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Phylogenetic Distinctiveness of a Threatened Aquatic Turtle (*Sternotherus depressus*)

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Abstract: *The musk turtle (Sternotherus minor) is common throughout the southeastern United States. In 1955 a morphologically atypical form confined to the Black Warrior River System in Alabama was discovered and accorded full species status as S. depressus, the "flattened musk turtle." Questions remain about the taxonomic status and evolutionary history of the flattened musk turtle because (1) the geographic distribution of S. depressus is nested within the range of S. minor; (2) the flattened shell might be a recently evolved anti-predator adaptation; and (3) reports exist of intergrades between S. depressus and S. minor. We generated and employed sequence data from mitochondrial DNA to address the phylogenetic distinctiveness and phylogeographic position of S. depressus relative to all other musk and mud turtles (Kinosternidae) in North America. The flattened musk turtle forms a well-supported monophyletic group separate from S. minor. Genetic divergence observed between S. depressus and geographic populations of S. minor is no less than that distinguishing many kinosternid congeners from one another. These molecular genetic findings bolster rationale for the taxonomic recognition of S. depressus and, hence, for special efforts to protect this federally threatened species.*

Distintividad Filogenética de una Tortuga Acuática Amenazada (*Sternotherus depressus*)

Resumen: *Las tortugas acuáticas ("musk turtles") de la especie Sternotherus minor (familia Kinosternidae) están ampliamente distribuidas en la región sudeste de los Estados Unidos de Norteamérica. En 1955, una variedad de musk turtle morfológicamente atípica, con el caparazón notoriamente comprimido y restringida a la cuenca del río Black Warrior en Alabama, fue descrita y asignada a una especie nueva, S. depressus ("flattened musk turtle"). Posteriormente, el origen evolutivo y el estatus taxonómico de esta nueva especie han sido cuestionados debido a: (1) su restringida distribución geográfica esta anidada dentro del rango de distribución de S. minor; (2) El caparazón aplanado podría ser una adaptación anti-depredador recientemente evolucionada; y (3) existen reportes de morfologías intermedias entre S. depressus y S. minor. En el presente trabajo se emplean secuencias de ADN mitocondrial para evaluar la identidad genética, posición filogenética, y distribución filogeográfica de S. depressus en relación a las demás especies norteamericanas de la familia Kinosternidae. Los datos obtenidos confirman la identidad de S. depressus como una especie separada genéticamente de la poblaciones locales de S. minor, y de las otras especies de la familia Kinosternidae. Estos resultados sugieren el reconocimiento taxonómico de S. depressus y justifican los esfuerzos conservacionistas para proteger esta especie amenazada.*

Introduction

In the early 1950s a survey of turtles in United States rivers entering the northern Gulf of Mexico revealed a morphological form of *Sternotherus* that subsequently was listed as a new species, *S. depressus* (Tinkle & Webb 1955). The flattened musk turtle is similar to other musk

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turtles (*S. minor minor* and *S. m. peltifer*) but is distinguished by head pattern and a noticeably flattened carapace. *S. depressus* is confined to the Black Warrior River system above the fall line in Alabama (Estridge 1970; Mount 1975; Conant & Collins 1991), and its described range is encircled by that of *S. m. peltifer* (Fig. 1).

In the 1970s morphological intergrades of *S. m. peltifer* and *S. depressus* (Estridge 1970; Mount 1975) were reported from the upper Cahaba River system and western tributaries of the Black Warrior River. Estridge (1970) suggested that genetic exchange had taken place between *S. depressus* and *S. minor*, and for these reasons Mount (1975, 1981) listed the flattened musk turtle as a subspecies of *S. minor* (*S. m. depressus*), as did Wermuth and Mertens (1961), Ernst and Barbour (1972), and Pritchard (1979). Seidel and Lucchino (1981), however, interpreted allozyme and morphological data to suggest that *S. depressus* and *S. minor* were distinct genetically and that claims of introgression were unwarranted. Most of the literature have employed and continues to use the full species epithet for the flattened musk turtle (Conant 1975; Behler & King 1979; Conant & Collins 1991; Ernst et al. 1994), but admit that its evolutionary status is uncertain (Iverson 1977).

