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# MOLECULAR SYSTEMATICS, PHYLOGEOGRAPHY, AND THE EFFECTS OF PLEISTOCENE GLACIATION IN THE PAINTED TURTLE (*CHRYSEMYS PICTA*) COMPLEX

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*Abstract.*—The painted turtle, *Chrysemys picta*, is currently recognized as a continentally distributed polytypic species, ranging across North America from southern Canada to extreme northern Mexico. We analyzed variation in the rapidly evolving mitochondrial control region (CR) in 241 turtles from 117 localities across this range to examine whether the painted turtle represents a continentally distributed species based on molecular analysis. We found strong support for the novel hypothesis that *C. p. dorsalis* is the sister group to all remaining *Chrysemys*, with the remaining *Chrysemys* falling into a single, extremely wide-ranging and genetically undifferentiated species. Given our goal of an evolutionarily accurate taxonomy, we propose that two evolutionary lineages be recognized as species within *Chrysemys*: *C. dorsalis* (Agassiz 1857) in the southern Mississippi drainage region, and *C. picta* (Schneider 1783) from the rest of the range of the genus. Neither molecular nor recent morphological analyses argue for the hybrid origin of *C. p. marginata* as previously proposed. Within *C. picta*, we find evidence of at least two independent range expansions into previously glaciated regions of North America, one into New England and the other into the upper Midwest. We further find evidence of a massive extinction/recolonization event across the Great Plains/Rocky Mountain region encompassing over half the continental United States. The timing and extent of this colonization is consistent with a recently proposed regional aridification as the Laurentide ice sheets receded approximately 14,000 years ago, and we tentatively propose this paleoclimatological event as a major factor shaping genetic variation in *Chrysemys*.

*Key words.*—Control region, genealogical species, mitochondrial gene tree, North American phylogeography, polytypic species.

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The debate regarding the levels of intraspecific or interspecific variation required to recognize species and subspecies boundaries has received considerable attention in the last several decades (Cracraft 1983; Ball and Avise 1992). Decisions regarding species and subspecies boundaries can, and do, have dramatic impacts on species conservation and management (Shaffer et al. 2000). In theory and practice, nearly all populations of a species exhibit genetic differentiation to some degree (Avise 1994). Thus, the criteria used to justify the recognition of a species, subspecies, or any other rank-based category often depend on the biological attributes of the taxa in question and one's overall species concept.

Many recent studies that have addressed the boundary between inter- and intraspecific variation have used molecular data, particularly to test existing hypotheses of species-level relationships and boundaries. Although difficult to accomplish, recent detailed examinations of molecular variation within taxa with continental distributions offer a particularly exciting opportunity to address the reality of polytypic species and gain insights into the process by which speciation occurs. A striking feature of these studies is the extensive molecular diversity that is often present, but not currently recognized taxonomically. For example, recent work on species boundaries and the monophyly of the *Ambystoma tigrinum* complex (Collins et al. 1980; Shaffer 1984a,b, 1993; Shaffer and McKnight 1996; Irschick and Shaffer 1997) suggests that the previously recognized polytypic species *A. tigrinum* is composed of distinct clades within the United States encompassing the Great Plains/Rocky Mountains, eastern, and western United States, and that each of these lineages should be recognized as a distinct, monophyletic entity. At the same time, several previously recognized subspecies from the Great Plains and Rocky Mountains are genetically identical and morphologically overlapping, implying that they probably should not be recognized at any taxonomic level. Similar work on the snake *Pituophis melanoleucus* indicates that the traditional view of this single polytypic species (Smith and Kennedy 1951; Conant 1956; but see Wright and

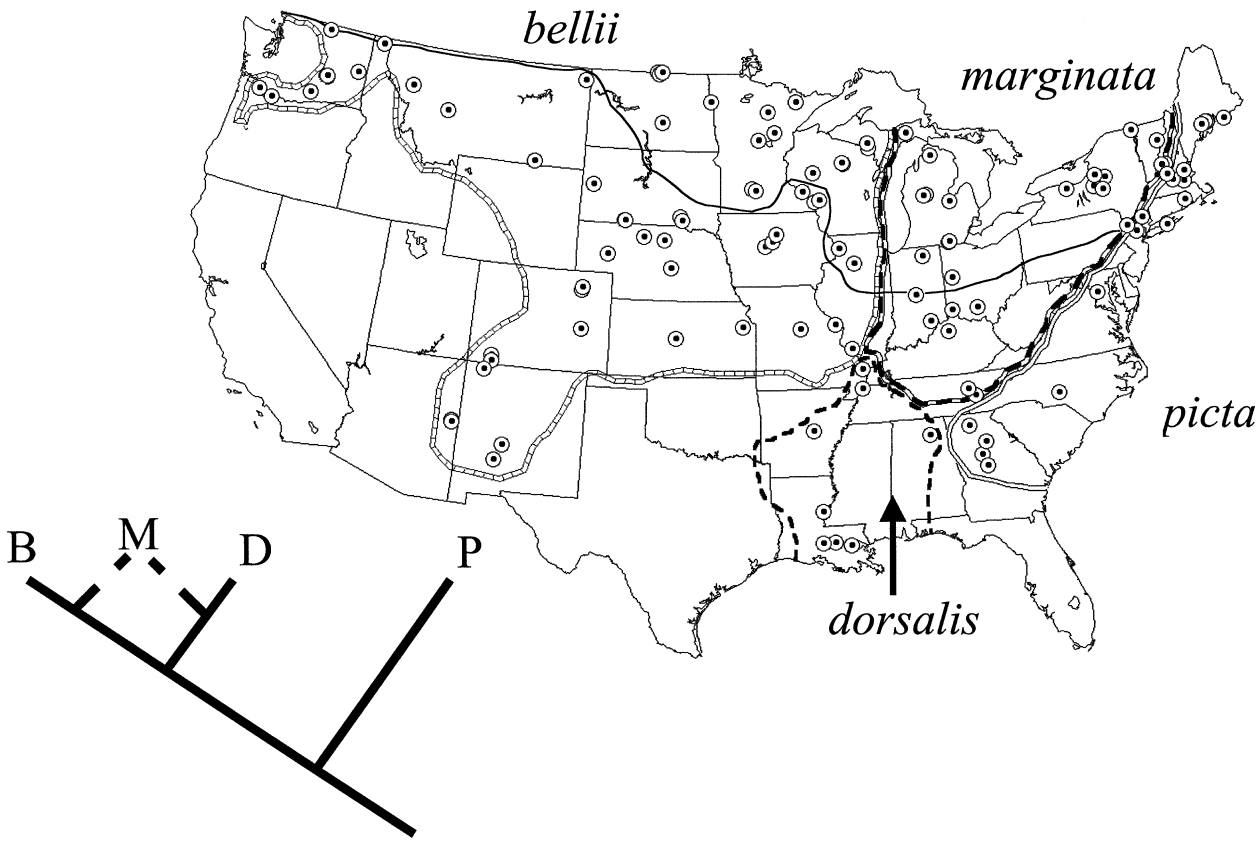


FIG. 1. The ranges of the four currently recognized subspecies of *Chrysemys*. The narrow black line running from east to west indicates the approximate extent of the Wisconsinan glaciation. All localities used in the present study are indicated by ⊙. Our interpretation of the hypothesis of Bleakney is presented as a cladogram.

Wright 1957) is inconsistent with the molecular evidence, and that recognizing three distinct species within the complex more accurately reflects the evolutionary history of the group (Rodriguez-Robles and De Jesus-Escobar 2000).

