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Morphological and molecular evidence indicates that the Gulf Coast box turtle (*Terrapene carolina major*) is not a distinct evolutionary lineage in the Florida Panhandle

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Four extant subspecies of *Terrapene carolina* in eastern North America, *Terrapene carolina bauri*, *Terrapene carolina carolina*, *Terrapene carolina triunguis*, and *Terrapene carolina major*, are recognized based on morphological studies. A fifth subspecies, *Terrapene carolina putnami*, has been described from Pleistocene deposits but is very similar morphologically to *T. c. major*. Questions concerning the relationship of the Gulf Coast box turtle (*T. c. major*) to other box turtles have been pervasive ever since it was described. We used a combined morphological and genetic analysis to address the status of *T. c. major* and other *T. carolina* lineages. *Terrapene c. bauri*, *T. c. carolina*, and *T. c. triunguis* are distinct based on a discriminate function analysis of 25 morphological characters, including characters traditionally used to assign subspecies. The results of the present study confirm that box turtles phenotypically diagnosed as *T. c. bauri*, *T. c. carolina*, and *T. c. triunguis* all occur within the hypothesized range of *T. c. major*, and that the latter does not possess a diagnosable morphology. The three morphological lineages also possess divergent mitochondrial haplotypes that are present within the hypothesized range of *T. c. major*. In addition, a fourth distinct mtDNA lineage co-occurs within the putative range of *T. c. major*. This unique lineage may include mitochondrial DNA variation from the Pleistocene *T. c. putnami*. Analysis of nine nuclear DNA microsatellites revealed no population structure in box turtles currently assigned to *T. c. major* from the Florida Panhandle, suggesting a complete admixture of lineages in this region. The results of the present study indicate that box turtles traditionally assigned to *T. c. major* based on phenotype are the result of introgression between eastern extant (predominantly *T. c. carolina*) and an extinct subspecies, *T. c. putnami*. Published 2011. This article is a US Government work and is in the public domain in the USA. © 2011 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2011, **102**, 889–901.

ADDITIONAL KEYWORDS: congruence – discriminate function analysis – d-loop – history – microsatellite – mitochondrial DNA.

INTRODUCTION

Understanding general patterns of biodiversity has depended on an accurate perception of species-level diversity. However, defining groups of populations into species or taxa below the species level has been common and controversial for over 70 years (Mayr, 1942; Frost & Hillis, 1990), and the subspecies rank has been criticized as being taxonomically convenient rather than evolutionarily informative (Piller, Bart & Hurley, 2008).

Historically, subspecies were defined by the presence of clinal polymorphisms of one or a few polytypic characters that display patterns of intergradation at zones of contact (Wilson & Brown, 1953; Smith, Chiszar & Montanucci, 1997). Increasingly, polytypic taxa have been examined for divergence using mitochondrial DNA (mtDNA). Eastern North America has

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numerous widespread polytypic vertebrate species, many of which have been examined using mtDNA (Burbrink, Lawson & Slowinski, 2000; Austin & Zamudio, 2008), although few studies have incorporated both independent morphological characters and nuclear DNA markers. Discrepancies between morphology and mtDNA have led some studies to question the validity of subspecies (Burbrink *et al*., 2000). Given the vagaries associated with delimiting taxa from uniparental genetic markers (e.g. mtDNA) or morphology alone, inferences of evolutionary history, population structure, and taxonomic status should be drawn from the analysis of multiple, independent genetic traits (Shaffer & Thomson, 2007).

Incorporating independent characters allows for a concordance approach (Avise & Ball, 1990) to determine the evolutionary validity of subspecies. This approach can be overly conservative, particularly when attempting to diagnose variation within species (Sites & Crandall, 1997). However, it may provide a more robust means of delineating species, particularly in taxa that are highly variable and have proven difficult to delineate systematically. In the present study, we examine mtDNA, nuclear microsatellite markers, and morphology in diagnosing current subspecies of eastern North American box turtles (*Terrapene carolina*) throughout their range and at an area representing a putative intergrade zone.

Terrapene is represented by four species: [*T. carolina* (Linnaeus), *Terrapene coahuila* (Schmidt & Owens), *Terrapene nelsoni* (Stejneger), and *Terrapene ornata* (Agassiz)]. Two of these are endemic to Mexico (*T. coahuila* and *T. nelsoni*) and the remaining species are distributed broadly across western (*T. ornata*) and eastern (*T. carolina*) North America. *Terrapene carolina* is a moderately sized emydid turtle with low adult dispersal. Adult *T. carolina* maintain relatively small home-ranges (Dodd, 2001) and typically do not venture vast distances (Schwartz & Schwartz, 1974; Iglay, Bowman & Nazdrowicz, 2007), although virtually nothing is known about the dispersal of young.

There are six extant recognized subspecies within *T. carolina*: [*Terrapene carolina carolina* (Linnaeus), *Terrapene carolina bauri* (Taylor), *Terrapene carolina major* (Agassiz), *Terrapene carolina mexicana* (Gray), *Terrapene carolina triunguis* (Agassiz), and *Terrapene carolina yucatana* (Boulenger)]. Four of these (*T. c. carolina*, *T. c. bauri*, *T. c. major*, and *T. c. triunguis*) are found east of the Mississippi River and intergrade zones among these subspecies have been proposed exclusively on the presence of phenotypic similarities and intermediate forms (Carr, 1952; Milstead, 1969). Ward (1980) suggested that these putative intermediates simply reflect the variation found within existing taxa. These four putative subspecies coexist along the southern Coastal Plain, and numerous studies (Carr, 1952; Ward, 1980; Minx, 1996; Dodd, 2001) have represented the ranges and areas of intergradation among them in Florida Panhandle differently (Fig. 1). Phenotypic characteristics that have been used to distinguish among the subspecies include carapace and plastron shape, coloration and patterning, the extent of concavity of the male plastron, eye, head, neck and leg coloration and pattern, and the number of hind toes (Dodd, 2001). Each of these characters is variable within subspecies, and their discriminatory power has not been quantitatively evaluated.

