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Conservation Genetics of the North American Box Turtle

Shehzad Batliwala

Alicia Gackle

Thomas Getz

Bradley Martin

John Placyk Jr.

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Shehzad Y. Batliwala, Alicia R. Gackle, Thomas J. Getz, Bradley T. Martin, and John S. Placyk, Jr..
Dept. of Biology, University of Texas at Tyler

UT TYLER HONORS PROGRAM
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Abstract

The North American Box Turtles (*Terrapene* spp.) are declining in numbers throughout their range and are listed as a species of special concern, threatened, or endangered in many states. Effective conservation efforts will require knowledge of the underlying genetics of the group both at the species/subspecies and population level. We used mtDNA sequence data in the form of the *cytochrome b* gene to construct a preliminary phylogeny for this taxon. Our data suggests that the Eastern Box Turtles are more distantly related to the Three-toed Box Turtles than previously thought. This is supported further by sufficient barcoding data measuring the degree of divergence between varying *Terrapene* species. Furthermore, they are distinct to such an extent that they may need to be regarded as separate species, rather than their currently held subspecies status. Our data also suggests that the Western varieties (Ornate Box Turtles and Desert Box Turtles) are more closely related than previously believed indicating that they may be clumped together as a single species without the subspecific designations. However, additional data is needed for a more accurate assessment. We intend to sequence additional mtDNA and nuclear genes through the use of microsatellite markers and genetic barcoding in the future to assess the genetic "health" of individual populations.

Three-toed Box Turtle (*Terrapene c. triunguis*) | Eastern Box Turtle (*Terrapene c. carolina*) | Ornate Box Turtle (*Terrapene o. ornata*) | Desert Box Turtle (*Terrapene o. luteola*)

Introduction

North American Box Turtles (*Terrapene* spp.) inhabit the majority of the United States (being absent only from the Western United States) and parts of Mexico (Dodd, 2001). Box Turtle populations throughout their range are declining due to low reproduction rates, human disturbance through commercial harvesting, highway mortality, habitat loss, increasing urbanization, and collection from the wild (Dodd, 2001). Conservation efforts are needed to prevent further declines in population. However, in order for conservation efforts to be most effective, the phylogeny of North American Box Turtles needs to be examined more thoroughly. Conservation plans are often species-based and current Box Turtle taxonomy is based almost solely on morphology, which may not be supported by more objective genetic data. Our objectives are to reassess the evolutionary history of the North American Box Turtles using molecular phylogenetic data and analyses and to recommend a revised Box Turtle classification system.

Methods

Tissue samples in the form of tail tips, blood, shell shavings, and toenails were either collected by Dr. Placyk, Dr. Koukl, or by volunteers and museums. DNA was extracted using the Qiagen DNeasy Blood and Tissue Kit. We amplified a 1200-bp region of mtDNA encoding the entire *cyt b* gene and part of the adjacent tRNA threonine (tRNAthr) via PCR using either primers GLUDG-L (Palumbi et al., 1991) and M-H (Shaffer et al., 1997) or CytbG and THR-8 (Spinks et al., 2004). We used the following thermal cycle parameters for 20 ul amplification reactions: 35 cycles of 1 min denaturing at 94°C, 1 min annealing at 51 °C, and 2 min extension at 72°C. We purified PCR products using the Qiagen QIAquick PCR Purification Kit and used purified template DNA in sequencing reactions using Beckman Coulter GenomeLab DTCS – Quick Start Kits and the same primers used in the initial PCR reaction. For some sequencing reactions we also used the internal primers Primus and Primus-rev (Feldman and Parham, 2002). Products were then sequenced using a Beckman Coulter CEQ-8000 automated sequencer.