Painted turtles (Testudines: Emydidae: *Chrysemys picta*) offer another excellent opportunity to explore the question of species boundaries within a widespread species complex. The genus *Chrysemys* as currently recognized contains a single extant species, *C. picta*. Four subspecies are generally recognized: *Chrysemys picta bellii*, *C. p. dorsalis*, *C. p. marginata*, and *C. p. picta* (Bishop and Schmidt 1931). *Chrysemys picta* has the largest geographic distribution of any turtle species in North America. It is distributed coast-to-coast across the northern United States, into southern Canada, and as far south as the United States Gulf Coast, with geographically isolated populations in river systems of Arizona, Colorado, New Mexico, and northern Chihuahua, Mexico (Conant and Collins 1991; Iverson 1992). There is an extensive fossil record for *Chrysemys* throughout the United States, with Miocene remains in Nebraska (Holman 1976; Holman and Sullivan 1981), Pliocene remains in Kansas (Wilson 1968), and material identified as *C. picta* from throughout the Pleistocene in areas as diverse as Michigan (Wilson 1967), Maryland (Holman and Grady 1989), Alabama (Holman et al. 1990), and Oklahoma (Preston 1979). These fossils indicate that *Chrysemys* was widely distributed over North America for at least two, and possibly more than five million years, and that the morphospecies *C. picta* inhabited much of its current range for much of that time.

Because much of the current range includes recently glaciated regions of the United States (Fig. 1), Bleakney (1958) considered a novel biogeographic hypothesis that simultaneously accounted for both the evolution of the subspecies of *C. picta* and post-Pleistocene invasions into previously glaciated areas of North America. According to this hypothesis, the evolution of the four subspecies of *C. picta* occurred over two time periods, yielding taxa of different phylogenetic ages and content. Bleakney postulated that two pre-Pleistocene forms, which were different in both size and color pattern, inhabited the Atlantic coastal plain (subspecies *picta* stock) and areas west of the Appalachian Mountains (non-*picta* stock). Although the details are not clear, Bleakney proposed that stocks for present-day *bellii* and *dorsalis* were isolated west of the Appalachians as a consequence of the Wisconsinan glacial advances approximately 20,000 years ago, and that the more ancient *picta* stock remained isolated east of the Appalachians. As the glaciers receded, each of these now distinct groups began a northward migration. At some undetermined point, but no earlier than 20,000 years ago, the *bellii* and the *dorsalis* stocks came into contact and hybridized to form an intermediate taxon, *C. p. marginata* (Bleakney 1958). This hybrid taxon colonized areas west of the Appalachian Mountains, as well as moving northeast. Finally, the *marginata* and the *picta* stocks came into contact

and formed the hybrid swarm that is hypothesized to characterize northern New England at present. Although Bleakney (1958) presented his hypothesis as an evolutionary scenario, we translated it into the phylogenetic hypothesis in Figure 1. Other than Bleakney, the only alternative view on (sub)speciation in the painted turtles is a brief hypothesis (Bishop and Schmidt 1931) suggesting that *picta* is an eastern and *bellii* a western derivative of *marginata*, with *dorsalis* derived from (i.e., sister to) *picta*.

We have two primary goals in this study. First, we use mtDNA sequence data to investigate the systematic relationships within *Chrysemys*, with an emphasis on potential species/subspecies boundaries. Our primary objective is to ask whether the concept of a single, continentally distributed species of painted turtle reflects the evolutionary history of this wide-ranging, variable taxon. Second, we test elements of the hypothesis proposed by Bleakney (and to a lesser extent, Bishop and Schmidt) regarding postglacial expansion of *Chrysemys* throughout the United States. We use the rapidly evolving control region (CR) of the mitochondrial genome, because it has proven reliable in resolving intraspecific variation in many vertebrates including turtles (Stewart and Baker 1994; Encalada et al. 1996; Shaffer and McKnight 1996; Holder et al. 1999), accurately identifies closely related emydid turtle species (Lamb et al. 1994), and is more variable than the protein coding ND4 gene in *Chrysemys* (Starkey and Shaffer, unpubl. data).

## Materials and Methods

### *Sampling Strategy*

We sampled extensively within the ranges of each subspecies of *C. picta*. Our sampling strategy involved multiple transects totaling 33 states, and 117 localities in the United States. These transects were constructed to (1) cross glacial boundaries as they are currently understood across most of North America, (2) cross subspecific contact zones among all recognized subspecies, and (3) sample geographically across all subspecies, including the western isolated populations in Arizona, New Mexico, and southwestern Colorado. Our sampling did not include individuals from southern Canada or northern Mexico; otherwise it constitutes relatively complete continental coverage (Fig. 1). From our collection of tissues, we randomly selected 1–8 individuals from each locality for sequence analysis, resulting in more than 200 animals sampled. We took a 5–10-mm long snip from the tail of each turtle, stored it in liquid nitrogen (occasionally in 95% ethanol) and immediately released all specimens at the site of capture. Representative individuals were photographed as identification vouchers, and all samples are catalogued and housed in the H.B. Shaffer tissue collection at the University of California, Davis (see Electronic Appendix, currently available from the *Evolution* Editorial Office at evolution@asu.edu).

### *Outgroups*

The choice of the appropriate outgroup(s) to use in rooting a phylogenetic analysis has received considerable attention in the last two decades (Watrous and Wheeler 1981; Farris 1982; Maddison et al. 1984; Smith 1994). We used multiple outgroups in a two-tiered strategy aimed at minimizing long-branch/homoplasy issues (Maddison et al. 1984; Smith 1994; Halanych and Robinson 1999; reviewed in Sanderson and Shaffer 2002). There is both morphological and molecular evidence suggesting that the family Emydidae is composed of two monophyletic subfamilies: the Emydinae and the Deirochelyinae (Gaffney and Meylan 1988; Seidel and Adkins 1989; Burke et al. 1996; Shaffer et al. 1997). In our initial analyses we sequenced *Terrapene* and *Emydoidea* (Emydinae) as well as representatives of all deirochelyine genera, to establish the monophyly of *Chrysemys* within the Deirochelyinae. Based on these initial analyses we used *Graptemys*, *Trachemys*, and *Pseudemys* as multiple outgroups that are closest to *Chrysemys* and therefore most appropriate to root the *Chrysemys* tree (Sanderson and Shaffer 2002). Locality data and specimen numbers for all outgroups are provided in the Electronic Appendix.

### *Mitochondrial DNA*

We amplified and sequenced a 720-basepair (bp) fragment of mtDNA from the 5′ end of the CR using the primers DES-1 (5′-GCA TTC ATC TAT TTT CCG TTA GCA-3′) and DES-2 (5′-GGA TTT AGG GGT TTG ACG AGA AT-3′), corresponding to positions 15,876–16,585 in the *Chrysemys picta* full mitochondrial sequence (Mindell et al. 1999). DNA extraction, purification and polymerase chain reaction (PCR) followed standard protocols (Maniatis et al. 1982). Polymerase chain reaction conditions were a ''hot start'' at 94°C for 3 min followed by 35 cycles of denaturing at 94°C for 30 sec, primer annealing at 55°C for 60 sec, primer extension at 72°C for 2 min, and a final extension at 72°C for 10 min. All taxa were sequenced directly from purified PCR products, using primers DES-1 and DES-2 on either an ABI 377 or 3100 automated sequencer (Applied Biosystems, Foster City, CA) at the University of California Davis Division of Biological Sciences sequencing facility.

