# Molecular phylogenetics and phylogeography of North American softshell turtles (*Apalone*)

by

David William Weisrock

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Graduate College
Iowa State University

This is to certify that the Master's thesis of

David William Weisrock

has met the thesis requirements of Iowa State University

Signatures have been redacted for privacy

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# INTRODUCTION

Testing the concordance of molecular and morphological characters in phylogenetic analysis has become an important focus in systematics (Larson, 1994; for examples, see Shaffer et al., 1991; Sites et al., 1996). Traditional systematic studies were solely based upon morphology and behavior and the resulting taxonomy reflects these characters. The advent of molecular biology techniques opened the door to a large number of characters that lie close to (i.e., allozymes) and at the heart (i.e., RFLPs and DNA sequence) of genetic change. The use of these molecular characters in theoretical and empirical phylogenetic analyses has been adopted widely (Hillis and Moritz, 1990; Avise, 1994). Molecular techniques have the benefit of generating data that are relatively independent of the environment and provide variation on a much finer scale than do morphological approaches. This allows for systematic studies of both macroevolutionary trends, and population level processes. These benefits of molecules do not preclude the use of morphology as a tool in phylogenetics because valuable information still exists in such characters and because a multi-faceted approach is essential for exploring evolutionary aspects such as species distinctions and taxonomy. The use of molecules in phylogenetics is also not without its own problems (i.e., homoplasy and lineage sorting). Because the majority of taxonomy is based on traditional morphological analyses, it is essential to corroborate postulated relationships with the use of molecular data. Assessing the concordance between these two types of information should be an important goal of systematists in taxonomic issues.

The use of DNA, in particular mitochondrial DNA (mtDNA) sequence, not only benefits the analysis of macroevolutionary relationships, but also aids in the systematic study of intraspecific taxa (Moritz et al., 1987). The ability to sample multiple populations from the extant range of a species can aid evolutionary biologists in a number of ways, from uncovering cryptic species that either lack substantial amounts of morphological variation or display some

level of convergence (Bruna et al., 1995) to understanding historical biogeographical events and dispersion of taxa associated with their intraspecific phylogenetic patterns (Avise et al., 1987). By analyzing the size and patterns of genetic variation within a species, one can begin to understand the past processes affecting it. For example, the lack of genetic variation among intraspecific populations in the northern part of the U.S. could be due to rapid range expansion following Pleistocene glacial recessions. Conversely, given what is known about the recent times of glacial retreats, the presence of substantial genetic structure among populations could indicate low dispersal rates and lack of gene flow. This approach to intraspecific phylogenetics has been utilized in a large number of studies of North American vertebrate taxa including salamanders (Phillips, 1994), fish (Turner et al., 1996), turtles (Walker et al., 1997), birds (Zink, 1996) and mammals (McKnight, 1995).

Molecular phylogeography (Avise et al., 1987; Avise, 1994) has been the theme of many recent publications and many of these studies have focused on herpetological systems. Most of the large scale efforts in turtles have been directed towards evaluating the global dynamics of sea turtles (Bowen et al., 1992; Bowen et al., 1994). Desert (*Xerobates*) and gopher (*Gopherus*) tortoises of the southwest and southeast U.S. (Lamb et al., 1989) and diamondback terrapins (*Malaclemys terrapin*) (Lamb and Avise, 1992) have also been studied moderately, but these species have limited ranges and cannot provide insight into the larger biogeographic history of the U.S. Map turtles (*Graptemys*) garnered attention in a systematic study of the entire genus (Lamb et al., 1994), which is widely distributed throughout the Mississippi, Missouri, and Ohio River drainages, but due to a very low level of interspecific variability, and an even lower amount of intraspecific variation, this study and system cannot elucidate broad scale U.S. biogeographical history. The most recent intraspecific studies of North American turtles focused on analyses of RFLPs in subspecies of snapping turtles (*Chelydra serpentina*) (Phillips et al., 1996) and in the stinkpot turtle (*Sternotherus odoratus*) (Walker et al., 1997). These studies have done much to further the understanding of

phylogeographic trends, but once again focus on relatively small sections of the greater U.S. and cannot provide insight into the more widely distributed turtle species.

Because of trends of minimal genetic differentiation detected in the above studies, turtles are thought to exhibit remarkably slow rates of mtDNA evolution. Evidence for this phenomenon has been found in some turtles (Avise et al., 1992; Lamb et al., 1994), but has not been surveyed in the majority of turtle families. However, the possibility of an evolutionary slowdown of mitochondria poses a problem for low level phylogenetic analyses of turtles in general, as mtDNA is the primary molecule used in such studies. Causes of variation in evolutionary rates have been attributed to intrinsic factors such as DNA repair mechanisms, generation time (Bromham et al., 1996), metabolic rates and thermal environments (Rand, 1994). Extrinsic forces, including population size and selection, also can have effects. In turtles, generation time seems particularly appealing as an explanation for slow rates of molecular evolution due to the long life spans of most turtles. However, designating all turtles as "slow evolvers" might be somewhat premature due to the relatively small number of families actually studied. Additionally, evolutionary rates have been shown to be quite variable at many levels of taxonomic rank (Li et al., 1987; 1990; Fieldhouse et al., 1997), highlighting the need to study many diverse members within families. Consequently, more data on additional and diverse families within the Testudines are needed before such a theory could be confidently applied to turtles in general.

**The Study System**

North American softshell turtles (*Apalone*) provide an excellent system with which to address many of these issues. The taxonomic history of New World softshells is diverse and, through the process of lumping and splitting, this group has acquired many different genera and species names over the years (Stejneger, 1944). The genus is currently divided into 3 species. The spiny (*Apalone spinifera*, LeSueur) and smooth (*Apalone mutica*, LeSueur)

softshells have very large, concordant ranges that span in the east from Quebec south into Florida and in the west from Montana south into Mexico. The third species, the Florida softshell (*Apalone ferox*, Schneider), has a distribution throughout Florida and its northern periphery.

Although spiny and smooth softshells occur in sympatry throughout much of their range, they are thought to occupy separate ecological niches. A study of softshells in Iowa concluded that smooth softshells inhabit only the larger areas of major rivers and streams whereas spinys occupy rivers, streams, and lentic waters (Williams and Christensen, 1981). Other differences existed in feeding behaviors and preferences, thermal preferences, and basking behaviors. Spiny softshells are typically more common throughout the U.S. and their distribution extends farther than those of *A. mutica*. *Apalone ferox* appears to share ecological preferences with *Apalone spinifera*. Complete descriptions of the natural history of *A. ferox* are given in Webb (1962), but no actual comparative studies are known.

Softshell turtles have a fossil record in North America that extends back into the late Cretaceous [approximately 80-65 million years ago (Mya)] (Holman, 1995). Closely related taxa to *Apalone* are thought to have been around since the Paleocene (approximately 65-55 Mya) (Holman, 1995). Fossil information for the first presence of any of the extant *Apalone* are non-existent. A smaller Pleistocene fossil record exists for *A. spinifera* and *A. ferox*, but no fossils are known for *A. mutica*.

The taxonomic relationships of North American softshells have been subject to numerous studies and reviews (Neill, 1951; Schwartz, 1956; Webb, 1962; Meylan, 1987) and have recently gone through revisions. The three species of *Apalone* formerly fell under a larger genus, *Trionyx*, which contained both Old and New World taxa. Webb (1962) conducted a thorough study of all three species, characterizing the morphological variation found among and within the three species and using this information to speculate on relationships among the three species and to Old World taxa. A cladistic analysis of the entire Trionychid family using

osteological characters (Meylan, 1987) provided evidence for the monophyly of the three New World species and placed them in their own genus, resurrecting the historical name *Apalone* (Rafinesque, 1832). Meylan further split the three species by including *A. spinifera* and *A. mutica* into the subgenus *Apalone* and placing *A. ferox* into its own subgenus, *Platypeltis* (Fitzinger). These phylogenetic results corroborated Webb's hypotheses as well as most other previous thoughts on the interspecific relationships in this genus.

**The Investigative Approach and Scope**

Meylan's (1987) intrafamilial phylogenetic analysis represents the most complete investigation of New World softshell turtles. Still, the splitting of *Trionyx* is not accepted by the entire herpetological community (e.g., Ernst et al., 1994; Webb, 1990), but until additional studies show otherwise and following the belief that taxonomy should follow phylogeny, I will use Meylan's phylogenetic approach identifying these turtles as *Apalone*.