Sequence Analysis

Sequence data was manually proofread and edited using Sequencher 4.9 and then aligned using ClustalX 2.0.11. Final editing was carried out using MacClade 4.08 and a maximum parsimony phylogeny was produced using PAUP* 4.0b10 (Swofford, 2003). Our final tree was created using TreeView X. We generated 15 *T. c. triunguis* sequences (9 from TX, 2 from MO, 3 from KS, and 1 from AR), 18 *T. o. ornata* sequences (1 from KS, 1 from NM, 1 from IL, 5 from CO, and 10 from WI), 55 *T. c. carolina* sequences (3 from IL, 5 from VA, 2 from KY, 10 from AL, 2 from TN, 14 from MA, 4 from MD, 1 from CT, 5 from WV, 2 from PA, 2 from OH, 1 from IN, 2 from GA, and 2 from FL), 4 *T. c. major* sequences (2 from FL, 1 from AL, and 2 from an unknown location), and 5 *T. o. luteola* sequences (all from NM). In addition, *cyt b* sequence data for two Mexican species of Box Turtle (i.e., the Spotted, *T. nelsoni*, and the Coahuilan, *T. coahuila*), 1 *T. o. luteola* from AZ, 1 *T. c. carolina* from NC and 1 *T. c. triunguis* were obtained from GenBank (Figure 1). A Spotted Turtle (*Clemmys guttata*) *cyt b* sequence from GenBank was used to root the tree, as Spinks et al. (2009) indicated that it was the sister group to the *Terrapene* genus.

DNA Barcoding

A 650 base pair region of the mtDNA *cytochrome oxidase subunit I (COI)* gene was amplified and sequenced for DNA barcoding purposes. The 20 µL *COI* PCR reactions consisted of the same volumetric ratios as with the *Cytb* and *GAPD* genes. Two primer sets were used to amplify and sequence the selected *COI* region. The first consisted of the forward primer L-turtCOI and the reverse primer H-turtCOIb (Stuart and Parham, 2004), and the second consisted of the forward primer VF2_t1 (Ward, et al., 2005) and the reverse primer FR1d_t1 (Ivanova, et al., 2007). The following PCR parameters were used with the L-turtCOI and H-turtCOIb primers: an initial denaturation of 95°C for 5 min, 35 cycles of 95°C for 45 sec, 55°C for 45 sec, 72°C for 45 sec; and a final extension of 72°C for 6 min. PCR parameters for the VF2_t1 and FR1d_t1 primers were as follows: an initial denaturation of 94°C for 2 min, 35 cycles of 94°C for 30 sec, 52°C for 40 sec, and 72°C for 1 min; and a final extension of 72°C for 10 min. K2P comparisons were utilized over Jukes and Cantor divergence comparisons to be consistent in comparisons with the Barcode of Life Data Systems (BOLD) database. Reid et al. (2011) indicated that interspecific *COI* sequences for many organisms (including turtles) are divergent by >2% and intraspecific percent sequence divergences are often <2%. This 2% sequence divergence was used as a reference value for *Terrapene* pairwise DNA sequence comparisons to evaluate their taxonomic status. As an *a posteriori* analysis based on the *Cytb* results, which showed two groups composed primarily of *T. c. major*, *T. c. major* was divided into two separate groups, with one consisting solely of *T. c. major* and one consisting of *T. c. major* and *T. coahuila*. This analysis was performed to assess whether *T. c. major* consisted of more than one phylogenetic lineage.

Results

To reduce the computational time, maximum likelihood analysis was performed on the 55 unique haplotypes rather than on all 101 individual sequences. Our maximum likelihood tree shows a *T. carolina* group and a *T. ornata* group (Fig. 2). Bootstrap values are shown at their corresponding branching sites.

Twenty-four DNA sequences were obtained for the *COI* gene pertaining to genetic barcoding differences. *COI* pairwise comparisons indicate that all taxa are divergent by more than the reference value of 2% (Reid, et al., 2011) except for *T. c. triunguis - T. c. mexicana*, *T. o. ornata - T. o. luteola*, and the *T. c. major* clade that did not include *T. coahuila* and *T. c. carolina* (Table 1).

