Chapter 4

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Molecular Insights into the Systematics of the Snapping Turtles (Chelydridae)

H. BRADLEY SHAFFER
DAVID E. STARKEY
MATTHEW K. FUJITA

SOME PROBLEMS IN SYSTEMATICS and phylogenetics are relatively easy to work out, and some are just plain difficult. There are many reasons why this may be the case, but the result is often the same—a few taxa seem to provide endless discussion, debate, and challenge for systematists, and frustration for taxonomists and comparative biologists who require well-resolved phylogenies. Among turtles, one of the most difficult problems has been the content and phylogenetic relationships of the family Chelydridae.

The relatively recent use of molecular markers in phylogenetics has greatly increased our knowledge of evolutionary relationships among organisms, from intraspecific genealogies to the tree of life. Molecular evidence is often most useful when it provides new insights into contentious phylogenetic questions that were previously intractable with more traditional sources of data. Although molecular similarities cannot replace strong morphological differences (any more than morphological similarity can override molecular divergence), new molecular data often force us to critically evaluate conclusions based on sparse sampling or poorly defined morphological characters. Protein electrophoresis (allozymes), chromosome morphology, and immunological cross-reactivity provided the first molecular data for phylogenetic analysis, and they continue to provide important insights into phylogenetic relationships. In general, however, these techniques were developed as a proxy for direct observations at the DNA sequence level. Because DNA sequencing has become increasingly straightforward for both mitochondrial (mtDNA) and nuclear (nDNA) genomes, DNA sequence data have grown to be the major source of molecular characters for phylogenetic inference. Optimal models of DNA evolution can be estimated for a set of sequences and incorporated into phylogenetic methods such as maximum likelihood

and Bayesian analysis. The combination of model-based phylogenetic methods, ease of data acquisition, and a virtually limitless source of characters has rendered mtDNA and nDNA sequences the data of choice for many vexing phylogenetic issues.

In this chapter, we review molecular data that are relevant to three questions in the systematics of the Chelydridae. The first is the relationship of the alligator snapping turtle, *Macrochelys temminckii,* to *Chelydra.* The alligator snapping turtle is geographically restricted to the central and southeastern United States, and some debate exists on whether *Macrochelys* plus *Chelydra* form a monophyletic group.

Second, we consider the relationships and classification of the genus *Chelydra.* As currently recognized, the single species *Chelydra serpentina* consists of four subspecies. *Chelydra s. serpentina* occurs commonly across the United States from the East Coast as far west as the Rocky Mountains, *C. s. osceola* is largely restricted to Florida, *C. s. rossignoni* is found in scattered localities from southern Mexico to northern Central America, and *C. s. acutirostris* ranges from southern Central America to northern South America. Although some have argued that the Florida snapping turtle, *C. s. osceola,* deserves species recognition, others have doubted the validity of even subspecific status. Neither chromosomal nor serological studies appear to distinguish *serpentina* from *osceola,* raising further questions about the validity of *osceola* as a distinct evolutionary form. More recently, mtDNA and allozyme data from a relatively limited sampling of the genus *Chelydra* have shed some light on the validity of these four subspecies, although controversy remains over the interpretation of these results.

Finally, we consider molecular insights regarding the relationships of the big-headed turtle, *Platysternon megacephalum,* because previous studies have disagreed about its phylogenetic position. Skeletal morphology as well as locomotor characters suggest to some researchers that *Platysternon* is allied with *Chelydra* and *Macrochelys,* whereas chromosomal, electrophoretic, and immunological precipitation data place it with Emydidae, Geoemydidae (= Bataguridae), and Testudinidae. Existing mtDNA data either weakly support a *Chelydra/Platysternon* relationship or are ambiguous, and combined mtDNA/morphological evidence strongly support a *Chelydra/Platysternon* sister group relationship (Shaffer et al. 1997). However, new nDNA data do not support this relationship (Krenz et al., unpublished). At least from a molecular perspective, the placement of *Platysternon* remains problematic, although new data reviewed below are shedding light on this problem.