At the intraspecific level, both *A. mutica* and *A. spinifera* are split into subspecies according to morphological variation across their geographic ranges (Webb, 1973a, b). In this paper however, I will not emphasize subspecific taxonomy, but rather will focus my attention on multiple geographic localities across the U.S. from which I sampled all three species. The goal of this wide scale sampling was to assess the patterns of mtDNA sequence variation across a large geographic region to understand how past biogeographic events and the dispersal abilities of these highly aquatic, riverine tetrapods have influenced the partitioning of their genetic variation.

As discussed above, one concern of an intraspecific analysis of mtDNA in turtles is the evidence suggesting that their mtDNA evolves unusually slowly relative to other vertebrates (Avise et al., 1992; but see also Martin et al., 1992). Thus, given the relatively recent recession of the Wisconsin ice sheet [approximately 15 thousand years ago (Kya)] at the end of the Pleistocene, slow evolutionary rates may not provide the necessary variation in mtDNA

needed to understand the phylogeography of northerly-distributed turtles like *Apalone*. The most recent evidence of such problems was encountered in an interspecific systematic study of the genus *Graptemys* (Lamb et al., 1994). Even among species in that study, very little variation at cytochrome *b* and control region loci was detected. This phenomenon would be even further magnified in a study at the intraspecific level. However, of all the studies in which these slow rates have been detected, none have used taxa representing the Trionychidae.

In this thesis I present the results of both an interspecific study of the genus *Apalone*, through the use of cytochrome *b* mtDNA and 12s rDNA mtDNA sequences, and an extensive intraspecific study of the genus *Apalone*, through analysis of cytochrome *b* mtDNA sequence. Both parsimony and distance-based analyses were directed towards three main objectives: 1) testing the hypotheses of interspecific relationships of *Apalone* proposed by morphological phylogenetic analyses, 2) assessing the patterns of genetic variation on an intraspecific level to address the phylogeographic history of the three *Apalone* species, and 3) evaluating the evolutionary rates of mtDNA in Trionychids and comparing these to other families of turtles to test the theory that turtles in general evolve at a "turtle's pace" (Avise et al., 1992).

# MATERIALS AND METHODS

## DNA Extraction and Sequencing

Tissue was collected from both laboratory-raised and field-collected turtles from across a large portion of the distribution of all three *Apalone* species (Fig. 1; Table 1). Samples used came in a variety of forms: skin wedges from the carapace, muscle, blood samples stored in lysis buffer, and liver. Genomic DNA was isolated from 27 individuals covering 16 different sampling locations and a *Trionyx triunguis* outgroup using a Proteinase K/SDS digestion and phenol/chloroform extraction method (Hillis and Moritz, 1990). Purified DNA was used in an initial PCR for both cytochrome *b* and 12s under the following thermal conditions: 95°C denature for one minute, 50°C anneal for 1 minute, and 72°C extension for two minutes for 35 cycles. PCR was conducted in 25µl volumes with 0.5-1.0 µg DNA, 1X PCR buffer (Tris-HCl, 1.5mM MgCl2 and 50 mM KCl), 0.1 mM dNTPs, 1.0 µM primers and 1 unit *Taq* polymerase (Boehringer Mannheim). Primers were developed to amplify an 800 base pair fragment of the mitochondrial cytochrome *b* gene. The forward primer (DW 2000; 5' ACA GGC GTA ATC CTA CTA A 3') was developed in the Janzen laboratory. The reverse primer sequence (DW 1594 5' TCA TCT TCG GTT TAC AAG AC 3') was obtained from M.L. McKnight (pers. comm.). The 5' end of DW 2000 corresponds to position 16594 of the *Xenopus* mtDNA genome (Roe et al., 1985) and the 3' end of DW1594 corresponds to position 17418. Universal primers (L1091 and H1478) were used for amplification of an approximately 400 base pair fragment of mitochondrial 12s rDNA (Kocher et al., 1989). The 12s primer numbers refer to their placement in the mitochondrial genome.

PCR product was run on a 1.5% low melt agarose TBE gel and the target DNA fragments were excised from the gel. The low melt fragment was suspended in 1 ml $dH_2O$ and

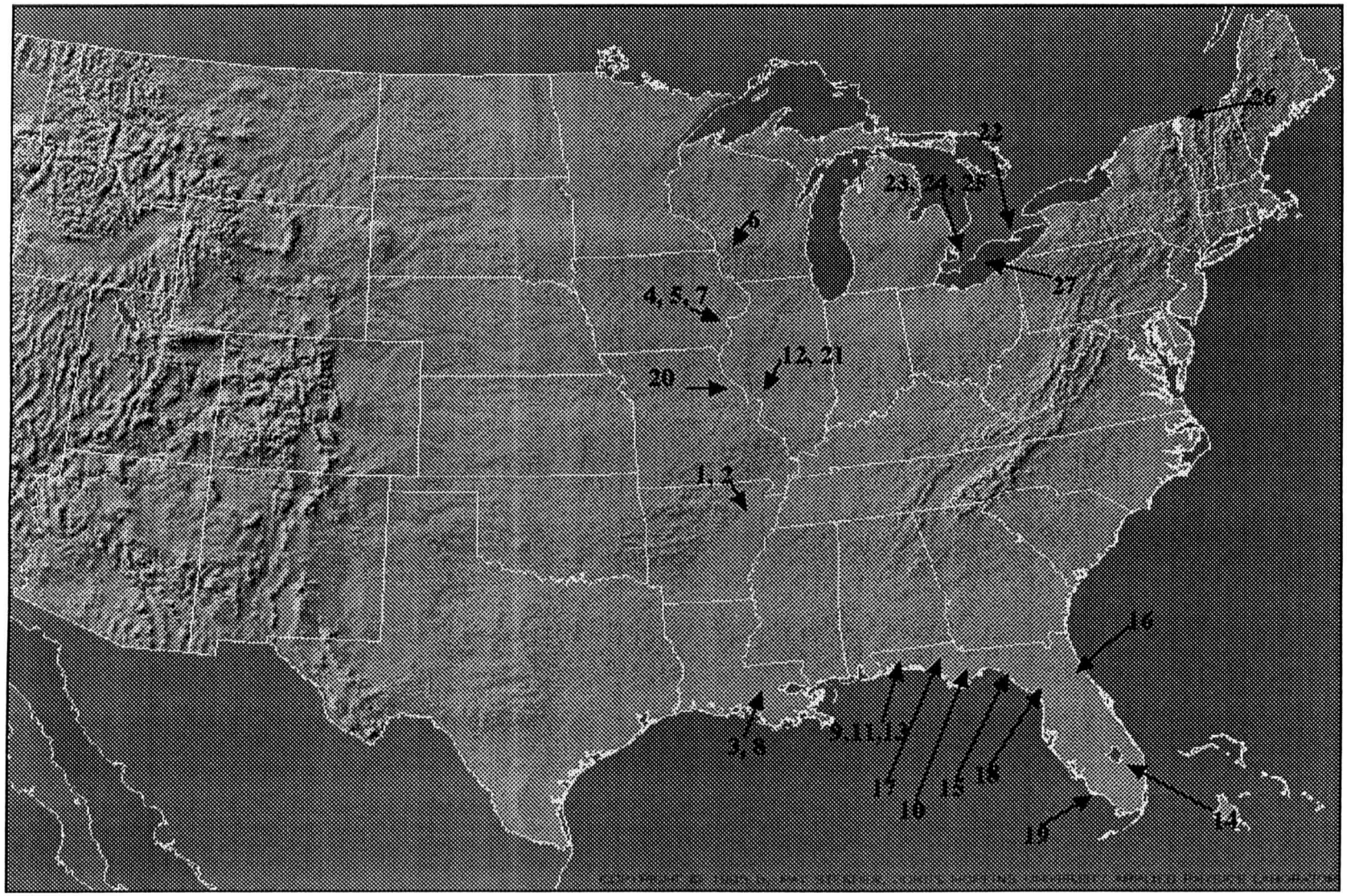


Fig. 1. Distribution of sampled populations included in this study. The numbers refer to the specific taxa and collecting locales listed in Table 1.