T. carolina

The *T. carolina* group is split into two distinct clades. One clade includes only *T. c. triunguis*, while the other includes *T. c. carolina*, *T. c. major*, and *T. coahuila*, which traditionally has been thought to be sister to the entire *T. carolina* group. The *T. c. major* group is a monophyletic sister to the *T. c. carolina* group. Additionally, one IL *T. o. ornata* is found in the *T. c. carolina* group.

T. ornata

The *T. ornata* group is a mix of the two currently recognized *T. ornata* subspecies and *T. nelsoni*, which traditionally has been considered the sister group of *T. ornata*. A distinct division was found between the *T. o. ornata* and *T. o. luteola* groups, albeit less pronounced than what is seen in the *T. c. carolina* and *T. c. major* groups.

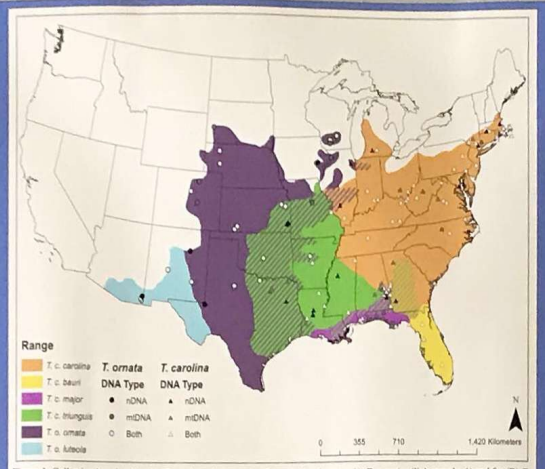

Figure 1: Collection locations for (A) *T. c. triunguis* (green; +), *T. c. carolina* (orange; *), *T. c. major* (light purple; ^) and for (B) *T. o. ornata* (dark purple; o) and *T. o. luteola* (light blue; L) used in our dataset.

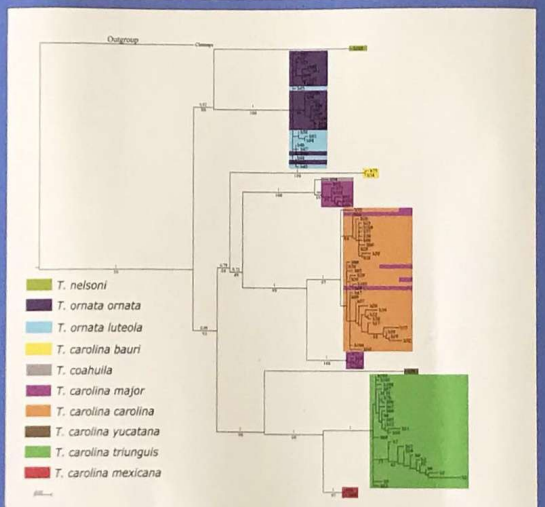

Figure 2: A phylogram representing 55 *cytochrome b* haplotypes generated from 101 *Terrapene* gene sequences. Bootstrap values are indicated by their corresponding branches. The phylogram is color coded (with *T. o. luteola* in light blue, *T. o. ornata* in dark purple, *T. c. carolina* in orange, *T. c. major* in light purple, and *T. c. triunguis* in green).