THE NEW WORLD CHELYDRIDS

We address two primary questions concerning the relationships of the New World snapping turtles. First, are *Chelydra* and *Macrochelys* sister taxa, as is generally implied in the literature? Second, is evolutionary diversification within the species *C. serpentina* and *M. temminckii* reflected in their current taxonomy?

Gaffney (1975) developed the intriguing hypothesis that *Platysternon* and *Macrochelys* are sister taxa, with *Chelydra* sister to that pair. Given the overall similarity in geographic range, morphology, and general biology of *Chelydra* and *Macrochelys* compared with *Platysternon,* this is a somewhat counterintuitive hypothesis, yet surprisingly little follow-up work has focused on the sister-group relationship of *Chelydra* and *Macrochelys.* Starkey (1997) provided mtDNA data strongly supporting the sister-group relationship of *Chelydra* and *Macrochelys,* as did Whetstone (1978b) on the basis of morphological characters. Starkey (1997) is the only completed molecular study of which we are aware that addresses this point, but his results are unambiguous. Based on a broad taxonomic sample of the living turtles of the world, he recovered a sister-group relationship of the New World chelydrids (*Chelydra* and *Macrochelys*) with very high statistical confidence (bootstrap proportion [BP] =100; Starkey, unpublished result). Unpublished data from our lab for a ~1–kb intron from the nuclear R35 neural transmitter gene also demonstrates, with similarly high bootstrap support, a *Chelydra–Macrochelys* sister-group relationship. We consider the combined mtDNA and nDNA results to be biologically reasonable and statistically extremely strong. Thus, for the remainder of this chapter we use the term "New World Chelydridae" to refer to this apparently monophyletic group.

At a lower phylogenetic level, the taxonomic content of *Chelydra* has been much more contentious. Phillips et al. (1996) studied 19 individual snapping turtles (10 *C. s. serpentina,* 5 *C. s. osceola,* 2 *C. s. acutirostris,* 2 *C. s. rossignoni*) using restriction endonuclease fragment patterns of mtDNA and isozyme variation. mtDNA fragment analysis uncovered two main groups, one in the United States (including *C. s. serpentina* and *C. s. osceola*) and a second comprising non-U.S. taxa (*C. s. rossignoni* and *C. s. acutirostris*). For the mtDNA, there were no diagnostic differences found between the fragment patterns in *C. s. serpentina* and *C. s. osceola,* and sequence divergence estimates between the two were extremely low, ranging from 0.0 to 0.3%. In contrast, *C. s. rossignoni* and *C. s. acutirostris* were strongly differentiated from the U.S. taxa in their mtDNA. Estimates of percent sequence divergence calculated from the restriction enzyme data ranged from ~3.0 to 4.5% for *C.* s. *acutirostris–C. s. serpentina* comparisons, and ~4.5 to 5.6% for *C.* s. *rossignoni–C. s. serpentina. C. s. acutirostris* and *C. s. rossignoni* were less divergent from each other, with pairwise divergences ranging from 1.7 to 2.6%.

Although limited in scope both in terms of number of loci examined and turtles sampled (see Sites and Crandall, 1997, for a critique), the allozyme data presented by Phillips et al. (1996) were generally consistent with their mtDNA results. They found no unique alleles in eight allozyme loci ex-

Table 4.1
Genetic distances among currently recognized subspecies of *Chelydra serpentina*.

	acutirostris	*rossignoni*	*osceola*	*serpentina*
acutirostris	—	5.45	8.39	8.39
rossignoni	0.319	—	6.03	6.03
osceola	0.347	0.008	—	0.23
serpentina	0.430	0.028	0.054	—

Note: Above the diagonal are the average uncorrected pairwise distance comparisons derived from mtDNA control region sequences described in the text. Below the diagonal are Nei's *D*-values calculated from eight allozyme loci reported in Phillips et al. (1996).