Table 1. Taxa and locale information for all *Apalone* samples included in the analyses.

| Sample | Taxa | Locale Information |
|---|---|---|
| 1 | *A. mutica* | White Co., Arkansas; White River near Georgetown |
| 2 | *A. mutica* | White Co., Arkansas; White River near Georgetown |
| 3 | *A. mutica* | East Baton Rouge Parish, Baker, Louisiana; Comite River at Highway 64, Comite Drive, and Dyer Road; 30° 30'N, 91 ° 04' W |
| 4 | *A. mutica* | Muscatine Co., Iowa; Near Weise Slough on Cedar River |
| 5 | *A. mutica* | Muscatine Co., Iowa; Near Weise Slough on Cedar River |
| 6 | *A. spinifera* | Vernon Co., Wisconsin; Pool 8 of Mississippi River near Stoddard |
| 7 | *A. spinifera* | Muscatine Co., Iowa; Near Weise Slough on Cedar River |
| 8 | *A. spinifera* | East Baton Rouge Parish, Baker, Louisiana; Comite River at Highway 64, Comite Drive, and Dyer Road; 30° 30'N, 91 ° 04' W |
| 9 | *A. spinifera* | Escambia Co., Florida; Escambia River near Florida-Alabama state line |
| 10 | *A. spinifera* | Liberty Co., Florida; Ochlocknee River, Whitehead Landing in Apalachicola National Forest Preserve |
| 11 | *A. mutica* | Escambia Co., Florida; Escambia River just north of state road 4 |
| 12 | *A. spinifera* | Madison Co., Illinois; Stump Lake near Alton |
| 13 | *A. spinifera* | Escambia Co., Florida; Escambia River just east of Century |
| 14 | *A. ferox* | Plam Beach Co., Florida |
| 15 | *A. ferox* | Lafeyette Co., Florida; Suwanee River |
| 16 | *A. ferox* | Volusia Co., Florida; De Leon Springs, Spring Garden Lake |
| 17 | *A. ferox* | Calhoun Co., Florida; Apalachicola River east of Blounstown |
| 18 | *A. ferox* | Marion Co., Florida; Rainbow Run near Dunnelon |
| 19 | *A. ferox* | Collier Co., Florida; US Highway 41, 3 miles east of junction with state road 29 |
| 20 | *A. spinifera* | St. Charles Co., Missouri; Airport Slough, near Mississippi River mile 220 |
| 21 | *A. spinifera* | Madison Co., Illinois; Piasa Island, near Mississippi River mile 210 |
| 22 | *A. spinifera* | Ontario, Canada; Long Point Provincial Park, Lake Erie |
| 23 | *A. spinifera* | Ontario, Canada; Thames River |
| 24 | *A. spinifera* | Ontario, Canada; Sydenham River |
| 25 | *A. spinifera* | Ontario, Canada; Thames River |

Table 1. (Continued)

| Sample | Taxa | Locale Information |
|---|---|---|
| 26 | *A. spinifera* | Quebec, Canada; Chapman Bay, Lake Champlain |
| 27 | *A. spinifera* | Ontario, Canada; Rondeau Provincial Park, Lake Erie |

heated at 95°C for 5 minutes. This mixture was then used as template in a second PCR to generate double stranded DNA for sequencing. The second PCR product was run on a 1% TBE agarose gel and the band was excised. DNA was purified from the gel slice with a 0.22 Micropure separator (Amicon) and then concentrated in an M-100 microconcentrator (Amicon). Template was sequenced at the Iowa State University DNA Sequencing Facility on an ABI PRISM model 377 automated sequencer. Double stranded DNA fragments were sequenced from both directions with the original PCR primers to verify the integrity of the sequence for each individual.

**Phylogenetic Analysis**

Forward and reverse sequences were assembled into a contiguous fragment with the use of Sequence Navigator version 1.0.1 (©Applied Biosystems, 1994). All sequences were subsequently aligned with the program Clustal W for the Power PC version 1.5 (Thompson et al., 1994). Phylogenetic parsimony and Neighbor-Joining (Saitou and Nei, 1987) analyses were performed with PAUP version 4.0.0d54 (Swofford, 1997). All trees were rooted with the homologous sequence from the African softshell turtle (*Trionyx triunguis*). *Trionyx triunguis* is an Old World species that is hypothesized to have a close ancestral relationship to members of the genera *Rafetus* and *Apalone* (Meylan, 1987).

Interspecific relationships of *Apalone* were assessed through the exhaustive search method on both cytochrome *b* sequence from a limited subset of populations for each species

and 12s sequence from a single representative individual of each species. Neighbor-Joining trees were constructed from both data sets utilizing uncorrected "p" distances.

Because of the large number of samples involved, a heuristic search option was used in the intraspecific analysis of a larger cytochrome *b* data set. In this case, the random addition option was utilized with 100 replicates. To account for possible intrapopulation variance, two samples from most populations were used in initial analyses. However, due to the lack of variation at this level, subsequent analyses typically used a single individual per locale. Neighbor-Joining trees were again constructed utilizing uncorrected "p" distances. The inter- and intraspecific taxa in these analyses were identical to those used in parsimony analysis.

Data were unweighted in both interspecific and intraspecific analyses. Bootstrapping (Felsenstein, 1985) was used to test the reliability of the data in finding the best tree. In all analyses bootstrapping was performed with 1000 replicates.

# RESULTS

## Interspecific Relationships

I obtained approximately 800 bases of cytochrome *b* sequence from 27 individuals and 400 bases of 12s sequence from one individual of each species. A *Trionyx triunguis* outgroup was also sequenced for both mtDNA loci. Interspecific analyses of a subset of the cytochrome *b* sequences and of the 12s sequences yielded similar trees both with a parsimony (Fig. 2A, 3A) and distance-based method (Fig. 2B, 3B). Maximum parsimony analysis produced single most parsimonious trees of 222 steps for the cytochrome *b* data set and 47 steps for the 12s data set. Both sets of analyses resolve *Apalone ferox* and *A. spinifera* as sister species. Neighbor-Joining trees including the same seven *Apalone* taxa and the *T. triunguis* outgroup for the cytochrome *b* data set and the 12s data set also resolve the sister relationship of *A. spinifera* and *A. ferox* with *A. mutica* as the outgroup taxon.

Sequence divergences for cytochrome *b* between *Apalone* and the outgroup *Trionyx triunguis* ranged from 15% (*A. ferox*) to 17% (*A. mutica* and *A. spinifera*). Sequence divergence between *A. mutica* and *A. spinifera* ranged from 7.5 % to 8.9%, between *A. mutica* and *A. ferox* from 7.3% and 7.8%, and between *A. spinifera* and *A. ferox* from 6.2% to 6.7%. Sequence divergence values were smaller for 12s, with *Apalone* being between 8.1% and 9.3% different than *T. triunguis*. Within *Apalone*, *A. mutica* had a sequence divergence of 3% with *A. spinifera* and 3.7% with *A. ferox*. *Apalone ferox* differed from *A. spinifera* by 2.2%.

I also compared the resulting interspecific trees with a parsimony tree constrained to the presently accepted interspecific relationships based on morphology. I constrained the cytochrome *b* parsimony tree by forcing *A. ferox* as the outgroup taxon to a sister clade of *A. mutica* and *A. spinifera*. This analysis produced a tree length of 224 steps, two steps longer