### *Phylogenetic Analyses*

Sequences were aligned with Clustal X (Thompson et al. 1997), and alignments were verified by eye and analyzed in PAUP* (Swofford 1998). Analyses were performed using neighbor joining (NJ), maximum parsimony (MP), and maximum likelihood (ML). All base positions were treated as unordered, equally weighted characters; gaps (which were rare) were treated as missing data or deleted if the alignment was ambiguous. For MP analyses, we conducted heuristic searches with 10 random addition sequence replicates, accelerated character transformation (ACCTRAN), branch swapping using tree bisection-reconnection (TBR), and the save-all-trees option (MULPARS). Neighbor-joining trees (Saitou and Nei 1987) were constructed using the Kimura 2-parameter distance correction (Kimura 1980). Nonparametric bootstrap probabilities (BP) based on 1000 or 100 replicates were used to determine relative support for internal nodes in NJ and MP analyses, and we summarized multiple most-parsimonious trees as majority rule consensus trees. We used Modeltest 3.0 (Posada and Crandall 1998) to identify the optimal model for ML analysis. As a starting tree, we identified the one topology among all most-parsimonious trees

with the highest likelihood score using the SH test (Shimodaira and Hasegawa 1999), estimated model parameters, then re-searched tree space to better ensure that our result was a global rather than a local optimum (Swofford 1998). We continued with this strategy until we identified successive trees with the same –*ln*L score and used this as our final result.

### *Saturation Curves and Hypothesis Testing*

Given that the control region is primarily noncoding (e.g., Brown 1983), saturation effects were only examined for transitions (ti) and transversions (tv). Alignments were unambiguous and required only a few small indels. To test for effects of saturation, estimates of uncorrected p-distances (pairwise sequence divergences) were plotted against total uncorrected p-distances for each class of substitution. Linear relationships were expected if the class of substitution in question was not saturated.

Multiple phylogenetic hypotheses are often assessed for differences in statistical support using the Templeton (Templeton 1983) and K-H (Kishino and Hasegawa 1989) tests implemented in PAUP* (Swofford 1998). However, recent studies have noted that many applications of these tests violate fundamental assumptions by comparing a priori (that is, previously stated) and a posteriori (the best tree derived from a dataset) hypotheses of relationship (Goldman et al. 2000; Buckley et al. 2001). If only a priori hypotheses are compared, then the assumptions of the tests are not violated (Buckley et al. 2001); if not, Shimodaira and Hasegawa (1999) proposed a new test (the S-H test, now implemented in PAUP*) that allows for the comparison of multiple a posteriori hypotheses by adjusting the differences in log likelihoods.

Because Bleakney (1958) proposed a scenario rather than a single phylogenetic hypothesis, it is difficult to characterize his hypothesis as a single phylogenetic tree. The key testable elements of Bleakney's hypothesis are: (1) the first split was between painted turtles from the eastern United States (*C. p. picta*) and all others; (2) the second, much more recent split was between western (*C. p. bellii*) and south-central (*C. p. dorsalis*) turtles; and (3) *C. p. marginata* in the central United States constitute a hybrid mixture of *bellii* and *dorsalis*, whereas painted turtles in northern New England are a hybrid swarm of *marginata* and *picta*. We constructed three sets of tests to evaluate whether our data were consistent with Bleakney's contraction-expansion hypothesis. First, we used constraint trees in which each of the four recognized subspecies was constrained to be sister to the remaining three, with no phylogenetic resolution among the three. For each of these four possibilities, we included one tree in which all subspecies were constrained to be reciprocally monophyletic, and one in which monophyly was not imposed on any subspecies. Bleakney proposed that the tree with *C. picta* sister to the remaining taxa is correct, and that this initial split was relatively ancient. Second, we repeated this analysis without *C. p. marginata*, since Bleakney proposed that they are of hybrid origin. This set of six trees more clearly examines Bleakney's proposition that *picta* is the sister group to *bellii* and *dorsalis* without the complicating influence of potential hybridization in *marginata*. Finally, we constructed the same set of six possible trees, but only using the southernmost populations of *picta*, *dorsalis*, and *bellii*, because these may most closely resemble the actual refugial populations proposed by Bleakney. Although no single mtDNA topology can unambiguously support or refute a hybrid origin of *marginata*, under sexually symmetrical hybridization our best tree should show no unique haplotypes for *marginata* turtles, but rather a complex intermixing of *bellii*, *dorsalis*, and *picta* haplotypes, depending on the geographic origin of the samples. Although far less completely stated, the hypothesis of Bishop and Schmidt (1931) suggests that the first *Chrysemys* split was between *marginata* and the rest, followed by *bellii*, and finally a most-recent *picta-dorsalis* divergence.

Because we exhaustively tested all possible topologies among the four or three relevant taxa, we did not violate the primary assumptions of the K-H approach, and we report these results here. We also conducted S-H tests, and in all cases found identical patterns of significance.

## RESULTS

### *Sequence Variation*

We amplified a total of 720 bp of CR mtDNA, of which 673 bp remained after removal of primer sequences. Heuristic analyses identified 173 variable characters, of which 133 were parsimony-informative in the total dataset (including both Emydinae and Deirochelyinae outgroups), 122 variable sites (60 parsimony-informative) in the deirochelyine dataset (*Chrysemys*, *Pseudemys*, *Trachemys*, and *Graptemys*), and 36 variable sites (26 parsimony-informative) within *Chrysemys*. We analyzed painted turtles from 117 localities, which contained 51 unique CR mtDNA haplotypes. The Electronic Appendix lists the location of each site (more detailed localities available from H. B. Shaffer), the individuals sequenced, and which mtDNA haplotypes were found in each sample. In general, localities that are in areas of contact between subspecies (for example, Horseshoe Lake, Illinois) had multiple haplotypes from multiple clades, whereas sites well away from presumed hybrid zones (for example, the Sandhills of western Nebraska) contained one or two very similar haplotypes.

Pairwise distance comparisons among nonidentical *Chrysemys* sequences populations ranged from 0.1 to 2.4% (uncorrected p-values, Table 1). The greatest pairwise distances among recognized subspecies were between *C. p. dorsalis* and all remaining subspecies, which ranged from 1.5 to 2.4%. Pairwise comparisons between the other three subspecies were much lower, ranging from 0.1 to 1.8% divergence. Within-subspecies divergences were also lower, ranging from a low of 0.15–0.45% in *dorsalis* up to 1.5% among a few *marginata* samples (Table 1). Based on visual inspection of saturation curves, neither transitions nor transversions appeared saturated, as would be expected from the low levels of observed sequence divergence. Consistent with most vertebrate mitochondrial genomes examined (Zhang and Hewitt 1996), we found a strong strand bias against guanine (base composition: A = 0.3132, C = 0.2021, T = 0.3449, and G = 0.1398). We always amplified a single band and our sequenc-

TABLE 1. Pairwise uncorrected p-distance comparisons among all unique populations of *Chrysemys picta* based on control region sequences. Names correspond to the recognized subspecies of *Chrysemys.*

| | *bellii* | *picta* | *dorsalis* | *marginata* |
|---|---|---|---|---|
| *bellii* | 0.151–1.368% | | | |
| *picta* | 0.453–1.511% | 0.151–1.208% | | |
| *dorsalis* | 1.512–2.419% | 1.511–2.268% | 0.151–0.454% | |
| *marginata* | 0.151–1.813% | 0.151–1.360% | 1.514–2.270% | 0.151–1.511% |

es align well with, and are very similar to, the full mitochondrial sequence for *Chrysemys* (Mindell et al. 1999).