amined between *C. s. serpentina* and *C. s. osceola*, demonstrating that the two are not diagnosably distinct for these loci. One of the eight loci (*Pnp*) showed a reasonably large frequency difference that may imply some differentiation between *C. s. serpentina* (frequency of the "a" allele = 0.67, *f*(b) = 0.33) and *C. s. osceola* (*f*(a) = 0.20, f(b) = 0.80), although it is difficult to make too much of this with the sample sizes available. Consistent with the mtDNA data, *C. s. acutirostris* was fixed for unique alleles for two of eight allozyme loci (*M-Icdh* and *S-Icdh*) based on the two individuals examined. However, the two *C. s. rossignoni* were virtually identical to the *C. s. serpentina* and *C. s. osceola* sampled for these eight loci. Thus, Nei's (1978) genetic distances (calculated by us using the data presented in Phillips et al. 1996) are quite large between *C. s. acutirostris* and the other three taxa (0.43 to 0.32; Table 4.1), but low among the remaining three taxa (0.008–0.05).

Walker et al. (1998) built on these results by examining mtDNA sequence variation among 66 *Chelydra* from ten states in the Southeast. They sequenced a 409–bp fragment of control region (CR) mtDNA, and found this fragment to be identical in 60 of the 66 individuals (91%) examined. The few variants differed from the common haplotype by a single base pair transition, or the single transition plus an additional single base pair indel event. Walker et al. (1998) provided excellent geographic coverage of both southern *C. s. serpentina* and *C. s. osceola*, as well as a transect across a phylogeographic transition zone in western Florida that is consistently deep for many other vertebrate taxa. This lack of variation within *Chelydra* across the southeastern United States is unique for turtles with similar distributions in the same region, leading these authors to agree with Phillips et al. (1996) that *C. s. serpentina* and *C. s. osceola* together represent a single evolutionary unit.

These studies point to a significant lack of diversity among common snapping turtles from the United States, with Phillips et al. (1996) hypothesizing a significant disjunction between non-U.S. and U.S. taxa. Although these results may appear straightforward, both studies have limits to their resolution. The fundamental conclusions of Phillips et al. are based on shared restriction fragment patterns, and not mapped restriction sites, which provide more accurate estimates of sequence divergence. Their allozyme data, while suggesting that *C. s. acutirostris* is a differentiated taxon, are based on very small samples of individuals (two each of the non-U.S. taxa) and loci (eight allozyme loci). Walker et al. (1998) provide compelling sequence evidence from the rapidly evolving CR that *C. s. serpentina* and *C. s. osceola* are virtually identical in the southeastern United States, but only limited population sampling for *C. s. serpentina* across its entire range and no data for the non-U.S. taxa.

To address the question of diversity within and among the United States taxa more completely (Shaffer, Starkey, and Fujita, unpublished data), we have sampled common snapping turtles from throughout their U.S. range (50 localities across 20 states). We analyzed 428 base pairs of CR mtDNA that is essentially the same fragment as that studied by Walker et al. (1998). The results of our study are consistent with the previous two studies in that we found almost no variation within U.S. common snapping turtles, with only a single new haplotype among the 50 or more turtles sampled. This new haplotype differed from the most common haplotype of Walker et al. (1998) by a single base pair insertion-deletion (indel) event, resulting in a maximum sequence divergence of 0.23% across a broad representation of *C. s. serpentina* and *C. s. osceola* (Table 4.1, Fig. 4.1). We also sequenced the same individuals of *C. s. rossignoni* and *C. s. acutirostris* used in Phillips et al. (1996), as well as two new samples of *C. s. rossignoni* from Mexico, one from Honduras, and a single new *C. s. acutirostris* from Panama. At the sequence level, we found even deeper divergences (ranging between ~5.4 and 8.4%) between these two taxa and the U.S. snapping turtles (Table 4.1). Thus, a relatively consistent molecular picture across several laboratories and data sets appears to be emerging. In the United States a single, virtually invariant lineage of *Chelydra* is broadly distributed, with no evidence of differentiation between the subspecies *C. s. serpentina* and *C. s. osceola*. This result is supported with excellent geographic sampling for mtDNA sequence data, thin sampling for allozymes, and currently no information for nDNA sequences. However, the Central American *C. s. rossignoni* and the South American *C. s. acutirostris* are deeply differentiated from each other and from the U.S. snapping turtles (Fig. 4.1), in particular, for mtDNA sequence data.

