Molecular phylogeography of *Apalone spinifera* (Reptilia, Trionychidae)

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The molecular evaluation of morphologically based species delimitations of many herpetofauna has improved the understanding of evolutionary processes and the rigor of conservation efforts. Previous evidence for a deep lineage divide between south-eastern + northern subspecies of the softshell turtle *Apalone spinifera* (*A. s. aspera* + *A. s. hartwegi* + *A. s. spinifera*) and western subspecies (*A. s. pallida* + *A. s. emoryi* + *A. s. guadalupensis*) was re-examined with a broader sampling using mitochondrial and nuclear markers. The south-eastern + northern clade and the western clade maintained mitochondrial reciprocal monophyly. We molecularly confirmed a geographical boundary between these two clades in Oklahoma, and developed a phylogeographical hypothesis that invokes stream capture. We evaluated whether these mitochondrial lineages represent distinct species by surveying these clades for divergence at the nuclear intron R35 and two nuclear genes, Cmos and recombination activating gene 1 (RAG-1). The nuclear loci showed no phylogenetic resolution and only the nuclear intron exhibited significant nearest neighbour statistics. Taken together, the nuclear data suggest that taxonomic elevation of the two mitochondrial clades would be currently unjustified.

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Introduction

The molecular phylogeographical analyses of geographically widespread taxa have enriched the understanding of evolutionary processes at both organismal and community levels (Moritz & Faith 1998; Lenk *et al.* 1999; Weisrock & Janzen 2000). Many of these studies have resulted in the creating and invalidating of species delimitations, thus providing invaluable information on biodiversity (Janzen *et al.* 2002; Starkey *et al.* 2003; Pellegrino *et al.* 2005; Spinks & Shaffer 2005). Herpetofauna have been the focus of many of these investigations, and morphological species and subspecies delimitations have been expanded by genetic work in some cases (e.g. Pellegrino *et al.* 2005) and rejected in others (e.g. Janzen *et al.* 2002). Natural history and demographics of each focal organism undoubtedly plays a role in whether traditional species delimitations are upheld, expanded or rejected by genetics. The softshell turtle *Apalone spinifera*, has specialized habitat requirements (obligate aquatic) and possibly high rates of molecular evolution (Weisrock & Janzen 2000). Thus, in this species, there could be relatively high potential for population subdivision and, perhaps, cryptic speciation to be supported by genetic evidence.

Spiny softshell turtles, *Apalone spinifera* (Le Sueur, 1827), are distributed widely across North America (Fig. 1). A previous molecular investigation of *A. spinifera* noted substantial geographically structured mitochondrial divergence throughout a large portion of its range (Weisrock & Janzen 2000). This split has divided south-eastern + northern subspecies (*A. spinifera spinifera*, *A. spinifera hartwegi* and *A. spinifera aspera*) from western taxa (*A. spinifera pallida*, *A. spinifera guadalupensis*, *A. spinifera emoryi*). Sampling for this previous study did not extend west of the Mississippi River, except for Texas, yet the range of this species extends westward as far as California (Ernst *et al.* 1994).

In this paper, we expanded the sampling to include additional western populations in Colorado, Nebraska, Kansas, Oklahoma and Mexico, and increased molecular sequence data with three goals: (i) to examine implications for the

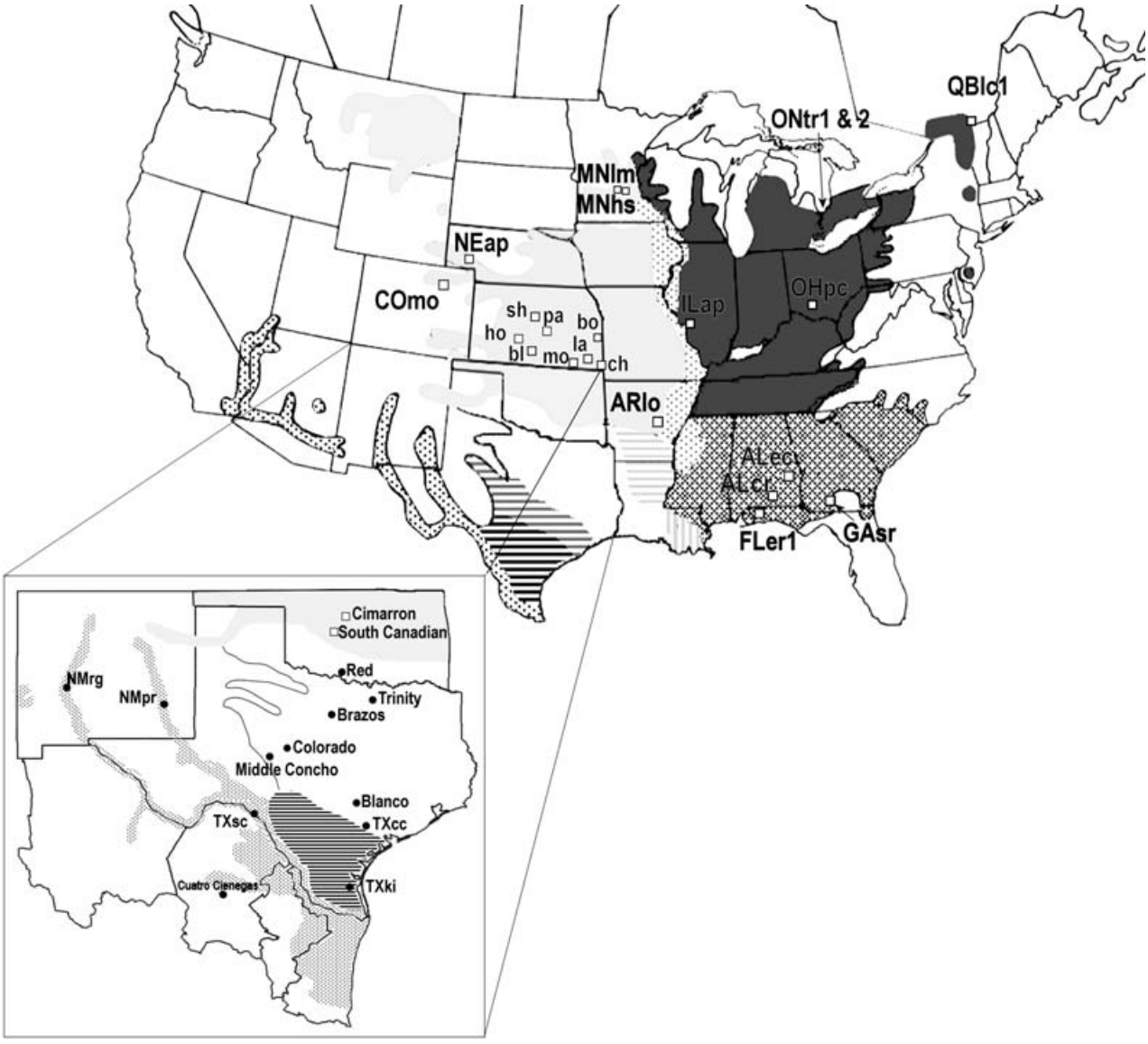

Fig. 1 Sample localities and range of *Apalone spinifera* subspecies, with emphasis on Texas, Oklahoma and Cuatrociénegas, Coahuila, Mexico (modified from Conant & Collins 1998). The western clade haplotypes are indicated by circles and the south-eastern + northern clade is represented by squares. For Kansas samples, the 'KS' subheading is dropped for visual clarity. Patterns indicating subspecies include: *A. s. spinifera*: dark grey; *A. s. aspera*: diamonds; *A. s. hartwegi*: light grey; *A. s. guadalupensis*: horizontal lines, *A. s. emoryi*: dots, unfilled areas to the east of the solid black line in Texas represent *A. s. pallida*. A small hybrid zone exists between *A. s. pallida* and *A. s. guadalupensis* and is represented by grey horizontal lines on the main image. Suspected hybrid zones in Louisiana of several *A. spinifera* subspecies (*A. s. hartwegi*, *A. s. pallida*, and *A. s. aspera*) are also represented by grey horizontal and vertical lines, and dots along the Mississippi River represent co-occurrence of *A. s. spinifera* and *A. s. hartwegi*. Evidence is presented here for a hybrid zone between *A. s. hartwegi* and *A. s. pallida* ranges in Oklahoma.

interpretation of the phylogeographical history of this widespread aquatic species; (ii) to evaluate the relationship of *A. s. hartwegi* to other *A. spinifera* subspecies because potential *A. s. hartwegi* from the previous study were limited to hybrid zones with *A. s. spinifera* near the Mississippi River (Conant & Collins 1998; Weisrock & Janzen 2000); and (iii) to evaluate the relationship of the subspecies of *A.* s. *atra*, a race endemic to the Cuatrociénegas basin in Coahuila, Mexico (McGaugh & Janzen in press), with range-wide samples of *A. spinifera*.