Additional morphological and protein analyses (Seidel et al. 1981; Seidel et al. 1986; Ernst et al. 1988; Iverson 1991) generally support the contention that *S. depressus* is differentiable from *S. minor*. The nature of information provided by such studies, however, has not permitted firm conclusions about the phylogenetic placement of the flattened musk turtle relative to geographic popu-

lations of *S. minor* or to other kinosternid taxa. One non-excluded possibility is that the flattened shape of the musk turtle is "merely" a recent adaptation (either genetically "hard-wired" or perhaps the result of developmental plasticity) and is not indicative of substantial phylogenetic separation from *S. minor*. Jackson (1988; see also Dodd 1986) emphasizes that *Sternotherus* turtles routinely squeeze into narrow crevices, a defensive behavioral strategy known as anachoresis (Edmunds 1974). The ability to enter narrow crevices probably confers upon musk turtles a strong fitness advantage, particularly in the Black Warrior River system where the stream beds are characterized by large stratified rocks.

We scrutinized control region sequences from mitochondrial (mt)DNA to (1) estimate matrilineal relationships for all nine species of North American mud and musk turtles (Kinosternidae), including geographic populations of *S. minor*, *S. odoratus*, *Kinosternon subrubrum*, and *K. baurii*; and (2) assessed the phylogeographic position of *S. depressus* within that framework. In this case, questions of taxonomic and phylogenetic status are of special importance because *S. depressus* is included on the federal list of threatened and endangered species (USFWS 1987, 1995). Indeed, *S. depressus* had the distinction of appearing in LIFE Magazine's (1974) montage of "the endangered 100" photogenic species in the United States.

Methods

Samples and Laboratory Procedures

Blood samples were taken from five *S. depressus* from two locations in Alabama: Blackwater Creek, Walker Co. ($n = 2$) and Sipsey Fork, Winston Co. ($n = 3$). In addition, heart, liver, and muscle tissues were taken (and frozen at -70°C for approximately 6 months) from one specimen found dead at the Sipsey Fork site. Total DNA was extracted from blood using the InstaGene Purification Matrix (Bio-Rad) and from other tissues following Lansman et al. (1981). The DNA sequencing was accomplished from PCR-based amplification products of a portion of the mtDNA control region. The control-region PCR primers were designed initially for marine turtles (Allard et al. 1994), but then were refined for *S. minor* (Walker et al. 1995). These primers (sequences presented in Walker et al. 1995) are located near the 5' end of the control region and extend into the adjacent tRNA^{Pro} gene. They amplify a fragment ca. 450 base pairs in length.

Sequences from *S. depressus* were generated from double-stranded PCR products using the fmol DNA Sequencing System (Promega) and compared to homologous sequences from a previous survey of *S. minor* ($n = 52$; Walker et al. 1995). All samples were sequenced in

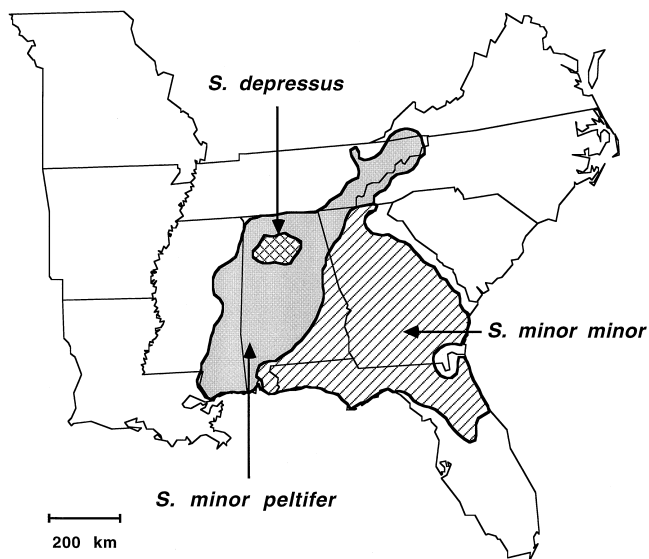


Figure 1. The described range of *Sternotherus depressus* (cross-hatched area in central Alabama) within the broader distribution of *S. minor*. For range maps of the other species considered in this report see Conant and Collins (1991).

