Editorial Comment. – This section presents research reports based on support provided by Chelonian Research Foundation through the Linnaeus Fund, its annual turtle research awards program. Named after CAROLUS LINNAEUS [1707–1778], the Swedish creator of binomial nomenclature, the fund honors the first turtle taxonomist and father of all modern systematics. Linnaeus Fund awards are granted annually to individuals for specific turtle research projects, with either partial or full support as funding allows. Priority is generally given to projects concerning freshwater turtles, but tortoise and marine turtle research proposals are also funded. Priority is given to the following general research areas: taxonomy and systematic relationships, distribution and zoogeography, ecology, natural history, and morphology, but other topics are also considered. Priority is also given to projects that demonstrate potential relevance to the scientific basis and understanding of chelonian diversity and conservation biology. The generally preliminary and summary reports in this section are not formally peer-reviewed, but are nonetheless subjected to editorial review.

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Molecular Phylogeography of the Western Pond Turtle (*Clemmys marmorata*): Preliminary Results. Linnaeus Fund Research Report

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In addition to demographic concerns, genetic variation is often viewed as an important consideration in determining the viability of natural populations (e.g., Lande, 1988). Low levels of genetic variation due to random genetic drift or inbreeding, exacerbated by reduced population sizes or limited migration from genetically distinct populations, may decrease individual viability or fecundity or increase the risk of population extinction when environments change. Indeed, very low levels of inter- and intra-populational genetic variation are often indicative of species that exhibit extreme fluctuations in population size over time, making them susceptible to declines. Overexploitation, habitat destruction, pollution, and climate change thus can each play a fundamental role in delimiting the extent of genetic variation in, and hence the viability of, natural populations. These factors are particularly relevant in a conservation context because threatened and endangered populations or species are characterized by limited numbers of individuals or reduced levels of interpopulational migration (i.e., fragmentation).

Examinations of intraspecific genetic variation in a wide variety of turtle species have typically documented low levels of among-population divergence (Seidel et al., 1981; Scribner et al., 1986; Lamb et al., 1989, 1994; Avise et al., 1992; Bowen et al., 1992; Karl et al., 1992; Lamb and Avise, 1992; Parker and Whiteman, 1993; Allard et al., 1994; Phillips et al., 1996). These observations have led to the suggestion that turtles in general may possess an intrinsically slower rate of molecular evolution than other taxa (e.g., Avise et al., 1992). Because low levels of genetic differentiation seem to characterize turtles and because many species are in need of conservation measures, identification of genetically distinct populations of turtles is singularly important in guiding conservation strategies of many taxa.

The main objective of this study was to evaluate molecular genetic differentiation among populations of the western pond turtle (*Clemmys marmorata*) (Fig. 1). A particular focus of this study was to identify potentially unique populations that might require special conservation management because *C. marmorata* is in serious decline throughout most of its range (reviewed in Gray, 1995). We first conducted an overview of genetic variation in the entire species, using individuals from populations in Washington, Oregon, Nevada, and California (USA), and Baja California (Mexico). Subsequently, we analyzed many populations from within a restricted geographic range (Oregon) to address possible microgeographic genetic differentiation in the species.

Materials and Methods. — The vast majority of tissue samples used in this study were collected throughout the range of *C. marmorata* by Dan Holland (Holland, 1992). The tissues were stored in liquid nitrogen before being shipped to the laboratory (University of California, Davis) where they were maintained at –80°C. For the initial molecular analyses reported here, we chose 76 representative individuals from throughout the entire range of the species (50 from Oregon and the rest from elsewhere).