In addition to increased sampling of cytochrome *b* (cyt *b*), which was used in prior studies, we also sequenced three nuclear loci, an intron and two protein nuclear genes, recombination activating gene 1 (RAG-1) and Cmos. The nuclear intron (R35) is known to show species-level divergence in the genus *Apalone* (Engstrom *et al*. 2004; Krenz *et al*. 2005) and was sequenced for nearly all localities sampled for cyt *b*. The two protein coding nuclear loci (RAG-1 and Cmos) are of unknown utility in the Trionychidae, but were also sequenced for at least one representative of each *A. spinifera* subspecies (Krenz *et al*. 2005; Le *et al*. 2006). One of these (Cmos) has shown species-level divergences in Testudinidae (Le *et al*. 2006).

Mitochondrial DNA offers only one perspective of an organism's history, and prudent species delimitations rely on information from both nuclear and mitochondrial markers (Shaw 2002; Ballard & Whitlock 2004; Rubinoff & Holland 2005). Thus, these nuclear genes were included to test the hypothesis that each of the two clades of the previously noted mitochondrial phylogeographical split in *A. spinifera* warrant taxonomic elevation to full species using the phylogenetic species concept. Such deep reciprocal monophyly at a mitochondrial locus would be congruent with species delimitations, not only by the phylogenetic species concept, but by several other operational criteria, such as gene flow based measures, if corroborated by nuclear data (Moritz 1994; Sites & Marshall 2004).

Materials and methods

Collection of samples

Samples of blood or tissue for this study were obtained from two sources: (i) donations from previous studies; and (ii) newly collected material from Oklahoma, Texas and Northern Mexico, which we obtained to specifically address questions arising from the previous analysis. Wild caught animals were trapped using hoop traps and rectangular lobster traps from 26 June 2000 to 25 July 2000 in Texas and Oklahoma and from 4 June to 5 July 2004 in Coahuila, Mexico (a complete list of samples, localities, and accession numbers for available photo vouchers is provided in the Appendix). Blood samples not exceeding 0.5 mL were drawn from the caudal vein of each animal, placed in buffer (0.01 M Tris, 10 mM EDTA, 0.01 M NaCl and 1% SDS), and frozen. When blood samples were not used, shell wedges were taken, stored in ethanol and frozen. Together these samples span nearly the entire range of the species from Florida to Canada in the east and from Coahuila, Mexico to Colorado in the west (Appendix).

DNA sequencing

DNA was extracted using High Pure Template Preparation Kit (Roche, Mannheim, Germany). Part of the last two-thirds of the mitochondrial gene cyt *b* (725 base pairs; forward primer SM2000 [5′-ACAGGCGTAATCCTACTAC-3′], reverse primer DW1594, see Weisrock & Janzen 2000) was sequenced from 48 new individuals and combined with data from nine individuals of the previous study. Forty-three individuals were sequenced for approximately 920 bp of the nuclear intron R35 (Fujita *et al*. 2004) and an additional sample was taken from GenBank (AY259580; Engstrom *et al*. 2004). The additional 13 samples required to make a data set matched to the cyt *b* alignment, were not sequenced due to unsuccessful cloning or sample exhaustion. All subspecies and nearly all localities from the cyt *b* data set were represented in the R35 data set.

In addition to these two data sets derived from markers with high expected rates of evolution (mitochondrial locus cyt *b* and nuclear intron R35), we also surveyed nuclear divergence at 616–622 bp of the first one-third of RAG-1 (primers RAG-1 F2 and RAG-1 R2 in Krenz *et al*. 2005) and 538 bp of the middle of oocyte maturation Cmos gene (see Saint *et al*. 1998 for primers) for 20 individuals across *A. spinifera*'s range, and for congeners *A. mutica* (Le Sueur, 1827) and *A. ferox* (Schneider, 1783).

PCR conditions followed protocols in original references and were conducted using Techne TC-412 and Eppendorf Mastercycler gradient thermocyclers. All PCR products were gel purified for the expected band size using QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). Some heterozygosity was observed in the R35 and RAG-1 sequences. As needed, separate alleles were cloned using pGEM-T Easy Vector System I (Promega, Madison, WI) and One Shot Mach I Competent Cells (Invitrogen, Carlsbad, CA). An average of seven, but no fewer than four, clones per individual were sequenced. Unique sequences were deposited in GenBank (accession numbers: cyt *b*: DQ529096–DQ529117; EU119874–EU119898; RAG-1: DQ529132–DQ529173, DQ785893; Cmos: DQ529174–DQ529206, DQ785894; R35: DQ529118–DQ529131, EU119899–EU119856).

Phylogenetic analyses

The mitochondrial phylogenetic analysis contained approximately 725 bp of cyt *b* from *A. s. aspera* ($N = 5$), *A. s. spinifera* ($N = 3$), *A. s. hartwegi* ($N = 20$), *A. s. emoryi* ($N = 4$), *A. s. pallida* ($N = 13$), *A. s. guadalupensis* ($N = 2$), *A. s. atra* ($N = 4$), an Illinois sample that was morphologically identified as *A. s. pallida* (Lamer *et al*. 2006), individuals from a potential hybrid zone of *A. s. hartwegi* and *A.* s. *pallida* in Oklahoma ($N = 3$), one sample of *A. ferox* (FLca; Weisrock & Janzen 2000) and one of *A. mutica* (LAcr1-*m*; Weisrock & Janzen 2000). Thus, in total, 57 individuals were included. Not all animals had field-noted subspecies designations or had photo-documentation. These animals were from ranges inhabited by only one subspecies of *A. spinifera*, and so the subspecies designations were inferred by the locality from which the sample was originally taken (referred heretofore as 'locality defined').

Apalone spinifera atra samples were restricted to those taken only from the type locality (Tío Candido, Cuatrociénegas, Coahuila, México; Webb & Legler 1960). A more expansive survey of the total drainages of the Cuatrociénegas basin is provided elsewhere (McGaugh & Janzen in press). We are confident that the section of cyt *b* represents a true mitochondrial sequence, and not a nuclear pseudogene, because no stop codons were present when the sequence was translated with the mitochondrial genetic code.

The nuclear analyses contained between 858 bp and 918 bp of the nuclear intron R35. Several indels accounted for the variability, but in total there were 926 bp characters including binary notation for indels (insertions or deletions). Individuals represented in the R35 phylogeographical analyses (total = 44) were from morphologically or locality identified *A. s. aspera* ($N = 4$), *A. s. spinifera* ($N = 1$), *A. s. hartwegi* ($N = 13$), *A. s. emoryi* ($N = 3$), *A. s. pallida* ($N = 12$), *A. s. guadalupensis* ($N = 1$), *A. s. atra* ($N = 6$), individuals from a potential hybrid zone of *A. s. hartwegi* and *A. s. pallida* in Oklahoma ($N = 2$), one sample of *A. ferox* (AY259580; Engstrom *et al*. 2004) and one of *A. mutica*. Three individuals contained only partial sequence due to poor sequencing results (GAsr allele a 678 bp, GAsr allele b 696 bp; Blanco 73 698 bp, Red93 allele a 453 bp).

Individuals (total $N = 22$) represented in the RAG-1 (622 aligned positions) and Cmos (538 aligned positions) analyses were morphologically or locality identified as *A. s. aspera* ($N = 3$), *A. s. spinifera* ($N = 1$), *A. s. hartwegi* ($N = 1$), *A. s. emoryi* ($N = 2$), *A. s. pallida* ($N = 8$), *A. s. guadalupensis* ($N = 2$), *A. s. atra* ($N = 2$), an individual from a potential hybrid zone of *A. s. hartwegi* and *A. s. pallida* in Oklahoma ($N = 1$), one sample of *A. ferox* and one of *A. mutica*.

Alignments were performed with CLUSTAL W (Thompson *et al*. 1994). Sequences were edited in BIOEDIT 7.0.0 (Hall 1999). Distance matrices were constructed for cyt *b*, RAG-1, Cmos and R35 in PAUP*4b10 (Swofford 2001) using parameters estimated by Akaike Information Criterion in MODELTEST 3.7 (Posada & Crandall 1998; Posada & Buckley 2004; models: transversional model + Γ [TVM + Γ], Hasegawa–Kishino–Yano [HKY85], Kimura81 with three substitution types [K3P], Hasegawa–Kishino–Yano + invariant sites [HKY + I], respectively).

Parsimony analysis was conducted for all loci separately with PAUP*4.0b10 (Swofford 2001) using a heuristic analysis with 10 random taxon addition replicates and the tree-bisection and reconnection branch swapping algorithm. Nonparametric bootstrapping created 1000 pseudoreplicates, and these were condensed into a majority rule consensus tree. Decay indices (Bremer 1988) were calculated using AUTODECAY 5.0 (Eriksson 2001).