Unlike the wide-ranging genus *Chelydra*, *M. temminckii* has a limited distribution in the southeastern United States. Roman et al. (1999) assessed variation among alligator snapping turtles utilizing a 420–base-pair fragment of CR mtDNA. They found considerably more variation within alligator snapping turtles than codistributed common snapping turtles, with estimates of pairwise diversity ranging from 1.7 to 2.4%. Furthermore, this variation was geographically structured, with 8 of 11 haplotypes restricted to single rivers, and major lineages identified in the western

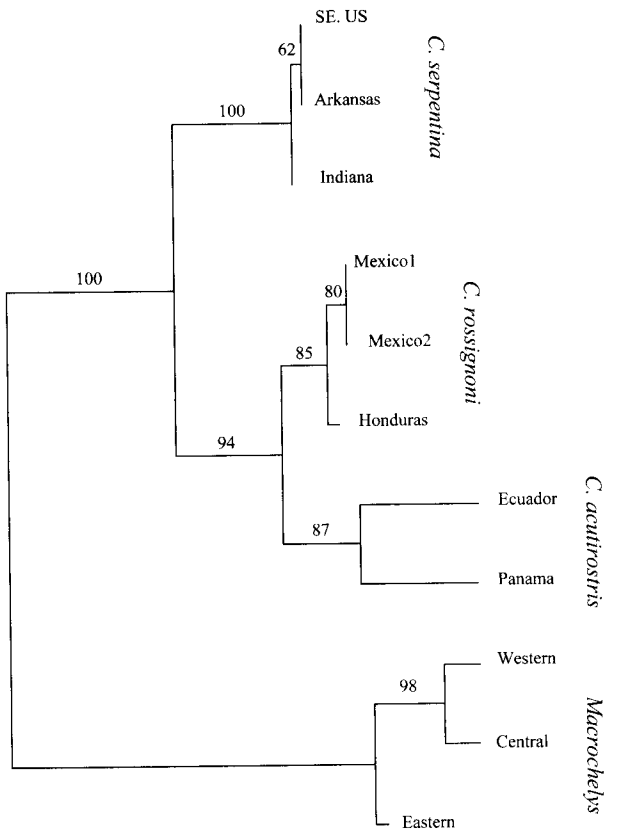

Fig. 4.1. Phylogram showing relationships of all unique sequences found in *Chelydra* and *Macrochelys*, based on 428 (462 including primers) base pairs of the mtDNA CR. The tree is a maximum likelihood reconstruction using the model HKY+Γ, with a–ln L = 1131.65. The following parameters were calculated from the data set using Modeltest 3.0 (developed by D Posada and KA Crandall in 2001): $t_i/t_v = 2.5462$, $\Gamma = 0.3921$, a = 0.2988, c = 0.2137, g = 0.1293, and t = 0.3582. Branch lengths are proportional to levels of mtDNA sequence divergence. Numbers above nodes are bootstrap proportions based on 1,000 bootstrap pseudoreplications. *C. s. osceola* is not shown because it was identical with the most common *C. s. serpentina* sequence.

(Mississippi), central (Apalachicola), and eastern (Suwannee) portions of the range. Roman et al. (1999) noted that the level of differentiation that they observed is similar to that found among species of map turtles in the same region and suggested that three evolutionarily significant units (ESUs) exist within *Macrochelys*. However, they did not recommend taxonomic recognition of these ESUs.

