reptile genomes. Still, the time is ripe for forays into the comparative genomics of turtles. In particular, the recent availability of a Bacterial Artificial Chromosome (BAC) library from a painted turtle (*Chrysemys picta*) paves the way for a scaling-up of genomic inquiries in turtles and for amassing large-scale information on the structure and organization of turtle genomes. BAC libraries are a means by which very long pieces of DNA (100,000 – 200,000 base pairs) can be isolated (cloned), sequestered from the remainder of the genome, and studied in detail. Although the sequencing of a turtle genome may still be several years away, BAC libraries will provide a useful resource in the interim for studying turtle genomics.

Applications of BACs to Chelonian Biology

Phylogenetics, Marker Development, and Genome Evolution. — Why is cloning long pieces of DNA of interest to the evolution, comparative genomics, molecular evolution, '**evo-devo**' and systematics of turtles and other vertebrates? First, the sheer size of pieces of DNA that can be isolated, and eventually sequenced (Harris and Murphy, 2001), means that a vast number of molecular characters are immediately available for study. Unlike short pieces of DNA amplified by PCR, BAC library inserts provide contiguous stretches of DNA, thereby permitting a more seamless integration of molecular systematics and genome evolution (Pollock et al., 2000; Edwards et al., 2005). The large amounts of contiguous sequence data (contigs) that can be characterized from BAC libraries in a phylogenetic context will yield new insights into phylogenetic analysis of genomic data. For example, Thomas et al. (2003) used contigs of the region containing the cystic fibrosis gene constructed from BAC clones to sequence up to 1.8 Megabases (Mb) of DNA from several mammals and a chicken. Such sequence data yielded abundant retroelements (such as SINEs and LINEs), which in turn serve as cladistic characters in a phylogenetic analysis (Shedlock and Okada, 2000). The alignment of these sequences also revealed numerous non-coding regions that were highly conserved between species, providing a detailed view of regions that could be important for regulation and genome stability. Another recent example of large-scale discovery of phylogenetically important information comes from comparative genomic studies of the coelacanth and bichir, a primitive ray-finned fish and basal tetrapod, respectively (Chiu et al., 2004; Noonan et al., 2004). BAC libraries have proved indispensable for identifying and characterizing multigene families that are important for development. For instance, one can examine conserved and nonconserved regions in these genes in comparison to sequenced organisms to elucidate possible noncoding, conserved function regions. Also, data mining and sequence analysis from BAC libraries can identify expansions or contractions of gene families (Miyake and Amemiya, 2004). In addition, BAC libraries ultimately pave the way for whole-genome sequencing as they can effectively serve as waypoints in the landscape of the genome.

BAC libraries are an efficient means for understanding broad-scale patterns within genomes without actually sequencing entire genomes, or even targeted regions. Features such as the frequency of various families of repetitive elements and retroelements, as well as base compositional and **isochore** structure, can be mined from BAC libraries in several ways. First, one can conduct hybridizations of specific genes or repetitive elements to filters on which the entire BAC library is spotted. In this way, one can obtain an estimate of the frequency of the particular element in the genome of the interrogated species. Second, one can survey the basic structure of a vertebrate genome by conducting a BAC-end sequencing survey, which consists of amassing thousands of sequence reads from the ends of BAC clones, primed using sequence in the BAC **vector**. Such a survey has been conducted for *Chrysemys picta*, leading to several new insights into turtle genome evolution and phylogeny (Shedlock et al., unpubl. data). An important spin-off for such BAC-end sequencing surveys (or end-sequence surveys of any type of clone) are the release of large numbers of loci for use in phylogeography and molecular systematics (Hare, 2001; Matthee et al., 2001; Jennings and Edwards, 2005). With any given clone-end read, one can immediately design primers for PCR for studying within- or between-species variation (see nuclear gene region section), although the phylogenetic resolution of any given sequence must be determined empirically. The loci typically recovered in a clone-end sequencing survey are noncoding and often 'anonymous' in so far as they do not match any known loci to a significant degree when data bases such as Genbank are interrogated by a BLAST or other similarity search. Such loci are of maximal interest to multilocus phylogeography because they will tend to be more variable than currently available markers.

Gene Function and Expression. — Another key feature of BAC clones is that they contain not only coding regions of genes but all of the noncoding, regulatory regions that affect gene expression. Such regions are frequently found immediately upstream of genes but can often be tens of kilobases away from the coding regions themselves. Thus BAC clones can often capture in a single clone all of the regulatory elements and coding regions of a particular gene or gene family. This makes possible a variety of experiments in developmental biology, such as expression of turtle gene families in developing embryos of model species to examine developmental consequences of gene misexpression (Heintz, 2000; Takahashi et al., 2000; Carvajal et al., 2001; Giraldo and Montoliu, 2001).

Chromosome Mapping. — Individual BAC clones are large enough to be visualized after fluorescent labeling and hybridization to metaphase chromosomes, as in the **FISH** technique (fluorescent in-situ hybridization). By contrast, individual PCR products and many cDNA clones are too short to use in FISH and often do not provide a reliably strong signal of hybridization to a target sequence on the chromosome. Thus BAC clones provide a critical tool for locating genes and gene families on turtle chromosomes. Such studies will provide an important window into turtle chromosome evolution. Thus far the resolution provided by hybridization of whole chicken chromosomes to turtle **karyotypes** has revealed that entire chromosomes found in turtles may have remained intact in birds, as in the example provided by hybridization of a chicken Z chromosome to the entirety of a turtle chromosome 5 and no other chromosomes (Graves and Shetty, 2001). However, even such evidence leaves room for small-scale genomic **translocations** that might not be detected using whole-chromosome hybridizations, particularly of single-copy regions that may not provide an amplified fluorescence signal. BAC clones are ideal for such purposes. Preliminary investigations of chromosome assignments

of several turtle genes are underway, particularly genes in the sex determining pathway and sex-linked genes (N. Valenzuela, unpubl. data; D. Janes, unpubl. data).

Future Directions. — Overall the prospects for robust comparative genomics of turtles are very strong provided that the appropriate resources are made available to the wider community. Ideally all such resources should be available through distribution centers; the *Chrysemys picta* BAC library and additional technical information can be accessed through the Joint Genome Institute (JGI) web site on available BAC libraries: http://evogen.jgi.doe.gov/second_levels/BACs/Our_libraries.html.

Using a 'community genomics' approach and the appropriate genomic resources, large scale projects in animal molecular systematics can be tackled by coordinated efforts of single-PI laboratories as well as genome centers, even for problems that are not of high priority to genome centers (Edwards et al., 2005). In fact, efforts are underway to identify SNPs and sequences that amplify across turtles from the *C. picta* BAC library, an endeavor that will make many more genetic markers available (Thomson, Edwards, and Shaffer, unpubl. data). Such large-scale genomics approaches are a natural complement to typical molecular systematics endeavors utilizing PCR, and will forge an even tighter link between genome evolution and systematics. With judicious use of the available BAC library, and continued attention to production of important genomic resources for turtles, the turtle community could lead the way in these important new directions.

Gene expression: cDNAs, ESTs, RT-PCR, Microarrays, Functional Assays, and RNAi

Firmly linking an organism's genotype to its phenotype is one of the most important, yet, ambitious goals of molecular genetics. Technological advances are now allowing for researchers to dissect at a molecular level fundamental questions, such as how organisms react to different environments and what contributes to morphological diversity among species. A useful starting point for such molecular studies is to profile gene expression. That is, outlining where the gene is expressed (i.e., what tissue or cell), when the gene is expressed (developmental stage, environment, season, etc.), the degree to which a gene is expressed relative to other genes or other treatments (treatment is used here to refer to a developmental stage, tissue, and environmental condition, etc.), and finally, what happens when the gene is purposefully over-expressed or physically turned off. Indeed, recent advances in assessing gene expression have allowed biologists to pinpoint the genetic basis of major evolutionary transitions (e.g., limblessness in snakes, Cohn and Tickle, 1999) and even adaptive traits contributing to species radiations (e.g., beak depth and length in Darwin's finches, Abzhanov et al., 2004, 2006).

Turtles may serve as an excellent system in which to analyze a wide array of biological phenomena, such as temperature-dependant sex determination, cold tolerance, and shell development, in a genetic and genomic context. Thus, we review technologies that allow researchers to profile gene expression.

The Candidate Gene Approach

The candidate gene approach is one where a gene shown to perform a particular function in model systems is examined for a similar role in non-model organisms. For example, several genes known to be involved in the sex determination pathway of mammals and birds were profiled in turtles and may have important roles in temperature-dependent sex determination (Spotila et al., 1998; Kettlewell et al., 2000; Place et al., 2001; Loffler et al., 2003; Murdock and Wibbels, 2003a,b; Place and Lance, 2004; Valenzuela et al., 2006; Valenzuela and Shikano, 2007). Candidate genes have also lead to a greater understanding of shell and body plan development in turtles (Gilbert et al., 2001; Loredo et al., 2001; Vincent et al., 2003; Ohya et al., 2005). Interestingly, examination of Hox gene expression, major controllers of anterior-posterior body axis in development, in *Pelodiscus sinensis* showed definite discrepancies in the way turtles, as opposed to mammals and birds, build their body (Ohya et al., 2005). The candidate gene approach is a relatively inexpensive way to discover expression pattern and level differences among lineages and treatments and can be imagined to help unravel the several turtle queries like the ones outlined below.

Future Directions. — Convergent evolution in head shape of the bigheaded turtle, *Platysternon megacephalum*, and the alligator snapping turtle, *Macrochelys temminckii*, could be explored using the same genes that partly control beak dimensions in Darwin's finches (Abzhanov et al., 2004, 2006 [bone morphogenetic protein–4 and calmodulin]) or molecular genetic effects of inhabiting polluted, fragmented landscapes could be assayed through examining levels of typical stress response genes (Evron et al., 2006; Grisaru et al., 2006; Song et al., 1991 [i.e. acetylcholinesterase and the glucocorticoid receptor]) in turtles living in degraded versus relatively pristine habitats. Although the candidate gene approach is extremely valuable, the opportunity to profile expression of thousands of genes in nonmodel organisms is becoming rapidly accessible through complementary techniques, some of which have actually been implemented in a turtle system (Kuraku et al., 2005; Storey, 2005).

Complementary DNA (cDNA) and Expressed Sequence Tags (ESTs)

Full-length cDNAs are DNA copies of messenger RNA (mRNA) transcripts created by a process called Reverse-Transcriptase PCR (**RT-PCR**; capable of reverse transcription up to about 20kb; Fig. 5). As DNA is inherently more stable than RNA, cDNA provides a way to keep a "library" of the organism's tissue/condition-specific transcriptome cloned into **plasmid** vectors (circular pieces of bacterial or phage DNA; detailed in Becker et al. [2003]). Expressed Sequence Tags (ESTs)

are generally created by one sequencing reaction from a cDNA clone, range between 200-800 nucleotides long, and provide a snippet of data with which one can identify genes that are being expressed in a certain treatment (Holloway et al., 2002). This technique allows for the relatively cheap, fast generation of large amounts of transcript data which can be an invaluable resource for studies of evolution and development. As of August 2007, over 45 million of these snippets from a variety of organisms and treatments were available through the national EST repositories (dbEST and Unigene databases from the National Center for Biotechnology Information [NCBI]).

Applications of cDNA and ESTs

Gene Discovery and Identification. — First developed in 1991 for use in human gene discovery, ESTs are one of the most useful tools for gene identification (Adams et al., 1991; Wolfsberg and Landsman, 1997). Since ESTs represent functional mRNA, they provide a gene expression profile from the treatment from which the mRNA was extracted (McCarter et al., 2000). Homologs and functional groups can be identified by comparing novel EST data to data created by other sequencing efforts (Ton et al., 2000). Full sequences of informative cDNAs can then be retrieved by sequencing the entire clone. For example, cDNA library screens were used to identify anoxia responsive genes in *Trachemys scripta elegans* and freeze responsive genes in *Chrysemys picta marginata* (Storey, 2005).

ESTs can also identify similar but unique transcripts of the same gene (i.e., isoforms). When aligned with genomic DNA, ESTs can illuminate splice variants, exon boundaries, and polymorphisms in untranslated regions (Wolfsberg and Landsman, 1997; Ulrich, 2000; Gemünd et al., 2001).

Marker Development. — Phylogenetic and phylogeographic studies are enhanced by the use of multiple, unlinked markers and existing EST projects as well as turtle specific EST projects, can generate primers to accomplish this (Brumfield et al., 2003). Because ESTs are copies of

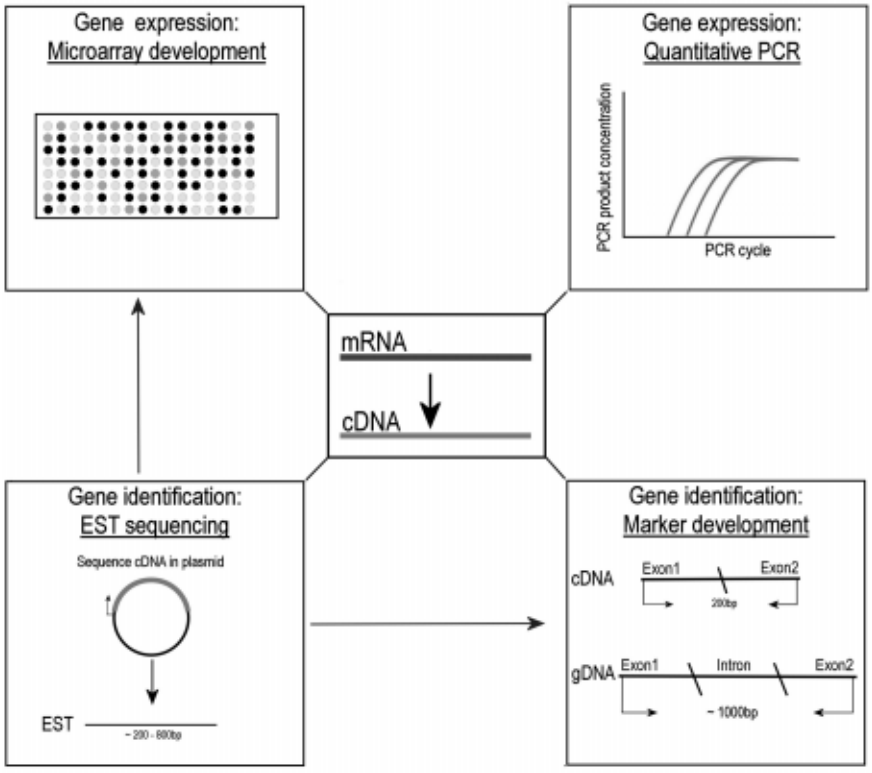

Figure 5. Messenger RNA (mRNA) is specific to the tissue and time it was taken from an organism. mRNA can be reverse transcribed into complementary DNA (cDNA) for a variety of uses. **Bottom Left:** cDNA can be cloned into a plasmid and sequenced to produce an expressed sequence tag (EST). An EST is one sequence read from an end of the cloned cDNA. When many ESTs are sequenced one can obtain a good estimate of which genes are expressed. **Top Left:** EST collections and cDNA clones can both be utilized to create a microarray. Thousands of these DNA seqments, called "probes," are printed on a specially treated glass slide. Each dot on the example slide represents a probe. Shown here is the result of an experiment using two conditions, for instance, warm and cold temperatures during sex determination. The lightest dots represent those probes that are over-expressed in the cold treatment relative to the warm treatment. The darkest dots represent those probes that are over-expressed in the warm treatment relative to the cold treatment. Dots of medium brightness represent genes that are relatively evenly expressed in both treatments. **Top Right:** Quantitative PCR assays gene expression for a limited number of genes. The relative starting concentrations of genes are measured by surveying the quantity of PCR product at each PCR cycle, leading to this method also being called real-time PCR. **Bottom Right:** EST sequencing can provide thousands of potential markers. One way to identify variable markers is to develop primers in two exons of a cDNA and use the same primers to amplify the gDNA. These primers will span an intron, an often variable nuclear region.

mRNA and do not include intron sequences, conserved primers can be anchored in ESTs that might amplify variable introns across disparate turtle groups (Fig. 5; similar to the strategy employed by Fujita et al. [2004] to discover the nuclear intron R35). SNPs can also be revealed by comparing ESTs between closely related species. In fact, empirical data suggest that each EST will contain *at least* one SNP (Brumfield et al., 2003). Lastly, ESTs can serve as probes for BAC libraries and isolate a gene of interest even when used from related species (McCarter et al., 2000).

Evolutionary Genetics. — The shear volume of ESTs generated by gene discovery projects provides a resource for surveys of genomic variation for evolutionary studies. For example, in a gene discovery project for chicken skeletal system development, over 6000 ESTs were generated (Jorge et al., 2004).

Jaramillo-Correa et al. (2001) used quantitative traits and markers developed from polymorphic ESTs to assay for signatures of population differentiation and compared these measures to investigate adaptive evolution (Q_{ST}- F_{ST} comparison) in white spruce, *Picea glauca*. Likewise, 95 microsatellite loci in noncoding regions of transcripts from Atlantic salmon, *Salmo salar*, were identified in EST databases and tested for signatures of selection, despite the fact that microsatellites are thought to evolve in a neutral fashion (Vasemägi et al., 2005). These authors also showed that some microsatellites displayed non-neutral patterns of evolution because they were tightly linked to genes under selection. Following loci with non-neutral patterns of evolution may be especially useful in identifying genes affected by selection in taxa such as turtles that lack extensive genetic resources or linkage maps.

Limitations. — Unlike genomic DNA, which will be relatively uniform in nearly every somatic cell in an organism's body, specific mRNAs will only be found in the specific tissues and during times when the gene is being expressed. This can make the acquisition of specific mRNAs difficult because RNA must be taken from the proper tissue during the treatment or developmental stage of interest, often requiring that specimens be sacrificed in the process. Further, RNA molecules are inherently more unstable than DNA, and in this respect, special care must be taken when handling samples in order to avoid contamination by somewhat ubiquitous RNA degraders called RNAses. Historically, tissue samples would be flash frozen in liquid nitrogen to preserve the molecule's integrity, but preservation products (e.g., RNAlater) have been developed and may provide better results if optimal harvest and storage conditions are not met. In addition, some studies have succeeded in extracting EST quality data from formalin-fixed, paraffin-embedded tissue that may enable the use of preserved specimens for gene identification, but not quantification (Lewis et al., 2001). Difficulties of formalin-fixed nucleic acid extraction have also been prohibitive, however, a successful extraction protocol could help researchers achieve a much deeper phylogenetic sampling, as the extensive turtle collections in museums are often times much easier to access than fresh field specimens.

Once the RNA is extracted and analyzed, researchers must take into account that the transcription of a gene into mRNA does not necessarily mean the mRNA is translated into a protein product. Several molecular mechanisms for silencing and stability reduction of transcripts are known (detailed in RNAi section below), and so upregulation of a gene's transcription is not sufficient to demonstrate that a gene is responsible for a certain phenotype. Functional assays are usually required to confirm that increased mRNA transcription is responsible, or partly responsible, for the phenotype exhibited. Most commonly, functional assays include inoculating the organism with a recombinant viral vector to over-express the gene of interest (detailed in Smith and Sinclair, 2004) or employing RNAi to turn off a gene's expression (see below).

Further, cDNA library construction is subject to contamination by bacteriophages, bias toward smaller, more abundant mRNAs, and is only truly relevant for the tissue, time, and development stage from which it is made (detailed in Becker et al., 2003). These problems are compounded in future applications like EST generation. The use of kits or contracting experienced companies can ameliorate contamination and biases while not contributing excessively to the cost of an experiment (Lucigen, AmpliconExpress, GATC Biotech [typical cost is approximately $6000 to supply a tissue sample and receive a complete library in return]). Also, although as many or as few ESTs can be generated from a cDNA library, typically several thousand sequence reads may be needed for the EST collection to have much utility and justify the cost of a relatively pricey cDNA construction. In such mass sequencing missions, ESTs are typically not checked for sequencing errors because minor mistakes usually do not prevent the matching of the EST to sequences of other organisms for identification (McCarter et al., 2000). This tolerance for inaccuracies may pose a problem if the ESTs are used for applications like SNP detection or protein sequence prediction. Lastly, sequencing of ESTs will result in redundant data. Although over 6000 ESTs were sequenced in the chicken development study mentioned above, only 2329 were unique after clustering (Jorge et al., 2004).

Future Directions. — Large amounts of EST data may be expected for future projects in chelonian gene discovery. Subsequently, thousands of potential phylogenetic markers will be generated by these large EST projects. Bapteste et al. (2002) illustrated the power of ESTs by identifying 123 orthologous genes which helped to resolve important, but previously unclear phylogenetic relationships in amoeboid lineages.

Real-Time PCR

Real-Time PCR (RT-PCR) or Quantitative PCR (Q-PCR) is capable of tracking the amount of amplified DNA produced at each cycle with the use of fluorescent dyes, thus

allowing the quantification of the initial RNA template (Fig. 5). The acronym RT-PCR is also commonly used for Reverse Transcriptase PCR, where an RNA template is converted to cDNA. These are simple, sensitive techniques for quantifying the relative number of gene transcripts in a particular tissue sample and are especially good for use with small sample volumes and discerning between related transcripts. The method is performed by measuring the PCR cycle at which the fluorescently labeled product can first be detected above background fluorescence. If, for instance, more copies of a particular gene are present in condition A than in condition B, then condition A's product will be detected at an earlier cycle, and it may be concluded that the gene being investigated is being up-regulated or over-expressed in condition A.

Gene Discovery and Comparative Gene Expression. — Comparing transcript levels is necessary in gene expression studies and is useful in understanding differences across treatments, individuals, species, etc. Differences in gene expression, along with knowledge of the suspected gene function in other species, can help infer the gene's role. For example, Kettlewell et al. (2000) used Q-PCR to assess expression levels of *Dmrt-1* in developing male and female embryos of a turtle with temperature-dependent sex determination (TSD), *Trachemys scripta*, and discovered that *Dmrt-1* shows higher levels of expression in males than in females. Because *Dmrt-1* also performs male specific functions in a range of taxa, and even has functional and sequence homologs in *Drosophila* and *Caenorhabditis elegans*, these authors suggested that *Dmrt-1* is important for sex determination in *T. scripta*. Further, comparative gene expression profiling has also provided insight into the evolutionary divergence of the developmental network underlying sex determination in turtles, helping identify candidate genes (e.g., *Sf1*) for the role of master TSD switch (Valenzuela et al., 2006; Valenzuela and Shikano, 2007). The utility of quantifying transcripts and making cDNAs for gene discovery can be expanded to identify genetic signatures of local and clinal adaptation, or to understand physiological processes, environmental response, ontogeny, and phylogenetic relationships (Gibson, 2002).

Limitations. — Because QRT-PCR is used to measure difference is transcript number, and because mRNA is so unstable, the QRT-PCR method is extremely sensitive to investigator error. For example, if one sample is fresher or bigger, then it might yield far more transcript copies than another (Wong and Medrano, 2005). To account for some of these issues, investigators should employ a normalization method. A conservative normalization method is to measure multiple housekeeping genes (i.e., genes that are constitutively 'on' and relatively evenly expressed across tissues and individuals along with each sample; Wong and Medrano, 2005).

Further, many genes are modified by transcription and translation machinery differently. That is to say, the same genomic DNA may make multiple mRNAs by using different translation or transcription start and stop sites and different intron splice sites, resulting in different 'isoforms' of the same gene (Weaver, 2005). All of these can contribute to functional differences in the mRNA's role. Therefore, when measuring the amount of mRNA with Q-PCR, primers should be designed that only amplify the functional isoform of interest. Otherwise, the number of transcripts being measured may be artificially inflated because investigators are actually measuring many related but functionally nonsynonymous transcripts.

Microarrays

Since their introduction in 1991 (Fodor et al., 1991), microarrays have been employed successfully to explore relative gene expression in many systems in a high-throughput way. As with Q-PCR and QRT-PCR, microarrays can tell researchers what genes are being expressed, when they are being expressed, where they are being expressed, and to some degree, how much they are being expressed. Microarrays, however, are not limited to small sets of genes of known sequence as in QRT-PCR. Furthermore, microarrays can be adapted to scan tens of thousands of genes, sometimes without knowing their sequence (anonymous cDNA microarrays). This high-throughput ability gives researchers enormous possibilities in understanding phenotypes and interactions between the genotype and the environment.

Traditional microarrays attach 'probes' (Fig. 5; cDNAs, oligonucleotides made from ESTs, genomic sequence, or even BACs, also called 'features') to a pretreated glass slide. These probes then hybridize to 'targets,' which are fluorescently labeled cDNAs made from the mRNA of the treatment of interest, in order to assay gene expression in that treatment. Stoughton (2005) and Holloway et al. (2002) offered comprehensive reviews of this technology, but multitudes of variations on this theme are present in the literature (for alternative microarray techniques see Brenner et al., 2000; Hegarty et al., 2005).

Applications of Microarrays

Gene Discovery. — Due to the large amount of sequence information required to construct the probes for oligonucleotide microarrays, such arrays hold potential for substantial gene discovery. Hybridization of the targets to the probes helps identify genes expressed in a particular treatment as well. For example, microarrays helped to identify a suite of genes responsible for a shift in worker to foraging behavior by honey bees (*Apis mellifera*; Whitfield et al., 2003) and nearly 100 genes that are candidates in social status modifications of cichlids (Renn et al., 2004). Further, a variation on the microarray, microbeads (detailed below), allowed researchers to identify genes involved in shell formation by targeting the carapacial ridge of the Chinese softshelled turtle, *Pelodiscus sinensis* (Kuraku et al., 2005). Another variation on the microarray technology, employing microarrays made from model organisms in-

stead of from the species of interest, was recently used to discover genes associated with cold tolerance in turtles (Storey, 2005).

Evolutionary Genetics. — Population level applications of microarrays can help uncover unique genetic variation or variable responses to environmental pressures in populations that may be extremely difficult to discover via candidate gene approach or other traditional DNA sequencing methods. Because phenotypic diversity without large DNA sequence divergence can still signify local adaptation, changes in gene expression and regulation may be illustrative of overall disparity between species (Schlötterer, 2002). Therefore, gene expression can greatly contribute to unique evolutionary trajectories of populations and species. Microarrays can help uncover these local, possibly adaptive differences in gene expression, thus identifying unique populations that warrant conservation (Turgeon and Bernatchez, 2003).

Limitations. — Microarrays are powerful tools, and require comparable levels of statistical and bioinformatic strength in analyzing the results (Stoughton, 2005). However, even with the help of a strong bioinformatics resource, extracting biological meaning from such a large and complex dataset is an arduous, on-going process (Butte, 2002). For quantification between two treatments, typically multi-chip experiments are required and statisticians are needed to design experiments with maximum power, as factors such as the day the chips were hybridized to the scanner used to view the fluorescence can add greatly to the variability of results. Interesting gene expression results are typically confirmed using QRT-PCR, because variation in microarray output data may be due to these experimental inconsistencies and not genuine gene expression differences (Pinhasov et al., 2004).

In addition, microarrays are expensive in terms of time and money. A start to finish project (i.e., development of an array from EST construction to confirmation of results) may take a lab studying a non-model organism two to four years, even when collaborating with high-throughput labs and computational specialists (detailed Holloway et al., 2002; Bowtell and Sambrook, 2003; Stoughton, 2005).

Future Directions. — A current alternative to using turtle-specific microarrays is to hybridize turtle mRNA to prefabricated microarrays from other model species such as chicken or human. Using nonspecific microarrays can provide an invaluable starting point in gene discovery. In fact, more than twelve genes involved in freeze tolerance and anoxia in *C. p. marginata* were identified by hybridizing turtle mRNA to human microarrays (Storey, 2005). Other cheaper and quicker alternatives to typical microarrays include anonymous cDNA microarrays, focused microarrays, and macroarrays. These arrays usually provide information of similar quality and may be viable alternatives for turtle investigators (Becker et al., 2003; Wurmbach et al., 2003; Hegarty et al., 2005).

Another alternative to using species-specific microarrays is the microbead library. Kuraku et al. (2005) used this technology for gene discovery in shell formation in *P. sinesis.* Here, cDNAs from the carapacial ridge (the region of interest for shell formation) and the thoracic region (a negative control) were "cloned" separately onto microbeads to create two libraries (Brenner et al., 2000). The two libraries were then hybridized together, automatically sorted, and ones that showed higher signals (i.e., higher expression) for the carapacial ridge were sequenced and further identified. Microbeads do not require *a priori* knowledge of sequences or chip layout design and therefore can circumvent the common prohibitive problems of cost, time, and limited tissue samples which may plague other turtle researchers interested in the microarray technology.

RNA Interference (RNAi)

RNA interference (RNAi), a type of gene silencing, can shed light on developmental and adaptive processes by "knocking down" or "knocking out" the expression of particular genes and allows observation of the effects that turning a specific gene down or off has on particular phenotypes (see Mello and Cante, 2004, for more technical information). RNAi takes advantage of an innate defense system used by the organism which degrades double stranded RNA in a sequence specific fashion (Guo and Kempheus, 1995; Fire et al., 1998; reviewed in Cogoni and Macino, 2000; Guru, 2000; Hammond et al., 2001). By introducing foreign dsRNA with sequence identical or nearly identical to the gene of interest, the cell machinery naturally converts them into small RNA (siRNA or microRNA [miRNA]), which target mRNA similar in sequence for degradation and reduced gene expression. Thus, the silenced gene is transcribed but rapid degradation of the transcripts prevents their accumulation and associated function. Small RNA can also down-regulate gene expression by transcriptional silencing, or translational inhibition of mismatched targets (Morris et al., 2004).

Applications of RNAi

Gene Function. — RNAi techniques are well suited for developmental and physiological studies to determine gene function, genetic pathway analysis, and to examine gene redundancy. As such, this technique can be extended to investigate fitness consequences associated with particular genes and gene functions, and thus to examine the genetic variability underlying adaptive variation and adaptive potential in particular taxa. Its main strength derives from being an experimental rather than a correlative approach to identifying genetic variation underlying target phenotypes with important fitness consequences. Although still incipient in its application to vertebrates *in vivo,* this and related techniques hold promise as tools to experimentally study target gene regulation and loss-of-function screening (Cullen, 2005). This derives from the fact that natural miRNA play a key role in regulating vertebrate differentiation and development and thus, RNAi loss of function screening can shed light on the connections and biological functions of biochemical pathways (Silva et al., 2005, Wienholds and Plasterk, 2005). Important functions that have been targeted

for study by this approach in vertebrates include DNA repair, apoptosis, cancer, and response to drugs among many others (Silva et al., 2005; Dickins et al., 2005) that may have significant therapeutic applications. Similar experimental analysis is plausible for biological phenomena relevant to turtles, such as temperature tolerance, courtship and nesting behavior, sex determination, and aging, among others, as this technique allows the experimental identification of those genes that are necessary and sufficient for particular phenotypes.

Limitations and Future Directions. — The main limitation of these methods is the high level of technical expertise and associated costs in time and money, making them unsuitable for the average ecological genetics laboratory (Mello and Cante, 2004, for technical information). RNAi is an increasingly powerful tool to determine gene function and its fitness consequences such that collaborative work should be considered to solve their logistic limitations to answer questions in developmental biology with significant implications for ecology, evolutionary biology, and conservation. A rising number of companies offer RNAi products and services (e.g., Ambion, Integrated DNA Technologies, Invitrogen) that parallel the expanding use of these techniques by research laboratories and derived publications, including a dedicated journal (Journal of RNAi and Gene Silencing) that can be found online.

Conclusion

In conjunction with ecological and behavioral studies, genetic and genomic data offer exciting possibilities for valuable insight into the evolution and biology of chelonians. The techniques presented here have been successful in other systems, and will help to explore how turtles fit into their ecological communities and are affected by their environment. With this understanding, we will be able to more fully appreciate the complexity of these animals and their unique biological interactions, ultimately ensuring more successful conservation efforts.

Currently 37% of the world's 309 turtle species are provided protection under the Convention on International Trade of Endangered Species, and of 181 species listed by the IUCN Red List, 69% are identified as threatened, endangered, or vulnerable (IUCN, 2004). As turtles are species of great conservation concern, additional information gleaned from the fields of molecular ecology and evolutionary biology can be incorporated directly and rapidly into conservation programs. Although this review has provided only a brief description of new technologies, the future implementation of molecular markers will provide great insights into the fundamental biology of turtles and potentially how best to ensure their survival.

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Glossary

AFLP – A genome–wide screen for dominant molecular markers through restriction enzyme digests, followed by selective PCR.

Allele – Different forms of the same gene (AA, aa), or if at a noncoding locus, this can refer to variation in DNA sequence.

Ascertainment bias – Systematic distortion in measuring the true frequency of a phenomenon due to the way in which the data are obtained. One example is illustrated by the empirical observation that microsatellite alleles found in a focal species may not amplify as well in related species and result in null alleles.

Autapomorphic – A derived characteristic exclusive to a given taxon or monophyletic group.

BAC – **B**acterial **A**rtificial **C**hromosome, an *E. coli* plasmid used as a vector to hold large inserts (up to 300,000 bp) of foreign DNA.

cDNA – A DNA copy complementary to a mRNA sequence made by the enzyme reverse transcriptase.

Cloning – A technique which refers to one of two things: 1) inserting a gene from one organism into another via a vector for propagation and investigation, or 2) identification of the location and sequence of a gene which is correlated with a certain phenotype.

Codominant marker – A locus whose alleles are co–dominant, i.e., the genotype of heterozygotes is readily recognizable from that of homozygotes.

Codon – A set of three nucleotides that specifies either termination of translation or a certain amino acid to be incorporated into a growing polypeptide (protein) during translation.

Demography – The study of size, structure, and distribution of populations, and their change over time due to births, deaths, migration, and ageing.

DNA – The material from which genes are made; deoxyribonucleotides linked with phophodiester bonds.

DNA fingerprinting – The use of multiple markers that provide unique DNA profiles for individual identification.

Dominant marker – A gene whose alleles are dominant, i.e., the genotype of heterozygotes is indistinguishable from that of the dominant homozygotes thus impeding the estimation of the heterozygote frequency.

Duplication – Doubling of a DNA sequence such as a dinucleotide repeat within a microsatellite, or as much as an entire gene, chromosome, or genome.

Electromorph – An allele identified by its unique mobility through gel electrophoresis, due to the specific molecular weight and conformation of the allele (e.g., DNA fragment, isozyme).

EST – **E**xpressed **S**equence **T**ags are a short cDNA sequence from one end of an expressed gene used to fish a gene out of the chromosomal DNA by matching base pairs.

Euchromatin – The less condensed part of the chromatin, as compared to heterochromatin; located away from the centromeres and telomeres of chromosomes.

Evo-Devo – A relatively new field called evolutionary developmental biology which takes a comparative look at the genetics behind developing organisms across all taxonomic levels.

Evolutionarily Significant Unit (ESU) or Management Unit (MU) – A group which has reciprocal monophyly in a mitochondrial marker and divergent allele frequencies at a nuclear marker; this designation should be assessed by genealogical concordance within and across genes within the species. This term should designate populations, species, or subspecies considered to have an independent evolutionary legacy. The definition of a manage-

ment unit is similar but does not typically require a large phylogenetic distance and instead only requires that the alleles frequencies be diverging.

FISH – **F**luorescent **i**n **S**itu **H**ybridization, a technique of hybridizing a fluorescently labeled DNA probe to whole chromosomes to determine the physical location of that marker.

Frame shift – A mutation that causes the reading frame of the codons to change; most commonly indels of 1 or 2 bases.

F_{ST} – A genetic measure of population subdivision that describes the variation in allele frequencies among different populations; typically an F_{ST} value of 0.25 is taken as evidence of substantial population differentiation.

Gene – A segment of DNA which performs a specific function such as coding for a protein, specifying a functional RNA molecule, or regulating other functions as in the case of DNA replication, chromosome segregation, or maintenance of chromosome integrity.

Gene tree – Contained within a species tree, it represents a branching pattern of evolution as the gene is passed on to more than one progeny per generation. Processes such as horizontal transfer, deep coalescence, and gene duplication or extinction can result in discordance between gene trees and species trees.

Genome – The complete genetic information contained in an organism.

Genotype – The particular allelic combinations found at a specific locus or loci of an individual (i.e., AA, Aa, aa).

Heterozygous – An individual with two different alleles for the same gene.

Homology – Sharing of characters because of their common ancestry.

Homoplasy – Characters that evolved more than once (e.g., as by convergent evolution) and were not present in the most recent common ancestor of the species sharing them.

Homozygous – An individual carrying two identical alleles of a given gene.

Indel – An insertion or deletion of nucleotides in a DNA segment.

Intron – A segment of noncoding DNA that separates coding parts (exons) within a gene.

Isochore – A region of genomic DNA sequence in which G+C compositions are relatively uniform.

Karyotype – The total set of all chromosomes of a cell of any living organism, displayed in pairs, and arranged by size, such that chromosomal aberrations and sex can be detected.

Locus – A delimited section chromosome housing a particular gene or other marker.

Marker – A gene, mutation, or other sequence that serves as an indicator of a known location in the genome.

Mendelian – Markers that are inherited under Mendel's laws of equal, random segregation and independent assortment during gamete production; examples include autosomal dominant, autosomal recessive, and sex–linked recessive and dominant genes.

Microarray – DNA sequences spotted on a microscope slide to which a labeled DNA pool of interest is hybridized in search for matching sequences.

Microsatellite – A DNA motif (2-6 bp long) repeated many times in tandem.

mtDNA – DNA of the mitochondria, typically about 16.5 kilobases (kb) for the entire genome. In animals, sequence evolution occurs more quickly than in most nuclear DNA. One exception includes nuclear microsatellites.

Neutral processes – Genetic processes which are not governed by selection (i.e., most commonly random genetic drift and random mutation).

Numt – Transferred pieces of mtDNA to nuclear chromosomal regions.

Ortholog – Homologous sequences where sequence divergence follow speciation.

PCR – **P**olymerase **C**hain **R**eaction is the exponential increase of DNA fragments *in vitro* using an enzyme (polymerase) that copies the DNA in between primers annealed to the flanking regions of the desired sequence.

Paralog – Homologous sequences that have arisen by a duplication event (i.e., hemoglobin and myoglobin). Each of the two duplicates are then on different evolutionary trajectories and are no longer comparable for phylogenetic analysis.

Phylogeny – The evolutionary relationships of groups of organisms, typically arranged in a branching diagram.

Phylogeography – The study of the patterns and processes responsible for the geographic distribution of genealogical lineages, particularly closely related species.

Plasmid – A double stranded piece of DNA that is separate from the chromosomal DNA; typically circular, ranging from 1–400 kb, and varying from one copy to several hundreds of copies in the cell.

Primer – An RNA or DNA fragment about 20 bp long that supplies the initial free end needed for DNA replication.

Pseudogene – A previously active gene which has accumulated a series of inactivating mutations.

QTL analysis – **Q**uantitative **T**rait **L**oci analysis, a statistical way to estimate the potential location on the genome coding for a complex or quantitative trait (i.e., height).

Restriction sites – A DNA sequence that is recognized by restriction enzymes which then cut the DNA molecule at or near that sequence.

RNA – A copy of DNA made into a polymer of ribonucleotides linked by phosphodiester bonds.

RT-PCR – **R**everse **T**ranscription-**P**olymerase **C**hain **R**eaction is a technique in which an RNA strand is reverse transcribed into its DNA complement, followed by amplification of the resulting DNA by PCR. **Real-Time PCR** – A PCR method capable of tracking the amount of amplified DNA produced at each cycle with the use of fluorescent dyes, thus allowing the quantification of the initial template (also called **Q**uantitative PCR or RT-PCR [QPCR or QRT-PCR]).

Saturation – Multiple nucleotide substitutions at a site that erase phylogenetic signal because conserved nucleotides cannot be distinguished from nucleotide sites that have independently mutated back to the same state (creating homoplasy).

SINE and LINE – Retrotransposons with utility as phylogenetic markers. SINEs (Short Interspersed Nuclear Elements) are nonautonomous, while LINEs (Long Interspersed Nuclear Elements) are autonomous (i.e., they can support their own transposition).

SNP – **S**ingle **N**ucleotide **P**olymorphism, a single nucleotide difference between two or more individuals at a particular locus.

Species tree – A phylogenetic tree representing the branching pattern among species lineages.

Transcriptome – The total set of mRNA transcripts produced in an individual at any given time.

Translocation – Movement of a section of DNA from its current location in a chromosome to a different chromosome.

Transposon – Sequences of DNA that can move around to different positions within the genome of a single cell and, in the process, may cause mutations and change the amount of DNA in a genome. They are also called jumping genes or mobile genetic elements.

Vector – A small DNA construct used in cloning, capable of carrying a foreign DNA fragment of interest into a host cell (such as *E. coli* bacteria) and facilitating its replication in that cell.

Defining Turtle Diversity: Proceedings of a Workshop on Genetics, Ethics, and Taxonomy of Freshwater Turtles and Tortoises
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Turtle Taxonomy: Methodology, Recommendations, and Guidelines

TURTLE TAXONOMY WORKING GROUP*

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ABSTRACT. – Taxonomy is the logical outcome of systematic research and knowledge; together, taxonomy and systematics form the basis for virtually all research in evolution, ecology, and conservation biology. Turtle taxonomy has been a very active field in the last few decades, particularly as new research has demonstrated that many traditionally recognized higher taxa are not monophyletic and therefore in need of revision. Unfortunately, there has been little consensus on how systematic research should be translated into taxonomic change, leading to a somewhat chaotic situation, with taxonomic and nomenclatural instability and a greatly reduced ability to communicate effectively with taxonomic names. We review the importance of a stable, efficient taxonomy for turtles, both for improved scientific communication and as the legal and scientific foundation of international conservation efforts, and suggest a set of guidelines for researchers to consider when name changes are being considered. These guidelines emphasize the crucial importance of a strong, well-supported phylogeny, clear criteria for species delimitation, and classifications that avoid monotypic higher taxa and unnecessary name changes. Finally, we briefly discuss the Phylocode and DNA barcoding as examples of the new directions in which taxonomy may be moving. We illustrate our points with examples from turtles, and implore the community of turtle researchers and conservationists to work together toward a stable taxonomy that will lead to both strong science and effective conservation.

KEY WORDS. – Reptilia; Testudines; taxonomy; nomenclature; systematics; turtles; Emydidae; *Actinemys*; *Clemmys*; *Emys*; *Emydoidea*; phylocode; DNA barcoding

Taxonomy has been defined as "the naming and assignment of organisms to taxa" (Futuyma, 1998) or "the theory and practice of classifying organisms" (Mayr and Ashlock, 1991). Taxonomy is one of the key elements of the study and protection of biodiversity. In disciplines ranging from conservation biology to biogeography to community ecology, we count, rank, classify, and study organisms and regions based on the names that we give to taxa. Taxonomic names, be they species, subspecies, or more inclusive groups like genera, families, or phyla, are both fluid (that is, they change frequently) and potentially informative. Like any set of names, taxonomies are most useful when the information that they convey is unambiguous, and when they are stable enough that we can use them to communicate that information efficiently. This dual goal, clarity of information content and stability over time, are the cornerstones of effective taxonomies, and in this paper we discuss these and other taxonomic issues with respect to turtles.

Our working group consists of individuals who approach taxonomy from a number of diverse perspectives, including conservation biology, evolutionary and population genetics, paleontology, and systematics. Some of us have proposed and implemented new names for turtles, and others have not. However, all of us have strong views on what names mean, why they are important from our individual research perspectives, and how they should be ap-

plied to turtles. If our group agrees on two things, they are that the names we use for species and higher groups are critically important, and that genetics often has a role to play in helping determine those names.

At the most fundamental level, the importance of taxonomy and nomenclature stems from the simple fact that we all need to communicate effectively if we are to conduct our more specialized research or achieve our management goals. To take one simple example, the western pond turtle (*"Clemmys" marmorata*) has been a candidate for listing under the US Endangered Species Act (USFWS, 1992), and the subject of at least one major recent genetic analysis (Spinks and Shaffer, 2005). For decades, the species was classified as *Clemmys marmorata* (Baird and Girard, 1852) in the widely distributed family Emydidae. However, as the genus *Clemmys* has been demonstrated to be paraphyletic (McDowell, 1964; Bickham et al., 1996; Lenk et al., 1999; Feldman and Parham, 2002), the names which have been assigned to the western pond turtle have included *Emys marmorata* (where the genus name *Emys* includes the species *blandingii*, *orbicularis*, and *marmorata*), *Actinemys marmorata* (where *Actinemys* includes only *marmorata*) and *Clemmys marmorata* (where *Clemmys* is retained in its previous usage to include *guttata, insculpta, muhlenbergii,* and *marmorata*). In this relatively simple case, the names *Actinemys, Clemmys,* and *Emys* lose their utility for communication when different researchers have different concepts of what those names mean. Also, searches of literature databases (e.g. Web of Knowledge) and DNA databases (GenBank) now yield a confusing combination of names that makes access to these important tools increasingly difficult. And perhaps most disturbing from a conservation perspective, regulatory agencies may no longer recognize the taxon as being listed on various protected species lists until the new name can be formally recognized and added to those lists. Although informatics tools are under development to efficiently untangle the confusion that stems from taxonomic instability (Patterson et al., 2006), the ideal solution is a stable taxonomy that enhances communication and information retrieval.

Our goal in this paper is to highlight some of the important issues to consider when thinking about taxonomy and classification, and in particular, when considering formal name changes. We focus on turtles, although most of our points apply equally well to any other group of organisms. One of the most difficult aspects of taxonomy is that it often includes a variety of opinions and points of view. However, for taxonomy to be most effective, a single set of names must be agreed upon and used, and that set should remain reasonably stable within the bounds of gradually expanding knowledge of the particular group of organisms. Although complete consensus among any group of users is probably impossible to achieve, we hope that reasonable agreement is possible, and emphasize its importance throughout this paper. One clear conclusion from our collective thinking is that taxonomic changes should be considered only when the science indicates that they are absolutely necessary (Dayrat, 2005), and even then, only when some attempt at consensus has been achieved. This approach has not been followed in the recent literature on turtles, leading to a somewhat chaotic taxonomy that neither facilitates communication nor leads to nomenclatural stability.

The Basics: Species and Subspecies are the Fundamental Units of Systematic Biology

Species. — The species is probably the most important level of classification and is the only level that has been suggested to have biological "reality". An important distinction exists between the conceptualization of species and the methods by which we recognize and delimit those species (de Queiroz, 2005). The biological species concept, or BSC (Mayr, 1963), has been historically important in turtles, and most systematists likely would concur that populations that are reproductively isolated due to the evolution of intrinsic reproductive isolating barriers should be considered as distinct species. Phylogenetic, lineage-based, and genealogical species concepts have been widely applied in the last decade or so, and at least some practitioners now feel that a unified "metapopulation lineage species concept" (that of an evolutionary lineage diverging through time) is broadly applicable as a universal species concept (de Queiroz, 2005). However, a wide range of criteria are used to delimit these lineages (Sites and Crandall, 2004), and accurate species delimitation is critically important to systematics, conservation, and evolutionary studies.

Whatever species criteria are applied to turtles, it is clear that the use of molecular genetic techniques has aided, and will continue to aid, in the identification of new species and the delimitation of existing ones. In a recent review of 12 current methods for delimiting species, Sites and Crandall (2004) noted that all 12 routinely rely on molecular data and at least 7 require it. As our understanding of the number of species of turtles and their geographic distributions improves, our ability to recognize and conserve biodiversity will increase. However, it is important to remember that species recognition is a double-edged sword. While reliable systematics studies based on appropriate data and analyses improves our understanding, poorly conducted studies can set back taxonomic progress and conservation efforts. We encourage chelonian systematists to be thorough in their approach, clear about their methods, and cautious in their conclusions (see also Dayrat, 2005). Studies in which new species are described should state what species concept/criterion is being followed, and be consistent in its application. Ideally, both the concept and criterion should be established *a priori* so that all biologists can evaluate the extent to which the data support a taxonomic

decision and new data can be used to test the hypothesis of species differentiation. We have formulated a set of guidelines that summarize what we consider to be important considerations in new descriptions and renaming of turtle taxa, and present these guidelines under "Guidelines for Best Scientific Practices" (see inset box).

Subspecies. — Subspecies are, at least for metazoans, the least inclusive taxonomic category that is recognized with a formal taxonomic rank. They have been defined as "a named geographic race; a set of populations of a species that share one or more distinctive features and occupy a different geographic area from other subspecies" (Futuyma, 1998).

Subspecies are historically important components of chelonian systematics and taxonomy and as such we are obliged to deal with them. Some systematists (including some of the members of our working group) take the position that this level of classification should be discarded—either a taxon is a diagnosable lineage, in which case it should be formally recognized as a species, or it

Guidelines for Best Scientific Practices for Revising Taxonomy

Accepting that multiple, scientifically valid philosophies exist with respect to both species and higher taxonomic categories, and that these are not likely to be reconciled any time soon, there are some recommendations that are broadly applicable to taxonomic revisions. We offer the following guidelines to workers in turtle taxonomy; think of them as caveats to keep in mind when embarking on a taxonomic revision.

1. Nomenclatural stability should be maintained as much as possible. Taxonomic changes are inevitable. However, introducing new or unfamiliar names creates a disjunction with the previous literature that leads to reduced, rather than enhanced communication about the contained taxa. For the sake of making information about turtles readily accessible, workers should try to maintain the continuity of turtle nomenclature with previously published literature unless widely supported data demands a change. The naming of new species, the accumulation of many species within a genus, or tentative data suggesting new phylogenetic relationships are not reasons to create new genera, or split up or merge existing ones.

2. Higher taxonomic names should represent monophyletic groups. We recommend that workers should only name higher level taxa that are demonstrably monophyletic, because modern systematics relies on monophyly as the primary criterion for the utility of a clade name. Anagenesis may help guide one on which monophyletic groups to name, but monophyly is the primary criterion.

3. Minimize naming new monotypic higher taxa. Monotypic higher taxa tell us nothing about shared ancestry, and therefore fail to convey interesting aspects of shared biogeography, comparative biology, and evolutionary history. Obviously, many monotypic genera and families are well established for turtles, and we feel that stability is more important than eliminating monotypic groups. In some instances monotypic taxa might be preferable due to uncertainty in their phylogenetic positions or because the rules of priority would require even more name changes if they were synonomized with their more inclusive sister-taxa. In addition, one must also remember that higher taxa containing a single living species may contain many described fossil species, in which case that taxon is not monotypic.

4. Names should not be changed unless there is *strong evidence* that the existing names do not reflect phylogenetic relationships. Although it may be tempting to name novel nodes recovered from a phylogenetic analysis, new or unfamiliar names can be deleterious to both communication and stability. This is especially true if these names are placed on poorly-supported nodes which are later refuted by additional study. Before naming a node, we recommend that workers consider the support for this node, both from a single data partition (i.e., mtDNA) and across data partitions (nDNA, morphology, behavior, etc.). To ensure stability of a name, workers should strive to seek concordance between independent data sets, with high bootstrap and Bayesian posterior support derived from each data set, before naming a new species or higher taxon.

5. Current taxonomy should be divorced from predictions about future changes in taxonomy. That is, defining a higher taxon and creating a new genus name based on the prediction that additional species will be discovered, and a genus-level name is needed to contain them, is ill advised.

6. New or redefined forms should be integrated into an existing taxonomic hierarchy unless the existing taxonomy is not adequate for the placement of the new form. For example "*Heosemys*" *leytensis* was recently placed into a phylogenetic analysis for the first time (Diesmos et al., 2005). It was found to be separate from other *Heosemys* and sister to the black marsh turtle, *Siebenrockiella crassicollis*. Rather than create a new monotypic genus, Diesmos et al. (2005) expanded *Siebenrockiella* to indicate that the two species form a clade.

is not, and should not be recognized with a name. However, other members take the view that subspecies classification can be useful and informative for delineating regional morphotypes that may fail to meet the criteria of full species under certain concepts.

We propose that subspecies classification, if used, should describe the major patterns of variation found within a species. A precise definition of "major" is elusive, but the formal subspecific description of small, isolated populations, particularly in low-vagility species, should be avoided unless there is strong reason to do otherwise. This could avoid the proliferation of named forms of small, isolated populations such as occurred with pocket gophers in western North America (Smith and Patton, 1988). However, the recognition of genetically divergent populations can have real value, and recognizing such populations as subspecies may be useful in some cases. For example, recognition of subspecies in the western USA salamander *Ensatina escholtzii* has been a key element of its interpretation as a ring-species in the midst of the speciation process (Wake, 1997, but see Highton, 1998). Among chelonians, the continued subspecific classification of Galapagos tortoises (*Geochelone nigra* ssp.) has focused attention on this insular radiation as an ongoing case study in speciation and adaptive radiation (Caccone et al., 1999, 2002; Beheregaray et al., 2004).

Genetic tools and datasets have been applied to problems at the intraspecific level, and at this point may represent the most important data for the recognition of intraspecific variation. For example, phylogeographic studies, employing mitochondrial DNA (mtDNA) and/or nuclear DNA (nDNA) sequences taken from geographically defined populations, can identify geographically restricted lineages that become candidates for subspecific recognition (Lenk et al., 1999; Engstrom et al., 2002; Starkey et al., 2003; Fritz et al., 2005; Spinks and Shaffer, 2005). Such studies can also reveal the presence of cryptic species or intraspecific groups like stocks, distinct population segments, evolutionarily significant units, or subspecies. What to call such differentiated populations will be determined by a number of factors including the level or degree of genetic differentiation (genetic distance) and the systematic philosophy of the investigator. In addition, a growing body of literature suggests that single gene analyses can often be misleading, and particular care should be taken when relying primarily on mtDNA (Funk and Omland, 2003). For this paper, suffice it to say that it may be appropriate to name subspecies of turtles where phylogeographic analyses indicate genetically differentiated populations that do not meet the requisites of species distinction, but whose recognition would aid in delineating the pattern of geographic variation within the species.

In summary, we recognize that both the "subspecies concept" and its use in systematics are controversial, and we do not seek here to resolve this controversy, even among our working group members. Rather, we emphasize that subspecies, if used, should convey real evolutionary information about lineages and geography, and must be based on field-collected specimens, adequate geographic sampling, appropriate statistical analyses of variation, and data that are reported in the primary literature and can be replicated by other researchers.

Higher Taxonomic Groups and How They Translate Into Taxonomies

Traditionally, species are grouped into genera, and genera into families; we term these collections of species "higher taxonomic groups". Generally, higher taxonomic groups are, or should be, based on phylogeny. Our understanding of turtle phylogeny is currently incomplete and changing rapidly, and future revisions of higher taxonomic groups are inevitable.

Virtually all workers agree that higher taxonomic groups should be monophyletic, and non-monophyletic groups are viewed as a problem to be fixed with taxonomic changes. However, the way in which a large, inclusive monophyletic group (like turtles, for example) should be divided into less inclusive monophyletic groups can be quite contentious. Within the turtle community, there are two distinct schools of thought guiding the creation of higher level names (we focus on genera in this discussion). The first is based on the view that genera should convey a certain level of evolutionary distinctiveness (Simpson, 1961). We call this view the 'anagenetic' perspective (anagenesis being defined as the "evolution of a feature over an arbitrary period of time"; Futuyma, 1998). The second emphasizes the utility of genera (and all other higher taxonomic names) to show hierarchical relationships only; we call this the 'phylogenetic' perspective (Hennig, 1966). The general practice in systematics has clearly moved toward the phylogenetic perspective (e.g., Cracraft and Donoghue, 2004), at least to the extent that all higher groups should be rendered monophyletic whenever possible, and we assume that most practicing turtle systematists use phylogenies as a guide in their higher-level taxonomic decisions.

However, even when all parties agree that monophyletic groups are important, there are fundamental differences between the anagenetic and purely phylogenetic viewpoints that can lead to conflicting taxonomic schemes. For example, under the anagenetic perspective, a well-accepted monophyletic genus could be split into many genera if subclades within that genus were deemed to be distinct enough. Such genera might contain one species or multiple species, but the decision on the number and content of genera would be based on their level of differentiation (genetic, morphological, or some other set of features). Under the phylogenetic perspective, the only compelling reason to split an existing genus is strong evidence that it is not monophyletic. The phylogenetic perspective claims that any measure of evolutionary distinctiveness is subjective, as indicated by the varying levels of distinctiveness that exist among animal genera (including turtles).

A few turtle examples illustrate these differences. In determining how to reclassify the apparently non-

monophyletic genus "*Clemmys,*" Holman and Fritz (2001) noted that "In all cladograms derived from molecular data, *Clemmys marmorata* is closer to the genera *Emys* and *Emydoidea* (taxa that have a plastral hinge) than to the hingeless *Clemmys guttata, C. insculpta,* and *C. muhlenbergii*... The most parsimonious nomenclatural way to resolve this situation is to put *C. marmorata* in a monotypic genus to avoid combining hinged and nonhinged species in a single clade. For this genus the name *Actinemys* Agassiz, 1857 is available" (p. 323). The reasoning behind this decision reflects the anagenetic view that the evolution of a plastral hinge (or the secondary loss of the hinge, as suggested by Holman and Fritz, 2001) is an important, genus-level character, and that hinged and non-hinged species should not be combined in the same genus. They used this reasoning as the primary justification for the recognition of *Emys, Actinemys,* and *Emydoidea* as genera, even though each contained only a single living species (*Emys trinacris* was described later, in Fritz et al., 2005). However, the plastral hinge varies within species of other turtles (Parham and Feldman, 2002; Chiari et al., 2005), leading other authors to conclude that the plastral hinge should not be viewed as a generic level character, and that a more informative taxonomy results if the clade containing the species *marmorata, orbicularis,* and *blandingii* is recognized as the genus *Emys*. Similar arguments hold for genetic data. For a given gene such as the widely used mitochondrial cytochrome *b* (cyt*b*), an anagenetic perspective might argue that there is a consistent percentage of sequence divergence among sister genera within a family, and divide existing monophyletic genera based on large levels of sequence divergence. The more purely phylogenetic view would emphasize that there is no single "genus level" of divergence for turtles, and therefore levels of divergence *per se* should not guide taxonomic decisions over the number and content of genera. For example, map turtles (*Graptemys*) and diamondback terrapins (*Malaclemys*) are sister genera that are less than 2.3% divergent for cyt*b* (Lamb and Osentoski, 1997), whereas average cyt*b* divergence among sister genera of softshell turtles (*Apalone* and *Rafetus*) is 13.4% (calculated from data in Engstrom et al., 2004).

One natural outcome of the phylogenetic view is that monotypic genera (and families) are largely uninformative, since they tell us little about phylogenetic relationships—in that sense, they are redundant with the fact that the contained single taxon is a species (Parham and Feldman, 2002; Spinks et al., 2004). Alternatively, the anagenetic view claims that monotypic genera and families are sufficiently distinct that they should be named, and that avoiding them obscures important evolutionary distinctiveness of some lineages. Extinct lineages add yet another dimension to this issue, because the fossil record indicates that many living monotypic groups are the lone survivors of more-diversified clades from the past (Carettochelyidae, Dermochelyidae, Dermatemydidae, and Platysternidae are all family-level examples, and *Emydoidea, Trionyx,* and *Erymnochelys* are examples at the genus-level). In such cases, these higher taxa are not monotypic, although their living representatives consist of a single species.

Guidelines for Taxonomic Changes

Like it or not, taxonomic change is an inevitable part of phylogenetic research; this is true for turtles as well as any other group. Taxonomic and associated nomenclatural changes are the logical result of advances in systematic biology. However, new insights into phylogenetic relationships can lead to a variety of taxonomic changes, including no change at all. In this section, we provide some guidelines on when, and how, to bring about taxonomic changes in turtles. We break the discussion into two related parts: a brief discussion of the more formal 'rules' governing nomenclatural changes, and what we consider to be 'best scientific practices' on how to proceed when one must propose a taxonomic change, given the systematic conclusions (see also Dayrat, 2005). In all cases, we hope that all researchers proposing changes will value the balance of communicating the newest taxonomic and/or phylogenetic results with the need to try to maintain stability of names.