RAG-1 and Cmos showed little to no divergence at the nucleotide level and no resolution with the parsimony trees. These loci were deemed inappropriately variable for further analyses. R35 also showed little to no divergence at the nucleotide level and little to no resolution with the parsimony trees, however, several indels were present in the alignment and so a maximum-likelihood analysis was performed as well. A combined analysis was also conducted for all nuclear loci using parsimony analysis with the exact parameters described for the separate parsimony analysis.

For cyt *b* and R35, maximum-likelihood phylogenetic trees were constructed with PAUP*4.0b10 (Swofford 2001) using parameters estimated with respect to the Akaike Information Criterion in MODELTEST 3.7 (Posada & Crandall 1998; Posada & Buckley 2004). For cyt *b*, the transversional model + Γ model (TVM + Γ) of sequence evolution with a Γ-shape parameter of 0.3379 was implemented and for R35 the model of Hasegawa–Kishino–Yano + invariant sites (HKY + I) was used. Maximum-likelihood estimates were bootstrapped for 100 pseudoreplicates with one random sequence addition. Congeneric species of *A. spinifera*, *A. mutica* and *A. ferox* were used as outgroups for all reconstructed phylogenetic analyses (Meylan 1987; Weisrock & Janzen 2000; Engstrom *et al*. 2004).

Bayesian analysis of cyt *b* was performed in MRBAYES 3.1.2 (Ronquist & Huelsenbeck 2003) using a GTR model with Γ-distributed rate variation across sites with no invariant sites, in line with the best fitting model predicted from MODELTEST 3.7. The Bayesian analysis was run for 8 000 000 generations with the first 10% of those removed as burn-in. Significant differences between Bayesian and maximum-likelihood most likely trees and Bayesian, maximum-likelihood, and parsimony consensus trees were assessed through Hasegawa–Kishino, Wilcoxon signed rank and winning-sites tests implemented in PAUP*4.0b10.

Hudson's nearest neighbour statistics (Snn) were calculated using DNASP v4.10.7 (Hudson 2000; Rozas *et al*. 2003). This statistic uses the infinite sites model to examine how often the nearest sequence neighbours are from the same population. To calculate a significance value for the Snn statistic, we performed a permutation test of a 1000-pseudoreplicates in DNASP. When Snn = 1, the two populations are highly genetically differentiated, whereas Snn near 0.5 indicates that the two populations are part of one interbreeding population. Two additional estimates (F_{ST} as calculated by Hudson *et al*. 1992 and χ^2 as calculated by Workman & Niswander 1970) were calculated with DNASP v4.10.7 in order to further understand geneflow levels and population differentiation indicated by the R35 data in selected cases. Haplotype networks were built using statistical parsimony in TCS 1.21 with gaps set as missing data (Clement *et al*. 2000).

Results

The 725 bp alignment of cyt *b* for 57 specimens, including outgroups, contained 599 invariant characters, 67 parsimony

uninformative characters and 59 parsimony informative characters. Of 926 total characters from 44 specimens for R35, 829 positions were invariant, 67 were parsimony uninformative and 30 were parsimony informative. RAG-1 consisted of 606 constant characters from 22 individuals, 11 parsimony uninformative variable characters and 5 parsimony informative characters. Cmos contained 525 constant characters from 22 individuals, 12 parsimony uninformative characters and 1 parsimony informative character. R35 phylogenetic reconstruction through parsimony and maximum-likelihood resulted in a polytomy at the crown node (trees available from the corresponding author) even though indels were counted as a character state. Further visual inspection of the alignment revealed that the indels and heterozygotes showed no geographical structuring and were thus not useful in this study. The phylogenetic analysis of each of the other two nuclear loci and the combined analysis of the nuclear data yielded no resolution (trees available from the corresponding author), so phylogenetic analysis of only cyt *b* is presented here.

Maximum-parsimony analysis of the 725 bp segment of cyt *b* recovered 136 equally parsimonious trees, with the consensus having 166 steps (CI = 0.819, RI = 0.966). Maximum-likelihood analysis recovered a single tree with the lowest –log-likelihood (–ln *L*) score = 1904.3546 (Fig. 2). In Bayesian analyses, the

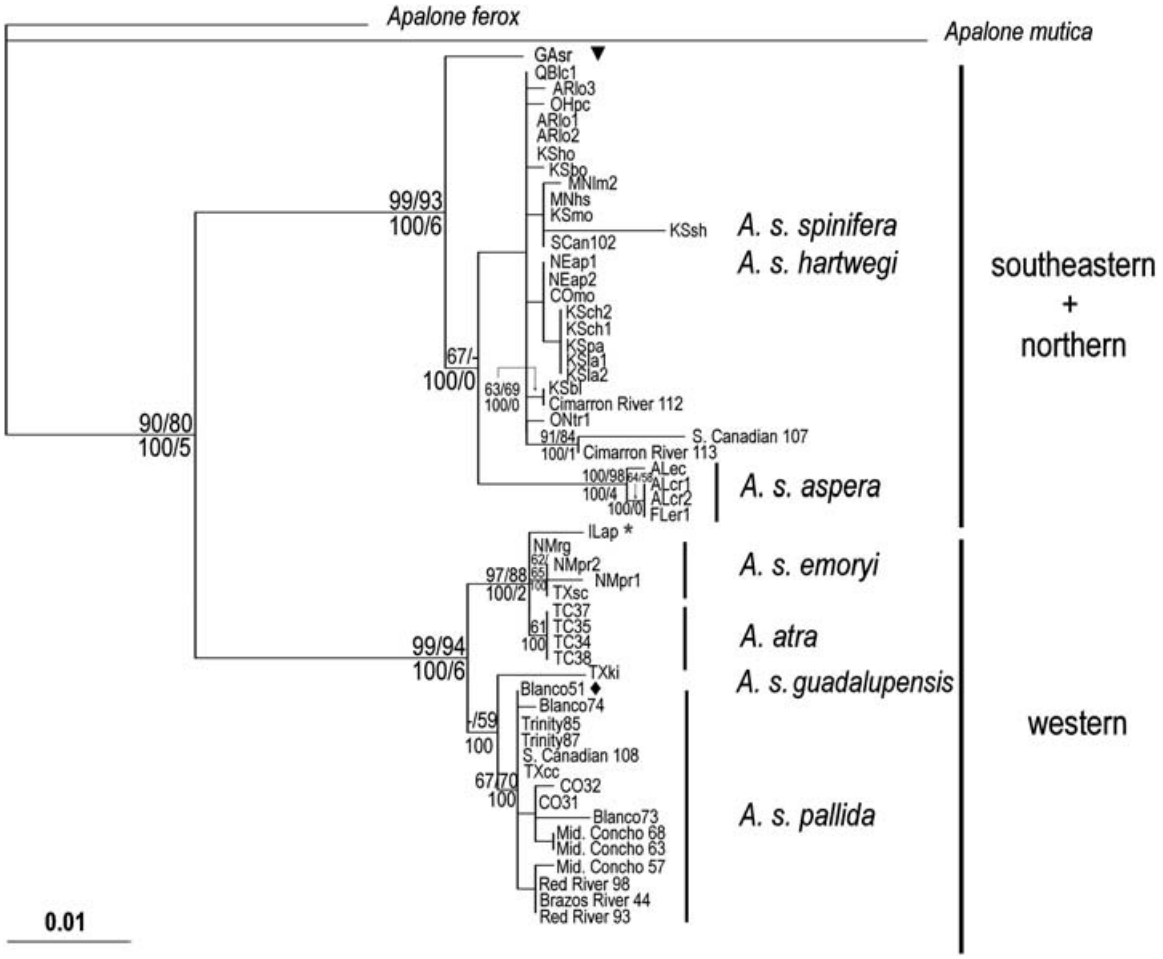

Fig. 2 Maximum-likelihood analysis of 725 base pairs of the mitochondrial gene cytochrome *b* for *Apalone spinifera*, fitting the TVM + Γ model of sequence evolution. The proportion of parsimony bootstrap pseudoreplicates supporting a particular node is given above the branch followed by the maximum-likelihood bootstrap pseudoreplicates. If either parsimony or maximum-likelihood reconstruction method did not support a particular branch a hyphen is given, except for the branch leading to *A. s. atra* individuals (prefix TC) where only parsimony supported that grouping (bootstrap = 61). Bayesian posterior probabilities and decay indices are given below the branch. A maximum parsimony tree was calculated, as well, and the topology matched that of the maximum-likelihood tree (most parsimonious tree length = 166, CI = 0. 819, RI = 0.966). The scale bar at the bottom is indicative of the number of substitutions per site. Designations that are not explicitly stated on the tree are denoted by a triangle: *A. s. aspera*, an asterisk: originally identified as *A. s. pallida* in an aberrant locality, and a diamond: morphologically identified as *A. s. guadalupensis* in a suspected hybrid zone with *A. s. pallida*.

average standard deviation of split frequencies reached only 0.023 after 8 000 000 generations, but the –ln *L* scores reached stationarity prior to this point (final –ln *L* = 2157.773). Maximum-likelihood and Bayesian trees and maximum-likelihood and parsimony trees were not significantly different (ML and Bayesian: Hasegawa–Kishino test $P = 0.655$, Wilcoxon test $P = 0.655$, winning-sites test $P = 1.00$; ML and parsimony: Hasegawa–Kishino test $P = 0.7319$, Wilcoxon test $P = 0.6845$, winning-sites test $P = 1.00$). Bayesian and parsimony trees were significantly different by Hasegawa–Kishino and Wilcoxon tests, but not so by the winning-sites test (Hasegawa–Kishino test $P = 0.0313$, Wilcoxon test $P = 0.0263$, winning-sites test $P = 0.1094$). Topology changes between the methods of tree reconstruction include that in the parsimony tree one sample of *A. s. guadalupensis* (TXki) is sister to the rest of the western clade and in the maximum-likelihood and Bayesian trees this sample is only sister to the *A. s. pallida* group which is located within the western clade. Likewise, an *A. s. emoryi* sample (NMrg) is grouped with *A. s. atra* samples in maximum-likelihood and Bayesian trees, but not in parsimony. Also, *A. s. aspera* sample, GAsr, is sister to all other south-eastern + northern clade members in the parsimony and Bayesian trees, but is included in a polytomy with other members of the south-eastern + northern clade in the maximum-likelihood tree.