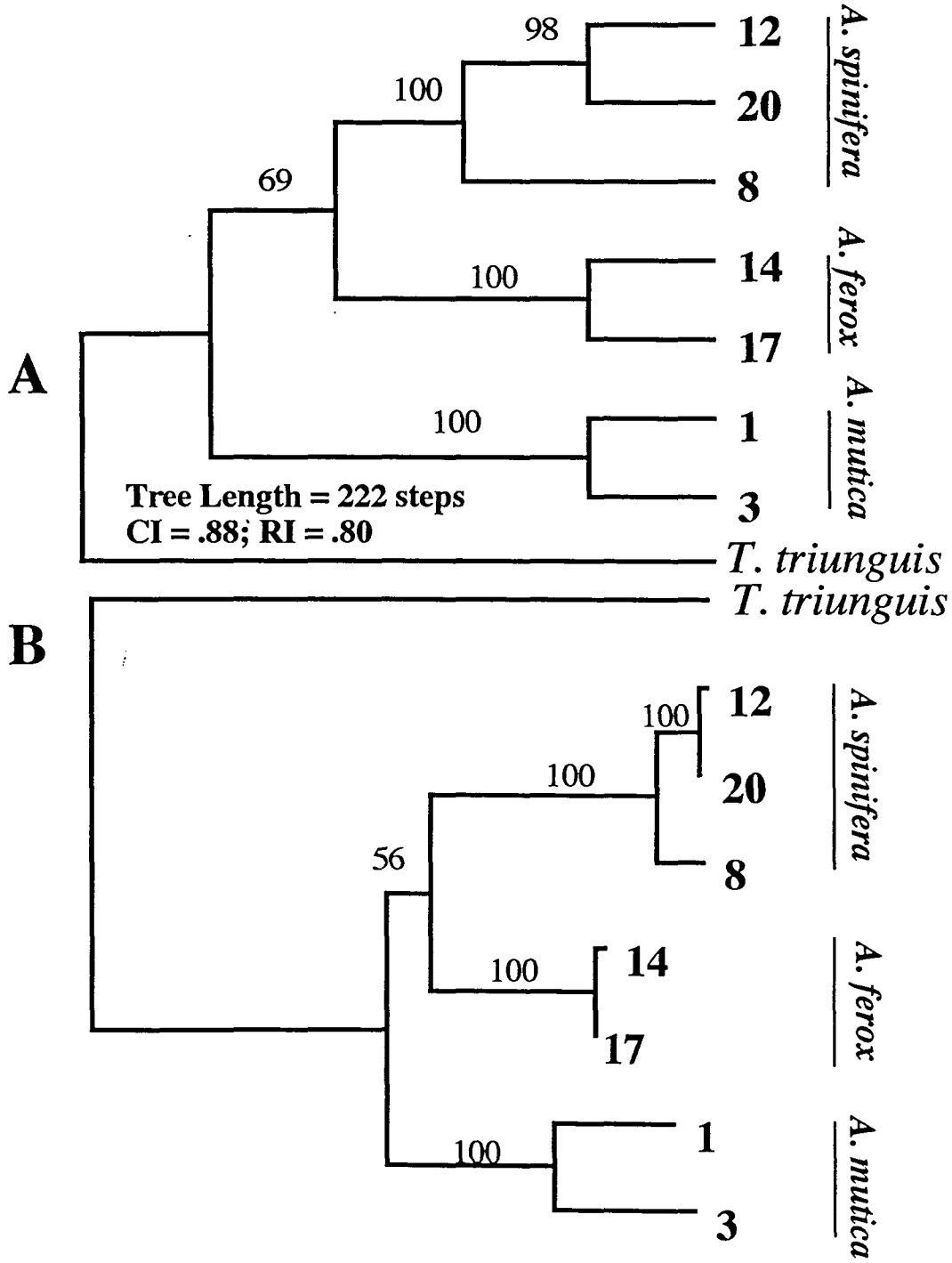


Fig. 2. Interspecific A) Parsimony tree and B) Neighbor-Joining tree for North American softshell turtles generated from 800 bases of cytochrome *b* mtDNA sequence. Numbers at ends of branches refer to sample numbers listed in Table 1. Numbers above branches indicate bootstrap level of support for the adjacent node. Consistency (CI) and retention (RI) indices for the parsimony analysis are listed below the tree length.

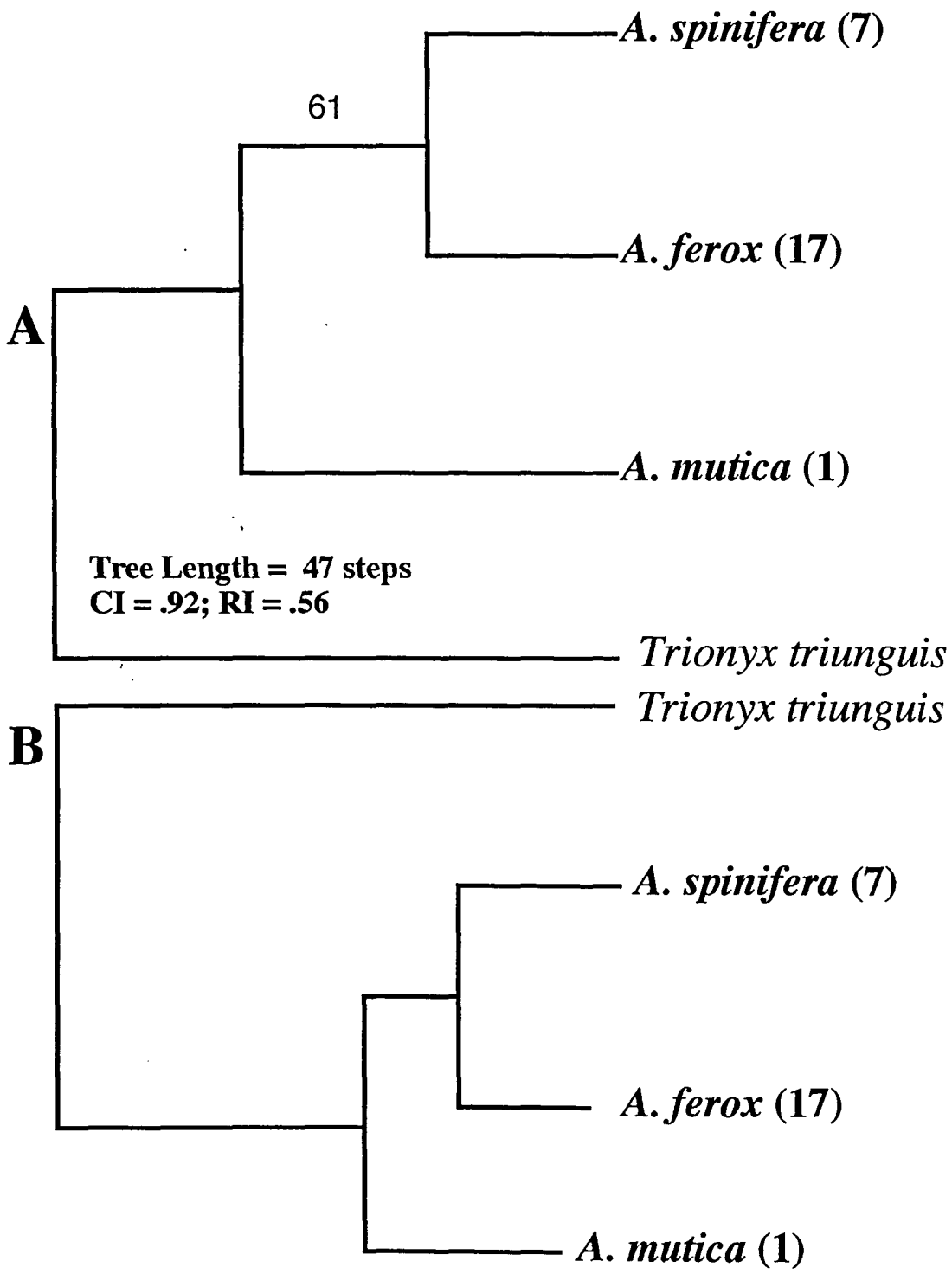


Fig. 3. Interspecific A) Parsimony tree and B) Neighbor-Joining tree for North American softshell turtles generated from 400 bases of 12s mtDNA sequence. Numbers in parentheses refer to the specific sample used (Table 1). Numbers above branches indicate bootstrap level of support for the adjacent node. Consistency (CI) and retention (RI) indices for the parsimony analysis are listed below the tree length.

than the most parsimonious tree generated in the unconstrained analyses (Fig. 2). When similar constraints were forced upon the 12s tree, the tree length increased one step.

Bootstrap analysis of the interspecific cytochrome *b* data set provides strong support for all nodes except for the break between *Apalone spinifera* + *A. ferox* and *A. mutica*. Strong support is judged as bootstrap values greater than 70% (Hillis and Bull, 1993).

**Intraspecific Relationships**

A heuristic parsimony analysis of 27 individuals representing multiple populations across the ranges of all three species produced four most parsimonious trees of 249 steps in length. A strict consensus of these results produced a tree (Fig. 4) that exhibits a significant amount of intraspecific structure and retains the interspecific relationships produced by the smaller, more exhaustive search (Fig. 2). The tree produced by Neighbor-Joining analysis (Fig. 5) produced identical interspecific relationships and very similar intraspecific relationships.

Within each species, varying amounts of genetic divergences were detected (Fig. 5; Table 2). The most substantial intraspecific divergence was found within *A. mutica*, where populations from the northern range (e.g., Arkansas and Iowa) are highly diverged from southern populations (e.g., Louisiana and Florida) by almost 4%. This genetic break occurs between populations north of, and including, Arkansas and populations south of Arkansas. This pattern detected in *A. mutica* was equally evident, but less pronounced, within *A. spinifera*. In this case, the northern populations exhibited small divergences approaching 1.5% from the southern populations.

Lower levels of sequence divergence within these regional clades of *A. mutica* and *A. spinifera*, as well as within the limited range of *A. ferox*, were also detected (Fig. 5; Table 2).