Table 1. A table representing the genetic barcoding differences present between *T. triunguis*, *T. coahuila*, *T. luteola*, *T. carolina*, *T. bauri*, *T. major-coah*, *T. major*, *T. nelsoni*, *T. ornata*, and *T. yucatana* as compared with one another. Results considered conclusive for species similarity are specified below.

	triunguis	*coahuila*	*luteola*	*carolina*	*bauri*	*major-coah*	*major*	*mexicana*	*nelsoni*	*ornata*	*yucatana*
triunguis	-										
coahuila	5.66	-									
luteola	6.36	5.14	-								
carolina	6.17	4.97	5.14	-							
bauri	5.35	4.65	4.99	5.32	-						
major-coah	5.49	0.23*	4.97	4.8	4.48	-					
major	6.08	4.97	5.06	0.78*	5.49	4.8	-				
mexicana	1.40*	6	6.08	6.68	5.85	5.83	6.5	-			
nelsoni	6.57	6	5	6	6.53	6.17	5.92	7.08	-		
ornata	6.45	5.14	0.23*	5.19	5.07	4.97	5.1	6.96	5.08	-	
yucatana	4.67	5.83	5.84	6.68	5.51	5.66	6.42	5	7.44	5.92	-

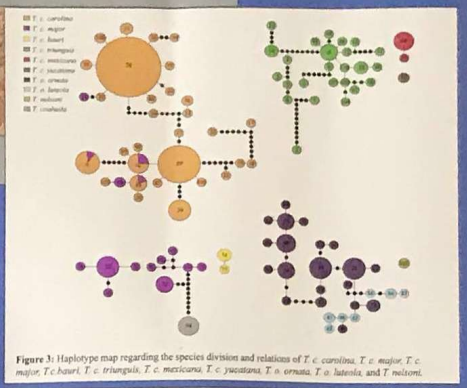

Figure 3: Haplotype map regarding the species division and relations of *T. c. carolina*, *T. c. major*, *T. c. bauri*, *T. c. triunguis*, *T. c. mexicana*, *T. c. yucatana*, *T. o. ornata*, *T. o. luteola*, and *T. nelsoni*.

Discussion

In general, our preliminary molecular dataset agrees with the phylogeny generated with morphological data (Minx, 1996). Specifically, there appear to be two main groupings of *Terrapene* species/subspecies, an Eastern group (including the *T. carolina* subspecies) and a Western group (including the *T. ornata* subspecies) with the exception of one IL *T. o. ornata* being found in the Eastern group. While this may have been a misidentified individual, given the morphological differences between *T. carolina* and *T. ornata*, this is unlikely. Rather, this may be evidence that supports claims that *T. carolina* and *T. ornata* are hybridizing where they are sympatric in IL. The phylogram may suggest that *T. c. triunguis* needs to be elevated to full species status, rather than their currently held subspecies status. The phylogeny also supports *T. c. major* remaining as a subspecies of *T. c. carolina* (Figure 2).

The ornate group shows a distinct separation between *T. o. luteola* and *T. o. ornata*, but they are much less genetically distant than the *T. c. major* and *T. c. carolina* group, which are also considered separate subspecies. This observation suggests that the separation between *T. o. ornata* and *T. o. luteola* is an indication of population divergence at a smaller scale than the subspecific level (Figure 3; Table 1). Thus, we propose that the subspecific designation may not be necessary and that the ornate group simply be clumped together as a single species, *T. ornata*, which disagrees with Herrmann and Rosen (2009). However, the small genetic distance may also indicate a high level of hybridization between the two subspecies and requires additional sampling. Lastly, the phylogram shows that *T. nelsoni* is sister to the two Western subspecies, which confirms Minx's (1996) earlier work.

The DNA barcoding results (Table 1) indicate that all taxa represent unique species except for *T. c. triunguis - T. c. mexicana*, *T. o. ornata - T. o. luteola*, and the *T. c. major* clade that did not include *T. coahuila* and *T. c. carolina*. The mutation rate of the *COI* gene is relatively high, and as such it is useful for delineating congenerics and conspecific groups (Cox and Hebert, 2001; Wares and Cunningham, 2001). DNA barcoding has been shown to be accurate in delineating species from one another (Hebert, et al., 2003), which is useful for our dataset because some of the congeneric relationships within *Terrapene* are in question. Our barcoding data agrees with all of the other analyses, and in light of this concordance and with the quantitative evidence that the DNA barcoding analysis provides, it is evident that taxonomic revisions are needed within *Terrapene*.