In addition to the extant subspecies, a seventh subspecies (giant box turtle, described as *T. putnami* but relegated to *T. c. putnami* by Auffenberg, 1958) was described from Pleistocene deposits in Florida (Hay, 1906). Even in the original description, Hay (1906) noted that there were no distinguishing characteristics to separate the Pleistocene fossils from extant *T. carolina* other than carapace size and the thickness of the shell. Subsequent to its description, no further distinguishing characteristics have been identified despite a wealth of osteological material, including fully intact skulls. This has led some studies to question the validity of the taxon and its relationship with *T. c. major*, the largest of the extant subspecies (Bentley & Knight, 1998). Fossils of *T. c. putnami* are known from New Mexico to Florida and as far north as Missouri and Kansas.

The taxon *T. c. major* has proven to be particularly difficult to delineate geographically as a result of its high degree of phenotypic variation (Minx, 1996). *Terrapene c. major* is distributed along the Gulf Coastal Plain from East Texas to the northwestern Florida peninsula. In the Florida Panhandle along the northern Gulf Coast, *T. c. major* comes into contact with *T. c. triunguis*, *T. c. carolina*, and *T. c. bauri*. Phenotypic variation along the panhandle may reflect complete intergradation among these subspecies (Milstead, 1969), although other studies have suggested that *T. c. major* is reproductively isolated and that variation is likely a result of environmental heterogeneity (Ward, 1980; Minx, 1996) within this complex biogeographic region.

We tested our ability to use morphological and genetic characters to discriminate among the eastern *Terrapene* subspecies. Our objectives were to test 25 morphological characters, including traditional subspecies diagnostic characters, aiming to determine their value in distinguishing among the eastern subspecies, and to assess whether phenotypes based on morphology are congruent with the distribution of mtDNA lineages. Concordance among morphology and mtDNA would provide substantiation of evolutionary isolation for sufficient time to consider

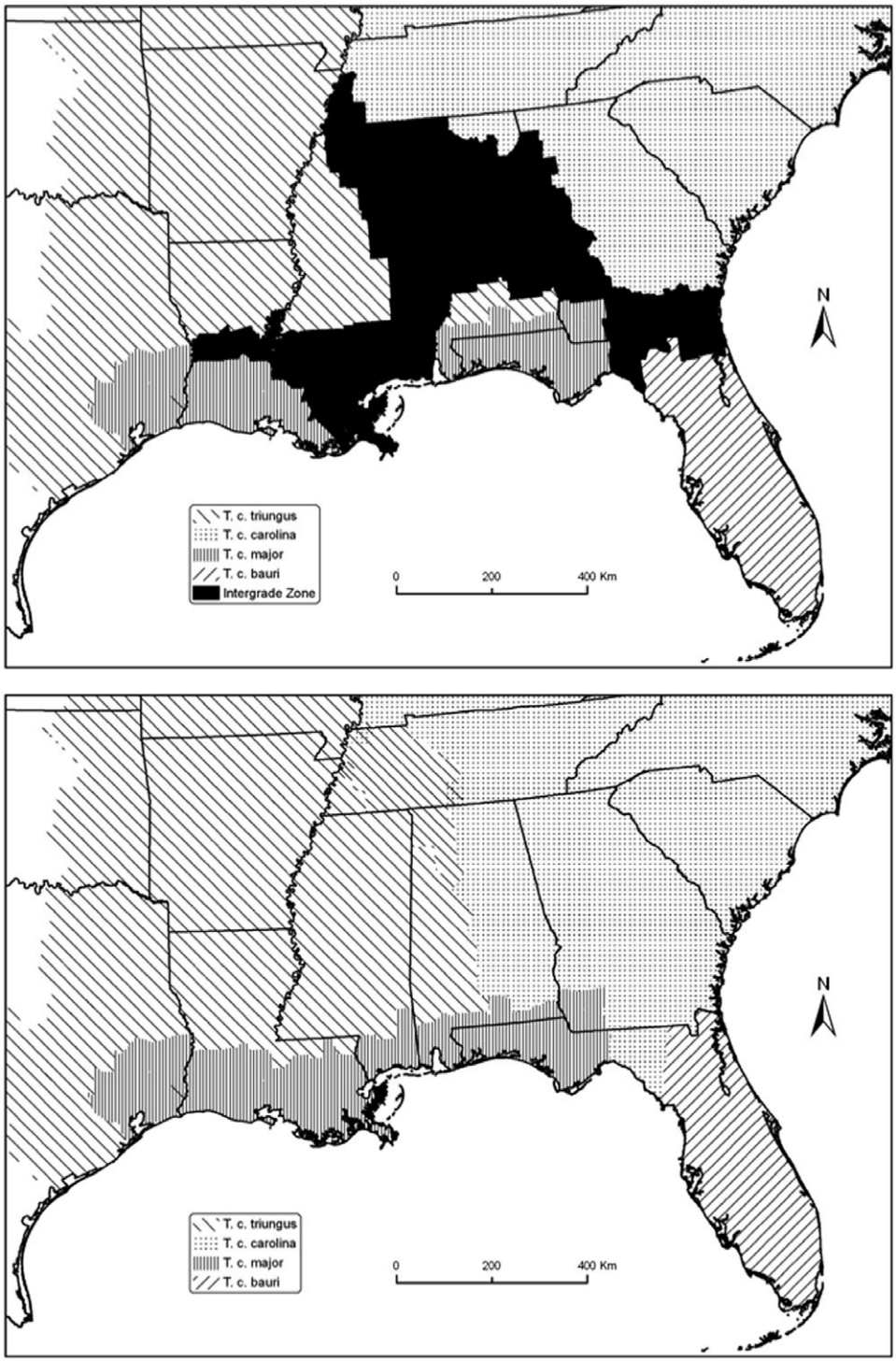

Figure 1. Map illustrating alternative interpretations of subspecies ranges and intergrade zones as proposed by Carr (1952) (top) and Ward (1980) (bottom).

taxonomic groups to be independent evolutionary trajectories. We examined nuclear DNA markers (microsatellites) and mtDNA to test for admixture and substructure among turtles along the Florida Panhandle. If *T. c. major* represents a reproductively isolated evolutionary lineage, we would expect to detect distinct morphological and mtDNA genetic lineages at this scale. The addition of nDNA permitted

further examination of substructure that might reflect nuclear admixture or restricted gene flow.

