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Molecular Systematics of Emydine Turtles. Linnaeus Fund Research Report

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The emydine turtles (Emydinae: genera *Clemmys*, *Emydoidea*, *Emys*, and *Terrapene*) are among the most familiar and well-studied chelonians in the world. This small turtle subfamily contains only ten species, yet exhibits greater ecological and morphological diversity than its more speciose sister group, the Deirochelyinae. Some species are fully aquatic (e.g., *Clemmys marmorata*) while others are almost entirely terrestrial (e.g., *Terrapene ornata*). In addition, species in the genera *Emydoidea*, *Emys*, and *Terrapene* possess shells with a movable plastron (plastral kinesis) while members of the genus *Clemmys* lack this trait.

Although emydines are extensively studied, popular, and of recent conservation concern, they lack a robust phylogeny. Morphological treatments of the Emydinae (Bramble, 1974; Gaffney and Meylan, 1988) hypothesized that the box turtles and other hinged genera form a monophyletic group (Fig. 1A). By default, the species without plastral kinesis were lumped into the genus *Clemmys*. Mitochondrial sequence data from the 16S ribosomal gene (Bickham et al., 1996) suggested that the genus *Clemmys* is not monophyletic (Fig. 1B). An attempt to combine these data with ecological, behavioral, biochemical, and additional morphological characters did not fully resolve the conflict between the morphological and molecular phylogenies (Burke et al., 1996). Despite the fact that some consensus has emerged from these studies, most hypothesized arrangements could be clarified and strengthened with additional molecular data.

Our objective was to shed light on the evolutionary history of emydines using all ten extant species, suitable sister taxa, and appropriately evolving molecular markers.

Materials and Methods. — We obtained liver tissue from museum specimens and blood samples from living zoo specimens for all 10 extant emydine species and 2 deirochelyine outgroup species (Appendix 1). We isolated genomic DNA from liver tissue and blood samples by standard proteinase K digestion and phenol/chloroform purification (Maniatis et al., 1982). We amplified a 1200 bp region of the mitochondrial genome encoding the entire cytochrome *b* gene and part of the adjacent transfer ribonucleic acid, threonine ($tRNA^{thr}$) via polymerase chain reac-