DNA was extracted from frozen turtle tissue (usually tail tips obtained nonlethally) using standard phenol/chloroform methods. We used universal primers (L14841 and H15149 from Kocher et al., 1989) to amplify a 307 bp region of the cytochrome *b* gene in the mtDNA, again following standard procedures (Kocher et al., 1989). For SSCP (single-strand conformation polymorphism) analysis (Orita et al., 1989), we ran samples of the double-stranded PCR products on non-denaturing 1X MDE gels with 5% glycerol for 12 hrs at 8 watts at 15°C. These gels were subsequently silver stained (Aguade et al., 1994) to

visualize the bands. We adopted this technique because it was faster and less expensive than direct sequencing for screening large numbers of individuals for genetic differences as small as a single base pair. Where SSCP analysis indicated a potentially new haplotype represented by either a new mobility class or a band with a different phenotype on a gel, we manually sequenced the entire 307 bp segment of cytochrome *b* following the methods of Shaffer and McKnight (1996) to characterize this genetic variation. This procedure was modified somewhat for the more intensive analysis of Oregon *C. marmorata* in that we amplified only a 180 bp region of cytochrome *b* using the primer H15149 from Kocher et al. (1989) and a primer developed in our laboratory, cyt *b* 4 (5' CTA CTG ATG AGA ATG CTA GT 3'), that occurs between the two universal Kocher primers. We used this smaller segment of cytochrome *b* for subsequent SSCP analysis rather than the full 307 bp fragment because it increased our likelihood of detecting subtle genetic variation between individuals in this region of the mtDNA.

Results. — Based on SSCP analysis, most individuals of *C. marmorata* exhibited the same genotype for cytochrome *b*. However, several unique genetic variants were identified, mainly from populations in southern coastal California and Baja California. Sequencing of this mtDNA segment for these unique populations has yet to be completed, but preliminary analyses suggest that the genetic differences may be small. For example, comparison of the 307 bp sequences from single turtles from Washington, Nevada, and San Luis Obispo, Tulare, and Santa Barbara counties in California indicated that only the Santa Barbara individual was distinct (by a single base-pair substitution).

The more extensive analyses of *C. marmorata* from Oregon largely confirmed the species-wide investigation. Of the 50 turtles examined from throughout the state (Benton, Douglass, Lane, Curry, and Wasco counties, and the Willamette Valley drainage), only two genotypes different than the common one were observed. One population from a west-flowing coastal stream in Curry Co. and another from the Willamette Valley were genetically distinct from the other Oregon populations examined. The level of sequence divergence for the Curry Co. population is currently unknown, because it has yet to be sequenced; the Willamette Valley population differed from the standard genotype by a single base-pair substitution.

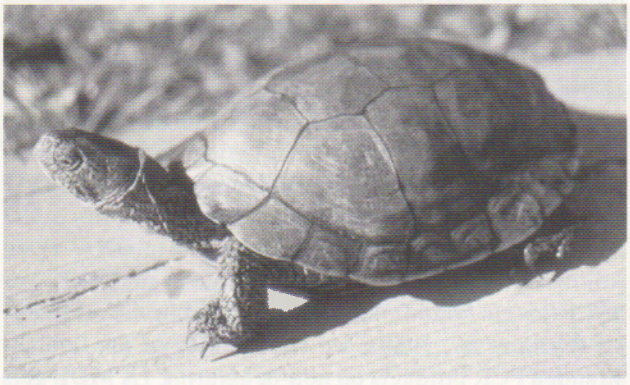

Figure 1. An adult *Clemmys marmorata* from Hastings Reservation, Monterey County, California. Photograph by H.B. Shaffer.

Discussion. — Based on our preliminary analysis, the SSCP and sequencing results are consistent with the current morphologically based description of two subspecies of *C. marmorata*, one north (*C. m. marmorata*) and one south (*C. m. pallida*) of central California. Furthermore, the Baja California turtles produced markedly distinct bands in the SSCP analysis, suggesting that these populations may be quite divergent as well.

Our results for *C. marmorata* are consistent with those of population-level molecular studies of other turtle species. As in our study, relatively little genetic differentiation has been detected among populations even across considerable geographic ranges for a broad assortment of turtle taxa (Seidel et al., 1981; Scribner et al., 1986; Lamb et al., 1989, 1994; Avise et al., 1992; Bowen et al., 1992; Karl et al., 1992; Lamb and Avise, 1992; Parker and Whiteman, 1993; Allard et al., 1994; Phillips et al., 1996). Of particular note, our findings are largely concordant with those obtained in a DNA fingerprinting study of *C. marmorata* (Gray, 1995). These genetic results overall may reflect an inherently lower rate of molecular evolution in turtles (e.g., Avise et al., 1992), recent migration events within the ranges of many turtle species (e.g., Hewitt, 1996), or both. In any case, future molecular studies of intraspecific relationships of turtle populations may need to adopt techniques like genome-wide AFLP analyses (e.g., Vos et al., 1995) that are used for inferring higher-level geneological relationships in other, faster-evolving taxa.