Why might the level of diversity be so different within *Chelydra* versus *Macrochelys* in the United States? They are both extremely aquatic turtles that can attain great size and age (Ernst et al., 1994). The range of the common snapping turtle extends much further north than that of the alligator snapper, and at least some of this range must represent post-Pleistocene expansion into previously glaciated habitat. While such a recent range expansion may explain the lack of genetic variation in the northern range of *Chelydra*, it does not explain the extraordinarily low levels found in the south where both *Chelydra* and *Macrochelys* coexist. Although both turtles are normally aquatic, common snappers display a much greater propensity for overland movement, which may result in greater gene flow among aquatic habitats. This difference in terrestrial ecology may explain the low levels of among-drainage differentiation in *Chelydra* compared with *Macrochelys*. However, even complete panmixia would not explain the lack of genetic diversity within and among populations of U.S. *Chelydra*. Two possible explanations are a massive, recent range expansion across the entire U.S. *Chelydra* population, or a selective sweep on the mitochondrial genome. Selective sweeps, where natural selection for a single haplotype leads to a specieswide fixation of that favored haplotype and a drastic reduction in genetic variation (Galtier et al. 2000; Przeworski 2002), is a particularly important mechanism for mtDNA because the entire molecule is a single recombinational unit. Differentiating between range expansion and selective sweeps requires data from nuclear genes, and we are currently collecting these data in our laboratory.

IS *PLATYSTERNON* A CHELYDRID?

More than 50 years after Williams's (1950) synthetic analysis of the higher-order phylogeny of the living turtles, the relationships of the big-headed turtle *Platysternon megacephalum* remain as controversial as ever. In many ways, the placement of this enigmatic species requires the full resolution of cryptodire relationships, since it appears to be an ancient lineage with no particularly close living relatives. Although we remain uncertain of the relationships of this key species, recent molecular evidence seems to be shedding light on parts of this deep phylogenetic puzzle, and the hope for future resolution is promising.

Three recent studies at the DNA sequence level have provided new insights on the possibility of a sister-group relationship of *Platysternon* with the New World Chelydridae, as proposed by Gaffney (1975) based on morphological evidence. Shaffer et al. (1997) examined two mitochondrial genes across 23 taxa spanning the breadth of living turtle families. They used 892 base pairs of the cytochrome *b* gene to provide resolution for the shallower nodes of the tree, whereas 325 base pairs of the slowly evolving 12S ribosomal gene were aimed at resolving deeper nodes within the Testudines. Both alone and in combination, the two genes provided little insight into the relationships of *Chelydra* and *Platysternon* to each other or to other turtles, with all relevant bootstrap proportions (BPs) <50 (see fig. 4a–c in Shaffer et al. 1997). Although statistical support was poor, the combined gene analysis placed *Chelydra* and *Platysternon* together, suggesting that weak molecular support for this relationship may exist. Alternatively, the weak support of these two relatively isolated lineages may reflect a "long branch attraction" problem in the molecular data. When combined with a data set of 115 morphological characters, the final molecules-plus-morphology analysis strongly supported (BP = 94) a sister-group relationship for *Chelydra* and

Platysternon, although this relationship was driven largely by the morphological data.

In a more detailed taxonomic sampling strategy, Starkey (1997) examined phylogenetic relationships among 72 recognized genera, and 230 species/subspecies of living turtles for a 991–base-pair fragment of the mitochondrial ND4 gene plus three adjacent tRNAs. Like the mitochondrial results in Shaffer et al. (1997), Starkey (1997) found little resolution for the relationships of *Platysternon* and the New World Chelydridae, apparently due to saturation of this relatively rapidly evolving mitochondrial gene. Two key results did emerge from Starkey's work, however. First, *Platysternon* is extremely divergent from all other turtles, and was reconstructed as a long, isolated branch near the base of the Cryptodira. Second, *Platysternon* and the New World Chelydridae showed no evidence of a sister-group relationship. Rather, the New World Chelydridae is sister (with a reasonable support, BP = 72) to Kinosternidae plus Dermatemydidae, and *Platysternon* is sister (BP <50) to the remaining cryptodires. Starkey's data are similar to Bickham and Carr's (1983) hypothesized relationships of *Platysternon* as close to the clade consisting of Testudinidae, Bataguridae (=Geoemydidae), and Emydidae (Testudinoidea of Shaffer et al. 1997), although Starkey's hypothesis also includes the marine turtles in this latter clade.