Table 1. Variable sites among 43 control region mtDNA sequences observed in kinosternid turtles.*

mtDNA haplotype (n)	Sequence positions												
	1111	1111111111	1111111111	1111111111	1111111111	1111111111	111111222	2222222222	2222222222	2222222222	2222222222	2222222222	2222222222
<i>S. minor</i> 1 (6)													
<i>S. minor</i> 2 (2)													
<i>S. minor</i> 3 (1)													
<i>S. minor</i> 4 (1)													
<i>S. minor</i> 5 (1)													
<i>S. minor</i> 6 (2)													
<i>S. minor</i> 7 (6)													
<i>S. minor</i> 8 (4)													
<i>S. minor</i> 9 (1)													
<i>S. minor</i> 10 (3)													
<i>S. minor</i> 11 (2)													
<i>S. minor</i> 12 (4)													
<i>S. minor</i> 13 (1)													
<i>S. minor</i> 14 (1)													
<i>S. minor</i> 15 (6)													
<i>S. minor</i> 16 (9)													
<i>S. minor</i> 17 (2)													
<i>S. odoratus</i> 1 (1)													
<i>S. odoratus</i> 2 (2)													
<i>S. odoratus</i> 3 (2)													
<i>S. odoratus</i> 4 (2)													
<i>S. carinatus</i> 1 (6)													
<i>S. depressus</i> 1 (2)													
<i>S. depressus</i> 2 (1)													
<i>S. depressus</i> 3 (2)													
<i>S. depressus</i> 4 (1)													
<i>K. baurii</i> 1 (1)													
<i>K. baurii</i> 2 (1)													
<i>K. baurii</i> 3 (1)													
<i>K. baurii</i> 4 (1)													
<i>K. baurii</i> 5 (1)													
<i>K. baurii</i> 6 (1)													
<i>K. subrubrum</i> 1 (1)													
<i>K. flavescens</i> 1 (1)													
<i>K. flavescens</i> 2 (1)													
<i>K. subrubrum</i> 2 (1)													
<i>K. subrubrum</i> 3 (1)													
<i>K. subrubrum</i> 4 (1)													
<i>K. subrubrum</i> 5 (2)													
<i>K. subrubrum</i> 6 (1)													
<i>K. subrubrum</i> 7 (2)													
<i>K. birupes</i> 1 (2)													
<i>K. sonortense</i> 1 (2)													

*Sequence positions correspond to nucleotide number counting from the 5' end of a reference sequence of *S. minor* deposited in GenBank (accession number U19540). Alignment gaps in the sequence are denoted by X.

both directions. We also obtained newly generated mtDNA sequences from the following species: *S. odoratus* ($n = 7$), *S. carinatus* ($n = 6$), *K. subrubrum* ($n = 9$), *K. baurii* ($n = 6$), *K. flavescens* ($n = 2$), *K. sonoriense* ($n = 2$), and *K. hirtipes* ($n = 2$). The individuals sequenced of *S. odoratus*, *K. subrubrum*, and *K. baurii* were chosen to represent the major intraspecific phylogeographic assemblages identified in previous restriction fragment length polymorphism (RFLP) studies of the entire mtDNA molecule (Walker et al. 1995, 1997, 1998). The newly acquired specimens were collected from the following locales: *S. carinatus*, Comite River, East Baton Rouge Parish, Louisiana; *K. flavescens*, Presidio County, Texas; *K. sonoriense*, Rock Creek, Cochise County, Arizona; and *K. hirtipes*, Presidio County, Texas.

Data Analyses

The DNA sequences were aligned using the Pileup program in the GCG package (Devereux et al. 1984). Each scored haplotype consisted of 402 nucleotide positions, with alignment gaps counted as a fifth base for purposes of comparing sequences. Phylogenetic analyses were based on maximum parsimony and minimum evolution (Kidd & Sgaramella-Zonta 1971; Rzhetsky & Nei 1992) as implemented in PAUP* version 4.0.0d55 (Swofford 1997). The *Kinosternon* species were used as outgroups for the *Sternotherus* taxa. For the parsimony analyses, all characters and substitution types were assigned the same weight, and heuristic searches were done by stepwise random addition of taxa with at least 10 replications and TBR branch swapping (MULPARS option in effect). Overall consistency indices (CI and RI; Farris 1989) were calculated as a measure of fit between the data and the reported topologies.

Using empirical base frequencies, minimum evolution searches were based on the HKY85 + γ model (Hasegawa et al. 1985; Yang 1993), allowing rate heterogeneity among sites to follow a gamma-distribution and assuming an invariant-sites model. Values for the parameters required by this model (shape parameter and proportion of invariant sites) were estimated by maximum likelihood on the most parsimonious trees. Heuristic searches were performed on starting trees obtained via neighbor joining (Saitou & Nei 1987). Bootstrapping (Felsenstein 1985) was used to estimate confidence in the maximum parsimony and minimum evolution results (500 pseudoreplications).