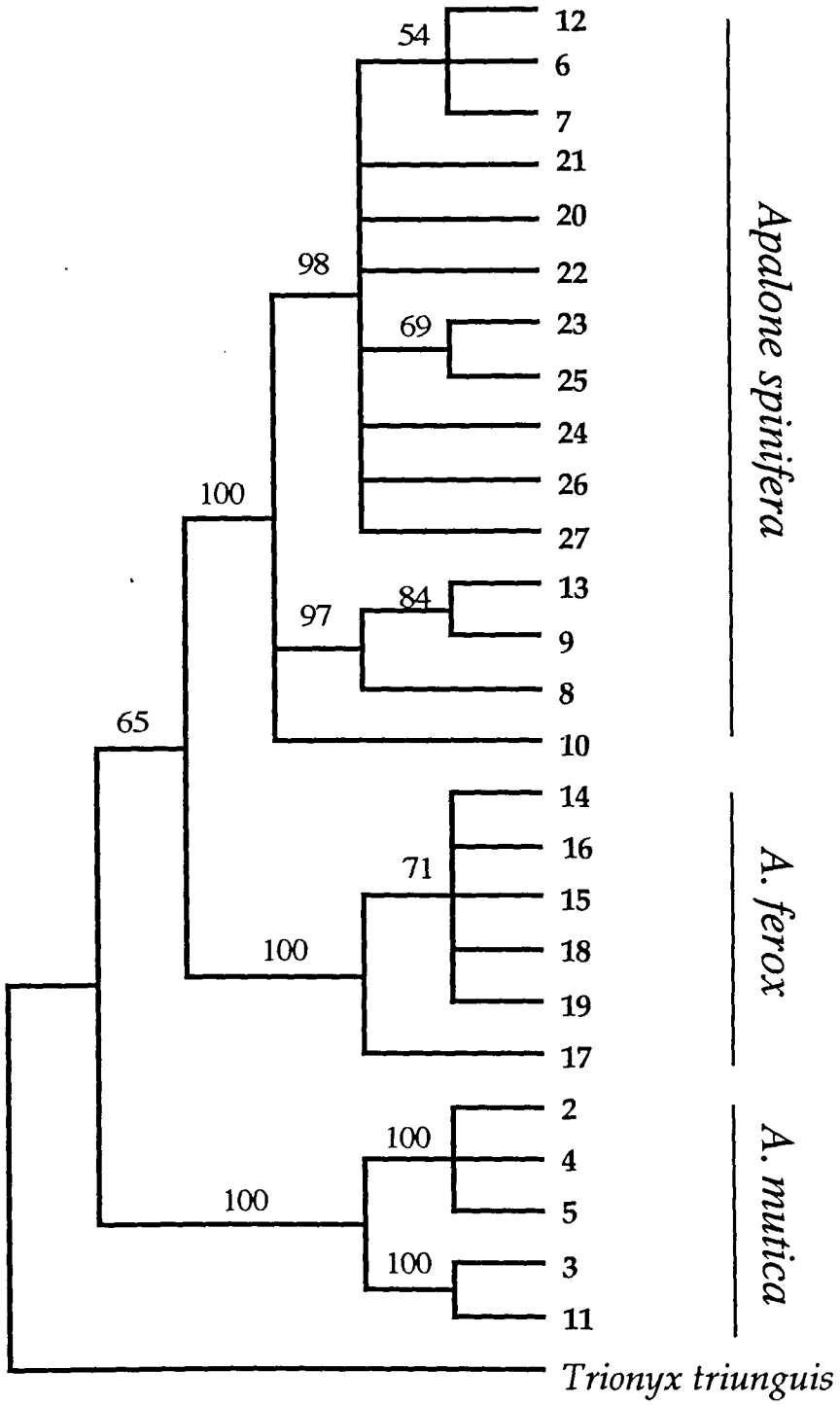


Fig. 4. Intraspecific strict consensus of four most parsimonious trees generated from 800 bases of cytochrome *b* mtDNA sequence. The tree length is 249 steps. The consistency and retention indices are .86 and .94 respectively. Numbers above branches indicate bootstrap levels of support for the adjacent node.

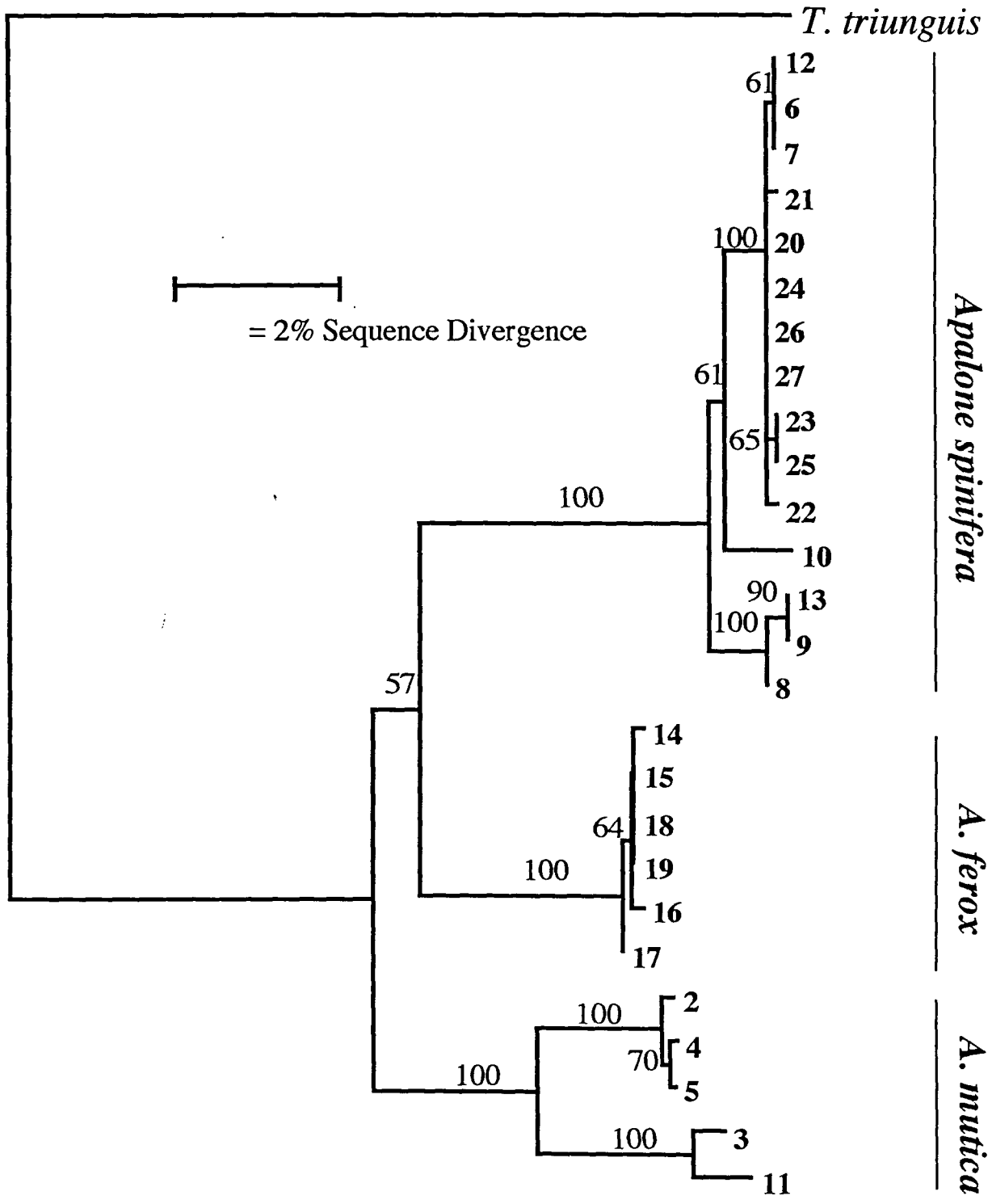


Fig. 5. Intraspecific Neighbor-Joining tree for North American softshell turtles generated from 800 bases of cytochrome *b* mtDNA sequence. Numbers above branches indicate bootstrap support levels for the adjacent node.