Both Shaffer et al. (1997) and Starkey (1997) used mtDNA for their sources of molecular data, and found little support at the deep nodes where the resolution of *Platysternon* relationships appear to reside. A recent analysis of 2,793 base pairs of the nuclear RAG-1 gene (Krenz et al. unpublished) for essentially the same set of taxa as in Shaffer et al. (1997) is a major addition to this literature. RAG-1 is a slowly evolving, single-copy, protein-coding gene that has proved very useful as a phylogenetic tool for deep relationships of birds and mammals, and provides a clean phylogenetic signal for the relationships of *Platysternon* to other turtle clades. Although Krenz et al. (unpublished) could not say with certainty the precise placement of *Platysternon* or the New World Chelydridae, they were able to reject (with reasonably strong statistical support) the hypothesis that the two form a monophyletic group. Based on RAG-1, *Platysternon* appears to be allied to the Testudinoidea, whereas *Chelydra* is close to a clade consisting of Kinosternidae, *Dermatemys*, and the marine turtles. This result is very similar to that of both Starkey (1997) and Bickham and Carr (1983), and is certainly not in conflict with the molecular results of Shaffer et al. (1997). However, the RAG-1 data are still not capable of toppling the morphological evidence suggesting a sister-group relationship of the New World Chelydridae and *Platysternon*, and the combined evidence tree still marginally supports the morphological view with weak bootstrap support (Krenz et al. unpublished).

To summarize the available molecular data, we constructed a supertree based on the molecular results of Bickham and Carr (1983), Shaffer et al. (1997), Starkey (1997), and Krenz et al. (unpublished). The resulting supertree, which is a summary of the topological relationships of the four input trees, is presented in Fig. 4.2. The key result is the distant relationship of *Platysternon* and *Chelydra* that consistently appears in most molecular analyses.

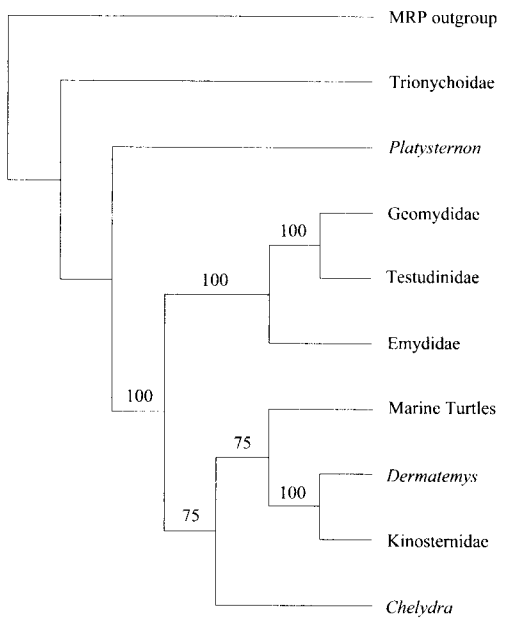

Fig. 4.2. A majority rule consensus of four equally parsimonious supertrees of some of the major clades of living turtles. For input trees, we used chromosomal data (Bickham & Carr 1983, their fig. 3), combined mtDNA from cytochrome *b* and 12S (Shaffer et al. 1997. their fig. 4c), mtDNA from ND4 plus adjacent tRNAs (Starkey 1997; new analysis based on the 23 taxa in Shaffer et al. 1997), and RAG-1 (Krenz et al. 2005; their fig. 5). We used the matrix representation with parsimony (MRP) supertree consensus method calculated in the program RadCon 1.1.5, and constructed a maximum parsimony tree in PAUP*. Numbers refer to the percentage of four equally parsimonious supertrees in which a given node was found. Trionychoidae is the monophyletic group including Trionychidae plus Carettochelyidae (Shaffer et al., 1997). Note that *Platysternon* and *Chelydra* are not sister taxa.

CLASSIFICATION RECOMMENDATIONS AND FUTURE RESEARCH DIRECTIONS

Within the genus *Chelydra*, existing mtDNA and allozyme data point to extremely weak differentiation between the U.S. subspecies *C. s. serpentina* and *C. s. osceola*. With less than a quarter of a percent sequence divergence in the rapidly evolving control region, and a minimal Nei's D of 0.05 (Table 4.1), no molecular evidence supports the recognition of these two taxa. Our broad sampling of mtDNA, combined with the published, detailed population-level analysis of southeastern U.S. populations all argue for a surprising lack of variation in U.S. *Chelydra*. Whether this eliminates *C. s. osceola* as a "good" subspecies (whatever that may mean) that is distinct from *C. s. serpentina* is still open to debate, and the final decision must include a careful analysis of mor-

phological variation (Feuer 1971). However, the molecular data imply that *C. s. osceola* and *C. s. serpentina* are not well-differentiated evolutionary lineages.