The Rules of Nomenclatural Changes

In principle, taxonomic changes are based on an objective review of all available evidence and a solid theoretical foundation. In practice, however, there is no universal agreement on systematic theory, and little consensus on how phylogenies translate into names. In general, the informal rule in taxonomy is that the latest published revision is valid until refuted. Unfortunately, this rule is not always realistic or followed—some published revisions may be known to be incorrect, but rigorous refutation often requires as much or more time and effort than the original study did, rendering correction a slow process. An important consideration for all taxonomists is that taxonomy has acquired importance beyond the biological sciences; conservation actions, legislation, and public awareness do not have the understanding, interest, tolerance, or time required to stay updated on taxonomic developments, but instead risk being confused or hindered by scientific name changes and unclear taxon definitions. It is also important to recognize that some taxonomic revisions are published based on inadequate data and/or incomplete descriptions, and we tend to view them as hypotheses to be tested, rather than changes to be accepted. It is to recognize and perhaps define such cases that we emphasize the following rules:

1. Proposed nomenclatural changes must be in accordance with the regulations set forth in the most recent edition of the International Code of Zoological Nomenclature (ICZN) (http://www.iczn.org/iczn/index.jsp).

2. Taxonomic and associated nomenclatural changes should be published in widely-available, peer-reviewed scientific publications that are indexed in the Zoological Record. Peer-reviewed publications are defined as publications that regularly publish the names of their editorial review board and external reviewers. A widely available publication is defined as a publication that is open to public subscription and purchase of individual issues, and which makes reprints of its contained articles available in paper and/or electronic format for authors to distribute person-to-person upon request. Obviously, "widely-available, peer-reviewed scientific publications" are somewhat subjective terms, and the ICZN is not strict on these issues. We strongly recommend that only peer-reviewed scientific journals that are available at libraries and other institutions be considered appropriate outlets for taxonomic changes. We also recommend that these journals be accessible through the major scientific online search engines whenever possible.

3. The taxonomic and/or species concept and criteria used to identify taxa should be clearly indicated in the publication, and the methodology used should be clearly and fully described. The methodology should be appropriate to the taxonomic group under investigation, and should ideally include a wide range of approaches (e.g., morphological, genetic, behavioral). Methods and results should be fully presented, and taxonomic conclusions must be solidly based on these results.

Proposed taxonomic changes that meet the three criteria above are more likely to be accepted into wide usage. Proposed taxonomic changes that do not fully meet all three criteria should probably not be adopted without additional independent research and debate. Until such time, the previous, 'traditional' taxonomic arrangement should probably be retained for practical purposes.

There are no simple formulas or rules for making taxonomic revisions. However, we hope that workers will take these guidelines into consideration before proposing changes. Ideally, workers would explicitly address all of these issues as part of their justification for proposed taxonomic changes.

Why it Matters: The Relationship Between Taxonomic Decisions and Conservation Effects

Taxonomy is the logical outcome of systematic research, and conservation must be based on and guided by the best-available taxonomy. In this sense, taxonomy (and systematic biology) assumes a critical role in guiding the management of species at risk, and 'getting the taxonomy right' is essential (Lovich and Gibbons, 1997). This is particularly critical at the species level, since it is a major focus of conservation actions. It is just as harmful to not recognize distinct species that exist in nature as it is to incorrectly recognize taxa that do not exist in nature—the former can lead to extinction due to neglect, whereas the latter can lead to the squandering of conservation resources on invalid taxa. We discuss these and other issues below.

The Focus of Conservation on Species. — In the realm of conservation, including conservation-related legislation in many countries, the basic unit is usually the species. Most regulatory agencies focus on the species-level unit, with subspecies or other less inclusive but diagnosable lineages (Evolutionarily Significant Units, Distinct Population Segments, etc.) considered to be of lesser or no importance. The level of concern directed at sub-specific taxa or lineages varies greatly across the world, with most nations and inter-governmental organizations (e.g., CITES, IUCN, FAO, CBD, CMS) paying little or no attention to any taxonomic units below the level of species. In the USA and some other countries, mechanisms exist to recognize and address conservation needs of lower taxonomic units. However, rightly or wrongly, sub-specific classification units garner proportionally less emphasis than do species. Similarly, supra-species classification units such as genera, families, and orders, are rarely taken into account by regulatory processes, although some conservation value is placed on species contained in monotypic higher taxa compared to species in polytypic genera and families (which further emphasizes that monotypic taxa should not be created arbitrarily).

Recognizing that conservation and legislative priorities focus on the species level, it is particularly important that thorough evaluations of potentially distinctive forms below the species level are carried out to ascertain whether they may warrant recognition as species. Parallel efforts should also be made to encourage the conservation and regulatory communities to encompass intra-specific units within their scope of activities. For example, IUCN is moving towards regional evaluations of taxa to facilitate regional conservation efforts, but remains focused on the species level.

Taxonomy Driven by Politics and Opportunism. — Recognizing species diversity is a fundamental requirement for conservation actions. The importance of conservation and management as a motivation for taxonomic revision at the species level is often recognized in the scientific literature, and has been taken to extremes by some taxonomists. As global biodiversity loss rose to the top of the global environmental agenda during a period of economic constraints, declining scientific and conservation funding was re-focused onto biodiversity conservation at the expense of traditional museum-based taxonomy. Predictably, taxonomy redefined itself to some extent as "biodiversity research". During the same period, theoretical developments in systematics led many taxonomists to abandon the traditional biological species concept, and adopt phylogenetic/evolutionary species concepts (Frost and Hillis, 1990). An ideological dislike for the concept of subspecies developed, with the logical result that if a taxon was recognizably different and perceived to be on an independent evolutionary path, some authorities 'automatically' regarded it as a distinct species (Collins, 1991; Grismer, 1999).

In cases that can be interpreted and justified either way (lumping or splitting at the species or subspecies ranking), some conservation-oriented biologists may find it tempting to err on the side of splitting or elevating a taxon, presumably because a higher-ranked or more diverse taxon could garner additional scientific and conservation attention. Finely-split taxa also are more likely to be endemic to a single political jurisdiction, rendering them easier to protect and manage.

There are dangers inherent to proposing, supporting, or retaining exaggerated taxonomy. Taxonomy is a public science, and subject to more intense peer scrutiny than many other branches of the biological sciences. On the one hand, fellow taxonomists understand and sympathize with taxonomic decisions made in good faith based on best available information and a solid theoretical framework, even when subsequent data and/or improved theoretical understanding later demonstrate these decisions to have been inappropriate. Recent cases include the recognition, based on subsequent genetic data, that a number of recently-described Asian geoemydid turtles were actually human-created hybrids rather than valid species (Parham et al., 2001; Spinks et al., 2004; Stuart and Parham, 2007). On the other hand, taxonomists who knowingly employ doubtful taxonomic practices or incomplete datasets degrade taxonomy and run the risk of being seen as less than objectively scientific by their colleagues, the general public, and legislative and regulatory authorities. We cannot emphasize enough the importance to conservation of bringing the strongest, most objective science possible to the table when taxonomic decisions are being made. In addition, it is critically important that when doubt exists over the validity of taxa that are receiving conservation attention, the best available taxonomic tools, which are likely to be genetic, should be brought to bear to help resolve these issues. Examples of such taxa might include the Plymouth red-bellied turtle (*Pseudemys rubriventris "bangsi*"; Iverson and Graham, 1990) and the Cat Island slider (*Trachemys terrapen "felis*"; Seidel and Adkins, 1987). Other entire clades that receive high conservation priorities remain in need of further work on species boundaries; the Asian box turtles (*Cuora*) are a case in point (Parham et al., 2001; Spinks and Shaffer, 2007).

Taxonomy and Legislation. — Regulatory authorities (and non-systematist conservationists) abhor changes to the names of taxa. Taxa of conservation and regulatory interest are usually managed from codified lists; altering the names on such a list is often a slow, laborious, and convoluted process, sometimes requiring parliamentary approval and lengthy delays. In some legislative processes, a taxonomic definition is given when including a taxon in a list. When systematic progress changes the taxon name or scope, the original intent and definition of the taxon remain subject to the regulation. For example, the genus *Podocnemis* was listed under CITES Appendix II in 1975 (http://www.cites.org/eng/app/appendices.shtml). When subsequent taxonomic revision split *Podocnemis* into *Podocnemis* plus *Peltocephalus* plus *Erymnochelys*, these names were 'automatically' included in the same list (Inskipp and Gillett, 2005). Alternatively, if genus A is listed in CITES, but genus B is not, and taxonomic research shows that genus B is a member of genus A, then the species originally included in genus B are not automatically included in CITES. For example, the keeled box turtle was long known as *Pyxidea mouhotii* but recently shifted to *Cuora* (Honda et al., 2002). The entire genus *Cuora* was listed in CITES in 2000, when nine species were recognized and before *mouhotii* was transferred out of *Pyxidea*. If *Pyxidea mouhotii* had not been listed independently in 2002, then *Cuora mouhotii* would not have been included in the CITES listing.

In most jurisdictions, a listed species is not defined; rather, the taxonomic name is a placeholder for the biological entity that is being listed. If that listed species name is changed by scientific revision, existing legislation may no longer protect the biological entity that was originally intended. This is certainly a problem for nomenclatural name changes, as when a listed species changes genus (e.g., the shift of *Trionyx swinhoei* to *Rafetus swinhoei*, Meylan, 1987), and this may require changes in legislation to clarify the transfer of legal protection. An even greater problem exists for cases where an existing species is split into two or more sibling species. For example, recent taxonomic analysis of *Malayemys subtrijuga* identified two distinct taxa, *M. subtrijuga* and *M. macrocephala* (Brophy, 2004). *Malayemys subtrijuga* was already listed as protected in Thailand, however, *M. macrocephala* will not be protected there until the Thai Wild Animals Reservations and Protection Act is amended to include that name. Other countries address taxonomic/nomenclatural change by including additional new names for existing taxa, in effect making the law a list of synonyms. Indian legislation would, for example, name *Cyclemys mouhotii*, *Pyxidea mouhotii*, and *Cuora mouhotii* in its list of protected species.

In summary, we recognize that taxonomic changes are necessary as our understanding of the evolutionary history and diversity of turtles matures, and some changes are both necessary and desirable. However, taxonomic changes also lead to confusion, a lack of ability to communicate effectively, and unanticipated changes in conservation status and international protection. There is value to increased taxonomic understanding, and with it comes the necessity for nomenclatural change, and we provide some guidelines on when to implement such changes. However, in today's world, where a species' name has implications far beyond the traditional biological scientific community, it is imperative that systematists also remember the wider implications of their taxonomic and nomenclatural decisions.

The Future of Taxonomy? Rank-Free Classification, the Phylocode, and DNA Barcoding

We end our discussion with a description of two new directions in taxonomy that purport to solve many of the problems inherent with our current way of conducting taxonomic research. Each has strong advocates and equally

strong detractors. In presenting them, the TTWG takes no position on them, since we have members who span the range of opinions on these topics. However, in the spirit of keeping abreast of new developments in the field, we present them as important future directions in taxonomy.

Rank-Free Classification and Phylocode.—Within the general systematics community, there is now broad consensus that classifications above the species level should be based on monophyletic groups (defined as an ancestor and all of its descendant taxa). In this sense, the majority of current systematists, including most turtle systematists, embrace the idea that classifications should be phylogenetic.

Although the monophyly criterion represents one of the most broadly agreed-upon concepts in current systematics, the concept of a stable, monophyly-based classification is often at odds with Linnaean, or rank-based classification methods. As pointed out by de Queiroz and Gauthier (1990, 1992, 1994), rank-based methods of classification are typological—a type specimen is assigned to define a species, and a higher taxon is defined with reference to a type species. Although this approach to naming genera, families, and other rank-based higher taxa has been in effect for over 200 years, it leads to a number of undesirable features as systematists attempt to create phylogenetic classifications (de Queiroz and Gauthier, 1990, 1992, 1994). Primary among these features are: 1) instability of names, 2) either poorly defined higher taxa or changes in the definition of a named taxon over time, and 3) a tendency for taxa to become monotypic with revision. Several of these problems have become quite severe in turtle classification. For example, using the checklist compiled for this volume of the world's turtles (Turtle Taxonomy Working Group, this volume), the number of species per genus overall now stands at between 3.05 and 3.48 (depending on how certain contentious genera are resolved); within the Pleurodira, that number is 3.74-5.0, while the Cryptodira have 2.86–3.16 species per genus on average. Perhaps more telling, the number of monotypic genera (that is, genera that contain only a single species) now stands at about 45% (range is 40–46% depending on taxonomy), or nearly half of the ca. 100 recognized turtle genera.

The reason for this largely stems from the consequences of applying the Linnaean rank-based system to phylogenetic classifications. For example, when a genus is found to have another genus nested within it, then either the nested genus must be synonymized into the more inclusive one, or the more inclusive genus must be split into several smaller genera.

Recent work on the old genus "*Clemmys*" demonstrates this point. Phylogenetic analyses (Bickham et al., 1996; Lenk et al., 1999; Feldman and Parham, 2002) have demonstrated that the four species that previously comprised the genus "*Clemmys*" (*guttata, muhlenbergii, insculpta,* and *marmorata*) are paraphyletic with respect to *Emydoidea*, *Emys*, and *Terrapene* (Fig. 1A). Given that these latter three genera cannot be contained within the genus "*Clemmys*" under the Linnaean rank-based system, some taxonomic change is required. One suggested solution (Fig. 1B) has been to resurrect two old genera (*Actinemys* and *Glyptemys*) to accommodate three species (*Actinemys marmorata, Glyptemys insculpta, G. muhlenbergii*), and leave *guttata* in the now-monotypic genus *Clemmys* (Holman and Fritz, 2001). Although all genera under this solution are monophyletic, it results in five genera to contain a total of seven living (and two fossil) species. If one of the goals of higher-level taxonomy is to convey phylogenetic information about how species are related (the phylogenetic view of taxonomy discussed above), this solution is at odds with that stated goal. Interestingly, it appears to also be at odds with the anagenetic goal; if *marmorata* and *guttata* are sufficiently distinct to be placed in different genera, why were they originally placed in the same genus? Another, recently proposed alternative by Feldman and Parham (2002), would recognize the monophyly of *blandingii, marmorata,* and *orbicularis* in the more inclusive genus *Emys* by relegating the old genus *Emydoidea* to the synonymy of *Emys* and shifting *marmorata* from *Clemmys* to *Emys* (Fig. 1C, Fig. 2). The final alternative, to include all species previously assigned to *Emys, Emydoidea, Clemmys*, and *Terrapene* to a single genus has not been seriously proposed because of the number of name changes it would entail. While each of the first two solutions is justifiable and has its proponents (e.g., Stephens and Wiens, 2003; Spinks and Shaffer, 2005), the primary point is that both require a substantial set of nomenclatural changes purely as a consequence of Linnaean ranks. If the names *Emys, Emydoidea,* and *Clemmys* were not of equal rank, then no name changes would necessarily be required as phylogenetic resolution continues to improve.

As a radical solution to this and other problems stemming from the Linnaean rank-based system, an alternative scheme has evolved over the last 10 years known as the Phylocode (http://www.ohiou.edu/phylocode/). Although the details of Phylocode are still being worked out, the system has reached a relatively mature state, with a codified set of standards for naming taxa at all levels in the hierarchy of life. Essentially, Phylocode proposes that taxa be defined with reference to a phylogenetic tree, rather than with respect to type specimens. It also proposes that ranks (but not named groups) be abandoned, since they are a primary source of instability in the Linnaean system. Thus, a named taxon might be defined as "the monophyletic group defined by the most recent common ancestor of an eastern box turtle (*Terrapene carolina*) and a painted turtle (*Chrysemys picta*), and all species derived from that ancestor", and it might be called Emydidae. Using such a definition, Emydidae will always be monophyletic—it has to be, since its very definition is based on monophyly. As a consequence, two important aspects of a named taxon—definition and monophyly—remain stable under Phylocode. However, the content of a group may change as phylogenetic hypotheses change. In the above example, based on the current state of knowledge,

Emydidae would contain 48 species (as noted in our other chapter in this volume, Turtle Taxonomy Working Group, 2007). If subsequent research demonstrated unequivocally that the eastern box turtle and the painted turtle were sister species (an unlikely result, obviously), then Emydidae as defined would consist solely of those two species. The definition would remain unchanged, and Emydidae would still be monophyletic, but its content would be quite different.

One of the natural (but not absolutely essential) consequences of the phylogenetic method of naming taxa embodied in the Phylocode is to abandon ranks. It is important to be clear on exactly what this means, and the costs and benefits of Linnaean ranks in a phylogenetic context. The greatest downfall of ranks is clearly demonstrated in the "*Clemmys*" example (Fig. 1). Because *Emydoidea* and *Clemmys* are both genera, the discovery that the former is nested within the latter (Fig. 1A) means that a nomenclatural change must follow—either *Emydoidea* must be synonymized (Fig. 1C), or *Clemmys* must be divided into additional genera (Fig. 1B). Whatever solution one chooses demands multiple name changes and taxonomic destabilization; the sole reason for those changes is the identical rank of *Emydoidea* and *Clemmys*.

Under a rank-free system, different nodes can be named (or not), and authors can use the full list of names associated with a terminal species (or not). Thus, if *Clemmys* were defined as "the group containing the most recent common ancestor of the terminals *guttata* and *orbicularis* and all of its descendants", and *Emys* were defined as "the group containing the most recent common ancestor of the terminals *orbicularis* and *marmorata* and all of its descendants", this would imply (given our current phylogenetic understanding) that *marmorata* is a member of both *Emys* and *Clemmys* at different phylogenetic levels. Because these are rank-free names, there is no conflict in one being nested within the other, and there are no necessary name changes if future phylogenetic research implies a different set of relationships. The same principle holds for all taxonomic levels.

Rank free classifications following the Phylocode have been proposed several times for turtles in the literature to date. The first was by Joyce et al. (2004) who used 25

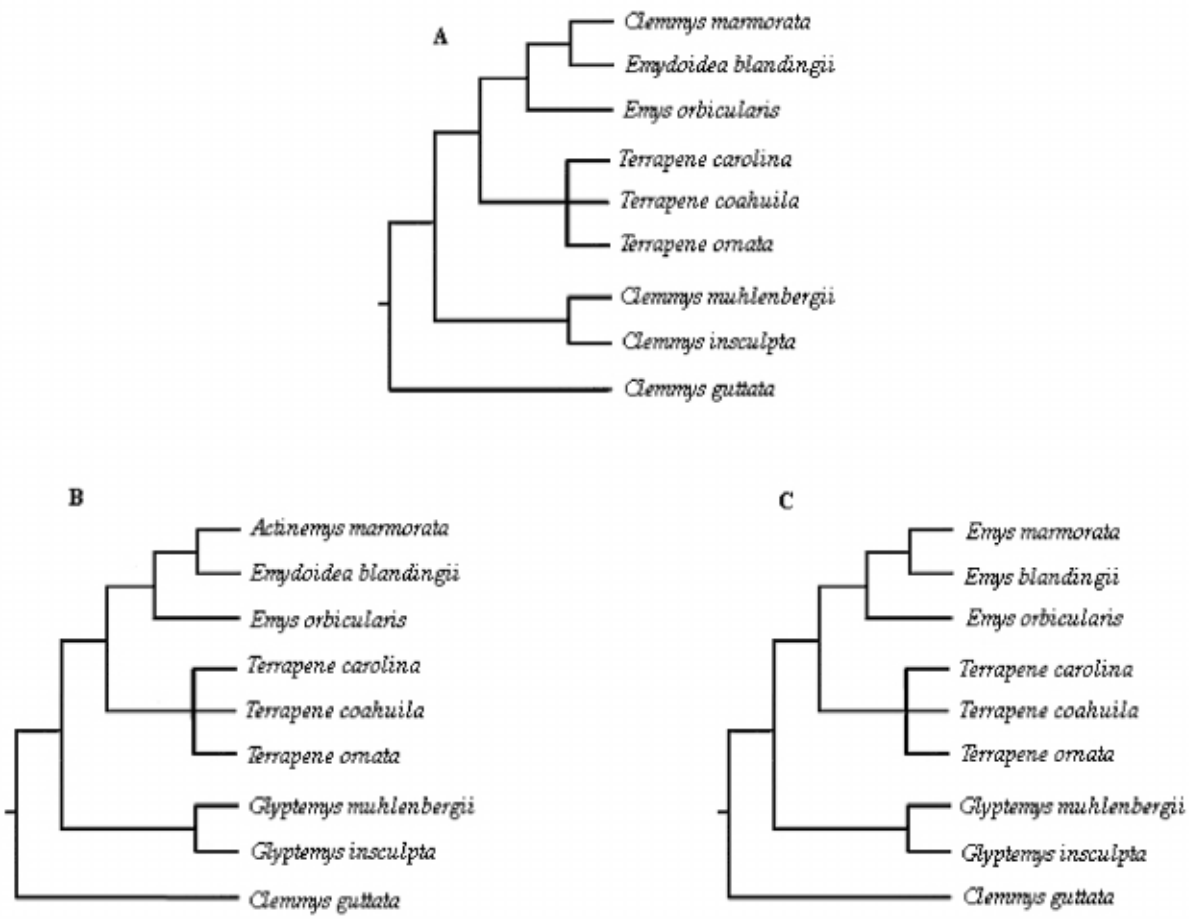

Figure 1. A current phylogeny of ten species of emydine turtles, and three alternative taxonomic schemes (after Feldman and Parham, 2002). The recently-named *Emys trinacris* (Fritz et al., 2005) is not shown, since it was not described at the time that this tree was developed; it would presumably be the sister species to *orbicularis*. Panel A shows the previously-used names, and the paraphyly of the old name "*Clemmys*" as applied to the four species *guttata, insculpta, marmorata,* and *muhlenbergii;* virtually all systematists recognize that this non-monophyly requires taxonomic changes. Panel B solves this problem by proposing two new generic names, leading to a total of three name changes and three monotypic genera, whereas Panel C solves the same problem by proposing a total of four name changes and one monotypic genus. See text for details.

relatively inclusive clades (down to the Linnaean rank level of family) of turtles as a test case to explore the challenges of converting well-established rank-based names into a rank-free taxonomic system. As an example from the other end of the phylogenetic spectrum, Engstrom et al. (2004) proposed a rank-free classification for the 26 species of softshell turtles (the traditional family Trionychidae) based on their molecular and morphological phylogenetic analysis. These two examples span a broad range of taxonomic levels, and deal with the challenges inherent in switching to a rank-free classification.

Figure 2. The three species that comprise the "*Emys* complex". **Top:** *Emys orbicularis* from Iran (photo by James Parham). **Middle:** *Emys* or *Actinemys marmorata* from California (photo by Jerome Maran). **Bottom:** *Emys* or *Emydoidea blandingii* from Michigan (photo by Michael Benard).

Like most of the systematics community, our Turtle Taxonomy Working Group includes a range of opinions on the costs and benefits of ranked vs. rank-free classifications, and whether or not the principles embodied in the Phylocode represent a net benefit or not to solving taxonomic issues with turtles. The literature similarly includes a full range of opinions from well respected taxonomists working across the tree of life. We make no explicit recommendations, other than the obvious one—that the community of turtle systematists should make every effort to track the new advances that are taking place in the larger systematics community and be open to meeting the challenges of refining and stabilizing the taxonomy of turtles.

DNA Barcoding. — DNA barcoding refers to the idea that species identification for an individual can potentially be determined by a small fragment of DNA sequence from that individual. The Consortium for the Barcode of Life (http://barcoding.si.edu/DNABarCoding.htm) has recommended that the cytochrome c oxidase subunit 1 mitochondrial region (COI) be used as the standard barcode region for all "higher" animals. This recommended region is short (about 650 base pairs in length), well characterized, and easy to use.

DNA barcoding can potentially contribute to two important empirical problems in taxonomic research and its applications. The less controversial is the rapid, inexpensive identification of organisms and their products when more traditional characters are either unreliable or inapplicable. For turtles, this might include pieces of meat, shell, or medicinal powders, hatchlings or eggs, and melanistic or otherwise unrecognizable specimens, to name a few examples (e.g., Roman and Bowen, 2000). Situations ranging from forensic analysis in criminal cases such as illegal trade, to the repatriation of captive specimens to the wild, all require rapid, accurate identification, and DNA barcoding could provide critical identifications for these and other important activities. Much more controversial is the idea that new, cryptic species might also be identified from DNA barcode data. This application is closely linked to the idea that species differ by a constant, minimal threshold level of COI sequence divergence. If, for example, species were generally 2% sequence divergent for COI, and a genetic survey from across a species range found populations that were more than 2% divergent from the rest of the species, those populations would be targeted as possibly new, cryptic species. This strategy has been explicitly advocated for poorly-known, hyperdiverse taxa like insects (Smith et al., 2006) and crustaceans (Lefebure et al., 2006), although serious issues have also been raised with the strategy (Rubinoff, 2006).

The Turtle Taxonomy Working Group recognizes that the application of DNA barcoding is a potentially useful management and forensics tool for many species. However, we also recognize that relying on a single mitochondrial gene is fraught with problems (Funk and Omland, 2003; Rubinoff, 2006), and that progress will rely on adequate characterization of known-locality specimens from across the range of each species as a precursor

to reliable DNA barcoding efforts. We do not recommend DNA barcoding as a mechanism for discovering new species, given the range of sequence divergence currently known for turtle species (compare Lamb and Osentoski, 1997, and Engstrom et al., 2004). We further recognize that some closely-related species may not be amenable to barcoding identification, and that the unusual situation imposed by hybridization in turtles (Parham et al., 2001; Spinks et al., 2004; Stuart and Parham, 2007) will further challenge the utility of the approach.

Concluding Thoughts

Taxonomy is clearly an active field with a variety of opinions and scientific strategies. Our working group includes many diverse opinions that cover this broad spectrum of science. However, we are absolutely united in our view that taxonomy and nomenclature are critical to the future of both science and conservation involving turtles. This leads us to the unified position that taxonomic revisions and usage must reflect the strongest available science, based on clear and unambiguous interpretations of that science, and published in the appropriate, widely-available, and peer-reviewed scientific literature. We feel that when the guidelines of our "Best Scientific Practices" are followed, and when reasonable consensus of the turtle community is sought, that the taxonomy of turtles will become the essential tool for communication and conservation action that it should be. We hope that all practitioners of turtle taxonomy, whether working at the intraspecific level or the deepest phylogeny of the group, will work together to achieve a stable classification of turtles that is maximally informative, based on the best available science, and reflective of the broadest possible consensus within the turtle community.

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In Search of the Tree of Life for Turtles

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ABSTRACT. – Based on a thorough review of the literature, we provide a bibliography of papers featuring phylogenetic hypotheses for living turtles, a composite tree of all turtle species based on those hypotheses, a compilation of the most rigorously derived trees from those papers (i.e., using contemporary methods with bootstrapping), and supertrees for selected families of turtles using input trees from those most rigorous trees. These outputs allow us to identify the branches of the tree of life for turtles that are best supported as well as those most in need of study. With the exception of the Platysternidae and Chelydridae, the phylogenetic relationships among turtle families seem to be well-resolved and well-supported. Within families, the relationships among most genera are also well-resolved; however, the reciprocal monophyly of the South American and Australian chelids, the relationships among the genera allied to the chelid genera *Batrachemys* and *Mesoclemmys*, and the monophyly of the emydid genus *Trachemys* remain problematic. The relationships among species of trionychids, geoemydids, and testudinids are best resolved (since they are based on morphology, multiple mitochondrial genes, and at least one nuclear gene), and those for the podocnemids and pelomedusids are the least understood (with no complete published tree for either). The relationships among species in the following genera are most in need of additional phylogenetic study (highest need first): *Pelusios, Podocnemis, Testudo, Kinosternon, Batrachemys* (and close relatives), *Elseya, Trachemys, Graptemys*, and *Pseudemys*. Future work should endeavor to include the broadest taxonomic and geographic sampling possible (including type specimens) in order to maximize our understanding of the evolution of modern turtle diversity. A comprehensive multilocus approach (with numerous mtDNA and nDNA genes) will clearly be the best strategy for fully resolving the tree of life for turtles.

KEY WORDS. – Reptilia; Testudines; phylogenetics; supertree; mitochondrial DNA; nuclear DNA; morphology

Although turtles have been evolving for over 200 million years, the phylogenetic relationships among them have been discussed for less than 200 years, and most of the resolution of relationships has been provided in the last 20 years. The oldest hierarchical classification of turtles appears to be that of Dumeril (1806: Fig. 1), although it enumerated only four genera and was not intended to represent an explicitly historical perspective. Many other hierarchical classifications of turtles appeared in the 1800s (reviewed by Gaffney, 1984), but the first explicit phylogenetic tree for the major groups of fossil and living turtles was published by Hay (1908; Fig. 2). However, despite the increasing acceptance of Darwin's theory of evolution by natural selection, and even the rise of the "modern synthesis" in the 1930s and 1940s, explicit phylogenetic hypotheses for turtles in the form of branching diagrams (or phylogenetic trees) were nearly absent before the 1970s (for early exceptions see Zug, 1966; Pritchard, 1967).

Fueled by the insights on phylogenetic systematics provided by Hennig (1966), and the associated emergence of cladistic methodology (reviewed by Nelson and Platnick, 1981), Gaffney (1972, 1975a,b, 1976, 1977, 1979a,b) pioneered the application of those techniques to the phylogenetics of both extant and fossil turtles. The emergence and development of DNA sequencing techniques and methods for the analysis of molecular and morphometric data (Felsenstein, 2003) has led to an exponential increase in the number of papers that have included phylogenetic trees for various turtle groups (Fig. 3; see also Fig. 2 in FitzSimmons and Hart, 2007). As a result of this activity, the phylogenetic

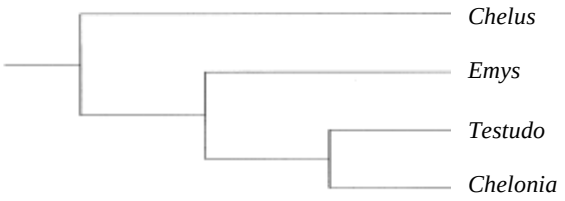

Figure 1. Phylogenetic "hypothesis" derived from Dumeril's (1806) hierarchical classification of turtles.

relationships among the families of living turtles have been fairly well resolved (Fig. 4), although some controversy remains (Krenz et al., 2005; Parham et al., 2006a; see below). Progress at lower taxonomic levels has been substantial, though significant gaps still exist in coverage. For example, in Iverson's (1992) checklist of turtles, of 87 recognized genera, 26 (30%) contained more than two species, but only 18 of those (69%) had a published phylogenetic hypothesis for most of the included species. However, at the end of 2005, about 104 genera were recognized, the increase due primarily to taxonomic splitting (only two previously unknown genera, *Elusor* and *Leucocephalon*, have been described since 1992; see TTWG, 2007b). Of those, 35 (34%) included more than two species, and at least one published phylogenetic hypothesis is available for all but 4 of those 35 (89%; not *Pelochelys* [3 species], *Batrachemys* [6 species], *Pelusios* [18 species], or *Podocnemis* [6 species]).

Despite this demonstrated proliferation in phylogenetic hypotheses for most clades of turtles, an attempt to produce an all-inclusive tree of all recognized living chelonian taxa has not been forthcoming (but see Gaffney and Meylan, 1988; Cracaft and Donoghue, 2004; Moen, 2006). Such a tree for turtles is desperately needed in order to 1) provide a working hypothesis of higher and lower level relationships among turtles; 2) identify the turtle taxa most in need of additional phylogenetic attention; 3) facilitate the identification of appropriate outgroups for future phylogenetic studies of turtles (e.g., compare Honda et al., 2002a, with Spinks et al., 2004); 4) facilitate studies of character evolution

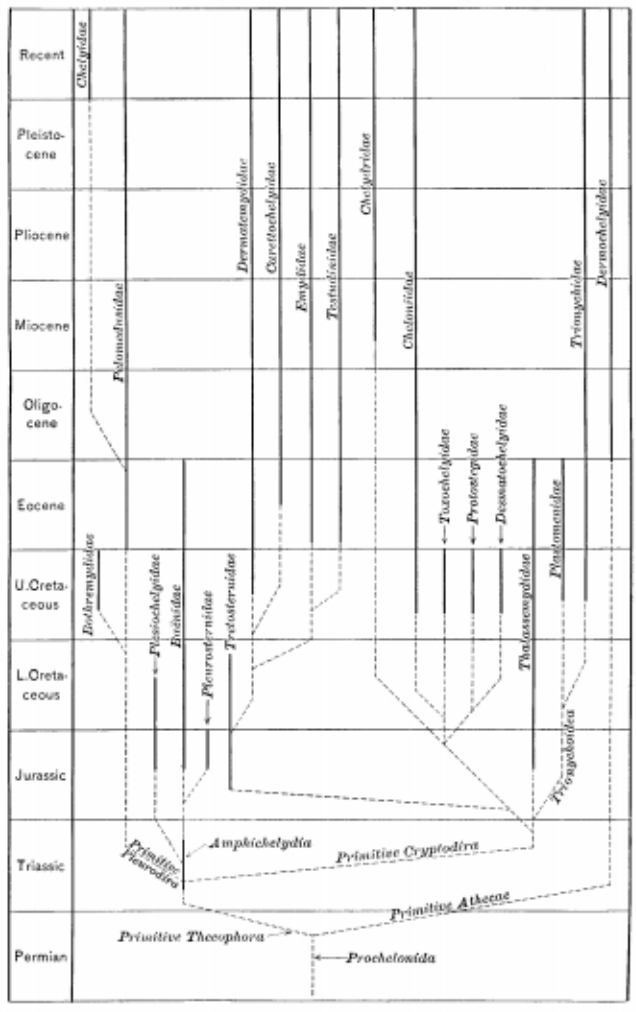

FIG. 8.—Phylogenetic chart showing supposed relationships of the families and higher groups of turtles.

Figure 2. Earliest explicit phylogeny of higher taxa of living and extinct turtles, published by Hay (1908).

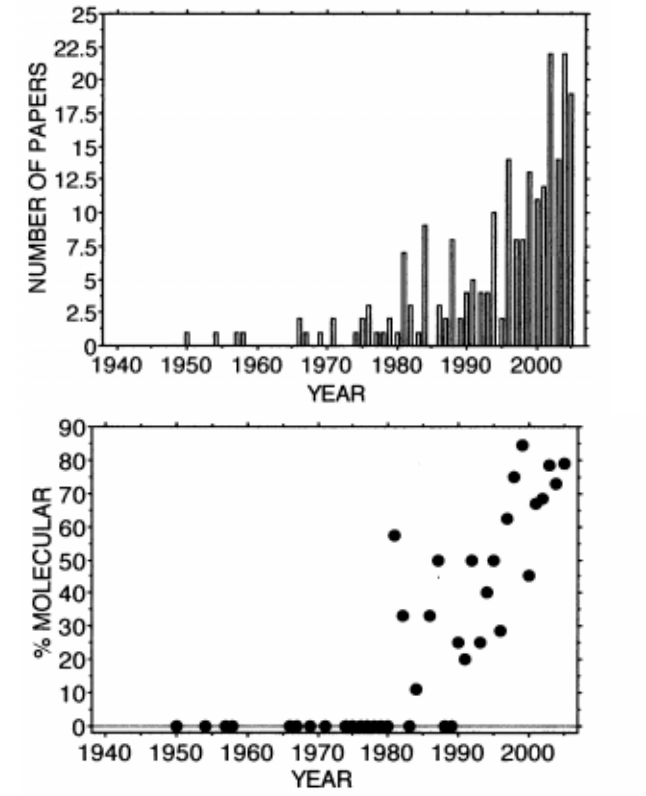

Figure 3. Publication dates of papers that included phylogenetic trees for turtle taxa at or above the species level. Dissertations and theses were excluded. **Top:** total frequency by year. **Bottom:** proportion of total papers that were primarily molecular (excluding karyotype papers). Key stimuli for increases were the synthesis of phylogenetic systematic philosophy by Hennig (1966), the first turtle cladistics paper by Gaffney (1972); the development of DNA sequencing methods (Sanger et al., 1977; Maxam and Gilbert, 1977); the pioneering of computer-based methods of phylogenetic reconstruction in the early 1980s (perhaps the biggest stimulus; reviewed by Swofford and Olsen, 1990); the development of Polymerase Chain Reaction methods (Mullis and Faloona, 1987; Saiko et al., 1988); and the development of Bayesian algorithms for phylogenetic reconstruction (Li, 1996; Mau, 1996). Only papers published through 2005 are plotted.

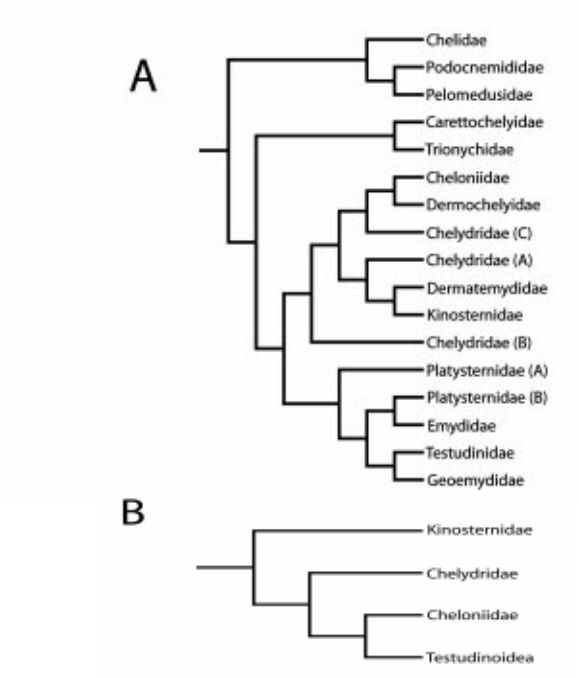

Figure 4. Current phylogenetic hypotheses of the relationships among the families of turtles. Ambiguity is illustrated by multiple placements of the families (1) Chelydridae: in Fig. **4A**, A after Cervelli et al., 2003 [ML], Near et al., 2005; B after Cervelli et al., 2003 [MP], Shaffer et al., 1997; C after Krenz et al., 2005; and in Fig. **4B**, after Parham et al., 2006a; and (2) Platysternidae: in Fig. **4A**, A after Krenz et al., 2005, Near et al., 2005; and B after Parham et al., 2006a.

in turtles (e.g., Stephens and Wiens, 2003b); 5) facilitate phylogenetic approaches to the study of zoogeography in turtles (e.g., Ronquist, 1998; Ree et al., 2005; Stephens and Wiens, 2003a); and 6) direct the appropriate setting of priorities for conservation initiatives (i.e., to conserve maximum genetic diversity of turtles; e.g., Krajewski, 1994; Engstrom et al., 2002; Fritz et al., 2005; Georges and Thomson, 2006).

With the intent of addressing the first two of these deficiencies, and further stimulating the investigation of the others, we provide herein our current best synthesis of the relationships among all recognized turtle species, and identify the clades with the weakest support (and hence most in need of further study).

METHODS

We reviewed the literature and compiled a bibliography of all locatable papers containing phylogenetic trees (or networks) that included turtles as terminal taxa (Appendix A). Based on the phylogenetic hypotheses generated in those papers, we identified the most recent and strongly supported trees for each family clade, giving preference to those with the most extensive character and taxon sampling (Appendix B). We then generated a compiled tree for all extant turtle species by concatenating this phylogenetic information (e.g., see Beck and Beck, 2005, and Jonsson and Fjeldsa, 2006, for justifications of this method).

For comparison with the compiled tree, we undertook a supertree analysis (Bininda-Emonds, 2004b) based on the "best" (see below) available trees. First, we compiled a list of candidate trees by higher taxon and tallied the character of the input data set and the methods of analysis (Appendix C). From that subset of potential input trees, in an attempt to maximize independence of our selected trees (Bininda-Emonds, 2004b:363), we first discarded redundant trees (e.g., trees in the same or different papers based on data partitions when a combined analysis was also available), as well as those based strictly on morphological characters. We next gave preference to trees with extensive character and taxon sampling and that used maximum parsimony analysis that included bootstrap values for nodes (or where those values could be calculated by our reanalysis of the reported data). We also discarded as redundant trees from separate papers that exhibited extensive overlap in genetic markers. Our purpose in doing so was to prevent disproportionate representation of any one kind of genetic data that might bias a supertree analysis if the majority of input trees were derived from the same class of DNA sequence data (see Bininda-Emonds, 2004c, for a discussion of issues relevant to data quality in supertree construction). An unfortunate

Figure 5. Current phylogenetic hypothesis of the relationships within the turtle family Chelidae.

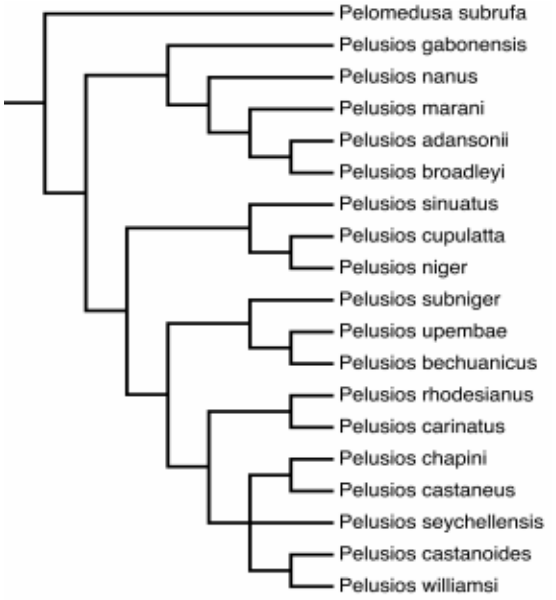

Figure 6. Current phylogenetic hypothesis of the relationships within the turtle family Pelomedusidae.

consequence of this necessary approach was that an adequate sample of input trees (only 22 total) was available for only five families (Cheloniidae, Kinosternidae, Geoemydidae, Emydidae, and Testudinidae). For simplicity, we have included only extant taxa in this first supertree analysis for turtles.

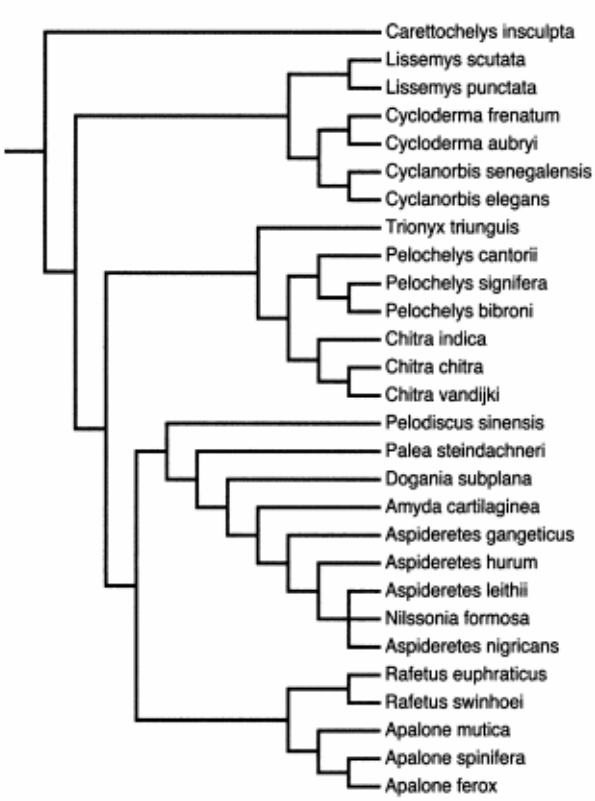

Figure 8. Current phylogenetic hypothesis of the relationships within the turtle family Trionychidae. The monotypic genus *Carettochelys* is included as the only representative of the family Carettochelyidae.

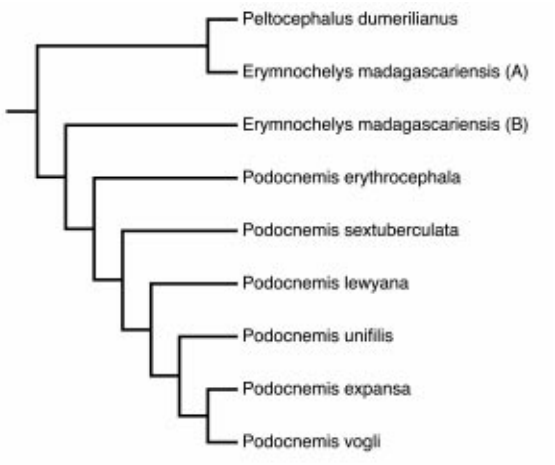

Figure 7. Current phylogenetic hypotheses of the relationships within the turtle family Podocnemididae. Ambiguity is illustrated by the double placement of *Erymnochelys madagascariensis* (A after Meylan, 1996, and Starkey et al., unpublished; and B after Georges et al.. 1998, Noonan, 2000, and Noonan and Chippindale, 2006).

Although there is considerable discussion about the most robust method for supertree construction (Wilkinson et al., 2005), we used matrix representation with parsimony (MRP), because it is generally accepted as one of the best current methods (Sanderson et al., 1998; Bininda-Emonds, 2004a,b; Burleigh et al., 2004), and because it has been applied productively in a number of recent studies (Salamin et al., 2002; Ruta et al., 2003; Davies et al., 2004; Kerr, 2005).

Exploratory MRP matrices for this study were initially constructed using SuperTree 0.85b (Salamin et al., 2002; http://www.tcd.ie/Botany/NS/SuperTree.html), and the Baum/Ragan coding scheme was used with nodes weighted by bootstrap support values (Davies et al., 2004). Final MRP matrices were constructed using r8s (Sanderson, 2004). For trees published without bootstrap support, we reanalyzed the

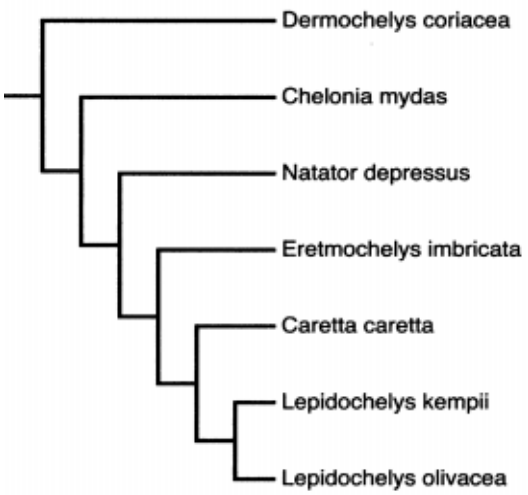

Figure 9. Current phylogenetic hypothesis of the relationships within the turtle family Cheloniidae. The monotypic genus *Dermochelys* is included as the only representative of the family Dermochelyidae. The topology of the single perfect supertree was identical to that illustrated here.

original dataset to obtain those values with 1000 MP replicates using PAUP 4.0B (Swofford, 2001). Weights were calculated following Farris (in Salamin et al., 2002) and manually input into PAUP files using TreeEdit (evolve.zoo.ox.ac.uk/software/TreeEdit/main.html).

The binary matrices were analyzed with PAUP 4.0B using weighted parsimony. We performed heuristic searches with 250 replicates of random taxon addition, subtree pruning-regrafting and branch swapping, holding 10 trees at each replicate. These saved trees served as starting trees in a second search using tree bisection-reconnection with a tree limit of 10,000 equally most parsimonious trees (Davies et al., 2004). Majority rule (50%) and strict consensuses (both constrained so that previously recognized families were monophyletic) were used to explore agreement between saved tree populations.

Finally, we have attempted to match names at the tips of our trees to those recognized through late 2006 by the Turtle Taxonomy Working Group (TTWG, 2007b). However, undescribed taxa are included in some trees (e.g., Chelidae, Testudinidae), because the additional forms have been identified in the literature, and more recent 2007 taxonomic changes have been included in the published list by the TTWG (2007b) since we generated our trees.

RESULTS AND DISCUSSION

Although phylogenetic trees including living turtle taxa have appeared in at least 142 publications (Appendix A), relatively few have included more than a few species,

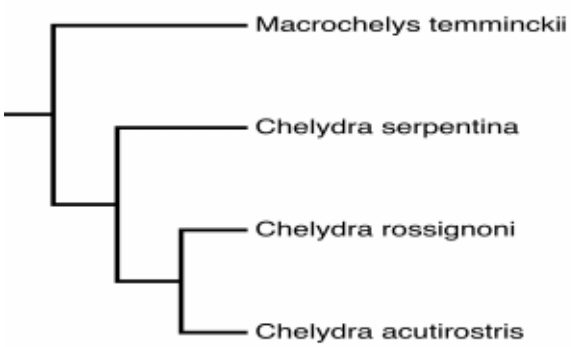

Figure 10. Current phylogenetic hypothesis of the relationships within the turtle family Chelydridae.

applied rigorous methods of phylogenetic reconstruction, provided support values for nodes using multiple reconstruction algorithms, and made objective comparisons of trees based on individual data partitions (e.g., cytb vs. ND4 vs. 12S/16S rRNA vs. Rag1 vs. morphology; see Table 1). In addition, there has been an obvious increase in the number of studies based primarily on molecular work, whereas the numbers of primarily morphology-based papers has remained fairly constant (Fig. 3). Nevertheless, we were able to compile at least preliminary trees for all living turtle families and species (Figs. 4-14) However, because of incomplete taxon sampling, the paucity of trees for several families, and discordance among trees within several families, our attempt to generate a single supertree for all turtle taxa was not successful (in that most families were not

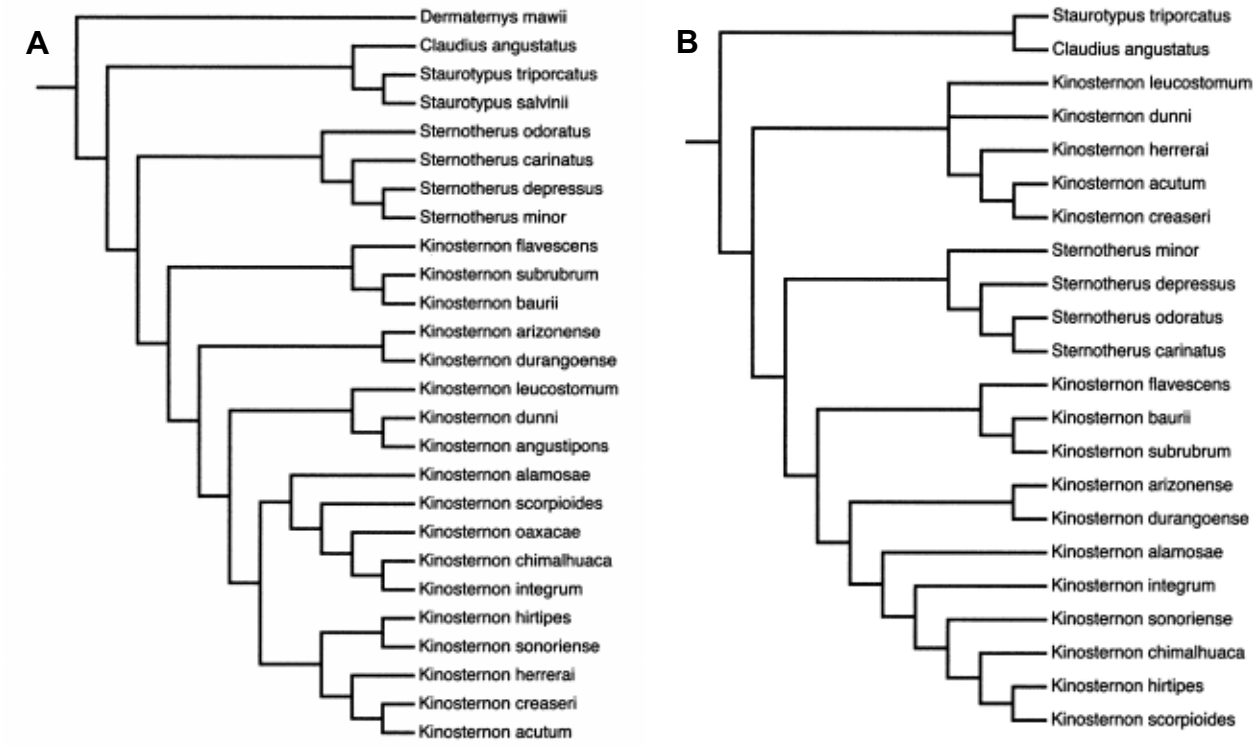

Figure 11. Current phylogenetic hypotheses (A = compiled tree; B = single perfect supertree) of the relationships within the turtle family Kinosternidae. The monotypic genus *Dermatemys* is included as the only representative of the family Dermatemydidae.

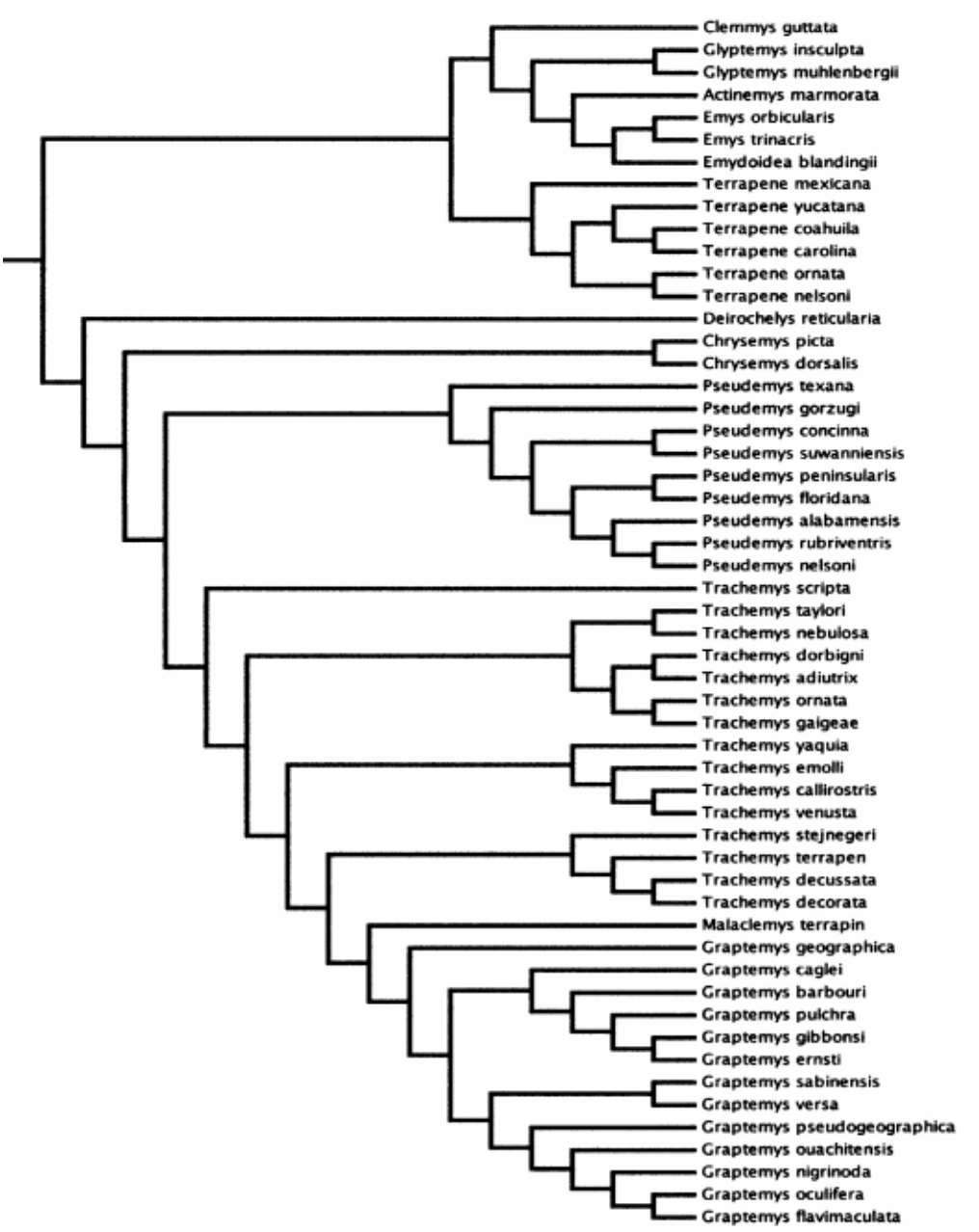

Figure 12. Current phylogenetic hypothesis of the relationships within the turtle family Emydidae.

resolved as monophyletic). Appropriate input trees (in number and taxonomic diversity) were available for supertree analysis within only five families: the Cheloniidae (Fig. 9), Kinosternidae (Fig. 11B), Geoemydidae (Fig. 13B), Emydidae, and Testudinidae (Fig. 14B).

Compiled Trees

Inter-Familial Relationships. — The monophyly of each of the two living subclasses of turtles (Cryptodira and Pleurodira) is well-supported in nearly all recent phylogenetic reconstructions, whether based on morphologic or molecular data (Gaffney and Meylan, 1988; Shaffer et al., 1997; Cervelli et al., 2003; Fujita et al., 2004; Krenz et al., 2005:Fig. 5B; Near et al., 2005; Parham et al., 2006a; but see Wu et al., 1999; and Krenz et al. 2005:Fig. 5A). Furthermore, with the exception of the placement of the Chelydridae and the Platysternidae, the phylogenetic relationships among most of the rest of the families is also well-resolved (Fig. 4).

Once considered to be closely related to the Chelydridae (e.g., Gaffney and Meylan, 1988; Shaffer et al., 1997), the monotypic family Platysternidae has recently (Krenz et al., 2005; Near et al., 2005) been considered to be sister to the Testudinoidea (= Emydidae + Geoemydidae + Testudinidae) based on combined nuclear (RAG-2) and mitochondrial (cytochrome *b* and 12S) DNA sequence data. However, based on the entire mitochondrial genome, Parham et al. (2006a) found support for the Platysternidae as sister to the Emydidae (Fig. 4A). In addition, that study also revealed a novel placement for the sea turtles (Cheloniidae) and the

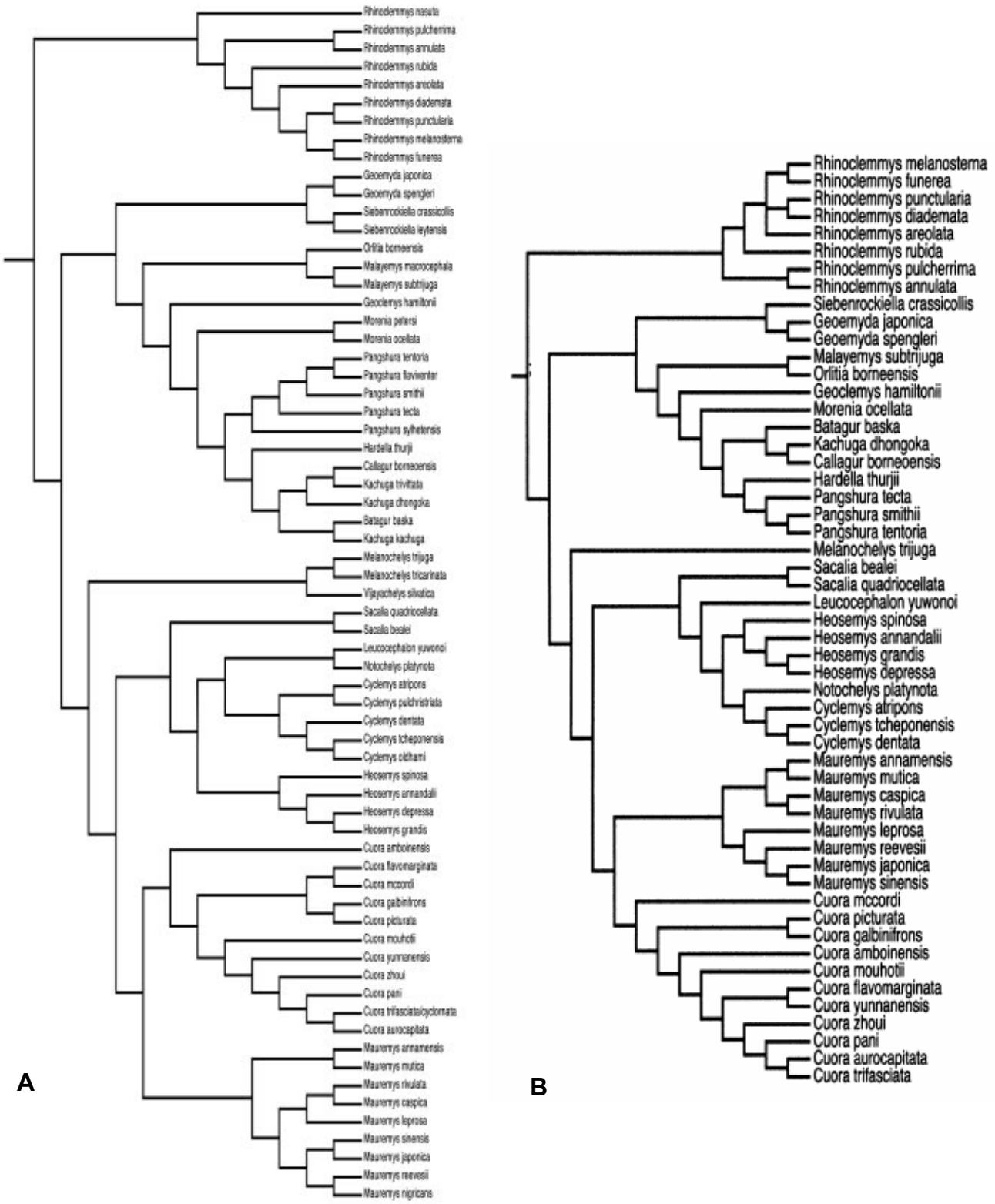

Figure 13. Current phylogenetic hypotheses (A = compiled tree; B = 50% majority rule supertree based of 3186 equally parsimonius trees in second search; 461 trees revealed by initial search) of the relationships within the turtle family Geoemydidae.

snapping turtles (Chelydridae) (Fig. 4B). As is evident from the various positions of the Chelydridae in Figs. 4A and 4B, its phylogenetic position among the Cryptodira is the least resolved of all turtle families. Final resolution of the phylogenetic position of these two families will require broader taxon and character sampling (i.e., from both the nuclear and mitochondrial genomes, as well as from morphology). A reconsideration of the shared morphology of chelydrids and platysternids in light of recent paleontological data may also prove useful.