Geographic separations

Our phylogeographical data (cyt *b* only) confirms that the boundary of the south-eastern + northern vs. western clades runs east to west through Oklahoma, separating the ranges of *A. s. hartwegi* (south-eastern + northern clade in northern Oklahoma) and *A. s. pallida* (western clade in northern Texas and southern Oklahoma; Fig. 1; Conant & Collins 1998). This boundary provides a clear geographical separation of the south-eastern + northern clade and western clade found by Weisrock & Janzen (2000). Samples from the two geographical ranges exhibited strongly supported reciprocal monophyly and highly significant nearest neighbour statistics (Snn) (Fig. 2; Table 1; Weisrock & Janzen 2000). Bootstrap support revealed that *Apalone* from the Cimarron River (in northern Oklahoma; Cim 113, Cim 112) were strongly classified in the south-eastern + northern clade, whereas specimens from the Red River (boundary line between Texas and Oklahoma; Red93, Red98) were unambiguously classified as members of the western clade subspecies *A. s. pallida*. In other words, specimens from northern Oklahoma were more closely related to those from Florida or Quebec thousands of kilometres away than they were to those from southern Oklahoma and Texas only about 200 km away. Phylogenetic signal obtained from *Apalone* from the South Canadian River (nested between the Cimarron River and Red River, Fig. 1) was split between south-eastern + northern (Scan107, Scan102) and western clades (Scan108), indicating a hybrid zone or admixture.

Table 1 Hudson's nearest neighbour statistics (Snn) for mitochondrially defined clades and subspecies of *Apalone spinifera*. Nearest neighbour statistics were calculated with DnaSP v4.10.7 and significance values were determined by performing a permutation test of a 1000-pseudoreplicates. Two populations are interpreted to be highly genetically differentiated when Snn equals one and part of panmictic population when Snn is near 0.5. If no polymorphism occurred between the two groups an N/A is used. South-eastern + northern vs. western refers to two mitochondrially distinct clades hypothesized to be separate species detailed in the text.

	Cytochrome *b*	R35	RAG-1	Cmos
South-eastern + northern vs. western	Snn = 1.00 $P < 0.01$	Snn = 0.64 $P < 0.01$	Snn = 0.65 $P < 0.54$	Snn = 0.57 $P < 0.11$
A. s. spinifera vs. *A. s. hartwegi*	Snn = 0.77 $P < 0.23$	N/A	Snn = 0.36 $P < 0.68$	Snn = 0.33 $P < 1.0$
A. s. spinifera + *A. s. hartwegi* vs. *A. s. aspera*	Snn = 0.96 $P < 0.01$	Snn = 0.78 $P < 0.05$	Snn = 0.63 $P < 0.13$	Snn = 0.44 $P < 0.93$
A. s. emoryi vs. *A. s. atra*	Snn = 0.92 $P < 0.01$	Snn = 0.67 $P < 0.11$	Snn = 0.60 $P < 0.30$	N/A
A. s. emoryi + *A. s. atra* vs. *A. s. pallida*	Snn = 1.00 $P < 0.01$	Snn = 0.59 $P < 0.13$	Snn = 0.66 $P < 0.03$	Snn = 0.50 $P < 1.00$

Lack of nuclear support for species hypothesis for mitochondrial clades tested by phylogenetic species concept

The distinctness of the south-eastern + northern (*A. s. aspera*, *A. s. spinifera*, *A. s. hartwegi*) and the western clades (*A. s. pallida*, *A. s. guadalupensis*, *A. s. emoryi*, *A. s. atra*) of *A. spinifera* identified by Weisrock & Janzen (2000) is confirmed by our more extensive sampling of cyt *b* (Fig. 2). The reciprocally monophyletic south-eastern + northern and the western clades were well-supported (93–100 bootstrap proportions) in parsimony, maximum-likelihood, and Bayesian analyses. Moreover, Hudson's nearest neighbour statistics (Snn) for cyt *b* unambiguously found that the nearest sequence neighbour was within the geographical boundaries defined by the south-eastern + northern and western sample localities (Snn = 1.00, $P < 0.01$). Samples from the hybrid zone (Scan107, Scan102; Scan108) and the individual identified as *A. s. pallida* found in Illinois were excluded from these estimates.

The phylogenetic division between the south-eastern + northern and western clades was accompanied by molecular divergences similar to between-species levels observed in other well-characterized species. *Apalone spinifera* subspp. are 10.96%–13.90% and 6.82%–9.57% divergent from *A. mutica* and *A. ferox*, respectively; by comparison, *A. spinifera* from the south-eastern + northern clade are 5.16%–8.01% divergent from specimens from the western clade (Table 2). Haplotype analyses revealed 17 fixed substitutions between the two clades (Fig. 3).

Table 2 Distance matrices for cytochrome *b*, RAG-1, Cmos, and R35 for seven subspecies of *Apalone spinifera* were constructed using models TVM + Γ, HKY85, K3P and HKY + I, respectively. *Apalone mutica*, and *A. ferox* are given for comparison. When data from more than one specimen or allele was available, the highest and lowest range values are given. Numbers in cells on the diagonal are within-subspecies divergences. *Apalone mutica* and *A. ferox* were 11.07%, 0.53%, 1.19 % and 0.92% divergent for cytochrome *b*, RAG-1, Cmos and R35, respectively. *Apalone spinifera atra* was 7.32%, 0.9%, 0.02%, 0.57%–0.68% divergent from *A. ferox* and 13.05%, 0.43%, 0.94%, 1.03%–1.15% different from *A. mutica* at cytochrome *b*, RAG-1, Cmos and R35, respectively.

	Divergence between *Apalone spinifera* subspecies					
	hartwegi	*emoryi*	*pallida*	*guadalupensis*	*aspera*	*spinifera*
hartwe						
Cyt *b*	0–1.66					
RAG-1	0.29–0.44					
Cmos	0–0.19					
R35	0–1.63					
emoryi						
Cyt *b*	5.63–8.01	0–0.42				
RAG-1	0–0.14	0–0.14				
Cmos	0–0.19	0				
R35	0–1.16	0–0.23				
pallida						
Cyt *b*	5.34–7.61	0.88–1.62	0.00–1.61			
RAG-1	0–0.93	0–0.70	0–1.18			
Cmos	0–0.37	0–0.19	0–0.19			
R35	0–1.40	0–0.69	0–0.92			
guadalupensis						
Cyt *b*	5.18–7.25	0.28–1.94	0.14–1.46	0.86		
RAG-1	0.14–0.59	0.14–0.29	0.14–0.93	0.29		
Cmos	0–0.37	0–0.19	0–0.37	0.19		
R35	0.11–0.92	0.11–0.34	0.11–0.70	0		
aspera						
Cyt *b*	1.30–2.26	5.59–7.09	5.34–6.93	5.34–6.82	0–2.24	
RAG-1	0–0.49	0.14–0.32	0.88	0.44–0.50	0	
Cmos	0–0.75	0–0.56	0–0.75	0–0.75	0–0.75	
R35	0–1.39	0.23–1.38	0–1.62	0.34–1.27	0–1.39	
spinif						
Cyt *b*	0–1.46	5.60–6.49	5.56–6.56	5.56–5.80	1.30–1.93	0.14–0.28
RAG-1	0–0.43	0–0.14	0–0.76	0.14–0.29	0.14–0.32	0.14
Cmos	0–0.19	0	0–0.02	0–0.02	0–0.56	0
R35	0–0.93	0.46–0.69	0.16–0.93	0.58	0.16–1.15	0
atra						
Cyt *b*	5.21–7.07	0.14–0.57	1.00–1.60	1.00–1.16	5.17–6.20	5.39–5.63
RAG-1	0–0.44	0–0.29	0–0.77	0.14–0.44	0.14–0.33	0–0.29
Cmos	0–0.20	0	0–0.20	0–0.20	0–0.56	0
R35	0.11–0.98	0–0.34	0–0.68	0.11–0.23	0.23–1.27	0.46–0.58
mutica						
Cyt *b*	10.96–13.99	12.75–13.68	11.16–11.34	11.44–12.3	12.50–13.10	11.98–12.28
RAG-1	0.17–0.53	0.34–0.51	0.17–1.13	0.70	0.70	0.34–0.51
Cmos	0.94–1.13	0.94	0.94–1.13	0.94–1.13	0.94–1.51	0.94
R35	0.80–1.64	1.03–1.27	0.90–1.51	1.16	0.80–1.74	1.05
ferox						
Cyt *b*	7.49–9.57	7.09–7.78	6.82–7.39	7.05–7.49	7.20–8.10	7.67–7.94
RAG-1	0.60–1.10	0.77–0.90	0.57–1.52	1.10	0.50–0.60	0.77–0.92
Cmos	0.20–0.40	0.18–0.40	0.18–0.40	0.18–0.40	0.18–0.80	0.18
R35	0.34–0.92	0.57–0.8	0.49–1.51	0.69	0.34–1.27	0.57