The situation for *C. s. acutirostris* and *C. s. rossignoni* is quite different. MtDNA and allozyme data both demonstrate deep differentiation between *C. s. acutirostris* and all other *Chelydra,* with diagnostic characters and an overall level of differentiation that is frequently found among well-differentiated turtle species. The same is true for *C. s. rossignoni* based on mtDNA sequence data, but not allozymes. Given that these taxa have allopatric distributions, we favor species designations based on character differentiation rather than inferred interbreeding. Based on both mitochondrial and allozyme evidence, *C. s. acutirostris* appears to qualify as a good phylogenetic species. One reasonable solution is to withhold judgment on the status of *C. s. rossignoni* pending additional nuclear DNA evidence, given the lack of allozyme differentiation between it and *C. s. serpentina.* Alternatively, the relatively deep mtDNA differentiation and allopatric distribution both argue that *C. s. rossignoni* is a separate evolutionary lineage, and therefore is best viewed as a separate species as well.

Based on currently available molecular evidence, we favor recognizing a monotypic, widespread *C. serpentina* across the continental United States and southern Canada, and abandoning *C. s. osceola* as an evolutionary entity. We recognize that there may be morphological evidence indicating that the Florida populations are somewhat differentiated, and when a full morphological analysis is complete, there may be sound reasons to recognize *C.* s. *osceola* at either the specific or subspecific levels. However, the available molecular data do not indicate any substantial differentiation between these taxa. We further recommend elevating the Central and South American forms to full species status as *C. acutirostris* and *C. rossignoni.* This was the recommendation of Phillips et al. (1996) based on incomplete sampling, and it has been substantiated and enhanced, at least for mtDNA, with additional samples. We concur with Sites and Crandall (1997) that additional sampling of *C. acutirostris* and *C. rossignoni* would be extremely valuable, and we provide limited additional sampling in Fig. 4.1. In total, the molecular evidence currently in hand is consistent with the three-species interpretation advocated by Phillips et al. (1996).

At the family level, the molecular evidence available strongly supports the interpretation of *Chelydra* and *Macrochelys* as sister taxa, and their placement in a monophyletic Chelydridae is clearly warranted. The situation for *Platysternon* is less clear, and the resolution of the molecular and morphological conflict over the relationship of *Platysternon* requires additional data and analysis. However, the available molecular evidence points to *Platysternon* as allied with the Testudinoidea, not Chelydridae. The prudent choice at this point is probably to place *Platysternon* in a monotypic family Platysternidae pending additional information. If and when the phylogenetic placement of *Platysternon* stabilizes, then we would favor relegating it to a more inclusive family that reflects its phylogenetic relationships, rather than placing it in a monotypic family that simply emphasizes its uniqueness.

Both for understanding relationships within *Chelydra,* and in particular for the resolution of the placement of *Platysternon,* multigene nuclear data are clearly vital. For relationships within *Chelydra,* the key issue centers on the validity of *rossignoni* and *acutirostris* as distinct from *serpentina.* The possibility that *osceola* is a real evolutionary lineage still should be considered, although it appears less likely based on mtDNA and allozymes. We are currently sequencing several nuclear introns, and they should shed light on this set of problems. At the deep phylogenetic level of *Platysternon* relationships, the RAG-1 data imply that nuclear exons may be informative, and unpublished intron data from our lab also appears promising. All available evidence currently points to *Platysternon* and Chelydridae as critical phylogenetic taxa that will require considerable sequence data before we fully understand their relationships to each other and other turtle lineages. In addition, if the nonmonophyly of these two taxa is even more strongly supported with additional molecular evidence, understanding the apparent homoplasy in several morphological characters that have previously been interpreted as homologies emerges as a fascinating problem in morphological evolution.

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