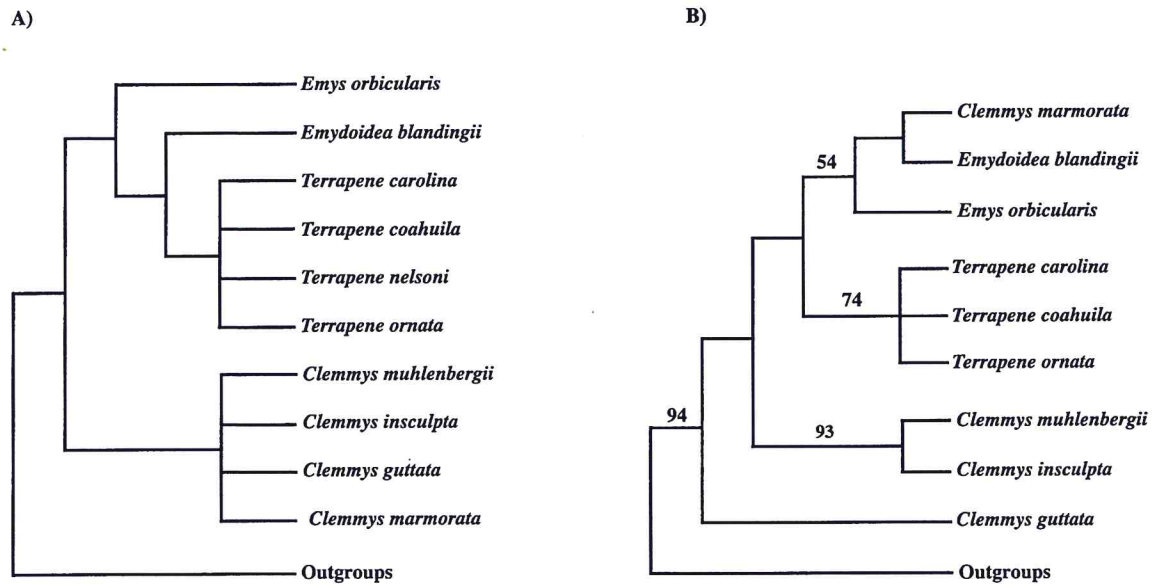

Figure 1. Previous phylogenetic hypotheses for the Emydinae. **A.** Phylogeny based on five osteological characters (Gaffney and Meylan, 1988). **B.** Phylogeny based on mitochondrial rRNA sequence data (Bickham et al., 1996) shown with bootstrap support.

tion (PCR; Saiki et al., 1988) using the primers GLUDG-L (Palumbi et al., 1991) and M (Shaffer et al., 1997) (Table 1). We amplified an additional 900 bp region of mtDNA encoding a portion of the nicotinamide adenine dinucleotide dehydrogenase subunit four gene (ND4) and flanking tRNA histidine ($tRNA^{his}$), serine ($tRNA^{ser}$), and leucine ($tRNA^{leu}$), using the primers ND4 and Leu (Arevalo et al., 1994) (Table 1). We used the following thermal cycle parameters for 50 µl amplification reactions: 35 cycles of 1 min denature at 94°C, 1 min anneal at 50–52°C, and 2 min extension at 72°C. We purified PCR products using the Wizard Prep Mini Column Purification Kit (Promega, Inc.) and used purified template in 10 µl dideoxy chain-termination reactions (Sanger et al., 1977) using ABI Big Dye chemistry (Perkin-Elmer Applied Biosystems, Inc.) and the primers listed in Table 1. We ran cycle-sequenced products on a 4.8% Page Plus (Ameresco) acrylamide gel using an ABI 377 automated sequencer (Perkin-Elmer Applied Biosystems, Inc.). We sequenced all samples in both directions.

We aligned DNA sequences with the sequence analysis program Sequencher™ 3.0 (Gene Codes Corp.). We translated protein coding nucleotide sequences into amino acid sequences using MacClade 3.06 (Maddison and Maddison, 1992). We identified tRNA genes by manually reconstructing their secondary structures using the criteria of Kumazawa and Nishida (1993). We deposited all mitochondrial DNA sequences in GenBank (Appendix 1).

We used maximum parsimony (MP; Swofford et al., 1996) and maximum likelihood (ML; Felsenstein, 1981) phylogenetic methods to infer the evolutionary relationships of emydine species. We conducted all phylogenetic analyses in PAUP 4.0b4a* (Swofford, 1998). We combined the cyt *b* and ND4 data sets and analyzed them together on the basis of total evidence (Eernisse and Kluge, 1993). We polarized the phylogeny via outgroup comparison (Maddison et al., 1984) using the chicken turtle, *Deirochelys reticularia*, and the painted turtle, *Chrysemys picta*.

We executed MP analyses with the branch-and-bound search algorithm (Hendy and Penny, 1982) using unordered characters. To assess the robustness of individual nodes, we used the bootstrap resampling method (Felsenstein, 1985) by employing 1000 replicates of closest searches in PAUP*. Additionally, we calculated branch support (Bremer, 1994) for internal nodes using the program TreeRot 2 (Sorenson, 1999).

To determine the most appropriate model of DNA substitution for reconstructing emydine relationships under ML, we executed a hierarchical likelihood ratio test (LRT; Felsenstein, 1993; Goldman, 1993; Yang, 1996) in the program Modeltest 3.0 (Posada and Crandall, 1998). The

Table 1. Oligonucleotide primers used to amplify and sequence turtle mtDNA in this study. The 3' end of the primers match nucleotide positions of the heavy strand of the mitochondrial genome of the deirochelyine turtle *Chrysemys picta* (Mindell et al., 1999). Ambiguity codes: R = A or G, Y = C or T.