The three nuclear loci (R35, RAG-1 and Cmos) showed little to no phylogenetic structure in the parsimony consensus trees. No resolution was achieved by combining the nuclear data and rerunning the parsimony analysis. No additional resolution was achieved by maximum-likelihood analysis with R35. Snn between the south-eastern + northern and western clades for the nuclear loci reiterated the low resolution noted in the phylogenetic analyses, but was significant

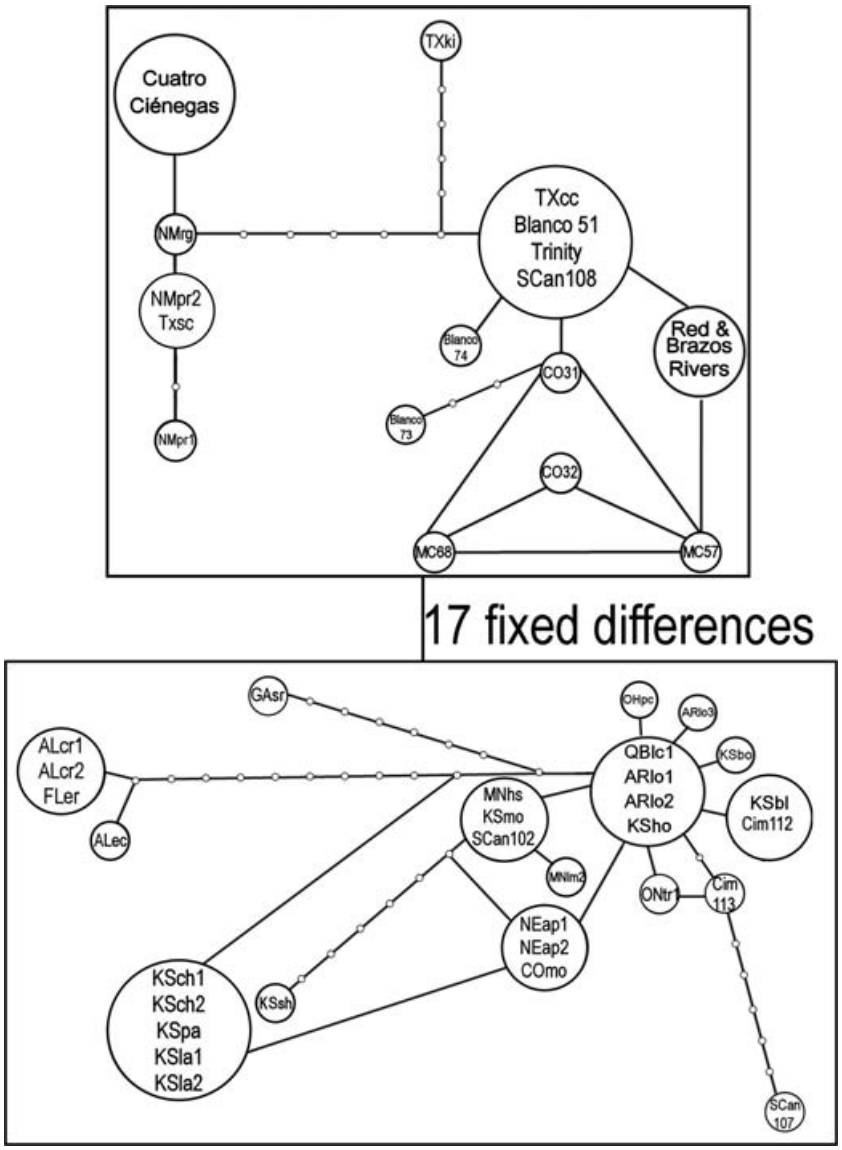

Fig. 3 TCS haplotype network of 725 base pairs of the mitochondrial gene cytochrome *b* for *A. spinifera* as calculated by statistical parsimony with gaps in the data set coded as missing. Haplotype frequencies are proportional to circle area and small, empty circles on branches are representative of haplotypes missing from the data set. Individuals in each haplotype are listed within the circle using the same notation as in Figs 1 and 2. The top network encompasses the western clade and the bottom network represents the south-eastern + northern clade.

for R35 (R35: Snn = 0.64, $P < 0.01$; RAG-1: Snn = 0.65, $P < 0.54$; Cmos: Snn = 0.57, $P < 0.11$).

Nuclear markers revealed some divergence between *A. spinifera* and its sister taxa *A. mutica* and *A. ferox* (R35: 0.80%–1.74%; RAG-1: 0.17%–1.13%; Cmos: 0.94%–1.13% and R35: 0.34%–1.20%; RAG-1: 0.50%–1.52%; Cmos: 0.18%–0.80%, respectively). In contrast, divergence levels between the south-eastern + northern and western clades within *A. spinifera* all included zero (RAG-1: 0%–1.18%; Cmos: 0%–0.75%; R35: 0%–1.62%), and did not exhibit any apomorphies congruent with clades defined by cyt *b*. Further, using R35, gene flow between the two clades was estimated to be high ($F_{ST} = 0.10$, $Nm = 2.39$) and another estimate of population level genetic differentiation, χ^2, was not significant ($\chi^2 = 35.96$, $P < 0.18$).

Evaluation of genetic diversity in subspecies

Apalone s. spinifera and *A. s. hartwegi* were not supported as reciprocally monophyletic for cyt *b* (Fig. 2) and were not significant for Snn (*A. s. spinifera* and *A. s. hartwegi*: 0%–1.46% divergent; Snn = 0.77, $P < 0.23$). *Apalone spinifera aspera* samples, aside from GAsr, which is sister to all other south-eastern + northern clade individuals (in Bayesian and parsimony), showed high bootstrap support (98%–100%) as a separate, reciprocally monophyletic clade from the *A. s. spinifera* + *A. s. hartwegi* group (1.30%–3.23% divergent; Snn = 0.96, $P < 0.01$). Divergences in R35 also supported *A. s. aspera* being distinct from *A. s. hartwegi* + *A. s. spinifera*, although this was weakly significant (Snn = 0.78, $P < 0.05$).

Table 3 Morphological subspecies and notable species distinguishers of key *Apalone* taxa following Ernst *et al*. (1994) and Conant & Collins (1998).

Subspecies	Location of spines	Carapace markings	Other defining characteristics
A. s. spinifera	Anterior edge	Dark ocelli in males and juveniles. Females blotched. One dark marginal line	Markings on feet, nostril ridge, tiny sandpaper projections on entire carapace
A. s. hartwegi	Anterior edge	Small dark spots, ocelli on the carapace	All other characteristics like *A. s. spinifera*
A. s. aspera	Anterior area	Two or more dark marginal lines	Lines from eye and the jaw unite
A. s. pallida	Posterior half	No black ocelli. Brown olive colour	Largest of the *A. spinifera* races
A. s. guadalupensis	Entire carapace	Small black dots. Overall dark pigment	
A. s. emoryi	Rear one-third	Light carapace marginal line. Brown-olive colour	
A. s. atra	Flesh coloured tubercles on posterior of males	Dark pigmentation	Corrugations on the posterior of carapace. Dark markings on plastron

Divergences were low among subspecies in the western clade (0.86%–1.62%; Table 2). *Apalone spinifera emoryi* and *A. s. atra* were classified as statistically distinct from each other at cyt *b* by Snn (0.14%–0.57% divergent; Snn = 0.92, $P < 0.01$), but Snn for R35 was not significant (Snn = 0.67, $P < 0.11$) and phylogenetic support for distinctness is only supported in the parsimony analysis, and weakly so at that (parsimony bootstrap = 61). With such low divergence between *A. s. emoryi* and *A. s. atra*, we combined *A. s. emoryi* with *A. s. atra* and tested for distinctness of these two entities from *A. s. pallida*. Reciprocal monophyly and statistically significant Snn values were exhibited between *A. s. emoryi* + *A. s. atra* and *A. s. pallida* at cyt *b* (0.86%–1.62% divergent; Snn = 1.00, $P < 0.01$), although this distinction was not supported by R35 analyses (Snn = 0.59, $P < 0.13$).