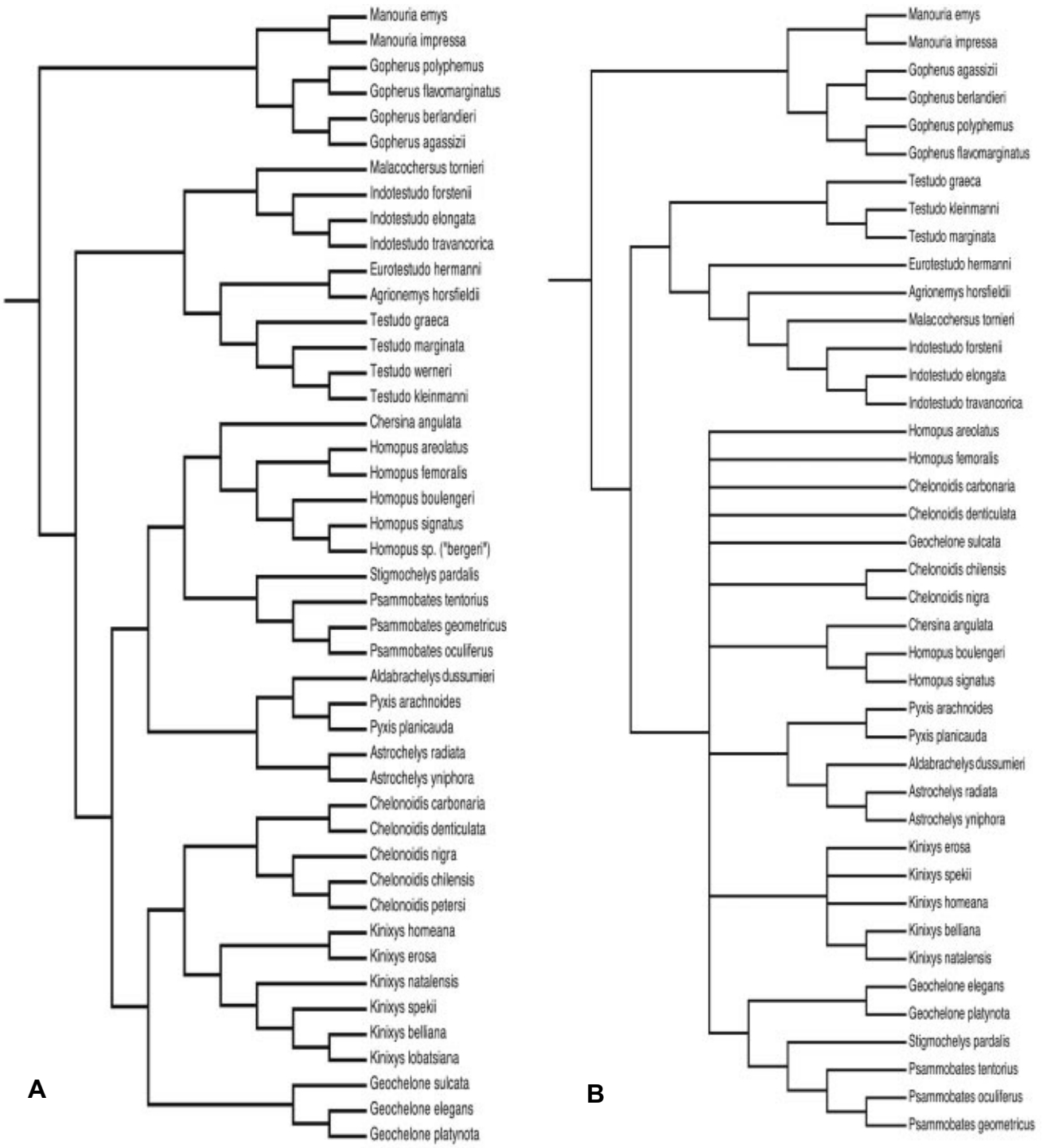

Figure 14. Current phylogenetic hypotheses (A = compiled tree; B = 50% majority rule supertree based on 10,000 equally parsimonious trees; 325 trees revealed by initial search) of the relationships within the turtle family Testudinidae.

Although there is no recent disagreement that the testudinids and geoemydids are closely related (i.e., belong to the monophyletic Testuguria; e.g., Parham et al., 2006a), recent analysis by Spinks et al. (2004:Fig. 3) reconstructed the Geoemydidae as paraphyletic with respect to the testudinids (though with low support), suggesting that the genus *Rhinoclemmys* might deserve familial status in order to preserve a monophyletic taxonomy. However, Le and McCord (in press) resolved *Rhinoclemmys* as sister to the rest of the geoemydids, and recommended its recognition as a subfamily of the Geoemydidae.

At this time five family pairs appear to be firmly supported as sister taxa: Podocnemididae–Pelomedusidae, Carettochelyidae–Trionychidae; Cheloniidae–Dermochelyidae; Dermatemydidae–Kinosternidae; and Testudinidae–Geoemydidae. The Chelidae is strongly supported as the sister group of the Podocnemididae–Pelomedusidae (= Pelomedusoides) as a monophyletic Pleurodira, and the Trionychidae–

Carettochelyidae (= Trionychia) is strongly supported as the sister group of the other living Cryptodira. The major remaining higher level questions for turtles are the phylogenetic relationships among the three other cryptodire family pairs and the Emydidae, Chelydridae, and Platysternidae.

Chelidae. — Resolution of the relationships among most of the chelids in Fig. 5 should be considered tentative, because of incomplete taxon sampling (Georges et al., 1998), reduced character sets (Seddon et al., 1997; McCord et al., 2002; Bour and Zaher, 2005), disagreements over character scoring (compare McCord et al., 2002; and Bour and Zaher, 2005), unreported bootstrap support for resolved nodes (Georges et al., 1998), and considerable undescribed (Georges and Thomson, 2006) and recently described (Bour and Zaher, 2005; Thomson et al., 2006) diversity. Particularly problematic are the relationships within the polyphyletic genus *Elseya* (Georges and Thomson, 2006) and the clade including the older genera *Batrachemys* and *Mesoclemmys* and the recently described or resurrected monotypic genera *Rhinemys*, *Ranacephala*, and *Bufocephala* (McCord et al., 2002). Despite this uncertainty, a consensus does appear to be emerging that the family includes three monophyletic groups, the Australasian long-necked turtles (*Chelodina* and *Macrochelodina*), the Australasian short-necked turtles (*Elseya* and relatives), and the South American species (with *Hydromedusa* as sister to the other South American forms; compare Gaffney and Meylan, 1988). However, the reciprocal monophyly of the Australian and South American taxa is still not resolved. Work currently underway should soon resolve the relationships among at least the Australian species (A. Georges, N. FitzSimmons, *pers. comm.*).

Pelomedusidae. — The genus *Pelomedusa* has been considered to be sister to the genus *Pelusios* by all recent authors (Fig. 6); however, no rigorous phylogenetic study to date has included *Pelomedusa* along with reasonable sampling within the speciose genus *Pelusios* (with at least 18 species; TTWG, 2007b). In fact, no phylogenetic hypothesis has previously been published for the species of the genus *Pelusios*. The tree provided in Fig. 6 is based entirely on morphology, as hypothesized by Roger Bour (unpubl. data). In addition, the description of two new cryptic species of *Pelusios* in the last six years (Appendix B) suggests that undescribed diversity remains in this genus [only the genus *Testudo* potentially includes more diversity; but see below]. Even a preliminary molecular phylogeny within this genus is sorely needed.

Podocnemididae. — Recognition of this clade as a separate family is a relatively recent concept (following de Broin, 1988), but well-supported phylogenetically (see references above under inter-family relationships). However, resolution among the genera and species is still unclear (Fig. 7). The position of *Erymnochelys* as sister to *Peltocephalus* is supported by Meylan (1996) and Starkey et al. (unpubl. data), but placement of *Erymnochelys* as sister to *Podocnemis* is supported by Georges et al. (1998), Noonan (2000), and Noonan and Chippindale (2006). A well-supported tree for the members of the genus *Podocnemis* is needed, and is currently underway (Starkey et al., unpubl. data).

Trionychidae and Carettochelyidae. — Following the work of Meylan (1987; based on morphology) and Engstrom (Engstrom et al., 2002, 2004; based on nuclear and mitochondrial DNA sequences and morphology), resolution of the relationships among the softshell turtles and their sister relationship to the monotypic family Carettochelyidae are quite well supported (Fig. 8). However, despite these comprehensive analyses, one clade remains poorly resolved, that including the genera *Aspideretes* and *Nilssonia*. Broader genomic sampling might clarify that last problematic softshell clade.

Cheloniidae and Dermochelyidae. — The position of *Dermochelys* as sister to the rest of the living marine turtles has long been supported (e.g., Gaffney and Meylan, 1988). In addition, the three most recent phylogenetic analyses of sea turtle species all supported the tree illustrated in Fig. 9 (Bowen and Karl, 1997; Dutton et al., 1996; Parham and Fastovsky, 1997). Nevertheless, additional genomic sampling (since only mtDNA data are currently available), analyzed by algorithms developed after those studies were published, should provide the definitive test of this hypothesis.

Chelydridae. — The relationships among the taxa in this family (Fig. 10) are well-resolved (Phillips et al., 1996; Shaffer et al., in press), and additional cryptic diversity seems unlikely to emerge (Shaffer et al., in press).

Kinosternidae and Dermatemydidae. — No recent disagreement exists concerning the relationships among the genera in these two families (Fig. 11A), whether based on morphology (Hutchison, 1991; Iverson, 1991, 1998) or molecules (Iverson, 1998; Krenz et al., 2005; Fujita et al., 2004). However, published phylogenetic studies to date either had reasonably comprehensive taxon sampling but minimal character sampling (Iverson, 1998) or minimal taxon sampling and only slightly better character sampling (Serb et al., 2001; Walker et al., 1998). In addition, to date only mitochondrial DNA has been sampled. As a result, there is considerable uncertainty in the relationships within even the two best-studied clades, *Sternotherus* (compare Iverson, 1998 and Walker et al., 1998) and the *Kinosternon flavescens* species complex (compare Iverson, 1998, Walker et al., 1998, and Serb et al., 2001). Because of this poor resolution, a more comprehensive study of nuclear and mitochondrial genes and morphology is underway (Iverson and Le, unpubl. data).

Emydidae. — Except for the genus *Trachemys*, the monophyly of and the relationships among the other genera in this family appear well resolved (Fig. 12), despite the fact that no data are yet available from the nuclear genome. As is evident from the compiled tree, *Trachemys* as currently constituted appears to be paraphyletic, and the relationships among the included species are tentative at best (compare Seidel, 2002 versus Stephens and Wiens, 2003b). Resolution among species in the genera *Pseudemys* and *Graptemys* is also unclear and will require extensive intraspecific (i.e., geographic) and interspecific sampling. For example, the tree generated by Stephens and Wiens (2003b) did not include all recognized taxa in the genus *Pseudemys*, and *Graptemys o. ouachitensis* and *G. o. sabinensis* were resolved in separate clades in that paper. Finally, although

there is some agreement (Minx, 1996; Feldman and Parham, 2002; among others) that the genus *Terrapene* includes two monophyletic clades (*ornata/nelsoni* and *carolina/coahuila/mexicana/yucatana*), the relationships among the taxa in the latter clade are poorly resolved (Stephens and Wiens, 2003) and will also require extensive geographic sampling to clarify.

Geoemydidae. — Several taxa of geoemydid turtles were described in the 1990s based on turtles supplied by animal dealers. Despite their being morphologically distinguishable and purportedly field-collected (with some of them being shipped in large numbers and capable of producing fertile, identical F1 offspring), six have been shown to be of hybrid origin (see Parham et al., 2001; Spinks et al., 2004, and Stuart and Parham, 2007; and papers cited therein). Whether those hybridizations were the result of human husbandry or natural events (or both) remains to be determined definitively. Three other new taxa appear to be valid species based on genetic and morphological analysis, but have not yet been field collected (Stuart and Parham, 2007). Further study of the propensity of turtles in this family to hybridize, even between members of distant clades (e.g., *Sacalia* and *Cuora*), will be essential for a full understanding of the evolution of the turtles in this family.

Despite the confusion caused by the hybrid descriptions, the relationships among most of the genera and species of geoemydid turtles have been well resolved (Fig. 13A; Spinks et al., 2004; Le, 2006; and other references in Appendix B). Nevertheless, several problematic clades do remain (e.g., the genera *Cyclemys*, *Cuora*, and *Mauremys*, each *sensu lato*). Recent morphological and molecular work (e.g., Guicking et al., 2002; and references therein) has suggested that instead of including only two species (Iverson, 1992), the genus *Cyclemys* may include as many as nine species (note that only five of these are included in Fig. 13A, because the species boundaries are so unclear). Only thorough geographic and genetic sampling can clarify the actual number of species in this genus. However, their historic transport in the food and pet trades, and hence opportunity for genetic contamination through escape and hybridization, may complicate those efforts.

Within the genus *Cuora*, molecular sampling within *C. amboinensis* will no doubt reveal that it is a species complex (C. Ernst, *pers. comm.*), and more complete taxon and geographic sampling will be necessary to sort out relationships within the *C. trifasciata*/*C. cyclornata* complex (compare Blanck et al., 2006, and Spinks and Shaffer, 2006). The fact that *C. trifasciata* hybridizes easily with at least six other species (Vetter and van Dijk, 2006) complicates this work, as does the very recent evidence for mitochondrial introgression and nuclear-mitochondrial pseudogenes in that species (Spinks and Shaffer, 2006).

Finally, within the genus *Mauremys*, the relationships among the European species have been the only significant area of recent contention (Spinks et al., 2004; Feldman and Parham, 2004; Fritz et al., 2006; Le, 2006). Thorough geographic and molecular sampling will be necessary to test the most parsimonious biogeographic hypothesis of monophyly of the European taxa (e.g., see Le, 2006). Resolution of this problem has significant taxonomic implications (e.g., compare Spinks et al., 2004, and Vetter and van Dijk, 2006).

Testudinidae. — As a result of the recent work by Le et al. (2006), Parham et al. (2006b) and other sources cited in Appendix B, the phylogenetic relationships among the genera of tortoises are quite well resolved in the compiled tree (Fig. 14A), even if the generic nomenclature is not (see TTWG, 2007b). However, rigorous phylogenetic hypotheses for species in several problematic genera (e.g., *Homopus*, *Kinixys*, *Psammobates*, *Aldabrachelys/Dipsochelys*, and especially *Testudo*) are still lacking. Because of the tremendous uncertainty surrounding species boundaries in the genus *Testudo* (5 species recognized in Iverson, 1992; 22 recognized in Guyot Jackson, 2004), and concern for conservation in that genus (e.g., Ballasina, 1995), a thorough molecular phylogenetic study of that genus is desperately needed.

Supertree Analyses

Our attempt to produce a single informative supertree for all turtles was unsuccessful. This was in large part due to the necessary restriction of input trees to those produced by maximum parsimony analysis, with reported bootstraps,

Table 1. Summary of primary data partitions on which published trees for turtle families have been based. See Appendix C for full source material. Available but yet unpublished data are indicated with an x.

		Mitochondrial genes					Nuclear genes			
Family	Morphology	cytb	ND4	12/16S rRNA	Control	CO1	cmos	R35	Rag1	Rag2
Chelidae	+	-	-	+	-	+	+	-	-	-
Pelomedusidae	-	-	-	-	-	-	-	-	-	-
Podocnemididae	-	x	x	-	-	-	-	-	-	-
Trionychidae	+	+	+	-	-	-	-	+	-	-
Kinosternidae	+	+	+	-	+	-	-	-	-	-
Cheloniidae	+	+	+	-	+	-	-	-	-	-
Emydidae	+	+	+	+	+	-	-	-	-	-
Geoemydidae	+	+	+	+	-	+	x	+	x	x
Testudinidae	+	+	+	+	+	+	+	-	-	+

and to those with minimal redundancy in character sets, but also to the dearth of published trees for several families and the fact that most molecular phylogenies are based on only a few mitochondrial genes (Table 1). Hence, well-resolved supertrees could not be generated for all families. However, for the cheloniids the supertree and compiled trees were identical (Fig. 9), reflecting the concordance of all three input trees. Unfortunately, as mentioned previously, the nuclear genome has not been sampled for marine turtles.

For the kinosternids, the single perfect supertree (Fig. 11B) differed from the compiled tree in suggesting a paraphyletic genus *Kinosternon*, the placement of the *K. herrerai* clade with the *K. leucostomum* clade, alternative relationships among the species of *Sternotherus*, the incorporation of the *K. hirtipes* group within the *K. scorpioides* group, and alternative relationships among the members of the latter two groups. These disparities apparently reflect the differences between the cytb (Iverson, 1998), ND4 (Starkey, 1997), and control region (Walker et al., 1998; Serb et al., 2001) gene trees included in the supertree analysis. The inclusion of additional genetic data (especially from nuclear genes) will most likely be necessary to resolve these conflicts.

The majority rule supertree for the geoemydids (Fig. 13B) is generally very similar to the compiled tree, with the primary differences being the placement of *R. areolata* within the genus *Rhinoclemmys*; the placement of the monotypic genera *Hardella*, *Notochelys*, and *Leucocephalon*; the basal relationships within the genus *Mauremys*; and the positions within the genus *Cuora* of *C. mccordi*, *C. amboinensis*, and *C. flavomarginata*. Most of the discrepancy between the compiled and supertree was a result of basing the former primarily on published and unpublished multi-locus studies with extensive taxon and character sampling (Spinks et al, 2004; Diesmos et al., 2005; Le, 2006; Le and McCord, in press), whereas the latter was based entirely on three published studies with minimal overlap in gene sampling (Honda et al., 2002a; Spinks et al., 2004; Parham et al., 2004), only one of which (Spinks et al., 2004) included a nuclear gene. Publication of the work by Le (2006) and Le and McCord (in press) may provide nearly complete resolution of the relationship within this family.

Both the consensus and 50% majority rule supertrees produced for the family Emydidae were nearly completely unresolved. For example, neither was able to resolve even the genus *Graptemys* as monophyletic. Hence, those trees are not illustrated nor discussed further.

The input trees for the supertree analysis of the Testudinidae were based primarily on 12S and 16S rRNA and cyt b mtDNA (only Le et al., 2006 included nuclear data), and the resulting majority rule tree was quite different from the compiled tree (Fig. 14A vs. 14B). In addition to not being fully resolved, the majority rule did not recognize the genera *Homopus*, *Geochelone*, or *Chelonoidis* as monophyletic. It also differed from the compiled tree in the placement of *Agrionemys*, *Eurotestudo*, and *Aldabrachelys*; the relationships within *Kinixys*; and the poor resolution among the more derived genera. Additional taxon sampling to supplement that of Le et al. (2006) should clarify these uncertainties.

These preliminary supertree analyses for turtles generally corroborated the results of the compiled tree approach. Discrepancies apparently reflected the incongruence among input trees which were based on variable gene partitions (sometimes overlapping and sometimes not). Our compiled tree approach had the possible advantage of relying more heavily on the most recent, most inclusive phylogenetic analyses, whereas by default the supertree analyses often included trees based on a single gene alongside trees based on multiple genes (sometimes both mitochondrial and nuclear). In any case, the exercise did demonstrate that most recent phylogenetic studies of turtles have focused on but a few mitochondrial genes (Table 1; Appendix C). This has produced some disparity in the resulting trees, particularly among poorly supported nodes. The more recent inclusion of multiple gene datasets (both mt and nDNA; e.g., Engstrom et al., 2004; Spinks et al., 2004; Diesmos et al., 2005; Le et. al., 2006) has produced better resolution in trees, although evaluation of individual gene trees is needed in order to determine which genes contributed most strongly to that resolution. Once both taxon and gene sampling are more complete for turtles, comparisons among single gene trees, trees based on total evidence, and supertrees based on individual gene trees as input should be very informative.

Conclusions

The last decade has seen amazing progress in the search for the "tree of life" for turtles, and this progress has had many ancillary benefits to turtle taxonomy and conservation. However, for this progress to continue, the next decade must see greater attention paid to comprehensive sampling of both markers and taxa in molecular studies (including subsampling within species). The value of many otherwise excellent studies over the past decade has been diminished because closely related taxa were not adequately sampled, because outgroups were inappropriately chosen, or because analysis relied too heavily on small regions of the genome. Emerging genetic resources show promise in overcoming the marker limitation issue. Engstrom et al. (2007) compiled all known primer pairs for turtles and found that many mtDNA primer pairs are known to be useful across turtles, but that nuclear sequence markers are in short supply. A bacterial artificial chromosome (BAC) library was recently constructed for *Chrysemys picta bellii* and has been employed to develop a set of 96 new nuclear markers, many of which appear to be useful across turtles (Shaffer and Thomson, 2007; R.C. Thomson, S.V. Edwards, and H.B. Shaffer, unpubl. data). These resources, coupled with increasing cooperation in assembling tissue banks within the academic and herpetocultural communities, make an attempt at recovering the tree of life for all turtle species using a comprehensive multi-marker approach a reason-

able goal in the near future. We hope that this summary of current phylogenetic hypotheses for turtles will guide future investigators appropriately.

We also conclude by offering two comments concerning the impact of phylogenetics on turtle taxonomy. First, we understand the temptation of authors to propose taxonomic changes (sometime extensive) whenever a new well-resolved tree is at variance with current taxonomy (e.g., see the discussion regarding the genus name *Emys* by the Turtle Taxonomy Working Group, 2007a). However, for the sake of nomenclatural stability, we recommend restraint in proposing taxonomic changes until taxon and character sampling are adequate to provide robust support for such changes. To do otherwise will add confusion to an already complex literature (see Frazier, 2006, and Bour, 2006, for one example), and may even hamper conservation efforts for this unique and imperiled clade of vertebrates (TTWG, 2007a).

Second, because zoological taxonomy is still operating under the rules of ICZN (but see TTWG, 2007a), binomial nomenclature is ultimately based on type specimens. It is therefore essential that future workers take seriously the goal of including type specimens in their analyses, if for no other reasons than to be sure that taxonomic names are being applied appropriately (e.g., see Guicking et al., 2002; Parham et al., 2004; Blanck et al., 2006 and Lehn et al., 2007) and that we are not overlooking cryptic diversity in turtles.

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APPENDIX A

Literature that includes phylogenetic trees for Recent taxa of turtles through 2006. Paleontological papers with minimal focus on living taxa are excluded (including a number by Auffenberg and Gaffney). Papers reporting networks of relationships are also included.

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APPENDIX B

Literature sources on which the compiled trees for turtles were based. Most full citations appear in Appendix A; those listed here lacked phylogenetic trees.

Family level (based primarily on Near et al., 2005; Fujita et al., 2004; Shaffer et al., 1997; and Noonan, 2000; but see Krenz et al., 2005, and Parham et al., 2006a, for the positions of the Chelydridae and Platysternidae, respectively).

Chelidae (based primarily on Georges and Thomson, 2006, McCord et al., 2002, and a 50% majority rule tree based on a parsimony analysis of the data matrix in Bour and Zaher, 2005). Additional sources included Derr et al. (1987), Georges et al. (1998), and the following:

THOMSON, S., GEORGES, A., AND LIMPUS, C.J. 2006. A new species of freshwater turtle in the genus *Elseya* (Testudines: Chelidae) from central coastal Queensland, Australia. Chelonian Conservation and Biology 5:74-86.

Pelomedusidae (based primarily on a preliminary interpretation of morphology from Bour, 1983 and unpublished). Additional sources included Noonan (2000), and the following:

BOUR, R. 1983. Trois populations endémiques de genre *Pelusios* (Reptilia, Chelonii, Pelomedusidae) aux îles Seychelles; relations avec les especes africaines et malgaches. Bull. Mus. Natl. Hist. Natur. Paris 4(5):343-382.
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BOUR, R. 2000. Une nouvelle espèce de *Pelusios* du Gabon (Reptilia, Chelonii, Pelomedusidae). Manouria 3(8):1-32.
BOUR, R., AND MARAN, J. 2003. Une nouvelle espèce de *Pelusios* de Côte d'Ivoire (Reptilia, Chelonii, Pelomedusidae). Manouria 6(21):24-43.

Podocnemididae (based mainly on Starkey et al., unpublished MS;

Noonan, 2000; and Noonan and Chippindale, 2006).
Trionychidae (based on Engstrom et al., 2002 and 2004).
Cheloniidae (based primarily on Bowen and Karl, 1997); additional sources included Dutton et al. (1996), and Parham and Fastovsky (1997).
Chelydridae (based on Phillips et al., 1996; and Shaffer et al., in press).
Kinosternidae (based primarily on Iverson, 1998); additional sources included Hutchison (1991); Serb et al. (2001); and Walker et al. (1998).
Emydidae (based primarily on Stephens and Wiens, 2003b); additional sources included Fritz et al. (2005); Seidel (2002); Starkey (1997); and Starkey et al. (2003).
Geoemydidae (based primarily on Spinks et al., 2004; Le, 2006; Le and McCord, in review); additional sources included Barth et al., (2004); Diesmos et al. (2005); Feldman and Parham (2004); Guicking et al. (2002); Parham et al. (2004); Praschag et al. (2006); Stuart and Parham (2004), and the following:

MOLL, E.O. 1986. Survey of the freshwater turtles of India. Part I: The genus *Kachuga*. Journal of the Bombay Natural History Society 83:538-552. [*Kachuga*]

MOLL, E.O. 1987. Survey of the freshwater turtles of India. Part II: The genus *Kachuga* . Journal of the Bombay Natural History Society 84:7-25. [*Kachuga*]

Testudinidae (based primarily on Le et al., 2006); additional sources included Baard (1990); Cunningham (2002); Fritz, et al. (2005); Iverson et al. (2001); Loveridge and Williams (1957); Parham et al. (2006b); Reynoso and Montellano-Ballesteros (2004); and the following:

BROADLEY, D.G. 1993. A review of the southern African species of *Kinixys* Bell (Reptilia: Testudinidae). Annals of the Transvaal Museum 36(6):41-52.

PERÄLÄ, J. 2001. A new species of *Testudo* (Testudines: Testudinidae) from the Middle East, with implications for conservation. Journal of Herpetology 35:567-582.

APPENDIX C

Compilation of candidate trees for supertree analysis. These studies each involved extensive character and taxon sampling, and either reported bootstraps or included raw data that allowed us to calculate bootstraps by resubmitting the data to maximum parsimony analysis ("reran"). For each entry, citation is followed by the text figure depicting the tree, a summary of the data set on which the tree was based, and the method of phylogenetic anlysis used (MP = maximum parsimony; ML = maximum likelihood; NJ = neighbor joining; and MB = MrBayes). Figure numbers in **bold** are those chosen as input trees for the supertree analyses. Some trees were collapsed to species level (so indicated).

Family level			
Shaffer et al. (1997)	Fig. 4a	892 cytb	MP
	Fig. 4b	325 12S rDNA	MP
	Fig. 4c	892 cyt b and 325 12S rDNA	MP
	Fig. 4d	115 morphology	MP
	Fig. 5a	892 cytb, 325 12S rDNA, 115 morphology	MP
	Fig. 5b	115 morphology with fossils	MP
	Fig. 5c	115 morphology with fossils	MP
	Fig. 5d	892 cytb, 325 12S rDNA, 115 morphology with fossils	MP
Cervelli et al. (2003)	Fig. 7 right	270 U17 snoRNA	MP (bootstraps w and w/o indels)
Fujita et al. (2004)	Fig. 4	1093 R35 nuclear intron	ML/ML/MP/MP
Krenz et al. (2005)	Fig. 4A	2793 RAG-1	MP
	Fig. 4B	2793 RAG-1	MB
	Fig. 5A	2793 RAG-1, 892 cyt b, 325 12S rDNA	MP
	Fig. 5B	2793 RAG-1, 892 cyt b, 325 12S rDNA	MB (Note: Fig 1 is Shaffer et al., 1997 with bootstraps)
Near et al. (2005)	Fig A1	892 cytb, 2790 RAG-1, 1009 R35	MB (bootstraps only >95%)
Parham et al. (2006a)	Fig. 3	7.2-16.2kb mtDNA	MP
Chelidae			
Seddon et al. (1997)	Fig. 3	411 12S rRNA	MP
Georges et al. (1998)	Fig. 1	394 12S rRNA, 474 16S rRNA, 345 CO1, 365 c-mos	MP weight/MP not/ML (only >70% bootstraps)
	Fig. 2	12S rRNA, 474 16S rRNA	MP weight/MP not/ML (only >70% bootstraps reported)
	Fig. 3	394 12S rRNA, 474 16S rRNA, 345 CO1	MP weight/MP not/ML (only >70%)
	Fig. 4	consensus of Figs 1-3	MP weight/MP not/ML (no bootstraps)
McCord et al. (2001)	Fig. 2	18 morphological	MP (no bootstraps; JBI reran)
Bour and Zaher (2005)	Fig. 7	19 morphological	MP (no bootstraps; JBI reran)
Pelomedusidae/Podocnemididae			
Noonan (2000)	Fig. 1	921 12S and16S rRNA	MP (and ML)
Starkey et al. (unpubl.)	Fig.	cytb and ND4	MB
Trionychidae			
Meylan (1987)	Figs. 31-34	no bootstraps, but see Engstrom et al 2004	
Weisrock and Janzen (2000)	Fig. 1	806-811 cytb	MP (collapsed)
	Fig. 2	806-811 cytb	NJ
Engstrom and McCord (2002)	Fig. 1	731 ND4/Hist	ML/MP
Engstrom et al. (2004)	Fig. 1	reanalysis of Meylan 1987 with bootstraps	MP
	Fig. 4	735 ND4/His, 1144 cyt b, 1063 R35 separate & combined	MB
	Fig. 5a	3 genes plus morphology	MP
	Fig. 5b	DNA data only	ML
	Fig. 5c	DNA data only	MB
	Fig. 5d	DNA plus morphology	MB
Kinosternidae			
Starkey (1997)	Fig. 19	992 ND4-Leu	NJ ("*leucostomum*" sample is bad)
	Fig. 20	992 ND4-Leu	MP ("*leucostomum*" sample is bad)
Walker et al. (1998)	**Fig. 2**	402 control region	Min evol method (but MP bootstraps)
Iverson (1998)	**Fig. 2**	290 cytb, 34 protein, 27 morphological	MP
Serb et al. (2001)	**Fig. 2**	1158 control region	MP
	Fig. 3	1158 control region	NJ

Cheloniidae/Dermochelyidae			
Bowen et al. (1993)	**Fig. 1 right**	503 cytb	MP bootstraps (but only > 85%) (collapsed)
Dutton et al. (1996)	Fig. 3a.	907 ND4-LEU	MP
	Fig. 3b	526 control region	MP
	Fig. 4b	ND4-LEU, cytb (from Bowen et al. 1993)	MP
	Fig. 4a	ND4-LEU, cytb, control	MP
Bowen and Karl (1997)	Fig. 2.1 top	repeat of Dutton et al 1996	MP
	Fig. 2.1 low	"anonymous mtDNA" (Karl et al. unpublished)	MP
Parham and Fastovsky (1997)	**Fig. 4**	24 morphological	MP no bootstraps (JBI reran)
Emydidae			
Lamb et al. (1994)	**Fig. 6**	74 restriction sites, 380 cytb, 344 control region	MP
Bickham et al. (1996)	**Fig. 3**	556 16S rRNA	MP
	Fig. 4 top	556 16S rRNA	MP
	Fig. 4 bottom	556 16S rRNA	MP
Starkey (1997)	**Fig. 15**	992 ND4-Leu	MP
	Fig. 16	992 ND4-Leu	NJ
	Fig. 17	992 ND4-Leu	MP (positions weighted)
Lamb and Osentoski (1997)	Fig. 3	386-440 cytb, 216-246 control region	MP
Feldman and Parham (2001) (and 2002)	**Fig. 2 (left)**	1200 cytb/threonine, 900 ND4/His/Ser/Leu	MP
Seidel (2002)	Fig. 2	23 morphological	MP (collapsed)
Stephens and Wiens (2003b)	Fig. 7	225 morphological, 345 control region, 1181 cytb	MP
[Note: this paper includes 12 other trees with bootstraps for small partitions of overall data set, e.g. a gene at a time]			
Geoemydidae			
Yasukawa et al. (2001)	Fig. 3	35 morphological	MP (no bootstraps; JBI reran)
	Fig. 4	35 morphological	NJ (no bootstraps)
Parham et al. (2001)	Fig. 3 top	700 CO1, 900 ND4/His/Ser/Leu	MP (lower: ML w/o bootstraps)
Honda et al. (2002a)	Fig. 2a	410 12S, 472 16S rRNA	NJ (all with bootstraps > 50%)
	Fig. 2b	410 12S, 472 16S rRNA	ML (all with bootstraps > 50%)
	Fig. 2c	410 12S, 472 16S rRNA	MP (all with bootstraps > 50%)
Guicking et al. (2002)	Fig. 2	982 cytb	MP (collapsed)
Spinks et al. (2004)	Fig. 2	1140 cytb	ML (but MP bootstraps)
	Fig. 3	1140 cytb, 400 12S rDNA, 1000 R35	ML (but MP bootstraps / MB >95%)
Parham et al. (2004)	**Fig. 1**	831 CO1, 892 ND4/His/Ser/Leu (mtDNA)	MP
Feldman and Parham (2004)	Fig. 1A	831 CO1, 892 ND4/His/Ser/Leu (mtDNA)	MP (collapse)
	Fig. 1B	831 CO1, 892 ND4/His/Ser/Leu (mtDNA)	MB
Stuart and Parham (2004)	Fig. 1	831 CO1, 892 ND4/His/Ser/Leu (mtDNA)	MP
	Fig. 2	831 CO1, 892 ND4/His/Ser/Leu (mtDNA)	ML
Barth et al (2004)	Fig. 2	1080 cytb/threonine	MP/ML/NJ
	Fig. 3A	1080 cytb/threonine	MP/ML (different taxa)
	Fig. 3B	1080 cytb/threonine	ML/MB/NJ
Diesmos et al. (2005)	Fig. 2	cytb, 12S, R35 from Spinks et al (2004) with *leytensis*	MP
Le and McCord (in press)	Fig. 4	1140 cytb, 409 12S, 580 16S, 602 cmos, 642 Rag1	MP
Testudinidae			
Lamb and Lydeard (1994)	Fig. 3A	352 cytb	MP (unweighted)
	Fig. 3B	352 cytb	MP (transversions weighted)
Caccone et al. (1999)	Fig. 2 top left	401 12S rRNA	MP
	Fig. 2 top rt	568 16S rRNA	MP
	Fig. 2 low left	386 cytb	MP
	Fig. 2 low rt	combined	MP (bootstraps in Table 3)
Meylan and Sterrer (2000)	Fig. 8	28 morphology	MP (no bootstraps; ML reran)
Gerlach (2001)	Fig. 5	66 morphological	MP (bootstraps "92-100%"; JBI reran)
Iverson et al. (2001)	**Fig. 1**	1094 cytb	MP/NJ
van der Kuyl (2002)	**Fig. 2A**	404 12S rRNA	MP (collapsed)
	Fig. 2B4	404 12S rRNA	ML (no bootstraps)
	Fig. 2C	404 12S rRNA	NJ
Palkovacs et al. (2002)	Fig. 2A	386 cytb, 403 12S rRNA, 568 16S rRNA	MB
	Fig. 2B	386 cytb, 403 12S rRNA, 568 16S rRNA	ML
	Fig. 3A	386 cytb, 403 12S rRNA, 568 16S rRNA	MP
	Fig. 3B	386 cytb, 403 12S rRNA, 568 16S rRNA	NJ
Caccone et al. (2002)	Fig. 4	430 12S, 553 16S, 416 cytb, 934 control, 1790 ND5, 520 ND6	ML/MP/NJ/MB
Cunningham (2002)	**Fig. 5.8**	1167 cytb+ND4	MP
Perälä (2002)	Fig. 3	61 morphological	MP
	Fig. 4	61 morphological	MP (only outgroup differs from Fig. 3)
Semyenova et al. (2004)	Fig. 5	213 RAPD fragments	UPGMA
Fritz et al. (2005)	Fig. 2	1124 cytb	NJ
	Fig. 3	1124 cytb	MP (collapsed)
	Fig. 5	84 ISSR fingerprints	NJ
Le et al. (2006)	**Fig. 2**	1140 cytb, 408 12S, 583 16S, 602 cmos, 654 Rag2	MP
	Fig. 3	1140 cytb, 408 12S, 583 16S, 602 cmos, 654 Rag2	ML/MB
Parham et al. (2006b)	**Fig. 3**	14858 complete mtDNA	MP/ML/MB

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ABSTRACT. – Freshwater and terrestrial turtles are among the most imperiled biota on the planet, with nearly half of all extant taxa threatened with extinction. Active science-based management is required for the persistence of many species. Evolutionary genetic principles are often overlooked in the development of conservation and management plans, yet genetic data and theory can be critical to program success. Conservation biologists are encouraged to consider using genetic data and concepts when developing conservation strategies for turtles. We identify general areas where genetic principles and empirical data can be profitably used in conservation planning and provide examples from the turtle literature. Finally, we suggest important areas for future research in chelonian conservation genetics.

KEY WORDS. – Reptilia; Testudines; adaptive potential; conservation; forensics; genetic diversity; genetic drift; gene flow; inbreeding; management units; mating systems; outbreeding; taxonomy; trade; turtle

Turtles and tortoises are threatened globally. Approximately 40% (129 taxa) of over 300 extant taxa are regarded as vulnerable or endangered, and many face extinction if effective conservation measures are not implemented. Widespread declines in abundance and distribution documented in recent decades have been caused by habitat destruction, pollution, and overexploitation for trade in meat, pets, and traditional medicines (Gibbons et al., 2000; van Dijk et al., 2000; Turtle Conservation Fund, 2002; Moll and Moll, 2004). The number and intensity of pressures continue to mount, with climate change looming as a new threat, particularly for species with temperature-dependent sex determination (Janzen, 1994; Davenport, 1997; Nelson et al., 2002; Miller et al., 2004; Booth, 2006). Removal or amelioration of immediate threats does not necessarily ensure the persistence of endangered taxa or populations. Remnant populations are more often than not, small and highly fragmented, attributes that exacerbate their vulnerability to extinction from stochastic events and loss of genetic diversity (Lande, 1998; Hager, 1998).

Genetic diversity represents the raw material to facilitate adaptation to changing environmental conditions through natural selection. Hence, loss of genetic diversity can result in the loss of adaptive potential. Global environmental change is occurring at a rate unseen in the history of our planet (Hare and Meinshausen, 2006; Lenton, 2006; Li et al., 2006). If chelonian species are to adapt and persist in the face of future changes, they will likely require active human intervention. Maintaining required levels of genetic diversity is only possible through conservation planning.

Knowledge of genetics is increasingly recognized as a critical element of conservation biology (Moritz, 1994; Soltis and Gitzendanner, 1999). Molecular techniques and methods of statistical analysis derived from evolutionary theory can be used to estimate how genetic diversity is apportioned spatially, how rapidly diversity will be lost over time, to identify crucial forces (anthropogenic or otherwise) contributing to present and future loss of diversity, to gain insight into fundamental aspects of an organism's biology, and to provide informed guidance for conservation and management (Moritz, 1999; Reed and Frankham, 2003; DeYoung and Honeycutt, 2005; Whiteley et al., 2006). Despite the clear importance of genetics as a foundation for understanding turtle biology and directing turtle conservation actions, there is a paucity of turtle genetic studies relative to many other taxa.

We describe how population genetic theory and data can contribute to greater understanding of turtle biology and how this knowledge can be applied to achieve conservation objectives. We address eight major genetic issues that we believe are most relevant to turtle conservation: 1) genetic diversity and potential for future adaptation; 2) genetic drift; 3) inbreeding and outbreeding; 4) selection; 5) gene flow and identification of management units; 6) clarifying taxonomy;

7) elucidating aspects of species' behavior and ecology; and 8) forensics. We provide a glossary of terms (highlighted in bold in the text) that are widely used in population genetics but may not be well known to biologists interested in turtles. Boxes are also included to emphasize several important concepts discussed in the text.

We have written the text to be accessible to the non-specialist and have minimized the use of technical terms. Background theory and concepts are developed and empirical examples are presented to show relevance in areas of turtle conservation. We conclude by suggesting future priorities and directions. We advocate the use of genetics as only one component of a comprehensive conservation toolkit. Genetic principles and data should be complemented with biological, ecological, zoogeographic, socio-economic and other relevant data in order to better direct decisions regarding chelonian conservation and management.

Genetic Diversity and Adaptive Potential

Genetic diversity is a fundamental component of life on earth. Without it, there can be no evolution, no diversification, and thus, little or no biodiversity at any level of biological organization. In a contemporary sense, without genetic diversity, populations cannot respond to biological or environmental changes through natural selection, be those changes natural or anthropogenic in origin (Frankham, 1995a, 2005; Amos and Balmford, 2001).

The **phenotype** of an organism (its observable properties) is determined by an individual's **genotype**, the expression of which is modified by the environment. Adaptation occurs when the phenotypic composition of a population shifts in response to environmental change. The new generation will preferentially represent the genetic composition of parents best able to cope with changes through their ability to survive and leave offspring. The resulting shift in genetic composition of the population reflects adaptation by **natural selection** (Orr, 2005). In the lifetime of an individual, responses to environmental change occur via **phenotypic plasticity** (non-heritable changes in phenotype such as faster growth when conditions are favorable). However, the capacity of an individual to be plastic also has a genetic basis. Variation is required at the level of **genes** coding for traits (Via, 1993; Bradshaw, 2006). Thus, phenotypic plasticity is itself an evolved trait.

The rate of adaptive **microevolution** is roughly proportional to the **additive genetic variance**. Loss of genetic diversity is a fundamental concern in conservation biology because a populations' ability to evolutionarily adapt to changing conditions is reduced when additive genetic variation is depleted (Amos and Balmford, 2001; Frankham, 2005). Given current rates of environmental change, the adaptive potential of populations will be critically linked to their probability of long-term persistence.

Levels of genetic diversity can be assayed by measuring variances and covariances in phenotypic traits among individuals. The field of **quantitative genetics** apportions variation in phenotypic traits resulting from complex interactions between heritable genetic and environmental sources of variation. **Quantitative trait loci** (QTL) are the most relevant targets of genetic studies of phenotypic adaptation (Falconer and MacKay, 1996; Lynch and Walsh, 1998; Barton and Keightley, 2002). However, quantitative genetic studies are difficult to conduct. Established pedigrees and/or large sample sizes are required to disentangle the effects of environment and genotype on quantitative traits (Falconer and MacKay, 1996; Lynch and Walsh, 1998; Barton and Keightley, 2002; Kirkpatrick and Meyer, 2004). It is often impossible to obtain large sample sizes from small wild populations, and establishing pedigrees is difficult and time-consuming. Small population sizes, long generation times, secretive mating habits, and the potential for long term sperm storage by females render turtles difficult subjects for quantitative genetic studies.

Genetic studies that employ **neutral genetic markers** are easier to conduct than quantitative genetic analyses. These two approaches differ because variation at neutral loci is presumably not subject to natural selection, but governed primarily by drift, **mutation**, and migration (Merila and Crnokrak, 2001; Holderegger et al., 2006). The adaptive potential of populations has frequently been inferred from population characteristics identified using neutral genetic markers, under the assumption that neutral and adaptive variations are positively correlated. Some empirical studies suggest that neutral markers can be predictive of variation at quantitative trait loci (Merila and Crnokrak, 2001), whereas other studies found no significant correlation (Reed and Frankham, 2001). The degree of correlation between the two measures of genetic variation will depend on the force of selection pressures on quantitative traits. Traits under the strongest local selection are expected to exhibit the greatest divergences from neutral variation. Traits that are not under selection will be largely shaped by the same microevolutionary forces as neutral regions (McKay and Latta, 2002). Neutral markers therefore must be evaluated carefully to infer adaptive variation. New emerging molecular technologies such as **genome**-wide scans will aid in development of measures of adaptive variation because these techniques can detect loci under selection in the absence of *a priori* knowledge of gene function (Schlotterer, 2003; Luikart et al., 2003; Nielsen, 2005; Storz, 2005; Kohn et al., 2006; see also McGaugh et al., 2007).

An on-going debate in conservation biology concerns the relative importance of adaptive versus neutral genetic variation when weighing conservation options (Merila and Crnokrak, 2001; McKay and Latta, 2002; Holderegger et al., 2006). **Heritability** measured for QTLs and **heterozygosity** (a measure of variation assayed using neutral molecular or biochemical markers) may both be related to current population **fitness** (Reed and Frankham, 2003). Thus, neutral genetic variation and trait heritability may both be useful as surrogates of population fitness and may be used to prioritize populations for conservation. The value of each approach for

conservation and management of chelonians will be highlighted by brief discussion of two published examples.

Janzen (1992) estimated the **heritability** of pivotal temperature (T_{piv}) determining sex (i.e., the incubation temperature that produces a 1:1 sex ratio) for common snapping turtles (*Chelydra serpentina*). A standard quantitative genetic breeding design was not possible because *C. serpentina* takes around 10 yrs or more to reach reproductive maturity (Iverson et al., 1997). Instead, eggs from 15 clutches were incubated near the T_{piv} for the population, such that the among-clutch variation in sex ratio could be interpreted statistically as quantitative genetic variation. Under controlled conditions, heritability of T_{piv} was estimated as 0.76 (possible range of 0 to 1) at 28ºC, suggesting substantial quantitative genetic variation for sex ratio. In nature, the temperatures of turtle nests are influenced by the environmental conditions in the area of the nest (e.g., soil moisture, canopy cover, aspect, etc.). When accounting for variations in the temperature of nests in a natural population of *C. serpentina* the *effective* heritability of T_{piv} reduced to 0.05, implying that genetic factors have a minimal effect on sex ratios compared to environmental factors. Anthropogenic habitat alterations to nest thermal environments can greatly influence offspring ratios in turtles with temperature-dependent sex determination. Active management may be required to maintain equitable sex ratios for populations nesting in thermally-altered habitats.

Molecular and/or biochemical genetic markers can also provide estimates of levels of genetic diversity. Beheregaray et al. (2003) used two different neutral genetic markers (nuclear **microsatellites** and mitochondrial DNA [**mtDNA**]) to estimate levels of genetic variability within and among four island populations of Galápagos tortoises (*Geochelone nigra*). Use of markers with different rates of mutation to new alleles facilitates estimation of the relative importance of contemporary vs. historical factors on population levels of genetic diversity. Microsatellites, with their faster rates of mutation, will illuminate the more contemporary situation compared to mtDNA (Avise et al., 1992). Analyses of sequence variation in the mtDNA control region revealed long-term evolutionary divergence *among* populations on the four islands that was concordant with the geographic history of the region. Interestingly, for the island of Pinzón, there was evidence of historical population growth and retention of high levels of diversity (estimated from 10 microsatellite loci) *within* the population despite the populations' near extinction in the 1920s from predation by the introduced black rat. Survivors of the island population had maintained higher levels of genetic diversity than expected from population genetic theory. Hence, conservation efforts for Galápagos tortoises may be best directed at retaining the relatively high existing genetic variability in two populations (Pinzón and La Caseta), and intensively managing to reduce further loss in two genetically depauperate populations (San Cristóbal and Cerro Fatal). Genetic studies as described above can be used to assess the merits of alternative management actions.

Genetic Drift

Genetic drift arises from chance fluctuations in **allele** frequencies from one generation to the next. Even if individuals mate randomly within populations, changes in **allele frequency** will occur each generation. Due to chance alone, not all alleles will be present in the next generation, because not all individuals will successfully reproduce. Genetic drift is often described as a 'sampling effect' in which individuals produced in each generation represents a sample of the alleles in the ancestral **gene pool** of previous generations. Genetic drift is greater in smaller relative to larger populations (Nei et al., 1975). For example, assume on average 70% of a turtle population is at a reproductive age. Not all sexually mature individuals will produce progeny for a given year for a variety of reasons, such as not finding a mate, poor nest site choice, predation of eggs, etc. Hence, effectively, only a fraction of the population will contribute genetically to the next generation and represents the **effective population size** (see Box 1). If the effective population size is small, then there is a greater chance that the "sample" will diverge in allelic composition from that of the overall gene pool. Thus the allele frequencies in the gene pool will drift.

If population numbers decline dramatically (i.e., the population experiences a **bottleneck**) or sex ratios become heavily skewed, or variance in male or female reproductive success is high, the effective population size (Ne) will be small and the probability that offspring represent a random sample from the original gene pool will be low. As a consequence of low Ne, alleles will be lost, particularly those present at low frequencies. When few alleles are present in the gene pool, opportunities for **heterozygous** combinations of alleles at a **locus** are reduced, and overall diversity will decline with each successive generation (see Box 2 for more detail). The rate of loss of diversity in a bottlenecked population depends on several related factors, including population size, severity and duration of the bottleneck, generation time, and gene flow (Allendorf, 1986; Hedrick and Miller, 1992; Richards and Leberg, 1995; Newman and Pilson, 1997; Garza and Williamson, 2001).

Kuo and Janzen (2004) used **neutral genetic markers** to compare the genetic diversity of a small, isolated population of imperiled ornate box turtles (*Terrapene ornata*) to that of a large population located within the main range of the species. Theory predicts that the small population size of the isolated population should over time lead to reduced genetic diversity due to the effects of genetic drift, relative to the large population. Genetic diversity was assessed using 11 polymorphic, nuclear microsatellite DNA loci for ca. 75 turtles from each population. Contrary to expectations, measures of genetic diversity did not differ between the two populations. However, the small population had a genetic signature that indicated a bottleneck in population size (that

Box 1: Calculating Effective Population Size

The effective population size is the number of individuals in an "ideal" population having the same magnitude of random genetic drift, or loss of genetic diversity, or increase in inbreeding as observed. Effective population size is often less than the total population size due to the fact that not all individuals contribute equal numbers of progeny to the next generation. Effective population size can be estimated either using population genetic data or demographic parameters.

N_e estimated using demographic data-- If the number adult males and females is known, effective population size can be estimated as:

$$N_e = 4N_mN_f/(N_m+N_f)$$

where N_m and N_f are the number of breeding age males and females respectively (Nunney and Elam, 1994). This equation defines the probability that 2 randomly selected genes in the current generation are copies of the same parental gene.

N_e estimated using empirical genetic data.-- Population allele frequencies change over time as a function of N_e and elapsed time in generations (t). Over small time intervals ($t<<2N_e$), and assuming that changes in allele frequency are due to drift, the expected variance in allele frequency [E(Fc)] is approximately $t/(2N_e)$. Using adults for a species which exhibits discrete non-overlapping generations, Waples (1989) defined the variance in allele frequency (Fc) between the 2 samples, which can be estimated for each locus as:

$$Fc = \left(\frac{1}{k}\right)\sum_{i=1}^{k}\frac{(x_i - y_i)}{(x_i + y_i)/2 - x_iy_i}$$

where x_i and y_i are the allele frequencies of the i^{th} of k alleles for adults in time periods t and t+1, respectively. Thus, Fc can be used to estimate N_e. Fc (variance in population allele frequency) must be estimated by Fc' (variance in sample allele frequency), which is also affected by random sampling errors in computing sample allele frequencies. Effective population size can be estimated by incorporating the variance in allele frequency due to the finite population size (genetic drift) and due to variation as a function of the finite number of samples used to estimate allele frequencies.

$$N_e = \frac{t}{2F_c - 1/(2S_o) - 1/(2S_t) + 1/N]}$$

where S_0 and S_t are the number of individuals samples in generations 0 and t. We can also estimate the effective number of breeders (not effective population size) using parent-offspring data (i.e., where t=1). This number can be adjusted to estimate effective population size. For example, for anadromous salmonids, Waples (1990) has shown that $N_e \sim gN_b$ where N_b is the number of breeders and g is the generation length (or average age of breeders) in the adult breeding population. With overlapping generations (i.e., breeding adults of several age classes contributing progeny to the next generation), estimating expected genetic drift becomes more difficult. N_e as defined above based on the temporal method must be corrected based on estimates of age-specific fecundity and survival (see Jorde and Ryman, 1995 for a review and for calculations).

had occurred based on theoretical expectations). Why was there no detectable difference in levels of genetic diversity between populations differing in current numerical abundance despite a bottleneck persisting for 100–200 yrs?

Ornate box turtles have a relatively long lifespan, living on average 22 yrs in the wild (Metcalf and Metcalf, 1985). This longevity, long generation times, and overlapping generations are life-history traits characteristic of turtles that might retard the negative effects of drift on population levels of genetic diversity. The long duration of the bottleneck spanning hundreds of years (and several generations) may have also influenced the retention of genetic diversity. Short, but severe bottlenecks were found by England et al. (2003) to have a greater impact on loss of alleles than bottlenecks of lower severity occurring over several generations.

Not all turtles have retained high levels of genetic diversity after experiencing population bottlenecks. Similar to the ornate box turtle, the gopher tortoise, *Gopherus polyphemus*, in the southeastern United States has suffered a bottleneck persisting for more than a century due to habitat destruction of favored longleaf pine forests, *Pinus palustris*, and harvesting of turtles for food. Populations were reduced numerically by up to 80% (Auffenberg and Franz, 1982). Schwartz and Karl (2005) estimated levels of genetic differ-

Box 2: Predicting the loss of genetic diversity in populations from drift.

Expected loss of genetic diversity from the effects of drift, as measured by heterozygosity, can be predicted based on the population size. Population measures of heterozygosity can be measured as the proportion of individuals heterozygous at a locus. The expected proportion of original heterozygosity remaining after a generation of drift is [1-1/2N]. If population size remains constant over many generations the heterozygosity after t generations (H_t) can be estimated as:

$$H_t = (1-1/2N)^t H_o$$

where H_o is the population heterozygosity in the present population, and N is the adult breeding population size.

Population size and stochastic changes in allele frequency due to drift also have demonstrable effects on other population measures of genetic diversity such as the number of alleles per locus. Consider a diploid locus with n alleles present in frequencies p_1, p_2, p_3……p_n. The expected number of alleles remaining after a single generation (n') of random mating by N adults is:

$$E(n') = n - \sum_{i=1}^{n} (1 - pi)^{2N}$$

The probability that an allele will be lost is a function of the frequency of the allele in the population. Thus, alleles at greatest risk of loss are those that are rare (Allendorf, 1986).

entiation among and diversity within gopher tortoise populations in Florida and Georgia using nine microsatellite loci. Genetic divergence among populations in both regions were high (average pairwise F_{ST} of 0.37 ± 0.17 and 0.14 ± 0.05 among Florida and Georgia populations, respectively). Values of F_{ST} greater than 0.10 are considered to be high (Wright, 1969) indicating restricted migration or **gene flow** (see below and glossary). Populations which are reproductively isolated, for example within highly fragmented landscapes, are more susceptible to loss of genetic variation due to drift.

Founder effects have been well documented, where newly established populations have substantially reduced levels of genetic variance compared to sources (Leberg, 1992; Hedrick et al., 2001). For example, only a small proportion of animals in the captive breeding program of Galápagos tortoises (evaluated for 15 microsatellite markers) contributed to the repatriated population on the island of Española (Milinkovitch et al., 2004). Variance in adult contributions can be attributed to several factors, most likely acting in concert, such as unequal access to mates, variance in fertility, unequal sex ratios, and differential survivorship of offspring. Re-evaluation of the breeding adults to equalize contributions of breeders will ensure that diversity is not compromised in the supplemented island population by the 'sampling effects' (Ramirez et al., 2006; Sigg, 2006).

Inbreeding and Outbreeding

Matings can occur between relatives, even if mating occurs at random and the population size is large. Inbreeding can have severe genetic consequences. The probability of matings between relatives will increase when populations are small in size, particularly if population size remains small over several generations, and in the absence of behavioral mechanisms to preclude inbreeding such as kin avoidance during mate selection. The primary effect of inbreeding is to change genotypic frequencies in favor of homozygous **genotypes** (see Box 3). Inbreeding can also lead to decreased fitness (**inbreeding depression**) due to the expression of **deleterious recessive alleles** through matings with close relatives. Inbreeding depression and the loss of heterozygosity probably contribute to many components of phenotype and fitness, including metabolic efficiency, growth rate, reproductive physiology, and disease resistance (Gilpin and Soule, 1986). The detrimental effects of inbreeding in captive (Ralls and Ballou, 1983) and natural populations (Keller and Waller, 2002) are widely accepted.

Population risk of extinction is related to population intrinsic rate of increase (Lande, 1988). Declines in reproductive output and survival (the basic components affecting population growth) increase proportionally with levels of inbreeding (Falconer and MacKay, 1996). There is a considerable literature from case studies on captive populations (Lacy, 1997), laboratory populations (Frankham, 1995b; Reed et al., 2002), natural populations (e.g., Frankham, 1997; Crnokrak and Roff, 1999; Keller and Waller, 2002), and from meta-analyses (review in Frankham, 2005) and population viability simulations (Brook et al., 2002) that document the negative impact of inbreeding depression and loss of genetic diversity on probabilities of population persistence.

Inbreeding can be a major concern in natural and captive populations of turtles, particularly if populations are small and there is little or no exchange among populations. For many populations, exchange of individuals and genes among populations is becoming infrequent or impossible

Box 3: Estimating Inbreeding in populations.

There are numerous definitions and ways to estimate inbreeding (reviewed in Templeton and Read, 1996). At the population level, inbreeding (F) is a measure of deviation from random mating (Hardy-Weinberg). Population levels of inbreeding can be quantified empirically using molecular or biochemical markers by estimating the excess or deficiency of observed heterozygosity (H_o) relative to heterozygosity expected if populations were mating at random (i.e., under Hardy-Weinberg). For example, expected heterozygosity (H_e) for a locus with 2 alleles with frequencies p and q = (1-p) would be 2pq. F can be estimated as:

$$(H_e - H_o) / H_e \Longrightarrow 1 - (H_o/2pq)$$

Thus, if F is a measure of the proportional deviation of observed from expected heterozygosity observed heterozygosity can be expected to diminish as

$$H_o = 2pq\,(1 - F)$$

and the frequency of homozygous and heterozygous genotypes in the next generation can be estimated as:

Genotypes	AA	Aa	aa
Hardy Weinberg frequencies	p^2	2pq	q^2
Frequencies with inbreeding	$p^2 + pqF$	$2pq(1 - F)$	$q^2 + pqF$

due to habitat fragmentation and human development creating impenetrable barriers to gene flow (see below). Isolated populations of turtles are at high risk of loss of genetic diversity through drift and inbreeding. Since adults of many species are long-lived and have reproductive life spans extending over long periods of time, there is the potential that they could mate with their sons and daughters, even grandsons and granddaughters, as adults. If there are no mechanisms to prevent mating with close relatives (i.e., kin recognition), inbreeding would accelerate loss of genetic variability and could result in expression of lethal recessive alleles leading to lower probabilities of population persistence. Levels of inbreeding will accrue in captive populations with high probability, so considerable attention has been devoted to design of captive breeding programs (Miller and Hedrick, 1993; Ebenhard, 1995; Philippart, 1995; see also Syed et al., 2007).