### *Phylogenetic Analyses*

Each of the phylogenetic methods recovered the same set of major nodes, although NJ and ML resolve additional (and virtually identical) branches that are unresolved with MP. Maximum parsimony heuristic searches, using *Graptemys*, *Trachemys*, and *Pseudemys* as outgroups with all characters weighted equally, recovered 3225 trees of 194 steps, with a consistency index (CI) of 0.716. A MP strict consensus tree grouped the 51 variable *C. picta* haplotypes into a monophyletic group sister to *Pseudemys concinna* (100% BP), regardless of whether emydine or deirochelyine outgroups were used (MP tree not shown, all BPs >50% for the MP analysis shown on Fig. 2). The initial split within *C. picta* identifies two clades. The first includes all samples currently recognized as *C. p. dorsalis*, including a few individuals from areas of potential intergradation at the northern margin of *dorsalis*'s range (BP 99%). The second, which is much less strongly supported (BP = 61%), includes all remaining subspecies (*bellii*, *marginata*, and *picta*). Neighbor-joining analysis (Fig. 2) recovered the same well-supported nodes as MP regardless of outgroup rooting, with similar BPs (Fig. 2). Modeltest identified the HKY'85 I + $\Gamma$ model (Hasegawa et al. 1985) as optimal, with $\Gamma = 0.7617$, I = 0.5844, and a Ti:Tv ratio of 4.1462. The resulting ML phylogram (not shown) was nearly identical to the NJ topology for both well- and poorly supported nodes.

Across analyses we found consistent groupings of four geographically contiguous sets of haplotypes (clades 1–4 on Figs. 2, 3) although clades 2–4 had low statistical support. Individual HBS 28620 from Colorado floated in position across analyses, but individuals from the same population (HBS 28603, 28610) fell in clade 4, where we assigned this population on Figure 3. Clade 1 encompasses the south-central United States, an area currently recognized as the range of *C. p. dorsalis*. Clade 2 encompasses areas within the range of *C. p. marginata*, although the eastern half of *C. p. marginata* is not included within the geographical range of this clade (Figs. 1, 3). Much of this region was glaciated during the latest Pleistocene (Fig. 1), including parts of Michigan, Indiana, Ohio, and New York. Clade 3 encompasses part of the range of *C. p. marginata* and all of *C. p. picta*, and is recovered as a shallowly differentiated, monophyletic group that includes all of the glaciated northeastern United States. Finally, clade 4 includes the entire range of *C. p. bellii* and part of the northern range of *C. p. marginata* (Fig. 3, Electronic Appendix 1). Geographically, clade 4 includes all samples from west of the Mississippi River excluding *C. p. dorsalis*, and accounts for approximately half the continental United States.

### *Hypothesis Testing*

To construct phylogenies for hypothesis testing, we used the parameters identified in Modeltest 3.0 (Posada and Crandall 1998). Using MacClade 3.05 (Maddison and Maddison 1995), we constructed constraint trees compatible with each of the alternative hypotheses previously outlined. We divide our hypotheses into: (1) four-taxon tests, with or without each subspecies constrained to be monophyletic; (2) three-taxon tests (excluding *marginata*), with or without each subspecies constrained to be monophyletic; and (3) three-taxon tests (excluding *marginata*), but only using the southernmost populations of each subspecies. For each of these three sets of hypotheses, we conducted tests using likelihood, parsimony, and NJ trees, all of which yielded identical results.

*Four-taxon tests.*—Among the eight possible trees compared, the one with *dorsalis* sister to the rest (and all taxa unconstrained to be monophyletic), was identified as the best. Only the tree with *bellii* sister to the rest (all taxa unconstrained to be monophyletic) could not be rejected as significantly worse (K-H; T = 1.08, $P = 0.14$). The remaining six alternatives were all found to be a significantly poorer fit to our data ($P \leq 0.05$). Thus, when all taxa are considered, the trees consistent with Bleakney's hypothesis (placing *picta* as sister group to the remaining taxa), and Bishop and Schmidt (*marginata* as sister to the other three) are strongly rejected.

*Three-taxon, all population tests.*—The results for three subspecies are very similar to the four-subspecies tests. With *marginata* removed from the dataset, the K-H test found that once again the tree with *dorsalis* sister to *bellii* and *picta* (all taxa nonmonophyletic) was best supported. Of the remaining five trees, all were rejected ($P \leq 0.01$) except for *bellii* as sister to the rest (all taxa nonmonophyletic). Once again, the trees that best support both Bleakney's and Bishop and Schmidt's hypotheses are significantly rejected.

*Three-subspecies, southern population tests.*—As a final test we included only those samples corresponding to Bleakney's (1958) hypothesis of late Pleistocene southern refugia for *picta*, *dorsalis*, and *bellii* during the Wisconsinan glaciation. The hypothesis of a monophyletic *dorsalis* sister to monophyletic *bellii* and *picta* is best supported under parsimony, whereas the same relationship with all taxa nonmonophyletic is best supported under likelihood. However, in both cases none of the five alternative trees can be statistically rejected, reflecting the limited statistical power in this very restricted dataset.