The morphologically defined *A. s. guadalupensis* sample, Blanco51, from a population sympatric with *A. s. pallida* had an *A. s. pallida* cyt *b* haplotype (Fig. 3). This result is taken to indicate hybridization between these two subspecies because two main morphological identifiers for *A. s. guadalupensis* (dark pigmentation and spines distributed over the entire carapace) were present on the sampled individual. The other *A. s. guadalupensis* sample (TXki) maintained a weakly supported separation from samples in the *A. s. pallida* clade (bootstrap proportion for maximum-likelihood separation from other western samples = 70; 0.14%–1.46% divergent from other *A. s. pallida* clade samples, Fig. 2).

Lastly, cyt *b* data indicate that the aberrant individual originally identified as *A. s. pallida* collected outside typical range by Lamer *et al*. (2006) in Jersey Co., IL, was actually more similar to *A. s. emoryi* (Fig. 2). Close inspection of the illustration in Lamer *et al*. (2006) reveals that the spines are restricted to the back one-third of the carapace, a characteristic of *A. s. emoryi* (Ernst *et al*. 1994; Table 3), which naturally occurs in the Rio Grande region (Fig. 1).

Discussion

The morphologically based systematic status of many herpetofauna is increasingly subject to scrutiny by molecular methods (e.g. Zamudio *et al*. 1997; Karl & Bowen 1999; Janzen *et al*. 2002; Rawlings & Donnellan 2003; Starkey *et al*. 2003; Stuart & Parham 2004; Fritz *et al*. 2005). Such approaches are crucial for confirming previous designations or identifying new ones, thereby improving the understanding of evolutionary processes and the rigor of conservation efforts. Our detailed molecular study of *A. spinifera* provides significant advances on both fronts.

Phylogeographical interpretation

We detected substantial mitochondrial divergence within *A. spinifera*. In some cases, the divergence within *A. spinifera* approaches or exceeds the level detected between *A. spinifera* and *A. ferox* for the same segment of cyt *b* (see Results and Table 1). Moreover, the variation in cyt *b* sequences exhibits striking geographical patterns. Perhaps the most notable pattern confirms the split between a south-eastern + northern clade and a western clade noted by Weisrock & Janzen (2000). Our finer scale sampling, in the western portion of the range, highlights an especially remarkable spatial feature of the variation. Over a short geographical range (< 200 km between Cimarron River and Red River sites in Oklahoma), *A. s. hartwegi* and *A. s. pallida* diverge considerably in cyt *b* sequence (5.40%–6.56%). In contrast, the geographical distance spanning the ranges of *A. s. hartwegi* and *A. s. spinifera* is over 2500 km, but only 0.42% sequence divergence exists between the two groups (when comparing Oklahoma and Quebec samples).

This phylogeographical pattern reveals how the North American turtle fauna might have been impacted by changes in drainage systems of the Great Plains in the Miocene and in subsequent glaciations. Throughout the preglaciation period, the drainages of the central and north plains stayed relatively separate from those of the western Gulf slope (reviewed in Cross *et al*. 1986). This could have afforded substantial allopatry for the lineages representing present day south-eastern + northern and the western clades. Repeated cycles of reconnection during, and recolonization after, glaciation may have prevented great divergence in *A. s. spinifera*

and *A. s. hartwegi* populations that are presently separated by great geographical distances. This secondary contact by recurring range constrictions was probably caused as streams that formerly drained into the Hudson Bay and Atlantic Ocean instead drained into the Mississippi River (Cross *et al.* 1986; Hewitt 1996; Bernatchez & Wilson 1998). The phylogeographical pattern described here has also been described in fish (Conner & Suttkus 1986; Kreiser *et al.* 2001). This shared pattern suggests that stream captures may have happened in a manner that kept some fauna in drainages of the western Gulf slope, such as the Red and Pecos Rivers, relatively separate from those in the Cimarron and Arkansas River drainages (Conner & Suttkus 1986; Kreiser *et al.* 2001). In addition, the sharing of haplotypes in *Apalone* populations throughout rivers in north Texas is indicative of stream captures by the ancestral Red River. The monophyly of *A. s. emoryi* (found in the Rio Grande and Pecos Rivers) + *A. s. atra* (in Cuatro Ciénegas) with respect to *A. s. pallida* and *A. s. guadalupensis* (found in the Red River and other central, south-east and north-east Texas drainages; Fig. 1) further supports the hypothesis that the Rio Grande-Pecos Rivers had limited contact with the Red River drainage (Conner & Suttkus 1986; Smith & Miller 1986).

Of special note is the reciprocal monophyly of the majority of *A. s. aspera* samples to *A. s. spinifera* + *A. s. hartwegi*. This phylogenetic structure suggests a uniqueness of *A. s. aspera* when compared to *A. s. spinifera* + *A. s. hartwegi* and cryptic variation within what is traditionally thought of as *A. s. aspera*. The *A. s. aspera* sample that did not fall within the subspecies concordant clade, GAsr, is placed sister to the rest of the south-eastern + northern clade (Bayesian and parsimony trees) or within the south-eastern + northern clade maximum-likelihood and is clearly revealed in the haplotype analyses to be quite divergent from the other samples in the south-eastern + northern clade (Fig. 3). The geographical location of GAsr (Fig. 1) occurs on the eastern side of the Apalachicola River, a well-documented phylogeographical divide for multiple other herpetofauna (reviewed in Walker & Avise 1998; Burbrink *et al.* 2000). Further sampling in eastern and southern Florida may uncover stronger support for this phylogeographical trend in softshell turtles and cryptic variation within *A. s. aspera*.

Tentative rejection of the species hypothesis for the two major mitochondrial clades

Mitochondrial data may obscure true phylogenetic history through a host of demographic processes, and thus ideally taxonomic changes should await corroboration of the mitochondrial signal by nuclear loci (Ballard & Whitlock 2004; Ballard & Rand 2005). The south-eastern + northern and the western clades maintained strongly supported, geographically concordant reciprocal monophyly in mtDNA and statistically significant nearest neighbour statistics in both mitochondrial and nuclear loci. However, the lack of nuclear apomorphies or phylogenetic structure that is concordant with mtDNA defined clades, suggests that the delimitation of the south-eastern + northern clade (*A. s. hartwegi* + *A. s. spinifera* + *A. s. aspera*) and the western clade (*A. s. pallida* + *A. s. guadalupensis* + *A. s. emoryi* + *A. s. atra*) as two separate species through the phylogenetic species concept or even evolutionarily significant units (ESU's *sensu* Moritz 1994) would be unjustified at this point. and although statistically significant nearest neighbour estimates were calculated for R35, gene flow between the two clades was estimated to be high (F_{ST} = 0.10, Nm = 2.39 via Hudson *et al.* 1992 calculated in DnaSP v4.10.7) and another estimate of population-level genetic differentiation, χ^2, was not significant (χ^2 = 35.96, P < 0.18; Workman & Niswander 1970). We therefore regard the nuclear support for these lineages, at least at the loci examined here, to be weak.

The paucity of nuclear divergence in Testudines, in extreme contrast to rapidly evolving mitochondrial DNA, has been noted by other authors (Caccone *et al.* 2004; Spinks & Shaffer 2005) and has been treated as an exceptionally difficult problem for turtle biology (Spinks & Shaffer 2005). It is likely that future studies using more rapidly evolving nuclear loci such as ISSR (Fritz *et al.* 2005) or single nucleotide polymorphisms (SNPs) could reveal significant nuclear divergence.

Any future taxonomic recommendations should ideally be supported by at least one substantive morphological distinction as well. One such morphological characteristic is the placement of the spines on the carapace. The shell-coloured 'spines' in the south-eastern + northern clade are located only on the anterior portion of the carapace, whereas prominent, white or shell-coloured tubercles cover the entire carapace (*A. s. guadalupensis*) or are apparent on the posterior part of the carapace (*A. s. pallida*, *A. s. emoryi*, *A. s. atra*) in the western clade (Table 3; Winokur 1968; Ernst *et al.* 1994; Conant & Collins 1998). In addition, the carapace of the south-eastern + northern clade is patterned with small circular spots or ocelli in males and juveniles, whereas the western clade lacks these (Ernst *et al.* 1994; Conant & Collins 1998). Thus, should corroborating evidence arise from new studies of nuclear loci, the taxonomic status of the mitochondrial clades of *A. spinifera* should be revisited.