MATERIAL AND METHODS

Taxon sampling

We examined 723 specimens of *T. carolina* from four museum collections for a suite of meristic shell pattern characters (see Supporting information, Appendix S1). Specimens represent each subspecies in eastern North America (Fig. 2) with an emphasis on turtles from the Florida Panhandle, an area that represents an important putative intergrade zone within the range of *T. c. major*. We used only ethanol-preserved specimens because skeletal specimens can lose characters during maceration. Tissue samples were obtained from museum and personal collections; most were collected from dead-on-the-road (DOR) specimens. Because few properly preserved tissue collections exist, few DNA samples were obtained from morphological specimens. When coordinates were not available, we used TOPO, version 6.0 (DeLorme) to georeference each specimen based on collection locality data. Specimens whose locality could not be determined within 10 km were omitted from analysis.

Morphology

We examined 25 characters on each specimen including continuous, discrete, and sexually dimorphic traits (see Supporting information, Appendix S1). We included both common subspecific-diagnostic characters (e.g. rear toe count, plastron, and carapace pattern) and additional characters to examine their discriminatory power. Continuous characters were measured with vernier calipers to the nearest 0.01 mm. Each measurement was taken three times (noncontinuously) to estimate repeatability (Yezerinac, Lougheed & Handford, 1992) using a nested analysis of molecular variance design (Bailey & Byrnes, 1990). Repeatability (r) was measured as $r = SS_{among}/(SS_{within} + SS_{among})$. In all cases, repeatability was greater than 99%; means from the three measurements were used for subsequent analyses. Characters were excluded that did not have significant ($P \geq 0.05$) discriminating probability.

We corrected for body size by computing a regression for all characters against shell volume (calculated from the product of depth, width, and length) and used the residuals for analyses (Reist, 1986). Lineage-diagnostic morphological characters were determined through discriminate function analysis (DFA) of carapace and plastron measurements corrected for shell volume. Males were identified by an enlarged tail; females lacked a concave posterior plastral lobe, enlarged tail, and enlarged rear claws. Stepwise DFAs were performed on combined and sex-specific datasets. Characters were examined both with and without log-transformation. Initial analysis of transformed and raw datasets did not produce different results, so we used nontransformed datasets for all analyses. Furthermore, discriminatory characters did not differ between sexes, so we conducted final analyses on the combined male and female dataset. Outliers may also impact results from DFAs (McGarigal, Cushman & Stafford, 2000). We identified only two prominent outliers that were removed from the dataset.

We identified eleven regions (Fig. 3) containing 20–30 morphological specimens within a 50-km^2 radius and used DFA to explore among- versus within-region morphological variation of box turtles. If morphology reflects phylogeny, then specimens from within the same geographic distribution of a particular subspecies should overlap in canonical space. Each of the regional concentrations was well within the defined subspecies ranges, so we assumed that most, if not all, samples represented the corresponding lineage. The exceptions were regions 4 through 6, within the range of *T. c. major*, that may or may not represent distinct, diagnosable lineages (see below). We applied a hierarchical approach to discern the impact of inclusion of the different regions in discriminatory analyses. We refined the model by grouping regional samples based on the observed overlap in the mean confidence limit ellipses (MCLE) of the eleven regional samples (see Results). Finally, we omitted three Florida Panhandle regional samples (regions 4, 5, and 6; Fig. 3) based on the increased discriminating power of this second model, aiming to explore the potential impact of hybrid morphology on DFA scores. In each case, we omitted from further model development, specimens with a classification probability lower than 95%. Using the DFA model derived from the analysis of regional samples, we subsequently assigned all remaining morphological specimens (i.e. those not included in the 50-km^2 radius of regional samples) to their most appropriate lineage ($N = 409$).

We used a novel application of Delaunay triangulation implemented in ALLELES IN SPACE (Miller, 2005) to generate a connectivity network among morphological samples. A geographical regionalization procedure (Monmonier, 1973) was applied to detect contiguous morphological distances (average proportion of morphological differences between geographic samples; Miller *et al*., 2006) along the connectivity network, allowing for the discovery of larger than average distances where species boundaries might exist. We interpolated morphological distance between samples across a uniform 100×100 grid and a distance weighting value of $\alpha = 0.5$.