One way to avoid inbreeding is to **outbreed**. The opposite of inbreeding depression is outbreeding enhancement, which is often referred to as **heterosis** or hybrid vigor (Lerner, 1954). Individuals from different populations are not likely to be homozygous for the same recessive alleles. Thus, outbreeding among individuals from different populations (wild or captive) can lead to masking of different **deleterious recessive alleles** present in different populations. If offspring from outbred matings subsequently contribute reproductively in future generations, and if the deleterious recessive alleles are present in low frequency, then these alleles are likely to be randomly lost from the population after several generations due to simple **Mendelian segregation** and genetic drift. The fitness of individuals and the long-term viability of an outbred population can be higher than that of either parental population due to the reduced frequency of these deleterious recessive alleles.

Outbreeding up to some threshold level (i.e., perhaps between individuals from lineages of divergent populations) would be expected to result in increased population mean fitness. If such a simplistic perspective were indeed true, one universal conservation prescription for turtle populations of conservation concern would be to advocate mating individuals from different populations. However, while inbreeding is essentially a concept formulated on a single locus basis, we

Box 4: Outbreeding depression causes a breakdown in co-adapted gene complexes.

Consider an outbreeding situation demonstrated using two loci. One locus has two alleles (A and a) and the second locus also has two alleles (B and b). There are two populations living in two different environments.

	Pop1	x	Pop2	F1 progeny	Progeny in later generations
Locus 1	AA	x	aa	Aa	AA or Aa or aa
Locus 2	bb	x	BB	Bb	BB or Bb or bb

Individuals in population 1 have 2 locus genotypes AA/bb whereas individuals in population 2 have genotypes aa/BB. If individuals from both populations inter-breed, offspring (F1 progeny) would all be Aa/Bb. The mixing of new alleles within the genetic background that has evolved within the environments inhabited by population 1 and population 2 can lead to problems. In the first generation, we may indeed see an increase in population fitness. If alleles A and B are primarily dominant to alleles a and b, then either AA or Aa genotypes or BB or Bb genotypes will still express the same phenotype. The initial reductions in the frequencies of homozygous recessive genotypes through outbreeding may actually be beneficial. However, expectations are that reductions in population fitness would be seen in later generations, where through Mendelian segregation, potentially maladaptive multi-locus genotypes (e.g., AA/BB, aa/bb) are present in the population.

need to consider outbreeding in the context of the entire **genome**. Declines in fitness can be realized over a much broader spectrum of outbred mating scenarios.

The phenomenon of **outbreeding depression** can be expressed in several ways. Under one scenario, declines in fitness for hybrids or outcrossed genotypes can occur due to "genetic swamping" of locally adaptive genes through gene flow or directed matings from another population that evolved under different ecological settings. We can consider two genotypes AA and BB that evolved in environments 1 and 2, respectively. AA has higher fitness in environment 1 than the BB genotype. Conversely, genotype BB has the higher fitness in environment 2. Hybrid genotype AB is not well adapted to either environment. The presence of inferior hybrid genotypes as a consequence of gene flow and subsequent reproduction will result in decreased population fitness.

The second way in which outbreeding depression can occur is by the breakdown of physiological or biochemical compatibilities between genes that have evolved in different populations. Interactions among alleles at several loci (**epistasis**) collectively affect fitness. Organisms have evolved in the context of specific environments and have evolved suites of genotypes across many genetic loci that are co-adapted to each environment. If new alleles are introduced via gene flow into the genetic background of the resident population, a loss in fitness may result from physiological or biochemical incompatibilities introduced through disruption of these co-adapted gene complexes (see Box 4). The fitness of the entire population could be compromised because outbred progeny are maladapted to either parental environment.

Outbreeding depression and inbreeding depression can occur simultaneously in a population. Fluctuations in population size and gene flow (either natural or directed) of maladaptive alleles can result in inbreeding or outbreeding depression, respectively, in natural populations, potentially reducing population fitness. Ultimately, in the design of breeding strategies, one must weigh the effects of potential past inbreeding in the population (which may have purged some deleterious alleles) relative to the effects of outbreeding on locally adaptive genotypic combinations. For many species of turtles, populations are numerically depressed, and in some cases, the species is only represented in captive populations, potentially represented by few individuals originating from geographically different locales, or even from different taxonomically recognized subspecies or **evolutionarily significant units**. Decisions to breed across genetically and ecologically differentiated groups must weigh the potential detrimental consequences of both inbreeding and outbreeding to probabilities of species persistence.

Selection

Natural selection acts on the phenotypic composition of a population, altering it via the differential survival and reproduction of individuals (Lande and Arnold, 1983). Phenotypes that are better adapted to their environment (i.e., individuals with greater 'fitness') will be preferentially transmitted to the next generation. When the characters under selection have a genetic basis and are inherited, natural selection may result in the differential success of genotypes passing gametes to future generations (Nielsen, 2005). Selection can be decomposed into components, by taking a cohort born at the same time and following changes in the phenotypic and/or genetic characteristics of this cohort through each stage of the life cycle. Selection components include *viability selection* (differential survivorship), *sexual selection* (differential mating success), and *fertility selection* (differential production of offspring).

Selection may be introduced by humans through environmental changes to biotic and abiotic features. In captive populations, selection may be intentional such as a deliberate selection program designed to change some characteristic of the population. Selection can also be an inadvertent side effect of sampling or husbandry procedures, for instance, by selecting a small segment of a population as breeders to produce the next generation. Selecting individuals with specific characteristics or phenotypes may increase the intensity of selection, and lead to loss of genetic variance. For example, in captive colonies of the Mallorcan midwife toad *Alytes muletensis* maintained as breeding stock for reintroductions, allelic richness and heterozygosity both declined in long-term captive bred stocks compared to short-term stocks and wild populations (Kraaijeveld-Smit et al., 2006). The consequences of selection may be a depression in fitness-related traits (e.g., fertility, disease resistance, growth rate) such as those that are related to survival and reproductive success. Consequences of selection in captive breeding programs are most important in situations where captive-reared individuals are released back into their native environment or when there is the possibility of breeding with wild individuals. Genetic monitoring of captive breeding and reintroduction programs is important to ensure that artificial selection does not impede continued success. For turtles and tortoises, there is currently little or no genetic monitoring of successful captive breeding and reintroduction programs (Ballou and Lacy, 1995; see also Syed et al., 2007).

Humans exert an ever-increasing influence on the direction and force of selection acting on species. Average global atmospheric temperatures have increased by approximately 0.6ºC from pre-industrial times to the year 2000, a rate of change much larger than that seen in the past 10,000 yrs (Houghton, 2005). By the year 2100, average global atmospheric temperatures are projected to rise by 2 to 6ºC (Mann and Jones, 2003). To put this predicted shift into perspective, this degree of climate change is one third of that seen in the last ice age that lasted a period of approximately 100,000 yrs (Houghton, 2005). Such dramatic climatic changes will exert strong selective pressure on species to evolve. For instance, even moderate temperature shifts (i.e., as little as 2ºC for the painted turtle, *Chrysemys picta*) can drastically skew sex ratios in reptiles with temperature-dependent sex determination (Janzen, 1994). Skewed sex ratios can result in smaller **effective population sizes**, elevating risks of inbreeding and loss of diversity via drift. Behavioral modi-

fications, such as nest-site choice and altered timing of the initiation of nesting, may compensate for the effects of these local climatic shifts on sex determination (Doody et al., 2006), although selection would also act on other aspects. For example, juvenile mortality may increase as turtles experience prolonged higher temperatures; reduced hatchling recruitment was found in *Chrysemys picta* after a particularly long hot summer in 1988 (Janzen, 1994). Given these startling projections, can turtles and tortoises evolve at a pace that is rapid enough to compensate for the negative fitness consequences of global warming?

Theory predicts that the maximum rate of sustainable evolution for a population, or conversely, the maximum rate of environmental change that can be tolerated, can be inferred on the basis of the interactions of evolutionary forces on quantitative genetic variation (Lynch and Walsh, 1998). In the absence of immigration, the rate of phenotypic evolution can become limited by the availability of **additive genetic variance**. If the rate of environmental change is too high, selective pressures (e.g., impacting survival and/or fecundity) could exceed a population's capacity to assimilate new genetic variation via mutation and maintain a positive growth rate, especially for organisms with long generation times such as turtles. If so, the inevitable outcome would be extinction. If the rate of environmental change is sufficiently slow, and if the amount of genetic variation relative to environmental variation is sufficiently high, the population may be able to evolve very rapidly in response to this change. Overall, the capabilities of turtles to respond to and survive the impacts of environmental change such as global warming will depend on the rate of climatic change (i.e., the intensity of selection) and the degree of genetic variance within each population for the key traits. In the face of global warming, maximizing the adaptive genetic diversity at the population, landscape, regional, and species scales is paramount to the survival of turtles and tortoises in the 21st century and beyond.

Gene Flow and Management Units

Gene flow is defined as the movement of alleles from one population to another. Such migration is an evolutionary force that counters the effects of genetic drift and inbreeding within each population. Gene flow among populations is often summarized as the average fraction of individuals in each population in each generation that has contributed genes derived from another. Gene flow can be measured directly from field techniques of mark-recapture and tracking individuals, and indirectly by applying various mathematical models of population structure to genetic data (i.e., the island model vs. stepping stone model vs. isolation-by-distance model).

There are several reasons to expect that direct measures of movements may differ from indirect measures of gene flow (Slatkin, 1985). First, gene flow in the strict sense refers to the transfer of genes from one population to another. Migration, as quantified by direct observations, documents the physical presence of an individual in more than one population at two or more time periods. Direct observations provide no information about the likelihood of breeding, and thus actual gene flow per se. Further, inferences from direct observations are only germane to those populations where observations were made. Gene flow can occur over much broader areas and the indirect genetic-based estimates can provide accurate measures from population to landscape scales.

Further, direct observations chronicle the extent of movements only over the period of observation but provide no information regarding historical levels of dispersal. Genetic measures of gene flow report the cumulative effects of past and contemporary gene flow. However, for many populations of conservation or management concern, present levels of gene flow are of special interest. If rates of gene flow and/or effective population size had historically been high, then estimates of gene flow may not reflect present conditions. For example, high levels of gene flow and little population genetic structuring (panmixis) were documented for the geometric tortoise (*Psammobates geometricus*). Populations of *P. geometricus* are now severely fragmented, and the indirect measures of gene flow reflect the historical high levels of connectivity rather than the current fragmented condition. In contrast, direct and indirect methods for estimating gene flow yielded similar results in the freshwater turtle *Hydromedusa maximiliani*, with very restricted movements suggesting a **metapopulation** structure within drainages (Souza et al., 2002).

Understanding the use of terrestrial and aquatic habitats by local breeding populations of amphibians and reptiles is critical for conservation and management (Semlitsch and Bodie, 2003). Freshwater turtles often require different habitats to carry out all life-history functions. Turtles often live and forage in temporary wetlands that are some distance from permanent wetlands. They use upland habitats to disperse seasonally between wintering, breeding, and foraging sites, for purposes of aestivation, feeding, and hibernation, and females use upland habitats to nest (Burke and Gibbons, 1995). For example, high levels of gene flow in the estuarine diamondback terrapin (*Malaclemys terrapin*) within estuaries are most likely promoted by mating aggregations during the breeding season and high juvenile dispersal (Hauswaldt and Glenn, 2005). These movements were not detected in long-term mark recapture studies (Gibbons et al., 2001) and may be important for inbreeding avoidance and maximizing genetic diversity in estuaries.

Landscape connectivity, the degree to which landscape features facilitate or impede movements and gene flow between populations (Taylor et al., 1993), is an essential feature of landscape structure because of effects on movements among populations, population persistence, and probabilities of recolonization. Landscape connectivity can be quantified in a relative sense based on indices that characterize the spatial dispersion of landscape habitat types and account for the proportional contributions of each landscape type to landscape matrices between populations. The degree

of genetic differentiation among populations has been widely used in wildlife studies as a surrogate measure of dispersal (Scribner et al., 2005). For example, Scribner et al. (1986) used protein allozymes to estimate genetic relationships among populations of slider turtles (*Trachemys scripta*) that were separated by different types of intervening habitats. Based on estimates of inter-population variance in **allele frequency**, these authors presented compelling evidence for higher rates of gene flow among populations from different embayments along contiguous lake shoreline relative to interspersed (but aquatically connected) riverine habitat. Populations in small ponds separated by upland terrestrial habitat had the lowest rates of gene flow compared to those in the other intervening habitat types.

Management strategies for populations need to account for the dispersal capabilities and natural history of the species. Where panmixis occurs, the populations may be managed as a single entity with a focus on maintenance of size and habitat quality. In contrast, where there is a high degree of structuring, each population contributes to overall species diversity. Managing these populations as separate units is important to ensure diversity is retained within each, and that overall species diversity is not compromised from increased gene flow and resultant genetic homogenization (DeYoung and Honeycutt, 2005; Moritz, 1994; Moritz, 1999). Mixing genetically differentiated populations can also cause outbreeding depression (see above). Management can be guided by the extent to which populations have diverged, with issues of outbreeding depression and isolation being of greatest concern among the most divergent units, referred to as **evolutionarily significant units** (ESUs; Moritz 1994), in comparison to less divergent populations referred to as **management units** (MUs).

Spinks and Shaffer (2005) defined management units for the vulnerable western pond turtle (*Emys [= Actinemys] marmorata)* with analyses of 1372bp of *ND4* and *tRNA* mitochondrial genes. Populations in northern California and farther north were genetically similar and formed a single management unit, whereas drainages farther south exhibited more structuring. In central and southern California, a large proportion of intraspecific diversity could be attributed to two populations. To retain diversity, these two populations should be a priority for conservation and management of the species.

Defining management units was a greater challenge for the giant Amazon river turtle, *Podocnemis expansa*. This species has an impressive dispersal capability, with females known to traverse up to 400 km between nesting beaches and feeding areas (Hildebrand et al., 1988). As predicted from theory, because of its dispersal capabilities and lack of barriers to dispersal, high levels of gene flow were found within basins (Pearse et al., 2006a). Based on this mtDNA analysis, an entire basin represents a management unit. Lack of structuring in basins was confirmed for nine microsatellite loci but these markers also revealed recent reductions in population size. Extensive harvesting has decimated populations of *P. expansa* and its continuation will result in loss of genetic diversity. Given the harvesting pressures, the units of management would be more appropriate at the population level to ensure local nesting beaches are not overexploited for eggs and mature females of *P. expansa.* Conservation biologists thus need to consider all threatening aspects from local to landscape scales when defining units for management in chelonians.

Clarifying Taxonomy

Inadequately informed management plans and a limited knowledge of biological richness are often the result of misunderstanding taxonomic status and relationships among taxa. If the units of evolutionary significance or taxonomic importance have not been identified and prioritized for conservation, biological diversity may not be protected adequately. Molecular methods are particularly amenable to resolving taxonomic relationships and identifying units for conservation, because they can uncover diversity in taxa not apparent from morphological analyses. Phylogenetics is a discipline that often uses genetic information to delimit species boundaries and divergent lineages within species, and then to estimate the evolutionary relationships amongst those units (Davis and Nixon, 1992; Avise and Wollenberg, 1997; Nei and Kumar, 2000; Iverson et al., 2007; Turtle Taxonomy Working Group, 2007a). We will illustrate how phylogenetics has contributed to resolving taxonomic issues in chelonians.

Taxonomy has traditionally used morphological characters to delimit species where a **holotype** is used as a reference specimen. However, the propensity of some turtles to hybridize with other species can result in difficulties. For example, at least two "species" of rare Chinese turtles were described from specimens purchased from the Hong Kong animal trade. Scientists were unable to find these animals in the wild and began to question their taxonomic validity. Allozyme and **mitochondrial** DNA analyses revealed that these "taxa" were not representative of species but rather they were distinct morphological forms resulting from **hybridization** events (Parham et al., 2001). Hybridization and **introgression** are fairly common in freshwater turtles (e.g., Georges et al., 2002; Stuart and Parham, 2004; Spinks and Shaffer, 2005). Neutral genetic markers may effectively resolve these taxonomic issues and have advantages over morphological traits as they are less subject to plasticity and presumably selection.

Phylogenetic studies can redefine taxonomies. Taxonomies have been refuted or supported by genetic evidence where phylogenetic criteria are used to delimit species and genera (reviewed in Turtle Taxonomy Working Group, 2007b). Delimiting species on the basis of combined molecular and morphological criteria is considered the best approach for resolution of taxonomies (Seberg et al., 2003; Blaxter, 2004; Dayrat, 2005). For turtles and tortoises, delimiting species boundaries can be even more difficult because interspecific hybridization frequently occurs even amongst distantly related taxa (e.g., Georges et al., 2002).

Phylogenetic methods can identify such instances of hybridization and resolve taxonomies to define groups constituting genera or species (Templeton, 2001; Sites and Marshall, 2004). For example, in a phylogenetic study of the Geoemydidae, not all recognized species appeared to be of the same evolutionary lineage. This suggested misclassification of several species (by some criteria), and instances of interspecific hybridization were documented. Based on this genetic evidence, taxonomic revision of this group was required (Spinks et al., 2004).

Phylogenetic or phylogeographic studies can identify cryptic species. Cryptic species are named because they comprise distinct genealogical lineages but in the absence of molecular or behavioral evidence, lack distinguishing morphologic characteristics or other diagnostic features to warrant recognition as species. For purposes of conservation, cryptic species are important units of diversity and may represent threatened taxa, previously unknown to conservation biologists (Georges and Adams, 1996; Georges et al., 1998; Walker et al., 1998; Fritz et al., 2005). In Asian softshell turtles, two species were formally recognized in the *Chitra* genus: *C. indica* and *C. chitra*. MtDNA sequence data revealed three deeply divergent **monophyletic groups** in *Chitra* (Engstrom et al., 2002). The third and previously unidentified form was subsequently named as a distinct species (*C. vandijki*) based on additional morphological data (McCord and Pritchard, 2002), and is a critically endangered species that warrants greater protection (Engstrom et al., 2002). As protection is usually only conferred to recognized species or subspecies in wildlife legislation, it is imperative that taxonomies are clearly defined for effective conservation (Soltis and Gitzendanner, 1999; George and Mayden, 2005; Turtle Taxonomy Working Group, 2007a).

Insights into Species Biology

Biologists have traditionally explored various aspects of the natural history of a species through observation. Turtles are notoriously difficult subjects for some observational studies, yet knowledge of many aspects of a species' biology is critical for successful conservation efforts. **Molecular markers** are providing new insights into turtle mating systems, dispersal (sex-specific or otherwise), population connectivity, and fluctuations of population sizes that can be difficult to ascertain from field and observational studies alone.

Female turtles have sperm storage structures in the oviducts (Gist and Jones, 1989), and captive females held in the absence of adult males have been known to produce viable eggs for as long as 7 yrs (Ewing, 1943; Magnusson, 1979). Molecular marker studies have revealed that freshwater turtles and tortoises in natural populations frequently use stored sperm to fertilize eggs (e.g., Gist and Congdon, 1998; Pearse and Avise, 2001; Roques et al., 2004). Indeed, microsatellite DNA analyses have revealed that some *Chrysemys picta* will produce fully-fertile clutches of eggs in nature without re-mating for 3 yrs (Pearse et al., 2002). However, lower hatching success and hatchling mass were found in clutches fertilized from stored sperm in the European pond turtle (*Emys orbicularis*), suggesting deterioration of stored sperm for some species (Roques et al., 2006).

The vast body of literature documents a substantial frequency of multiple paternity in non-marine turtles and tortoises (examples include Galbraith, 1993; Palmer et al., 1998; Moon et al., 2006), but there are exceptions. Low incidences of multiple paternity (less than 10% of clutches) have been documented for *Emys orbicularis*, resulting perhaps from competition of viable stored sperm to fertilize eggs (Roques et al., 2006). This finding contradicted observations of multiple *E. orbicularis* males mounting a single female during the breeding season (Rovero et al., 1999). Mating systems may also differ between populations of the same species. *Podocnemis expansa* exhibited 100% multiple paternity in smaller samples (Valenzuela, 2000) and 10 to 20% in larger samples (Pearse et al., 2006b). Molecular markers thus can shed light on mating systems in turtles and tortoises that may not be apparent from observational data.

Reproductive success is critical to population persistence. Only recently, based on applications of biochemical markers, have turtle biologists been able to extend estimates of annual recruitment to quantify reproductive contributions of individual adult males and females. Variance in reproductive success will greatly affect Ne and generational rates of loss of genetic diversity. Importantly, knowledge of phenotypic, demographic, and geographic (e.g., habitat) variables that can be linked to reproductive success and to inter-annual variation in recruitment will greatly aid in the development of conservation plans. Scribner et al. (1993) used allozymes to examine relationships between inter-annual variation in reproductive success and juvenile cohort measures of genetic diversity in *Chrysemys picta* that inhabits the E.S. George Reserve, a large protected wetland complex in southeastern Michigan. During years where few females successfully reproduced, offspring from these cohorts were characterized by higher **inbreeding coefficients** (F), lower heterozygosity (H), and higher genetic correlations among individuals (θ) compared to cohorts recruited in years when greater proportions of females contributed progeny. For conservation biologists, these findings emphasize that factors affecting inter-annual variation in recruitment also can impact cohort levels of genetic diversity.

Ecological characteristics are not alone predictive of how genetic variation is apportioned within and among populations. Closely related turtle species may display substantial variation in connectivity and structure that reflect important differences in natural history among species. For example, Roman et al. (1999) found strong phylogenetic structuring for the highly aquatic alligator snapping turtle (*Macrochelys temminckii*) across basins in a mtDNA control region analysis, suggesting limited dispersal of turtles. In contrast, *Chelydra serpentina* lacked structure for allozyme and mtDNA, reflecting its greater tendency to disperse over land and long distances in water (Phillips et al., 1996). Each species is different. The most informed conservation deci-

sions are formulated based on knowledge of fundamental aspects of a species' biology derived from joint studies of genetic structure and natural history.

Estimating the size of a population from mark-recapture analyses can be difficult and time-consuming, particularly for species that are difficult to capture or at low population densities. Obtaining genetic samples can be easier because individuals do not need to be subsequently re-caught to obtain data for estimating population size.

Molecular data can be used to estimate the effective population size, which is the size of the population that is actually reproducing, a parameter that may be more meaningful for conservation than the census size. The effective population size (Ne) can be monitored by assessing temporal changes of allele frequencies in the population (Richards and Leberg, 1995; Luikart et al., 1999). Genetic techniques can also provide point estimates of the number of breeding individuals in a population (Nb) from paternity (or maternity) microsatellite data. Pearse et al. (2001) developed a technique for estimating current reproductive size of a population of *Chrysemys picta* and provided additional information, such as the movement of breeding individuals, which was not possible based on capture-mark-recapture studies alone.

Forensics

Trade in turtles has increased dramatically and is considered to be the greatest threat to their survival (Asian Turtle Working Group, 1999; van Dijk et al., 2000). Turtle and tortoise trade can be classified into three main categories: trade for human consumption, pet shop trade, and traditional medicines (van Dijk et al., 2000; Turtle Conservation Fund, 2002). Consumption of turtles is by far the largest scale trade, and larger, more mature individuals tend to be targeted. Due to their life-history characteristics (great longevity, high juvenile mortality, and late onset of maturity), this type of trade probably has the greatest negative impact on chelonian populations (Smith, 1993; van Dijk et al., 2000). Exploitation of chelonians for the pet shop trade favors juveniles of unusual species and, as commodity values are often driven by rarity, this can rapidly contribute to the extinction of rare and endangered species (Ceballos and Fitzgerald, 2004; Gamble and Simons, 2004; Cheung and Dudgeon, 2006; Gong et al., 2006; Stuart et al., 2006). Finally, large numbers of turtles are frequently harvested primarily for their shells, which are ground to a powder or jelly, and sold for its alleged positive effects on longevity and virility in humans (van Dijk et al., 2000; Hsieh et al., 2006; Lo et al., 2006).

DNA-based forensic methods can be used to monitor illegal trade by verifying taxonomy and providing information on geographic origin of seizures. Traditionally, morphological characteristics were used for species identification. However, often seizures include small fragments of eggshells, carapace, cooked meat, or powdered turtle shell, where standard diagnostic features are no longer discernible. Molecular methods are ideal for forensics because they can be used on degraded or processed specimens, and can elucidate species, and even regional or population origins (Randi, 2003). Where commercial industries are established, genetic techniques may be the only means by which products derived from legal trade can be reliably distinguished from poaching activities. Further, genetic methods have the resolution to 'tag' individuals and establish paternities or maternities, technologies that are particularly useful for monitoring activities of licensed reptile breeders. The application of molecular techniques for wildlife forensics is still in its infancy. Approaches tend to be handled on a case-by-case basis and standard protocols have not been adopted. Currently only a few studies have applied molecular techniques for forensic issues in freshwater turtles and tortoises.

Legitimacy of turtle meat trade in Florida and Louisiana were investigated by Roman and Bowen (2000). Species composition was determined from 36 turtle meat products purported only to contain *Macrochelys*. The majority did not contain *Macrochelys*, but were predominantly *Chelydra serpentina*, as revealed by analyses of the control region and cytochrome *b* genes of mtDNA (394bp and 256bp respectively). This shift in trade to a species that is 50 kg lighter in weight and less favored for its flavor is speculated to reflect depletions of *Macrochelys* populations. With more catch effort required by harvesters to meet demand from these depleted populations, the market shifted to the more readily available *Chelydra*. In addition, softshell turtles (*Apalone* spp.) were present in a small proportion of the products. Impacts of this trade have not been investigated for any of these species, although current harvest rates may not be sustainable. Further research on the effects of harvesting and continued genetic monitoring of processed trade goods is recommended to prevent overexploitation or to minimize its impact in these species.

Molecular methodologies have analyzed species composition in cooked meat, eggs (Moore et al., 2003), and powdered turtle shell (Lo et al., 2006). Preparations of turtle shell in the Taiwanese market were analyzed with mitochondrial 12s ribosomal RNA and cytochrome *b* sequences (Lo et al., 2006). Reassuringly, CITES (Convention on International Trade of Endangered Species of Wild Fauna and Flora) listed species were not present in these turtle shell and jelly preparations. Also in Taiwan, methods have been developed for determining the presence of a CITES-listed endangered turtle (*Kachuga tecta*) in shell preparations (Hsieh et al., 2006).

Identifying geographic origins or provenance of seizures is required to repatriate animals to their wild populations without disrupting existing genetic structure or elevating risks of outbreeding depression. Molecular techniques can also be used for assessing origins of individuals. In the case of the Indian star tortoise (*Geochelone elegans*), the origins of 92 individuals seized from the Singapore airport were determined using mtDNA (control region, cytochrome *b*) and six microsatellites (Gaur et al., 2006). The rescued group of tortoises was found to be a mix of individuals from

different populations in southern India and possibly Sri Lanka. Exact localities for many of the individuals could not be identified because sampling was limited and not all diversity has been characterized across the range of *G. elegans*. With more extensive sampling, these methodologies will be able to identify source populations of seized chelonians, enabling them to be returned to their original geographic location(s). Overall, these studies highlight the power of molecular methods to monitor trade directly from a range of trade products for species identification and provenance delineation.

The utility of genetics in forensics is hindered by the limited markers available for chelonians. With more markers becoming available from genome sequencing projects, such as that proposed for *Chrysemys picta* (see http://www.reptilegenome.com for more information), genetics will play an ever-increasing role. New technologies, such as single nucleotide polymorphisms (SNP) markers will enable analyses of samples from more highly degraded samples, more rapidly and with greater resolution for addressing forensic issues. Advances in genetic technologies and marker development will pave the way for development of DNA registers for routine monitoring of trade activities. Such inventories are urgently required if we are to assess the threats of overexploitation to turtles and tortoises worldwide.

Concluding Remarks

We have discussed important genetic issues that conservation biologists should consider when planning and executing projects involving turtles. We have highlighted the importance of genetic diversity for future adaptive evolution and we outlined processes by which diversity is lost. Anthropogenic effects can exacerbate loss of genetic diversity owing to increased habitat fragmentation and diminished population size. Genetic approaches can be used to detect and monitor these effects at various temporal and spatial scales.

Understanding historical and contemporary evolutionary processes, at scales ranging from an individual to an entire landscape, provides valuable knowledge for development of short-term and long-term conservation plans. Conservation priorities can be identified and program success can be monitored using molecular methodologies. Aspects of turtle biology and mating systems that are exceedingly difficult or impossible to ascertain from field studies can be illuminated using genetic markers. Further, molecular methods are an emerging crime investigation tool for monitoring the turtle trade. Despite these applications and the inherent importance of genetic diversity to long-term viability of turtle populations, there is a general paucity of such genetic studies on freshwater turtles and tortoises (reviewed in FitzSimmons and Hart, 2007).

Due to the lack of studies, there is a limited repertoire of molecular markers currently available for turtle geneticists (Engstrom et al., 2007). With the ongoing genomic revolution, the number of available markers, their information content, and range of applications for chelonian conservation will greatly increase. For example, new genomic approaches offer exciting possibilities to investigate whether variation within specific gene regions can be tied to phenotypic or other traits that are tied to probabilities of survival or reproductive success. Emerging technologies hold great promise to link increasingly assessable modern technology to fundamental problems in turtle biology and conservation. Other technological advancements will enhance efficiency of DNA fingerprinting technologies and enable high throughput analyses, such as SNPs (**single nucleotide polymorphisms**) and **microarrays** (reviewed in McGaugh et al., 2007).

We conclude by listing what we perceive to be three crucial future directions in turtle conservation genetics:

1. Reconciling taxonomic uncertainties and identification of genetic discontinuities at landscape and species levels to delineate management units.

2. Predicting effects of landscape-level changes and concomitant changes in population demography and movement patterns on apportionment of genetic diversity within and among populations.

3. Monitoring trade and directing enforcement to protect overexploited turtle populations.

Each issue is a global concern that potentially influences every turtle species. While substantial progress has been made, the geographic and taxonomic coverage has been uneven and not necessarily focused on species of greatest concern (reviewed in FitzSimmons and Hart, 2007). Turtle geneticists should work closely with biologists, managers, local communities, and conservation organizations to bring state-of-the-art technology and methods of statistical inference to bear on pressing issues in turtle conservation.

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GLOSSARY

Additive Genetic Variance. – Genetic variance that arises from the additive effects of genes on the phenotype.

Allele. – Alternative forms of a gene at a given locus on a chromosome.

Allele Frequency. – Also termed gene frequency. The proportion of an allele (or gene) in a population relative to other alleles (or genes) at its locus.

Allelic Richness. – The number of alleles in a population corrected for sample size. Used as a measure of genetic diversity.

Allozymes . – Forms of an enzyme that differ in amino acids and have different electrophoretic mobilities.

Chromosome. – A strand of DNA with associated proteins that is visible as a rod-shaped structure in cells that have been stained during cell division. Chromosomes contain the heritable genetic information within the DNA.

Deleterious Recessive Alleles. – The phenotypic effects of recessive alleles are masked in the phenotype of heterozygotes, and expressed in homozygotes. Deleterious alleles have negative fitness effects on individuals.

Effective Population Size. – The average number of breeding individuals in a population which are assumed to contribute equally to the next generation.

Epistasis. – The interaction between two nonallelic genes, such that one gene alters the expression of the other at a different locus.

Evolutionarily Significant Unit. – A population (or group of populations) reproductively isolated from other conspecific population units for long enough duration to display genetic isolation, and is an important component in the evolutionary legacy of the species.

Fitness. – The ability of an individual to produce offspring in a given environment. In a genetic sense, the relative reproductive success of a genotype.

Founder Effects. – The loss of genetic diversity when a new colony is formed by a very small number of individuals from a larger population; a form of genetic drift.

Gene. – A basic unit of inheritance transmitted through the gametes from generation to generation, occupying a specific locus on a chromosome and with a specific function.

Gene Pool. – All the genes available among reproductive members of a population at a given point in time.

Genetic Drift. – Changes in allele frequencies of populations due to random sampling effects because not all individuals (and their genes) will reproductively contribute to the next generation.

Gene Flow. – Movement of genes from one population to another by interbreeding or migration.

Genotype/genotypic. – The genetic constitution or expression of an individual.

Genome. – The entire complement of genetic material in a cell. In eukaryotes this refers to the genetic material in a single set of chromosomes.

Genotypic Frequency. – The proportion of a genotype in the population relative to all other genotypes.

Heritability. – The proportion of phenotypic variability for a given trait that is quantitatively genetically based; expressed as the ratio of phenotypic variance to genetic variance.

Heterosis. – Superiority or vigor of hybrid individuals compared to either parental stock.

Heterozygote. – A diploid individual with different alleles at a particular locus.

Holotype. – The single specimen designated or indicated as the name-bearing type of a nominal species or subspecies by the original author.

Homozygote. – A diploid individual with identical alleles at a particular locus.

Hybridization. – Crossbreeding of individuals of different genetic composition, typically belonging to different species or varieties to produce hybrid offspring.

Inbreeding. – Mating of related individuals.

Inbreeding Coefficient. – The probability that an individual contains copies of the same ancestral gene from both its parents because they are related.

Inbreeding Depression. – Reduction of fitness by increased homozygosity as a result of inbred matings.

Introgression. – The spread of genes from one species to another via hybridization and backcrossing.

Locus/loci. – The specific region on a chromosome where a gene is located.

Management Units. – Demographically independent sets of populations identified to aid short-term conservation management. Genetically divergent but not to the extent as observed in evolutionarily significant units.

Meiotic Drive. – Preferential production of certain gametes during meiosis (germ cell production). This alters the expected Mendelian segregation ratios in heterozygotes.

Mendelian Segregation. – Mendel's first law. The principle that the two different alleles of a gene pair segregate from each other during meiosis; each resultant gamete has an equal probability of obtaining either allele.

Metapopulation. – A group of spatially separated populations from the same species connected by immigration and emigration.

Microevolution. – Evolutionary events occurring over a shorter period of time, such as the changes in the gene pool of a population.

Microsatellites. – Tandem repeat motifs of DNA sequence interspersed throughout the eukaryotic genome in which the repeat unit is typically five or fewer bases in length.

Molecular marker. – A genetic polymorphism with multiple alleles and a simple mode of inheritance. Useful in pedigree studies, disease studies, studies of the distribution of genes in populations and linkage mapping.

Mutation. – A change in a gene or chromosome.

Microarrays. – A technique used to monitor gene expression in which genes or gene fragments are deposited typically on a glass, filter, or silicon wafer in a predetermined spatial order allowing them to be made available as probes.

Migration. – Movement of an individual or group from one location to another.

mtDNA. – Mitochondrial DNA: The circular, double-stranded DNA of the mitochondria. It typically has matrilineal inheritance, although paternal leakage has been documented for some taxa.

Monophyletic Group. – A group comprised of a single ancestral species and all its descendants. Also called a clade.

Natural Selection. – A primary mechanism for evolution in which individuals best suited to their environment have greater survival and reproductive success, thereby transmitting their genetic characteristics to succeeding generations.

Neutral Genetic Markers. – Genetic markers presumably not under the forces of natural selection and often residing in non-coding genomic regions.

Outbreeding. – The breeding of genetically unrelated or distantly related individuals.

Outbreeding Depression. – A reduction in the fitness of progeny from matings of individuals from different populations, possibly from the breakdown of co-adapted gene complexes or 'swamping' of locally adaptive genes.

Panmictic. – Pertaining to a genetically unstructured randomly mating population.

Phenotype/phenotypic. – The observed properties of an organism, resulting from the interaction of its genotype with the environment.

Phenotypic Plasticity. – The ability of an organism's phenotype to change in response to changes in the environment.

Population Bottleneck. – An evolutionary event resulting in a decrease in the size of a population and subsequent loss of genetic diversity via the effects of genetic drift.

Quantitative Genetics. – The study of the genetic basis of traits showing continuous variation.

Single Nucleotide Polymorphism. – Variations in DNA sequence that occur when a single nucleotide base (adenine, guanine, cytosine, or thymine) is altered via a mutation event.

Vicariance. – The splitting of closely related groups of taxa or biota by the formation of a natural barrier.

Defining Turtle Diversity: Proceedings of a Workshop on Genetics, Ethics, and Taxonomy of Freshwater Turtles and Tortoises
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A Compendium of PCR Primers for mtDNA, Microsatellite, and Other Nuclear Loci for Freshwater Turtles and Tortoises

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ABSTRACT. – Molecular markers have proven to be a powerful tool for research on turtles. In particular, the application of the polymerase chain reaction (PCR) has increased the availability of molecular technologies while decreasing the cost. However, the cost, time, and expertise associated with developing and testing primers for a particular species can still present a significant barrier, especially to researchers less experienced with molecular methods. In this paper we provide the primer sequence, genomic location, and taxa for 202 PCR primers spanning the entire mitochondrial genome. We also report primers for 11 nuclear coding genes and introns. Finally, we provide primer sequence, amplicon size, and number of observed alleles for 181 microsatellite loci from all major clades of living turtles. We hope that this nearly comprehensive compilation of freshwater turtle and tortoise PCR primers can reduce some of the initial difficulties for beginning turtle geneticists and further facilitate research in existing labs.

KEY WORDS. – Reptilia; Testudines; turtle; PCR; primer; mtDNA; nuclear DNA; microsatellite; STR

The power and utility of genetic tools for the study of turtle biology and conservation is evidenced by the extensive and rapidly growing literature on the past, present, and future use of such molecular tools reviewed in this volume. The increasing availability and decreasing cost of molecular technologies, specifically the polymerase chain reaction (PCR), is making genetic analysis more accessible to researchers. However, the cost, time, and expertise associated with developing and testing primers for a particular species can still present a significant barrier, especially to researchers inexperienced with molecular methods. In this paper we hope to reduce some of the initial difficulties or frustration for turtle biologists by providing a thorough compilation of published (and some unpublished) information on PCR primers developed specifically for turtle studies.

We have organized our discussion of molecular markers into three categories: (1) mitochondrial DNA, (2) nuclear loci (including both protein-coding genes and introns), and (3) microsatellite loci (Simple Tandem Repeats). For each marker type we provide a brief description of its strengths and limitations, and the kind of study for which it may be most appropriate. In Tables 1 and 2 we list each primer's region (gene or locus), name (or names), primer sequence, original reference, and a selective (non-exhaustive) list of citations for studies that have used that primer. Because some primers have been used on multiple taxa, we have also included a list of species (when possible) or a summary of the major clades in which the primers have been successfully applied. For the mitochondrial primers (Table 1), we include the orientation and 5' primer position relative to the published *Chrysemys picta* mitochondrial genome (Mindell et al., 1999) along with a genomic map (Fig. 1A-E) to compare primer coverage and provide estimates of predicted product size of different primer combinations. Due to space limitations not all primers are depicted in the figure. We encourage readers to consult the figure to find primers in the region of interest and then reference the tables for a more complete listing of available primers in that region and taxa in which those have been used. Our summary of primers focuses on freshwater turtles and tortoises, and largely excludes the extensive literature on marine turtles. However, because of the demonstrated inter-species cross-amplification of many microsatellite loci, we have also included a non-exhaustive list of marine turtle primers. Also for the microsatellite markers, we have included an indication of the expected size and level of variation of the amplified product in the target species and a list of non-target species in which the locus has been tested and failed to amplify.

A paper of this nature (reporting a set of available primers) will already be out of date on the day it is published. This is unavoidable in a hard copy publication, but can be avoided by establishment of an open access database for turtle researchers to report their primers as they are devel-

oped, provided of course that researchers are willing to post their new primers or the application of existing primers to new species on the database. We have established such a database in a companion website for this publication, which can be accessed at http://www.csuchico.edu/biol/personnel/engstrom/turtleprimers.htm. We hope that compiling this information in a single reference will aid in the rapid diffusion of information on new, useful primers and new applications of existing primers. We hope that this will facilitate research and accelerate progress toward understanding the phylogeny and population genetics of turtles, by guiding researchers to molecular markers that will (1) be applicable their particular study animal, (2) harbor levels of variation appropriate to their question, and (3) be comparable to previous studies. However, to ensure that appropriate credit accrues to the researchers who have performed the hard work of developing markers, we remind anyone using primers listed in this publication or the companion website to cite the primary references for those primers or to contact those who developed previously unpublished primers for updated citation information. We reiterate that publications by the original developers of the molecular markers should be considered the primary references, NOT this summary report or its companion website.

Mitochondrial DNA

Mitochondrial DNA (mtDNA) sequence data have been and continue to be particularly informative in both in phylogeography and in systematics (Hillis et al., 1996). The mitochondrial genome has a highly conserved gene content and gene order (Boore, 1999, but see Parham et al., 2006a,b), lacks introns, lacks significant recombination (Avise, 1994, 2004; Moore, 1995; Sunnucks, 2000), and is present in multiple copies per cell, thus rendering the acquisition and analysis of mtDNA sequence data relatively easy and straightforward compared with the more complex nuclear genome (see below). The overall rate of nucleotide substitution in the mitochondrial genome is relatively rapid (Brown et al., 1979), providing a rich source of variable characters. However, this organelle also offers a mix of fast evolving genes, useful for studies of recently diverged lineages (e.g., within species, among closely related species), and slowly-evolving genes suitable for studies of more ancient divergences (e.g., among genera or families [Graybeal, 1994]). Mitochondrial DNA has a small effective population size relative to the nuclear genome, resulting in a shorter average coalescent time (Moore, 1995), albeit with a high variance (Hudson and Turelli, 2003). This combination of attributes renders mtDNA useful for a wide variety of genetically based studies. However, as a maternally-inherited, single locus, mtDNA provides a somewhat limited perspective on the evolutionary and ecological history of a species. The demonstration of hybridization (Parham et al., 2001; Stuart and Parham, 2007; Spinks et al., unpubl. data) and potential differences in male and female behavior (FitzSimmons et al., 1997), for example, may often require nuclear data to test mtDNA-based hypotheses. Thus, while mtDNA has provided and will continue to provide an invaluable tool, it is also important to identify independent markers that complement those in the mitochondrial genome.

The 202 turtle mtDNA primers listed in Table 1 have been used to amplify and sequence all regions of the turtle mtDNA genome, including all 13 protein-coding genes, 22 tRNAs, 2 rRNAs, and the control region/d-loop. The most frequently used genes in deep phylogenetic studies are the slowly evolving 12s rRNA (e.g., Shaffer et al., 1997), and moderately evolving Cytochrome *b* (Cytb) (e.g., Shaffer et al., 1997; Spinks et al., 2004). Cytb, NADH 4 (also commonly abbreviated ND4) and other protein coding genes have been most useful for studies among closely related species (e.g., Caccone et al., 1999a,b; Engstrom et al., 2002; Feldman and Parham, 2002) or for phylogeographic studies within species (e.g., Starkey et al., 2003; Spinks and Shaffer, 2005). The control region is widely used in population and intraspecific level studies because of its high rate of mutation (Stewart and Baker, 1994; Starkey et al., 2003; Pearse et al., 2006); however, some studies have noted equal or greater levels of variation in protein coding genes (Spinks and Shaffer, 2005).

Nuclear Loci

Because of the recognized limitations of mtDNA, increased attention is being paid to the nuclear genome as an additional, independent source of data for phylogenetic, phylogeographic, and population genetic analyses (e.g., Bruford and Wayne, 1993; Groth and Barrowclough, 1999; Hare, 2001). The three sources of nuclear data most commonly used include size polymorphisms at microsatellite loci (discussed below), and sequence data from nuclear protein-coding genes and introns. In contrast to mtDNA, nuclear protein-coding genes and introns tend to evolve more slowly (Prychitko and Moore, 1997, 2000; Groth and Barrowclough, 1999; Birks and Edwards, 2002; Caccone et al., 2004; Engstrom et al., 2004; Fujita et al., 2004), making them less prone to excessive homoplasy—a common problem among mitochondrial genes over deeper divergences. Nuclear introns have the further advantage of being free from many of the evolutionary constraints imposed on protein-coding sequences, resulting in little base compositional bias, relatively low transition/transversion ratio, and little among-site rate heterogeneity (Armstrong et al., 2001; Prychitko and Moore, 2003; Fujita et al., 2004). One disadvantage of nuclear DNA is that the slow rate of evolution, which minimizes homoplasy on long timescales, can also reduce variation on shorter timescales (Birks and Edwards, 2002). This characteristic can limit its utility in phylogeographic and population genetic studies of turtles (Spinks and Shaffer, 2005).

Because they can be more difficult to develop compared with mtDNA loci, relatively fewer primers have been de-

Figure 1. The five panels depict map of 5' position and orientation of turtle primers listed within Table 1 relative to the sense strand (L) and loci of the *Chrysemys picta* mitochondrial genome (GenBank accession AF069423).

scribed for nuclear protein coding genes and introns. In Table 2 we list primers for 6 introns and 3 protein-coding genes. Intron sequence has shown great utility in interspecific phylogenetics (Engstrom et al., 2004; Fujita et al., 2004), but due to their lack of functional constraint they can be difficult to align across deep phylogenies (Fujita et al., 2004; but see Loytynoja and Goldman, 2005) Protein-coding genes have proven useful in interspecific phylogenies at many levels (Georges et al., 1998), and will be crucial in testing the location of the root of the turtle tree (Krenz et al., 2005; Near et al., 2005) and in understanding the placement of turtles relative to other amniotes (Hedges and Poling, 1999). Because nuclear introns and protein coding genes are bi-parentally inherited, detection of heterozygotes is a useful tool in the identification of interspecific hybrids (Stuart and Parham, 2007; Spinks, unpubl. data). Another less-explored source of nuclear gene data is the rapidly growing field of developmental genetics. Many genes have been cloned from complementary DNA (cDNA) libraries constructed for studies of sex determination (e.g., Valenzuela et al., 2006), morphological development and gene expression (Chien et al., 2005, 2006) and chromosome evolution (Kuraku et al., 2005, 2006; Matsuda et al., 2005). Complimentary DNA is synthesized using the enzyme *reverse transcriptase* to make DNA copies of all of the mature mRNA transcribed in a tissue sample. Although primers for detection of genes identified in these cDNA libraries have been published, we have decided not to include this exten-

Table 1. Primers currently available for amplification of mitochondrial loci of tortoise and freshwater turtles. Each primer is listed by locus, strand orientation (O*) (H = heavy, L = light), and 5' position relative to the *Chrysemys picta* mitochondrial genome (GenBank accession AF069423) except in cases where the primer does not align with *Chrysemys*, in which case the primer is aligned either with mitochondrial genomes of *Dogania subplana* (NC002780) and indicated with a "D" or *Pelomedusa subrufa* (AF039066) and indicated with a "P". No location is given for several primers designed for amplification of the control region in kinosternid turtles, which did not align well with other turtle genomes. Groups of taxa successfully amplified and associated references are listed in the final columns. Contact information for unpublished primer sequences: TNE (tengstrom@csuchico.edu), NNF (Nancy.FitzSimmons@canberra.edu.au), MRJF (mf@txstate.edu). Key to taxa: CR = Suborder Cryptodira, Chely = Family Chelydridae, TE = Superfamily Testudinoidea, Test = Family Testudinidae, Geo = Family Geoemydidae, Emy = Family Emydidae, TR = Superfamily Trionychoidea, Car = Family Carettochelyidae, Trio = Family Trionychidae, K = Superfamily Kinosternoidea, Derma = Family Dermatemydidae, Kino = Family Kinosternidae, Platy = Family Platysternidae, C = Superfamily Chelonioidea, Chelo = Family Cheloniidae, Dermo = Family Dermochelyidae, PL = Suborder Pleurodira, Cheli = Family Chelidae, P = Superfamily Pelomedusoidea, Pelo = Family Pelomedusidae, Podo = Family Podocnemididae.

Primer Location	O*	Pos.+	Primer Name	Primer Sequence (5'-3')	Orig. Ref.	Taxa	References Citing Primer
tRNA-Phe	L	19	L1	AAAGCACGGCACTGAAGATGC	135	Geo	
tRNA-Phe	H	28	KNPH 35R	GCCGTGCTTTGATATAAGCT	148	Kino	
tRNA-Phe	H	50	GT12STR	ATCTTGGCAACTTCAGTGCC	28	Test	23, 27
tRNA-Phe	H	50	Phe-H26	TACCCATCTTGGCAACTTCAGTGCC	119	Test	
12S rRNA	H	78	DL3Rev	AATATTTGAGTTGTCGTGGG	15	Test	
12S rRNA	H	128	12S-57R	GATACTTGCATGTGTAAGTTT	148	Kino	
12S rRNA	H	143	H10	TTCACTGGTTATGCAGATACTT	135	Geo	
12S rRNA	L	497	N/12SA	AAACTGGGATTAGATACCCCACTAT	120	TE	150, 154
12S rRNA	L	491	L1091	AAAAAGCTTCAAACTGGGATTAGATACCCCACTAT	82	Geo, Test, Cheli	4, 25, 27, 74, 75, 90, 98, 118, 147, 163, 164, 172
12S rRNA	L	501	LGL 284	TGGGATTAGATACCCCACTAT	33	Test	114
12S rRNA	L	508	12SXLF	GATTAGATACCCCACTATGCTTAG	153	Geo	
12S rRNA	H	582	TCR2	GCTCGTAGTTCTCTGGCGG	113	Podo	151
12S rRNA	L	626	O	CCTAGAGGAGCCTGTTC	150	TNE	
12S rRNA	H	939	P/12SB	GAGGGTGACGGGCGGTGTGT	120	TE	150, 154
12S rRNA	H	947	H1478	TGACTGCAGAGGGTGACGGGCGGTGTGT	82	Geo, Test, Cheli	4, 25, 27, 74, 75, 118, 147, 163, 164, 172
12S rRNA	L	1058	L2	AAAGCATTCAGCTTACACCTGA	135	Geo	
16S rRNA	H	1255	H1	TTTCATCTTTCCTTGCGGTAC	135	Geo	
16S rRNA	L	1639	L2606	GGCCTAAAAGCAGCCACCTGTAAAGACAGCGT	70	Geo	74, 75
16S rRNA	L	1954	LGL 381	ACCCCGCCTGTTTACCAAAAACAT	16	Emy	
16S rRNA	L	1958	16Sar/AR	CGCCTGTTTATCAAAAACAT	120	Test	27, 28, 118
16S rRNA	L	2002	M89 (L)	AGGAGTGATGCCTGCCCAGTGAC	63	PL	
16S rRNA	H	2073	LGL 283	TGATTATGCTACCTTTGCACRGT	33	Test	114
16S rRNA	L	2124	L3	GTCTCTTACAAATAATCAGTGA	135	Geo	
16S rRNA	H	2207	H2	AAGTTCCACAGGGTCTTCTCG	135	Geo	
16S rRNA	H	2485	M90 (H)	CCTTAATAGCGGCTGCACCATTAGGA	63	PL	
16S rRNA	H	2560	16Sbr/BR	CCGGTCTGAACTCAGATCACGT	120	Test	27, 28, 90, 118
16S rRNA	H	2562	H3056	CTCCGGTCTGAACTCAGATCACGTAGG	70	Geo	74, 75
16S rRNA	H	2589	LGL 286	AGATAGAAACCGACCTGGAT	16	Emy	
ND1	L	P2457	Podmt2	TTGCTGTAGAATCTGACATCC	151	Podo	
ND1	H	P3549	Tur d-loop R	GGAAGTGTATATGAAACCTGGGT	174	Pelo	
ND1	L	2899	ND1F	GGMTAYATACAACTTCGAAAAGG	153	Geo	
ND1	L	3169	L11	TCCGGTTGAGCTTCAAACTC	135	Geo	
ND1	H	3340	H3	ACTATTCCTGCTCAGGCTCCG	135	Geo	
ND1	H	3829	ND1R	GGTTTTAGCCTCTATTATTCACCC	153	Geo	
tRNA-Met	L	3928	LGL 562	TAAGCTATCGGGCCCATACC	114	Test	
ND2	L	4374	L4	ACCTGACAAAAACTAGCCCCA	135	Geo	
ND2	H	4506	H11	GTAGTTGGGTTTGGTTTAGTCC	135	Geo	
ND2	L	4842	1613-ND2	CTAAGCCTATTCTTCTA	149	Emy	
ND2	L	4923	CS2	GGACGCCATAACACAAT	167	Chely	
ND2	H	5084	ND2R	GAGGTTCTATCTCTTGTTTGGGGC	153	Geo	
tRNA-Tyr	L	5379	L-turtCOI	ACTCAGCCATCTTACCTGTGATT	157	Geo	24, 126, 153
tRNA-Tyr	L	5396	L-turtCOIc	TACCTGTGATTTTAACCCGTTGAT	157	Geo	24, 126, 153
CO I	L	5420	L5	THTTCTCYACTAACCATAAAG	135	Geo	
CO I	L	5424	LCO-1490	GGTCAACAATCATAAAGATATTGG	51	Geo	45, 125
CO I	H	5436	912-COI	GTGGTTGGTTGAGAATAATCA	149	Emy	
CO I	H	5486	H4	ACTATTCCTGCTCAGGCTCCG	135	Geo	
CO I	H	5839	H-COIint	TAGTTAGGTCTACAGAGGCGC	157	Geo	24, 126
CO I	L	5956	L-COIint	TGATCAGTACTTATCACAGCCG	157	Geo	24, 126
CO I	L	6106	M72 (L)	TGATTCTTCGGTCACCCAGAAGTGTA	63	PL	
CO I	H	6128	LGL 452	ACTTCAGGGTGCCCAAAGAATCA	114	Test	
CO I	H	6131	HCO-2193	TAAACTTCAGGGTGACCAAAAAATCA	51	Geo	45, 125
CO I	H	6265	H-turtCOI	CCCATACGATGAAGCCTAAGAA	157	Geo	24, 126
CO I	H	6272	H-turtCOIc	TGGTGGGCTCATACAATAAAGC	157	Geo	24, 126
CO I	H	6326	H-turtCOIb	GTTGCAGATGTAAAATAGGCTCG	157	Geo	24, 126
CO I	L	6337	L12	CTCATCCCCAACAGGAGTAAAA	135	Geo	
CO I	H	6551	M73 (H)	CCTATTGATAGGACGTAGTGGAAGTG	63	PL	
CO I	H	6579	H5	AAATCYTGCTATGATGGCGAA	135	Geo	
CO II	L	7594	L6	AAACAGACGCARTCCCAGGCAC	135	Geo	
CO II	H	7795	H12	GTCATCCTGTTTAGCTTCTCTAG	135	Geo	
ATPase 8	L	8659	L13	GCCTCTACCTACAAGAAAC	135	Geo	
ATPase 6	H	8766	H6	GTTATTAGTAGTTGCTGCTGTGC	135	Geo	
CO III	L	9038	DW 2000	ACAGGCGTAATCCTACTAA	168	Trio	
CO III	L	9209	TCox3_551F	CTACAAGCCATAGAGTATTACGAAGC	TNE	Trio, Geo	
CO III	L	9379	TCox3_716F	CTTTGGGTTTGAAGCAGCTGC	TNE	Trio, Geo	
CO III	L	9386	L10647	TTYGAAGCMGCMGCMTGATACTG	106	Emy	107
tRNAGly	L	9481	New Gly	ATAAGTACAATGMYTTCCA	5	Test	20

tRNA-Gly	L	9482	Raeu GlyF	CCAATACAAATGACTTCCAATC	TNE	Trio	
tRNA-Gly	L	9483	TGlyF1	TAGTAYAARTGACTTCCAATCA	TNE	Trio, Geo	
tRNA-Gly	L	9485	L7	AGTACAAATGACTTCCAATCA	135	Geo	
tRNA-Gly	L	9492	TGlyF2	TGACTTCCAATCAYTMAGTTT	TNE	Trio, Geo	
NADH3	H	9717	H13	GAAGAATCGAATTGAGAATGG	135	Geo	
NADH3	H	9884	H11100	TCTGCYCAYTCTARKCCTCCYTG	106	Emy	107
tRNA-Arg	L	D9924	ArgF1	GATTGATAAAACATGGTTACCC	TNE	Trio	
tRNA-Arg	L	9929	ArgF2	TAAAACATGGTTACCCTATGACACC	TNE	Trio	
NADH4	H	D10286	Raeu ND4-42R	GTATCATATGTGTGTTGGTTTGG	TNE	Trio	
NADH4	H	10239	ND4_43R	GGTTTAGGTTTTGTAGGTGGCTTG	TNE	Geo	
NADH4	H	10483	T_ND4_288R	TAGGATTATTAGTGGAGTAAGTCAGC	TNE	Trio, Geo	
NADH4	L	10508	L15	GAACCCCTATCACGAAAACG	135	Geo	
NADH4	H	10677	H7	TTTGATTWCCTCATCGTGTGTG	135	Geo	
NADH4	L	10886	ND4	CACCTATGACTACCAAAAGCTCATGTAGAAGC	5	Emy, Geo, Test	20, 41, 45, 46
NADH4	L	10892	ND4/ ND4_672(f)	TGACTACCAAAAGCTCATGTACAAGC	43	Emy, Trio	42, 44, 152
NADH4	L	10910	L-ND4	GTAGAAGCCCCAATCGCAG	157	Geo	24, 126, 153
NADH4	L	10918	L-ND4c	CCAATCGCAGGATCAATAATC	157	Geo	24, 126
NADH4	H	10921	Nap2	TGGAGCTTCTACGTGRGCTTT	5	Test	20
NADH4	L	11000	Turt1	GATCCTCTATCAAAAACACT	MRJF		
NADH4	L	11079	ND4 #2	TACGACAAACAGACCTAAAATC	5	Test	96
NADH4	H	11389	H-ND4int	GGTTAGCTCTCCTATTAGGTTGAT	157	Geo	24, 126
NADH4	L	11534	L-ND4int	ACCCATACACGAGAACATCTACT	157	Geo	24, 126
tRNA-His	H	11674	Hist-ND4	CCTATTTTAGAGCCACAGTCTAATG	43	Trio	42
tRNA-His	H	11675	Hist	CCTATTTTTAGAGCCACAGTCTAATG	44	Trio	
tRNA-Leu	L	11772	L8	AGGATAGAAGTAATCCAATGG	135	Geo	
tRNA-Leu	L	11775	LGL 763	AATAGTTTATCCRTTGGTCTTAGG	34	Test	114
tRNA-Leu	H	11821	H-Leu2	ATTTGCACCAAGGGTTAATGG	157	Geo	24, 126
tRNA-Leu	H	11836	H-Leu	ATTACTTTTACTTGGATTTGCACCA	157	Geo	24, 126, 153
tRNA-Leu	H	11837	Leu	CATTACTTTTACTTGGATTTGCACCA	5	Geo, Test	20, 45, 46, 96, 125
NADH5	L	P11901	Podmt3	TCACAGACATAACCATAAGCAC	151	Podo	
NADH5	H	11956	H15	GCTGTTTTTACGGCTGTTTTTTG	135	Geo	
NADH5	L	12454	ND5_619F*	ACCACGTTTAGGTTCATTTTCATTAC	45	Emy	*“Leu” in 45
NADH5	L	12812	L16	CATACACGCCTTCTTTAAAGC	135	Geo	
NADH5	H	12899	H8	TATCTTTCGAATTGCTTGTTC	135	Geo	
NADH5	H	13488	ALD-DLBRev	ACGATGTGCAGTGGGAGTGGTTG	119	Test	
NADH5	H	13590	ND5_1755R	AGATTAAGGAGATTCGGTGGAG	TNE	Trio	
NADH6	L	14118	ND6 346F	GAATAAGCAAAAACCACTAACATACCCCC	44	Trio	
tRNA-Glu	L	14349	L14724	CGAAGCTTGATATGAAAAACCATCGTTG	105	Emy, Test, Geo	4, 76, 87, 88, 104, 114
tRNA-Glu	L	14358	GLU	TGACATGAAAAAYCAYCGTTG	116	Test	25, 118
tRNA-Glu	L	14358	Gludg/GLUDGE/A/ Forward 14724	TGACTTGAARAACCAYCGTTG	120	CR,PL	7-9, 27, 28, 44, 45, 89, 118, 150, 154
tRNA-Glu	L	14368	CytbG	AACCATCGTTGTWATCAACTAC	154	Emy, Geo, Test	36, 77, 90, 103, 153
tRNA-Glu	L	14369	L9	AACCACCGTTCTATTCAACTA	135	Geo	
tRNA-Glu	L	14370	L14735t	CCATCGTTGTAATCAACTAC	76	Geo	
tRNA-Glu	H	14381	LGL 764	TTACAACGATGGTTTTTCATRTCA	34	Test	114
Cyt b	L	14462	MT-a	CTCCCAGCCCCATCCAACATCTCAGCATGATGAAAC	60	Test	
Cyt b		14462	mt-a-neu	CTCCCAGCCCCATCCAACATCTCAGCATGATGAAACTTCG	56	Geo	
Cyt b	L	14462	L14841	AAAAAGCTTCCATCCAACATCTCAGCATGATGAAA	82	Emy, Chely	1, 78, 138
Cyt b	L	14471	B/Cyt b 1	CCATCCAACATCTCAGCATGATGAAA	120	TE	1, 94, 150
Cyt b	L	14473	Forward 14841	ATCCAACATCTCAGCATGATGAAA	8	Test	7, 9
Cyt b	L	14476	mt-A	CAACATCTCAGCATGATGAAACTTCG	93	Geo, Emy	1, 13, 14, 91, 92, 144
Cyt b	L	14478	Forward 14848	CATCTCAGCATGATGAAACTTCGGA	8	Test	7, 9
Cyt b	H	14532	Reverse 14854	TGTAGGATTAAGCAGATGCCTAGT	8	Test	7, 9
Cyt b	H	14513	H16	CTAATAGTGATCCGAAGTTTCAT	135	Geo	
Cyt b	L	14581	C / 14946	ACTAGCATTCTCATCAGTAG	150	Test	7-9
Cyt b	L	14612	D	CGAGATGTTAATAACGGCTG	150	TE	
Cyt b	H	14635	Reverse 14957	AAGTCATCCGTATTGTACGTCTCG	8	Test	7, 9
Cyt b	H	14641	Reverse 14966	TCGGATAAGTCACCCGTACTG	8	Test	7, 9
Cyt b	L	14658	E	GCGCCTCATTCTTCTTTATCT	150	TE	
Cyt b	L	14678	Forward 15045	TGCATTTACCTCCAYATYGGCCG	8	Test	7, 9
Cyt b	L	14678	“F”/CB94lt	TGCATCTACCTTCACATYGGMCG	150	TE	44
Cyt b	H	14723	Reverse 15048	GGTAAGAGCCGTARTAAAGTC	8	Test	7, 9
Cyt b	L	14792	mt-C	TAYGTCCTACCATGAGGACAAATATCATTCTGAGG	170	Emy, Geo	11, 66, 91, 171
Cyt b	L	14804	Primus	TGAGGCCAAATATCCTTCTGAGGTGCAACCG	45	Emy	
Cyt b	L	14805	mt-c2	GAGGACAAATATCATTCTGAGG	13	Geo	
Cyt b	L	14804	G	TGAGGACAAATATCATTCTGAGGGGCTGCAG	150	TE	
Cyt b	H	14824	Reverse 15145	TCAGAATGATATTTGTCCCCATGGT	8	Test	7, 9
Cyt b	H	14827	mt-B	ACCTCAAAAGGATATTTGTCCTCA	14	Geo	
Cyt b	H	14827	CB2-3'/15149/”I”/ Cytb2	CCCTCAGAATGATATTTGTCCTCA	121	Emy, Test, Trio	9, 14, 27, 28, 44, 89, 94, 150
Cyt b	H	14834	Primus-rev	CGGTTGCACCTCAGAAGGATATTTGGCCTCA	45	Emy	
Cyt b	H	14836	no name(1)	AACTGCAGCCCCTCAGAATGATATTTGTCCTCA	138	Chey	
Cyt b	H	14837	H15149	AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA	82	Emy, Test	4, 14, 78, 87, 88
Cyt b	H	14837	H15149	TAACTGTAGCCCCTCAGAATGATATTTGTCCTCA	76	Geo	
Cyt b	H	14843	mt-B	TTGTGATTACTGTAGCACCTCAAAATGATATTTGTCCTCA	170	Emy, Geo	14, 66, 91, 171
Cyt b	H	14850	TestudRi3	AGTAGGTTGGTGATGACAGTGGC	13	Geo	
Cyt b	H	14852	H15197	CCGATATAAGGGATTGCTGA	76	Geo	
Cyt b	L	14912	CB534f	GACAATGCAACCCTAACACG	44	Trio	
Cyt b	L	14995	Rush	TTCCTACATGAAACCGGATCAAACAACCCAAA	45	Emy	
Cyt b	L	14996	H	TTCCTWCACGAAACAGGNTCAAACAA	150	Test	
Cyt b	L	15009	MT-c-emys	CCGGATCAAACAAYCCAACAGG	60	Test	
Cyt b	L	15011	TCR1/CytbJSi	GGATCAAACAACCCAACAGG	113	Emy, Geo, Test, Podo	14, 36, 77, 103, 151, 154
Cyt b	H	15027	Rush-rev	GTTGGGTTGTTTGATCCGGTTTCATGTAGAAA	45	Emy	
Cyt b	H	15030	J	CCTGTTGGGTTYTTTGAKCC	150	TE	
Cyt b	H	15030	CytbJsSr	CCTGTTGGGTTGTTTGATCC	154	Emy, Geo, Test	36, 77, 103