Subspecies delimitations

The use of subspecies is controversial for taxonomic and conservation purposes (e.g. Burbrink *et al.* 2000; Zink 2004; Phillimore & Owens 2006). Recent evaluations of other Testudines have found that subspecies designations are not always congruent with molecular data (e.g. Starkey *et al.* 2003; Spinks & Shaffer 2005). Our study finds the distinctness of *A. s. spinifera* and *A.s. hartwegi* is not well supported by the

four loci sequenced here and we concluded there to be very little utility in recognizing these two northern subspecies as separate entities. Instead, we suggest referring to these two groups as simply *A.s. spinifera*. Support exists for the molecular distinction of *A. s. aspera* and *A. s. spinifera* + *A. s. hartwegi*. Some support exists for cryptic molecular diversity within *A. s. aspera*, indicating that not all of the genetic variation has been capture by a single subspecies designation for the gulf coast softshells. In the western clade, weak support exists for the separation of *A. s. emoryi* and *A. s. atra*, and a companion study confirms that the original species delimitation of *A. s. atra* is not supported genetically (McGaugh & Janzen in press). *Apalone spinifera pallida* maintains a well supported separation from *A. s. emoryi* + *A. s. atra*, whereas morphs of *A. s. guadalupensis* are polyphyletic, with one individual (TXki) situated as sister to the *A. s. pallida* clade or to the entire western clade, depending on the phylogenetic reconstruction method used, and the other morphologically identified sample of *A. s. guadalupensis* (Blanco51) grouping within the *A. s. pallida*. We suspect that the *A. s. pallida* haplotype of the morphological *A. s. guadalupensis* sample (Blanco51, indicated in Fig. 2 by a diamond) is the result of introgression with *A. s. pallida* (Funk & Omland 2003). Lastly, the geographically discordant sample *A. s. emoryi* sample from Illinois (Lamer *et al*. 2006) could be potentially an introduction from the pet-trade, as we see no evidence for *A. s. emoryi* haplotypes in any locations except the Texas, New Mexico and Cuatrociénegas. Non-native morphologically identified *A. s. pallida* has been noted in California as well, although these samples were never analysed genetically (Spinks *et al*. 2003).

Conclusions

Cryptic or unexpectedly high levels of genetic variation are oftentimes found in wide-scale examination of a species range (e.g. Spinks & Shaffer 2005), and splitting taxa along these molecularly defined lines can be strongly motivated by conservation implications (Hey *et al*. 2003; Isaac *et al*. 2004). However, reliance on specific taxa as units of conservation is inherently difficult because species concepts are non-equivalent (Hey *et al*. 2003; Sites & Marshall 2004). What is a 'species' by one definition does not necessarily entail the same level of evolutionary or ecological uniqueness or significance as may be implied by another (Sites & Marshall 2003, 2004; de Queiroz 2005). In contrast, comparative phylogeography may greatly inform conservation priorities by identifying geographical regions that harbour genetic variation for multiple species (Moritz & Faith 1998), regardless of what taxon names are attached to that variation. With this idea in mind, we emphasize that our decision to retain the current taxonomic level between the south-eastern + northern and western clades of *A. spinifera* should not overshadow the existence of real and important mitochondrial divergence and slight nuclear genetic diversity detected, as well as the apparent phenotypic diversity. Further genetic exploration of other aquatic or semi-aquatic fauna between the Red River (TX) and Cimmarron River (OK), within the range of *A. s. guadalupensis*, and across the range of *A. s. aspera* on the gulf coast would be valuable for future comparative studies. Lastly, increasing emphasis on preserving genetic uniqueness across Florida's gulf coast will be important for maintaining current diversity throughout *A. spinifera*'s range.

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Appendix Localities, sample names and morphological subspecies identification. GenBank accession numbers are given for cytochrome *b*, RAG-1, Cmos and R35, respectively and separated by semi-colons for each gene. Alleles for each gene are separated by commas. Iowa State University herpetology accession numbers are given for photo vouchers for samples new to this study when photos were available. For those samples where no photo vouchers could be obtained, subspecies designations were inferred from range data and locality of collection. Individuals and sequences previously used by Weisrock & Janzen (2000) and Engstrom *et al*. (2004) are underlined. Donated samples are indicated by and asterisk.

Sample	Locality and Genbank accession numbers
LAcr1m	Comite River 30°30′N, 91°04′W; East Baton Rouge Parish, Baker, LA
	(J51306); *Apalone mutica*
	Cyt *b*; DQ529173; DQ529206; EU119955, EU119956
Ferox	US Highway 41, approximately 5 km east of junction with state road 29, Collier Co., FL
	(J53743); *Apalone ferox*
	Cyt *b*; DQ785893; DQ785894; *R35*
FLer1	Escambia River, Escambia Co., FL (J52172); *A. s. aspera*
	Cyt *b*; DQ529172; DQ529204, DQ529205; EU119904, EU119905
Alec	Euphapee Creek, Macon Co., AL (J20044); *A. s. aspera*
	Cyt *b*; DQ529161; DQ529194, DQ529195; EU119903
ONtr1	Thames River, north of London, Ontario, Canada
	(J53773); *A. s. spinifera*
	Cyt *b*; DQ529141, DQ529142; DQ529179; R35 no data

Appendix *Continued*.

Sample	Locality and Genbank accession numbers
ONtr2	Thames River, north of London, Ontario, Canada
	(J53776); *A. s. spinifera*
	Cmos only: DQ529180, R35: EU119953
TXki	Kingsville, Kleber Co., TX (J20042); *A. s. guadalupensis*
	Cyt *b*; DQ529145, DQ529146; DQ529183, DQ529184; DQ529124
TXsc	Sycamore Creek, Valverde Co., TX (J20045); *A. s. pallida*
	Cyt *b*; DQ529147, DQ529148; DQ529185, DQ529186; DQ529125
TXcc	Coleto Creek, Goliad Co., TX (J20047); *A. s. pallida*
	Cyt *b*; DQ529149, DQ529150; Cmos no data; DQ529126
NMrg	North Elephant Butte Reservoir, Socorro Co. NM (J20013); *A. s. emoryi*
	Cyt *b*; DQ529151; DQ529187; DQ529127
GAsr	Suwanee River, Lanier Co., GA (J20034); *A. s. emoryi*
	Cyt *b*; DQ529152; DQ529188, DQ529189; EU119901, EU119902
QBlc1	Chapman Bay, Lake Champlaim, Quebec, Canada
	(J53779), *A. s. spinifera*
	EU119874; RAG-1 no data; Cmos no data; R35 no data
SCan102	South Canadian River, Caddo Co., OK, 35°32′20′′N, 98°19′W
	(J22036; CME102) ISUA20065; *A. s. spinifera hybrid* zone with *A. s. hartwegi*
	DQ529116; RAG-1 no data; Cmos no data; EU119930
SCan107	South Canadian River, Caddo Co., OK, 35°32′20′′N, 98°19′W
	(J22037; CME107) ISUA20066; *A. s. spinifera hybrid* zone with *A. s. hartwegi*
	DQ529096; DQ529138, DQ529139; DQ529177; DQ529120
SCan108	South Canadian River, Caddo Co., OK, 35°32′20′′N, 98°19′W
	(J22038; CME108) ISUA20067; *A. s. spinifera hybrid* zone with *A. s. hartwegi*
	DQ529115; RAG-1 no data; Cmos no data; EU119952
Cim113	Cimarron River, Kingfisher Co., OK, 35°57′N, 97°54′30′′W
	(J22039; CME113) ISUA20068, *A. s. hartwegi*
	DQ529097; DQ529168, DQ529169; DQ529200, DQ529201; EU119924, EU119925
Cim112	Cimarron River, Kingfisher Co., OK, 35°57′N, 97°54′30′′W
	(J22040; CME112) ISUA20069, *A. s. hartwegi*
	DQ529117; RAG-1 no data; Cmos no data; R35 no data
Blanco51	Blanco River, Hays Co., TX, 29°57′N, 98°9′30′′W
	(J22041; CME51) ISUA200610, *A. s. guadalupensis*
	DQ529098; DQ529170, DQ529171; DQ529202, DQ529203; R35 no data
Blanco74	Blanco River, Hays Co., TX, 29°57′N, 98°9′30′′W
	(J22042; CME74), *A. s. pallida*
	DQ529099; DQ529164, DQ529165; DQ529197, DQ529198; EU119926
Blanco73	Blanco River, Hays Co., TX, 29°57′N, 98°9′30′′W
	(J22043; CME73), *A. s. pallida*
	DQ529107; DQ529166, DQ529167; DQ529199; EU119945
Trinity 85	Trinity River, Denton Co., TX 33°19′N, 97°2′30′′W
	(J22044; CME85) ISUA200611, *A. s. pallida*
	DQ529100; RAG-1 no data; Cmos no data; EU119931, EU119932
Trinity 87	Trinity River, Denton Co., TX 33°19′N, 97°2′30′′W
	(J22045; CME87) ISUA200612, *A. s. pallida*
	DQ529101; DQ529162, DQ529163; DQ529196; EU119933, EU119934
MidCon68	Middle Concho River, Irion Co. TX, 31°23′N, 100°47′W
	(J22046; CME68) ISUA200613, *A. s. pallida*
	DQ529102; DQ529143, DQ529144; DQ529181; EU119939
MidCon63	Middle Concho River, Irion Co. TX, 31°23′N, 100°47′W
	(J22047; CME63) ISUA200614, *A. s. pallida*
	DQ529103; DQ529157, DQ529158; DQ529192; EU119940, EU119941
MidCon57	Middle Concho River, Irion Co. TX, 31°23′N, 100°47′W
	(J22048; CME57), *A. s. pallida*
	DQ529105; RAG-1 no data; Cmos no data; EU119943, EU119944
CO31	Colorado River, Runnels Co., TX, 31°43′N, 100°03′W
	(J22049; CME31) ISUA200615, *A. s. pallida*
	DQ529106; DQ529155, DQ529156; DQ529191; DQ529121