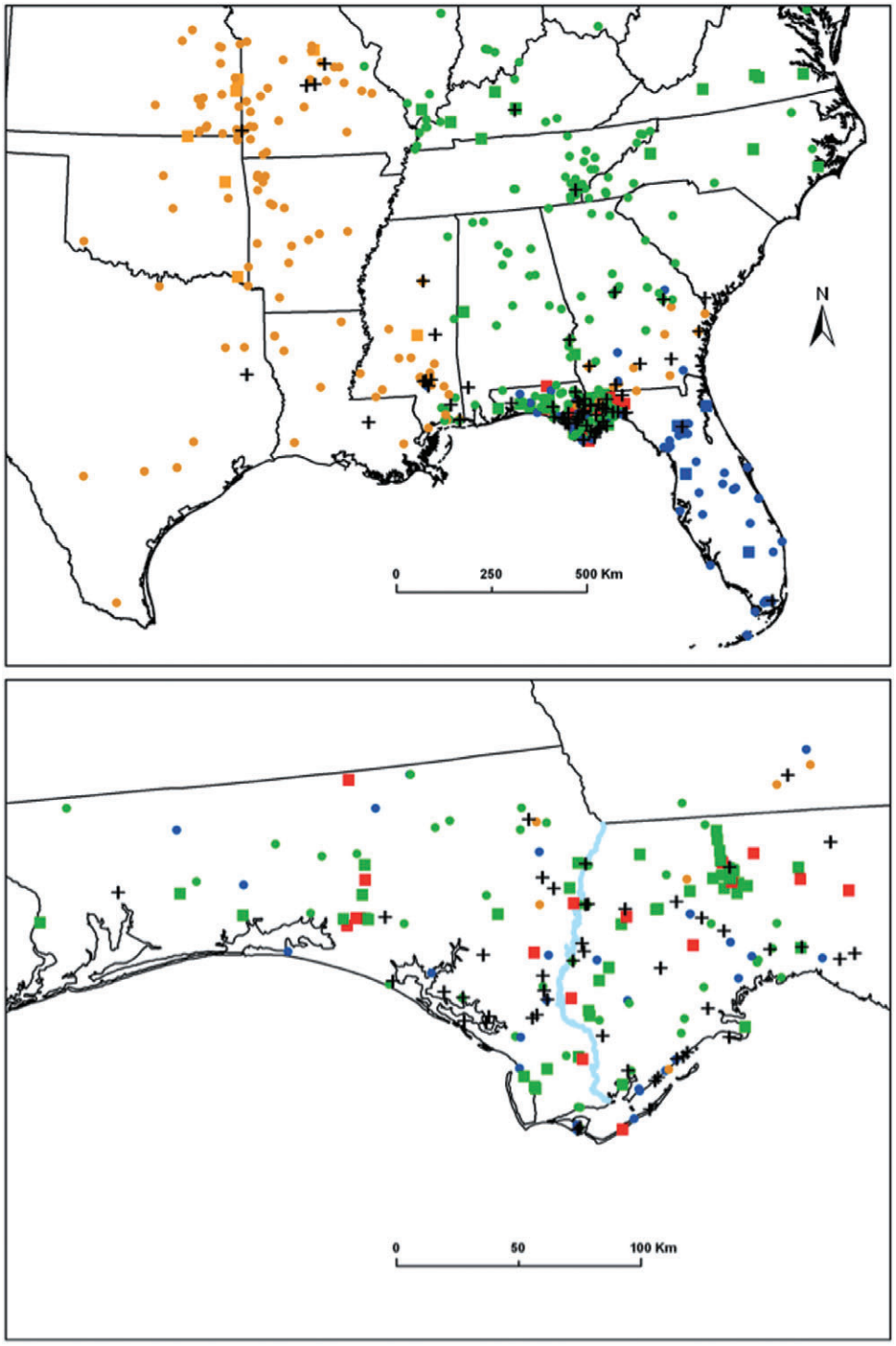

Figure 2. Distribution of morphological and tissue specimens examined for range-wide lineage distribution. Circles, morphological specimens assigned to lineage based on DFA. Unassigned individuals are indicated by '+'. Note that most unassigned individuals are located in the Florida Panhandle region (see text). Squares, mtDNA lineages. Coloured circle and square symbols correspond to the lineage: *Terrapene carolina triunguis* (yellow), *Terrapene carolina carolina* (green), *Terrapene carolina bauri* (blue), putative *major*/*putnami* haplotypes (red). The detailed sampling of the Florida Panhandle is illustrated in the bottom part (the Apalachicola River, a major biogeographic break, is highlighted in blue).

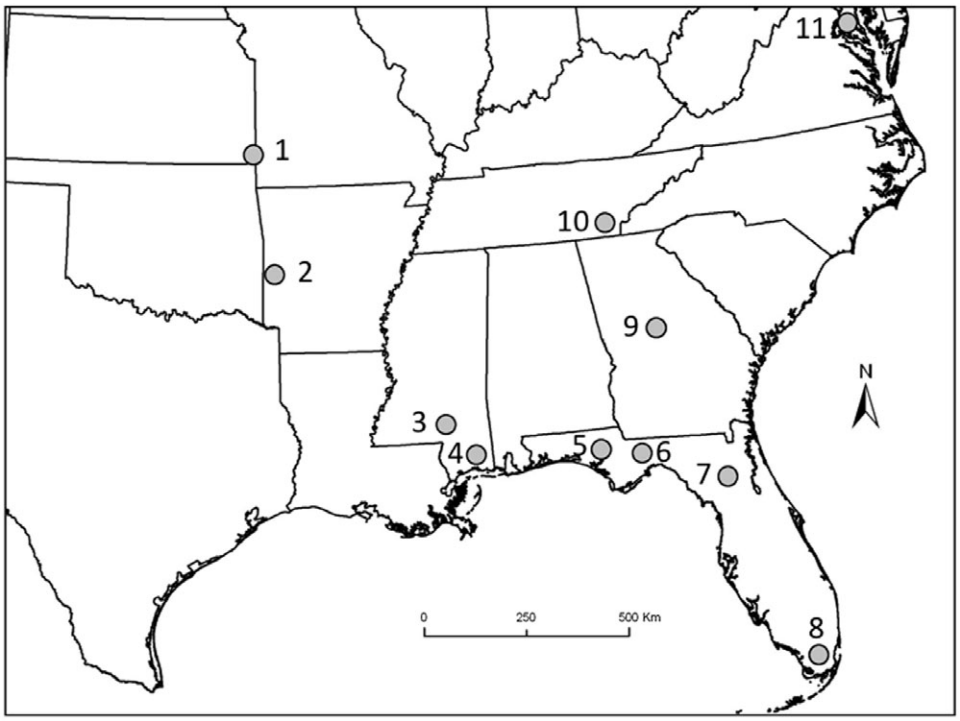

Figure 3. Sample regions for morphological discriminate function model development. Each circle represents a group of 20–30 specimens collected from within a 50-km^2 radius. Regional samples are grouped into three groups based on their overlapping mean confidence limit ellipses in the discriminate function analysis.

Molecular data

Total genomic DNA was extracted using standard phenol-chloroform techniques (Sambrook & Russell, 2001) following a Proteinase-K digestion. An approximately 700-bp fragment of the mitochondrial displacement loop (d-loop) region was amplified for 114 specimens representing the panhandle and samples from deep within the ranges of each of the subspecies. Amplification and bidirectional sequencing was conducted using primers DES1 and DES2 (Starkey *et al*., 2003). Polymerase chain reaction (PCR) profile consisted of an initial denaturation for 5 min at 94 °C; 35 cycles of 45 s at 94 °C, 45 s at 52 °C, and 45 s at 72 °C; followed by a 5-min extension at 72 °C. PCR product was visualized on 10% agarose gels and unincorporated nucleotides removed using 1 U each of exonuclease I and shrimp alkaline phosphatase. Cycle sequencing reactions consisted of: 0.5 μL of BigDye Terminator (Applied Biosystems), 1.5 μL of 5× sequencing buffer (400 mM TRIS pH 9.0, 10 mM $MgCl_2$), 0.12 μL of primer (2.5 μmol), 1–2 μL of amplified product, and ddH_2O for a total reaction volume of 5 μL. Sequencing reactions were cleaned with Sephadex (Sigma-Aldrich) and electrophoresed on a 3130xl Applied Biosystems capillary sequencer. Opposing sequences were assembled, base calls confirmed, and final alignment produced using CLC COMBINED WORKBENCH, version 3.0 (CLC Bio A/S). Unique sequences were deposited in GenBank under accession numbers HQ638982–HQ639016.