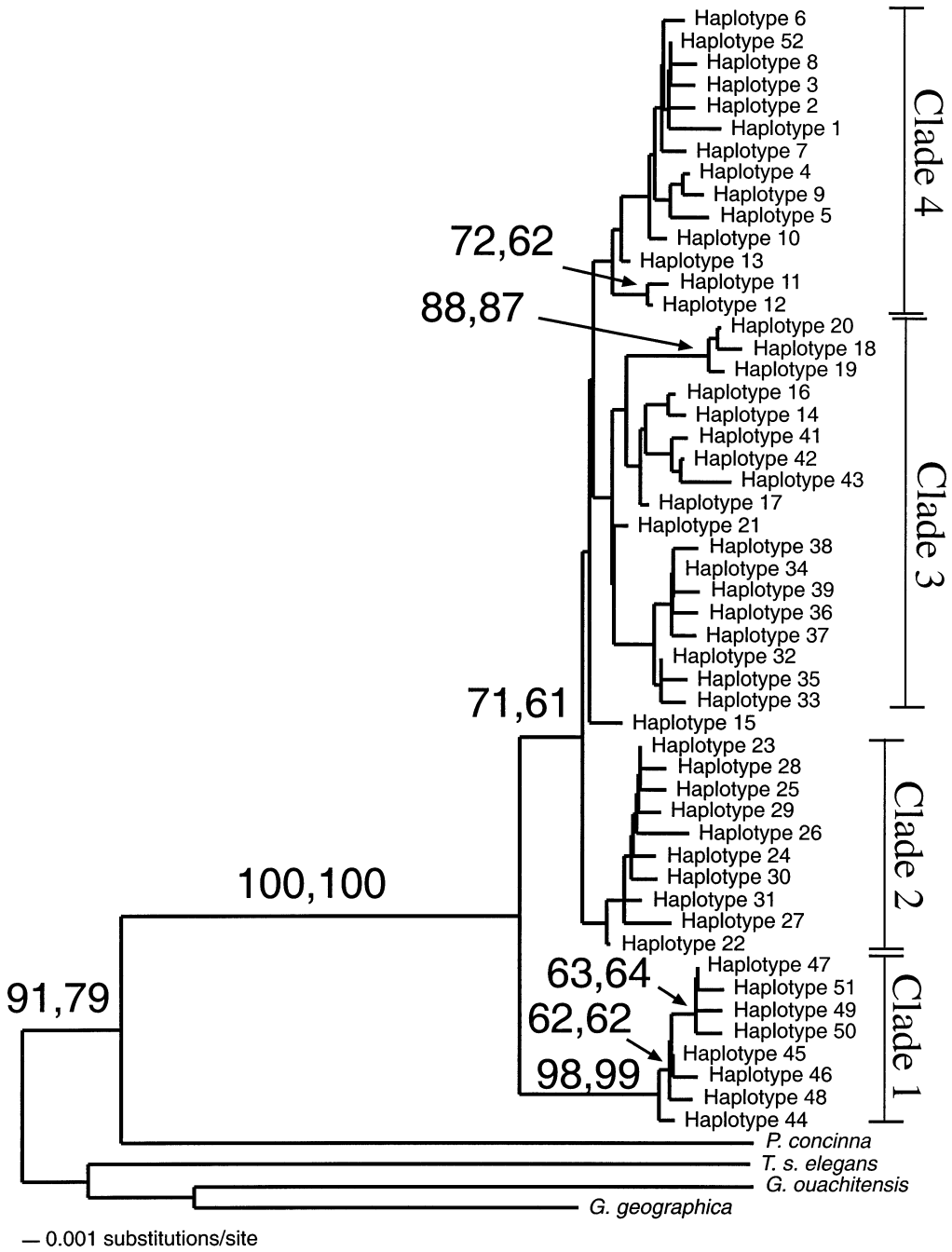


FIG. 2. Neighbor-joining (NJ) bootstrap analysis showing the relationships among the 51 unique *Chrysemys* haplotypes and four outgroups. For the geographical location of all haplotypes, see Electronic Appendix. Numbers on each branch correspond to bootstrap values based on 1000 NJ pseudoreplicates, followed by bootstrap proportions for 100 maximum-parsimony pseudoreplicates. Four geographically contiguous clades labeled 1–4 are indicated on the tree and mapped in Figure 3.

## DISCUSSION

### *Species Boundaries: Is Chrysemys picta a Single Species?*

Clearly, there is no consistent or objective degree of genetic or morphological differentiation that unambiguously determines whether two taxa are distinct species. We adhere to the genealogical species school (Baum and Shaw 1995) in that taxa at any level should be diagnosable and monophyletic, preferably for multiple character sets. If they demonstrate the evolution of intrinsic reproductive isolating mechanisms (and are therefore good biological species), so much the better, although we do not view this as a necessary prerequisite to species recognition. One important consequence of genealogical views of species is that intraspecific variation is generally not recognized taxonomically. This is reflected in the dissatisfaction with the ''subspecies concept''

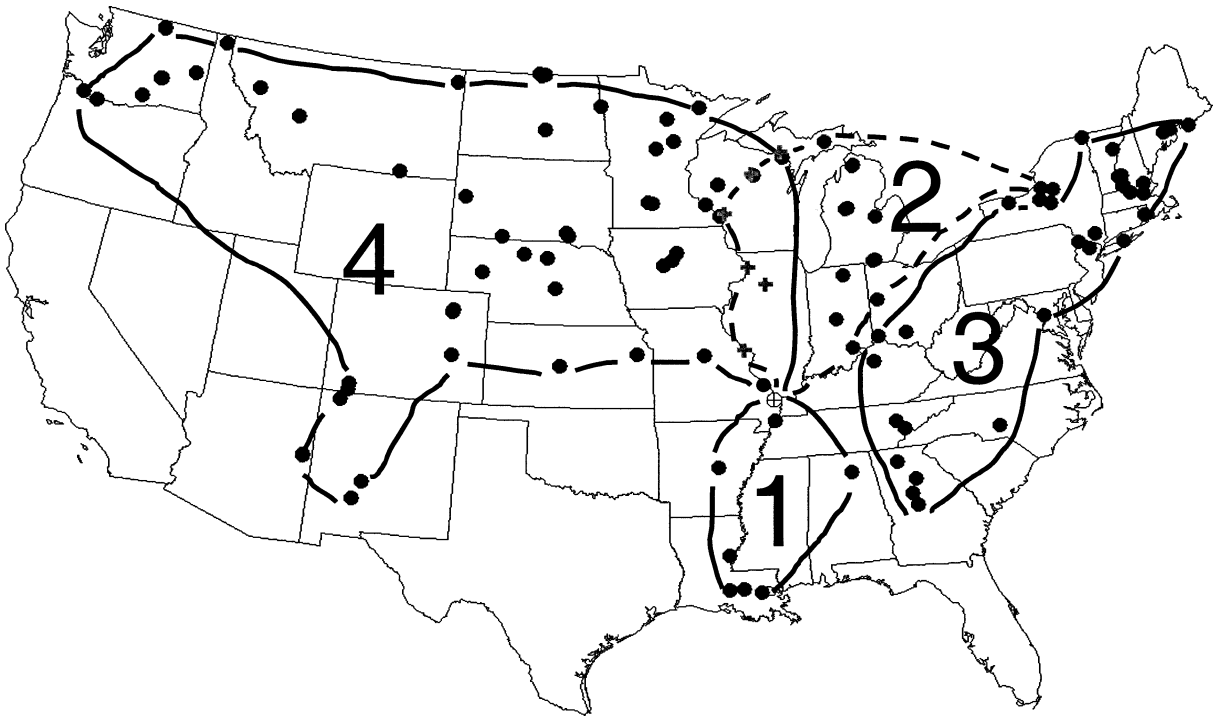


FIG. 3. Map showing the U.S. distribution of the four clades recovered for *Chrysemys* based on neighbor-joining and maximum-likelihood analyses of 241 individuals from 117 localities. Solid/dashed lines indicate clades identified in Figure 2; clade 2 is outlined with a dashed line for clarity only. Crosses indicate localities that contain haplotypes present in clades 2 and 4. The open circle with a cross is a population from Horseshoe Lake, Illinois, that contains haplotypes from clades 1, 2, and 4.

found in much of the current systematics literature; if taxa are recognizable, the minimum level of recognition is the species.

Our mtDNA data indicate that two reciprocally monophyletic clades exist within the painted turtle complex. *Dorsalis* is well supported (BP = 99% under parsimony, 98% under NJ) and 23 apomorphic mtDNA characters (five unambiguous) diagnose it. All of the remaining painted turtles also form a clade (BP = 61% under parsimony, 71% under NJ), diagnosed by 18 apomorphic mtDNA characters (two unambiguous). At 1.5–2.4% sequence divergence, *dorsalis* is as different from other *Chrysemys* as some species of the speciose genus *Graptemys* (based on p-distances that we calculated from the short CR sequences presented by Lamb et al. 1994), and is roughly equivalent to CR divergence between the box turtles *Terrapene carolina* and *T. ornata* (Starkey and Shaffer, unpubl. data). Given its likely sister-group relationship to the remaining members of *Chrysemys*, the mtDNA data indicate that recognizing *C. dorsalis* as an evolutionary species distinct from *C. picta* (as originally proposed by Agassiz 1857) may be justified. The broad, red middorsal stripe of *dorsalis* provides one striking morphological apomorphy for this species (Carr 1952), and *dorsalis* is instantly recognizable morphologically. In a series of comparative studies on hibernation physiology of painted turtles, Ultsch and colleagues have shown that *dorsalis* uniquely perishes from anoxia after about 50 days, whereas the remaining subspecies of *Chrysemys* generally live over 120 days under identical experimental conditions (Ultsch et al. 1985, 1999). Although these morphological and physiological differences do not confirm species status, they do indicate that *dorsalis* is divergent across several data types.