Results

Notwithstanding a few scattered nucleotide gaps (usually 1-bp long each, except for a single instance of a 2-bp

deletion), all mtDNA control-region sequences could be aligned unambiguously. The assayed mtDNA sequences from some conspecific individuals were identical such that the 86 samples total displayed 43 different mtDNA haplotypes (Table 1). Seventeen of these haplotypes were from a previous study of *S. minor* (Walker et al. 1995); the remainder are newly reported. Among the 402 base pairs sequenced, 91 (23%) were variable, and 71 (78%) of the latter were phylogenetically informative (i.e., not confined to a single haplotype).

Phylogenetic analyses applied to these data identified 56 equally parsimonious trees (length 202 steps; CI = 0.58; RI = 0.86) and a single best minimum evolution tree (Fig. 2). A strict consensus of the 56 maximum par-

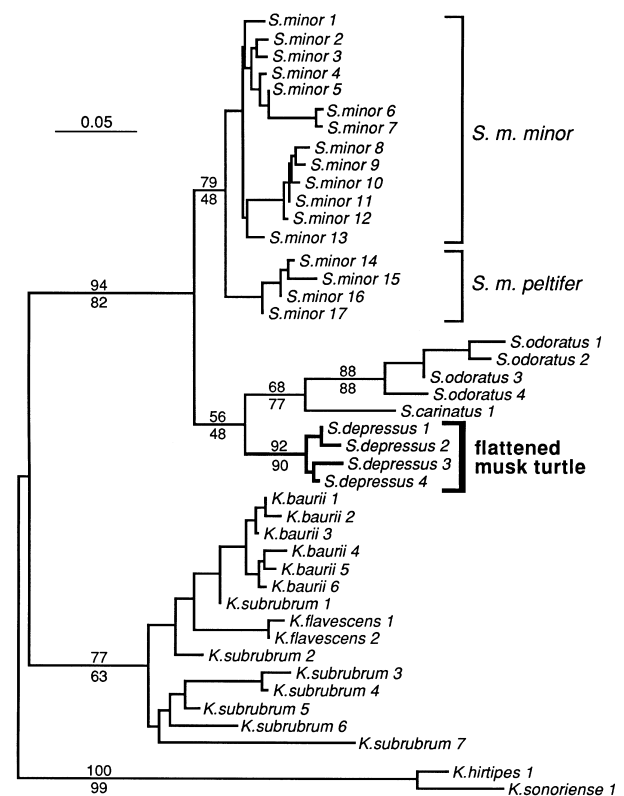


Figure 2. Phylogenetic tree estimated using the minimum evolution method assuming HKY85 + γ distances (tree length = 1.58 expected substitutions per site). Model parameters (estimated from the data) are proportion of invariable sites = 0.62 and gamma shape parameter = 0.78. *Kinosternon sonoriense* and *K. hirtipes* were used as outgroup taxa. Branch lengths are proportional to the number of changes (scale bar indicates 0.05 expected substitutions per site). Numbers above and below branches are bootstrap values obtained by maximum parsimony and minimum evolution, respectively (only shown for nodes of particular interest). Haplotypes for *S. minor* are numbered in agreement with Walker et al. (1995).

simony trees contained the same major clades of interest as presented in Fig. 2. Disagreements among the most parsimonious trees (as evidenced by polytomies in the strict consensus tree) involved relationships among particular *Kinosternon* haplotypes and among haplotypes within *S. minor minor*.

Four major phylogenetic patterns emerged consistently, regardless of method of data analysis (Fig. 2). First, all assayed *Kinosternon* species formed a clade distinct from all four *Sternotherus* species. Second, within *Sternotherus*, each recognized species constituted a distinct lineage. Third, specimens from the two recognized subspecies of *S. minor* (*S. m. minor* and *S. m. peltifer*) formed two separate monophyletic groups, as was true in restriction site analyses (Walker et al. 1995). Fourth, *K. baurii* appears as a recognizable sublineage within the broader phylogenetic assemblage of *K. subrubrum*-like mtDNA haplotypes. This last observation is consistent with results from previous mtDNA restriction site assays (the taxonomic and evolutionary ramifications are discussed by Walker et al., 1998).