Table 2. Percent sequence divergence values for all taxa. Numbers on the top row and left column refer to taxa listed under Table 1.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2 | 0.0 | | | | | | | | | | | | | | | | | | | | | | | | | |
| 3 | 3.7 | 3.7 | | | | | | | | | | | | | | | | | | | | | | | | |
| 4 | 0.2 | 0.2 | 3.6 | | | | | | | | | | | | | | | | | | | | | | | |
| 5 | 0.4 | 0.4 | 3.7 | 0.1 | | | | | | | | | | | | | | | | | | | | | | |
| 6 | 7.7 | 7.7 | 8.5 | 7.8 | 7.7 | | | | | | | | | | | | | | | | | | | | | |
| 7 | 7.7 | 7.7 | 8.5 | 7.8 | 7.7 | 0.0 | | | | | | | | | | | | | | | | | | | | |
| 8 | 8.2 | 8.2 | 8.7 | 8.3 | 8.2 | 1.4 | 1.4 | | | | | | | | | | | | | | | | | | | |
| 9 | 8.2 | 8.2 | 8.7 | 8.3 | 8.2 | 1.6 | 1.5 | 0.2 | | | | | | | | | | | | | | | | | | |
| 10 | 8.3 | 8.3 | 8.9 | 8.4 | 8.3 | 1.4 | 1.2 | 1.7 | 2.0 | | | | | | | | | | | | | | | | | |
| 11 | 4.0 | 4.0 | 1.0 | 3.8 | 4.0 | 8.9 | 8.8 | 9.0 | 9.0 | 9.3 | | | | | | | | | | | | | | | | |
| 12 | 7.7 | 7.7 | 8.5 | 7.8 | 7.7 | 0.0 | 0.1 | 1.4 | 1.6 | 1.4 | 8.9 | | | | | | | | | | | | | | | |
| 13 | 8.2 | 8.2 | 8.7 | 8.3 | 8.2 | 1.6 | 1.5 | 0.2 | 1.6 | 2.0 | 9.0 | 7.8 | | | | | | | | | | | | | | |
| 14 | 7.5 | 7.5 | 7.8 | 7.4 | 7.5 | 6.5 | 6.4 | 6.4 | 6.7 | 6.3 | 7.9 | 6.5 | 6.7 | | | | | | | | | | | | | |
| 15 | 7.4 | 7.4 | 7.7 | 7.3 | 7.4 | 6.4 | 6.3 | 6.3 | 6.5 | 6.2 | 7.8 | 6.4 | 6.5 | 0.1 | | | | | | | | | | | | |
| 16 | 7.5 | 7.5 | 7.8 | 7.4 | 7.5 | 6.5 | 6.4 | 6.4 | 6.7 | 6.3 | 7.9 | 6.5 | 6.7 | 0.2 | 0.1 | | | | | | | | | | | |
| 17 | 7.3 | 7.3 | 7.5 | 7.2 | 7.3 | 6.3 | 6.2 | 6.2 | 6.4 | 6.0 | 7.7 | 6.3 | 6.4 | 0.2 | 0.1 | 0.2 | | | | | | | | | | |
| 18 | 7.4 | 7.4 | 7.7 | 7.3 | 7.4 | 6.4 | 6.3 | 6.3 | 6.5 | 6.2 | 7.8 | 6.4 | 6.5 | 0.1 | 0.0 | 0.1 | 0.1 | | | | | | | | | |
| 19 | 7.4 | 7.4 | 7.7 | 7.3 | 7.4 | 6.4 | 6.3 | 6.3 | 6.5 | 6.2 | 7.8 | 6.4 | 6.5 | 0.1 | 0.0 | 0.1 | 0.1 | 0.0 | | | | | | | | |
| 20 | 7.5 | 7.5 | 8.4 | 7.7 | 7.8 | 0.1 | 0.0 | 1.2 | 1.5 | 1.2 | 8.8 | 0.1 | 1.5 | 6.4 | 6.3 | 6.4 | 6.2 | 6.3 | 6.3 | | | | | | | |
| 21 | 7.7 | 7.7 | 8.3 | 7.7 | 7.7 | 0.1 | 0.2 | 1.4 | 1.6 | 1.4 | 8.7 | 0.2 | 1.6 | 6.5 | 6.4 | 6.5 | 6.3 | 6.4 | 6.4 | 0.1 | | | | | | |
| 22 | 7.7 | 7.7 | 8.5 | 7.8 | 7.7 | 0.2 | 0.2 | 1.4 | 1.6 | 1.4 | 8.9 | 0.2 | 1.6 | 6.6 | 6.4 | 6.6 | 6.3 | 6.4 | 6.4 | 0.1 | 0.2 | | | | | |
| 23 | 7.7 | 7.7 | 7.8 | 7.8 | 7.7 | 0.2 | 0.2 | 1.4 | 1.6 | 1.4 | 8.9 | 0.2 | 1.6 | 6.5 | 6.4 | 6.5 | 6.3 | 6.4 | 6.4 | 0.1 | 0.2 | 0.2 | | | | |
| 24 | 7.5 | 7.5 | 8.4 | 7.7 | 7.5 | 0.1 | 0.1 | 1.2 | 1.5 | 1.2 | 8.8 | 0.1 | 1.5 | 6.4 | 6.3 | 6.4 | 6.2 | 6.3 | 6.3 | 0.0 | 0.1 | 0.1 | 0.1 | | | |
| 25 | 7.7 | 7.7 | 8.5 | 7.8 | 7.7 | 0.2 | 0.2 | 1.4 | 1.6 | 1.4 | 8.9 | 0.2 | 1.6 | 6.5 | 6.4 | 6.5 | 6.3 | 6.4 | 6.4 | 0.1 | 0.2 | 0.2 | 0.0 | 0.1 | | |
| 26 | 7.5 | 7.5 | 8.4 | 7.7 | 7.5 | 0.1 | 0.1 | 1.2 | 1.5 | 1.2 | 8.8 | 0.1 | 1.5 | 6.4 | 6.3 | 6.4 | 6.2 | 6.3 | 6.3 | 0.0 | 0.1 | 0.1 | 0.1 | 0.0 | 0.1 | |
| 27 | 7.5 | 7.5 | 8.4 | 7.7 | 7.5 | 0.1 | 0.1 | 1.2 | 1.5 | 1.2 | 8.8 | 0.1 | 1.5 | 6.4 | 6.3 | 6.4 | 6.2 | 6.3 | 6.3 | 0.0 | 0.1 | 0.1 | 0.1 | 0.0 | 0.1 | 0.0 |

Among northern populations small amounts of sequence divergences were detected (Fig. 5; Table 2). Southern populations, however, displayed larger amounts of genetic divergences (Fig. 5; Table 2). This is particularly evident in *A. mutica* where populations from Louisiana and Florida have a sequence divergence of close to 1%. Similar east-west divergence patterns were detected in southern populations of *A. spinifera* and *A. ferox* (Fig. 5; Table 2).

The intraspecific relationships found with both tree-constructing analyses differ only in the placement of a Florida *A. spinifera* population (#10). The strict consensus parsimony tree leaves this population as part of an unresolved polychotomy with other assemblages *within A. spinifera* (Fig. 4), whereas the Neighbor-Joining tree places it as a sister group to the regional clade containing all the northern distributed samples (Fig. 5). Among the four most parsimonious trees, two place it in an ancestral position to all other *A. spinifera* assemblages, while the other two place it in the position found in the Neighbor-Joining tree.

Bootstrap analysis of the larger intraspecific cytochrome *b* data set provides similar values of strong support (>70%)(Hillis and Bull, 1993) for the interspecific relationships as found in the smaller cytochrome *b* and 12s data sets (Figs. 2, 3). Intraspecific bootstrap values decrease below the 70% level in some of the end nodes, probably as a result of smaller levels of character support.

# DISCUSSION

## Interspecific Phylogenetics

Previous systematic studies of North American softshell turtles have been solely based on morphology. The most recent study was performed by Meylan (1987) through the use of parsimony analysis on a suite of osteological characters that covered the entire Trionychid family. The study split apart the lumped genus *Trionyx*, revived the use of the genus name *Apalone* for North American softshells, and hypothesized relationships of the three species of that genus, with *A. ferox* as the outgroup to a sister clade of *A. mutica* and *A. spinifera*. Molecular evidence from this study verifies the monophyly of each of these species, but does not support Meylan's interspecific relationships. My results from multiple mtDNA loci and different phylogenetic analyses consistently resolve *A. mutica* as the outgrouped lineage to a sister clade of *A. ferox* and *A. spinifera*.

Despite the consistency of the phylogenetic results, the lengths of the molecular-generated parsimony trees change little when topological constraints are forced into the morphologically-proposed relationships. The parsimony tree produced by cytochrome *b* data (Fig. 2) only increases by two steps when the morphological based relationships were imposed upon it. A similar amount of change occurs in the 12s tree, which only increased in length by one step. The 12s parsimony tree is expected to give small differences due to the low amount of synapomorphic characters supporting the observed relationships as a function of the extremely conserved nature of the 12s locus. However, the cytochrome *b* data set should be expected to yield a significantly large difference in tree length under different, less parsimonious topological constraints.

Thus, the lack of a large difference in tree lengths signals the need for caution in constructing phylogenetic hypotheses. The small differences in tree lengths also suggest the

need for further phylogenetic testing utilizing evolutionary models that more accurately describe the loci used in this study.