Appendix *Continued.*

Sample	Locality and Genbank accession numbers
CO32	Colorado River, Runnels Co., TX, 31°43′N, 100°03′W
	(J22050; CME32) ISUA200616, *A. s. pallida*
	DQ529104, RAG-1 no data; Cmos no data; EU119942
Red98	Red River, Jefferson Co. OK, 34°13′30′′N, 98°3′W
	(J22051; CME98) ISUA200617, *A. s. pallida*
	DQ529108; DQ529140; DQ529178; R35 no data
Red93	Red River, Jefferson Co. OK, 34°13′30′′N, 98°3′W
	(J22052; CME93) ISUA200618, *A. s. pallida*
	DQ529110; DQ529136, DQ529137; DQ529176; EU119950, EU119951
Brazos44	Brazos River, Pale Pinto Co., TX, 32°51′N, 98°24′W
	(J22053; CME44) ISUA200619, *A. s. pallida*
	DQ529109; RAG-1 no data; Cmos no data; EU119948, EU119949
TC34mt	Cuatrociénegas, Coahuila, Mexico, 26°52′N, 102°04′W
	(J21907) ISUA200620, *Apalone atra*
	DQ529113; RAG-1 no data; Cmos no data; DQ529130
TC35mt	Cuatrociénegas, Coahuila, Mexico, 26°52′N, 102°04′W
	(J21906) ISUA200621, *Apalone spinifera atra*
	DQ529112; RAG-1 no data; Cmos no data; DQ529131
TC37mt	Cuatrociénegas, Coahuila, Mexico, 26°52′N, 102°04′W
	(J21909) ISUA200622, *Apalone spinifera atra*
	DQ529111; RAG-1 no data; Cmos no data; DQ529128, DQ529129
TC38mt	Cuatrociénegas, Coahuila, Mexico, 26°52′N, 102°04′W
	(J22054) ISUA200623, *Apalone spinifera atra*
	DQ529114; DQ529132, DQ529133; DQ529174; DQ529118
TC36mt	Cuatrociénegas, Coahuila, Mexico, 26°52′N, 102°04′W
	(J22054) ISUA200625, *Apalone spinifera atra*
	Cyt *b* no data; RAG-1 no data; Cmos no data; DQ529119
TC45mt	Cuatrociénegas, Coahuila, Mexico, 26°52′N, 102°04′W
	(J21904) ISUA200624, *Apalone spinifera atra*
	Cyt *b* no data; DQ529134, DQ529135; DQ529175; EU119954
ALcr1*	Conecuh River at River Falls; Covington Co., AL
	HBS107771, *A. s. aspera*
	EU119880; RAG-1 no data; Cmos no data; R35 no data
ALcr2*	Conecuh River at River Falls; Covington Co., AL
	HBS107772, *A. s. aspera*
	EU119890; RAG-1 no data; Cmos no data; EU119927
ALcr3*	Conecuh River at River Falls; Covington Co., AL
	HBS10770, *A. s. aspera*
ARlo1*	Joe Hogan State Fish Hatchery; Lonoke Co., AR
	HBS23258, *A. s. hartwegi*
	EU119885; RAG-1 no data; Cmos no data; R35 no data
ARlo2*	Joe Hogan State Fish Hatchery; Lonoke Co., AR
	HBS23335, *A. s. hartwegi*
	EU119887; RAG-1 no data; Cmos no data; EU119921
ARlo3*	Joe Hogan State Fish Hatchery; Lonoke Co., AR
	HBS23259, *A. s. hartwegi*
	EU119879; RAG-1 no data; Cmos no data; EU119922
OHpc*	Paint Creek State Park 6mi S of Greenfield; Highland Co., OH
	HBS27218, *A. s. spinifera*
	EU119883; RAG-1 no data; Cmos no data; EU119928
COmo*	Fishing Lake 3 mi N of Brush along Hwy 71 near S Platte River; Morgan Co., CO, HBS28098, *A. s. hartwegi*
	EU119886; RAG-1 no data; Cmos no data; EU119923
NMpr1*	Oxbow off of Pecos River Bitterlakes NWR; Chaves Co., NM
	HBS28629, *A. s. emoryi*
	EU119882; RAG-1 no data; Cmos no data; R35 no data
NMpr2*	Oxbow off of Pecos River Bitterlakes NWR; Chaves Co., NM
	HBS28633, *A. s. emoryi*
	EU119881; RAG-1 no data; Cmos no data; EU119946, EU119947

Appendix *Continued.*

Sample	Locality and Genbank accession numbers
KSmo*	37°01′32.18′N, 95°57′19.50′′W; Montgomery Co., KS
	CS8770, *A. s. hartwegi*
	EU119894; RAG-1 no data; Cmos no data; EU119929
KSch1*	37°02′25.84′′N, 94°38′08.27′′W; Cherokee, KS
	CS8787, *A. s. hartwegi*
	EU119889; RAG-1 no data; Cmos no data; EU119908, EU119909
KSpa*	38°14′24.76′′N, 98°57′16.34′′W; Pawnee, KS
	CS9005, *A. s. hartwegi*
	EU119891; RAG-1 no data; Cmos no data; EU119910, EU119911
KSbl*	37°18′34.34′′N, 99°41′05.10′′W; Bluff River, Clark Co., KS
	CS9173, *A. s. hartwegi*
	EU119878; RAG-1 no data; Cmos no data; EU119918, EU119919
KSho*	38°04′08.90′′N, 100°00′01.80′′W; Hodgeman Co., KS
	CS9303, *A. s. hartwegi*
	EU119892; RAG-1 no data; Cmos no data; EU119899, EU119900
KSbo*	37°51′20.92′′N, 94°38′22.78′′W; Bourbon Co., KS
	CS9332, *A. s. hartwegi*
	EU119896; RAG-1 no data; Cmos no data; EU119935, EU119936
KSla1*	37°07′40.47′′N, 95°09′06.52′′W; Labette Co., KS
	CS9614, *A. s. hartwegi*
	EU119893; RAG-1 no data; Cmos no data; EU119912, EU119913
KSla2*	37°09′41.02′′N, 95°10′15.46′′W; Labette Co., KS
	CS9617, *A. s. hartwegi*
	EU119895; RAG-1 no data; Cmos no data; EU119937, EU119938
KSch2*	37°10′47.53′′N, 95°04′12.83′′W; Lighting River, Cherokee Co., KS
	CS11737, *A. s. hartwegi*
	EU119888; RAG-1 no data; Cmos no data; EU119906, EU119907
KSsh*	38°42′53.30′′N, 99°09′42.98′′W; Smoky Hill River, Ellis Co., KS
	CS11950, *A. s. hartwegi*
	EU119898; RAG-1 no data; Cmos no data; R35 no data
ILap*	38°55′59.16′′N, 90°17′35.38′′W; Jersey, Co., IL
	INHS2358, aberrant locality: *A. s. pallida*
	EU119897; RAG-1 no data; Cmos no data; R35 no data
MNlm1*	Lake Maria State Park, Wright Co. MN
	JFBM1, *A. s. hartwegi*
MNlm2*	Lake Maria State Park, Wright Co. MN
	JFBM2, *A. s. hartwegi*
	EU119875; RAG-1 no data; Cmos no data; EU119914, EU119915
MNhs*	Horseshoe Lake S. of Minnesota River; Brown Co., MN
	HBS23552, *A. s. hartwegi*
	EU119884 (under name MNls); RAG-1 no data; Cmos no data; EU119920
NEap1*	Rattlesnake Pond, Garden Co., NE
	JI 2–3, *A. s. hartwegi*
	EU119876; RAG-1 no data; Cmos no data; R35 no data
NEap2*	Rattlesnake Pond, Garden Co., NE
	JI 2–5, *A. s. hartwegi*
	EU119877; RAG-1 no data; Cmos no data; EU119916
NEap3*	Rattlesnake Pond, Garden Co., NE
	JI 3–6, *A. s. hartwegi*
	Cyt *b* no data; RAG-1 no data; Cmos no data; EU119917