Cyt b	L	15050	mt-D	AAAATCCCATTCCACCCCTACTACTCCACAAAAGA	170	Emy, Geo	66, 91, 154, 171
Cyt b	H	15066	CB649r	GGGTGGAATGGGATTTTGTC	44	Trio	
Cyt b	L	15089	Mau-F	CTAGGCCTCATCTTAATACT	56	Geo	
CytB	H	15149	Ri-neu	GTGAAGTTGTCTGGGTCTCCTAG	56	Geo	
Cyt b	L	15171	CB791f	CACCMGCYAACCCACTATC	44	Trio	
Cyt b	L	15206	mt-e	AAACCAGAATGATACTTCCTATTTGC	13	Geo	
Cyt b	H	15231	mt-E	GCAAATAGGAAGTATCATTCTGG	13	Geo, Test	
Cyt b	H	15232	CB3-3'/"K"	GGCAAATAGGAARTATCATTC	121	Trio	150
Cyt b	L	15237	L-15601	CCATTCTACGCTCAATCCC	91	Emy	57-59, 91
Cyt b	L	15237	Podmt1	CAATGCTGCGATCCATCC	151	Podo	
Cyt b	H	15435	L	TCTTCTACTGGTTGTCCTCCGATTCA	150	TE	
Cyt b	L	15457	L14	AGCAGCCTCCATCCTTTTACTT	135	Geo	
tRNA-Thr	L	15557	CYTTOR/ CytoR4	GCTTAACTAAAGCACCGGTCTTG	28	Test	15, 23, 91
tRNA-Thr	L	15567	LGL 283	TACACTGGTCTTGTAAACC	87, 91	Emy	89, 91, 114
tRNA-Thr	L	15569	Thr-L15569	CATTGGTCTTGTAAACCAAAGACTG	119	Test	
tRNA-Thr	H	15569	H-15909/MT-f-na	AGGGTGGAGTCTTCAGTTTTTGGTTTACAAGACCAATG	91	Emy, Geo, Test	56-60, 91, 144
tRNA-Thr	H	15585	THR-8	GGTTTACAAGACCAATGCTT	154	Emy, Geo, Test	36, 77, 91, 103, 153
tRNA-Thr	H	15591	Tcytbthr	TTCTTTGGTTTACAAGACC	44	Trio	
tRNA-Thr	H	15593	H-15909	CAGTTTTTGGTTTACAAGACCAATG	14	Geo	
tRNA-Thr	H	15593	"M"/DW1594/THR	TCATCTTCGGTTTACAAGAC	150	Emy, Geo, Test, Trio	36, 45, 77, 91, 103, 154, 168
tRNA-Thr	L	15565	TCRThr	AAAGCAYTGGTCTTGTAAACC	TNE	Chelo, Podo, Trio	
tRNA-Thr	L	15573	PounCRThr	GGTCTTGTAAACCAAAAACTG	TNE	Podo	
tRNA-Thr	H	15593	H9	CAATCTTTGGTTTACAAGACC	135	Geo	
tRNA-Thr	L	15605	no name(2)	TCTTCCTAGAATAATCAAAAG	139	Chely	138
tRNA-Thr	L	15609	CS1	CTAGAATAATCAAAAGAGAAGG	167	Chely	
tRNA-Pro	L	15624	myt001	GAGAAAGACTTAAACCTTC	164	Test	
tRNA-Pro	L	15629	ALD-DLAFor	AGACTCAAACCCTCATCTCCGG	119	Test	
tRNA-Pro	H		DW1	CCCTTTGATAAAAGATACGGATCTTACGGC	165	Kino	166
Control	L	15863	Tur d-loop F	GGCTATGTACGTCGTGCATTCAT	174	Pelo	
Control	L	15876	DES-1	GCATTCATCTATTTTCCGTTAGCA	155	Emy	152
Control	L	15780	MS1F	CAAGGGTGGATCGGGCATAAC	54	Emy	
Control	H	15884	CR12H	ATGAATGTACAATTATACATA	93	Emy, Geo	66, 91, 92, 164, 171
Control	H	15884	CR12H	ATGAATGTACAATTATACAT	92	Emy	
Control	L	15902	H16464	CTTACTAACAAGGTTGCTAATT	105	Test	114
Control	L	P15949	Tur d-loop F1	TCTTCAGGATACCTCTGGCTGTT	174	Pelo	
Control	L	16048	KNCR 271F	ATCGTTATACATGGTTATCTATT	148	Kino	
Control	L	16088	EbF1	CGAGARATAAGCAACCCTTGT	2	Emydoidea	
Control	L	16163	L10	AACTGATTTATTCTGGCCTCT	135	Geo	
Control	L	16166	Ald-DL1FR	GATCTATTCTGGCCTCTGG	119	Test	
Control	H	16176	TCR500	CCCTGAAGAAAGAACCGAGGCC	44	Podo, Trio, Chelo	
Control	H	16188	MS1R	GTGCCTGAAAAAACAACCACAGG	54	Emy	
Control	H	16194	myt003	GACAAAACAACCAAAGGCCAG	164	Test	
Control	H	16202	LGL 1115	ATGACCCTGAAGAAAGAACCAG	87	Emy, Chely	89, 104, 114, 138, 139, 164
Control	H	16237	PounCR500	GAACCAGAGGCCTCTTAAAAAG	TNE	Podo	
Control	H	16269	DW2	GATTAATAGTCTAGAACTTACTGACCAAAGGC	165	Kino	166
Control	L	16288	KNCR 562F	GGTCTTACTTGCATATCGTAG	148	Kino	
Control	H	16294	Ald-DL2Rev	TAAAAGCGCAATATGCCAGG	119	Test	
Control	H	16308	KNCR 581R	CTACGATATGCAAGTAAGACC	148	Kino	
Control	L	16332	ChelProF	CCGGTCCCCAAAACCGGAAC	3	Kino	148
Control	H	16374	H14	CAGTCTTCATTGAGTTGGCAG	135	Geo	
Control	H	16583	EbR1	ATTTAGGGGTTGYCGAGA	2	Emydoidea	
Control	H	16585	DES-2	GGATTTAGGGGTTTGACGAGAAT	155	Emy	152, 153

sive list of potentially very useful loci in this review because these primers have not been tried on genomic DNA, and cDNA cloning techniques are not as accessible to many molecular biologists. However, we strongly encourage readers to consult the original references and explore the utility of this rich source of phylogenetically informative genetic loci.

Microsatellite Simple Tandem Repeat (STR) Loci

Microsatellites have become popular genetic markers for determining population structure and revealing differentiation among populations and individuals (Bruford and Wayne, 1993). Microsatellites, or simple tandem repeats (STRs), are non-coding repetitive DNA sequences composed of a variable number of tandemly repeating motifs. On average, STRs have mutation rates between 10^{-2} and 10^{-5} per gamete per generation (Page and Holmes, 1998) and thus can provide the resolution to differentiate individuals and populations, even within small geographic areas. Microsatellites are bi-parentally inherited (unless associated with a sex chromosome) and co-dominant, thereby allowing both alleles at a locus to be identified in heterozygotes. Microsatellites are generally considered selectively neutral (but see McGaugh et al., this volume) and their simple Mendelian transmission makes them useful for assessing genetic diversity. In freshwater turtles and tortoises, microsatellites have been used in studies of population genetics (e.g., Ciofi et al., 2002; Kuo and Janzen, 2004), conservation genetics (e.g., Sites et al., 1999; Cunningham et al., 2002; Pearse et al., 2006), as well as paternity and mating systems (e.g., Valenzuela, 2000; Roques et al., 2006; Pearse et al., in press). In addition, STRs are well-suited to address future concerns in turtle biology such as interspecies hybridization (Roy et al., 1994, 1996; Williams et al., 2005) and forensic detection of wildlife poaching (e.g., Manel et al., 2002).

The process of finding microsatellite markers can unfortunately be very time-consuming and expensive. The methods for locating STR loci have improved (Zane et al.,

Table 2. Primers currently available for amplification of nuclear loci of tortoise and freshwater turtles. Groups of taxa successfully amplified and associated references are listed in the final columns (cited references listed below). Key to taxa: CR = Suborder Cryptodira, Chely = Family Chelydridae, TE = Superfamily Testudinoidea, Test = Family Testudinidae, Geo = Family Geoemydidae, Emy = Family Emydidae, TR = Superfamily Trionychoidea, Car = Family Carettochelyidae, Trio = Family Trionychidae, K = Superfamily Kinosternoidea, Derma = Family Dermatemydidae, Kino = Family Kinosternidae, Platy = Family Platysternidae, C = Superfamily Chelonioidea, Chelo = Family Cheloniidae, Dermo = Family Dermochelyidae, PL = Suborder Pleurodira, Cheli = Family Chelidae, P = Superfamily Pelomedusoidea, Pelo = Family Pelomedusidae, Podo = Family Podocnemididae.

Target Locus	Primer Name	Length (bp)	Primer Sequence(5'-3')	Ref.	Taxa	References citing primer
Actin intron	ACT I-5'	20	GCTGTTTTCCCGTCCATTGT	121	Test	26
Actin intron	ACT II-3'	24	GTCCTTCTGCCCCATACCSACCAG	121	Test	26
aldolase intron	Ald1-5'	23	TGTGCCCAGTATAAGAAGGATGG	121	Test	26
aldolase intron	Ald2-3'	29	CCCATCAGGGAGAATTTCAGGCTCCACAA	121	Test	26
Calmodulin intron	cal1	23	GCCGAGCTGCARGAYATGATCAA	38	Test	26
Calmodulin intron	cal2	26	GTGTCCTTCATTTTNCKTGCCATCAT	37	Test	26
c-mos oncogene	G136 (F)	20	AAGCAGGTGAAGAAATGCAG	63	PL	
c-mos oncogene	G137 (R)	19	TCCAATCTTGCACACACCC	63	PL	
c-mos oncogene	CM1	23	GCCTGGTGCTCCATCGACTGGGA	12	Test	90
c-mos oncogene	CM2	25	GGGTGATGGCAAAGGAGTAGATGTC	12	Test	90
c-mos oncogene	Cmos1	26	GCCTGGTGCTCCATCGACTGGGATCA	90	Test	
c-mos oncogene	Cmos3	23	GTAGATGTCTGCTTTGGGGGTGA	90	Test	
Creatine kinase intron 6	CK6-5'	24	GACCACCTCCGAGTCATCTCBATG	121	Test	26
Creatine kinase intron 6	CK7-3'	21	CAGGTGCTCGTTCCACATGAA	121	Test	26
GAPDH	GapdH950	27	CATCAAGTCCACAACACGGTTGCTGTA	55	Emy	152
GAPDH	GapdL890	26	ACCTTTAATGCGGGTGCTGGCATTGC	55	Emy	152
HNF-1a intron 2	HNFAL-F	20	GCAGCCCTCTACACCTGGTA	131	Geo	153
HNF-1a intron 2	HNFAL-R	20	CAATATCCCCTGACCAGCAT	131	Geo	153
ITS-1	RNA-1	29	TCCGTAGGTGAACCTGCGGAAGGATCATT	95	Test	26
ITS-1	RNA-2	29	CACGAGCCGAGTGATCCACCGCTAAGAGT	95	Test	26
ITS-1	RNA-3	19	GCGTTCCGGCGCGGAGGTT	95	Test	26
ITS-1	RNA-4	19	AAACCTCCGCGCCGCAACG	95	Test	26
R35 Intron 1	R35 Ex1	21	ACGATTCTCGCTGATTCTTGC	61	Emy, Geo, Trio	36, 44, 152-154
R35 Intron 1	R35 Ex2	24	GCAGAAAACTGAATGTCTCAAAGG	61	Emy, Geo, Trio	36, 44, 152-154
R35 Intron 1	L-R35int	25	AGCATTACTACATTTTGATGCAATG	158	Geo	
R35 Intron 1	H-R35int	21	CCAGCAAAGGACTCACTTGTA	158	Geo	
R35 Intron 1	R35In1CF	20	TTKVTGBAATKTATGGRRAG	153	Geo	
R35 Intron 1	R35In1CR	20	CTYYCCATAMATTVCABMAA	153	Geo	
RAG1	RAGF1	20	CCWGAWGARATTCAGCAYCC	83	TE	
RAG1	RAGF2	21	GAGATCATTYGAAAAGGCACC	83	TE	
RAG1	RAGF3	21	AGAACCTGCATCCTRAAGTGC	83	TE	
RAG1	RAGF5	21	GAGATGTCAGYGAGAAGCATG	83	TE	
RAG1	RAGR1	22	GCAAGATCTCTTCATCRCATTC	83	TE	
RAG1	RAGR2	22	GATGTTCAGGAAGGATTTCACT	83	TE	
RAG1	RAGR3	21	CTCAGGATGGCTGTCAGAGTC	83	TE	
RAG1	RAGR4	21	TGCAACACAGCTCTGAATTGG	83	TE	
RAG1	RAGR5	20	GACATCCTCCATTTCATAGC	83	TE	
RAG2	F2 (Rag2)	23	CAGGATGGACTTTCTTTCCATGT	90	Test	
RAG2	F2-1(Rag2)	19	TTCCAGAGCTTCAGGATGG	90	Test	
RAG2	R2-1(Rag2)	25	CAGTTGAATAGAAAGGAACCCAAGT	90	Test	
Reelin intron 61	RELN61F	30	TGAAAGAGTCACTGAAATAAACTGGGAAAC	153	Geo	
Reelin intron 61	RELN61R	26	GCCATGTAATTCCATTATTTACACTG	153	Geo	

2002), yet, even for the experienced worker, laboratory procedures may require substantial time and money. Commercially, it can cost from $10,000 to $15,000 per species to develop an STR library. In addition, even after loci have been identified, there is no guarantee the loci will be polymorphic (i.e., exhibit multiple alleles) and therefore be informative to the research question. Although costs are high in the development phase, this expense is offset by relatively low costs associated with later phases (i.e., genotyping) and by the potential utility of the markers for future studies of the target species or other closely related taxa. Because the cost of commercially synthesized primers is low (ca. $0.30/bp), assessing the utility (i.e., polymorphism) of primers already developed for taxa closely related to the target species is far more cost effective. In Table 3 we have compiled the primer sequences for 160 STR loci from all major clades of turtles. Many of these loci have already exhibited successful amplification in other species.

Turtles are suggested to have conservative genomes and therefore may be particularly well suited to inter-species primer amplification (Avise et al., 1992; FitzSimmons et al., 1995; King and Julian, 2004). However, conservation of the sequence flanking the STR (i.e., where the primer attaches) does not necessarily imply that the STR motif has also been conserved. Therefore, we offer a few cautionary tales to stress the importance of sequencing polymorphic loci developed in a non-target species before making assumptions regarding utility of a marker, even if it is to be used in a closely related species. Sequencing also allows for uniformity of datasets by different researchers. For example, despite hundreds of millions of years of evolutionary change, primers developed for a microsatellite locus in *Chelonia mydas* amplify the same locus (verified by comparing flanking sequences) in *Gopherus agassizii* and exhibit moderate variability, although the repeat motif is dramatically different (Edwards et al., 2004) (Tables 3 and 4). Motif changes can also be observed within a genus (e.g., locus GP81 identified in *Gopherus polyphemus* and successfully amplified in *Gopherus agassizii* [Tables 3 and 4; Schwartz et al., 2003]), or even within a species (e.g., locus GP61 originally

Table 3. Primer pairs developed for microsatellite loci in turtles. Taxa Described = original species in which the loci were identified. Additional Taxa = Taxa in which the locus has been successfully amplified. Additonal References = Studies in addition to the original reference which have used the locus. Key to Taxa: Apsp = *Apalone spinifera;* Caca = *Caretta caretta*; Cain = *Carettochelys insculpta*; CHEL = Famly Cheloniidae; Chmy = *Chelonia mydas*; Chpi = *Chrysemys picta*; Chru = *Chelodina rugosa*; Chse = *Chelydra serpentina*; Deco = *Dermochelys coriacea*; DERM = Family Dermochelyidae; DIPS = *Dipsochelys* spp.; ELSE = *Elseya* sp.; Embl = *Emydoidea blandingii*; Emma = *Emydura macquarii*; EMYD = Famly Emydidae; Erim = *Eretmochelys imbricata*; Erma = *Erymnochelys madagascariensis*; GEOC = *Geochelone* spp.; Gera = *Geochelone radiata*; Glin = *Glyptemys insculpta*; Glmu = *Glyptemys muhlenbergii*; Goag = *Gopherus agassizii*; GOPH = *Gopherus* spp.; Gopo = *Gopherus polyphemus*; Grko = *Graptemys kohnii*; Leke = *Lepidochelys kempi*; Leol = *Lepidochelys olivacea*; Mate = *Malaclemys terrapin*; Poex = *Podocnemis expansa*; Tegr = *Testudo graeca*; Tehe = *Testudo hermanni;* Teho = *Testudo horsfieldii*; Tema = *Testudo marginata*; Tewe = *Testudo weissingeri*; Teor = *Terrapene ornata*. Information for unpublished primer sequences: Unpub01 = Arthur Georges(georges@aerg.canberra.edu.au) primers for *Chelodina rugosa* purchased under contract from Jane Hughes (Griffith U) optimized by Erika Alacs (U. Canberra); Unpub02 = FitzSimmons et al. (Nancy.Fitzsimmons@canberra.edu.au); Unpub03 = FitzSimmons and Georges; Unpub04 = Peter H. Dutton (Peter.Dutton@noaa.gov).

Locus	GenBank Acc. No.	Repeat Motif	Forward and Reverse Primer sequence (5'_3')	Amplicon Size (bp)	Obs. Alleles	Taxa Descr.	Add. Taxa	Orig. Ref.	Add. Refs.
Ah01		GA	F: TGCAGTTTGCTGAGCTTAGAG R: TGTTGGCTGGTCTCATGTTC	120-160	6	Teho		79	
Ah02		GA	F: AGGGGTGGGGATAGATTG R: GCAGAGAGCAGAGGTTTGACC	123-137	7	Teho		79	
BTCA2	AY335787	$(CA)_8N_{14}(CA)_7$	F: CTTAAAAAGACATTAAAATATCTT R: AACTCTCCCTAAAACCACAG	184–192	3	Embl	Chpi, Chse	97	
BTCA5	AY335788	$(GA)_{11}$	F: GCTGCTTAGCACAACTCATAA R: CTTTTGTATTTAATCCATGATGAA	146–154	3	Embl	Chpi, Chse	97	
BTCA7	AY335789	$(CA)_{12}$	F: TGGAATTAGATGTTTTGCAGTT R: TCATTTCTGTTTTCCACACTG	154–158	2	Embl	Chpi, Chse	97	
BTCA9	AY335790	$(CA)_9$	F: TACTCAAGATTTGAAGCAGATACA R: GGCTTGATTCTACTGTCACTTAC	148–184	9	Embl	Chpi, Chse	97	
BTGA2	AY335791	$(GGA)_5N_3(GA)_3$	F: ATGATCTAATGGTCCCTTCTG R: CTGTTAGCTTATTCTTCTGCAA	144–148	3	Embl	Chpi, Chse	97	
BTGA3	AY335792	$(GA)_{11}$	F: CCTAGATTTTGTCTGGCTATTA R: TATCTCAGTAATAATCCCCTTAG	108	1	Embl	Chpi, Chse	97	
BTGA4	AY335793	$(GA)_{11}$	F: CTCATAAAGTAAGGACGGGAA R: CCTAGAGATGGAATCTTTGTATT	146–154	3	Embl	Chpi, Chse	97	
Cc117		$(CA)_{17}$	F: TCTTTAACGTATCTCCTGTAGCTC R: CAGTAGTGTCAGTTCATTGTTTCA			Caca	CHEL, DERM	49, 50	32, 48, 50
Cc-136		$(GT)_{21}$	F: ACCAATCATTCAATGCCTTAGG R: CTTTGCTAGGTATTTATACACACAG	124-228	44	Caca		Unpub02	
Cc141			F: CAGCAGGCTGTCAGTTCTCCAC R: TAGTACGTCTGGCCTGACTTT			Caca		Unpub02	19, 32, 110
Cc7		$(CA)_{14}$	F: TGCATTGCTTGACCAATTAGTGAG R: ACATGTATAGTTGAGGAGCAAGTG	165-217	20	Caca	EMYD, GOPH	47	19, 32, 39, 40, 110
Ccar176	AF333763		F: GGCTGGGTGTCCATAAAAGA R: TTGATGCAGGAGTCACCAAG	186–220	16	Caca		110	
CCM2			F: TGGCACTGGTGGAAT R: TGACTCCCAAATACTGCT			Caca		53	19, 110
Ci-107		$(CT)_6T(CT)_3(CA)_{10}TA(CA)_8$	F: CCAGGAATTTCTTCATGCCAC R: GTTTAACATGCCTTGGCTCCTTC	288	1	Cain		Unpub03	
Ci-123		$(CA)_3CG(CA)_{13}$	F: GTTTGCAGGCAACCATCATATAGTC R: GGAACATTTCAACCCATCAGG	172	1	Cain		Unpub03	
Ci-124		$(CA)_4CN(CA)_{32}$	F: AAACAAATCTGCTATCATGCC R: GTGGAGATACAACCTTTATGATGAC	150-210	16	Cain		Unpub03	
Ci-125		$(CA)_{17}$	F: ACACAGCATATTATGATTTGG R: TTGTGTCTTTGCTATTTTAGTC	194-196	2	Cain		Unpub03	
Ci-126		$(CA)_{16}$	F: GGGATCAAACCATGCAAGTATG R: GTTTTCCAGATTTGTCCCTCCA	192-194	2	Cain		Unpub03	
Ci-128		$(CA)_{17}$	F: GTTTCCATCCCTATTAAGTTATCAC R: TTATGGGAGTTGCTCTTTGCC	283	1	Cain		Unpub03	
Ci-130		$(CA)_{12}GA(T)_7$	F: GTTTACAATACCTGCACTTTCTC R: TTAGGCAATTAACACTTCTC	103	1	Cain		Unpub03	
Ci-145		$(CA)_{13}$	F: GTTTGGGCACCTGTCTCTTATAG R: GGGCTTTCAGGCATCTTCAC	147	1	Cain		Unpub03	
Cm3		$(CA)_{13}$	F: AATACTACCATGAGATGGGATGTG R: ATTCTTTTCTCCATAAACAAGGCC			Chmy	CHEL, DERM	49, 50	32, 48, 137
Cm58		$(CA)_{13}$	F: GCCTGCAGTACACTCGGTATTTAT R: TCAATGAAAGTGACAGGATGTACC			Chmy	CHEL, DERM, Goag	49	41, 48, 137
Cm72		$(CA)_{33}$	F: CTATAAGGAGAAAGCGTTAAGACA R: CCAAATTAGGATTACACAGCCAAC			Chmy	CHEL, DERM	49	48, 137
Cm84		$(CA)_{15}$	F: TGTTTTGACATTAGTCCAGGATTG R: ATTGTTATAGCCTATTGTTCAGGA			Chmy	CHEL, DERM	49	32, 73, 80, 137
Cp10			F: GGTGCAGCAAGTTCAGGAGAC R: GGTGTTAATGCACTGGAGAATCA		~24	Chpi		129	
Cp2			F: CTCTAAGGGTTGCACTTCTCAAA R: GAGGTGGCATCAAAACATCAT		~24	Chpi		129	
Cp3			F: ATCTTTAAGTCTGTGAACTTCAGGG R: CTGTCTCATGCAAAGCTGGTAG		~24	Chpi		129	
Dc107			F: GTCACGGAAAGAGTGCCTGC R: CAATTTGAGGTTATAGACC	158–186	11	Deco	Caca	Unpub04	19
Dc99			F: CACCCATTTTTTCCCATTG	130–140		Deco		19	32
Eb05	AF416293	AAT	F: GCCAGGAACAATGTTTTA R: TTGGCATTCTACACATAATAA R: ATTTGAGCATAACTTTTCGTGG	45–57	5	Embl	Chse, Gopo	115	
Eb09	AF411049	CA	F: TTGAATTAGCTCATAAGCAC R: TCATAATGTGAATTGGTCTC	128–160	15	Embl	Gopo	115	108

Eb11	AF411050	CA	F: GAGGATCAGAATGTTCAGAC R: TCTGACTTGAATTAAACCTC	172–204	13	Embl		115	108
Eb12	AF416294	CA	F: GTCCCTAGATTTAACTGATAAACTTG R: AGGGTGGAGGAAGAGGAATAG	119–141	7	Embl	Chse, Chpi	115	
Eb15	AF411051	CA	F: AATTGATCCCTTGATCCG R: TCAGGACTATGAGGAAGC	147–186	34	Embl	Chse, Chpi, Gopo	115	
Eb17	AF416295	AAT	F: CCCACAAAAGTAGACACCTAT R: GGCACTGAAATAAGAGAAAGTA	94–109	5	Embl	Chse, Chpi, Gopo, Trsp	115	108
Eb19	AF416296	AAT	F: AGGGCTCTGAAGCACTAAAGTAA R: CTCCGGCTTTTCATTTGTGT	100–109	3	Embl	Chse, Chpi, Apsp	115	108
Ei8		$(CA)_{19}$	F: ATATGATTAGGCAAGGCTCTCAAC R: AATCTTGAGATTGGCTTAGAAATC			Erim	CHEL, DERM	49	32, 73, 80, 108, 137
GAL100		$(CA)_{26}$	F: TCTTAATAAATTCCATGAGTTGAGCT R: AGGGTGATTTCATAAACAAACAGAA	100-156	19	GEOC	DIPS	31	15, 119
GAL127		$(CA)_{21}$	F: TAACTATAAACATCAACTGGCAGAA R: GTTTAGTGTCATCTGCATATGC	97-175	31	GEOC		31	15, 62
GAL136		$(CA)_{20}$	F: ATGAGATGTATGTACAGAAAATATA R: CTGGAGGGAAGTAAGAATC	73-101	12	GEOC	DIPS	31	15, 119
GAL159		$(CA)_{24}$	F: AATATTTGAAGATACTCATCCTCGA R: TTATGTGCTTGTGTCATCTTTTT	83-123	19	GEOC		31	15
GAL247		$(CA)_{39}$	F: ATTAACTGATTTGAGCAGTCATCCA R: TGCTGTGAATAGTAACTGAGC	69–93	3	GEOC	DIPS	119	
GAL263		$(CA)_{17}$	F: GGGAAAGTACTATTTCCAGAGCTGG R: GCTGAGGCTAGCTAATTTTTATGT	80-164	25	GEOC	DIPS	31	15, 119
GAL45		$(CA)_{17}$	F: TATCTCCTTCCACACGGAGATGGG R: CCCCAAAGTAAAGTTAGCTCTCTCA	87-123	13	GEOC		31	15
GAL50		$(CA)_{24}$	F: TGGGACAGGCAAACTAACAAAACTT R: TGCAGAAGTTAATCCCTTTCTCCTT	96-182	37	GEOC	DIPS	31	15, 119
GAL73		$(CA)_{24}$	F: ATTATGTGCTTGTGTCATCTTTTTC R: TTGAAGATACTCATCCTCGATACA	78-126	20	GEOC	DIPS	31	15, 119
GAL75		$(CA)_{24}$	F: GAAGCCATTTACCACAAACTTATT R: GTTACCATAGCATTCCTGATTATAG	73-149	22	GEOC		31	15
GAL85		$(CA)_{22}$	F: TGTGGGGCATGGAAGGGCC R: CACCAAGAGAGGAAAATAATGCTGGG	81–91	3	GEOC	DIPS	119	
GAL94		$(CA)_{18}$	F: CTTCTATTTCCCAACCATCT R: AACTTTATATTTGTGTGCATATT	85-111	13	GEOC	DIPS	31	15, 119
GmuA18	AF337648	$(GT)_{14}$	F: TATCAGGGAAAGCAATGTAAGG R: AGTGAAACAAGCAGTTATGGTG			Glmu	EMYD	81	
GmuA19	AF517227	$(GA)_7(GT)_{14}$	F: TAAGAGACAGATGCTCAGCAAG R: GTACATAACACGCACCCAATG			Glmu	Teor	81	84
GmuA32	AF517228	$(GT)_{33}$	F: TTATATTGCCTGCTGCTATCAC R: ATGAAAGTGTGCCTTTCACTG			Glmu	EMYD	81	
GmuB08	AF517229	$(TAC)_{10}$	F: CTCTGAGACCCTTATTCACGTC R: AGCCTTTGTCTGTAAGCTGTTC			Glmu	Teor	81	79, 84, 140
GmuB12	AF517230	$(TAC)_7$	F: TCAATCTTCCAGCCTAACTGTG R: AGGGATGTGTTTTGCAACTGG			Glmu	Teor, Tegr	81	84
GmuB21	AF517231	$(TAC)_{10}$	F: CTAGTTCGAAACAGGACCGTTG R: CCACACGACAGTTTGATGTCAG			Glmu	Teor, Glin	81	84, 160
GmuB67	AF517232	$(TAC)_{13}$	F: ACTCAAGCACTGACACACAATC R: CCAGTATTTGTGAGAATTTCCTTC	151–168	3	Glmu	Teor	81	84
GmuB80	AF517233	$(ATCT)_{16}$	F: TTATTGTGCATTGTATCATGGG R: CGCTACCATCATGTAACTAAGAG			Glmu	EMYD	81	
GmuB91	AF517234	$(TAC)_6$	F: TCAGGGAAGCAATAGAACACTC R: TCTCATCCCTAAGTAAACCCAC	139–142	2	Glmu	Teor	81	84
GmuD107	AF517250	$(ATCT)_{15}$	F: GACAAACATGAACAGGAGAAGAG R:ATTAGAGAGACAGATAGATAGGACTTG	189–209	5	Glmu	EMYD	81	
GmuD114	AF517251	$(ATCT)_{13}$	F: ATAGACATAGTGCATATAGACATAGCC R: ACGTTCTTGCAGGGTCAGAG	92–128	6	Glmu	EMYD	81	141
GmuD121	AF517252	$(ATCT)_8$	F: GGCAAATATCCAATAGAAATCC R: CAACTTCCTCGTGGGTTCAG	138–154	5	Glmu	Teor	81	84
GmuD16	AF517235	$(ATCT)_{19}$	F: ATCCCTGAAATTTTGTGTGTTC R: TTTACTCTAGAAGGGGCAATCC	188–228	9	Glmu	EMYD, Glin, Tegr	81	84, 160
GmuD21	AF517236	$(ATCT)_{15}$	F: GCAGTTAGGCATTACTCAACATC R: AGGGTATGAATACAGGGGTGTC	163–199	5	Glmu	Teor	81	84
GmuD28	AF517237	$(ATCT)_{15}$	F: AGCTGTTTGTCATCATACACTCTC R: TGGCCCTCATGTTTTATAAGTG	208–236	6	Glmu	EMYD	81	
GmuD40	AF517238	$(ATCT)_{22}$	F: TTTGTCATATCATCCACTCACC R: TTTGTCACAGATGGGAATTAGC	157–201	9	Glmu	EMYD, Glin	81	160
GmuD51	AF517239	$(ATCT)_{52}$	F: GTTGGGCACTAGATAGATTCG R: CATTCAAGTCAACGGAAAGAC	307–359	10	Glmu	EMYD, Tegr	81	79, 140, 141
GmuD55	AF517240	$(ATCT)_{10}$	F: GTGATACTCTGCAACCCATCC R: TTGCATTCAGAATATCCATCAG	212–224	4	Glmu	Teor	81	84
GmuD62	AF517241	$(ATCT)_{11}$	F: GGTGGTATAGAAAATCCTAAAATGG R: GTGCAAACTGTCTGGAAATAGG			Glmu	Teor	81	84
GmuD70	AF517242	$(ATCT)_8$	F: AGTGTAGTCATGGCATAGAGAGG R: ATCAAATTCTTCCAACCCTACC	185-205	5	Glmu	EMYD	81	
GmuD79	AF517243	$(ATCT)_{10}$	F: GCCCTGTTCCATTCTTATTCTG R: ATCCCCTTAGTCGTCTCTTTTC	164–192	3	Glmu	Teor	81	84
GmuD87	AF517244	$(ATCT)_{22}$	F: AAACCCTAAGACATCAGACAGG R: CAAATCCAGTACCCAGAAAGTC	260–292	8	Glmu	Teor, Glin	81	79, 84, 140
GmuD88	AF517245	$(ATCT)_{18}$	F: AACAATGCCTGAAAATGCAC R: TAGGCTACCTCTGAAAATGCTG	154–178	17	Glmu	Teor	81	84
GmuD89	AF517246	$(ATCT)_7$	F: GCTCGCTGTAACTAGCTCTAACTC R: CCAGGCAGCTTTGTTTAATG	112–124	3	Glmu	EMYD	81	
GmuD90	AF517247	$(ATCT)_9$	F: ATAGCAGGACAATTACCACCAG R: CCTAGTTGCTGCTGACTCCAC	122–134	3	Glmu	Teor	81	84

GmuD93	AF517248	$(ATCT)_{18}$	F: AGACTCTCTTGACCAGATTTTCTC R: TCTGCCTTCTATCACTCTCCTG	185–389	10	Glmu	Teor, Glin	81	84, 140, 141
GmuD95	AF517249	$(ATCT)_{17}$	F: AGGTACGAGACAGGACAAAGTG R: TGAATGCAGTGTAACATTTGAG	153–177	4	Glmu	Teor	81	84
*Goag*3	AY317141	$(CAA)_6$	F: CTGATTGGTCTGACTCCCT R: CCTGATTGCTTCCTGACAC	375-381	3	Goag		40	
*Goag*32	AY317147	$(AC)_6$	F: GTGCTGCCTTGATAAGTAA R: ATAGTTTTCTTTCCTACACAT	177-179	2	Goag		40	
*Goag*4	AY317142	$(CAA)_{24}$	F: CTCAACAAAAGGTAAGTGATG R: GCATAAAAGTAAACAGTAAAGTA	110-188	17	Goag		40	
*Goag*5	AY317143	$(GAT)_{17}$	F: AGGCAAGTGGGTGGTAATG R: GCGATTTTGAGGCTTCTTTC	257-365	27	Goag		40	
*Goag*6	AY317144	$(TC)_8(AC)_{11}$	F: TAAGGGCTATGAGGAAGAAT R: GTAATGGTGTGGGTGGGA	360-442	15	Goag		40	
*Goag*7	AY317145	$(AC)_3(GC)_5(AC)_{11}$	F: TCAATCCATTAGTCTTCACCC R: TTTCTGTTTATGCTCCGTATTA	261-281	8	Goag		40	
*Goag*8	AY317146	$(CA)_{14}TA(CA)_3$	F: ATGCTGACAATAGAACAAGA R: ACATCTGGGGCTAAAGTG	192	1	Goag		40	
GP102	AF546890	$(GT)_5(CT)_{13}(CA)_5$	F: AGCTGCCTGACTGCTATGCT R: GCATAATCAGCATCAACAACAAA	299–339	15	Gopo	GOPH, Grko	146	54, 109, 145
GP15	AF546895	$(GA)_{15}(GT)_8$	F: CCTATTTTTCCCCCTCACAGT R: GAAAATAAAAACAGTCCCAACCA	207–269	19	Gopo	GOPH, Grko	146	54, 109, 145
GP19	AF546891	$(GT)_9/(GT)_3(GA)_6$	F: GCAGGACAGTGCCACACTA R: CAGCCATATTAATGACAATCTG	252–256	3	Gopo	GOPH, Grko	146	54, 109, 145
GP26	AF546892	$(GT)_{12}$	F: GACAACCATCTTTACCCACA R: TCCCAAGACATAAGTCAGTAGC	358–370	6	Gopo	GOPH, Grko	146	54, 109, 145
GP30	AF546889	$(GT)_{13}$	F: GAATGCAGCACTGCTTGGTA R: CGAAGAGGGAGCACGTTTAG	194–232	10	Gopo	GOPH, Grko	146	54, 109, 145
GP55	AF546893	$(GT)_9$	F: TTAGGGATTTTCTGTCTACTTCAG R: CGCAATGTGACACGCTATT	265–271	2	Gopo	GOPH, Grko	146	54, 109, 145
GP61	AF546896	$(GT)_{12}$	F: GCATTAAACCATTGTGCCTCA R: AGTGGTGGTCGAAGTGGAAC	197–245	7	Gopo	GOPH, Grko	146	54, 109, 145
GP81	AF546894	$(GT)_{11}(GA)_{10}$	F: TCACACAAACCCCATCCATA R: TCCATTGAATTGCCATCTGA	397–415	7	Gopo	GOPH, Grko	146	54, 109, 145
GP96	AF546888	$(GA)_{11}$	F: TCAGTTACCGGATAATGTTCAGTG R: TGCTGTTACCTCGTGCATGT	141–157	8	Gopo	GOPH, Grko	146	54, 109, 145
Klk314		$(CA)_5$	F: GGTGCCAAGGAGGACGCTG R: CATGCTCGCCCCTGGAAAG	109	1	Leke		80	
Klk315		$(CA)_8$	F: AGACAAACTCCCCCTTGCTAGG R: CCCAGAAGGTGAAGAAATACCAAA	135	10	Leke	Leol	80	
Klk316		$(CA)_{22}$	F: TACATCCATACATGCAGCCCCCTGA R: GGTGCTAGGGTGAGTATTGAGCACT	132	3	Leke	Leol	80	32
Klk325		$(CA)_8$	F: CCCAGTTCCTTTCAACCAAGTA R: CTTGAGCTTTAACAGATGACAAAA	155	1	Leke		80	
MR-1	AY934859	(AC)11	F: TTTCTGCACCTGCTTAACTT R: CTCATGGAGGTGGTGTTACT	222–234	5	Mari		101	
MR-2	AY934860	(AC)9	F: ACGGAATCCTGATTAATTCC R: CTTCCCTCAATACAATGGTT	199–229	5	Mari		101	
MR-3	AY934861	GT)8	F: CATTTTCTTTATCGCCTCAC R: CTTTCACAGCACAAGTCTCA	181–189	5	Mari		101	
MR-5	AY934862	(GA)18	F: TCTAGGGTCGCCCCTGTAGG R: CTGGGAATGTTCTGCGGTTG	149–189	10	Mari		101	
MR-8	AY934863	(GT)32(GA)12	F: TGCCCTCTGATGCTCTGGTG R: GCCCAAATGTCTACAACTGTGG	154–194	18			101	
MR-9	AY934864	(CT)16	F: CCAATGCTCCAGGCGTG R: GCCAGTCTTACTGCTGAACC	97–105	5			101	
OR-1	AY325422	$(CAAA)_{16}$	F: CCCCTTGTGTTCTGAAATCCTATGA R: CAGGCATAGGGAAAAATCAGAGGTA	150–202	24	Leol	Chmy, Erim	1	
OR-2	AY325423	$(GT)_8GCC(GT)_5$	F: GCTCCTGCATCACTATTTCCTGTT R: TGCTGCCCCCACACCCTCTG	153–185	12	Leol	Chmy, Erim, Deco	1	
OR-3	AY325424	$(TC)_9(AC)_6GC(AC)_2$	F: TTGTTTTATTTTTATTGGTCATTTCAG R: GCACCTTTTCACGTTGTCCACATGT	146	1	Leol	Chmy, Erim, Deco	1	
OR-4	AY325425	$(TG)_9/(TG)_{23}$	F: AGGCACACTAACAGAGAACTTGG R: GGGACCCTAAAATACCACAAGACA	122–172	18	Leol	Chmy, Erim	1	
OR-7	AY325427	$(GT)_6(GA)_7$	F:GGGTTAGATATAGGAGGTGCTTGATGT R: TCAGGATTAGCCAACAAGAGCAAAA	185–219	16	Leol	Chmy, Erim, Deco	1	
OR-8	AY325428	$(TC)_{23}$	F: GCACTGGTGGGAAAATATTGTTGT R: GCTGGGCTAATAAAATGTTGTGCA	148–166	8	Leol	Chmy, Erim, Deco	1	
PE1075	AF141138	$(AC)_{11}$	F: ATGAGCCTGAAGAGTTGGAA R: AACTTAGGCTGCATGAGTTG	247–283	6	Poex		161	127, 128
PE344	AF141136	$(AG)_{13}$	F: ATCCTGAGTTTAAAGGTGA R: AACTCTTCAAACTCCTCTAG	144–208	10	Poex		161	127, 128
PE519	AF141137	$(CT)_7(CA)_8(CG)_2(CA)_8$	F: GCTGAGCTAGACTAACATGC R: GTAAATTGCCATACTTGGAG	239–327	8	Poex		161	127, 128
Pod1		$(CA)_{32}$	F: GATCTTTCTTTACAGGTGCAGTTC R: CACAACTAAATTACAGCACTCCG	154-204	21	Poex		151	127, 128, 161
Pod128		$(GT)_{27}(GC)_7$	F: GTGTCAGGGCTACCATCAAGATTG R: CCAGTAAAATTCACTACCAGCATG	140-209	23	Poex		151	127, 128, 161
Pod147		$(GT)_{16}(A)_{20}$	F: GTGACAGCAGCATCTCATTTTCTC R: ATGACACATTACCATCCCATAGG	181-249	19	Poex		151	127, 128, 161
Pod62		$(GT)_{11}(TA)_5$	F: ATGAGTGTGGAATGAGAGGAAC R: CCCATCCACAGAAGCAAATTCC	182-214	9	Poex		151	127, 128, 161
Pod79		$(CT)_{13}(CA)_{16}$	F: GGGAGAGCATTGCTGGTTGGTG R: CAATGTCATCACCGCAGAACCC	220-260	16	Poex		151	127, 128, 161
Pod91		$G_9(GT)_{17}(GA)_8$	F: TCATTTTGGTTAGAAGTGAAGGC R: GGTTGTTCATCTTTTAGATTCACC	111-255	40	Poex		151	127, 128, 161

RAD14	AY900651	$(CT)_{12}(AC)_{14}$	F: GATCCCCAACTGTCACCAC R: AAAATGTTGCTCTCCTAAATGC	218–262		Gera		122	
RAD27	AY900652	$(TG)_7TA(TG)_{16}$	F: AAAATCTACCAAGGTCTGCAAAG R: TTACAGAGCATCAGCAAGGC	230–270		Gera		122	
RAD284	AY900653	$(GT)_{22}$	F: GTGCTGAACAGAGGCTGATG R: CACACACACAGACAGAAGATTATT	209–243		Gera		122	
RAD313	AY900654	$(GT)_{12}GAG(GT)_3(GA)_6(GT)_5(GA)_{11}$	F: AGTTGTTTTCCCACCCCC R: TCCCCAAGACACCTGCTG	220–292		Gera		122	
RAD542	AY900655	$(CA)_{13}$	F: TCCTGTGATTGTTTCATAGAACG R: TCTGCTCCTTCCTGTGTGC	148–196		Gera		122	
RAD573	AY900656	$(CA)_8(TGCA)_2(CA)_2CG(CA)_4$	F: TGAACAGAACGATCCTCCCC R: GGGAAAGCCAGGGCACTAG	199–225		Gera		122	
RAD891	AY900657	$(CT)_{12}(CA)_6CG(CA)_9$	F: TATTCACCCACGAAAGCTCA R: GGTTGTTGGAGAAAGGAGGA	194–242		Gera		122	
RAD932	AY900658	$(GT)_{15}$	F: GGTAGATAGTTCCTTCAGCCTTG R: TCCCCTCTTTTTCTGTCTCATAG	152–204		Gera		122	
T12		$(CAG)_9/GAG/(CAG)_3$	F: GGGATCACTCGGCCACTCTGG R: ACCCAAGAATACCCGTCACCG	157-163	3	Chru		Unpub01	
T14		$(TGC)_8$	F: TAGGCTCAGGGATATGATAGC R: CTCCAGCGACAGTTGCAACAG	120-129	4	Chru		Unpub01	
T17		$(TGC)_7$	F: AACAGTATTATGGATGCAGAC R: GACACAAAAGGTACCATTCCC	121-130	4	Chru		Unpub01	
T26		$(GCA)_7$	F: CAGTGATTTTTGCTACCAAGG R: GCAAAACAGTATTATGGATGC	158-167	4	Chru		Unpub01	
T42		$(ACC)_8$	F: CCAAACTTGAACACTGCTGTG R: GGACTCCCAGATTATGGTCTC	158-164	2	Chru		Unpub01	
T44		$(AGC)_7$	F: AAGGCAGTTGAGAACCAGGTG R: GTAGATGCCACCCATGTTGTC	131-142	5	Chru		Unpub01	
T50		$(GCA)_8$	F: TGCTGCCTGCCATTAGCTTAC R: CTGCATTTGAGCAATTCGCTG	134	1	Chru		Unpub01	
T58		$(CAC)_7$	F: TCCTGAAAGGGTGGGCAAAGG R: CTAGATGATTCTCAGTCTTTC	157-166	3	Chru		Unpub01	
T79		$(TGC)_7C(TGC)_1$	F: TTCCCCCCACAAGTCACTTTC R: TGTATTACTCTCCGTGTCTCG					Unpub01	
T80		$(TGC)_7$	F: CTCACCTGCAGCCTCTTTCTC R: AGGACCTTTCAGGACCCTCAC	138-159	6	Chru		Unpub01	
T87		$(TGC)_9$	F: CAGCACTGATCTGCAAGTACC R: GCTACACCAGTTTCACTCTGC	136-148	3	Chru		Unpub01	
TerpSH1	AY156709	$(AGAT)_{15}$	F: CCACTGGGATCTAATCACTT R: GGCAACTTAGCAT	254–302	12	Mate		68	69
TerpSH2	AY156710	$(AGAT)_{12}$	F: GCCAGCAGGAGTAATG R: CTATTAGGGCAGAGACGAG	171–227	12	Mate		68	69
TerpSH3	AY156711	$(CAAA)_{14}$	F: TCCCCCAATGCACAC R: CTGC*CCAATCCATTTAGA	283–311	8	Mate		68	69
TerpSH5	AY156713	$(CTAT)_{12}$	F: TTGCTGCTATATGCTTAAT R: CCTCCCTGCCTATTGA	157–189	8	Mate		68	69
TerpSH7	AY156715	$(AGAT)_{13}$	F: CACACACACTGTATTTTGATA R: CTATGCCCTTTCTAGTTTG	97–137	10	Mate		68	69
TerpSH8	AY156716	$(GA)_{19}$	F: CCAAATTAAATATCTACC R: AGCCTTTCCAGTATTCAGTA	193–221	14	Mate		68	69
Test10	AY822052	$(AC)_{15}(TA)_2(GA)_2$	F: AGACTCTCTGTGATGGTAATAGCA R: GATTTTCATTGGCATATAAGACACA	194–228	10	Tehe		52	
Test21	AY822048	$(CA)_{10}(CT)_5$	F: AAACTGGCTGAAACCCAGC R: TTGGGAGTTTGACTGATCTAGGA	203–235	9	Tehe		68	
Test56	*AY822049*	$(CT)_6GCT(CA)_{12}$	F: GATATGCAGGCAAACAGGCT R: CAGGAATCTGTGCATGATTGA	199–205	3	Tehe		68	
Test71	AY822050	$(AC)_9$	F: GATTGTGGTCACATATAGAGGAGG R: TGTTGTACTTAGCTGTTCTGATCTATT	126–130	3	Tehe		68	
Test76	AY822051	$(CA)_8$	F: GAATTCTAACTTTTCTCTGTGGAGC R: TCTTATTGCATATCTGAGTACAGAAGA	116–118	2	Tehe		68	
Test88	AY822053	$(TC)_{10}(AC)_8$	F: TTTCCACAGAAAGGAGGAGC R: CAAATTGAATAAACAGAGTTTTCCC	181–209	5	Tehe		68	
tle10f		AC	F: TTCTGCTTCTGTGGTTCCACC R: CTGTATTTCAAGGACTCTGCC	139-155	6	Emma	ELSE	Unpub01	
tle13.1		TG	F: TGGGTCTAATTCAGTGAAGAG R: TGAGTTTCAGGCATCTCCTCG	197-221	20	Emma	ELSE	Unpub01	
tle13.3		TG	F: GTGTCAGCCCTCCAGAATGTC R: TCAACGAGAAGCAAATTGAAG	110-168		Emma		Unpub01	
tle6.2		GT	F: GTTTACAGTTCACCTCTTCAG R: TCAATCTAACGTAATTGTGCC	97-129	22	Emma		Unpub01	
tle7.2		CA	F: ACAGCCATCACGTTTAGCCAC R: GCCAATTTGTTTACATATCCC	121-141	12	Emma		Unpub01	
tle16.31		AC	F: GACCCTAATCCCCTCCTAATCC R: CCAACCCTTCTGACTCTCACTC	231-309	36	Emma	ELSE	Unpub01	
tle19.1		CTT	F: CTACCACCTGCTTTACCAACC R: GTGAAACCCGATGCTCTTGAACC	181-202	8	Emma	ELSE	Unpub01	
tle19.3		AC	F: CAGCGTTTTGCCCATGGTAAG R: GTGCTAAACCAGTCTCATTGTG	253-299	24	Emma		Unpub01	
tle23.41		$(GCT)_4CCT(GCT)_4$	F: CACCCAAGAATACCCGTCACC R: GTACACCCAATGATCACTCG	176	1	Emma		Unpub01	
tle28.21		AC	F: GCTTTGCCTATCATCCTCTTGC R: CCTGGTCTCATTCAGAAAGG	133-173	17	Emma	ELSE	Unpub01	
tle31.1		$(TC)_{14}(AC)_{10}$	F: TAACGGAAGGTCTTCAAAGGTC R: GTAGTGTGTCCCAGGCGATTCGAC	270-384	26	Emma		Unpub01	
TWS190	DQ398951	(TC)9	F: TTGTTCTGCCATCAGTCAGC R: ATCCCCTTACCACCAACTCC	091–097	3	Tewe	Tema	130	

TWL61	DQ398949	(CA)13	F: CCAACCCTGTAGGACTGAAGC R: GTTCCGAGCACTGCAACC	137–171	9	Tewe	Tema	130
TWR106	DQ398952	(CT)11(CA)19	F: ACAATCCCACACTCCTTTGC R: CTCACCTTTGGCCCTTCC	171–227	10	Tewe	Tema	130
TWL221	DQ398955	(TG)12 (TCTG)6TC	F: TGCTGGCTGAAGTTTACAGAG R: CCAGAAGCTGAAGCAACTCC	217–267	6	Tewe	Tema	130
TWMD51	DQ398956	(AC)7	F: CACTGGGCAGAAACCAGAAG R: GCTGCATGTGGCTCTTTTAC	249–251	2	Tewe	Tema	130
TWI61	DQ398953	(GT)11(GA)10	F: TATTTCAGGCGTGGAGCAAC R: CAATGGGCTACTTGCCTACC	242–344	19	Tewe	Tema	130
TWT113	DQ398954	(TC)10	F: CTTTTAGGCTGGGCTGATTG R: ATGCAACCCCAGTACCTCTG	276–286	5	Tewe	Tema	130
TWQ113	DQ398950	(CT)12	F: CAGAGGACGTGAGCGAGAG R: TTGAGGATGTTGTAGAGGATGC	281–293	6	Tewe	Tema	130
59HDZ131	DQ464448	(CA)12	F:AAGTTCAGACTGGGCAGGG R:CCACCTTCAGACACACACTCAC	204–220	4	Erma		136
59HDZ188	DQ464447	(CA)9	F:CTCAAACCAGGGGCTAAAG R:CTATTTCAGGCTGTGGGAGG	208–214	3	Erma		136
59HDZ196	DQ464449	(GT)21	F:AGGATTCAAACAGTGGAGTGC R:CCCAGACAATGACTAACAAACC	196–220	5	Erma		136
59HDZ234	DQ464450	(CTTT)5	F:CTCCCACGAAATCTCATGC R:TGTAAGATGCTGGCAAAAGTG	231–235	3	Erma		136
59HDZ242	DQ464451	(GT)17	F:AGCGGAGAGAGGGGGAAC R:TGAAACAAAGGGGCAATCC	078–094	5	Erma		136
59HDZ327	DQ464452	(TC)8(AC)7AAAA(TC)8(AC) 8AATT(TC)9TT(TC)8(AC)11	F:ACACAGGGTCCATCCACTTC R:TCAGCAAAACAAGCAACGAG	308–316	4	Erma		136
59HDZ397	DQ464453	(GT)7	F:GAACGCACCAGAACGCAG R:CCCAGAACGCTCCTACATTG	140–160	4	Erma		136
59HDZ499	DQ464454	(CA)9GA(CA)3 GC(CA)14	F:GTGAGCCCCCAAATSCCC R:TGCTGGACAACTAATCTTTTCTATC	187–205	8	Erma		136
59HDZ669	DQ464455	(GT)9	F:CCAGGACATCTTAGACTACTGTTCC R:CACTATTTAGGCTTTTCATTCTGC	225–229	4	Erma		136
59HDZ777	DQ464456	(CA)20	F:GAAAAAAAAAGGGGTGGGG R:AGGGAGTTAGGGGTTGTAGGAG	134–148	7	Erma		136
59HDZ897	DQ464457	(GT)13	F:TGTGTGGAGAGGGATGGTTC R:GTATGCTTAACCCCCACCTC	147–159	6	Erma		136

described in *G. polyphemus* [Schwartz et al., 2003]). Locus GP61 exhibits two different motif states in *G. agassizii*; alleles having greater than 16 repeats have a simple dinucleotde motif, $(GT)_{16+}$, but alleles that score in the range of 10–12 repeats possess a compound motif, $(GT)_4AT(GT)_6$ (Edwards, unpubl. data; Tables 3 and 4). For this locus a single *G. agassizii* individual can be homozygous for either motif or heterozygous for both motifs. Knowledge of the different allelic states can help researchers choose the best model for their analysis, such that an infinite allele model might be a better choice for analyses of these data than a stepwise model of evolution.

While motif differences among species may not affect the utility of a marker within a species, changes that occur across populations within a species might reveal more significant evolutionary changes that would be masked during fragment analysis without subsequent sequencing. For example, locus Goag05 was originally described in *Gopherus agassizii* from samples collected in the Sonoran Desert (Tables 3 and 4; Edwards et al., 2003). Fragment analysis of this locus in *G. agassizii* samples collected from the Mojave Desert reveal amplicon lengths in the range of those observed in the Sonoran samples. However, comparison of locus sequences from both populations revealed fixed differences in the motif indicating that there has been significant evolutionary change between the populations and that gene flow does not occur (Edwards, unpubl. data; Tables 3 and 4). It might also be implied that the motif observed in the Mojave Desert samples is derived from the Sonoran Desert motif. The nucleotide sequence of the flanking regions surrounding the motif also revealed single nucleotide polymorphisms (SNPs) between the two populations. Although microsatellites are generally best applied to genetic studies within a species, these examples suggest that sequencing STR loci and their flanking regions can reveal potentially neutral, autosomal SNPs that imply deeper evolutionary changes and are applicable for inter-species phylogenetic studies.