Our molecular data do not support recognition of the remaining three subspecies of painted turtle as separate evolutionary lineages. Three morphological characters have traditionally been used to define subspecies of *C. picta*: degree of carapacial scute disalignment, plastral patterning, and width of the light anterior margins of the second costal scutes (Hartman 1958). A recent study analyzed variation in these characters for 1164 *Chrysemys* specimens from throughout the United States and Canada, including geographic representation of all four subspecies (Ultsch et al. 2001). The conclusions of this extensive study were mixed, but generally concluded that subspecies were poorly differentiated based on these characters. Although the means of the four subspecies all differed statistically, even in the center of their ranges subspecies were not diagnosable, and north to south clinal variation was extensive within subspecies.

Our conclusions on species boundaries are still tentative since we lack evidence from nuclear genes. However, based on our mtDNA analysis and the published comparative morphological and physiological literature, we propose that *Chrysemys* be recognized as two monotypic taxa pending future analysis: *Chrysemys dorsalis* (Agassiz 1857) in the southern Mississippi drainage (Fig. 1) and *Chrysemys picta* (Schneider 1783) from the remainder of the range of the genus. Whether our results conclusively demonstrate that subspecies within *picta* should be abandoned or retained is debatable, as is the more general role of subspecies in evolutionary taxonomy.

Our fundamental conclusion for *Chrysemys* species bound-

aries is that the previous notion of a single, continentally distributed species is not supported, but neither should all of the previously recognized subspecies be considered distinct evolutionary species. A similar conclusion has been reached over the last decade for several ectothermic vertebrates, including the tiger salamander complex (Shaffer and McKnight 1996), the leopard frog complex (Hillis 1988), the gopher snake *Pituophis* (Rodriguez-Robles and De Jesus-Escobar 2000), and the black rat snake complex (Burbrink et al. 2000). Unlike these other recent studies, our mtDNA work supports the concept of a continentally distributed species for *C. picta* (sensu lato), although we feel that *C. dorsalis* should not be a part of that wide-ranging species. Still, our data indicate that *C. picta* is virtually unique among continentally distributed ectothermic lineages examined to date in that a large, transcontinentally distributed species remains after detailed molecular analysis.

### *Tests of Alternate Hypotheses: Was Bleakney Right?*

Our hypothesis tests all indicate that the first divergence within *Chrysemys* was between *dorsalis* and all other populations, and strongly reject the notion of eastern *picta* as the first to diverge within the complex. We thus find no support for this fundamental aspect of Bleakney's model, based on either the four- or three-taxon tests. We similarly reject Bishop and Schmidt's concept of an ancestral *marginata* giving rise to the remaining taxa. The more intriguing element of Bleakney's model, the hybrid origin of *marginata*, is more difficult to test given the lack of strong support for the monophyly of southern populations of *picta* and *bellii*. That is, because we cannot easily recognize these two taxa as distinct evolutionary lineages, we cannot ask whether *marginata* is a mixture of the two. The intermingling of what have been traditionally considered *marginata* populations with *picta* in New York and New England (our clade 3) does indicate a close relationship between some, but not all *marginata* and *picta* sequences, consistent with possible hybridization in this region. However, resolution of this component of Bleakney's model requires more informative molecular data before any firm conclusions can be drawn.

### *Rapid Radiations and the Effects of Glaciers*

Less than 20,000 years have elapsed since the Wisconsinan Ice Age covered vast tracts of North America. These and earlier Pleistocene glaciations have been the subject of intensive investigations as mechanisms generating evolutionary diversification and speciation (Coope 1979; Bermingham et al. 1992; Zink and Slowinski 1995; Riddle 1996; Avise and Walker 1998; Hewitt 1999; Knowles 2000). For *Chrysemys*, the overall amount of diversity (about 2.0–2.5% sequence divergence) indicates a relatively old origin of diversification. Although we lack a calibration for *Chrysemys*, biogeographic evidence (Avise et al. 1992) suggests that turtle molecular clocks may be slow, with overall mtDNA divergence rates in the deirochelyine genus *Graptemys* estimated at 0.36–0.46%/million years/lineage (Lamb et al. 1994). Fossil material referable to the genus *Chrysemys* date back to the Miocene in Nebraska (Holman 1976; Holman and Sullivan 1981), and the oldest *C. picta* material of which we are aware is an Irvingtonian II site from Cumberland Cave, Maryland (400,000–900,000 years ago; Holman 1977), a Rancholabrean site from northeastern Mississippi (10,000–120,000 years ago; Holman 1995) and sites from the plains of Nebraska and Kansas from throughout the Irvingtonian (400,000–1,900,000 years ago; Preston 1971, 1979; Holman 1995). Thus, consistent with the molecular evidence, these fossils indicate that the *C. picta* morphospecies extends at least to the earliest Pleistocene, and that the *dorsalis*/*picta* split could date to 3.47–2.7 million years, implied by the *Graptemys* clock calibration.

Two additional points relevant to the effects of late-Pleistocene glaciation on *Chrysemys* genetics also emerge from our analyses. First, there is clear evidence of at least two separate invasions into glaciated northern regions from our data, one in New England (clade 3; Figs. 2, 3) and the other in the upper Midwest (clade 2; Figs. 2, 3). Second, and more unexpectedly, our data point to an additional, recent invasion into a vast tract of the central Great Plains and Rocky Mountain region that is not predicted from glacial coverage alone. Clade 4 consists of 62 identical sequences and 28 more that are within one or two mutational steps from this widespread haplotype, distributed across 16 states from Michigan to Washington, Montana to Arizona and New Mexico, and Minnesota to Missouri (Figs. 2, 3). This widespread clade containing virtually no variation implies that a single, recent range expansion occurred over this region of central and western North America, even though the boundaries of the Wisconsinan ice sheets were more than 1500 km north of the southern margins of this area. Fossil evidence confirms that *C. picta* has been present on the Great Plains at scattered localities in Nebraska and Kansas since the earliest Irvingtonian, (900,000–1.9 million years ago; Holman 1995), suggesting that there has been ample time for the accumulation of genetic variation if these populations had been stable. Thus, the lack of variation over this region suggests that painted turtles were present in, extirpated, and recently recolonized the Plains region. An alternative explanation, that a selective sweep eliminated mitochondrial variation in the same region, can be distinguished with nuclear DNA data, and we are collecting this information for a nuclear intron.

Although the reason for this lack of variation in the Great Plains/Rocky Mountain populations of *Chrysemys* is not known with certainty, one intriguing possibility is that a brief period of aridification associated with the retreat of the Laurentide ice sheets may be involved. Bartlein et al. (1998) modeled the paleoclimatology changes across North America over the last 20,000 years as the most recent ice sheets retreated, and postulated a period of extreme aridification over the Great Plains/Rocky Mountain region that reached its maximum 14,000 years ago and receded rapidly thereafter. If correct, this reconstruction predicts that the maximal eastward extent of aridification was a line from roughly present-day Chicago south to Houston (Fig. 1)—a line that matches the easternmost extent of clade 4 plus the mixed populations from Illinois very closely (Fig. 3). Shaffer and McKnight (1996) noted a virtually identical pattern for populations of the tiger salamander *Ambystoma tigrinum*, another aquatic species that is predicted to have been strongly affected by such an aridification event. Fossil evidence that would be

necessary to test this model requires a complete record for the Great Plains over the last 20,000 years, and such a fine-scale record is not currently available. A radiocarbon dated *Chrysemys* from the Jones Fauna, Kansas dated at 26,700–29,000 years ago places the species in this region during the late Pleistocene immediately prior to the hypothesized period of aridification (Lundelius et al. 1983). Unfortunately Great Plains sites from the critical period between 26,000–10,000 years ago are not available (Holman 1995). Sites from Texas spanning the period from 9,000–19,000 years ago lack *Chrysemys* fossils (Holman 1995), although these sites are outside of the current distribution of *C. picta*, and may never have been occupied by the species. A more in-depth analysis of some 700 CR sequences currently underway in our lab, combined with ongoing nuclear DNA studies, should shed additional light on this intriguing model of Wisconsinan extinction-recolonization dynamics.