Primer	Gene	Sequence	Position	Reference
(L) ND4	ND4	5'-CAC CTA TGA CTA CCA AAA GCT CAT GTA GAA GC-3'	10,919	Arevalo et al., 1994
(H) Leu	$tRNA^{leu}$	5'-AC CAC GTT TAG GTT CAT TTT CAT TAC-3'	11,837	Arevalo et al., 1994
(L) GLUDG	$tRNA^{Glu}$	5'-TGA CTT GAA RAA CCA YCG TTG-3'	14,378	Palumbi et al., 1991
(H) Primus-rev	cyt *b*	5'-CGG TTG CAC CTC AGA AGG ATA TTT GGC CTC A-3'	14,804	This study
(L) Primus	cyt *b*	5'-TGA GGC CAA ATA TCC TTC TGA GGT GCA ACC G-3'	14,834	This study
(H) Rush-rev	cyt *b*	5'-GTT GGG TTG TTT GAT CCG GTT TCA TGT AGA AA-3'	14,996	This study
(L) Rush	cyt *b*	5'-TTC CTA CAT GAA ACC GGA TCA AAC AAC CCA AA-3'	15,027	This study
(H) M	$tRNA^{thr}$	5'-TCA TCT TCG GTT TAC AAG AC-3'	15,574	Shaffer et al., 1997

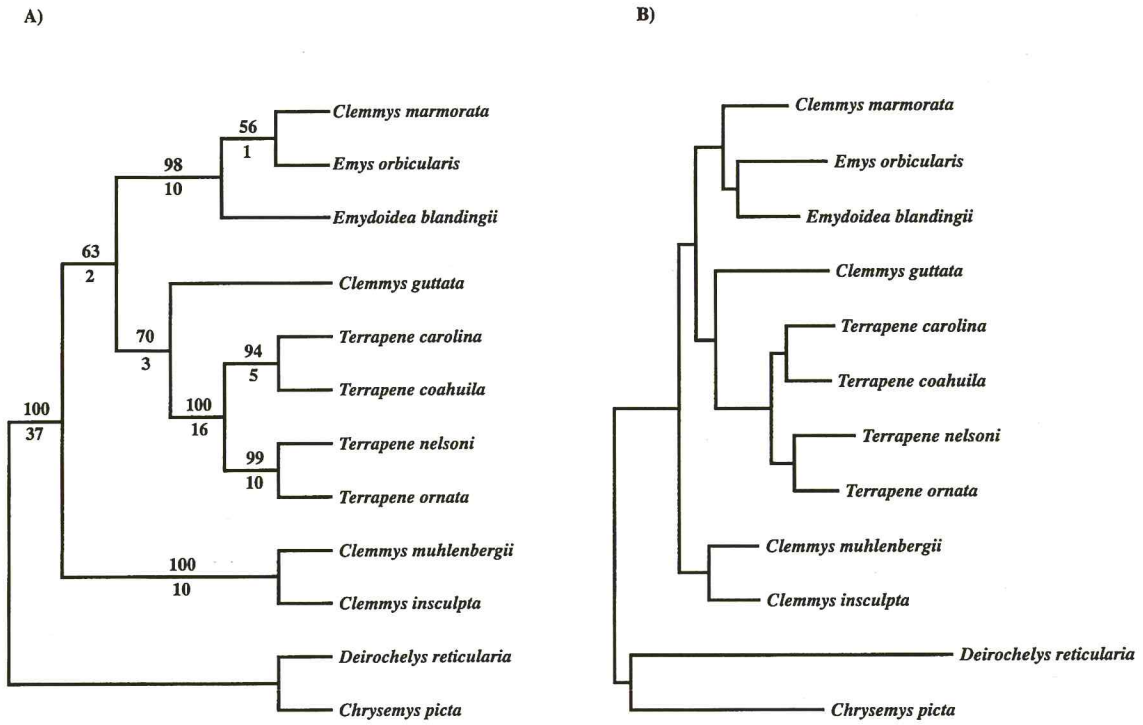

Figure 2. Phylogenetic trees for emydine and outgroup mtDNA lineages. **A.** Single most parsimonious tree (L =1088; CI = 0.642; RI = 0.466). Numbers above the nodes indicate bootstrap support while those below the nodes represent decay indices. **B.** Maximum likelihood estimate of emydine phylogeny (LnL = -7798.4184; α = 0.2766). Branch lengths are drawn proportional to the maximum likelihood estimates of genetic divergence.