The development of molecular tools for freshwater turtles and tortoises is not complete. Obviously there is great potential in exploring and applying entirely new molecular techniques, such as sequencing entire mitochondrial genomes (Parham et al., 2006a,b), development of additional informative nuclear markers (Fujita et al., 2004), or microarrays and beyond. Indeed, there are many questions and many species that will require development of new markers or new approaches. However, there is still much to be learned about the biology and conservation of freshwater turtles and tortoises by simply applying the wide array of molecular markers that are already available today. For the majority of common

Table 4. Observed motif differences from cross-species amplification of microsatellite loci.

Locus	Species	Motif
Cm58	*Chelonia mydas*	$(CA)_{13}$
	Gopherus agassizii	$(CA)_2CG(CT)_4$
GP81	*Gopherus polyphemus*	$(GT)_{11}(GA)_{10}$
	Gopherus agassizii	$(GT)_9GACA(GA)_8$
GP61	*Gopherus polyphemus*	$(GT)_{12}$
	Gopherus agassizii (allelic state 1)	$(GT)_{16+}$
	Gopherus agassizii (allelic state 2)	$(GT)_4AT\ (GT)_6$
Goag05	*Gopherus agassizii* (Sonoran)	$(GAT)_{6\text{-}38}$
	Gopherus agassizii (Mojave)	$GACGAA(GAT)_2GACGAA$

applications in most species, all the tools needed already exist and are consolidated here. It should be noted, however, that the tables we provide here are incomplete, as many researchers have not included in their publications information such as GenBank accession numbers, STR motifs, expected amplicon size, or other species that a primer might have utility in. We urge those in the research community contributing such data to the scientific literature to include as much information as possible. We are entering a new era in which the cost and time associated with the development of molecular markers should not hinder researchers hoping to apply molecular approaches to important challenges in turtle biology and conservation.

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Non-Standard Sources in a Standardized World: Responsible Practice and Ethics of Acquiring Turtle Specimens for Scientific Use

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Abstract. – Many of the world's turtle species are seriously threatened in the wild, calling in question the need to collect and preserve whole animals for research activities. However, there are new opportunities for collecting samples from non-standard sources. In this paper we define standard and non-standard materials and discuss guidelines for their ethical and responsible collection and use.

Key Words. – Reptilia; Testudines; turtles; tortoises; ethical collecting; non-standard materials; standard materials; legal requirements; laws; regulations

Many of the world's turtle species are seriously threatened in the wild by habitat destruction and overexploitation by humans (Thorbjarnarson et al., 2000; van Dijk et al., 2000). This decline increases the urgency for studies on the conservation, systematics, and population biology of turtles, and calls in question the need to collect whole animals for these research activities. In addition, the threatened status of some populations and species makes destructive sampling of turtles for research even more difficult to justify. The exploitative use of turtles for food, traditional medicine, religious purposes, and pets ironically creates new opportunities for collecting research samples from non-standard sources. This paper defines 'standard' and 'non-standard' material for use in scientific research on turtles, addresses ethical issues, and sets out guidelines for responsibly collecting and using these materials.

We preface this discussion with the explicit recognition that it is the responsibility of individual researchers to be aware of and to comply with the relevant laws and regulations of the jurisdictions where samples are collected and transported. We also explicitly recognize that ethical decisions are ultimately the responsibility of the researcher. Ethical issues are contentious, and even among the authors of this paper there are varying opinions on particular issues; therefore this paper represents a consensus of our opinions. We present it as a discussion of the specific issues and to offer some general guidelines for making ethical decisions.

While we advocate minimizing the sacrifice of animals for science, we are not proposing that the use of non-standard samples replace standard methods of collection in all circumstances. Non-standard samples in conjunction with standard samples can allow for greater samples sizes, especially for hard-to-find or rare species. Non-standard samples are often of limited value, but in some circumstances non-standard samples may be all that are available. For example, the Southeast Asian box turtles *Cuora mccordi*, *C. picturata*, and *C. zhoui* are known to biologists only from commercial trade specimens and remain unknown in the wild (Parham et al., 2004; Stuart and Parham, 2007).

Definitions of Standard and Non-Standard Materials

Scientific researchers sample biological material from turtles for a variety of purposes, including ecological, population genetic, systematic, and genomic studies. Typically, this material is obtained by the researcher or an agent under his/her supervision, by collecting individuals in the wild, recording data on the circumstances of collection, and depositing the material, usually in association with a voucher specimen (see Lehn et al., 2007), in a natural history repository institution where it is curated for long-term preservation, and made available to other researchers. Hereafter, such materials with clear and documented provenance are referred to as 'standard materials'. Among other uses, such specimens are used to verify taxonomic identifications and to document where and when the species was found.

Non-standard sources of material may include live or dead turtles from the pet trade, markets, zoos, private

collections, salvage (such as trophies and trash), pharmacies, tourist items, archeological and anthropological artifacts, and other sources (see Pritchard, 2007). These might be obtained either *in-situ* (within the species' range) or *ex-situ* (outside the species' range). Two key features of non-standard specimens are that they do not always require the sacrifice of a specimen, and they may not have been collected by either the researcher or an agent of the researcher. Therefore non-standard specimens are often of unknown, uncertain, or very generalized provenance. While many non-standard materials have traditionally been accepted by museums, such materials have become significantly more important as systematic research has added molecular approaches to the traditional morphological research that historically placed greater emphasis on whole specimens (e.g., Engstrom et al., 2002).

The use of non-standard specimens is particularly important to turtle biology because of some special characteristics of turtles. Turtles are often large-bodied, making handling, preservation, and curation of whole specimens difficult. They have a relatively late age of maturity and low offspring survivorship, typically resulting in low recruitment levels and low sustainable harvest levels. These demographic characteristics typically form the basis for objections to collecting large series of specimens. Turtles are often kept in captivity for long periods, and are often sold for meat or medicinal purposes in either local or distant markets. Thus, many turtle species are now more readily available through secondary means than through direct capture in the field. These circumstances dictate that non-standard opportunities to collect specimens must be considered. Incorporating non-standard specimens into research presents the further advantage of collecting data on a species without sacrificing individuals, an important consideration for endangered, rare, and legally protected species.

Researchers' Responsibilities

Collection of both standard and non-standard specimens entails many ethical, legal, and practical issues (Duellman, 1999). In consideration of these complicated issues and regardless of historical practices, all specimens must be obtained and transported only in compliance with all relevant regulations and laws in all pertinent jurisdictions. Regardless of the type of specimens to be collected, it is the responsibility of the researcher to be familiar with all applicable regulations and laws and to follow them completely. This task may be difficult, especially where multiple countries, states, and agencies may have jurisdiction and conflicting interests. In many cases, legal collection and possession of biological materials requires that researchers obtain permits, which often require significant time and effort in advance (Duellman, 1999). Researchers should be aware that most regulations and laws apply to parts of animals in the same way they apply to whole animals, and therefore collecting non-standard specimens usually requires the same permitting procedures as for standard specimens. In some cases, export of non-standard materials is even more restricted than that of standard materials because of their perceived value as "genetic resources" (Duellman, 1999).

Specimens should only be collected under humane protocols, and where appropriate, previous approval should be obtained from Institutional Animal Care and Use Committees or Animal Ethic Committees. Researchers should follow professional protocols, such as the "Guidelines for Use of Live Amphibians and Reptiles in Field Research" (http://www.asih.org/pubs/herpcoll.html). If lethal collecting is necessary, extra effort should be made to sacrifice humanely, because turtles are very resilient (Pritchard, 2007). Finally, researchers have a responsibility to publish or otherwise disseminate their results to the people, organizations, and regulatory agencies that might make use of their results. Hopefully, this would help avoid unnecessary duplication of sampling efforts, thus minimizing impacts of research on wild populations.

Ethics of Non-Standard Material Collection

Although standard specimens usually provide the most complete information for use in research, in some circumstances using non-standard specimens presents ethical advantages over standard specimens. Examples of this approach include collecting salvage material from refuse sites or DNA from captive specimens, which might reduce the need to remove individuals from vulnerable wild populations. Obtaining turtles from meat or pet markets may provide opportunities to build assurance colonies, thus allowing these individuals to make genetic contributions to future generations. Non-standard sources such as markets may be valuable sources of natural history information, and also may present opportunities for scientists to present the case for conservation (Shine et al., 1998, 1999; Pritchard 2007). In all such cases, we encourage authors to be explicit about circumstances of acquisition of specimens utilized in research, for example, pet trade or wildlife trade specimens should be identified as such in publications.

Many museums have accepted market-collected specimens in the past, and this practice will probably become more common as markets become more prominent sources of specimens. Researchers should make every effort to avoid paying for specimens because of the possibility that they might stimulate either *in-situ* or *ex-situ* markets, thus negatively impacting wild populations. This risk might be reduced if the source is an already existing market, rather than a situation where the purchase of a sample may create a new market. It is important to note that market-obtained specimens are subject to laws that regulate collection for scientific purposes and international transport, even though the same materials may be legally sold locally for consumption or other uses, or sold illegally but without significant enforcement effort. Another limitation of market-collected speci-

mens is the lack of quality collection data. For example, the unreliability of locality data associated with turtle specimens allegedly purchased in markets has led to the erroneous assumption that some captive-produced hybrids represented naturally occurring species, thus confounding conservation efforts (Parham et al., 2001; Stuart and Thorbjarnarson, 2003).

Researchers may be tempted to conclude that if a species is sold in large quantities in markets, it must be abundant in the wild. This is not necessarily true—rare species are sometimes temporarily common in markets because a few centers of abundance have been discovered and exploited unsustainably by commercial interests. For example, *Leucocephalon yuwonoi*, endemic to the island of Sulawesi in Indonesia, appeared in large numbers in Chinese markets for a short period of time before becoming commercially extinct (Lau and Shi, 2000). Conversely, common species may be rare in markets because of low demand.

One ethical issue associated with non-standard materials is the consideration of whether there are any circumstances under which illegally collected specimens may be used in scientific research. For example, scientifically valuable photographs of specimens of uncertain provenance may become available, or illegal specimens may be widely and openly available in markets, and the researcher must make ethical decisions as to whether any kinds of data, including strictly observational, may be obtained. It might be argued that when a researcher acts without malice and does not stimulate markets, it is wasteful not to make use of available specimens, especially when it does not entail further losses from wild populations. A contrasting viewpoint is that once a specimen is collected illegally, its scientific value must be ignored so as not to encourage further illegal activities. We could not come to consensus on this issue, however, it should be noted that for liability reasons, many museums can only accept specimens with demonstrable legality.

Sometimes a researcher may subsample a specimen (such as taking a small amount of tissue for DNA analysis) while knowing that the specimen is likely to be maintained in captivity. In such cases the researcher should make every effort to ensure that the individual animal is photographed and uniquely marked for future identification, so that upon its death, it can be deposited in an appropriate museum as a voucher. Data associated with the previously collected subsample should be provided to the museum; in this way the non-standard and standard materials are linked.

Ethics of Standard Material Collection

There are situations where sacrificing turtles may be necessary. For example, type specimens of named taxa should be deposited in a permanent collection where they may be examined by other researchers. In other circumstances, it may be acceptable to conduct research that requires sacrifice of a few hatchlings, since they are often available in large numbers and have low survivorship in the wild. In some cases it is possible to estimate the number of required specimens statistically on the basis of a pilot study, thus reducing over-exploitation (Still, 1982; Eckblad, 1991).

We believe that ethical collecting is that which has no impact on the survival prospects of the population or species and does not needlessly cause injury or death to individual turtles. With the availability of non-standard specimens, it is appropriate to re-evaluate standard specimen collecting for some types of research on turtles. For example, 50 years ago diet studies were accomplished by collecting large series of turtles and examining their stomach contents by dissection. Today stomach flushing and fecal sample analysis are standard practice, reducing the need for lethal collecting for such studies. We recommend taking as few individual turtles as necessary for the scientific purpose intended, especially for vulnerable and threatened species. When possible, collecting of reproductive females should be minimized because of their value to the population. Before lethal collecting is undertaken, it should be determined that a non-standard specimen will not suffice for the scientific purpose intended, that the samples needed are not already available in collections, and if not, that a secure repository for the specimens to be collected is identified. Those few museums that restrict themselves entirely to turtles (including the Nanjing Turtle Museum in China, the Chulalongkorn University Turtle Lab and Museum in Thailand, the Leatherback Turtle Museum at Playa Grande, Costa Rica, and the Chelonian Research Institute collection in Oviedo, Florida) have a uniform policy of not sacrificing live turtles, yet some have large holdings that are extremely valuable for many kinds of scientific research.

While it is essential that researchers adhere to applicable laws, this is not always easy to accomplish and can be frustrating. We have experienced situations where laws are broadly stated, and therefore open to multiple interpretations and practice; where they conflict with common sense; where multiple agencies have seemingly conflicting regulations; and where regulations to protect resources inadvertently and nonproductively impede scientific inquiries. For example, one of us (RLB) recently began a research project with the goal of establishing the population of origin of diamondback terrapins (*Malaclemys terrapin*) sold in food markets in Chinatown, New York City. This research necessitated collecting tissue samples from terrapins that were legally purchased in markets by a third party. A wildlife conservation officer later informed RLB that while the purchase of the live terrapins for food was legal, taking blood samples from the purchased terrapins required a permit "just to be safe". Another of us is aware of situations where collecting whole animals is relatively easily permitted, but collecting tissue samples for genetic research requires additional permits that are difficult to obtain. In some countries it is currently legal to export animals for

the pet or wildlife trade, but illegal to export standard or non-standard specimens for scientific research. Also, the Museum National d'Histoire Naturelle (Paris) has a number of important turtle specimens, including the type specimens of both *Emys geoffroyana* and *Testudo gigantea*, that were taken from the King of Portugal's collection by Napoleon's forces nearly 200 years ago (Wilcken, 2004). By modern standards, these specimens are not "legal", but they are invaluable nevertheless and cannot be ignored.

Furthermore, situations exist where scientific collecting is prohibited but regulation of large-scale commercial consumption is not enforced. Species will continue to decline in the face of protective regulation if those regulations do not target the major sources of population decline and are not uniformly applied. We recognize the necessity of intelligent discretion at the point of application of conservation law, but too often we observe that activities genuinely harmful to the persistence of populations or species have *de facto* exemption.

Our Influence and Recommendations on Existing Regulations and Laws

Many turtle researchers have played important roles in the development of laws, regulations, and treaties designed to protect wildlife in general and turtles in particular. We hope to continue to influence these regulations in an atmosphere of mutual respect. We wish to emphasize the non-commercial nature of our work, and would point out to regulators charged with protecting natural resources that we share their goals. Unfortunately, regulations concerning biological samples often block legitimate research without advancing species protection, and are sometimes unnecessary. We believe authorities should make all efforts to make confiscated material available to the scientific community, rather than destroy it. Similar consideration also should be given to material that may have been held by individuals or in private collections that is later being offered for scientific use, though the legality of the original acquisition of such material should be ascertained.

We believe that collecting for scientific purposes should not require permits more elaborate than those for collecting for commercial purposes. Permits for sampling that does not involve removing or threatening individuals in the wild should be granted readily for accredited research.

We hope that journal editors and peer reviewers will work to encourage legal compliance in the publishing of scientific works, in an effort discourage those who break the law, and we recommend that authors be required to state that materials utilized in submitted papers were acquired legally to the best of their knowledge. For example, the instructions for submissions to the journal *Herpetologica* require that article submissions be accompanied by letters indicating that "the authors have observed appropriate ethical and legal guidelines and regulations…when obtaining subjects, especially endangered species (e.g., proper collecting permits or use of reputable dealers)". Furthermore, "Submitted studies that obviously deviate from acceptable practices, when noted by the editorial staff, are subject to rejection." In many cases these goals can be accomplished by requiring museum accession numbers for specimens, given that most museums now require legal documentation before accepting specimens. Another positive outcome of this recommendation would be that specimens would be deposited in museums before results are accepted for publication. Finally, while we recognize the growing relevance of intellectual property rights issues to this discussion, we feel it is beyond the scope of our discussion.

Recommendations

1. Every researcher must make his/her best effort to be aware of all laws and regulations (LARs) relevant to his/her research. We are aware that some relevant LARs may be relatively unknown, and we describe examples where LARs are confusing and even contradictory, but LAR investigation should be considered part of field research.

2. Some of us felt that it was reasonable to collect specimens in some cases where LARs are confusing, contradictory, or even grossly unfair. These authors pointed out that in these circumstances it is not possible to identify a procedure by which all LARs can be followed meticulously, and that therefore some options can be considered permissible while others are not. Some of us felt that following both the spirit and the letter of LARs was necessary, because non-compliance with any part of the LARs leaves the researcher and associated institution open to legal consequences. These authors felt that where LARs were not clear, field research was not advisable until LARs were clarified officially. All of us recommend that scientists advise and work with regulators to clarify LARs so that these ambiguities are resolved.

A case in point is the opportunistic discovery of rare salvage material of obvious value to science, but where collection of such material is illegal under comprehensive bans on wildlife collecting of any kind. Authorities and institutions in such countries may be able to receive such material once it has been explained what the material is and why it is valuable. Where LARs are confusing, contradictory, or appear to unfairly discriminate against scientific collecting, we encourage scientists to work with relevant authorities to correct these problems. We point out that, at least theoretically, regulatory agencies and scientists share important goals, and this should provide common ground for resolving difficulties.

3. We strongly encourage the use of non-lethal collection wherever this will provide appropriate materials for research. Adult turtles are especially important to the persistence of wild populations, and therefore removal or sacrifice of adults, especially adult females, is to be particularly discouraged.

4. We recommend that editors and reviewers require that authors state the source of their specimens (both standard and non-standard material) where practical, that au-

thors list permits for any collections made, and that scientific works discussing specimens be required to publish museum accession/catalog numbers. These recommendations necessarily involve museums in the process of verifying that researchers have obtained appropriate permits.

5. We recommend that editors and reviewers require that authors verify that relevant approvals from Institutional Animal Care and Use Committees or other official bodies were obtained prior to field work.

6. Whenever non-standard or standard materials are collected, we recommend that researchers make every effort to deposit voucher material in the permanent collection of a recognized museum or similar institution along with all appropriate field data. We encourage the use of photographs of specimens in the field and/or in life as part of the deposited field data.

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Responsible Vouchering in Turtle Research: An Introduction and Recommendations

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ABSTRACT. – Voucher specimens are critical to the advancement of research efforts on turtles, and by association, for conservation efforts associated with this group. This paper addresses the importance of voucher specimens and provides recommendations for responsible practices associated with voucher specimens. For the purposes of this paper, a voucher specimen is defined as a biological specimen, the primary function of which is to provide verification for the taxonomic identification assigned to an animal and any eventual published or reported scientific investigations associated with it. A traditional voucher specimen for a turtle consists of a fluid-preserved specimen or a complete skeleton and its associated data appropriately preserved for permanent storage and housed in a curated collection for posterity. Although not optimal, a non-traditional voucher may also provide verification for taxonomic identification and may include image or acoustic data, eggs or eggshells, or tissue samples. Examples are given of when a traditional voucher specimen deposited in a curated collection is recommended and when alternatives to the traditional voucher specimen, such as an *e*-voucher, may be used. In addition, a worldwide survey of curated collections holding turtles was conducted and the percentage of turtles represented in reptile collections is reported.

KEY WORDS. – Reptilia; Testudines; turtles; traditional voucher specimen; non-traditional voucher specimen; *e*-voucher; curated collection; tissue sample

Turtles[1] (Order Testudines) comprise just 3.7% of all named extant reptile species (307 turtle species out of 8240 total named reptiles; Uetz and Hallermann, 2007). Extant turtles are a highly distinctive group characterized by several features, including a secondarily anapsid skull, a shell that encloses both limb girdles, an external ear supported by a large, semicircular quadrate, and toothless jaws (Ernst and Barbour, 1989; Meylan, 2001). Approximately 40% of all extant turtle species are considered threatened and listed as either Critically Endangered, Endangered, or Vulnerable by the IUCN - World Conservation Union (Khamsi, 2004; IUCN, 2007). Key threats to turtles include direct mortality by collection for food, traditional medicine, and the pet trade, in addition to incidental mortality caused by road kills, habitat loss, and the introduction of predators and competitors (van Dijk et al., 2000; Khamsi, 2004).

Species boundaries play a crucial role in the prioritization of conservation efforts for turtle taxa (Avise, 1989; Remsen, 1995; Reynolds et al., 1996; Sites and Crandall, 1997; Soltis and Gitzendanner, 1999; DeSalle and Amato, 2004). Taxonomists use many different characters, including morphological and/or molecular, in the delineation of species and/or subspecies (Wiley, 1978; Frost and Hillis, 1990; de Queiroz, 1998, 1999). Over time, species concepts, criteria, and the characters used to distinguish species may change. It is therefore not uncommon that a species description may be reviewed and challenged over the years (e.g., Parham et al. 2001). Type specimens for new species and voucher specimens from published studies provide researchers with the option to use alternative methods or advancing technologies to re-examine previous descriptions or conclusions. Voucher specimens also allow independent verification of the taxonomic identification of individuals used to test the hypotheses generated in the study (Reynolds et al., 1996). It is for these reasons that voucher specimens are critical to the advancement of research efforts on turtles, and by association, for conservation efforts associated with this group.

This paper will address the importance of voucher specimens and provide recommendations for responsible practices associated with voucher specimens.

The Definition of a Voucher Specimen

A voucher specimen has previously been defined in the literature by authors representing various biological disciplines:

[1] For the purposes of this paper, a turtle refers to all species included in the Order Testudines, including turtles, tortoises, and terrapins.

Lee et al. (1982) stated that for a general biological voucher, "*A voucher specimen is one which physically and permanently documents data in an archival report by: 1). verifying the identity of the organisms(s) used in the study; and, 2). by so doing, ensures that a study which otherwise could not be repeated can be accurately reviewed or reassessed.*"

Yates (1985) defined a mammalian voucher specimen as one "*which serves to physically and permanently document data in an archival report by 1) verifying the identify of the organisms(s) used in the study and 2) by so doing, assuring the repeatability of the study which otherwise could not be repeated and/or accurately reviewed or reassessed. Thus, voucher specimens are the sole means to verify the data documented in a report and to make historical comparison possible*."

Reynolds et al. (1994) in referring to amphibians defined voucher specimens as "*Specimens that permanently document data in an archival report*" and described the role of vouchers, including to "*provide a basis for verification of identifications and thereby duplication of a study.*"

Reynolds et al. (1996) in discussing mammals defined voucher specimens as "*Specimens that permanently document data in an archival report. Such specimens and corresponding data assembled during field studies of mammals, particularly the small and medium-size species that are difficult to identify and often poorly known, are critical for accurate identification of the animals studied and for verification of the data gathered and reported as resulting from the investigation.*"

Winker et al. (1996) stated with regard to voucher specimens of birds that "*The study skin is the basis for identification in birds – not tissue specimens. When tissues are collected, good scientific procedure requires that a voucher specimen (i.e. a specimen that enables the identification of accompanying material) be preserved and deposited in a research collection. Voucher specimens serve as quality control for phylogenetic and population genetic analyses based on tissues.*"

Huber (1998) defined voucher specimens of invertebrates in the broad sense as "*..all biological specimens having the minimum information of collection locality (ideally specified by latitude, longitude, altitude) and date that are preserved to document biological research, including taxonomic research.*"

Barkworth and Jacobs (2001) defined plant voucher specimens as "*…specimens that are made from the biological entities used in a research project and deposited in a recognized, active herbarium or museum.*"

GenBank® (http://www.ncbi.nlm.nih.gov/projects/collab/FT/index.html; for the submission of sequence data) defines a specimen voucher as "*an identifier of the individual or collection of the source organism and the place where it is currently stored, usually an institution.*"

For the purposes of this paper, a voucher specimen is defined as: *a biological specimen - the primary function of which is to provide verification for the taxonomic identification assigned to an animal and any eventual published or reported scientific investigations associated with it. A traditional voucher specimen for a turtle consists of a fluid-preserved specimen or a complete skeleton and its associated data appropriately preserved for permanent storage and housed in a curated collection for posterity. Although not optimal, a non-traditional voucher may also provide verification for taxonomic identification and may include image or acoustic data, eggs or eggshells, or tissue samples.* Practical guidelines for the preservation of traditional voucher specimens for reptiles and amphibians may be found elsewhere (e.g., Simmons, 2002, and references therein).

"Curated collections" refer to natural history museums or other institutions with demonstrated long-term commitments to biological collections, including adequate staffing, protection from physical hazards, appropriate storage for specimens and samples, accessibility to the specimens by the research community, compliance with national and other regulations, and written policies for collection management (Lee et al., 1982). The practice of holding specimens in private and/or stand-alone collections or on solely web-based and/or temporary databases is strongly discouraged. Collections such as these are short-lived and are typically dependent on one individual's commitment, not an institution's, and therefore the prospects for long term preservation of specimens is not secure (Corthals and DeSalle, 2005; Hanner et al., 2005). Lists of curated collections may be found in Dessauer and Hafner (1984), Leviton et al. (1985, 1988), Prendini et al. (2002), and Corthals and DeSalle (2005).

The minimum required information for a specimen accessioned and catalogued into a curated collection includes: a unique sample designation, date and time of collection, sex, name of collector, taxonomic identification and standard measurements, in addition to any other relevant information regarding the collection of that specimen (Lee et al., 1982; Reynolds et al., 1996).

A voucher specimen should accompany any study when the scientific name assigned to individuals is significant to the content or results of the paper (Reynolds et al., 1996; Barkworth and Jacobs 2001). A published scientific study which lists all "specimens examined" within the publication provides that study with the potential for repeatability, a basic tenet of scientific practice (Ruedas et al., 2000). Ideally, within the publication the authors should list the collection where the specimen is stored and its catalog number, in addition to the locality information, date of collection, and name of collector (Prendini et al., 2002).

A search of nucleotide submissions to GenBank® on the National Center for Biotechnology Information's website (http://www.ncbi.nlm.nih.gov/) highlights some of the issues related to vouchers and their importance with regard to turtles. Our core nucleotide search for Testudines, conducted on 15 October 2007, found that 1311 sequences out of a total of 6751 (approximately 19%) provided voucher specimen information. Over one-half of these submissions were associated with one author (768/1311). Approximately

one-half of these submissions were tissue samples (620/1311) and a great majority of these samples (616/620) were associated with one author. One-third of these submissions (436/1311) were deposited during the first ten months of 2007 and no voucher specimens were associated with sequences submitted to GenBank® prior to 2001.

An example highlighting the importance of voucher specimens and of providing the resources necessary for the repetition of a study was found in a review of the aforementioned 6751 sequences. In Cervelli et al. (2003), sequence data was submitted to GenBank® for 16 chelonian species, however it was found that one of the sequence accession numbers, that listed for *Cyclemys dentata* (AJ310188), was actually the sequence for *Oncorhynchus mykiss*, a rainbow trout. We were unable to locate any sequence data in GenBank® for *Cyclemys dentata* associated with this manuscript (Cervelli et al., 2003). By providing information relating to voucher specimens in the publication, ideally for both traditional specimens and for tissue samples, Cervelli et al. (2003) would have provided subsequent researchers the ability to verify the data presented in their paper.

There are several possible reasons for reluctance by researchers to collect and preserve turtles as a traditional voucher specimen, including: 1) because turtles are long-lived (Gibbons, 1987); 2) copious amounts of formalin are required to preserve a large specimen (Forstner et al., 1997); 3) it may be difficult to find museum collections with adequate storage space, especially for large specimens (Gans, 1989); 4) concerns that sacrificing animals impacts populations (Shine, 1996; Stuebing, 1998; Patterson, 2002), and lastly, 5) there may be an unwillingness to sacrifice animals held as pets. These concerns should be carefully weighed against the increased value of the data associated with the specimen and the potential for future research as well the overall importance of museum collections in general to research and conservation efforts for all taxa (Remsen 1995; Earl of Cranbrook 1997; Shaffer et al., 1998; Suarez and Tsutsui, 2004).

Turtle Specimens in Natural History Museums Worldwide

We conducted a survey via email between September – December 2005 of selected museums in Africa, Asia, Europe, and North America, with known herpetological contents, regarding their reptile and turtle holdings. Responses were received from 63 institutions and are compiled in Appendix I. Data quality (accurate specimen counts for total reptiles and total turtles and a low backlog of unregistered material) was best for North American museums; elsewhere, accurate counts were received only from a few of the major museums. Most respondents from museums in Europe and Asia mentioned that no electronic database existed for their collections. Several could supply only a single catalogue number for a series of specimens or jars containing several specimens (these have not been listed), and two collections mentioned that a significant amount of additional material had not yet been catalogued. The percentage of turtles represented in reptile collections ranged from 0% up to 32%, with an average of 5% representation (Appendix I).

One major systematic collection for turtles, the Chelonian Research Institute, Oviedo, Florida has nearly 11,150 catalogued and 300 non-catalogued specimens of turtles (P.C.H. Pritchard, *pers. comm.*). On the other side of the spectrum, a few collections reported a few or even no turtle specimens in their collection. Wildlife Heritage Trust of Sri Lanka, the largest systematic collection in Sri Lanka, reported no turtles, and attributed this to the fact that none of the staff have worked on the group (R. Pethiyagoda, *pers. comm.*). Low figures were also found in regional collections located in areas with relatively depauperate or poorly studied turtle faunas (e.g., Pakistan, the Philippines, Israel, and Iran).

While the size of a collection (= total number of catalogued specimens) is an important consideration in judging the importance of a particular collection, other factors may also be taken into account, including taxonomic diversity, geographic representation, and historical collections (particularly type specimens). Using these criteria, smaller, regional holdings, particularly those that are national repositories, may be deemed important as repositories of turtle voucher specimens.

The prominent factors behind the acquisition of turtle specimens for a collection have been identified by this survey as individual research interests, as well as geographically determined turtle diversity and abundance. Critical for the advancement of knowledge, as well as for the continued existence and support for natural history museums, these specimens, once collected and accessioned into a curated collection, should be utilized for research and the museums and the specimens subsequently cited in publications (Suarez and Tsutsui 2004).

Traditional Voucher Specimens

As mentioned above, upon accessioning and cataloguing a traditional voucher specimen into a curated collection a minimal amount of information is required, however, it is critical that as much information be associated with the specimen as possible. For example, blood/tissue samples should be taken before the specimen is preserved, as well as photographs, recordings, etc. Table 1 provides examples of various voucher types and their characteristics.

Description of Species. — When a new taxon description (species or subspecies) is published or a revision to current classification is recommended, a voucher specimen should be deposited into a curated collection, whether the published evidence is based on morphological or molecular evidence or both. Although a (traditional) holotype specimen housed in a curated collection is not a mandatory requirement by the Fourth Edition of the International Code of Zoological Nomenclature for new taxon descriptions, it is nonetheless strongly recommended (Wakeham-Dawson and Morris, 2002; Dubois and Nemésio, 2007).

Table 1. Data characteristics and scientific value for various voucher types. In this table, the quality and reliability of the voucher increase in scientific value as one moves down the list. The value of all vouchers is increased with the inclusion of field notes, including habitat type, georeferenced locality data, date, collector, and standard measurements.

Voucher Type	General Category Data Characteristics	Scientific Value
No Voucher		
	Sighting/Description	Low
	Anonymous sighting	
	Specimen description	
	Drawing/illustration	
***e*-Voucher**		
	Image/Recording	Medium
	Single photo	
	Diagnostic audio recordings	
	Diagnostic video recordings	
	Diagnostic photos (series)	Medium-high
Voucher		
	Tissue	
	Blood/ tissue sample (no photo)	
	Blood/ tissue sample (with photo)	High
	Tissue samples (various organs; series)	
	Developmental tissue/expression library	
	Traditional Specimen Voucher	
	Diagnostic skeletal materials	
	Complete skeleton	Very high
	Fluid preserved complete specimen	

Genetic Studies. — When biological samples are used in systematic, taxonomic, and phylogeographic studies it is also recommended that traditional voucher specimens be accessioned and catalogued into a curated collection. Specimens used in such studies should be listed in a table, appendix or text along with the accompanying museum catalogue number (Ruedas et al., 2000). In these studies, independent verification of the identification of the taxon will most likely be required by researchers revisiting related questions in the future (Reynolds et al., 1994).

The importance of including voucher specimen information within publications is recognized by the Journal of Herpetology in its *Instructions to Authors* which "requires that all submissions from researchers reporting results of phylogenetic reconstruction and taxonomic decision be supplemented by in-text (if a shorter communication) or appendix (if a major paper) reference to voucher specimens" (http://www.ssarherps.org/pages/JHinstr.php).

Rare Animals in Captivity. — Animals housed in zoological parks, aquariums, and private collections offer the researcher a valuable resource and may provide the only readily available access to a specific taxon. Animals in captivity may also have associated data, including behavioral observations, veterinary records, and reproductive condition and history, that are not available from animals collected in the field and may be crucial to an interpretation of results. However, biological samples collected from living captive animals and used to generate data for scientific publications also pose a special situation with regard to voucher specimens and one for which the researcher must weigh the costs and benefits. Arrangements between the owner of the animal and a curated collection should be made for the disposition of the animal upon its death and it is recommended that all arrangements be made well in advance of the animal's demise (Lehn, 2005). It is also recommended that the animal be permanently marked, e.g., microchip or PIT tag, in order to ensure accurate identification in the future. A voucher specimen is also especially critical in those instances when provenance data are unavailable or unreliable, as is the case with many captive-held animals, including animals held by private collectors and/or purchased from the pet trade, food markets, or from animal dealers (Ruedas et al., 2000). Private collectors and zoological parks and aquariums should be encouraged to collaborate with a curated collection, especially in cases when very rare and/or endangered animals are being held.

Alternatives to the Traditional Voucher Specimen

Although it is optimal to collect a traditional voucher specimen in the field and preserve it in a curated collection, there are various reasons or circumstances when this may not be feasible or deemed ethical. In these situations non-traditional alternatives may be preferred. Examples of non-traditional vouchers may include image data (photographs, either digital or hardcopy), acoustic data, eggs and eggshells, and tissue samples (Monk and Baker, 2001).

To be valuable as a voucher specimen, a digital voucher (or *e*-voucher) should comprise an image showing the entire specimen and/or body parts diagnostic for taxon identification (Reynolds et al. 1996; Barkworth and Jacobs, 2001; Monk and Baker, 2001) (Fig. 1). One set of guidelines for the photodocumentation of turtles may be found in Bender (2001). In addition, a list of available resources for digital imaging and best practices may be found on the American Museum of Natural History's website (http://library.amnh.org/diglib/resources/index.html).

Limitations to Tissue Samples and DNA Barcoding. — Although providing a blood/tissue sample as the sole voucher for a molecular study is preferable to providing no voucher at all, these samples should be considered complements to the traditional voucher specimen and not alternatives (Peterson and Lanyon, 1992; Monk and Baker, 2001). By making the tissue sample available to the research community, future researchers are provided with the capability to repeat the molecular study, however, independent verification of taxonomic identification using morphological characters is impossible using such vouchers (Ruedas et al., 2000). An additional limitation associated with a tissue sample collected as a voucher is that its subsequent use is destructive and will lead to its eventual consumption, it is therefore imperative that the researcher collect tissue in amounts sufficient for the immediate needs of the research, as well as for the future needs of the community.

DNA barcoding promises to revolutionize taxonomic identifications by using a single gene sequence from the mitochondrial gene, cytochrome oxidase I (COI; Hebert et al., 2002) to uniquely identify species. At present, this is far

from being a reality for many taxa, including turtles. GenBank's® database currently holds only 107 sequences for turtles for this mitochondrial gene and only 23 species in 4 genera are represented. All of these sequences have associated voucher specimens, however the majority of these vouchers (58/107) are tissue samples. The barcoding initiative will only be useful when all taxa from all geographical regions are represented by sequences and will then only be useful to the extent that voucher specimens are available and at the appropriate taxonomic level. Additionally, since the mitochondria are inherited only through the maternal lineage, the barcoding of a mitochondrial gene may not always be useful for the identification of hybrid individuals (Karl et al., 1995), which may be particularly relevant for some turtle taxa [but see Spinks et al., 2004 and Stuart and Parham, 2004 for examples when mitochondrial DNA was useful in identifying hybrid individuals].

Population Studies. — An example when a traditional voucher specimen is not feasible or recommended from each individual is for population genetic and phylogeographic studies when potentially hundreds of biological samples across the range of a single taxon may be collected. In these studies, researchers usually avoid sacrificing entire individuals by using minimally invasive or non-lethal sampling techniques to collect blood, skin, tail tips, shell, or toe clips (Haskell and Pokras, 1994). It may be appropriate to collect a traditional voucher specimen from a representative individual for a locality or region. We strongly recommend, however, that researchers consider exhaustively *e*-vouchering each individual from which a sample has been taken. This will allow for independent verification of species identification of all samples included in the study. In cases where exhaustive *e*-vouchering is not possible or too laborious, we recommend that a photograph be taken of individuals representative of the population, e.g., at each collection locality, and of color or morphological variants. This practice will alleviate problems that may arise subsequently with closely-related taxa being inadvertently misidentified and sampled and will greatly assist identifications in those cases when individuals are genetically divergent from all others in the study.

Damaged Specimens. — A traditional voucher may also not be possible when genetic samples are taken from heavily damaged or partially degraded roadkills or other unsalvageable specimens. In such instances, if genetic samples are taken, the dead animal should be photographed and standard collection notes and locality data (including georeferenced positional data, if possible) should accompany the photo (Monk and Baker, 2001). Additionally in these instances, any salvageable portion of the specimen (e.g., the skull) should be collected and deposited in a museum whenever possible.

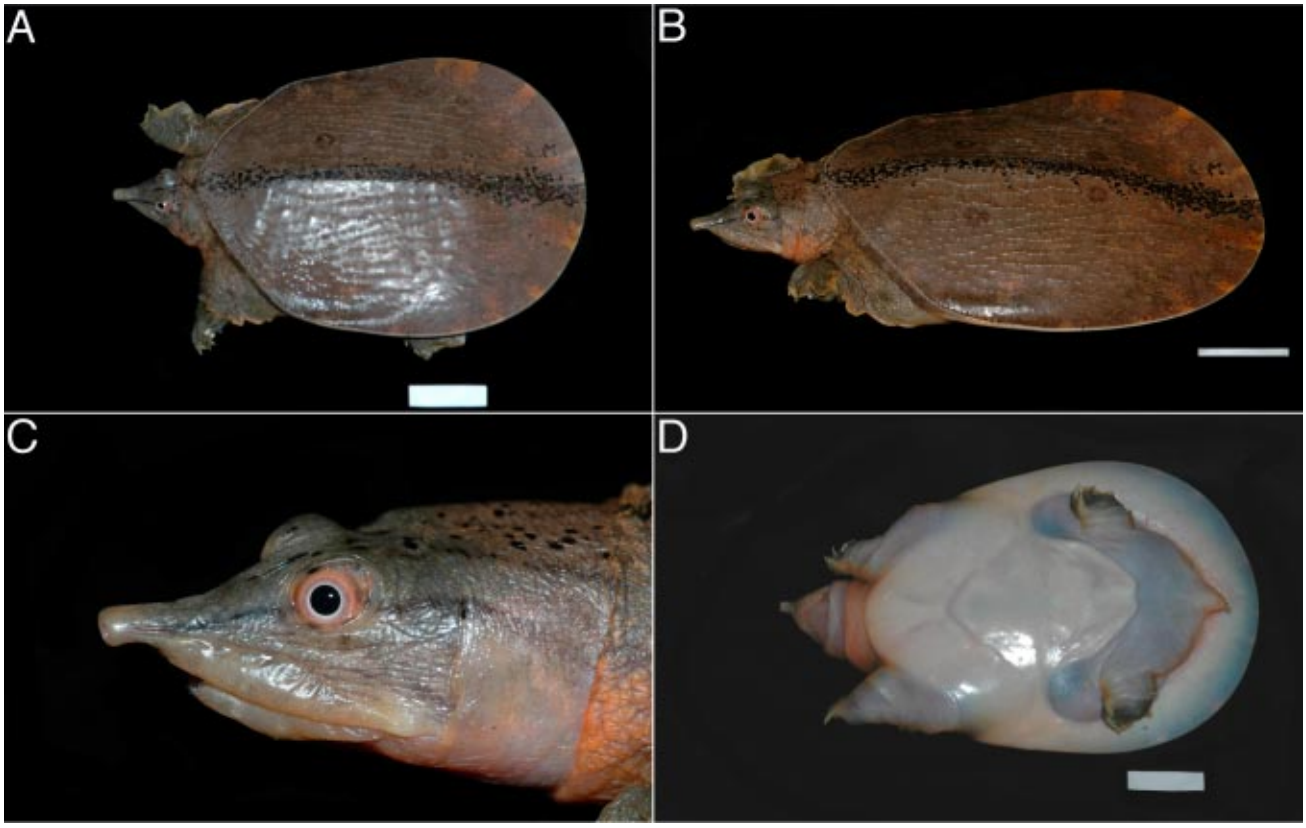

Figure 1. Example of a digital voucher and associated data for a turtle specimen. *Dogania subplana*, subadult male (straight carapace length = 102.3 mm; straight carapace width = 83.1 mm; measurements including cartilaginous flap), from Sungei Pueh (01º43'38.5''N, 109º43'25.7"E, datum WGS 84), near Kampung Sebako, base of Gunung Pueh, Sematan District, Kuching Division, Sarawak, Malaysia (Borneo). USDZ [IMG] 2.63. Body in dorsal **(A)** and lateral **(B)** views; lateral view of head **(C)**; and ventral aspect of plastron **(D)**. Scale markers = 20 mm. Photos: I. Das.

Storage or Handling Concerns. — It may also not be feasible to collect traditional specimens from extremely large specimens because of storage space concerns in the museum's collection (Gans, 1989) or for handling in the field, in these instances a digital voucher can be a suitable alternative to a traditional voucher specimen.

Living specimens. — Captive collections of living animals may provide very valuable resources for the researcher and considerations to be taken at the time of the animal's death have already been discussed. However, biological samples collected from living animals and used to generate data for scientific publications also pose a special situation with regard to traditional voucher specimens (Monk and Baker, 2001). In these instances, the live animal should be fully photo-documented, PIT tagged or marked by some other unique or individual-specific identifying means, and an aliquot of the sample along with images and accompanying data should be deposited into a curated collection.

In some cases, a natural history museum may not be willing to accept or even be capable of accepting captive specimens for reasons such as storage constraints, a lack of data associated with a captive animal, or the destruction of critical characters during necropsy and it is in these circumstances that non-traditional vouchers are extremely valuable (Monk and Baker, 2001).

As stated above, the impact on the population from collecting animals for preservation is usually minimal, however, there are cases when the number of individuals of a given taxon that remain alive are so low that the collection of even a single animal may reduce the probability of its continued survival. One well-known example is the case of "Lonesome George" (*Geochelone* [= *Chelonoidis*] *nigra abingdoni*), the sole survivor of his subspecies (Nicholls, 2004; Fig. 2). We recommend that in these rare cases, non-traditional vouchers, especially photographic vouchering (accompanied by complete data), be collected and arrangements be made prior to the death of the animal to have it preserved in a curated collection immediately following its death.

Regulatory Restrictions. — There are numerous regions and protected areas where collecting biological specimens is prohibited by local law, but where the collection of non-traditional vouchers may be possible (Prendini et al., 2002). In cases where specimens are sampled from protected areas or where a researcher wishes to document the presence or distribution of a species that cannot be collected, *e*-vouchers are recommended. One might also envision cases where researchers make incidental or unexpected observations and where collecting the animal at that particular time may be illegal or otherwise unfeasible. In these instances images in the form of *e*-vouchers are preferable to no documentation at all.

Legal and Ethical Concerns

Any time a traditional or non-traditional voucher is taken from an animal it is imperative that it is done legally and in a humane fashion. In today's international environment, there will be circumstances when collecting permits may not be granted by particular countries or permit-issuing authorities (Prendini et al., 2002). If researchers wish to document the distribution of species or the occurrence of a given species in a particular area of interest, the alternative to collecting may be to adopt non-traditional vouchers. All vouchered specimens, whether traditional vouchers or tissue samples, must be accompanied by the appropriate permits. If possible, every attempt should also be made to deposit a portion of the collected specimens into curated collections within the country of origin (Reynolds et al., 1996). A more thorough discussion of the legal and ethical concerns associated with the acquisition of animals for research may be found in Burke et al. (2007).

Summary and Conclusions

A traditional voucher specimen for a turtle consists of a fluid-preserved specimen or a complete skeleton and its associated data appropriately preserved for permanent stor-

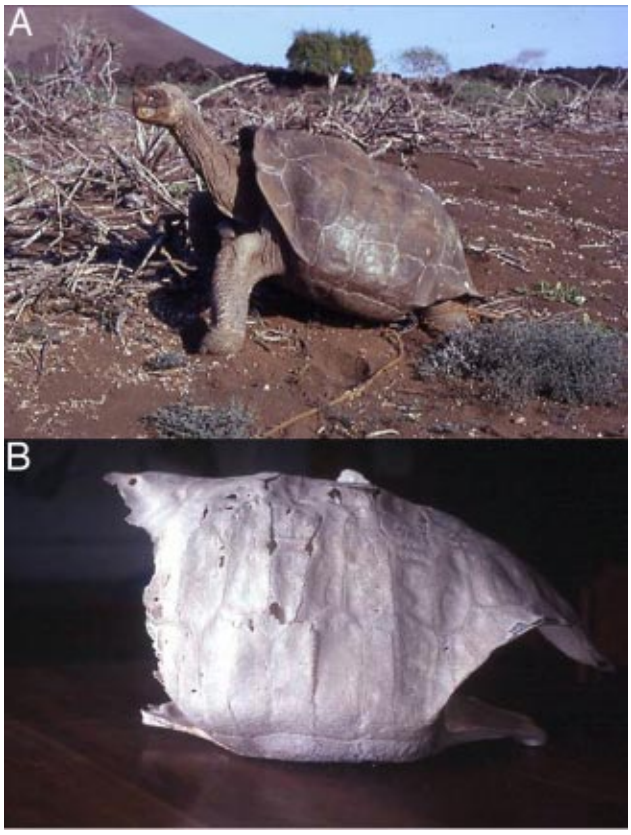

Figure 2. A. The sole survivor of the Abingdon Island tortoise (*Geochelone* [= *Chelonoidis*] *nigra abingdoni*), a subspecies classified by IUCN as Extinct in the Wild. This photograph was taken shortly before this individual was removed from Pinta Island in 1972. The plight of this individual animal, popularly dubbed "Lonesome George," is often evoked during debates of the many ethical and philosophical issues associated with collection of voucher specimens in turtle systematics and taxonomy. **B.** The bony shell of a specimen of *G. n. abingdoni*, found on Pinta in 1964 and deposited in the collection at Charles Darwin Research Station. Photos courtesy of P. Pritchard.

age and housed in a curated collection for posterity. The importance of voucher specimens has been documented and appreciated by researchers in various disciplines for many years, however, the practice of vouchering is still far from routine for researchers working on turtles. Many reasons may account for reluctance by researchers to sacrifice animals for preservation in a curated collection, however, the value of such specimens for taxonomic verification and repeatability of research in addition to the provisions of future research far outweigh many concerns. Digital photography provides a viable option for non-traditional vouchering techniques, particularly in the case of population studies involving large numbers of individuals. In addition, researchers collecting samples from captive collections should also be aware of the importance of obtaining voucher specimens. There are instances when there is no truly adequate substitute for the deposition of a traditional voucher specimen into a curated collection, e.g., new taxon descriptions and phylogenetic studies. It is our hope that a strong emphasis on the importance of voucher specimens by professional colleagues and journals will strengthen an appreciation by the research community on the importance of voucher specimens for responsible science.

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APPENDIX I

Contents of the reptile collections of natural history museums in Africa, Asia, Australia, Europe, North and South America, that responded to an email survey conducted between September - December 2005, showing the proportion of turtles represented[1]. Museum abbreviations after Leviton et al. (1985; 1988), where available; other abbreviations may be found in the Acknowledgments. Asterisk (*) indicates approximate numbers.

Museum (Country)	Total Reptiles	Total Turtles	% Turtles
AMS (Australia)	116,000	2,411	2.1
AMNH (USA)	149,687	8,527	5.7
BMNH (UK)	120,000*	4,000*	3.3*
BNHM (India)	5,070*	90	1.8*
CAS (USA)	168,374	2,464	1.5
CRI (USA)	11,450	11,450	100.0
CIB (China)	14,300*	185	1.2*
CM (USA)	88,109	28,652	32.5
FHGO-USFQ (Ecuador)	3,537	30	0.8
FML (Argentina)	20,550	77	0.4
FMNH (USA)	118,167	5,870	4.9
FRIM (Malaysia)	578*	1	0.2*
HNHM (Hungary)	3,163	145	4.6
HUJ (Israel)	21,000*	539	2.6*
KRSU(Kyrgyzistan)	4,589	31	0.7
KU (USA)	131,730	5,249	4.0
KUZ (Japan)	47,000*	350*	0.7*
LSUMZ (USA)	88,791	3,672	4.1
MCN (Brazil)	2,157	23	1.1
MCZ (USA)	183,977	4,517	2.5
MHNG (Switzerland)	34,800*	658	1.9
MHNLS (Venezuela)	6,000*	61	1.0
MLP R (Argentina)	5,200	61	1.2
MNKhNU (Ukraine)	10,000*	100*	1.0*
MNHN (France)	110,000*	3,700*	3.4
MSNM (Italy)	4,004	774	19.3

MVZ (USA)	99,031	2,840	2.8
MZB (Indonesia)	8,902	336	3.8
NMK (Kenya)	8,100	94	1.2
NHM (Denmark)	407	36	8.8
NHMC (Crete)	5,000*	100*	2.0*
NHMK (Nepal)	530*	17	3.2*
NMB (Switzerland)	23,400	817	3.5
NMW (Austria)	40,640	5,170	12.7
NMNS (Taiwan)	4,500	130	2.9
NSMT (Japan)	38,000*	86	0.2
NTNU (Norway)	250*	10	4.0
OMNH (Japan)	2,200*	34	1.6*
PEM (South Africa)	17,000*	1,109	6.5*
PNHM (Pakistan)	2,000*	35	1.8*
PNM (Philippines)	4,000*	128*	3.2
QM (Australia)	43,011	1,853	4.3
RBINS (Belgium)	51,120	1,143	2.2
RMNH (Netherlands)	34,060*	4,000*	11.7*
ROM (Canada)	16,310	1,060	6.4
RUZM (Iran)	4,000*	45	1.1*
SBC (Malaysia)	111	2	1.8
SM (Malaysia)	1,728	182	10.5
SMF (Germany)	87,000*	4,000*	4.6*
SNHM (Germany)	5,000*	80	1.6
TAU (Israel)	14,422	740	5.1
TMU (Norway)	38	3	7.9
TNHC (USA)	31,088	1,672	5.3
UF (USA)	80,513	11,211	13.2
UMMZ (USA)	134,421	8,904	6.6
USDZ (Singapore)	6,165	398	6.5
USNM (USA)	175,388	18,513	10.6
VNM (Vietnam)	5,650*	150*	2.7*
WHT (Sri Lanka)	1,400*	0	0
YPM (USA)	15,049	1,027	6.8
ZDEU (Turkey)	13,936	426	3.1
ZMA (Netherlands)	15,000–20,000*	506	3.4–2.5
ZMB (Germany)	70,000*	2,600	3.7
ZMH (Germany)	35,000*	1,200*	3.4*
ZMUC (Denmark)	40,000*	700*	1.8
ZSI (India)	25,622*	1,173	4.6*

[1]Carnegie Museum of Natural History (28,652 specimens, representing 32.5%), reported the highest number and proportion of turtles (except for CRI at 100%), which is composed mostly of embryological specimens from the work of its late Curator, Clarence McCoy, 1935–1993 (see Bull et al., 1982; Vogt et al., 1982; McCoy et al., 1983). Another large collection of mostly hatchling turtles was received by Carnegie Museum from the embryological work of Michael A. Ewert (1938–2005), but these are yet to be accessioned in the collection (S.R. Rogers, *pers. comm.*). Other collections with large holdings of turtles also represent a special interest in the group by one or more former staff, e.g., at the United States National Museum, Leonard Stejneger, and more recently, Carol Ruckdeschel, Jack Frazier, George Zug and Tom Fritts [G. R. Zug, *pers. comm.*], at the Florida Museum of Natural History, Walter Auffenberg, Archie Carr, John Iverson and Peter Meylan, and at the Naturhistoirisches Museum, Wien, Friedrich Siebenrock (1853–1925). Three respondents mentioned a lack of funding support for their collections, hence the charge of herpetology given to other Divisions. One major herpetological collection in Vietnam has no official status, and therefore its contents are to be dispersed after the studies by its collector are completed. The survey did not specifically inquire about sea turtles, and suspect that this group, in addition to other large-bodied group of turtles (e.g., gigantic members of the Testudinidae and some Geoemydidae) would be relatively underrepresented in collections. Gans (1989) mentioned that museum curators are averse to accepting large turtles and crocodilians in their collection, with the result that most of the studies on internal anatomy of these species have been conducted on juvenile specimens.

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Genetic Considerations for Captive Breeding and Translocation of Freshwater Turtles and Tortoises for Conservation

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Abstract. – Approximately 40% of the living tortoises and freshwater turtles of the world are considered threatened species and several are critically endangered due to a variety of anthropogenic causes. Captive breeding and the creation of assurance colonies, with subsequent translocation, is an important conservation strategy for some of these species and will likely become more important for others. At present there is a diversity of programs, including those done *in-situ* (within the natural range of threatened species), or *ex-situ* (out of natural range). Captive breeding occurs in large commercial farms and small intensive non-commercial ventures. In order to help achieve success, these programs need to include genetic management of their captive populations and understand the genetic implications of their actions to eventual translocated wild populations. Concerns include the loss of genetic diversity within small populations, including inbreeding depression and inappropriate mixing of turtles belonging to different genetic lineages and loss of cryptic lineage diversity. Although many captive breeding programs do not include genetic management of their populations at present, those that do will have greater conservation value in the future. As wild populations of species disappear, captive populations may provide stock for reintroductions. Comprehensive and conscientious record keeping and data management over the long time that populations are held captive are of utmost importance. This paper reviews the various captive breeding and translocation activities that can be put to use in the conservation of freshwater turtles and tortoises.

Key Words. – Reptilia; Testudines; captive breeding; turtles; tortoises; conservation; *in-situ* preservation; *ex-situ* preservation; translocation; Species Survival Plan (SSP); studbook

There are over 300 extant species of freshwater turtles and tortoises worldwide, of which 136 taxa are listed as threatened according to the 2006 IUCN Red List of Threatened Species. Worldwide, many species have been exploited indiscriminately without regard to sustainability and many populations are declining. Six taxa are believed extinct and two are at least extinct in the wild. Thirty-one taxa are critically endangered (http://www.iucnredlist.org; Turtle Conservation Fund, 2002). Turtle habitats may be fragmented, destroyed, developed, polluted, and invaded by exotic competitors and predators. Additional threats they face include harvesting as food, use in traditional medicine (eggs, juveniles, adults, or body parts), and other commercial trade collections (van Dijk et al., 2000). Many species are also collected for the exotic pet trade.

Captive breeding and the creation of assurance colonies will likely be one aspect of an overall conservation strategy for many species. These programs have historically been performed by zoos and government management authorities; however, conservation organizations, in collaboration with individuals or community-level organizations, are playing an increasing role (Hudson and Buhlmann, 2002; Turtle Conservation Fund, 2002). To be successful, most of these programs will need to integrate high-quality husbandry, health care, genetic management, and small population management principles. Communication and cooperation among captive-breeding programs, conservation organizations focused on protected areas, and university research efforts are critical parts of coordinated species recovery (van Dijk et al., 2000).

The translocation of wild populations has often been used as a mechanism to simply move individual animals out of the way of development or because of uncontrollable hunting. In many of these cases, the goal seems to be to save individual animals, and less emphasis has been placed on reestablishing viable populations. However, translocations could also be used as a technique for achieving conservation goals.

Reestablishing new populations or augmenting existing ones in protected areas may be one of the long- term goals of assurance colony management. However, introducing turtles to a new site—whether it be by translocating wild individuals from another site or introducing animals from a captive-reared assurance colony—should only be conducted after

many factors are considered. These may include: 1) whether the initial cause(s) of decline have been remediated, 2) whether the population will be protected from future loss of habitat or illegal harvest or poaching, 3) whether the translocation is attempting to establish a new population or augment an existing one, and 4) whether there is commitment and support for monitoring to determine success (i.e., site fidelity, survival, reproduction). These questions must all be answered before translocations or reintroductions occur. It is not the intention of this paper to discuss the concerns over translocation projects and the reader is referred to the following papers on the subject (Dodd and Seigel, 1991; Griffith et al., 1989; Tuberville et al., 2005; Wolf et al., 1996).

However, should translocation be deemed a viable conservation strategy under certain circumstances, then appropriate genetic management of assurance colony stock becomes a critical factor. It has been used as an augmentation tool for small isolated populations; the introduction of new co-specifics can alleviate genetic concerns such as inbreeding depression. Translocation has been also used to create additional populations in order to offset the loss of other populations. However, single translocation events using small founding population sizes, may eventually result in founder effects, such as loss of alleles (variation), or fixing of certain alleles over several generations. Potential effects of small population size and subsequent inbreeding include reduced fecundity and survival rates; both of which have been observed in captive populations (Bodkin et al., 1999).

Captive Breeding

The ideal strategy for the long-term protection of biological diversity is the *in-situ* protection of natural communities, processes, and populations in the wild. Specifically, this refers to protection of native habitat, and direct conservation measures for a species in its native range. Within the context of captive breeding, "*in-situ*" conservation refers to facilities that operate within the range of the species in question and often strive to enhance the breeding success of nearby wild populations by enhancing juvenile survivorship by "headstarting".

In-situ preservation may not be effective if the last remaining population of a rare or endangered species is too small to maintain itself in the wild, is found outside protected areas, or continues to decline despite conservation efforts. It is likely that the only way species in such circumstances can be prevented from going extinct is to maintain individuals in artificial conditions under human supervision, often in facilities located at some distance away, including facilities outside of the historic range of the species. This strategy is known as *ex-situ* or off-site preservation (Kleiman et al., 1996, in Primarck, 2004).

The long-term goal of many *ex-situ* conservation programs is the eventual reestablishment of new populations in the wild through reintroduction, when sufficient numbers of offspring are produced by assurance colonies and suitable protected habitat becomes available. *Ex-situ* and *in-situ* conservation are complementary strategies. Thus, under appropriate design and management strategies the individuals produced by *ex-situ* populations can be periodically released into the wild to augment or re-establish extirpated populations (Kleiman et al., 1996, in Primarck, 2004).

There is a considerable range of interpretation as to what comprises an *ex-situ* or *in-situ* program. Captive breeding programs under artificial conditions established within the natural range of the species may technically represent *in-situ* efforts, but depending on the conditions such efforts might actually be better interpreted as *ex-situ* conservation. For our purposes we will consider *ex-situ* programs as those that are either located outside of the natural range of the species, or that do not include regular exchange of animals with a local wild population.

For captive breeding to be successfully applied to global turtle conservation, two important aspects are highlighted: 1) breeding programs must define the goals of each project, whether it be reintroduction or simply increasing the numbers of individuals in captivity, and 2) record keeping is critical, including genetic data to facilitate an awareness of the evolutionary consequences of captive management.

In-situ Headstarting Programs. — These programs include those efforts that take place in the wild environment within the historical distribution of the species under management (Table 1). *In-situ* headstarting and release programs may include 1) the collection of gravid females that lay eggs in a protected environment, with subsequent release of the adult female and hatchlings, and 2) the collection of nests (clutches) and/or hatchlings from the wild environ-

Table 1. Examples of *in-situ* conservation used for turtles and tortoises. Some of these projects represent historical activity and have varying degrees of current activity.