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## Literature Cited

Agassiz, L. 1857. Contributions to the natural history of the United States of America. Little, Brown, and Company, Boston, MA.

Avise, J. C. 1994. Molecular markers, natural history and evolution. Chapman and Hall, New York.

Avise, J. C., and D. Walker. 1998. Pleistocene phylogeographic effects on avian populations and the speciation process. Proc. R. Soc. London B 265:457–463.

Avise, J. C., B. W. Bowen, T. Lamb, A. B. Meylan, and E. Bermingham. 1992. Mitochondrial DNA evolution at a turtle's pace: Evidence for low genetic variability and reduced microevolutionary rate in the testudines. Mol. Biol. Evol. 9:457–473.

Ball, R. M., Jr.,and J. C. Avise. 1992. Mitochondrial DNA phylogeographic differentiation among avian populations and the evolutionary significance of subspecies. Auk 109:626–636.

Bartlein, P. J., K. H. Anderson, P. M. Anderson, M. E. Edwards, C. J. Mock, R. S. Thompson, R. S. Webb, and C. Whitlock. 1998. Paleoclimate simulations for North America over the past 21,000 years: Features of the simulated climate and comparisons with paleoenvironmental data. Quat. Sci. Rev. 17:549–585.

Baum, D. A., and K. L. Shaw. 1995. Genealogical perspectives on the species problem. Pp. 289–303 *in* Proceedings of the fifth international symposium of the International Organization of Plant Biosystematics. Monographs in systematic botany; experimental and molecular approaches to plant biosystematics. Missouri Botanical Garden, St. Louis, MO.

Bermingham, E., S. Rohwer, S. Freeman, and C. Woods. 1992. Vicariance biogeography in the Pleistocene and speciation in North American wood warblers: a test of Mengel's model. Proc. Natl. Acad. Sci. USA 89:6624–6628.

Bishop, S. C., and F. J. W. Schmidt. 1931. The painted turtles of the genus *Chrysemys*. Field Mus. Nat. Hist. Publ. Zool. Ser. 18: 123–139.

Bleakney, S. 1958. Postglacial dispersal of the turtle *Chrysemys picta*. Herpetologica 14:101–104.

Brown, W. M. 1983. Evolution of animal mitochondrial DNA. Pp. 62–88 *in* M. Nei and R. K. Koehn, eds. Evolution of genes and proteins. Sinauer Associates, Sunderland, MA.

Buckley, T. R., C. Simon, H. Shimodaira, and G. K. Chambers. 2001. Evaluating hypotheses on the origin and evolution of the New Zealand alpine cicadas (*Maoricicada*) using multiple-comparison test of tree topology. Mol. Biol. Evol. 18:223–234.

Burbrink, F. T., R. Lawson, and J. B. Slowinski. 2000. Mitochondrial DNA phylogeography of the polytypic North American rat snake (*Elaphe obsoleta*): A critique of the subspecies concept. Evolution 54:2107–2118.

Burke, R. L., T. E. Leuteritz, and A. J. Wolf. 1996. Phylogenetic relationships of emydine turtles. Herpetologica 52:572–584.

Carr, A. 1952. Handbook of turtles: The turtles of the United States, Canada, and Baja California. Comstock Publishing, Ithaca, NY.

Collins, J. P., J. B. Mitton, and B. A. Pierce. 1980. *Ambystoma tigrinum*: A multispecies conglomerate? Copeia 1980:938–941.

Conant, R. 1956. A review of two rare pine snakes from the Gulf Coastal Plain. Am. Mus. Novit. 1781:1–31.

Conant, R., and J. T. Collins. 1991. The Peterson field guide series: A field guide to reptiles and amphibians eastern and central North America. Houghton Mifflin, Boston, MA.

Coope, G. R. 1979. Late Cenozoic fossil *Coleoptera*: evolution, biogeography, and ecology. Annu. Rev. Ecol. Syst. 10:249–267.

Cracraft, J. 1983. Species concepts and speciation analysis. Pp. 425 *in* R. F. Johnston, ed. Current ornithology. Plenum Press, London.

Encalada, S. E., P. N. Lahanas, K. A. Bjorndal, A. B. Bolten, M. M. Miyamoto, and B. W. Bowen. 1996. Phylogeography and population structure of the Atlantic and Mediterranean green turtle *Chelonia mydas*: A mitochondrial DNA control region sequence assessment. Mol. Ecol. 5:473–483.

Farris, J. S. 1982. Outgroups and parsimony. Syst. Zool. 31: 328–334.

Gaffney, E. S., and P. A. Meylan. 1988. A phylogeny of turtles. Pp. 157–219 *in* M. J. Benton, ed. Phylogeny and classification of the tetrapods. Clarendon Press, Oxford, England.

Goldman, N., J. P. Anderson, and A. G. Rodrigo. 2000. Likelihood-based tests of topologies in phylogenetics. Syst. Biol. 49: 652–670.

Halanych, K. M., and T. J. Robinson. 1999. Multiple substitutions affect the phylogenetic utility of cytochrome *b* and 12S rDNA data: Examining a rapid radiation in leporid (Lagomorpha) evolution. J. Mol. Evol. 48:369–379.

Hartman, W. L. 1958. Intergradation between two subspecies of painted turtle, genus *Chrysemys*. Copeia 1958:261–265.

Hasegawa, M., H. Kishino, and T. A. Yano. 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. J. Mol. Evol. 22:160–174.

Hewitt, G. M. 1999. Post-glacial re-colonization of European biota. Biol. J. Linn. Soc. Lond. 68:87–112.

Hillis, D. M. 1988. Systematics of the *Rana pipiens* complex: puzzle and paradigm. Annu. Rev. Ecol. Syst. 19:39–63.

Holder, K., R. Montgomerie, and V. L. Friesen. 1999. A test of the glacial refugium hypothesis using patterns of mitochondrial and nuclear DNA sequence variation in rock ptarmigan (*Lagopus mutus*). Evolution 53:1936–1950.

Holman, J. A. 1976. The herpetofauna of the lower Valentine formation north-central Nebraska. Herpetologica 32:262–268.

———. 1977. The Pleistocene (Kansan) herpetofauna of Cumberland Cave, Maryland. Ann. Carnegie Mus. 46:157–172.