model of DNA evolution that best fit our sequence data was the general time reversible model (GTR; Rodriguez et al., 1990) of nucleotide substitution in conjunction with gamma (Γ; Yang, 1994a,b). The GTR + Γ model accommodates unequal base composition by using the empirical base frequencies, estimates the uneven ratio of each type of nucleotide substitution, and accounts for the heterogeneous rates of nucleotide substitutions across all sites.

Results. — Of the 2092 aligned base pairs, 609 were variable and 339 were parsimony informative. Among the ingroup taxa, 461 base pairs were variable and 251 were parsimony informative.

The branch-and-bound, equally weighted MP analyses produced a single most parsimonious tree (Fig. 2A), 1088 steps in length (CI = 0.642; RI = 0.466). The ML GTR + Γ reconstruction also yielded one tree (LnL = -7798.4184; α = 0.2766) nearly identical to the most parsimonious tree (Fig. 2B). In all analyses the emydine turtles group to the exclusion of the deirochelyines (100% bootstrap; 37 decay index) and phylogenetic relationships were well resolved and well supported for most nodes of the tree.

The four North American box turtles (genus *Terrapene*) form a monophyletic group (100% bootstrap; 16 decay index) in which the two western species, *T. ornata* and *T. nelsoni*, form one clade (99% bootstrap; 10 decay index) and the aquatic *T. coahuila* and widespread *T. carolina* form another (94% bootstrap; 5 decay index). The genus *Clemmys* is not monophyletic. Instead, *C. marmorata* belongs to a clade containing *Emydoidea blandingii* and *Emys orbicularis* (98% bootstrap; 10 decay index). However, the relationships among these three taxa are not well resolved, as indicated by the conflict between the MP and ML reconstructions; the MP tree connects *Emys orbicularis* to *C. marmorata* (56% bootstrap; 1 decay index) while the ML tree links *Emys orbicularis* to *Emydoidea blandingii*. Additionally, the spotted turtle (*C. guttata*) does not group with the other eastern US *Clemmys*, *C. insculpta* and *C. muhlenbergii*. Instead, *C. guttata* gains some support as the sister taxon to the box turtles (70% bootstrap; 3 decay index) in both the MP and ML reconstructions. Finally, *Clemmys insculpta* and *C. muhlenbergii* form a robust monophyletic group (100% bootstrap; 10 decay index). Both MP and ML phylogenetic methods suggest that *C. insculpta* and *C. muhlenbergii* are the sister clade to all other emydines. This phylogenetic hypothesis, however, is poorly supported (63% bootstrap; 2 decay index).

In summary, the Emydinae can be divided into four well-supported clades: 1) *Terrapene*; 2) *Clemmys guttata*; 3) *C. insculpta* and *C. muhlenbergii* and; 4) *C. marmorata*, *Emys orbicularis*, and *Emydoidea blandingii*. Unfortunately, relationships between these emydine clades remain enigmatic. Both MP and ML phylogenetic analyses yield the same topology, placing *C. insculpta* and *C. muhlenbergii* as the sister group to a monophyletic clade containing the rest of the emydine turtles.