Taxon	Location	Activity	Reference
Aspideretes gangeticus	India	headstarting	P.P. van Dijk, pers. comm. 2005; Whitaker and Andrews, 1997
Batagur baska	India, Malaysia	headstarting	Quinn, 2002
Callagur borneoensis	Malaysia	headstarting	Sharma and Tisen, 2000
Chitra indica	Chambal river, India	headstarting juvenile rearing	Choudhury et al., 2000
Gopherus agassizii	Mojave Desert, USA	nest protection	Morafka, 1989
Gopherus flavomarginatus	Mapimi, Mexico	headstarting	Aguirre et al., 1997; Morafka et al., 1994
Podocnemis expansa	Manaus, Brazil Orinoco River, Venezuela	headstarting	R.C. Vogt, pers. comm. 2005; Cantarelli, 1997 Licata and Elguezabal, 1997
Testudo hermanni	Massif des Var, France	headstarting	Devaux and Stubbs, 1997; villagetortues.com

ment that are moved into a protected environment and reared prior to release. These programs seek to diminish predation on the vulnerable egg and hatchling life stages to increase survivorship of these stages. These programs may be housed within captive breeding centers with explicit conservation goals or at farms that have primarily commercial purposes (food and pet trade). The program for protection of *Batagur baska* in India and Malaysia is an example of the former approach (Quinn, 2002). Each year thousands of eggs are collected and incubated in artificial nests. The nests are protected protected from predation and confine the hatchlings. The hatchling success rate is from 40–85%. When the animals are 4 yrs old, they are released into their respective river systems. Since 1968, more than 30,000 turtles have been released (Quinn, 2002).

The northern red-bellied turtle , *Pseudemys rubriventris,* has been the subject of a head-starting program in Massachusetts, USA (Haskell et al., 1997). In this program, hatchlings are collected from a single donor population, raised in captivity and released at 9 months of age into several ponds. Survival and growth of the head-started turtles is monitored and evaluated at each pond through annual recapture studies (Haskell et al., 1997); it was found that the survivorship of animals released during the first year of life was lower than the survivorship of the animals released in following years.

A program that protects gravid females and nests of the Amazon river turtle, *Podocnemis expansa,* is conducted by the government of Brazil. The program releases thousands of hatchlings every year into the rivers, and genetic management is considered through DNA analysis of animals at the different localities (Cantarelli, 1997; R.C. Vogt, *pers. comm.*, 2005).

Genetic Concerns in Headstarting Programs. — In-situ head-starting programs provide the fewest genetic concerns, as these programs include the collection of animals from natural nest sites with subsequent release of head-started animals into the same area. However, human alteration of the genetic structure of the population may occur if the enhanced survivorship of hatchlings through head-starting is only provided to a few gravid females. If space to house and head-start hatchlings is limited, then an equal proportion of hatchlings from every clutch should be head-started. Secondary genetic concerns of rearing hatchlings in captivity include selection for survival in captive conditions, but not necessarily conditions in the wild. Consideration of the potential for artificial selection, through diet, temperature or other environmental conditions in captivity should be taken into account. Rearing in protected, but "semi-wild" enclosures or pond facilities prior to release can reduce artificial selection pressures while also minimizing the threat of predation.

In-situ Captive Breeding Programs. — These programs include management of small to relatively large populations in semi-controlled conditions within the natural range of the species distribution. Throughout the world, animal rescue centers often harbor turtles that were confiscated from illegal trade. Turtles from these rescue centers may be useful to conservation-based captive breeding programs, but intensive health screenings and genetic analyses are necessary since the origins of most individuals are unknown.

There are several successful examples of *in-situ* captive breeding programs, including one to preserve the Madagascar ploughshare tortoise, *Astrochelys yniphora*. The program has produced more than 100 captive-bred offspring in six years. Efforts have more recently been focused on protection of some wild areas adequate for their reintroduction and several individuals have been released into the wild environment (Durrell, 1994, 1998; Juvik et al., 1997).

Another example of this kind of program is being conducted in Mapimi Biosphere Reserve in Mexico for the Bolson tortoise, *Gopherus flavomarginatus*. Genetic samples have been collected from tortoises to determine levels of variation, help with pairing recommendations, and to evaluate new stock from zoos or the wild. (T. Edwards, *pers. comm*., 2006). Thirty-two tortoises have recently been reintroduced to New Mexico, USA onto private land.

In-situ captive breeding and head-starting programs have also been implemented at the Center for Herpetology/ Madras Crocodile Bank (MCB) in India. MCB has bred six species of Indian turtles and tortoises and has head-started a large softshell turtle, *Aspideretes gangeticus*, since 1987. Due to its ecological role as a scavenger, increasing populations of this species are also helping to improve the Ganges river water quality (Whitaker and Andrews, 1997).

One program that combines *in-situ* captive breeding with *ex-situ* breeding is the conservation program of the Galapagos tortoise, *Chelonoidis nigra*. The *in-situ* captive breeding program began in 1974 with 14 animals of the subspecies *C. n. hoodensis* (12 females and 2 males), and in 1977 an additional male was added. At the Darwin Station on Santa Cruz Island, eggs belonging to all different subspecies of Galapagos tortoises are incubated, hatched, and head-started. Hatchlings from Santa Cruz tortoises are released in their natural environment, as part of an *in-situ* conservation effort, while those belonging to subspecies from Española and Pinzón islands are transferred to those islands, and released as part of an *ex-situ* conservation effort. A program to eradicate goats from the islands aimed at restoring native vegetation is also being conducted (Cayot and Morillo, 1997) and illustrates the need for captive breeding programs to work with land management programs.

In Europe, the species recovery program for Hermann's tortoise (*Testudo hermanni*) has reintroduced more than 6000 tortoises to 15 different sites in southern France since 1988. The reintroduced animals include a variety of age classes. The program also integrates habitat management measures to ensure appropriate habitat conditions in the area where these tortoises live (Devaux and Stubbs, 1997).

Genetic Concerns for in-situ Captive Breeding Programs. — We have observed, at least in the case of the Central American river turtle, *Dermatemys mawii* in Mexico, and *Batagur baska* in Malaysia and Thailand, that appropriate genetic management may not be occurring in captive breeding programs. In the case of *Batagur baska*, the animals

Figure 1. *Dermatemys mawii* from a captive breeding farm in Tabasco, Mexico. This critically endangered turtle could be a candidate species for reintroduction into rivers within its historic range, but unsustainable hunting pressures must first be curtailed, and phylogeographic genetic analysis performed on wild populations as well as captive animals being considered for repatriation, in order to ensure long-term viability of reintroduced and native populations.

coming from the Andaman Sea and the Malacca Strait could be genetically different from the South China Sea population. Since the captive offspring result from the mating of animals from these different regions, offspring may be prone to out-breeding depression, and their fitness may be reduced. In addition, offspring have been released at a number of sites in Thailand, including places that are not appropriate for their survival (Quinn, 2002).

In Mexico, more than 800 specimens of the Central American river turtle *Dermatemys mawii* (Fig. 1) housed at a turtle farm in Tabasco have been held together for breeding in the same pond for several years without any consideration of their genetic characteristics or location of origin. If the offspring of these captive animals are to be introduced within their natural range, they could actually reduce the overall fitness of the natural population, posing a new threat to these already endangered populations (Frankham et al., 2000; H. Quinn, *pers. comm*., 2005). Unfortunately, in cases where founder stock originates via government confiscations or donations via the pet trade, knowledge of the original localities of individuals (founders) is usually unknown.

Genetic investigation could potentially determine the geographic origin of individuals, but this is only possible if prior work has been done to identify phylogeographic genetic structure among river systems or across landscapes, and if these populations have unique genetic markers at a relevant geographic scale (e.g., Roman et al., 1999; Berry et al. 2002; Souza et al., 2002; Spinks and Shaffer, 2005). If this is the case, then captive breeding programs can be conducted to maintain the genetic integrity of identified populations. However, in some cases the situation is so grave that few individuals remain at all, and any reproduction supercedes these issues. The most extreme such example is the case of Lonesome George, the sole surviving member of one Galapagos tortoise subspecies, *C. n. abingdonii*. Here genetic studies are driving the search for the most closely related females to mate with him, as well as helping to establish the guidelines for captive breeding and repatriation of other endangered Galapagos tortoise subspecies (Burns et al., 2003). In others, such as the Australian western swamp turtle (*Pseudemydura umbrina*), rapid expansion of the captive population and multiple reproductions by founders are central to successful recovery (Burbidge and Kuchling, 1994).

Hybridization has recently become more of a concern in the captive conservation management of turtles, partly because the application of genetic markers has documented previously unrecognized hybridization in wild populations (e.g., Parham et al., 2001; Georges et al., 2002). For such issues, genetic investigations are critical to the integrity of reintroduction projects (e.g., FitzSimmons et al., 2002).

Captive Breeding in Commercial Farming Programs — Turtle farming is an economic activity, undertaken for financial gain and typically without conservation objectives, that has affected native turtle populations in mostly negative ways. In some cases, conservation efforts can be aided by the reduction of wild harvest of a species, if a farming operation is self-sustaining, as may be the case with *Batagur baska* in Malaysia (H. Quinn, *pers comm.*, 2005) or *Pelodiscus sinensis* elsewhere in Asia (P.P. van Dijk, *pers. comm.*, 2005). However, farming species within their natural range seems to create pressure on the wild populations. As a species becomes more rare, the economic value of that species increases, usually resulting in local extirpation or at least commercial extinction of founder stock from the wild (van Dijk et al., 2000). When farming is undertaken outside of the native range of a species, intentional or unintentional releases may occur that produce new feral populations. For example, in the farming of *P. sinensis* in Asia, escaped breeding stock has established non-native populations in the Ryukyu Archipelago in Japan (Sato and Ota, 1999). In China, the three-striped or golden-coin turtle (*Cuora trifasciata)* is farmed for traditional medicines. This has increased the monetary value of the species, but farming has not reduced the collection pressure on wild populations (van Dijk et al., 2000). However, in Taiwan, small-scale farms use local wild-caught *Mauremys sinensis* as founders and produce offspring for the pet trade and for repatriation back into ponds in local Buddhist temples and rivers (especially the Tansui River in Taiwan). These farms do not appear to have had negative effects on the wild populations (Ades et al., 2000).

Turtle farms began operating at the turn of the last century in both Japan and the USA (Mitsukuri, 1904). In Tokyo, softshell turtles (most likely *Pelodiscus* sp.) were raised as part of an enterprise that also included goldfish, carp, and eel as early as 1875 (Mitsukuri, 1904), and today, central Japan continues to supply the majority of the Japanese markets. The Asian turtle farming industry currently produces several turtle species for meat, including the softshell turtle, *P. sinensis*, in Japan, Taiwan, China, Singapore, Malaysia, Thailand, Vietnam, and Indonesia. In China and Taiwan, these animals are extensively farmed, producing 5,000 to 10,000 metric tons of turtles per year, and exceeding the amount of trade of wild-captured turtles in these areas

(van Dijk et al., 2000). Another softshell turtle, *Palea steindachneri* is also farmed in China (P.P. van Dijk, *pers. comm.*, 2005) as well as *Cuora trifasciata*, *Mauremys mutica*, and *Mauremys sinensis*, though to a lesser degree than *Pelodiscus* (Lau and Shi, 2000). In Malaysia, both *Chitra chitra* and *Pelochelys cantorii* are currently farmed for the pet trade (P.P. van Dijk, *pers. comm.*, 2005).

In the USA, the diamondback terrapin (*Malaclemys terrapin*) farming industry was developed by the Bureau of Fisheries and private individuals during the early 1900s in North Carolina and Maryland. At present, there are no large-scale commercial terrapin farms in operation in the USA (Nash, 1991). However, the USA does have more than 20 species of turtles produced by commercial breeders, almost entirely for the pet market, the primary species being the red-eared slider, *Trachemys scripta elegans*. Pond cultivation of the red-eared slider in Louisiana for the pet industry began in the 1960s and while some fraction of the total eggs hatched are still collected from wild populations or from founder females taken from the wild, the vast majority of hatchlings sold are now from captive bred "semi-wild" enclosures. States have wildlife laws that apply to individual collectors and the commercial pet trade within the USA, imposing size and number restrictions on wild-caught turtles, but the international export of many US species, including red-eared sliders, snapping turtles, and softshells, is a significant proportion of the overall market, and has not been well regulated.

Genetic Concerns for Commercial Farming Programs. —The primary concerns of commercial farming programs are the successful reproduction and survival of founder stock, high fecundity, and high survivorship of eggs and juveniles. Usually these farming programs do not include genetic management of their population even though awareness of the genetic consequences of captive management could enhance commercial productivity (Lutz-Carillo et al., 2006). However, genetic management in farms for high fecundity and rapid growth or for novel forms may be in conflict with conservation goals. Hybridization often occurs within farm facilities, and is even encouraged by farm managers, because these animals may bring higher prices among collectors or they can be offered erroneously as "new" species (van Dijk et al., 2000; Ota, *pers. obs.*). These turtles have no value for conservation and may pose risks to the native populations if released to the wild. Unfortunately, it is likely not a rare occurrence that some turtles escape from facilities. If the escaped captive-bred animals are hybrids or from different genetic lineages, they may alter the wild population through the introgression of foreign genes (Shi and Parham, 2000; Ota, *pers. obs.*). In some cases, natural disasters, or simply the failure of a particular farm as a business venture could lead to large numbers of animals being introduced into the natural habitat near a farm. Clarification of the taxonomic status for several questionable captive-bred forms remains urgent (van Dijk et al., 2000; Parham et al., 2001; Schilde et al., 2004). Even if captive animals are retained within commercial farms, once live turtles are sold, whether as pets, for food, or other purposes, there is absolutely no control over where they may end up in the wild (e.g., Spinks et al., 2003).

Taken as a whole, commercial farming operations may have conservation value in the future. Should entire wild populations of a species disappear, captive populations will be the stock used to repopulate the wild environment. Genetic analyses and management of these specimens are important, and conveying this to commercial farm managers should be a key strategy for creating and incorporating a conservation value for farming operations. Obtaining information about wild turtle populations and their underlying genetic structure is an increasingly relevant conservation goal in helping to direct the management of captive populations.

Ex-situ Captive Breeding Programs. — The majority of conservation-focused assurance colony projects are currently conducted outside of the natural range of the target species. Some of these programs involve the cultivation of non-native species in zoos (e.g., Asian brown tortoise, *Manouria emys*, and impressed tortoise, *Manouria impressa*, Minnesota Zoo; black-breasted leaf turtle, *Geoemyda spengleri*, Denver Zoo; painted terrapin, *Callagur*

Table 2. Examples of current and historic *in-situ* captive breeding programs for freshwater turtles and tortoises.

Taxon	Location	Activity	Reference	Comments
Astrochelys yniphora	Madagascar	Captive breeding	Durrell, 1994, 1998; www.durrell.org	Reproduction and reintroduction
Batagur baska	Malaysia	Captive breeding Headstarting	Quinn, 2002	Headstarting and release conducted for more than 20 yrs
	Thailand	Captive breeding	Quinn, 2002	No data
Chelonoidis nigra duncanensis *Chelonoidis nigra hoodensis*	Ecuador	Captive breeding	darwinfoundation.org Cayot and Morillo, 1997	Focused genetic conservation using related stocks and intense management
Chitra chitra	Malaysia Thailand Myanmar	Captive breeding Headstarting	chitrachitra.com Win Ko Ko et al., 2006	Some releases completed and more planned for next two years
Dermatemys mawii	Tabasco, México	Captive breeding	Syed, pers. obs.	No releases as yet
Gopherus flavomarginatus	New Mexico, USA	Captive breeding	T. Edwards, pers. comm. 2005	No releases as yet, genetics analyses of stock on-going
Hardella thurjii	India	Captive breeding	Choudhury et al., 2000	Reproductive success
Kachuga sp.	India	Captive breeding	Choudhury et al., 2000	Reproductive success
Pseudemydura umbrina	Australia	Captive breeding	Kuchling, 1997	Reproduction and reintroduction
Vijayachelys silvatica	India	Captive breeding	Choudhury et al., 2000	As yet limited success

borneoensis, Fort Worth Zoo; river terrapin, *Batagur baska,* Cleveland Metroparks Zoo; and Burmese star tortoise, *Geochelone platynota,* Bronx Zoo; Table 3). All of these are small programs with conservation objectives, often performed in facilities under manipulated environmental conditions.

In general, the same concerns can be applied to these programs as to *in-situ* captive programs, with emphasis on founder stock origin, population genetics, but with artificial selection (i.e., different climate, temperature regime, nesting season) becoming increasingly important for these populations at these facilities (see Appendix 1). While not all *ex-situ* programs have the goal of successful reintroduction of offspring to the wild, managing them as such provides greater conservation value.

In North America, Australia, Europe, Japan, New Zealand, South Africa, and Central America, Species Survival Plans (SSPs) have been put in place for many endangered species (Wiese and Hutchins, 1994). These SSPs are cooperative breeding and conservation programs that involve coordinated management of all captive individuals held by cooperating institutions. Regional and international studbooks are used to make recommendations on which animals should breed, with what partner, how often, and where offspring should be transferred for future breeding opportunities (Wiese and Hutchins, 1994; Frankham et al., 2003). To provide opportunity for use in reintroductions, all of these programs actively manage genetic diversity. Consequently, all SSP programs attempt to maintain as much representation of a population's wild genetic diversity as possible (Wiese and Hutchins, 1994).

Among the ongoing SSPs involving turtles are programs for Egyptian tortoise, *Testudo kleinmanni*, Jersey Zoo, UK (Syed, *pers. obs.*); Galapagos tortoise, *Chelonoidis nigra,* Henry Doorly Zoo,Omaha, Nebraska and San Diego Zoo (both USA); flat-tailed spider tortoise, *Pyxis planicauda,* Bronx Zoo; and Coahuilan box turtle, *Terrapene coahuila,* Houston Zoo, Bronx Zoo, and Jersey Zoo (Cerda and Waugh, 1992). Additional zoo programs in the USA and in Europe are listed in Table 3.

We recommend that *ex-situ* captive breeding programs established with the goal of potential reintroductions adhere to the following guidelines:

1) Undertake appropriate studies of geographic variation in morphology and genetics for the target species;

2) Establish the captive population with genetically appropriate stock;

3) Grow the captive population(s) to a secure size and identify the numbers of offspring, and age structure needed for reintroduction;

4) Manage the founder captive population and produce offspring from the founders for as long as possible;

5) Select offspring from founders for reintroduction;

6) Select secure sites for reintroduction and address factors that caused the original population demise;

7) Manage the reintroduced population in the wild through long term monitoring to determine success;

8) Define reintroduction success, which should include site fidelity, survival, and reproduction.

Since the founder population size is usually small, it is important to understand husbandry techniques for the species. Management at the initial stage is often focused on basic research to develop husbandry, and efforts to ensure survival and reproduction of the founders. It is critical to establish a system of individual identification (shell-notching, PIT-tagging) and keep accurate records to insure that all

Table 3. Some turtle and tortoise studbooks currently (as of late 2006) in place in the USA. Many separate turtle and tortoise studbooks are kept in Europe by the European Studbook Foundation (www.studbooks.org) and Homopus Research Foundation (www.homopus.org).

Common name	***Scientific name***	**Studbook keeper**	**Institution**
Alligator snapping turtle	*Macrochelys temminckii*	Patricia Shoemaker	Houston Aquarium
Annam leaf turtle	*Mauremys annamensis*	Adam Stern	Miami Metrozoo
Asian box turtles (8 spp.)	*Cuora* spp.	Heather Lowe	Turtle Survival Alliance
Asian brown tortoise	*Manouria emys*	Karla Anderson	Minnesota Zoo
Black-breasted leaf turtle	*Geoemyda spengleri*	Rick Haeffner	Denver Zoo
Burmese star tortoise	*Geochelone platynota*	Bill Holmstrom	Bronx Zoo
Chinese stripe-necked turtle	*Mauremys (Ocadia) sinensis*	Alan Baker	Charles Paddock Zoo
Chinese three-striped box turtle	*Cuora trifasciata*	Heather Lowe	Turtle Survival Alliance
Coahuilan box turtle	*Terrapene coahuila*	Beth Moorhead	Houston Zoo
Egyptian tortoise	*Testudo kleinmanni*	Karen St. John	National Aquarium in Baltimore
Fly River (pig-nosed) turtle	*Carettochelys insculpta*	Steve Conners	Miami Metrozoo
Flat-shelled spider tortoise	*Pyxis planicauda*	Michael Ogle	Knoxville Zoo
Galapagos tortoise	*Chelonoidis nigra*	Ed Louis	Henry Doorly Zoo
Impressed tortoise	*Manouria impressa*	Karla Anderson	Minnesota Zoo
Indian star tortoise	*Geochelone elegans*	Tommy Owens	San Diego Zoo
McCord's box turtle	*Cuora mccordi*	Heather Lowe	Turtle Survival Alliance
Painted terrapin	*Callagur borneoensis*	Diane Barber	Fort Worth Zoo
Pancake tortoise	*Malacochersus tornieri*	Andy Daneault	Disney's Animal Kingdom
Radiated tortoise	*Astrochelys radiata*	Bill Holmstrom	Bronx Zoo
Red-necked pond turtle	*Mauremys (Chinemys) nigricans*	Dino Ferri	Jacksonville Zoo
Reeves' turtle	*Mauremys (Chinemys) reevesii*	Dino Ferri	Jacksonville Zoo
River terrapin	*Batagur baska*	Brad Poynter	Cleveland Metroparks Zoo
Roti Island snake-necked turtle	*Chelodina mccordi*	Liz Burke	Columbus Zoo and Aquarium
South American river turtle	*Podocnemis unifilis*	Tina Sals	Brookfield Zoo
Spider tortoise	*Pyxis arachnoides*	Michael Ogle	Knoxville Zoo

founders are represented in subsequent generations. In the maintenance stage, the population is managed at zero population growth, at a size determined by the genetic and reintroduction goals of each program. These healthy captive populations will serve as reservoirs of genetic material and sources for eventual reintroductions (Wiese and Hutchins, 1994). Then, in the growth phase, the focus is on the rapid reproduction and expansion of the population in multiple facilities.

Genetic Management of ex- situ Captive Breeding Programs. — For all species, one consequence of captive propagation is the reduction of the genetic variability of the captive population over generations due to random genetic drift and selection. Smaller populations will experience some of these genetic effects (particularly drift) to a greater degree than larger populations. In captive populations with small numbers of animals, this process can quickly reduce genetic variation, leading to an increase in homozygosity as well as the potential for inbreeding depression (Hedrick and Kalinowski, 2000; Frankham et al., 2004). This can have negative effects on population persistence and has been linked to increased rates of extinction in wild populations as well as in captive ones (Hedrick and Kalinowski, 2000; Frankham et al., 2004). In the long term, increasing homozygosity may limit the ability of a population to adapt to future habitat changes. Captive populations therefore need to maintain as much of the genetic variability of the founding population as possible (Frankham et al., 2003). Which founders reproduce, how many offspring they produce, and whether all founders reproduce before they die becomes a crucial aspect of subsequent genetic representation.

Several aspects of turtle and tortoise biology provide advantages in achieving these genetic goals (Kuchling, 1999). Turtles and tortoises are long-lived vertebrates, with reproductive ability expected for many years after achieving adult size. By integrating the long reproductive life of turtles and tortoises into captive reproduction goals the long-term success of subsequent generations can be enhanced by retaining as much of the founder variation as possible. Burbridge and Kuchling (1994) have successfully applied this strategy in the western swamp tortoise (*Pseudemydura umbrina*) recovery program. Founders are actively managed to produce offspring for as long as possible, using as many different founders as possible. Thus, the F1 generation (which will retain the greatest amount of the founders' genetic variation) is very large and quite likely to be genetically robust in representing the original genetic variation.
Turtles in breeding programs should be individually identified for record keeping and reproductive history. A variety of means to accomplish this includes photo documentation (Bender, 2001), physical shell marking (Cagle, 1939), and metal tags or microchip implantation (Camper and Dixon, 1988). Thus, it is possible and necessary to retain records for each individual and to follow the reproductive history of the population. Even in cases where individual matings may not be easily identified (e.g., colony breeding designs), it might be possible to use new technologies (nesting area web cams for female identification and clutches) or application of genetic marker systems to provide both maternity and paternity assignments for offspring (Moon et al., 2006) or to select appropriate breeders (e.g., Burns et al., 2003).

Overall, *ex-situ* management requires intense management to alleviate the genetic issues associated with small populations. A variety of data management designs and analytical tools are available to assist with this aspect of husbandry. Some of these include conceptual frameworks like studbooks and pedigree analysis, which can be data intense and require a basic understanding of both inheritance and population genetics (Frankham, 1999). A studbook database system records a given captive population's history in captivity (Primarck, 2004). These databases track all individuals in the managed population. They provide a direct means of determining ancestry and relatedness of all animals in the studbook. Within the studbook each animal is assigned a unique numerical identifier or studbook number that provides the mechanism to track individuals, facilitate population analyses, breeding designs, and recommendations. Relevant examples of freshwater turtles and tortoise studbooks are in Table 3.

The studbook database enables analyses of the population that are otherwise impossible. Studbooks make possible summary pedigree analyses based on the data assembled for that captive population over time. Pedigree analyses of this type require significant information regarding founders, parentage, offspring, and mortality. Hence, consistent and accurate record keeping is the single greatest advantage a captive population can have alongside conscientious husbandry. Studbook data provide answers to questions such as (1) what is the number of founders? (2) how is that original genetic variation distributed (on average) in the population today? and (3) what is the genetic relationship among individuals in the population?

Modern computer software also provides the opportunity to discern the pattern of genetic variation and determine how well or poorly that variation is being retained in the population (or may be retained) under a given management strategy. For example, two analyses, Gene drop (MacCluer et al., 1986) and Peeling (Cannings et al., 1978) simulate the distribution and partitioning of genetic variation in the population under various models of population growth, decline, or breeding. It is important to emphasize the depth of time in these management programs. In designing and implementing a captive management program for a species, the normal timeline seeks to maintain the founding genetic diversity for a minimum of 100 years.

For private collections the broader usefulness of captive breeding often relates to the ability to integrate with global management initiatives. From a genetic standpoint, the significant number of captive specimens held by private individuals represents a potentially important resource base. It is simultaneously a boon to resource allocation that these facilities are independently supported, but unfortunately, it potentially represents a tremendous loss if record keeping is insufficient to allow integration of these living collections

into global conservation programs (Snyder et al., 1996). As collaborations grow stronger, the expertise of private collectors and their considerable live holdings may be better integrated with record keeping and population management strategies of zoos and aquaria.

It is also important to consider the time scale of the life span of turtles because often the owners die before their animals, hence private collectors are urged to collaborate with professional organizations, if for no other reason than to establish a future home for the turtles (and records) after the owner's death.

Translocation

Translocation is a generic term used to encompass any release of animals in a new location (Caldecott and Kavanagh, 1988). Konstant and Mittermeier (1982) suggested the following definitions:

Translocation: the capture and transfer of feral animals from one part of their natural range to another, with minimal time spent in captivity.

Introduction: the release of animals into a habitat in which they have never occurred naturally. Introduction usually involves wild-caught individuals, but may sometimes be attempted with captive-born animals. Introduction may be intentional or inadvertent.

Reintroduction: the release of either wild caught or captive-born animals into an area in which they have either declined or disappeared as a result of human pressures.

Frankham et al. (2003) defined translocation as the movement of any individual from one location to another as a result of human actions. This activity may eventually play an important role in turtle conservation. Tuberville et al. (2005) concluded that translocation can be successfully implemented to relocate or repatriate gopher tortoises, (*Gopherus polyphemus*). Their study found that wild-caught adult tortoises are more likely to exhibit high site fidelity to the new location if they are subjected to long-term penning (9–12 mo) prior to release (i.e., removal of pen walls). Following penning, site fidelity appears to remain high as long as habitat quality is maintained. Tuberville et al. (2005) also suggested that the most effective translocation techniques and protocols will vary among species and require an in-depth understanding of the species behavior and ecology. We also suggest that the appropriate age class (hatchlings, juveniles, or adults) to be used in reintroductions will vary with the species. Multiple yearly releases may be necessary to establish a stable population demography.

Turtles should be released within the historical range of the species, with special attention to genetic lineages. If the turtles to be released differ in geographic origin from the wild population still living in the release area, genetic studies should be done to understand the underlying genetic structure of both the wild and translocated populations (e.g., Bock et al., 2001; Souza et al., 2002; Spinks and Shaffer, 2005; Gaur et al., 2006). Ideally both populations should belong to the same genetic lineage. It will be important to monitor the release population, using techniques such as radio-tracking, to determine whether the new individuals remain on site and become part of the breeding population. As emphasized above, all programs require that accurate and comprehensive records be maintained, and the availability of those records should be assured beyond the life of the program itself.

Conclusion

As turtles and tortoises continue to decline, actions toward conserving them are required through a combination of traditional landscape-scale conservation efforts (i.e., protected areas) and by integration of captive management programs into global designs. Captive management of any species requires significant consideration of the processes acting on the captive population. All small populations are subject to a variety of population genetic processes that can act to decrease genetic variation over generations (Whitlock and McCauley, 1990). Captive breeding designs and programs that seek to enhance conservation efforts must account for and minimize these processes strategically over many years. Explicit guidelines for genetic management of a given turtle species or population depends on a number of life history and population biology considerations, and will vary for each taxon. It may be appropriate to recommend maintaining 500 star tortoises (*Geochelone elegans*) in a captive colony (a species with a small adult size), but quite another to suggest the same for giant river turtles (e.g., *Orlitia borneensis*). However, there are several guidelines that transcend the taxon-specific realities in management. Thorough, dedicated, accurate record keeping for captive breeding programs is the single most important aspect necessary to allow integration of those populations within a conservation framework. The next crucial step is applying those records to population management and actively seeking to overcome the subtle but relentless forces acting on the genetics of small populations. No effort toward the goal of turtle and tortoise conservation should be wasted as resources are too precious and the problems too vast, and we must seek to do no further harm, either by catastrophe or by failing to consider the genetics underlying the very populations we seek to conserve.

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APPENDIX 1

The Turtle Survival Alliance (2004):
Record Keeping and Population Management Guidelines

It is important to manage the *ex-situ* populations as if they were small ones, in order to conserve their genetic diversity and avoid inbreeding, thus maintaining a demographically suitable

structure. These principles for maintenance of a viable population over long periods of time will enhance the ability of these populations to survive in the wild, if release programs are established in the future.

A managed population should start with about 25–30 breeding, founder (wild-caught) turtles, with an equal number of males and females. More are not necessary. Each founder pair should produce at least 12 hatchlings (which will survive to adulthood) for the managed population. Thus, the first generation population should contain around 150–180 individuals. Such a population is necessary since not all hatchlings will survive to reproduce, and to minimize genetic drift (random change of a population's genetic structure due to its small size).

Eight principles to follow when managing turtle assurance colonies:

1. Keep comprehensive, complete, accessible records for all of the captive animals in the colony.

2. Avoid inbreeding the population for as long as possible (more than 50 years is feasible).

3. Get all founders (wild-caught) to breed, have them produce at least 12 hatchlings (F1s) per pair for the managed program (see Important Note above), and try to have the founders all contribute about the same number of offspring . If possible, keep the founders in pairs, and don't interchange animals among pairs (i.e., retain the pairs throughout their lives). This may not be realistic for a number of reasons, but it is an optimal solution, if possible. If small groups are required (i.e., 1.2 or 2.3), keep these groups together without interchange.

4. Do not select the biggest, prettiest, healthiest hatchlings for the managed population. If only a given proportion of the hatchling turtles produced are destined for the managed population, they should be selected randomly.

5. Do not breed turtles with lots of relatives in the population to turtles that have none or very few relatives.

6. When pairing adult turtles initially, do so randomly. Do not select pairs based on color, size, vigor, or other characters.

7. If a particular turtle species demonstrates temperature-dependent sex determination, incubate the eggs at temperatures that will produce an equal sex ratio.

8. Stretch-out the generation time as long as possible. For example, if the initial population contains all founders, it is desirable to produce as many offspring as are needed in the managed population right away. These offspring constitute the first generation, or F1s, and approximately 300 of them (see Important Note above) are desired. The founders can continue to produce offspring, but these are not needed in the managed population. However, if in a few years the original (now older) F1s begin to be replaced with hatchling F1s (that the original founders are continuing to produce), the generation time will be extended and more space in captivity will be created (young turtles take up less space than adults). This is an important reason for retaining the genetic variation in the population for the longest period possible. The older F1s can then be entered into reintroduction maintenance programs or surpluses, thus the managed population has room for the younger F1s. This process should continue until the founders are no longer productive or begin to die. At that time the process should be repeated in forming the F2 generation. However, it would be advisable to produce a few F2s (through appropriate pairings, following the above guidelines) prior to this to make sure it can be done.

GLOSSARY

Assurance colony: Captive population of a threatened species, intended to maintain the long term genetic diversity of a species. Eventually could be reintroduced within the wild environment, ensuring the species or the population survival.

Cryptic lineages: Two or more lineages that are morphologically very similar; but show reproductive isolation, or clear genetic distinctiveness.

Ex-situ preservation: refers to maintaining individuals in artificial conditions under human supervision, often in facilities located at some distance outside of the historic range of the species.

Founder stock: First individuals used for reproduction in a captive breeding program, originally taken from the wild environment.

Genetic drift: Genetic changes and losses that occur by chance, especially evident in small populations.

Genetic lineage: Group of individuals with a number of genetic characteristics unique to the group and different from other groups.

Headstarting: An *in-situ* preservation technique that attempts to preserve a species by reducing the exposure of eggs and or juvenile animals to predation until after they have hatched or reached a certain age.

Hybridization: Offspring resulting from mating involving animals of two different genetic lineages.

Inbreeding depression: Lowered reproductive rates or production of offspring with lowered survival and reproduction following mating among close relatives.

In-situ preservation: refers to facilities that enhance the breeding success of nearby wild populations through captive breeding or by enhancing juvenile survivorship by headstarting.

Introduction: The release of animals into a habitat in which they have never occurred naturally. Usually involves wild-caught individuals, but may sometimes be attempted with captive-born animals; introduction may be intentional or inadvertent.

Out-breeding depression: Lowered reproductive rates or production of offspring with lowered fitness following mating among individuals of different species or widely separated populations.

Reintroduction: the release of either wild-caught or captive-born animals into an area in which they have either declined or disappeared as a result of human pressures

Repatriation: same as reintroduction.

Restocking: periodic release of individuals produced by *ex-situ* populations into the wild in order to supplement or re-establish extirpated populations.

Species Survival Plans (SSPs): Strategies for wildlife conservation that involve coordinated management of all captive individuals of endangered species held by cooperating zoos and aquaria.

Translocation: Translocation is a generic term used to describe the intentional release of individuals of a species at a within-range location different from their capture location in order to "establish, reestablish, or augment a population" (Griffith et al., 1989).

Defining Turtle Diversity: Proceedings of a Workshop on Genetics, Ethics, and Taxonomy of Freshwater Turtles and Tortoises
H. Bradley Shaffer, Nancy N. FitzSimmons, Arthur Georges, and Anders G.J. Rhodin, Eds.
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Internet Resources for Turtle Research and Conservation

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Abstract. – Three challenges must be met to provide useful Internet resources for turtle research and conservation: continuous content update, quality assurance/quality control, and synthesis and integration. Website design can be improved by analyzing a set of trade-offs related to these three challenges. Consideration of these design issues leads us to propose a considerable extension to the current EmySystem website to be achieved through the creation of a suitable permanent institution that will provide funding and organize personnel.

Key Words. – Reptilia; Testudines; taxonomy; phylogeny; World Wide Web; data sharing; database management; bioinformatics; quality assurance/quality control

With over 100,000,000 websites, the Internet, or World Wide Web, is an astonishing mixture of chaos and insight, data, and misinformation. As a resource it is both ultimately frustrating and rewarding. Here we discuss the Internet as a resource for work on the biology and conservation of turtles, as undertaken by the international community of turtle scientists and conservationists. We do not provide a list of websites that are currently useful; such existing sites are better found with an Internet search engine (such as Google; www.google.com). Rather, we discuss some of the strategic issues facing the turtle research and conservation community by focusing on three of the overarching challenges that must be met in developing Internet resources. We address these challenges by discussing a series of trade-offs that frame the decisions that must be made in the process of improving our Internet resources.

The ideas presented here are largely a result of our experience in building and maintaining the EmySystem (emys.geo.orst.edu) (Fig. 1) that is a web-based extension of Iverson (1992). The goal of the site is to provide a comprehensive account of the taxonomy of all turtle species of the world (with the exception of sea turtles) and, through map-based displays, provide all known locality records from both museums and the literature. The site was constructed from 1998 to 2001 and in its current form dates from late 2001. There are 263 species, 90 genera, 14 families and 37,025 georeferenced (to the nearest minute) locality records. There are 20,768 citations to 2,010 different publications and 57,239 museum specimens from 347 museums. Each taxon page reflects the information given in Iverson (1992) updated through 2001. The site is now considerably out-of-date. We are in the process of updating the taxonomy and locality data. We would like to expand the site to cover new topics such as molecular databases and phylogenies. Our ideas for this expansion are given below. These ideas are the results of the feedback we have received on the site since 2001 and from our interviews with colleagues at the Workshop.

The three challenges are:

- Continuous Content Update
- Quality Assurance/Quality Control (QA/QC)
- Synthesis and Integration

The design trade-offs are:

- Ease of use vs. functionality
- Standardization vs. flexibility
- The turtle community vs. the overall biodiversity research and conservation community
- Centralized vs. distributed content management
- Open source vs. proprietary data and software

We describe a set of potential future developments in terms of some of the problems facing turtle workers. Our thesis is that hard problems in the turtle world are mirrored by hard problems in computer science and that the solutions to turtle problems will require help from computer science. We conclude by discussing the social engineering or human aspects for developing and using web resources. These issues are the genuinely hard problems – much harder than the technical computer science problems. We finish by recommending the creation of an international institution whose job will be to solve the problems we discuss and create truly powerful Internet resources for the international turtle community.

The Challenges

1. Continuous Content Update. — It does not seem to be hard to create a website that many users will visit once. The most important single challenge in website design is to create a site that users will return to again and again. The first law of websites is "Users only come back to websites that are changing." Without good content and new content websites

Figure 1. EmySystem website (emys.geo.orst.edu), showing Home Page and a sample species page (*Phrynops gibbus*; currently classified as *Mesoclemmys gibba*, see Turtle Taxonomy Working Group, 2007b).

become stale and ultimately ignored. All of the tools of website production and design are worthless without real content that is continuously improving.

2. Quality Assurance/Quality Control (QA/QC). — Users of a website must be able to learn why they should trust the content of a site or at least feel that they can evaluate the reliability of a website. We should remember that the goal of QA/QC is not necessarily data of high quality, but rather data of *known* quality. The ongoing discussion about *Wikipedia* (en.wikipedia.org) both on the Internet (Stvilia 2006) and off (Schiff 2006) is a good example of how the issues of QA/QC on the Internet play out. The strength of *Wikipedia* is its ability to harness the knowledge of many thousands of authors. Its weakness is that it is sometimes hard to know how to evaluate what is written. This weakness is magnified when the topic is controversial and writers with different views are competing for authorship. Analogously, there are many different lists of the turtle species of the world besides the EmySystem on the Internet, such as www.chelonia.org/Turtle_Taxonomy.htm and www.flmnh.ufl.edu/herpetology/turtcroclist.But it is often difficult to evaluate them because the bases of the decisions that have had to be made to create such a list are not also given or are not clear. In contrast, the list of terminal taxa given in this volume (Turtle Taxonomy Working Group 2007b) is careful to note the basis of its decisions and to emphasize areas of uncertainty or controversy. This kind of audit trail makes it possible for a reader to grasp the quality of the work and therefore know better how to use it.

3. Synthesis and Integration. — Serving raw data of known quality on the Internet is the starting point for developing web resources. This community curation of data is in itself no easy task. But the further challenge is to provide tools that enable both individual investigators and communities of collaborators to synthesize these data into new knowledge. For individual investigators we must provide tools for data analysis and visualization, or at least make the data amenable to easy export into local statistical and visualization packages. Also, collaborative authoring tools are a necessity. Tools such as *Google Documents* are available and generally well understood. Our final challenge will be to develop tools that enhance our abilities to jointly create syntheses. While these tools are not yet well developed, programs that would permit interactive dynamic presentation of visualizations over the Internet would be a start. A simple example would be a graphics application that lets two Internet users simultaneously markup a given chart or graphic as if they were both using the same piece of paper.

Design Trade-Offs

1. Ease of Use vs. Functionality. —People vary greatly in what they expect of any computer-based system. For example, in the case of statistical or phylogenetic inference packages many prefer software that is easy to use and simply provides "the answer." Others prefer programs with greater functionality that allow them to tune the analysis to their particular needs at the expense of having a steeper learning cure. Some scientists are wary of any black box program whose inner workings they cannot see. As with all of the trade-offs, there is no one correct answer, but web resource design must be conscious of its intended audience and their multiple needs and varying backgrounds.

New Internet technology (generally referred to as Web 2.0) makes possible the construction of websites that have the functionality associated with desktop programs. Use of such new technologies makes it easier to create websites that have greater functionality with greater ease of use. However, the trade-off still remains an issue because user demands develop as well.

2. Standardization vs. Flexibility. — The Internet currently offers a blizzard of standards for the types of data that we wish to share. Some of the standards that might be evaluated in the design of a turtle research and conservation webpage are given in Table 1.

In considering these and many other existing standards, the turtle research community must decide between adopting general standards to begin with, or creating our own standards. Such decisions are not easy because there is a trade-off between standardization and flexibility. Furthermore, many of

the standards listed in Table 1 are works in progress (e.g. PhyloCode) and adopting them would mean working with the relevant sponsoring organizations and this activity, while certainly laudable, could be very time consuming.

As an example of the trade-off between standardization and flexibility, consider the simple question: "Is there a standard list in English of the countries of the world that a turtle may be from? Certainly the International Standards Organization (ISO) provides one: ISO Standard 3166 "English country names and code elements" at www.iso.org. In general, adopting this standard is a good idea. However, if we follow out Parham et al. (2006) we find the complete mitrochondrial genome of *Geochelone pardalis* (GenBank NC_007694) to be based on a specimen collected by T.J. Papenfuss with the GenBank locality of /isolation_source="Somaliland, Awday Region" and we further find that ISO 3166 does not list Somaliland as a country. Indeed we know there is some controversy about the existence of Somaliland as a country separate from Somalia. On the one hand we would like to use the standard, but on the other hand anyone wishing to return to this locality would be well advised to respect the interests of the inhabitants of the country they regard as Somaliland.

The general issue of standardization vs. flexibility is an important research area in current computer science. Almost everyone, not just turtle workers, faces this issue. The emergence of the XML (Extensible Markup Language) family of technologies is due to the ubiquity of this problem. One of the promises of XML is that a much wider variety of data can be managed in a consistent, self-identifying format. Michael Kay (2004, p. 691) puts it this way: "Data that fits neatly into rows and columns, to my mind, isn't interesting enough to be worth studying; and what's more, it's likely that the only reason it fits neatly into rows and columns is that a lot of important information has been thrown away in order to achieve that fit. With XML, we can do better." For example, with XML we can easily identify exceptions to standards as part of the flexibility protocol that would solve the Somaliland problem. We can design data structures that support multiple taxonomies. We can build a tool that will take whatever format any museum decides to use for specimen data (as long as it is more or less internally consistent) and transform it into a common format for viewing and analysis. With XML many more resources will become data in the sense that there is a known structure (metadata) and quality associated with information.

Table 1. Some Internet standards relevant to turtle research and conservation.

URL	Description Provided by the Site
www.tdwg.org	International Working Group on Taxonomic Databases (TDWG)
www.iso.org	English country names and code elements Standard 3166
lsid.sourceforge.net	The Life Sciences Identifier (LSID) is an I3C and OMG Life Sciences Research (LSR) Uniform Resource Name (URN) specification in progress.
www.gbif.org	Facilitating digitization and global dissemination of primary biodiversity data, so that people from all countries can benefit from the use of the information, is the mission of the Global Biodiversity Information Facility (GBIF).
www.itis.gov	ITIS, the Integrated Taxonomic Information System! Here you will find authoritative taxonomic information on plants, animals, fungi, and microbes of North America and the world.
www.ohiou.edu/phylocode	The PhyloCode is a formal set of rules governing phylogenetic nomenclature. It is designed to name the parts of the tree of life by explicit reference to phylogeny.

An important aspect of standardization and flexibility for an international project is the choice of languages that it will support. For now English is the obvious choice, although support for multiple languages is a desirable long-term goal. However, even if English is used, it is important to provide support for citations in other languages. At a minimum this means there must be support for the orthographies and fonts of the world. Such support is now easy using the Unicode standard (www.unicode.org). This standard should be adopted by any international effort.

3. The Turtle Community vs. the Overall Biodiversity Research and Conservation Community. — The Internet needs of the turtle community are not necessarily those of the broader community. For example, the turtle community might be much more open to taxonomic instability and ambiguity resulting from publication of new data than the overall biodiversity research and conservation community. The negative reaction of the general conservation community (Smith and Chiszar 2006) to the recent sweeping revisions to amphibian taxonomy (Frost et al. 2006) illustrates the potentials for conflicts arising from this trade-off. We can also characterize this trade-off as one of stability vs. accuracy. Consider the taxon formerly known as *Clemmys marmorata*. The use of *Clemmys* has been stable for over 100 years while either *Actinemys* or *Emys* is more accurate given our current understanding of phylogeny and the desire to name only monophyletic groups (Turtle Taxonomy Working Group 2007a). The needs of the turtle taxonomy community may be best met by keeping the controversy between *Emys* and *Actimemys* alive; the needs of the "public" users of taxonomy are perhaps best met with a consensus decision which deemphasizes the complexity and uncertainty of the interpretation of phylogenetic data. These considerations lead us to conclude that support for multiple taxonomies is critical to any future website.

In general, biodiversity-oriented websites may be either vertically or horizontally integrated. By vertical integration we mean that the primary focus is on the taxon (turtles) and the goal is to integrate information from many scientific disciplines from molecular biology to ecology. For the turtle

community this means providing "one stop shopping" for turtle data at a single site or linked set of sites. By horizontal integration we mean that the focus is on some scientific topic integrated across many taxa (including turtles). GenBank is an example of a horizontally integrated site. Clearly a vertically integrated site can link to and integrate appropriate horizontally integrated sites.

4. Centralized vs. Distributed Content Management.—A major lesson from our experience with the EmySystem is that the management of museum specimen data and its associated georeferencing information presents severe QA/QC problems. John Iverson spent decades assembling the original data on which the site was based and the amount of effort required to convert them to digital format was enormous. Most of Iverson's effort in the creation of the website went into QA/QC even though we had developed relatively sophisticated data validation routines. At the time we developed the EmySystem (2001) few museums had their data available online. Today many museums do have their data online and it now makes much more sense to leave the content management of these data to the museums that curate the specimens. Presumably, the museums, as professional guardians of the data, are best prepared to manage the QA/QC of the data. Museums will, however, have to undertake georeferencing of their specimens. Under this model a request to the EmySystem would cause it to search all museums that hold turtles for the relevant data, convert it to a standard format and display it. The role of the EmySystem would shift to integration rather than content management.

In contrast to museum data, there does not seem to be a set of institutions that could perform distributed content management for literature citations and their contents. It is more likely that data derived from publications will continue to be managed in a centralized database. Adding georeferencing to such data will continue to be a major task. Many publications do now contain georeferencing (often more accurate than that used in the current EmySystem) and hopefully this trend will continue.

A comprehensive effort to centrally manage turtle literature citations to the publications themselves is being undertaken by the Chelonian Research Foundation (www.chelonian.org) as the *Bibliotheca Testudinum*. A long-term goal of this effort is to make the citations available on the Internet and linked to or part of the EmySystem.

5. Open Source vs. Proprietary Data and Software.—While this trade-off is pervasive throughout all uses of computers, it is the one that for our purposes does not require sophisticated analysis. We simply believe that both data and software should be open-source as much as possible.

Data sharing is the core of our Internet efforts. In 1985, the United States National Research Council (NRC) produced an important report on sharing research data (Fienberg et al. 1985). The many benefits of data sharing are detailed there. The report also surveyed the many contentious issues associated with such sharing and produced a set of recommendations. Of great importance to our concerns is *"Recommendation 3: Data relevant to public policy should be shared as quickly and widely as possible."* Although in the past scientists and institutions may have been able to regard species data as proprietary, we believe that this practice is counterproductive. Because of the current concern for the future of biodiversity, species taxonomy, phylogeny, and locality data are now *ipso facto* policy relevant. Therefore, data should be shared as quickly and widely as possible. The NRC (1997) also produced a more extensive report entitled *Bits of Power: Issues on Global Access to Scientific Data*. This report emphasizes the need for sharing to occur across all national boundaries. The need for international sharing of policy relevant data is the major motivation of our Internet efforts.

Open source software is preferable because it is equally available to all parties regardless of their economic resources. It is also more valuable to the scientific process because the exact nature of the program can be determined. For example, there are phylogenetic inference programs that are open source and those that are proprietary. For the scientist who is interested in the exact details of how a calculation was made, only examination of the source code will give the final answer. Further, open source programs are subject to continuous public peer review and are ultimately less likely to have bugs. One of the best examples of open source software is the statistics and visualization package R (www.r-project.org) that surpasses its proprietary rivals in comprehensiveness and support.

It is worth noting that the papers for this proceedings volume were initially prepared in Microsoft Word, a proprietary program and format that make sharing over the Internet

Table 2. Proposed topics for an upgraded EmySystem.

Taxonomy	Nomenclature, synonymies, PhyloCode, alternative taxonomies
Biogeography	Locality data from museum specimens and literature
Phylogenetics	Trees and their visualization
Vouchering	Classical and contemporary standards; media-based vouchers
Primers	Known primers and their availability
Sequences	Link to GenBank
Gene Expression	Proteins, regulation, networks
Molecular Genetic Tools	Standard references and new approaches
Conservation	*In-situ* and *ex-situ* protection; links to current projects
Literature	Centralized database
Museum Specimens	Distributed database
Turtle Workers	Voluntary form
Projects and Proposals	Document sharing and collaborative authoring
Identification	Keys, photographs
Morphology	Data files, images
Demography	Birth, death, immigration, emigration; sex ratio
Hybridization	Individual cases, species, captive populations

very difficult. Microsoft has announced that it will adopt an open XML format for its files in the near future. This decision will make collaborative work over the Internet much easier.

Proposal

Our response to the challenges and trade-offs discussed above is the following proposal that has three parts:

1. Adopt the general strategy of vertical integration of all relevant turtle data focusing on needs and standards of the international turtle community.

The idea of a vertically integrated website is the heart of our proposal. We believe this approach makes the most sense given the strength of the international turtle community as demonstrated by these proceedings. Further, there is a lack of good functional examples of successful vertical integration for any taxon and the lessons we will learn attempting this strategy with turtles may well be useful for workers in other taxa.

2. Adopt the tactical approach of building on the existing EmySystem by expanding it to include the topics covered in this workshop and volume.

Table 2 presents the types of information that should be included in an expanded EmySystem. This list was compiled from the current information on the website, from experience gained while managing the current website, and based upon ideas generated at the workshop. The topics overlap and interact with each other in complex ways, so a more detailed account of the structure of the website must await further work.

3. Create an institutional base for the EmySystem.

We believe that the challenges reviewed here can best be met by the creation of an institution whose task it is to provide the kind of Internet resources that we envision. Such an institution would provide the basic structure so that the work of individual contributors could best be used. This institution would have elements of both a foundation and a journal. As a foundation it would secure the physical basis of the website and provide mechanisms for fund raising and coordination. As a journal it would function to provide QA/QC for all data by subjecting them to peer review. As both it would provide syntheses of knowledge with explicit regard for the bases of decisions and for alternative views of the results.

The amount of human labor required for the effort we envision is enormous. The quantity and quality of our web resources will depend directly on the number of people involved. The institution and website must be designed to encourage participation and be tolerant of turnover as people's interest and availability change. The social engineering requirements implied by this proposal are surely the most difficult part of its implementation.

The results of the conference presented here form the intellectual and societal structure for such an institution and website. They demonstrate the power of our international community to marshall data, create knowledge through synthesis and integration, and present alternative views.

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An Annotated List of Modern Turtle Terminal Taxa with Comments on Areas of Taxonomic Instability and Recent Change

TURTLE TAXONOMY WORKING GROUP*

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ABSTRACT. – We compiled a list of the named terminal taxa for the world's modern turtle fauna that would summarize recent changes in turtle nomenclature. We provide an annotated list of 465 currently recognized modern terminal taxa (319 species plus 146 additional subspecies) in a hierarchical framework. In order to be as objective as possible we strive to uncritically record the most recent assignment of terminal taxa. For higher-level changes, we show competing schemes equally without endorsing any arrangement. In both cases (terminals and higher taxa) we direct readers to the systematic works that discuss taxonomic revisions. We anticipate that this annotated list will be a useful resource for everyone interested in turtles and their nomenclature. In addition to clarifying some issues or points of confusion, this list should also provide an impetus for future work aimed at clarifying and resolving areas of taxonomic disagreement and/or uncertainty.

KEY WORDS. – Reptilia; Testudines; turtle; tortoise; taxonomy; nomenclature; genera; species; subspecies; synonymization

Turtles, perhaps more than any other reptile group, have been the subject of numerous comprehensive lists (e.g., Wermuth and Mertens, 1977; Iverson, 1992; David, 1994; Iverson et al., 2001a; Joyce et al., 2004; Fritz and Havas, 2006, 2007; also see Pritchard, 1990, and Adler, 2007, for historical reviews). But despite their relatively modest extant diversity, turtle nomenclature is in a constant state of flux and, in places, wrought with differing opinions and directly conflicting arrangements. Consequently, it is impossible to compile a comprehensive list that is not already partially obsolete (or disagreed upon) by the time it is published. We took on the challenge of compiling a list of the named terminal taxa for the world's modern turtle fauna that would summarize some of this recent dynamism in turtle nomenclature. We decided to use Iverson's 1992 checklist of recognized turtle taxa as a starting point, since it was published in hardcopy form and widely disseminated and accepted (e.g., as opposed to starting from the Iverson et al. 2001a web-based checklist), and we agreed on a general format, using a hierarchical, *mostly* rank-free list.

As the title of this work implies, this list attempts to serve two functions. First, it is a list of all currently recognized named terminal taxa for modern turtles. This aspect is meant to be comprehensive as of November 2007 for extant or recently extinct turtle taxa (using the IUCN and CREO criteria of 1500 AD as the cutoff date for recent extinctions, see annotation number 1 below). This comes to a total of 465 modern turtle taxa, comprised of 319 species and 146 additional subspecies (see Table 1). By 'currently recognized' we simply mean those terminals that have not been explicitly refuted or synonymized. In order to be

as objective as possible we accepted the most recent changes relatively uncritically and direct readers to the systematic works that discuss these terminals. However, our one criterion for accepting a proposed change was that it be accompanied by data or at least arguments explaining the taxonomic revision. Consequently, some species lists published with major ad hoc revisions (e.g., from the herpetoculturist and web-based literature) were not incorporated.

The second aspect of this list highlights areas of instability or recent change, especially at the genus level, but also some higher-level categories. In contrast to the terminal list, we do not always accept the most recently proposed changes. Instead, we try to highlight these areas of instability and direct readers to the papers that discuss these controversies, and make no specific recommendations as to which terminology should be used. For a discussion of higher-level phylogenetic relationships, including consensus supertrees and unresolved controversies, see Iverson et al. (2007, this volume) for details.

In addition, we document those taxa that have been described as new or resurrected since Iverson (1992), plus those taxa still recognized as distinct by Iverson (1992) or other subsequent authors, that have subsequently been synonymized under current names for various reasons. In many cases those synonymizations have been well supported by morphologic or genetic analysis, but some have not. These recently synonymized taxa, in addition to the many previously synonymized taxa documented in Iverson (1992) and Fritz and Havas (2006, 2007), represent a wealth of potential diversity at lower levels of distinctiveness (e.g., possible Evolutionarily Significant Units or Management Units) or possibly valid terminal taxa simply in need of more detailed analysis.

Our list is not a complete historical review of all taxonomic changes to turtles, but does aim to be complete for the time since Iverson (1992). Moreover, it should not be taken as our opinion on the validity of any particular name. We fully expect that some of the terminals listed here will be synonymized based on future work while some excluded names will later be considered valid.

The format of this list is an indented hierarchy (by phylogeny) of turtle clades with modern terminal taxa. See Krenz et al. (2005) and Parham et al. (2006a) for the phylogeny used to create this hierarchy. The major levels of the hierarchy are listed phylogenetically with lower levels (equivalent to families, subfamilies, genera, species, and subspecies) listed alphabetically. Terminal taxa (species or subspecies) are in bold. Nominotypical subspecific terminals are implied, but not listed separately. Competing genera are generally listed in the order that they were most recently proposed, so the sequence should not be used to infer preference for any name. Because we sometimes list multiple genera for several terminals we abandon the convention of placing authorities of transferred species names in parentheses. Comments on areas of instability or recent change or synonymizations are indicated by superscript numbers that refer to the annotations below, while terminal taxa that have gone extinct within modern times (since 1500 AD) are indicated by a (†).

We anticipate that this annotated list will be a useful resource for everyone interested in turtles and their nomenclature. In addition to clarifying some issues or points of confusion, this list should also provide an impetus for future work aimed at clarifying and resolving areas of taxonomic disagreement and/or uncertainty.

Table 1. How many modern turtle species are there?
This table, modified and expanded from Adler (2007), records the number of modern turtle species, additional subspecies, and total taxa (species plus subspecies) listed as distinct by various authorities progressively through the years. As we have continued to discover and investigate more of the world's turtle populations, and applied increasingly refined morphologic and genetic characters and criteria for recognizing and documenting chelonian diversity, the number of distinct turtle species and total taxa have grown dramatically. Of the currently recognized turtle taxa, 6 species plus 3 additional subspecies (9 total taxa) have gone extinct since 1500 AD, leaving us currently with 313 living turtle species, 143 additional living subspecies, and 456 living turtle taxa (species and subspecies).