Interestingly, although the data are inconsistent with Meylan's morphological study, they are concordant with two external morphological characteristics. Both *A. ferox* and *A. spinifera* share the presence of spiny bumps on the anterior end of the carapace. In *A. ferox* these bumps meld into a ridge that continues down the edges of the carapace. This ridge has apparently been lost or never developed in *A. spinifera*. These two taxa also share the presence of a small ridge extending from their nasal septum into the nasal cavity. Neither of these two characteristics are found in *A. mutica*. In addition to different morphology, *A. mutica* exhibits a behavioral preference for swift flowing riverine conditions whereas *A. ferox* and *A. spinifera* both utilize ponds, lakes and marshes as well as rivers. These morphological and behavioral traits pale in comparison to the large numbers of characters used in Meylan's (1987) study, but are still useful in supporting my large molecular data sets and in providing field characters.

Unfortunately, the lack of a complete fossil record for this genus prevents me from dating the first presence of any of these species. Thus, at this time I have no additional evidence to corroborate the phylogenetic findings. The disparity in results between the morphological and molecular analyses needs to be addressed with further studies, including molecular analysis of nuclear loci.

Because this study was limited only to North American softshells, the results provide little inference into the dynamics of *Trionyx-Rafetus-Apalone* relationships. Indeed, because the recent taxonomic revisions are controversial, it would be interesting to test the recent splitting of *Trionyx* with molecular methods. With the absence of *Rafetus* the present study has little power to do so. However, this study does provide evidence that lends support to the splitting of the group. Sequence divergences between the *Trionyx triunguis* outgroup and the three *Apalone* species range from 15% to 17%. These are large genetic differences and suggest that the divergence of these genera was a very long time ago. The genetic distances separating

these taxa, coupled with their Old World vs. New World zoogeographic placement, lend credence to the splitting of the former *Trionyx* genus into the present *Trionyx*, *Rafetus*, and *Apalone* (Meylan, 1987).

**Major North American Patterns of mtDNA Variation**

One of the most surprising discoveries of this study was the detection of large genetic subdivisions within *A. mutica* and *A. spinifera*, suggesting that regional assemblages within these two species have been separated for a long time. These subdivisions are most pronounced in *A. mutica* where populations sampled from Arkansas and Iowa differed from populations in Louisiana and Florida by almost 4% sequence divergence. To put this result into context, previous studies of cytochrome *b* and other regions of the genome in turtles have not found such large differences even between genera (e.g., Lamb et al., 1994). The magnitude of these intraspecific divergences also seem large when compared to the interspecific divergence of *A. mutica* and *A. spinifera*, which is around 8%. Intraspecific differences of this size seem even more extraordinary given that cytochrome *b* codes for an essential protein and is not afforded the selective relaxation of neutral mitochondrial loci (i.e., control region) that are typically used in low level phylogenetic studies.

The large divergence found between these north-south clades is difficult to explain. A prominent and well studied biogeographic factor that may help explain the observed intraspecific genetic differences in many organisms distributed across North America is the cyclical pattern of Pleistocene glaciation that occurred from about 2 Mya until about 15 Kya (Holman, 1995). The most recent glacial procession southward in North America occurred from about 150 to about 15 Kya and reached a maximum southward range into the Great Lakes region and northern latitudes across North America. The glacial maximum and its associated climatological change south of the actual ice sheet caused extensive range shifts of plant and animal communities (e.g., Clark, 1993). This process could have been responsible for

splitting species' ranges and thus permitting genetic differentiation in allopatry. The true extent to which biotic communities were influenced south of the ice sheet is debated (Holman, 1995), but it is accepted that southern range shifts were common and functioned in fragmenting and isolating populations (Hocutt and Wiley, 1986). However, given the extent of the genetic differences between these northern and southern assemblages, it seems unlikely that relatively recent glacial events during the Pleistocene alone can adequately explain the observed phenomena, even if Trionychids do not exhibit slow rates of mtDNA evolution as thought to exist in other turtle families.

Without an established and reliable molecular clock for softshell turtle mtDNA, it is difficult to determine when this north-south split occurred. If their mitochondria are evolving at the slow rates of .25%/My as hypothesized for other turtle families by Avise et al. (1992), then their divergence occurred somewhere during the Miocene. Using standard vertebrate calibrations of 2%/My (Shields and Wilson, 1987) places the break during the Pleistocene. The Pleistocene contains the glacial intrusions and variable climates that could explain the formation of these separate lineages. The Miocene and eventual Pliocene, on the other hand, were times of warmer and more stable climates and do not seem to have possessed the climatological extremes needed to force allopatry, but this does not necessarily rule out the divergence of intraspecific lineages from occurring during these times.

An additional interesting phenomenon evident in the Neighbor-Joining tree is that, although there appears to be concordance in the intraspecific patterns of northern and southern populations of *A. mutica* and *A. spinifera*, a disparity exists in the sizes of their divergences. Divergences at this level are approximately 2% within *A. spinifera* whereas *A. mutica* has a north-south divergence of 4%. This difference could be explained by either separate allopatric events in the two species generating the observed patterns or through *A. mutica* having an increased molecular evolutionary rate of mtDNA. The latter explanation would mean that smooth softshells have a substitution rate twice that of spiny softshells.

**Regional Patterns of mtDNA Variation**

The levels of sequence divergence within the northern intraspecific lineages of both spiny and smooth softshells are consistent with the hypothesis of recent colonization following the retreat of the Wisconsin ice sheet. This is especially apparent in the northern clade of *Apalone spinifera* where populations from Iowa, Illinois, Wisconsin, Ontario, and Quebec all group together. Despite this close grouping, small amounts of genetic structure do exist among these populations. The strict consensus parsimony tree (Fig. 4) groups the Iowa, Wisconsin and one of the Illinois populations together. This clade falls into an unresolved polytomy with the remaining populations sampled from the more northern distribution. Members of an Ontario population from the Thames River also group together distinctly from the other members of the clade. The presence of these small clades, albeit formed on the basis of only one or two synapomorphic changes, indicates the possibility that substitutional change has occurred recently (i.e., within the last 15 Kya) following dispersal into these northern areas.

This is not the only explanation, however, as these changes could also be the product of localized founder effects following glacial retreats. Animals with large dispersal abilities might have spread north from the southern refugia following the newly-created suitable habitat. The smaller numbers of dispersing turtles on the edge of the southern refugia population might have reached northern areas and established new populations prior to the arrival of the majority of formerly southern individuals. Assuming there was genetic variation present in the larger refugium, this process could have allowed for the establishment of localized haplotypes. However, the cyclical process of glaciation in the northern populations has been proposed to promote genetic homogeneity (Hewitt, 1996). If so, genetic variation would be absent within southern refugia and the present genetic structure would be a function of recent post-colonization substitution events. Despite the multiple mechanisms that could give rise to the

current genetic pattern, the presence of genetic variation within such a recently colonized area is remarkable given previous studies of turtle phylogeography.

Low numbers of sampled populations in *A. mutica* ,which stem partially from the ability to obtain samples, prevent a complete comparison to the genetic trends found in *A. spinifera*. However, this reduced sampling also is correlated with the more constrained distribution of *A. mutica* and the limited population sizes found within its distribution relative to *A. spinifera*, which has a more extreme and extensive distribution and appears to be much more common than *A. mutica*. Nonetheless, detected patterns in *A. mutica* relationships were similar to those found in *A. spinifera*.

Evolutionary relationships of southern populations indicate the presence of a vicariant force separating Louisiana and Florida populations of *A. mutica* and *A. spinifera*. A similar pattern is evident in *A. ferox* where populations from the Florida panhandle are genetically distinct from peninsular Florida populations. Southern populations of spiny and smooth softshells both show concordant genetic divergence between populations from southeastern Louisiana and Florida panhandle populations. Populations from the panhandle of Florida, which represent the most southeastern distribution of *A. mutica* and one of the more southern populations of *A. spinifera*, exhibit greater divergence from other conspecific populations than do populations from Louisiana. Furthermore, the magnitudes of differences between Florida and Louisiana populations in *A. mutica* are considerably larger than those found in *A. spinifera* (Fig. 5), again suggesting that either this pattern was generated by separate vicariant events in the two species or that *A. mutica* has a molecular rate of mtDNA evolution that is approximately twice that found in *A. spinifera*.

The pattern of genetic divergence in southern populations is also evident in the intraspecific relationships of *A. ferox*. The Florida softshell is distributed throughout, and is almost limited to, the state of Florida. Panhandle populations from Florida appear to be distinct from those of peninsular Florida. Populations distributed throughout the peninsula exhibit low

divergence and suggest either recent colonization or gene flow between the assayed populations. Rising sea levels in past interglacial periods of the Pleistocene could account for the observed patterns. At one point the central peninsular portion of Florida was fragmented from the mainland due to high sea levels (Holman, 1995). Peninsular *A. ferox* would have been isolated from northern populations and this allopatric event could be the source of the genetic divergence found in the results. Although this scenario does not directly explain the divergences found between Louisiana and Florida populations of *A. mutica* and *A. spinifera*, similar processes could be invoked. Rising sea levels could have fragmented the populations along an east-west break and produced the present patterns of mtDNA divergence.