———. 1995. Pleistocene amphibians and reptiles in North America. Oxford Univ. Press, Oxford, U.K.
Holman, J. A., and F. Grady. 1989. The fossil herpetofauna (Pleistocene: Irvingtonian) of Hamilton cave, Pendleton County, West Virginia. NSS Bull. 51:34–41.
Holman, J. A., and R. M. Sullivan. 1981. A small herpetofauna from the type section of the Valentine formation (Miocene: Barstovian), Cherry County, Nebraska. J. Paleontol. 55:138–144.
Holman, J. A., G. Bell, and J. Lamb. 1990. A late Pleistocene herpetofauna from Bell cave, Alabama. Herpetol. J. 1:521–529.
Irschick, D. J., and H. B. Shaffer. 1997. The polytypic species revisited: Morphological differentiation among tiger salamanders (*Ambystoma tigrinum*) (Amphibia: Caudata). Herpetologica 53:30–49.
Iverson, J. B. 1992. A revised checklist with distribution maps of the turtles of the world. J. B. Iverson, Richmond, IN.
Kimura, M. 1980. A simple method for estimating evolutionary rate of base substitution through comparative studies of nucleotide sequences. J. Mol. Evol. 16:111–120.
Kishino, H., and M. Hasegawa. 1989. Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data and the branching order in Hominoidea. J. Mol. Evol. 29:170–179.
Knowles, L. L. 2000. Tests of Pleistocene speciation in montane grasshoppers (genus *Melanoplus*) from the sky islands of western North America. Evolution 54:1337–1348.
Lamb, T., C. Lydeard, R. B. Walker, and J. W. Gibbons. 1994. Molecular systematics of map turtles (*Graptemys*): A comparison of mitochondrial restriction site versus sequence data. Syst. Biol. 43:543–559.
Lundelius, E. L., R. W. Graham, E. Andersen, J. Guilday, J. A. Holman, D. W. Steadman, and S. D. Webb. 1983. Terrestrial vertebrate faunas. Pp. 311–353 *in* H. E. Wright and S. Porter, eds. Late Quaternary environments of the United States: The late Pleistocene. Univ. of Minnesota Press, Minneapolis, MN.
Maddison, W. P., M. J. Donoghue, and D. R. Maddison. 1984. Outgroup analysis and parsimony. Syst. Zool. 33:83–103.
Maddison, W. P., and D. R. Maddison. 1995. MacClade. Sinauer Associates, Sunderland, MA.
Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Mindell, D. P., M. D. Sorenson, D. E. Dimcheff, M. Hasegawa, J. C. Ast, and T. Yuri. 1999. Interordinal relationships of birds and other reptiles based on whole mitochondrial genomes. Syst. Biol. 48:138–152.
Posada, D., and K. A. Crandall. 1998. Modeltest: Testing the model of DNA substitution. Bioinformatics 14:817–818.
Preston, R. E. 1971. Pleistocene turtles from the Arkalon local fauna of southwestern Kansas. J. Herpetol. 5:208–211.
———. 1979. Late Pleistocene cold-blooded vertebrate faunas from the midcontinental United States, I. Reptilia; Testudines, Crocodilia. Univ. Mich. Mus. Paleontol. 19:1–53.
Riddle, B. R. 1996. The molecular phylogeographic bridge between deep and shallow history in continental biotas. Trends Ecol. Evol. 11:207–211.
Rodriguez-Robles, J. A., and J. M. De Jesus-Escobar. 2000. Molecular systematics of new world gopher, bull, and pinesnakes (*Pituophis*: Colubridae), a transcontinental species complex. Mol. Phylogenet. Evol. 14:35–50.
Saitou, N., and M. Nei. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4:406–425.
Sanderson, M. J., and H. B. Shaffer. 2002. Troubleshooting molecular phylogenetic analyses. Annu. Rev. Ecol. Syst. 33:49–72.
Schneider, J. G. 1783. Allgemeine naturgeschichte der schildkroten, nebst einem systematichen verseichnisse der einzelnen arten und zwei kupfren. Muller, Leipzig.
Seidel, M. E., and M. D. Adkins. 1989. Variation in turtle myoglobins (subfamily Emydinae: Testudines) examined by isoelectric focusing. Comp. Biochem. Physiol. B 94:569–574.
Shaffer, H. B. 1984a. Evolution in a paedomorphic lineage. I. An electrophoretic analysis of the Mexican ambystomatid salamanders. Evolution 38:1194–1206.
———. 1984b. Evolution in a paedomorphic lineage. II. Allometry and form in the Mexican ambystomatid salamanders. Evolution 38:1207–1218.
———. 1993. Phylogenetics of model organisms: The laboratory axolotl, *Ambystoma mexicanum*. Syst. Biol. 42:508–522.
Shaffer, H. B., and M. L. McKnight. 1996. The polytypic species revisited: Genetic differentiation and molecular phylogenetics of the tiger salamander *Ambystoma tigrinum* (Amphibia: Caudata) complex. Evolution 50:417–433.
Shaffer, H. B., P. Meylan, and M. L. McKnight. 1997. Tests of turtle phylogeny: Molecular, morphological, and paleontological approaches. Syst. Biol. 46:235–268.
Shaffer, H. B., G. M. Fellers, A. Magee, and S. R. Voss. 2000. The genetics of amphibian declines: Population substructure and molecular differentiation in the Yosemite Toad, *Bufo canorus* (Anura, Bufonidae) based on single-strand conformation polymorphism analysis (SSCP) and mitochondrial DNA sequence data. Mol. Ecol. 9:245–257.
Shimodaira, H., and M. Hasegawa. 1999. Multiple comparisons of log-likelihoods with applications to phylogenetic inference. Mol. Biol. Evol. 16:1114–1116.
Smith, A. B. 1994. Rooting molecular trees: Problems and strategies. Biol. J. Linn. Soc. Lond. 51:279–292.
Smith, H. M., and J. P. Kennedy. 1951. *Pituophis melanoleucus ruthveni* in eastern Texas and its bearing on the status of *Pituophis catenifer*. Herpetologica 7:93–96.
Stewart, D. T., and A. J. Baker. 1994. Evolution of mtDNA D-loop sequences and their use in phylogenetic studies of shrews in the subgenus *Otrisorex* (*Sorex*: Soricidae: Insectivora). Mol. Phylogenet. Evol. 3:38–46.
Swofford, D. L. 1998. PAUP*: Phylogenetic analysis using parsimony (*and other methods). Sinauer Associates, Sunderland, MA.
Templeton, A. R. 1983. Phylogenetic inference from restriction endonuclease cleavage site maps with particular reference to the evolution of humans and the apes. Evolution 37:221–244.
Thompson, J. D., T. J. Gibson, F. Plewnial, F. Jeanmougin, and D. G. Higgins. 1997. The Clustal X windows interface: flexible strategies for multiple alignment aided by quality analysis tools. Nucleic Acids Res. 25:4876–4882.
Ultsch, G. R., R. W. Hanley, and T. R. Bauman. 1985. Responses to anoxia during simulated hibernation in northern and southern painted turtles. Ecology 66:388–395.
Ultsch, G. R., M. E. Carwile, C. E. Crocker, and D. C. Jackson. 1999. The physiology of hibernation among painted turtles: The eastern painted turtle *Chrysemys picta picta*. Physiol. Biochem. Zool. 72:493–501.
Ultsch, G. R., G. M. Ward, C. M. LeBerte, B. R. Kuhajda, and E. R. Stewart. 2001. Intergradation and origins of subspecies of the turtle *Chrysemys picta*: Morphological comparisons. Can. J. Zool. 79:485–498.
Watrous, L. E., and Q. D. Wheeler. 1981. The outgroup comparison method of character analysis. Syst. Zool. 30:1–11.
Wilson, R. L. 1967. The Pleistocene vertebrates of Michigan. Pap. Mich. Acad. Sci. 52:197–234.
———. 1968. Systematic and faunal analysis of a lower Pliocene vertebrate assemblage from Trego county, Kansas. Contrib. Mus. Paleontol. Univ. Mich. 22:75–126.
Wright, A. H., and A. A. Wright. 1957. Handbook of snakes of the United States and Canada. Comstock Publishing Associates, Ithaca, NY.
Zhang, D.-X., and G. M. Hewitt. 1996. Nuclear integrations: Challenges for mitochondrial DNA markers. Trends Ecol. Evol. 11: 247–251.
Zink, R. M., and J. B. Slowinski. 1995. Evidence from molecular systematics for decreased avian diversification in the Pleistocene epoch. Proc. Natl. Acad. Sci. USA 92:5832–5835.

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