Subsequent to a screening of published microsatellite primers (King & Julian, 2004), ten loci (GmuB08, GmuB12, GmuD16, GmuD21, GmuD40, GmuD55, GmuD87, GmuD88, GmuD90, and GmuD121) originally isolated from *Glyptemys muhlenbergii* were used to genotype 83 samples collected from the Florida Panhandle region, and an additional 38 individuals representing samples from within the ranges of *T. c. carolina*, *T. c. bauri*, and *T. c. triunguis*. Microsatellite PCR amplifications were 15-mL reactions consisting of approximately 20 ng of genomic DNA, 2 mM MgCl2, 1× GoTaq PCR buffer, 0.2 U *Taq* (Promega) and equal concentrations of fluorescently labelled and unlabelled primer. D16, D40, D87, D88, D90, and D21 were amplified as simplexes using 0.5 mM primers at annealing temperatures of 60 °C and 55 °C, respectively). Two duplex reactions consisted of primers D121 and D55 (0.6 and 0.3 mM primer concentrations, respectively, annealing temperature 55 °C) and B08 and B12 (0.3 and 0.6 mM primer concentrations, respectively, annealing temperature 60 °C). PCR profiles were 94 °C for 3 min, 35 cycles of 94 °C for 45 s, annealing for 45 s and 72 °C

for 45 s, followed by a 5-min extension at 72 °C. Genotyping runs were performed on an ABI 3130xl using GeneScan-500 ROX size standard (Applied Biosystems). Scores were manually confirmed using GENEMARKER, version 1.75 (SoftGenetics).

We used MICRO-CHECKER, version 2.2 (Van Oosterhout *et al*., 2004) to examine for scoring error and allelic dropout. We tested the random union of gametes between all pairs of loci using the Markov chain approximation (5000 dememorization steps, 1000 batches of 5000 iterations) of an exact test implemented in GENEPOP, version 4.0 (Rousset & Raymond, 1995).

Analysis of mtDNA

We used Bayesian phylogenetic reconstruction, choosing our best-fit model of DNA substitution using MODELTEST, version 3.7 (Posada & Crandall, 1998). We applied the Bayesian inference method using MrBayes, version 3.1.2 (Huelsenbeck & Ronquist, 2001). We specified the general substitution model (nst = 2) and rate (invgamma) as suggested by the Akaike information criterion (Posada & Buckley, 2004). All substitution model parameters (gamma shape parameter, proportion of invariable sites, character state frequencies) were allowed to vary for two independent runs and prior defaults were used. Each Bayesian run consisted of 10 chains for 5.0×10^6 generations, sampling trees every 100 cycles, producing 5.0×10^4 sampled trees, of which 5.0×10^3 were discarded as burnin. TRACER, version 1.3 (http://evolve.zoo.ox.ac.uk) was used to examine the frequency distribution of sampled trees, and the effective sample sizes. For our Bayesian analysis, we included two deirocheline species as outgroups [*Chrysemys picta* (GenBank Accession Number AF069423) and *Trachemys scripta* (FJ392294)], and included two existing emydine sequences in the phylogenetic analysis [*Terrapene ornata* (GQ487257) and *T. coahuila* (HQ639017)]. We rooted our phylogenetic tree with deirocheline species rather than *T. ornata* or *T. coahuila* because of the poorly resolved relationship among *Terrapene*. ARLEQUIN, version 3.11 (Excoffier, Laval & Schneider, 2005) was used to calculate pairwise F_{ST} with significance determined using 1000 bootstraps.

Assaying structure across the Florida Panhandle

We assessed spatial autocorrelation structure of microsatellite genotypes and distance across the Florida Panhandle with a correlogram generated using GENALEX, version 6 (Peakall & Smouse, 2006). After preliminary analyses, we represented autocorrelation across nine cumulative distance classes. Multilocus genetic distances were calculated using the 'interpolate missing' option. We tested the significance of r using 999 random permutations and 10000 bootstrap replicates.

Global departures from Hardy–Weinberg across the 83 panhandle samples were determined using GENEPOP, version 4 (Rousset, 2008). Under scenarios where population substructure exists, heterozygote deficiency can result (Rousset & Raymond, 1995). Alternatively, in an admixed population, an excess of heterozygosity can occur (Milkman, 1975). We tested for spatial substructure using a Bayesian clustering approach implemented in STRUCTURE, version 2.2 (Pritchard, Stephens & Donnelly, 2000; Falush, Stephens & Pritchard, 2003) and TESS version 2.3 (François, Ancelet & Guillot, 2006; Durand *et al*., 2009). In STRUCTURE, individual genotypes are probabilistically assigned to one of K genetic clusters. We estimated K with the highest posterior probability given the data using the admixture model and correlated allele frequencies between populations (Falush *et al*., 2003). MCMC runs consisted of 1.0×10^5 burn-in iterations followed by 2.0×10^6 iterations. We explored K-values in the range 1–5 and performed ten replicates at each K-value to assess convergence. We also examined the impact of the starting value of α, the Dirichlet parameter for degree of admixture. Higher α (> 1) generally reflects that most individuals are admixed. We ran the analysis with starting α ranging from 1 (default) to 3. We also explored the impact of uniform and gamma priors (defaults settings used) for estimating α. For each model, we plotted $\ln P(D|K)$ against K to choose the value of K that corresponds to a plateau of the $\ln P(D|K)$ curve (Durand *et al*., 2009).