Discussion. — Our molecular phylogeny is both congruent and incongruent with previous estimates of emydine relationships (Gaffney and Meylan, 1988; Bickham et al., 1996; Burke et al., 1996). Importantly, the large number of informative characters (339) in our multi-gene data set

allows us to address various hypotheses of emydine taxonomy and evolution. We discuss these taxonomic and evolutionary questions at length elsewhere (Feldman and Parham, in press), but briefly highlight two important points of our proposed phylogeny here: the paraphyly of *Clemmys*; and the paraphyly of hinged emydines.

Our mtDNA data explicitly show that the genus *Clemmys* is paraphyletic (Fig. 2). We propose that the spotted turtle, *C. guttata*, is the closest living relative to the North American box turtles. Our data also suggest that *C. marmorata* is not closely related to other *Clemmys*, but shares a more recent common ancestor with *Emys orbicularis* and *Emydoidea blandingii*. Lastly, our phylogeny indicates that *C. muhlenbergii* and *C. insculpta* form a monophyletic group exclusive of, and sister to, all other emydine turtles.

A paraphyletic *Clemmys* stands in contrast to both the accepted taxonomy of the Emydinae (Collins, 1997; Crother, 2000) and the morphological phylogeny of the group (Gaffney and Meylan, 1988; Fig. 1A). A non-monophyletic *Clemmys*, however, is not an entirely original concept. Previous molecular (Bickham et al., 1996; Fig. 1B) and combined treatments (Burke et al., 1996) of the Emydinae have suggested a paraphyletic *Clemmys*. Thus, we propose a new taxonomy for the Emydinae in a more thorough summary (Feldman and Parham, in press). Our taxonomy is consistent with the Linnaean system of ranks as well as the informative scheme of phylogenetic taxonomy (de Queiroz and Gauthier, 1992).

The most notable result of our mtDNA phylogeny is the paraphyly of the hinged emydines. Emydine shell kinesis involves several morphological specializations: 1) an alignment of scales with plastral sutures and a reduction of sutural connections to form a hinge; 2) segmented scapulae that facilitate head and limb retraction; and 3) a closing mechanism modified from cervical musculature (Bramble, 1974). This particular combination of traits is unique among living chelonians and is thought to have evolved only once (Bramble, 1974; Gaffney and Meylan, 1988). However, our data suggest that shell kinesis evolved either twice (once in *Terrapene* and once in the *C. marmorata* + *Emys* + *Emydoidea* clade) or evolved once and was lost twice (in *C. marmorata* and *C. guttata*). Using information from the fossil record and data on the independent derivation of plastral kinesis in other living turtles (e.g., various batagurid genera), we hypothesize that plastral hinging evolved twice in parallel in the Emydinae (Feldman and Parham, in press).

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APPENDIX 1. Specimens used and GenBank Accession numbers for DNA sequence data. Acronyms are: MVZ = Museum of Vertebrate Zoology, Berkeley, California; CAS = California Academy of Sciences, San Francisco, California; ROM = Royal Ontario Museum, Toronto, Ontario; AF = GenBank (http://www.ncbi.nlm.nih.gov).

Clemmys guttata: MVZ 175961, AF258858, AF258870; *Clemmys insculpta*: ROM 1523, AF258864, AF258876; *Clemmys muhlenbergii*: zoo specimen, Wildlife Conservation Society, Bronx Zoo, New York, New York, AF258863, AF258875; *Emydoidea blandingii*: ROM 20922, AF258857, AF258869; *Clemmys marmorata*: MVZ 164994, AF258855, AF258867; *Emys orbicularis*: CAS 182905, AF258856, AF258868; *Terrapene carolina*: MVZ 137441, AF258859, AF258871; *Terrapene coahuila*: zoo specimen (T00228), Gladys Porter Zoo, Brownsville, Texas, AF258860, AF258872; *Terrapene nelsoni*: zoo specimen, Arizona Sonoran Desert Museum, Tucson, Arizona, AF258861, AF258873; *Terrapene ornata*: MVZ 137743, AF258862, AF258874; *Chrysemys picta*: MVZ 230532, AF258866, AF258878; *Deirochelys reticularia*: MVZ 230923, AF258865, AF258877.

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