Authority	Species	Additional Subspecies	Total Taxa
Linnaeus, 1758	11	0	11
Linnaeus, 1766	15	0	15
Schneider, 1783-92	20	0	20
Gmelin, 1789	33	0	33
Schoepff, 1792-1801	55	0	55
Daudin, 1801	58	0	58
Schweigger, 1812	78	0	78
Duméril and Bibron, 1835	121	0	121
Fitzinger, 1835	122	0	122
Gray, 1844	136	0	136
Gray, 1856b	154	0	154
Gray, 1873c	209	0	209
Boulenger, 1889	212	0	212
Siebenrock, 1909	232	33	265
Rust et al., 1934	252	45	297
Mertens and Wermuth, 1955	211	121	332
Wermuth and Mertens, 1961	212	112	324
Pritchard, 1967	232	95	327
Wermuth and Mertens, 1977	219	121	340
Pritchard, 1979	237	115	352
Iverson, 1986	246	115	361
Ernst and Barbour, 1989	257	125	382
Iverson, 1992	257	139	396
David, 1994	273	137	410
Fritz and Havas, 2006	313	148	461
Fritz and Havas, 2007	313	147	460
Present checklist	319	146	465

CHECKLIST OF MODERN TURTLE TAXA[1]

Testudines
- Cryptodira
  - Chelydridae
    - *Chelydra*[2]
      - ***acutirostris*** Peters 1862
      - ***rossignonii*** Bocourt 1868
      - ***serpentina*** Linnaeus 1758
        - ***osceola*** Stejneger 1918
    - *Macrochelys* [formerly *Macroclemys*][3]
      - ***temminckii*** Troost 1835
  - Chelonioidea
    - Cheloniidae
      - *Caretta*
        - ***caretta*** Linnaeus 1758
      - *Chelonia*
        - ***mydas*** Linnaeus 1758[4]
      - *Eretmochelys*
        - ***imbricata*** Linnaeus 1766
          - ***bissa*** Rüppell 1835[5]
      - *Lepidochelys*
        - ***kempii*** Garman 1880
        - ***olivacea*** Eschscholtz 1829
      - *Natator*
        - ***depressus*** Garman 1880
    - Dermochelyidae
      - *Dermochelys*
        - ***coriacea*** Vandelli 1761
  - Kinosternoidea
    - Dermatemydidae
      - *Dermatemys*
        - ***mawii*** Gray 1847
    - Kinosternidae
      - Kinosterninae
        - *Kinosternon*
          - ***acutum*** Gray 1831b
          - ***alamosae*** Berry and Legler 1980
          - ***angustipons*** Legler 1965
          - ***arizonense*** Gilmore 1922[6]
          - ***baurii*** Garman 1891
          - ***chimalhuaca*** Berry, Seidel, and Iverson 1996[7]
          - ***creaseri*** Hartweg 1934
          - ***dunni*** Schmidt 1947
          - ***durangoense*** Iverson 1979[6]
          - ***flavescens*** Agassiz 1857[6]
          - ***herrerai*** Stejneger 1925
          - ***hirtipes*** Wagler 1833
            - ***chapalaense*** Iverson 1981
            - ***magdalense*** Iverson 1981
            - ***megacephalum*** Iverson 1981 (†)
            - ***murrayi*** Glass and Hartweg 1951
            - ***tarascense*** Iverson 1981
          - ***integrum*** Le Conte 1854
          - ***leucostomum*** Duméril and Bibron 1851
            - ***postinguinale*** Cope 1887
            - ***oaxacae*** Berry and Iverson 1980
          - ***scorpioides*** Linnaeus 1766[8]
            - ***abaxillare*** Baur 1925
            - ***albogulare*** Duméril and Bocourt 1870
            - ***cruentatum*** Duméril and Bibron 1851
          - ***sonoriense*** Le Conte 1854
            - ***longifemorale*** Iverson 1981
          - ***subrubrum*** Bonnaterre 1789
            - ***hippocrepis*** Gray 1856a
            - ***steindachneri*** Siebenrock 1906b
        - *Sternotherus* [formerly in *Kinosternon*][9]
          - ***carinatus*** Gray 1856a
          - ***depressus*** Tinkle and Webb 1955[10]
          - ***minor*** Agassiz 1857
            - ***peltifer*** Smith and Glass 1947
          - ***odoratus*** Latreille 1801
      - Staurotypinae
        - *Claudius*
          - ***angustatus*** Cope 1865
        - *Staurotypus*
          - ***salvinii*** Gray 1864b
          - ***triporcatus*** Wiegmann 1828
- Testudinoidea
  - Emydidae
    - Deirochelyinae
      - *Chrysemys*
        - ***picta*** Schneider 1783[11]
          - ***bellii*** Gray 1830
          - ***dorsalis*** Agassiz 1857[11]
          - ***marginata*** Agassiz 1857
      - *Deirochelys*
        - ***reticularia*** Latreille 1801
          - ***chrysea*** Schwartz 1956
          - ***miaria*** Schwartz 1956
      - *Graptemys*
        - ***barbouri*** Carr and Marchand 1942
        - ***caglei*** Haynes and McKown 1974
        - ***ernsti*** Lovich and McCoy 1992
        - ***flavimaculata*** Cagle 1954
        - ***geographica*** LeSueur 1817
        - ***gibbonsi*** Lovich and McCoy 1992
        - ***nigrinoda*** Cagle 1954
          - ***delticola*** Folkerts and Mount 1969
        - ***oculifera*** Baur 1890
        - ***ouachitensis*** Cagle 1953
          - ***sabinensis*** Cagle 1953[12]
        - ***pseudogeographica*** Gray 1831b
          - ***kohnii*** Baur 1890
        - ***pulchra*** Baur 1893c
        - ***versa*** Stejneger 1925
      - *Malaclemys*
        - ***terrapin*** Schoepff 1793
          - ***centrata*** Latreille 1801
          - ***littoralis*** Hay 1904
          - ***macrospilota*** Hay 1904

- ***pileata*** Wied 1865
- ***rhizophorarum*** Fowler 1906
- ***tequesta*** Schwartz 1955

Pseudemys
- ***alabamensis*** Baur 1893a
- ***concinna*** Le Conte 1830[13]
  - ***floridana*** Le Conte 1830[14]
  - ***suwanniensis*** Carr 1937[15]
- ***gorzugi*** Ward 1984[16]
- ***nelsoni*** Carr 1938a
- ***peninsularis*** Carr 1938b[17]
- ***rubriventris*** Le Conte 1830
- ***texana*** Baur 1893a

Trachemys[18]
- ***adiutrix*** Vanzolini 1995
- ***callirostris*** Gray 1856b[18]
  - ***chichiriviche*** Pritchard and Trebbau 1984[19]
- ***decorata*** Barbour and Carr 1940
- ***decussata*** Gray 1830
  - ***angusta*** Barbour and Carr 1940
- ***dorbigni*** Duméril and Bibron 1835[18,20]
- ***emolli*** Legler 1990[18]
- ***gaigeae*** Hartweg 1939[18]
  - ***hartwegi*** Legler 1990[19]
- ***nebulosa*** Van Denburgh 1895[18]
  - ***hiltoni*** Carr 1942[19]
- ***ornata*** Gray 1830[18]
- ***scripta*** Schoepff 1792
  - ***elegans*** Wied 1839
  - ***troostii*** Holbrook 1836
- ***stejnegeri*** Schmidt 1928
  - ***malonei*** Barbour and Carr 1938
  - ***vicina*** Barbour and Carr 1940
- ***taylori*** Legler 1960[18]
- ***terrapen*** Bonnaterre 1789
- ***venusta*** Gray 1856b[18]
  - ***cataspila*** Günther 1885[19]
  - ***grayi*** Bocourt 1868[19]
- ***yaquia*** Legler and Webb 1970[18]

Emydinae

Clemmys
- ***guttata*** Schneider 1792

Emys or *Actinemys*
- ***marmorata*** Baird and Girard 1852 [formerly in *Clemmys*][21,22]

Emys or *Emydoidea*[21]
- ***blandingii*** Holbrook 1838

Emys[21]
- ***orbicularis*** Linnaeus 1758
  - ***capolongoi*** Fritz 1995
  - ***colchica*** Fritz 1994
  - ***eiselti*** Fritz, Baran, Budak, and Amthauer 1998
  - ***fritzjuergenobsti*** Fritz 1993
  - ***galloitalica*** Fritz 1995
  - ***hellenica*** Valenciennes 1832
  - ***hispanica*** Fritz, Keller, and Budde 1996
  - ***iberica*** Eichwald 1831[23]
  - ***ingauna*** Jesu, Piombo, Salvidio, Lamagni, Ortale, and Genta 2004
  - ***lanzai*** Fritz 1995
  - ***luteofusca*** Fritz 1989
  - ***occidentalis*** Fritz 1993
  - ***persica*** Eichwald 1831[24]
- ***trinacris*** Fritz, Fattizzo, Guicking, Tripepi, Pennisi, Lenk, Joger, and Wink 2005

Glyptemys [formerly in *Clemmys*][21]
- ***insculpta*** Le Conte 1830
- ***muhlenbergii*** Schoepff 1801

Terrapene
- ***carolina*** Linnaeus 1758
  - ***bauri*** Taylor 1895
  - ***major*** Agassiz 1857
  - ***mexicana*** Gray 1849[25]
  - ***triunguis*** Agassiz 1857
  - ***yucatana*** Boulenger 1895[25]
- ***coahuila*** Schmidt and Owens 1944
- ***nelsoni*** Stejneger 1925
  - ***klauberi*** Bogert 1943
- ***ornata*** Agassiz 1857
  - ***luteola*** Smith and Ramsey 1952

Platysternidae[26]

Platysternon
- ***megacephalum*** Gray 1831c[27]
  - ***peguense*** Gray 1870b
  - ***shiui*** Ernst and McCord 1987

Testudinoidae or Testuguria[28]

Bataguridae or Geoemydidae[29]

Batagur[30]
- ***baska*** Gray 1830[31]
- ***borneoensis*** Schlegel and Müller 1844 [formerly in *Callagur*][30]
- ***dhongoka*** Gray 1832 [formerly in *Kachuga*][30]
- ***kachuga*** Gray 1831a [formerly in *Kachuga*][30]
- ***trivittata*** Duméril and Bibron 1835 [formerly in *Kachuga*][30]

Cuora[32,33]
- ***amboinensis*** Daudin 1801
  - ***couro*** Schweigger 1812
  - ***kamaroma*** Rummler and Fritz 1991
  - ***lineata*** McCord and Philippen 1998
- ***aurocapitata*** Luo and Zong 1988
- ***flavomarginata*** Gray 1863d
  - ***evelynae*** Ernst and Lovich 1990
  - ***sinensis*** Hsü 1930[34]
- ***galbinifrons*** Bourret 1939b[35]
  - ***bourreti*** Obst and Reimann 1994
  - ***picturata*** Lehr, Fritz, and Obst 1998

- ***mccordi*** Ernst 1988
- ***mouhotii*** Gray 1862 [formerly in *Pyxidea*][32]
  - ***obsti*** Fritz, Andreas, and Lehr 1998
- ***pani*** Song 1984
- ***trifasciata*** Bell 1825[36]
- ***yunnanensis*** Boulenger 1906[37]
- ***zhoui*** Zhao 1990

Cyclemys[38]
- ***atripons*** Iverson and McCord 1997
- ***dentata*** Gray 1831b
- ***oldhamii*** Gray 1863d
- ***pulchristriata*** Fritz, Gaulke, and Lehr 1997
- ***shanensis*** Annandale 1918
  - ***tcheponensis*** Bourret 1939a

Geoclemys
- ***hamiltonii*** Gray 1830

Geoemyda[39]
- ***japonica*** Fan 1931
- ***spengleri*** Gmelin 1789

Hardella
- ***thurjii*** Gray 1831b[40]

Heosemys
- ***annandalii*** Boulenger 1903 [formerly in *Hieremys*][41]
- ***depressa*** Anderson 1875
- ***grandis*** Gray 1860b
- ***spinosa*** Gray 1830

Leucocephalon
- ***yuwonoi*** McCord, Iverson, and Boeadi 1995 [formerly in *Geoemyda* or *Heosemys*][42]

Malayemys
- ***macrocephala*** Gray 1859[43]
- ***subtrijuga*** Schlegel and Müller 1844

Mauremys[33,44]
- ***annamensis*** Siebenrock 1903a [formerly in *Annamemys*][44]
- ***caspica*** Gmelin 1774
  - ***siebenrocki*** Wischuf and Fritz 1997
  - ***ventrimaculata*** Wischuf and Fritz 1996
- ***japonica*** Temminck and Schlegel 1835
- ***leprosa*** Schweigger 1812[45]
  - ***saharica*** Schleich 1996
- ***mutica*** Cantor 1842
  - ***kami*** Yasukawa, Ota, and Iverson 1996
- ***nigricans*** Gray 1834 [formerly in *Chinemys*][44]
- ***reevesii*** Gray 1831b [formerly in *Chinemys*][44,46]
- ***rivulata*** Valenciennes 1833
- ***sinensis*** Gray 1834 [formerly in *Ocadia*][44]

Melanochelys
- ***tricarinata*** Blyth 1856
- ***trijuga*** Schweigger 1812
  - ***coronata*** Anderson 1879
  - ***edeniana*** Theobald 1876[47]
  - ***indopeninsularis*** Annandale 1913
  - ***parkeri*** Deraniyagala 1939
  - ***thermalis*** Lesson 1830

Morenia
- ***ocellata*** Duméril and Bibron 1835
- ***petersi*** Anderson 1879

Notochelys
- ***platynota*** Gray 1834

Orlitia
- ***borneensis*** Gray 1873a

Pangshura [formerly in *Kachuga*][48]
- ***smithii*** Gray 1863e
  - ***pallidipes*** Moll 1987
- ***sylhetensis*** Jerdon 1870
- ***tecta*** Gray 1830
- ***tentoria*** Gray 1834[49]
  - ***circumdata*** Mertens 1969
  - ***flaviventer*** Günther 1864[49]

Rhinoclemmys
- ***annulata*** Gray 1860a
- ***areolata*** Duméril and Bibron 1851
- ***diademata*** Mertens 1954
- ***funerea*** Cope 1876
- ***melanosterna*** Gray 1861
- ***nasuta*** Boulenger 1902
- ***pulcherrima*** Gray 1856b
  - ***incisa*** Bocourt 1868
  - ***manni*** Dunn 1930
  - ***rogerbarbouri*** Ernst 1978
- ***punctularia*** Daudin 1801
  - ***flammigera*** Paolillo 1985
- ***rubida*** Cope 1870
  - ***perixantha*** Mosimann and Rabb 1953

Sacalia[33]
- ***bealei*** Gray 1831b
- ***quadriocellata*** Siebenrock 1903a

Siebenrockiella
- ***crassicollis*** Gray 1830
- ***leytensis*** Taylor 1920 [formerly in *Heosemys*][50]

Vijayachelys
- ***silvatica*** Henderson 1912 [formerly in *Geoemyda*][51]

Testudinidae

Aldabrachelys or *Dipsochelys* [formerly in *Geochelone*][52,53]
- ***arnoldi*** Bour 1982[54]
- ***daudinii*** Duméril and Bibron 1835 (†)
- ***dussumieri*** Gray 1831b[55]
- ***hololissa*** Günther 1877

Astrochelys [formerly in *Geochelone*][52]
- ***radiata*** Shaw 1802

Astrochelys or *Angonoka* [formerly in *Geochelone*][52]
- ***yniphora*** Vaillant 1885a[56]

Chelonoidis [formerly in *Geochelone*][52]
- ***carbonaria*** Spix 1824
- ***chilensis*** Gray 1870a[57]
- ***denticulata*** Linnaeus 1766
- ***nigra*** Quoy and Gaimard 1824[58]

abingdonii Günther 1877
becki Rothschild 1901
chathamensis Van Denburgh 1907[59]
darwini Van Denburgh 1907
duncanensis Garman 1917[60]
hoodensis Van Denburgh 1907
(nigra Quoy and Gaimard 1824) (†)[61]
phantastica Van Denburgh 1907 (†)[62]
porteri Rothschild 1903[63]
vicina Günther 1875[64]
petersi Freiberg 1973[57]
Chersina
angulata Schweigger 1812
Cylindraspis
indica Schneider 1783 (†)[65]
inepta Günther 1873 (†)
peltastes Duméril and Bibron 1835 (†)
triserrata Günther 1873 (†)
vosmaeri Suckow 1798 (†)[66]
Geochelone[52]
elegans Schoepff 1795
platynota Blyth 1863
Geochelone or *Centrochelys*[52]
sulcata Miller 1779
Gopherus
agassizii Cooper 1863
berlandieri Agassiz 1857
flavomarginatus Legler 1959
polyphemus Daudin 1801
Homopus[67]
areolatus Thunberg 1787
boulengeri Duerden 1906
femoralis Boulenger 1888a
signatus Gmelin 1789
cafer Daudin 1801
solus Branch 2007[67]
Indotestudo
elongata Blyth 1853
forstenii Schlegel and Müller 1844
travancorica Boulenger 1907[68]
Kinixys
belliana Gray 1830[69]
domerguei Vuillemin 1972
nogueyi Lataste 1886
zombensis Hewitt 1931
erosa Schweigger 1812
homeana Bell 1827
lobatsiana Power 1927
natalensis Hewitt 1935
spekii Gray 1863c
Malacochersus
tornieri Siebenrock 1903b
Manouria
emys Schlegel and Müller 1844
phayrei Blyth 1853
impressa Günther 1882
Psammobates
geometricus Linnaeus 1758
oculifer Kuhl 1820
tentorius Bell 1828
trimeni Boulenger 1886
verroxii Smith 1839
Pyxis
arachnoides Bell 1827[70]
brygooi Vuillemin and Domergue 1972
oblonga Gray 1869
planicauda Grandidier 1867
Stigmochelys or *Psammobates* [formerly in *Geochelone*][52]
pardalis Bell 1828[71]
babcocki Loveridge 1935
Testudo[72]
graeca Linnaeus 1758[73]
armeniaca Chkhikvadze and Bakradze 1991
buxtoni Boulenger 1921
cyrenaica Pieh and Perälä 2002
ibera Pallas 1814
lamberti Pieh and Perälä 2004
marokkensis Pieh and Perälä 2004
nabeulensis Highfield 1990
soussensis Pieh 2001
terrestris Forsskål 1775
zarudnyi Nikolsky 1896
kleinmanni Lortet 1883[74]
marginata Schoepff 1793[75]
Testudo or *Agrionemys*[72]
hermanni Gmelin 1789[76]
boettgeri Mojsisovics 1889
horsfieldii Gray 1844[77]
kazachstanica Chkhikvadze 1988[77]
rustamovi Chkhikvadze, Amiranashvili, and Ataev 1990[77]

Trionychia
Carettochelyidae
Carettochelys
insculpta Ramsay 1886
canni Wells 2002a[78]
Trionychidae
Cyclanorbinae
Cyclanorbis
elegans Gray 1869
senegalensis Duméril and Bibron 1835
Cycloderma
aubryi Duméril 1856
frenatum Peters 1854
Lissemys
punctata Bonnaterre 1789
andersoni Webb 1980
scutata Peters 1868
Trionychinae
Amyda
cartilaginea Boddaert 1770
Apalone

ferox Schneider 1783
mutica LeSueur 1827
calvata Webb 1959
spinifera LeSueur 1827
aspera Agassiz 1857
atra Webb and Legler 1960[79]
emoryi Agassiz 1857
guadalupensis Webb 1962
hartwegi Conant and Goin 1948
pallida Webb 1962
Aspideretes or *Nilssonia*[80]
gangetica Cuvier 1825
hurum Gray 1830
leithii Gray 1872
nigricans Anderson 1875[81]
Chitra
chitra Nutaphand 1986
javanensis McCord and Pritchard 2003
indica Gray 1830
vandijki McCord and Pritchard 2003
Dogania
subplana Geoffroy Saint-Hilaire 1809
Nilssonia[80]
formosa Gray 1869
Palea
steindachneri Siebenrock 1906a
Pelochelys
bibroni Owen 1853
cantorii Gray 1864a
signifera Webb 2003
Pelodiscus[82]
axenaria Zhou, Zhang, and Fang 1991
maackii Brandt 1857
parviformis Tang 1997
sinensis Wiegmann 1835
Rafetus
euphraticus Daudin 1801
swinhoei Gray 1873b[83]
Trionyx
triunguis Forsskål 1775

Pleurodira
Chelidae
Acanthochelys
macrocephala Rhodin, Mittermeier, and McMorris 1984[84]
pallidipectoris Freiberg 1945
radiolata Mikan 1820
spixii Duméril and Bibron 1835
Chelodina[85]
canni McCord and Thomson 2002[86]
gunaleni McCord and Joseph-Ouni 2007
longicollis Shaw 1794
mccordi Rhodin 1994b
roteensis McCord, Joseph-Ouni, and Hagen 2007a[87]
novaeguineae Boulenger 1888b
oblonga Gray 1841[88]
pritchardi Rhodin 1994a
reimanni Philippen and Grossmann 1990
steindachneri Siebenrock 1914[85]
timorensis McCord, Joseph-Ouni, and Hagen 2007b[89]
Chelodina or *Macrochelodina*[85]
burrungandjii Thomson, Kennett, and Georges 2000
expansa Gray 1857
kuchlingi Cann 1997d[90]
parkeri Rhodin and Mittermeier 1976
rugosa Ogilby 1890[91]
Chelus
fimbriata Schneider 1783
Elseya[92]
albagula Thomson, Georges, and Limpus 2006
branderhorsti Ouwens 1914[93]
dentata Gray 1863a
irwini Cann 1997c
jukesi Wells 2007b[94]
lavarackorum White and Archer 1994
novaeguineae Meyer 1874
schultzei Vogt 1911[95]
stirlingi Wells 2007b[96]
Elseya or *Wollumbinia*[92]
bellii Gray 1844[97]
georgesi Cann 1997a
latisternum Gray 1867
purvisi Wells and Wellington 1985
Elusor
macrurus Cann and Legler 1994
Emydura
australis Gray 1841
macquarii Gray 1830[98]
binjing Cann 1998
dharra Cann 1998
dharuk Cann 1998
emmotti Cann, McCord, and Joseph-Ouni 2003
gunabarra Cann 1998
krefftii Gray 1871
nigra McCord, Cann, and Joseph-Ouni 2003
signata Ahl 1932
subglobosa Krefft 1876
worrelli Wells and Wellington 1985[99]
tanybaraga Cann 1997b
victoriae Gray 1842
Hydromedusa
maximiliani Mikan 1825
tectifera Cope 1870
Phrynops[100]
geoffroanus Schweigger 1812
hilarii Duméril and Bibron 1835
tuberosus Peters 1870
williamsi Rhodin and Mittermeier 1983

Batrachemys or *Mesoclemmys*[100]
dahli Zangerl and Medem 1958
heliostemma McCord, Joseph-Ouni, and Lamar 2001[101]
nasuta Schweigger 1812
raniceps Gray 1856b
tuberculata Luederwaldt 1926
zuliae Pritchard and Trebbau 1984
Mesoclemmys[100]
gibba Schweigger 1812
perplexa Bour and Zaher 2005
Mesoclemmys or *Bufocephala*[100]
vanderhaegei Bour 1973
Mesoclemmys or *Ranacephala*[100]
hogei Mertens 1967
Phrynops or *Rhinemys*[100]
rufipes Spix 1824
Platemys
platycephala Schneider 1792
melanonota Ernst 1984
Pseudemydura
umbrina Siebenrock 1901
Rheodytes
leukops Legler and Cann 1980

Pelomedusidae
Pelomedusa
subrufa Bonnaterre 1789[102]
Pelusios
adansonii Schweigger 1812
bechuanicus FitzSimons 1932
broadleyi Bour 1986
carinatus Laurent 1956
castaneus Schweigger 1812
castanoides Hewitt 1931
intergularis Bour 1983
chapini Laurent 1965
cupulatta Bour and Maran 2003
gabonensis Duméril 1856
marani Bour 2000
nanus Laurent 1956
niger Duméril and Bibron 1835
rhodesianus Hewitt 1927
seychellensis Siebenrock 1906c (†)[103]
sinuatus Smith 1838
subniger Bonnaterre 1789
parietalis Bour 1983
upembae Broadley 1981
williamsi Laurent 1965
laurenti Bour 1984
lutescens Laurent 1965

Podocnemididae or Podocnemidae[104]
Erymnochelys
madagascariensis Grandidier 1867
Peltocephalus
dumerilianus Schweigger 1812
Podocnemis
erythrocephala Spix 1824
expansa Schweigger 1812
lewyana Duméril 1852
sextuberculata Cornalia 1849
unifilis Troschel 1848[105]
vogli Müller 1935

ANNOTATIONS

1. Both IUCN (The World Conservation Union, http://www.iucnredlist.org) and CREO (Committee on Recently Extinct Organisms, http://creo.amnh.org) have designated 1500 AD as their official cutoff date for determining what constitutes a recently extinct species, and we follow their criteria in our checklist.
2. *Chelydra*: Phillips et al. (1996) elevated *acutirostris* and *rossignoni* to full species status and retained the subspecies *osceola*. See Shaffer et al. (in press) for a complete review.
3. *Macrochelys* [formerly *Macroclemys*]: Although *Macroclemys* has been the most commonly used name, Webb (1995) showed that *Macrochelys* is the oldest available name.
4. *Chelonia mydas*: Bowen et al. (1992) showed that recognition of the taxon *agassizii* Bocourt 1868 renders *mydas* paraphyletic, and *agassizii* is no longer generally recognized as either a distinct species or subspecies. See Parham and Zug (1996) and Karl and Bowen (1999) for a complete review.
5. *Eretmochelys imbricata*: Fritz and Havas (2006, 2007) did not list *bissa* as a valid taxon, but no argumentation for this opinion was given. Genetic data (Okayama et al., 1999) have suggested significant separation of Atlantic from Pacific stocks.
6. *Kinosternon* species: Serb et al. (2001) elevated two former subspecies of *flavescens* (*arizonense* and *durangoense*) to full species status.
7. *Kinosternon chimalhuaca*: This new species name appeared prematurely and erroneously first in the hobbyist literature, with the full original description published a few months later (Berry et al., 1996, 1997).
8. *Kinosternon scorpioides scorpioides*: Includes the previously recognized subspecies *seriei* Freiberg 1936 and *carajasensis* Cunha 1970 in synonymy (Cabrera and Colantonio, 1997).
9. *Sternotherus*: This genus was included as a junior synonym of *Kinosternon* by Iverson (1992) and David (1994) based on work by Seidel et al. (1986) and Iverson (1991). However, this view was never widely accepted, and Iverson (1998) showed that the species referred to either *Sternotherus* or *Kinosternon* formed reciprocally monophyletic clades and recommended that both genera be used.

10. *Sternotherus depressus*: Whereas some earlier authors had placed this taxon as a subspecies of *minor*, Walker et al. (1998) showed that *depressus* was genetically distinct from *minor*.
11. *Chrysemys picta dorsalis*: This subspecies of *Chrysemys picta* was elevated to full species status by Starkey et al. (2003), who recognized two distinct genetic lineages: *C. dorsalis* and *C. picta*. They did not find genetic support for the other subspecies of *C. picta* (*belli*, *marginata*) but did not recommend that they be abandoned. Fritz and Havas (2006, 2007) argued that full specific status of *dorsalis* was not fully demonstrated and retained it and the other two taxa as subspecies of *C. picta*, agreeing also with Ernst et al. (2006).
12. *Graptemys ouachitensis sabinensis*: Based on molecular and morphologic data, Stephens and Wiens (2003) suggested that *sabinensis* may not be closely related to *ouachitensis*. However, statistical support for this was weak, and they did not discuss or recommend a taxonomic change. Further study of this complex may warrant the elevation of the sympatric taxon *sabinensis* to full species status.
13. *Pseudemys concinna concinna*: Includes the previously recognized subspecies *hieroglyphica* Holbrook 1836, *mobilensis* Holbrook 1838, and *metteri* Ward 1984 in synonymy (Seidel, 1994).
14. *Pseudemys concinna floridana*: This taxon was previously considered a separate species, but was designated a subspecies of *concinna* by Seidel (1994). Jackson (1995) argued for the retention of *floridana* as a full species, but Seidel (1995) rejected this argument.
15. *Pseudemys concinna suwanniensis*: Previously considered a subspecies of *concinna*, Seidel (1994) argued for the elevation of this taxon to full species status, but Jackson (1995) argued for its subspecific status.
16. *Pseudemys gorzugi*: This taxon was previously considered a subspecies of *concinna*, but was elevated to species status by Ernst (1990) without argumentation, but then supported through analysis by Seidel (1994).
17. *Pseudemys peninsularis*: This taxon was previously considered a subspecies of *floridana*, but was elevated to species status by Seidel (1994). Jackson (1995) argued for the retention of *peninsularis* as a subspecies of *floridana*, but Seidel (1995) reaffirmed his recognition.
18. *Trachemys* species: Seidel (2002) recommended elevating nine Mesoamerican taxa, previously recognized as subspecies of *Trachemys scripta*, to species rank.
19. *Trachemys* subspecies: Seidel (2002) also recommended reassigning five taxa, previously subspecies of *scripta*, to subspecies of his various elevated *Trachemys* species.
20. *Trachemys dorbigni*: Includes the previously recognized subspecies *brasiliensis* Freiberg 1969 in synonymy, based on morphologic work (del Barco and Larriera, 1993).
21. *Emydoidea* and the turtles formerly known as *Clemmys*: The four traditional species of *Clemmys* (*guttata* [type], *insculpta*, *muhlenbergii*, and *marmorata*) do not form a monophyletic group with respect to the two monotypic genera *Emys orbicularis* and *Emydoidea blandingii* in phylogenies based on DNA data (Bickham et al., 1996; Burke et al., 1996; Lenk et al. 1999; Feldman and Parham, 2002). While there is a general agreement that *insculpta* and *muhlenbergii* are sister-species and should be placed in the genus *Glyptemys* (Holman and Fritz, 2001; Parham and Feldman, 2002), there are two schemes presented for *marmorata* and *blandingii*. Holman and Fritz (2001) recommended that *marmorata* be placed in the monotypic genus *Actinemys*, retaining both *Emys orbicularis* and *Emydoidea blandingii* as additional monotypic genera. Other authors (Bickham et al., 1996; Feldman and Parham, 2002; Parham and Feldman, 2002) recommended that *marmorata* and *blandingii* be placed into an expanded *Emys*, a scheme favored in the most recent analysis of variation in *marmorata* (Spinks and Shaffer, 2005).
22. *Emys* or *Actinemys marmorata*: Previously, two subspecies were distinguished, including *pallida* Seeliger 1945, but genetic analysis by Spinks and Shaffer (2005) demonstrated that the typical and previously recognized subspecies *pallida* were within the same phylogeographic clade and so *pallida* should not be considered valid.
23. *Emys orbicularis iberica*: Includes the recently described subspecies *kurae* Fritz 1994 in synonymy (Fritz, 1998).
24. *Emys orbicularis persica*: Includes the recently described subspecies *orientalis* Fritz 1994 in synonymy (Fritz, 1998).
25. Mexican *Terrapene carolina*: Stephens and Wiens (2003) suggested that Mexican subspecies of *T. carolina* may warrant full species status. While this convention has also been adopted previously (Smith et al., 1996), almost all other workers recognize these as subspecies.
26. Platysternidae: Krenz et al. (2005) confirmed that nuDNA placed *Platysternon* solidly within the Testudinoidea, and Parham et al. (2006a) supported this finding with mtDNA.
27. *Platysternon megacephalum*: Ernst and Laemmerzahl (2002) synonymized two subspecies of *megacephalum* (*vogeli* Wermuth 1969 and *tristernalis* Schleich and Gruber 1984) with the nominate subspecies.
28. Testudinoidae or Testuguria: Shaffer et al. (1997) coined the name 'Testudinoidae' for the clade that united Testudinidae with Bataguridae/Geoemydidae. Joyce et al. (2004) listed Testudinoidae as an undesirable derivative of *Testudo* being to similar to both 'Testudinidae' and 'Testudinoidea.' In that same paper, the authors coined the new clade name 'Testuguria' for that same clade (while neglecting to list Testudinoidae as an objective senior synonym). Parham et al. (2006a) explicitly argued for the use of Testuguria over Testudinoidae.
29. Bataguridae or Geoemydidae: Both names are being used to refer to this group of predominantly Asian testudinoids. McDowell (1964) used the name Batagurinae for this group (as a subfamily) which was

changed to Bataguridae (as a family) by Gaffney and Meylan (1988). Bour and Dubois (1986) showed that Geoemydidae has priority, and David (1994), Spinks et al. (2004) and others have embraced this view. However, this approach was questioned by Joyce et al. (2004) who, working in a rank-free phylogenetic taxonomy framework, recommended the continued use of Bataguridae. In the interest of reconciling phylogenetic nomenclature with traditional Linnaean rules of priority, Parham et al. (2006a) endorsed a phylogenetic codification of Geoemydidae.

30. *Batagur*: Praschag et al. (2007b) and Le et al. (2007) demonstrated that species of *Kachuga* were genetically paraphyletic with respect to those referred to *Batagur* and *Callagur* and recommended that only one genus be recognized, and the name *Batagur* has priority.
31. *Batagur baska*: The subspecies *ranongensis* Nutaphand 1979 is not well differentiated and has been synonymized under *baska* by Fritz and Havas (2006, 2007), but no specific morphologic or genetic analysis has yet been performed to formally evaluate the status of this taxon.
32. *Cuora*: Phylogenies based on DNA data (Honda et al., 2002a; Stuart and Parham, 2004; Parham et al., 2004; Spinks et al., 2004) have shown that continued recognition of the genus *Pyxidea* for *mouhotii* would render *Cuora* paraphyletic. All of these studies recommended expanding *Cuora* to include *mouhotii*. Other schemes for *Cuora* have not been published in the recent scientific literature, though there has been some use of *Cistoclemmys* for *flavomarginata* and *galbinifrons* (e.g., Zhao et al., 1997; Zhao, 1997; Yasukawa and Ota, 1999).
33. Hybrid species: The validity of six taxa of *Cuora*, *Mauremys* [including *Ocadia*], and *Sacalia* recently described from pet trade specimens has been refuted by genetic studies that have shown them to be based on hybrids (Parham et al., 2001; Wink et al., 2001; Spinks et al., 2004; Stuart and Parham, 2004, 2007). The taxa shown to be hybrids are: *Cuora galbinifrons serrata* Iverson and McCord 1992b, *Mauremys iversoni* Pritchard and McCord 1991, *Mauremys pritchardi* McCord 1997, *Ocadia glyphistoma* McCord and Iverson 1994, *Ocadia philippeni* McCord and Iverson 1992, and *Sacalia pseudocellata* Iverson and McCord 1992a.
34. *Cuora flavomarginata sinensis*: Some authors recognize this taxon as a valid subspecies (McCord and Iverson, 1991; Fong et al., 2002) while others synonymize it with *flavomarginata* (Yasukawa and Ota, 1999; Fritz and Havas, 2006, 2007).
35. *Cuora galbinifrons*: The taxa *bourreti* and *picturata*, originally described as subspecies of *Cuora galbinifrons*, were elevated to species rank by Stuart and Parham (2004) based on concordance of morphological with molecular differentiation. Fritz et al. (2006c) returned *bourreti* to subspecies rank based on osteological characters shown by market specimens, and suggested that *picturata* warrants the same ranking; Fritz and Havas (2006, 2007) subsequently listed *picturata* at subspecies rank based on morphologically intermediate pet trade specimens. Includes the previously recognized *hainanensis* Li 1958 in synonymy (Zong and Pan, 1989; Iverson and McCord, 1992b).
36. *Cuora trifasciata*: Blanck et al. (2006) recommended that *Cuora trifasciata* be split into two species (including their newly named species *cyclornata* and its new subspecies *meieri*) based on paraphyletic mtDNA haplotypes and morphological differences. Spinks and Shaffer (2007) showed that *trifasciata* as traditionally recognized is monophyletic based on nuDNA and therefore recommended that *cyclornata* should not be recognized, pending additional study.
37. *Cuora yunnanensis*: This species has been listed as extinct by the IUCN since 2000 (www.iucnredlist.org), based on several decades of not finding any surviving animals despite intensive searches. Recently, a pair of animals representing this species were found in markets (Zhou and Zhao, 2004; Zhou, 2005), with subsequent confirmation through genetic analysis (He et al., 2007).
38. *Cyclemys*: Iverson (1992) recognized two taxa of *Cyclemys* (*dentata* and *tcheponensis*). Later, *atripons* and *pulchristriata* were described and *oldhamii* was resurrected (Iverson and McCord, 1997; Fritz et al., 1997). Genetic analysis by Guicking et al. (2002) also supported the validity of *shanensis*.
39. *Geoemyda*: Yasukawa et al. (1992) elevated *japonica* to species status (previously considered a subspecies of *spengleri*).
40. *Hardella thurjii*: Praschag et al. (2007b) found no genetic or morphologic evidence for continued recognition of the subspecies *indi* Gray 1870b, and synonymized it under *thurjii*.
41. *Heosemys annandalii* [formerly in *Hieremys*]: Spinks et al. (2004) showed that *annandalii* was nested among species of *Heosemys*. Diesmos et al. (2005) formally moved *annandalii* into *Heosemys*.
42. *Leucocephalon yuwonoi* [formerly in *Geoemyda* or *Heosemys*]: Originally described as a species of *Geoemyda* (McCord et al., 1995), Fritz and Obst (1996) placed *yuwonoi* in *Heosemys*. McCord et al. (2000) showed that *yuwonoi* was not closely related to the type species of *Geoemyda* or *Heosemys*, but instead sister to *Notochelys platynota*, and erected a new genus, *Leucocephalon*, for *yuwonoi*.
43. *Malayemys macrocephala*: Brophy (2004) proposed the recognition of this species as distinct from *subtrijuga* based on morphological grounds.
44. *Mauremys* [including species formerly in *Annamemys*, *Chinemys*, or *Ocadia*]: Iverson and McCord (1994) included *annamensis* under an expanded *Mauremys*. Subsequent phylogenies based on DNA data (Honda et al., 2002b; Barth et al., 2004; Feldman and Parham, 2004; Spinks et al., 2004) showed that the genera *Ocadia* and *Chinemys* rendered *Mauremys* paraphyletic. Based on these results, some authors (Feldman and

Parham, 2004; Spinks et al., 2004) recommended synonymizing *Ocadia* and *Chinemys* under *Mauremys*. Barth et al. (2004) presented this same scheme as well as one that would retain *Chinemys* and *Ocadia* and further divide *Mauremys* into the genera *Cathaiemys* and *Emmenia*. Barth et al. (2004) did not favor one scheme over the other and a competing scheme for *Mauremys* has not been formally proposed in the scientific literature.

45. *Mauremys leprosa*: Fritz et al. (2006a) explicitly synonymized several subspecies of *leprosa* recently described by Schleich (1996) (*atlantica, erhardi, marokkensis, wernerkaestlei*, and *zizi*) plus *vanmeerhaeghei* Bour and Maran 1998, and only recognized *leprosa* and *saharica*.
46. *Mauremys reevesii*: Iverson et al. (1989) and Barth et al. (2003, 2004) refuted the validity of the terminal taxon *megalocephala* Fang 1934, but it has continued to be recognized by Chinese researchers (Guo et al., 1997; Zhao, 1997; Zhang et al., 1998), and Fritz and Havas (2006, 2007) listed it as a separate taxon with speculation about its relationships.
47. *Melanochelys trijuga edeniana*: The subspecies *wiroti* Reimann 1979 was recognized by Iverson (1992), but David (1994) suggested that it was synonymous with *edeniana*, and Fritz and Havas (2006, 2007) followed this arrangement.
48. *Pangshura* [formerly in *Kachuga*]: Das (2001) and Schleich and Kästle (2002) used the name *Pangshura* to refer to small-bodied *Kachuga*. A phylogeny based on DNA data (Spinks et al., 2004) showed that *Kachuga* was paraphyletic and so removed *flaviventer*, *smithii*, *sylhetensis*, *tecta*, and *tentoria* into the genus *Pangshura*. Praschag et al. (2007b) using mtDNA confirmed the well-supported monophyly of *Pangshura*.
49. *Pangshura tentoria flaviventer*: Schleich and Kästle (2002) elevated *flaviventer* to full species status based on sympatry with *circumdata*, but Praschag et al. (2007b) performed a phylogeographic analysis and retained *flaviventer* as a subspecies of *tentoria*.
50. *Siebenrockiella leytensis* [formerly in *Heosemys*]: Diesmos et al. (2005) placed *leytensis* into the genus *Siebenrockiella* based on strong genetic evidence for its sister relationship to *S. crassicollis*.
51. *Vijayachelys silvatica* [formerly in *Geoemyda*]: This species was originally named as a species of *Geoemyda*. However, a molecular study by Praschag et al. (2006) suggested a distant relationship with that genus and they recommended that it be placed in the new monotypic genus *Vijayachelys*.
52. The *Geochelone* complex: This generic complex includes the genera *Geochelone*, *Aldabrachelys*, *Astrochelys*, *Angonoka*, *Centrochelys*, *Chelonoidis*, *Dipsochelys*, and *Stigmochelys*. Lapparent de Broin (2000b), Gerlach (2001, 2004), Le et al. (2006), and Fritz and Bininda-Emonds (2007) recommended dividing the *Geochelone* complex into several genera, although their schemes differ somewhat. A general consensus on a generic-level revision for some members of the group is lacking while in other areas (e.g., *Astrochelys radiata*, *Chelonoidis*) there is agreement.
53. *Aldabrachelys* or *Dipsochelys*: Bour (1982) originally recommended that Aldabran tortoises (*dussumieri* or *gigantea*) be placed in the genus *Dipsochelys* instead of *Aldabrachelys*. However, *Aldabrachelys* is still widely used, including sometimes by Bour (Austin et al., 2003), though *Dipsochelys* is favored by others (Palkovacs et al., 2002, 2003; Gerlach, 2004). There is recent disagreement regarding the type specimen of *Testudo gigantea*, the type species of *Aldabrachelys*, that was presumed lost. Frazier (2006) designated a neotype for *T. gigantea*, an act that would seemingly validate the use of both *Aldabrachelys* and the terminal taxon *gigantea*. Around the same time, Bour (2006) rediscovered the original lost type specimen, which is actually an individual of the South American tortoise *Chelonoidis denticulata*. If this claim is correct, then the names *Aldabrachelys* or *gigantea* might not be applicable to Aldabran tortoises. Whether Frazier's neotype designation or Bour's specimen rediscovery prevails nomenclaturally remains a matter of ongoing debate, but since Bour (2006) was the most recently published authority we use the name *dussumieri* rather than *gigantea* in our list.
54. *Aldabrachelys* or *Dipsochelys* species: Gerlach and Canning (1998) recognized six species of tortoises in Aldabra, Madagascar, and the Seychelles (three of which were extinct: *abrupta*, *daudinii*, and *grandidieri*). The two species from Madagascar became extinct prior to modern times (*abrupta* Grandidier 1868 in ca. 1250 AD and *grandidieri* Vaillant 1885b in ca. 950 AD) so we do not include them in our list of modern taxa. Palkovacs et al. (2002, 2003) questioned the validity of multiple extant species based on their analysis of genetic data, recognizing only a single living taxon (*Dipsochelys dussumieri*). Gerlach and Bour (2003) reemphasized the validity of the extant species based on the observation that the hatchlings are diagnostic. Fritz and Havas (2006, 2007) recognized only one extant species of Indian Ocean giant tortoise which they referred to *Aldabrachelys gigantea*, but did not address the findings of Gerlach and Bour (2003) or Bour (2006). As we consider the issues surrounding the validity of these species as remaining unresolved, we list all these species as potentially valid.
55. *Aldabrachelys* or *Dipsochelys dussumieri*: Iverson (1992) listed this species as *Geochelone gigantea* Schweigger 1812. Many authors now use *dussumieri* for the Aldabra tortoise (see above), but others persist in using the older name *gigantea* (e.g., Fritz and Havas, 2006, 2007), and others have used the name *elephantina* Duméril and Bibron 1835 (David, 1994; Devaux, 2007).
56. *Astrochelys* or *Angonoka yniphora*: Le et al. (2006) named *Angonoka* for *yniphora* because of its uncertain phylogenetic position. Fritz and Bininda-Emonds (2007) recovered a weak sister relationship between *yniphora*

and *Astrochelys radiata* under some algorithms and recommended that *yniphora* be placed in *Astrochelys*.

57. *Chelonoidis petersi*: According to Cabrera (1998), citing morphologic and osteologic work by Fernández (1988), *Chelonoidis chilensis* should be divided into two species, *chilensis* and *petersi* Freiberg 1973, but he considered the taxon *donosobarrosi* Freiberg 1973 to be synonymous with *chilensis*. Fritz and Havas (2006, 2007) speculated that *petersi* may not be valid and synonymized it under *chilensis*, citing phenotypic plasticity in other tortoise species as a reason for not accepting the reported differences between *petersi* and *chilensis*.
58. *Chelonoidis nigra*: Most recent authors have considered the various taxa of Galapagos tortoises as subspecies of *nigra* (e.g., Pritchard, 1996; Caccone et al., 1999; Fritz and Havas, 2006, 2007), but Caccone et al. (2002) and Russello et al. (2005, 2007) treated them as distinct species. The nomenclatural and survival status of these taxa were discussed in detail by Pritchard (1996).
59. *Chelonoidis nigra chathamensis*: This taxon described from western Chatham Island (San Cristóbal) appears to have been extirpated from its original range, but a population of tortoises persists on eastern Chatham Island that was considered a possible separate subspecies by Pritchard (1996). Pending genetic analysis and resolution of this issue we continue to list *chathamensis* as the extant taxon from Chatham, whereas Fritz and Havas (2006, 2007) listed it as extinct, but made no mention of the extant population.
60. *Chelonoidis nigra duncanensis*: This taxon from Duncan Island (Pinzón) was historically usually referred to *ephippium* Günther 1875, but Pritchard (1996) demonstrated that *ephippium* was a synonym of *abingdonii* and therefore resurrected the old nomen nudum *duncanensis* Garman 1917.
61. The nominotypical subspecies *nigra* from Charles Island (Santa Maria or Floreana) is considered to be extinct and is therefore included separately on this list.
62. *Chelonoidis nigra phantastica*: This taxon was listed by Fritz and Havas (2006, 2007) as extant, but Pritchard (1996) considered it probably extinct.
63. *Chelonoidis nigra porteri*: This taxon from Indefatigable Island (Santa Cruz) has often been referred to *nigrita* Duméril and Bibron 1835, but most recent authors, including Pritchard (1996) and Fritz and Havas (2006, 2007) have used *porteri*.
64. *Chelonoidis nigra vicina*: This widespread taxon from Albemarle Island (Isabela) was previously recognized as one of several valid taxa on that island, including *becki* Rothschild 1901, *microphyes* Günther 1875, *guentheri* Baur 1889, and *vandenburghi* De Sola 1930. Pritchard (1996) synonymized *microphyes*, *guentheri*, and *vandenburghi* under *vicina*, and recognized only *vicina* and *becki* from Albemarle.
65. *Cylindraspis indica*: Includes the recently described *borbonica* Bour 1978 in synonymy, based on genetic work by Austin and Arnold (2001).
66. *Cylindraspis vosmaeri*: Fritz and Havas (2006) credited Fitzinger 1826 with authorship of this name, but corrected it to Suckow 1798 in their 2007 checklist.
67. *Homopus*: A separate taxon of *Homopus* was referred to *H. bergeri* Lindholm 1906 by Branch (1989). However, that name was a junior synonym of *Psammobates tentorius verroxii* Smith 1839 (Branch, 1992; Boycott and Bourquin, 2000), and the new taxon was recently described as *H. solus* by Branch (2007).
68. *Indotestudo travancorica*: This taxon was previously considered a subspecies of *forstenii* (Hoogmoed and Crumly, 1984; Iverson, 1992), but was resurrected to species status by Pritchard (2000) based on morphology, a conclusion supported by mtDNA analysis by Iverson et al. (2001c).
69. *Kinixys belliana*: Fritz and Havas (2006, 2007) recognized only *belliana* and *nogueyi*, following Broadley (1993) uncritically, but others (Iverson, 1992; David, 1994; Iverson et al., 2001a) also recognized *domerguei* and *zombensis*. As the phylogeography of this broadly distributed species complex has not been analyzed, we list the four most widely recognized subspecies.
70. *Pyxis arachnoides*: The three recognized subspecies have recently been confirmed as genetically distinct lineages (Chiari et al., 2005).
71. *Stigmochelys* or *Psammobates pardalis*: Based on genetic analysis, Le et al. (2006) recommended that this taxon be included in an expanded genus *Psammobates*. Fritz and Bininda-Emonds (2007) argued for the retention of a monophyletic *Psammobates* exclusive of *pardalis*. Le at al. (2006) also found a high level of mitochondrial divergence between two specimens assigned to the two subspecies *pardalis* and *babcocki*. In conjunction with morphological distinctions between these two taxa (Loveridge and Williams, 1957; Broadley, 1989), the preliminary genetic data suggest that they may be different at the species level.
72. *Testudo* or *Agrionemys*: The species *horsfieldii* and *hermanni* have been alternatively placed in the genera *Testudo* or *Agrionemys* (Khosatzky and Mlynarski, 1966; Gmira 1993, 1995) and *hermanni* also recently in *Eurotestudo*. Lapparent de Broin (2000a,b) and Parham et al. (2006b) supported the placement of *horsfieldii* in the genus *Agrionemys*, but suggested that a new genus name was needed for *hermanni*. Later Lapparent de Broin et al. (2006) created the name *Eurotestudo* for *hermanni*, but Fritz and Bininda-Emonds (2007) demonstrated that older genus names (*Chersine* and *Medaestia*) are available for that species. Fritz and Bininda-Emonds (2007) recovered a weakly monophyletic clade that included *horsfieldii*, *hermanni*, and the three core species of *Testudo* (*graeca*, *kleinmanni*, and *marginata*). Based on this phylogeny they recommended that all of these species be placed in the genus *Testudo*. The genetic support for some nodes within this clade is not strong and the decision to lump or split is subjective (e.g., whether *Agrionemys* should be used for

horsfieldii is open to debate), therefore the taxonomy of this group may remain in flux for some time.

73. *Testudo graeca*: This species complex has been the subject of massive taxonomic revisions at the species and subspecies level. These revisions have resulted in the naming and elevation of numerous taxa (e.g., Perälä, 2002a,b,c). Several studies (van der Kuyl et al., 2002, 2005; Harris et al., 2003; Carretero et al., 2005; Parham et al., 2006b,c; Fritz et al., 2007) have explicitly refuted the validity of many of these taxonomic acts. Fritz et al. (2007) proposed a taxonomic scheme that recognized five mitochondrial clades in the eastern part of the range of *T. graeca* as subspecies, but did not address the status of several North African subspecies. Since this is the most recent taxonomic suggestion, it is listed here. However, in their recent checklist, Fritz and Havas (2006, 2007) included not only the eleven taxa we list, but also *anamurensis* Weissinger 1987, *antakyensis* Perälä 1996, *floweri* Bodenheimer 1935, *nikolskii* Chkhikvadze and Tuniyev 1986, *pallasi* Chkhikvadze and Bakradze 2002, and *perses* Perälä 2002c. The relationships within this species complex remain uncertain and we expect its taxonomy to continue fluctuating.
74. *Testudo kleinmanni*: Baha el Din (2006), Siroky and Fritz (2007), and Attum et al. (2007) explicitly refuted the validity of *werneri* Perälä 2001 as a species distinct from *kleinmanni*.
75. *Testudo marginata*: Fritz et al. (2005b) explicitly refuted the validity of *weissingeri* Bour 1996 as a subspecies of *marginata*.
76. *Testudo hermanni*: Fritz et al. (2006b) explicitly refuted the validity of *hercegovinensis* Werner 1899 (previously resurrected by Perälä, 2002b) and recommended that *boettgeri* be considered a subspecies of *hermanni*.
77. *Testudo horsfieldii*: In a conference proceedings, Perälä (2002a) elevated two subspecies of *horsfieldii* (*kazachstanica* and *rustamovi*) to full species status. This was accepted by Lapparent de Broin et al. (2006), but warrants reconsideration, especially considering the evidence for unjustified taxonomic inflation in related tortoises in the same work (van der Kuyl et al., 2002, 2005; Fritz et al., 2005b, 2006b; Parham et al., 2006b,c).
78. *Carettochelys insculpta canni*: This subspecies from northern Australia described by Wells (2002a) was only weakly defined as different from the nominotypical subspecies from New Guinea. We list it tentatively pending further analysis, as did Fritz and Havas (2006), although they excluded it from their 2007 checklist.
79. *Apalone spinifera atra*: This taxon has usually been designated a subspecies of *spinifera* (usually with the original spelling *ater*), but others (e.g., Flores-Villela, 1993; David, 1994) have listed it as a full species, though usually without specific argumentation.
80. *Aspideretes* or *Nilssonia*: Engstrom et al. (2004) found *Aspideretes* to be paraphyletic with respect to *Nilssonia formosa* based on morphologic and genetic criteria. Praschag et al. (2007a) formally synonymized *Aspideretes* into an expanded concept of *Nilssonia* based on their analysis of mtDNA of all five included taxa.
81. *Aspideretes* or *Nilssonia nigricans*: Recent morphologic and genetic work on this species previously known only from a single captive population has demonstrated that it also occurs in the wild (Praschag and Gemel, 2002; Praschag et al., 2007a).
82. *Pelodiscus*: The genus has recently been recognized as including up to four separate species by some authorities (David, 1994; Zhao, 1997; Chen et al., 2005, 2006; Fritz and Havas, 2006, 2007). Relationships within the genus are far from resolved and also complicated by translocation and mixing of huge numbers of farm-raised individuals from many parts of the range.
83. *Rafetus swinhoei*: Includes the recently described *Pelochelys taihuensis* Zhang 1984 (Farkas, 1992) and *Rafetus leloii* Duc 2000 in synonymy (Farkas and Webb, 2003).
84. *Acanthochelys macrocephala*: Includes the recently described *Phrynops chacoensis* Fritz and Pauler 1992 in synonymy (Fritz and Pauler, 1999).
85. *Chelodina*: This genus was split into three genera by Wells and Wellington (1985), using *Chelodina* for the narrower-headed shorter-necked species (*longicollis*, *novaeguineae*), and establishing *Macrochelodina* for the broader-headed longer-necked species (*oblonga*, *expansa*, *rugosa*, *siebenrocki*), and *Hesperochelodina* for *steindachneri*. Iverson et al. (2001b) refuted the availability of the name *Hesperochelodina*, but validated *Macrochelodina*. Georges et al. (2002) retained *Chelodina* for the entire genus, but identified three phylogenetic clades within the genus and recommended recognition of three subgenera (but did not name them). Fritz and Havas (2006, 2007) accepted two of these clades (*Chelodina* and *Macrochelodina*) as separate genera.
86. *Chelodina canni*: This taxon is the same as the previously described *rankini* Wells and Wellington 1985, but that name was declared invalid as a *nomen nudum* by Iverson et al. (2001b). Wells (2007a) recently disputed this interpretation and redescribed *rankini*, but *canni* McCord and Thomson 2002 retains nomenclatural precedence and *rankini* Wells 2007a is therefore a junior synonym of *canni*.
87. *Chelodina mccordi roteensis*: This recently named subspecies described in the hobbyist literature needs genetic confirmation of its distinctiveness, but we recognize it pending further analysis.
88. *Chelodina oblonga*: Thomson (2000) showed that the holotype of *oblonga* Gray 1841 is a specimen of what is currently regarded as *Chelodina rugosa* Ogilby 1890. An application is before the International Commission for Zoological Nomenclature (ICZN) to conserve current usage of the name *C. rugosa* Ogilby 1890 for the northern snake-necked turtle and to apply the earlier available name *Chelodina collei* Gray 1856a to the long-necked species of southwestern Australia, while retaining the nomenclatural availability of the name

oblonga for potential future designation of distinct populations of *rugosa* (Thomson, 2006). Though no decision has yet been rendered by the ICZN, Fritz and Havas (2006, 2007) used the name *colliei* for this southwestern population. Georges et al. (2002) found support that this taxon represents a third subgenus under *Chelodina*, but did not formally establish it under a generic-level name.

89. *Chelodina timorensis*: This species recently described in the hobbyist literature by McCord et al. (2007b) was also described a few months later as a new subspecies of *mccordi* (‘*timorlestensis*’) by Kuchling et al. (2007), but the McCord et al. description has chronologic precedence. Concerns surrounding the history and methodology of the description of *timorensis* by McCord et al. are discussed by Kuchling et al. (2007) and serve to emphasize our recommendations (made in our other chapter in this volume) to follow certain procedural guidelines for descriptions of new taxa (Turtle Taxonomy Working Group, 2007).
90. *Chelodina kuchlingi*: This species was described from a single specimen, leading to doubts about its validity (Georges and Thomson, 2006; Fritz and Havas, 2006, 2007), but it remains listed pending further exploration of its remote area of provenance.
91. *Chelodina rugosa*: The species *siebenrocki* Werner 1901 was considered valid by Rhodin and Mittermeier (1976) and Rhodin and Genorupa (2000), but synonymized under *rugosa* by Georges et al. (2002) based on weakly differentiated allozymes within the broader *rugosa* complex.
92. *Elseya*: This genus has been recognized as consisting of two separate lineages (Georges and Rose, 1996; Georges and Thomson, 2006). It was subsequently split into two genera, *Elseya* and *Wollumbinia*, by Wells (2007c), with *latisternum* designated genotype of *Wollumbinia.* Papers by Wells (2002a,b; 2007a,b,c) and Wells and Wellington (1985) have been self-published without any peer review and also highlight our recommendations to follow certain procedural guidelines for descriptions of new taxa (Turtle Taxonomy Working Group, 2007).
93. *Elseya branderhorsti*: This species was considered valid by Rhodin and Genorupa (2000), Thomson et al. (2006), and Georges and Thomson (2006).
94. *Elseya jukesi*: The name *jukesi* Wells 2002b was a *nomen nudum* since no type specimen was designated, but the species was recently redescribed by Wells (2007b).
95. *Elseya schultzei*: This species was listed by Thomson et al. (2006) and Georges and Thomson (2006), but neither morphologic nor genetic data have been analyzed from the type population and its status remains unclear.
96. *Elseya stirlingi*: The previously named taxon *stirlingi* Wells and Wellington 1985 was declared invalid as a *nomen nudum* by Iverson et al. (2001b) (though spelled erroneously as *sterlingi*), but was recently redescribed as a valid species by Wells (2007b).
97. *Elseya* or *Wollumbinia bellii*: The taxon *dorriani* Wells 2002b is a *nomen nudum* without a type designation, but was recently considered a valid subspecies of *bellii* by Wells (2007c).
98. *Emydura macquarii*: The taxonomy of *E. macquarii* was previously reviewed by Georges and Adams (1996). Later, Cann et al. (2003) and McCord et al. (2003) described two new subspecies, but taxa previously described by Cann in 1998 (*binjing*, *dharra*, *dharuk*, and *gunabarra)*, plus *signata* Ahl 1932 were not specifically evaluated by those authors. However, these taxa were all recognized as subspecies of *macquarii* by Fritz and Havas (2006, 2007), and since phylogeographic variation in the *macquarii* species complex has not yet been fully resolved with adequate genetic work, we tentatively list all these subspecies as valid, pending further analysis.
99. *Emydura subglobosa worrelli*: Originally described as *Tropicochelymys worrelli*, this taxon was synonymized under *Emydura victoriae* Gray 1842 by Iverson (1992) and the nomenclatural validity of the species name confirmed by Iverson et al. (2001b). Cann (1998) considered it a distinct species, but Georges and Thomson (2006), partially based on electrophoretic work by Georges and Rose (1996), concluded that it was best referred to as a subspecies of *subglobosa* Krefft 1876. Fritz and Havas (2006, 2007) also listed it as a subspecies of *subglobosa*, but Georges et al. (2006) referred to it as a species, though without providing data or argument.
100. *Phrynops*: Wermuth and Mertens (1977) divided this genus into three subgenera: *Phrynops*, *Batrachemys*, and *Mesoclemmys*. Cabrera (1998) and Georges et al. (1998) elevated these subgenera to generic level. McCord et al. (2001) further divided the remaining monophyletic *Phrynops* into a total of four genera (*Bufocephala*, *Phrynops*, *Ranacephala*, and *Rhinemys*). Joyce et al. (2004) did not accept the taxonomic acts of McCord et al. (2001). Bour and Zaher (2005) synonymized *Bufocephala* and *Ranacephala* with *Mesoclemmys*, but recognized *Rhinemys* as distinct.
101. *Mesoclemmys heliostemma*: Rueda-Almonacid et al. (2007) questioned the validity of this taxon which is completely sympatric with *raniceps*, suggesting that it may simply represent a juvenile color morph of that taxon, and recommended genetic analysis.
102. *Pelomedusa subrufa*: Gasperetti et al. (1993) recommended that the two previously recognized subspecies (*nigra* Gray 1863b and *olivacea* Schweigger 1812) be abandoned.
103. *Pelusios seychellensis*: The taxonomic status of this species is unclear. Gerlach and Canning (2001) concluded that it is extinct.
104. Podocnemididae or Podocnemidae: Cope (1868) used the name Podocnemididae to refer to this clade. Baur (1893b) later referred to this group as Podocnemidae.

Joyce et al. (2004) phylogenetically defined Baur's name (Podocnemidae) to refer to this clade.

105. *Podocnemis unifilis*: This long-recognized species was briefly referred to as *P. cayennensis* Schweigger 1812 by David (1994), but that name was previously often used for what is now recognized as *P. erythrocephala* (Mittermeier and Wilson, 1974), and most authors have continued to use *unifilis*.

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TURTLE POETRY

Defining Turtle Diversity: A Light-Hearted Poetic View

Turtle Origins

JOSEPH W. GASTINGER[1]

One thought the turtle anapsid
But now perhaps they're diapsid
It's confusing to me
That old turtle tree
What the twigs and branches and sap did.

Turtle Names

ANDERS G.J. RHODIN[2]

Some have called you Chelonia
or Chelonii, an Order of Reptilia
some have known you as Testudinata
or the long-forgotten Cataphracta

But best you be Testudines
the name used by Linnaeus
the plural of the type Testudo
defines the group with ease.

[1] Composed April 1999, submitted by Martin A. Larson.
Written as a personal poetic reflection on the evolutionary question of turtle origins as published in Rieppel, O. 1999. Turtle origins. Science 283:945-946.

[2] Composed April 2001, revised November 2007.
Written as a personal poetic reflection on the nomenclatural question of what scientific name to use for the monophyletic group defining all turtles and tortoises.

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