Unlike STRUCTURE, TESS incorporates information on individual spatial coordinates as a prior. TESS computes the deviance information criterion (DIC; Spiegelhalter *et al*., 2002), a measure of model fit (low DIC values) penalized by an estimate of model complexity. We ran TESS for values of $K_{max} = 2$–5 and plotted the DIC versus K_{max} and, as above, chose the value that corresponded to a plateau of the DIC curve (Durand *et al*., 2009). We ran ten replicates, each consisting of and 5.0×10^5 sweeps following 2.0×10^5 burn-in sweeps. We used the admixture model with default values.

RESULTS

MORPHOLOGY

Discriminate function analysis conducted on the 11 sample regions identified 12 characters with significant discriminatory power (all $P < 0.05$; Table 1). Regions 1–3 (Fig. 3), all located within the

Table 1. Mean discriminate function analysis scores by group from the best model used to predict lineage assignment based on 12 morphological characters

	Character frequency								Mean residuals			
Region	Head/leg pattern	Notched beak	Dark carapace	Number of rear toes	Vertebral ridge	Streaks	Spots	Blotches	Posterior lobe	Interegular seam	Interhumeral seam	Depth
1, 2, 3	0.67	0.74	0.23	3.03	0.32	0.24	0.44	0.18	–3.51	0.98	–1.72	–0.87
7, 8	0.84	0.29	1.00	3.43	0.84	0.94	0.68	0.01	3.11	–1.80	2.69	4.49
9, 10, 11	0.64	0.28	0.98	3.97	0.49	0.21	0.51	0.88	–1.26	–0.05	–0.75	–3.06

Locations of group clusters can be found in Figure 3.

distribution of *T. c. triunguis* (Group I), had overlapping MCLE. Similarly, the two regions within the range of *T. c. bauri* (hotspots 7 and 8, Group II) overlap in canonical space. Finally, all DFA regions within the defined ranges of *T. c. carolina* and *T. c. major* overlapped together (hotspots 4–6, 9–11, Group III). The first two canonical functions of model 1 accounted for 71% of the discriminating power of the characters (Table 2) with misclassification of 23.6% of the specimens. Model 2, combining the 11 regional samples into three groups based on their MCLE values (Fig. 3), had 99% of the discriminating power explained by the first two canonical functions, and only 1.7% of specimens were misclassified. These results reflect individual misclassifications occurring predominantly among regions within groups rather than between groups in the first model. The third model excluded the panhandle regions (4–6) and had no misclassifications. In model 3, the first two canonical functions accounted for 100% of the discriminating power (Table 1). When model 3 was applied to all 409 remaining samples (i.e. those outside of regional samples), there was a strong association between morphologically assigned groups and a priori subspecies designation for *T. c. carolina*, *T. c. bauri*, and *T. c. triunguis*. The samples from the Florida Panhandle (within the geographic range of the putative *T. c. major*) were assigned predominantly to *T. c. carolina*, but also to *T. c. bauri* and *T. c. triunguis* (Fig. 2, inset). We also identified ten morphological specimens in southern Georgia as *T. c. triunguis* (Fig. 2). We were unable to classify 83 of the samples with 95% probability. The Florida Panhandle contained a majority of these unassigned individuals (53 of 83), with ten of the remaining 30 unassigned individuals located along the southern Atlantic and Gulf Coastal Plains (Fig. 2). Finally, our morphological landscape shape interpolation performed in ALLELES IN SPACE supported the existence of a highly variable region centered on the Florida Panhandle (see Supporting information, Fig. S1), supporting the evidence that this region contains high morphological variability.

Genetic structure

mtDNA variation and lineage identification

We sequenced 89 of 114 samples for 527 bp of the d-loop region, resulting in 35 unique haplotypes. A further 25 individuals were partially sequenced for a 332-bp portion of homologous d-loop region. The partial haplotypes were included in identifying lineage distributions but were excluded from the phylogenetic analyses.

Bayesian analysis (Fig. 4) identified three well-supported clades that correspond to the recognized

Table 2. Model summaries from discriminate function analysis of 12 morphological characters

Models		Canonical discriminating power			Misclassified
		Function 1	Function 2	Total	
Model 1	All regions	38%	33%	71%	23.6%
Model 2	Groups I, II, and III	55%	44%	99%	1.7%
Model 3	Groups I, II, and III – regions 4, 5, and 6 omitted	52%	48%	100%	0%

Sampling regions (1–11) correspond to those in Fig. 3. Groups correspond to enclosed regions represented in Fig. 3.