These same east-west patterns are detected in many other vertebrate species in the southeastern U.S. A summary of phylogeographic patterns across a wide range of taxa that include marine and freshwater fishes, birds, crabs, and turtles produced concordant genetic breaks between Atlantic and Gulf populations of marine and coastal species and eastern and western populations of mainland species (Avise, 1992). The results presented by Avise (1992) for a variety of freshwater fish, as well as emydid (Avise et al., 1992) and kinosternid (Walker, 1995; 1997) turtle species, distributed throughout the same area as the present study show remarkable concordance with my results for all three species of softshell turtles. Whatever the mechanism of vicariance, the concordance in the patterns of mtDNA variation, for both these three softshell species and those of other freshwater vertebrates, in this region are striking and further strengthen the possibility that similar phylogeographic forces have acted on all three species.

### Evolutionary Rates of Softshell Turtles

What can be said for the sizes of divergences both among species (6.2-8.9%) and within species (0-4%)? Because actual calibrations of a molecular clock are difficult due to unknown times of divergence, I can at best compare my results to published data for other

North American turtles that share similar distributions and habitat preferences. Map turtles (*Graptemys*) are a widely distributed genus in North America with three of twelve species occupying a large distribution throughout the Mississippi, Ohio, and Missouri River drainages. Despite this vast distribution, no genetic variation was found within any species for both mtDNA RFLP analysis as well as almost 400 bases of cytochrome *b* sequence (Lamb et al., 1994). Within *Graptemys ouachitensis*, this analysis encompassed a range extending from south-central Wisconsin to northeastern Alabama. *Apalone* species distributed across a similar range and distance exhibit intraspecific divergences as great as 1.6% in *A. spinifera* and 4% in *A. mutica*. This disparity may be a result of a relatively young North American *Graptemys* history. However, the shared riverine habitat and fossil evidence for *Graptemys* presence in the early Pleistocene (Ernst et al., 1994) indicate that they have possibly been subjected to the same climatological and biogeographic forces as *Apalone*.

Additional comparisons can be made with slider turtles (*Trachemys scripta*) in the southeast U.S. Small amounts of sequence divergence were found across a range from eastern Virginia to west of Louisiana (Avise et al., 1992). Sequence divergences within *Apalone* samples from the same general vicinity are small in *A. spinifera* at 0.2%, but almost twice as large as slider divergences in *A. mutica* at 1.0%. Furthermore, *Apalone* populations analyzed from this region were obtained across much smaller distances than those used in the*Trachemys* study. Because southern populations tend to show more genetic structure relative to northern populations in *Apalone*, this leaves open the possibility that divergences could be even greater across more distantly separated populations similar to the distribution of samples used in the *Trachemys* study.

Diamondback terrapins (*Malaclemys terrapin*) exhibit extremely low levels of genetic variation with a maximum sequence divergence throughout populations strung along the Atlantic and Gulf coast of 0.4% (Lamb and Avise, 1992). This comparison lends itself somewhat less useful due to the different habitat and distribution of *M. terrapin*. However, the

magnitude of this disparity between my extensive intraspecific results and the minimal divergences in the study of the widely distributed terrapin are worth noting.

Snapping turtles (*Chelydra serpentina*) also exhibit low amounts of sequence divergence within North America as evidenced by mtDNA RFLP analysis (Phillips et al., 1996). Maximum sequence divergence across populations from Illinois, Oklahoma, Missouri, and Florida was 0.5%. Furthermore, maximum genetic divergence found between the North American samples and populations from Central America was 4.5%, only slightly higher than the among population estimates of *A. mutica* within North America. Similar estimates of low intraspecific variability have also been found in desert tortoises and sea turtles (summarized in Avise et al., 1992).

In contrast, observed intraspecific patterns of mtDNA variation in the kinosternid turtles *Sternotherus minor* and *Sternotherus odoratus* (Walker et al., 1995; 1997) are more similar to my results. Both species exhibited intraspecific sequence divergences over 3% across their southeastern and central distributions. These large divergences parallel the size of genetic divergence found between the northern and southern populations of softshell turtles in this study. The families Trionychidae and Kinosternidae share a close phylogenetic relationship to each other relative to other turtle families belonging to the same superfamily (Gaffney and Meylan, 1988). The close phylogenetic relationship and comparable trends in intraspecific divergences suggest the possibility that both families of turtles may be evolving as a function of similar, perhaps intrinsic, causal factors.

The difficulty in establishing a molecular clock from intraspecific data is a function of an inadequate fossil record. Softshell turtle fossils have been reported from the Rancholabrean of the Pleistocene and some exist from even older deposits (Holman, 1995). However, using these to date the first occurrence of conspecific populations of turtles seems inadequate. Instead, using the same approach taken by Avise et al. (1992) and Avise (1992), I focus on the intraspecific trends exhibited within *Apalone* and their comparisons with other congeneric turtle

species. Comparisons of my results with those published for both sympatric and non-co-distributed species consistently point to substantially larger intraspecific divergences within *Apalone* species, with the possible exception of a related genus (i.e., *Sternotherus*).

**Rate Variation Within *Apalone***

*Apalone spinifera* and *A. mutica* share very similar distributions across North America, as well as similar patterns of mtDNA variation across these distributions. However, one aspect of mtDNA evolution that they do not share is the magnitude of detected variation. The smaller number of populations sampled for *A. mutica* prevent a complete comparison of the two species. Nonetheless, comparisons can be made for large scale patterns. Both species share similar genetic breaks across a northern-southern boundary, yet the size of mtDNA divergence in *A. mutica* across these groups is over twice that found in *A. spinifera*. The same pattern is found within southeastern populations of both species. Furthermore, both species share the east-west break exhibited by other turtles and freshwater fish (Avise, 1992). Once again, the size of mtDNA divergence in *A. mutica* across this break is twice as large as the corresponding divergence in *A. spinifera*. One explanation for this disparity is that these two species may have been separated by similarly acting but temporally different forces, thus allowing for the observed differences in mtDNA divergence. This could be the result of the different ecological preferences in *A. spinifera* and *A. mutica*. *Apalone spinifera* is much more of a generalist in terms of habitat. This generalist nature may have allowed spiny softshells to avoid population fragmentation due to early Pleistocene glacial expansion and habitat loss, a condition which would be expected to affect *A. mutica* more severely. Subsequently, a later, more intense glacial maximum could have pushed *A. spinifera* beyond its ability to maintain gene flow and, hence, produced the distinct north-south lineages that I detected as well as the disparity in sizes of divergence across the two species.

However, I detected similar trends and disparity in sizes of mtDNA evolution in multiple geographic regions. When viewed on a whole, it seems more parsimonious to assume that these species have been subjected to temporally and spatially similar vicariant forces. Furthermore, the trends detected in the southeast are corroborated with other vertebrate examples (Avise, 1992), again suggesting similar phylogeographic histories. This line of evidence suggests that smooth and spiny softshells may more likely have differing rates of mtDNA evolution.

Molecular rate variation is not an uncommon phenomenon and has been detected among a variety of taxonomic groups. Many of the studies describing mtDNA rate variation have been among high-level taxonomic groups (Adachi et al., 1993), although, rate variation for closely-related rodent species was detected in nuclear loci (Fieldhouse et al., 1997). The possibility of substantial rate variation within *Apalone* suggests that mtDNA rates may also differ for other closely-related species. *Apalone mutica* may exhibit a faster pace of mtDNA evolution for a number of reasons. Proposed determinants of evolutionary rates included DNA repair mechanisms, generation time, metabolic rates, selection and population size (Bromham et al., 1996; Fieldhouse et al., 1997). Because of the close phylogenetic relationships of these two species of turtles, it seems unlikely (but not impossible) that many of the intrinsic factors have much influence. However, one extrinsic factor, population size, does seem to be a possible difference between *A. mutica* and *A. spinifera*. *Apalone mutica* appears to be less common than *A. spinifera*, which could be a function of its more specialized ecological tendencies requiring large riverine conditions (Ernst et al., 1994). This behavior translates into smaller population sizes and increases the chance in *A. mutica* that a substitution will become fixed due to genetic drift.

The patterns of genetic variation and trends in mtDNA evolutionary rate found within softshell turtles are an intriguing alternative to the majority of studies in turtle phylogeography. The detected north-south and east-west patterns of divergence in these turtles hint at past

biogeographic forces separating intraspecific lineages of softshell turtles and, most likely, other co-distributed vertebrate taxa.

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