Future Directions

In order to further support this preliminary *Terrapene* phylogeny, more individuals and species/subspecies must be sampled, more sequences must be generated, and more markers must be evaluated. As with any phylogenetic tree, the one we present here is only one of many possible hypotheses as to the evolutionary relatedness of the *Terrapene* genus and until a more complete and comprehensive dataset is generated, we ask that this phylogeny and the implications that surround it be considered carefully.

Looking forward, we intend to incorporate this phylogenetic research into further efforts through the implementation of microsatellite markers, single nucelotide polymorphisms (SNPs), and other aspects that allow for species to be distinguished from one another at a mtDNA level. These markers will advance the phylogenetic research to determine the "health" of varying species along with further accuracy in species determination itself.

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Literature Cited

Cox, A.J. and P.D.N. Hebert. 2001. Colonization, extinction and phylogeographic patterning in a freshwater crustacean. Mol. Ecol. 10, 371-386.

Dodd, K.C. 2001. North American Box Turtles. University of Oklahoma Press, Norman.

Feldman, C.R. and J.F. Parham. 2002. Molecular phylogenetics of Emydine turtles: taxonomic revision and the evolution of shell kinesis. Molecular Phylogenetics and Evolution 22:388-398.

Hebert, P.D.N., A. Cywinska, S.L. Ball and J.R. deWaard. 2003. Biological identifications through DNA barcodes. Proc. R. Soc. Lond. B Biol. Sci. 270, 313-321.

Herrmann, H. and P.C. Rosen. 2009. Conservation of Aridlands Turtles III: Preliminary Genetic Studies of the Desert Box Turtle and Yaqui Slider. Sonoran Herpetologist 22(4): 38-43.

Minx, P. 1996. Phylogenetic Relationships Among The Box Turtles, Genus *Terrapene*. Herpetologica 52(4): 584-597.

Palumbi, S., A. Martin, S. Romano, W.O. McMillan, L. Stice and G. Grabowski. 1991. The Simple Fool's Guide to PCR. University of Hawaii, Honolulu.
Shaffer, H. B., P. Meylan, and M.L McKnight. 1997. Tests of turtle phylogeny: Molecular, morphological, and paleontological approaches. Syst. Biol. 46: 235-268

Parham, J.F., C.R. Feldman, and J.L. Boore. 2006. The complete mitochondrial genome of the enigmatic bigheaded turtle (*Platysternon*): description of unusual genomic features and the reconciliation of phylogenetic hypotheses based on mitochondrial and nuclear DNA. BMC Evol. Biol. 6, 11.

Reid, Bradley N., W. P. McCord, James B. Iverson, A. Georges, T. Bergmann, G. Amato, R. DeSalle, and E. Naro-Maciel. "Comparing and Combining Distance-based and Character-based Approaches for Barcoding Turtles." *Molecular Ecology Resources* 11 (2011): 956-67.

Spinks, P.Q., H.B. Shaffer, J.B. Iverson, and W.P. McCord. 2004. Phylogenetic hypotheses for the turtle family Geoemydidae. Molecular Phylogenetics and Evolution 32:164-182.

Spinks, P.Q., R.C. Thomson, G.A. Lovely, and H.B Shaffer. 2009. Assessing what is needed to resolve a molecular phylogeny: simulations and empirical data from emydid turtles. BMC Evolutionary Biology 9:5

Stuart, B.L. and J.F. Parham. 2004. Molecular phylogeny of the critically endangered Indochinese box turtle (*Cuora galbinifrons*). Mol. Phylogenet. Evol. 31, 164-177.

Swofford, D. L. 2003. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.

Wares, J.P. and C.W. Cunningham. 2001. Phylogeography and historical ecology of the North Atlantic intertidal. Evolution. 12, 2455-2469.