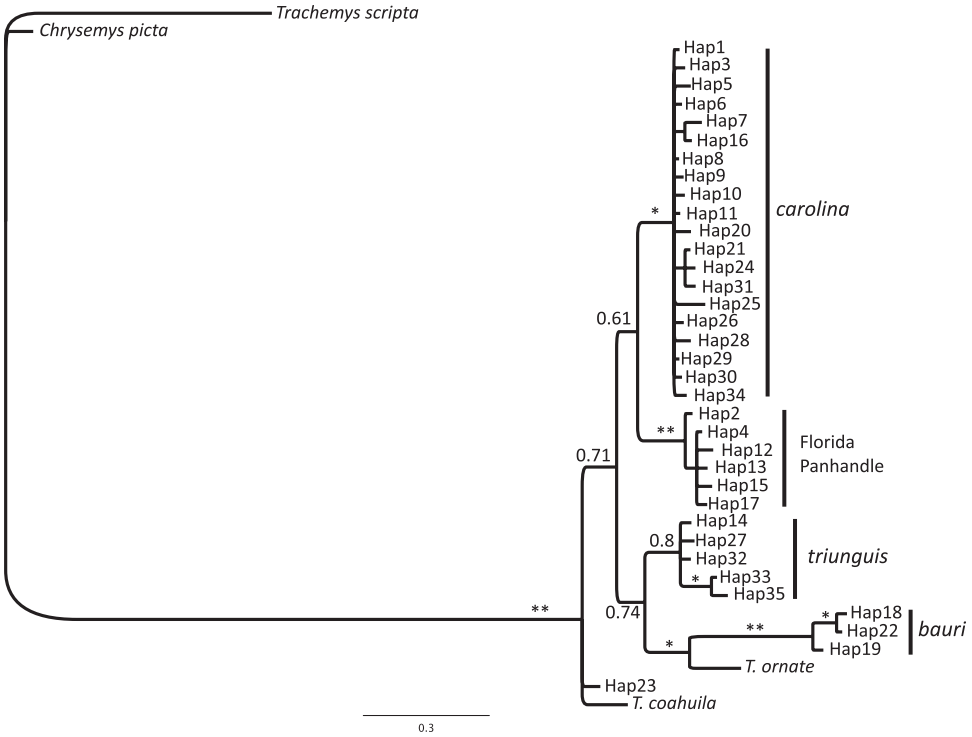

Figure 4. Bayesian phylogenetic hypotheses derived from 527 bp of d-loop sequenced from 89 *Terrapene carolina*, represented by 35 unique haplotypes. Well-supported nodes are represented by '*' (0.95–0.99) and '**' (1.0). Additional Bayesian support values for basal nodes are indicated for reference. Taxon designations were determined by geographic range (Fig. 2).

geographic distributions of subspecies. One clade representing samples from the region of *T. c. carolina* was found on the Gulf Coastal Plain and a second, sister-clade comprised of haplotypes sampled only from the Florida Panhandle (Figs 1, 4). The third well-supported clade consisted of three unique *T. c. bauri* haplotypes that were resolved in a sister-relationship with *T. ornata*. *Terrapene c. triunguis* was monophyletic, though with low support. One haplotype (Hap23) from Escambia County in the Florida Panhandle was almost identical to *T. coahuila*. Basal resolution of the major clades was poorly resolved (low posterior support). Lineages were significantly differentiated with pairwise F_{ST} estimates ranging from 0.12 (*carolina–bauri*) to 0.38 (*major–triunguis*). Average pairwise F_{ST} was 0.25 (SE = 0.035).

Microsatellite genetic structure

We successfully genotyped 116 individuals, 83 of which were sampled across the Florida Panhandle. There were 6–26 alleles per locus (see Supporting information, Table S1). No loci showed significant linkage disequilibrium. Locus specific tests of excess homozygosity were nonsignificant for seven of the ten loci. One locus, D90, had significant excess of homozygosity that was interpreted as the result of null alleles by MICROCHECKER. The remaining two (D40 and D88) did not display abnormal patterns that

would suggest amplification or scoring error. Subsequently, we removed D90 and conducted the remaining analyses on nine of the ten loci.

STRUCTURE results using different starting parameters for the level of admixture (α) were similar in identifying an optimal K for the given models at 1. Similarly, high LnL scores were found for $K = 2$ and $K = 3$, although the variance among replicates was relatively large at these K-values (see Supporting information, Fig. S2). Results from TESS indicated that the highest DIC score for the minimum number of partitions was 2. Because TESS does not evaluate the likelihood of a single partition, we were unable to determine whether $K = 1$ was a better model fit. The correlogram similarly reflected a lack of spatial autocorrelation across the entire region (see Supporting information, Fig. S2).

DISCUSSION

The results of the present study reflect limited support for the recognition of *T. c. major*. To our knowledge, this is the first attempt to quantitatively determine the utility of morphological traits to objectively distinguish among lineages of North American box turtles. Three of the four currently recognized subspecies of *T. carolina* (*T. c. triunguis*, *T. c. carolina*, and *T. c. bauri*) possess diagnostic multivariate morphological characteristics. However, high variability in putative *T. c. major* resulted in all of these specimens being assigned morphologically to one of the three other subspecies examined, most notably *T. c. carolina*. The three morphologically diagnosed lineages also possessed unique mitochondrial haplotypes with strong support for all but *T. c. triunguis*. A fourth clade restricted to the panhandle co-occurred with *T. c. carolina* haplotypes. In the absence of morphological evidence to the contrary, this fourth mtDNA lineage might have been ascribed to *T. c. major*. However, the affinity of many panhandle specimens to *T. c. carolina* and the large number of unassigned morphological specimens in the panhandle suggest that the evolutionary history of box turtles in the panhandle is complex and that currently recognized subspecies require further consideration.

The distribution of specimens assigned to morphological and mtDNA lineages is almost concordant with current subspecies distributions of *T. c. carolina* and *T. c. triunguis*. However, there is little evidence to consider *T. c. major* as a distinct evolutionary lineage. Our discriminant model identified *T. c. triunguis*, *T. c. carolina*, and *T. c. bauri* morphotypes within the Florida Panhandle, as well as a large number ($N = 53$) of unassigned morphotypes. These results reflect a long-standing ambiguity over the status of *T. c. major*. Additionally, d-loop sequences from most Florida Panhandle specimens are grouped with *T. c. carolina*. The panhandle was also home for a distinct haplotype lineage and the distinct haplotype 23. The high phenotypic variance together with the presence of multiple divergent mtDNA lineages supports the hypothesis proposed by Milstead (1969) suggesting that the currently recognized range of *T. c. major* represents an area of admixture, rather than a distinct lineage. We contend that the unique mtDNA lineage and the lack of morphological distinction of panhandle *Terrapene* reflects long-term introgression between what was an isolated historic *T. carolina* lineage (Florida Panhandle clade; Fig. 4) and other the eastern lineages to varying degrees. Alternatively, the panhandle lineage could represent mtDNA variation from the extinct *T. c. putnami*. This scenario would require considerable introgression among lineages to have preserved this variation until the present.

The genus *Terrapene* has a long evolutionary history in North America, with the oldest fossils dating from the middle Miocene (13–14.5 Mya) of Nebraska. Holman & Fritz (2005) suggested that fossils from 9–10 Mya in Kansas might be the earliest *T. carolina*. By 8.5 Mya, the giant box turtle *T. c. putnami* occurred in southeastern North America and was essentially identical to modern *T. c. major* in osteology, differing only in maximum size. A lack of defining characters led Bentley & Knight (1998) to suggest that *T. c. major* and *T. c. putnami* were conspecific.