With respect to the major focus of this report, *S. depressus* consistently appears as a phylogenetically distinctive unit (with strong bootstrap support) in all of the mtDNA analyses (Fig. 2). Furthermore, consistent with Tinkle (1958), its closest matrilineal ties are to other *Sternotherus* musk turtles (bootstrap support $\cong 90\%$). The position of *S. depressus* within the Sternotherine clade is not so clear as judged by bootstrap values (Fig. 2). For this reason, parsimony analyses were used to evaluate three competing hypotheses for the matrilineal relationships of *S. depressus* vis-à-vis the three other *Sternotherus* species. Among these three possibilities, a sister group relationship of *S. depressus* with *S. carinatus* + *S. odoratus* entails two or four fewer steps than do the two competing arrangements.

Discussion

Where decisions of conservation triage must be made, a general philosophy has been that higher priority should be placed on phylogenetically unique as opposed to minimally differentiated taxa (Vane-Wright et al. 1991, 1994; Brooks et al. 1992; Crozier 1992; Faith 1992, 1994; Krajewski 1994; Vane-Wright 1996). Molecular genetic approaches (notably those involving mtDNA) are well suited for assessing phylogenetic distinctiveness when taxonomic assignments at the species or subspecies levels have remained uncertain based on morphological or biogeographical evidence (Avisé 1989, 1994; Avisé & Hamrick 1996; Hibbett & Donoghue 1996; Smith & Wayne 1996). Examples exist in which mtDNA phylogeographic patterns have bolstered (Bowen et al. 1991) and in other cases diminished (Laerm et al. 1982;

Avisé & Nelson 1989; Wayne & Jenks 1991; Vogler & DeSalle 1993) implicit phylogenetic rationales underlying the taxonomies for endangered species toward which special conservation efforts have been directed.

In the current case, *S. depressus* proved to be highly distinct in mtDNA composition from all geographic populations of *S. minor* as well as from other musk and mud turtles in North America. *S. depressus* appears as a distinct and statistically supported matrilineal clade in all phylogenetic treatments (Fig. 2). Furthermore, the magnitude of its mtDNA sequence divergence (p) from other kinosternid species ($p = 0.041 - 0.073$) surpasses the mean ($p = 0.000 - 0.034$) and usually the maximum ($p = 0.000 - 0.053$) genetic distances measured by the same molecular yardstick among *any* conspecific samples assayed within these other taxa.

There are strengths and weaknesses to the current appraisal of the phylogeographic and taxonomic status of *S. depressus*. The extensive sampling of mtDNA geographic variation within several related species of Kinosternid turtles (Walker et al. 1995, 1997, 1998) bolsters and places in context the conclusion that the genetic distinctiveness of the flattened musk turtle surpasses intraspecific geographic variation normally characteristic of other species within the complex. This conclusion, however, is based on one "gene," and thus on only a small sample of the total genealogical history of these turtles (Avisé & Wollenberg 1997).

Several studies have documented notable disparities between a mitochondrial "gene genealogy" and a "species phylogeny" that may result either from stochastic lineage sorting from a polymorphic ancestor or from gender-asymmetric introgressive hybridization (reviewed in Avisé 1994). Such factors, however, usually result in the appearance of similar or identical mtDNA genotypes in related taxa when such sharing was not anticipated by other evidence. Here, the situation is reversed. The distinctive position of the flattened musk turtle in the mtDNA phylogenetic tree strongly suggests that the matrilineal lines within *S. depressus* have had a relatively long and separate history from those of other musk turtles.

The following have been identified as important environmental threats to the flattened musk turtle: siltation from agricultural runoff, forestry practices, mining, and industrial and residential development; water pollution by organic and inorganic chemicals; and hydrologic changes associated with mining and with navigation and flood control projects (Dodd et al. 1988; USFWS 1990). As is often the case for threatened and endangered species, the implementation of a species recovery plan (USFWS 1990) for *S. depressus* could have appreciable impacts (for better or worse) on local economies and human environmental practices. Thus, conservation plans should be well motivated and scientifically justified. The current molecular data are consistent with the view that the problematic taxon *S. depressus* is a relatively unique

and distinctive historical (phylogenetic) entity and from this perspective warrants continued attention as an object of special conservation concern.

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