Although we detected little overall genetic variation, our results do provide some insight into broadscale historical relationships among populations of *C. marmorata*. Southern populations, particularly in Baja California, may in fact be genetically different enough from the northern populations to warrant specific status. DNA sequencing has yet to be completed, but our SSCP results suggest that Baja California turtles are very distinct genetically from other populations. If the same criteria are used for *C. marmorata* as for other emydid species (e.g., *Graptemys*, see Lamb et al., 1994), even fixed differences of a few base pair substitutions between *C. marmorata* from Baja California and other populations may indicate species-level differentiation. This conclusion is also supported by the morphological differentiation between *C. marmorata* from Baja California and those from the rest of the range (Seeliger, 1945; Bury, 1970). In fact, Seeliger (1945), who named the two currently recognized subspecies of *C. marmorata* based on morphology, found that western pond turtles from Baja California differed so much from the others

that "no attempt will be made to assign them to either subspecies."

Regardless of whether western pond turtles from Baja California should be designated as a distinct species, the genetic uniqueness of many southern populations of *C. marmorata* suggests that special care should be taken to preserve and manage them. If turtles exhibit a slow rate of molecular evolution (e.g., Avise et al., 1992), any documentable genetic differentiation in cytochrome *b* is likely to signal relatively deep historical splits among populations and thus indicate their singular importance. Populations of western pond turtles have been declining for decades, and both northern and southern populations have experienced drastic reductions and local extinctions (Gray, 1995). Given this pattern of population loss, combined with restricted levels of genetic variation found throughout the species, we believe that protection of western pond turtle populations that exhibit genetic variation and differentiation represents an important component of the management of this declining species.

Beyond important conservation concerns, our results also have implications for the historical biogeography of western pond turtles. Based on fossil evidence, Brattstrom and Sturn (1959) hypothesized that progenitors of *C. marmorata* arose in the Paleocene of west-central North America. The turtles were then supposed to have split in the Eocene, with individuals dispersing northwest and southwest, eventually occupying the current range of the species by the beginning of the Pleistocene (Brattstrom and Sturn, 1959). Thus, populations of *C. marmorata* are hypothesized to have separated and then come back into secondary contact along the Pacific coast. This scenario is consistent with the distribution of the currently recognized subspecies and their intergrade zone in central California (Bury, 1970). However, the minimal levels of genetic variation within and differentiation among northern populations of *C. marmorata* (Gray, 1995; this study) may be more consistent with a scenario that invokes a recent northward invasion from a more ancient southern stock. This hypothesis could be tested by comparing homologous DNA sequences among species closely related to *C. marmorata* to determine the polarity of character changes and hence the phylogenetic relationships among western pond turtle populations. Southern populations should be more basal (and northern ones more derived) and paraphyletic with respect to a monophyletic northern group, if the northward migration hypothesis is correct.

We see at least two future directions for molecular conservation genetic research on western pond turtles. First, additional specimens from populations in the southern and coastal portions of the range need to be examined with SSCP methods and then all variants need to be sequenced. This approach would confirm or reject the hypothesis that these populations of *C. marmorata* are genetically distinct from the rest of the range and, if so, at what level. A second useful direction would be to employ a more rapidly evolving gene (e.g., mtDNA control region) or a more sensitive molecular technique (e.g., AFLP analysis) to examine population subdivision in *C. marmorata*. This approach might identify more subtle genetic differentiation among populations than can be detected with the more slowly evolving cytochrome *b* gene and the SSCP technique. In either case, the results of future molecular work on *C. marmorata* will be a crucial supplement to our current knowledge base for making intelligent management decisions regarding this unique, but rapidly declining, species.

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