Throughout the Plio-Pleistocene fossil record, there is a pattern of one large box turtle and one small box turtle occupying many regions (Auffenberg, 1958). In addition, there is a trend of size reduction in many Pleistocene vertebrate groups (Forsten, 1993; Hill, Hill & Widga, 2008), which may account for a decrease in body size of *T. c. putnami* to what is now called *T. c. major*. Therefore, an alternative to recent admixture (Milstead, 1969) is the ancient admixture of *T. c. putnami* with other members of the Carolina clade. The presence of a unique mtDNA lineage on the panhandle supports the *T. c. putnami* hypothesis. Rather than the result of a recent admixture event, *T.c. major* is the phenotypic remnant of a long-term series of contacts with varying levels of introgression interspersed with periods of isolation. Indeed, introgression continues today throughout much of the northern Gulf Coast resulting in much phenotypic variation but limited osteological and genetic differentiation. The lack of nuclear structuring in our microsatellite data supports the idea of long-term introgression. However, we cannot rule out the likelihood that the lack of structure is not biological but, instead, is a result of the limitations in sample size and the clustering models explored (Fogelqvist *et al.*, 2009).

Terrapene c. bauri of the Florida peninsula is distinct morphologically and genetically, although the strong association of the *T. ornata* haplotype makes the currently recognized *T. carolina* group non-monophyletic. The results of the present study and the highly disjunct geographic separation of *T. ornata* and *T. bauri* suggest that the latter should be elevated to species status. This in turn would leave *T. carolina* paraphyletic if further phylogenetic analysis supports the monophyly of *T. c. triunguis* and its sister relationship with *T. bauri*.

We were unable to use genetic and morphological approaches on the same samples as a result of a lack of tissue collections associated with museum samples. Accordingly, we are unable to determine whether mtDNA geographic outliers represent outliers in DFA of morphological lineages. Some of the road-kill specimens collected in the Florida Panhandle phenotypically resembled *T. c. carolina* but possessed unique d-loop haplotypes, whereas others that had been classified as *T. c. major* (based on head, limb and shell coloration, and size) possessed eastern d-loop haplotypes; descriptions of phenotypes are provided by Dodd (2001). Of particular interest are specimens from south Georgia (Fig. 2) that ally morphologically with the western clade, *T. c. triunguis*. These specimens may represent either intergrades of *bauri*/*carolina* or an eastern disjunct population of *T. c. triunguis*. Many museum specimens (USNM 60898–68901, FLMNH 4229–4445) collected from this area were referred to as *T. c. triunguis* by their collectors (Iverson, 1992).

The absence of spatial autocorrelation or any detectable biogeographic signal across the Florida Panhandle further supports the interpretation that gene flow in box turtles is high, or that drift plays a minor role in shaping existing microsatellite variation. The common mtDNA haplotype in both *T. c. carolina* (Hap1) and the Florida Panhandle clade (Hap4) was distributed widely, including both sides of the Appalachicola River. Genetic structuring of organellar DNA within the Florida Panhandle, often associated with the Apalachicola River, is evident among many taxa, including mammals (Ellsworth *et al*., 1994), fishes (Bermingham & Avise, 1986), plants (Maskas & Cruzan, 2000) and reptiles (Walker & Avise, 1998; Burbrink, 2001).

The infrequent long-distance dispersal in adult *T. carolina* (Schwartz & Schwartz, 1974; Iglay *et al*., 2007) should reflect fine-scale genetic structuring. However, box turtles can disperse independently of aquatic connections and their ranges do not appear to be greatly affected by stream capture or flooding (Dodd, 2001). Although most box turtles do not venture vast distances, transient males have been identified in some populations and there are records of individuals moving up to 10 km (Kiester, Schwartz & Schwartz, 1982). A long life span and gene flow by transients could result in genetic homogenization. Future studies with sufficient samples outside the panhandle may determine whether there are significant differences in allele frequencies among subspecies.

Conclusions

The results obtained in the present study corroborate the hypothesis of Milstead (1969) suggesting that *T. c. major* is not a distinct lineage but, instead, a mixture of extant taxa plus the extinct *T. c. putnami*. Paraphyly was detected within the recognized *T. c. carolina* group. Although *T. c. bauri*, *T. c. carolina*, and *T. c. triunguis* represent distinct mtDNA and morphological lineages, the specific status of *Terrapene* within the range of *T. c. major* is more complex. Although often possessing distinctive phenotypes, we contend that *major* does not represent an isolated evolutionary lineage. Because there are no specific identifying characters between fossil giant box turtles (*T. c. putnami*) and the *T. c. major* phenotype except for size, we concur with Bentley & Knight (1998) that these represent the same taxon. The name *T. c. major* has precedence over *T. c. putnami*, so that the latter name should be synonymized with the former. The name *T. c. major* should only be used for Pleistocene fossil giant box turtles, although the large extant box turtles of the Gulf Coast region might be considered as possessing a major or Gulf Coast phenotype. Additional research on the genetic structure and life history of the assemblage of box turtles along the northern Gulf Coast undoubtedly will yield new insights into the evolutionary history of *Terrapene*.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Results of morphological landscape interpolation implemented using ALLELES IN SPACE (Miller, 2005) using a 100×100 grid and distance weighting parameter of 0.5. The x- and y-axis correspond to the geographical landscape of eastern North America (Fig. 2). Peaks on the morphological distance (z-axis) indicate areas of greatest distance (in this case, corresponding to the Florida Panhandle). White trace indicates the Florida peninsula and panhandle region.

Figure S2. (Top) Number of *Terrapene carolina* populations detected from individuals genotyped for nine microsatellite loci across the Florida Panhandle ($N = 83$) and range-wide ($N = 38$). Bars represent the highest posterior probability expressed as the mean likelihood [$\log P(X|K)$], over ten runs for each K (1 to 5) estimated using STRUCTURE for three different α-values (black, $\alpha = 1$; grey, $\alpha = 2$; white, $\alpha = 3$). Error bars represent standard deviations (SD for $K = 1$ is 0.5). Results from TESS (DIC) are shown as points (mean ± SD) from $K = 2$ to $K = 5$. (Bottom) Correlogram representing the lack of spatial autocorrelation across the Florida Panhandle.

Table S1. Summary statistics for six microsatellite loci.

Appendix S1. The descriptions listed detail each discrete morphological character or measurement and include the source and potential diagnostic problems when relative (*traditionally used subspecies character for this group).

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