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Molecular Analysis of Hybridization between the Box Turtles *Terrapene carolina* and *T. ornata*

James C. Cureton, II[1,2], Anna B. Buchman[1,3], Raelynn Deaton[1], and William I. Lutterschmidt[1]

Hybridization of the box turtles *Terrapene carolina* and *T. ornata* has been reported throughout their sympatric range. Herein, eight polymorphic microsatellite loci and one mitochondrial polymorphism were used to assess patterns of introgression between these two species, both of which are of conservation concern. We estimated standard population statistics, population assignments, and ancestry for 28 *T. carolina*, 17 *T. ornata*, and ten putative *T. carolina*–*T. ornata* hybrids from southwest Texas. Both parental populations had high observed heterozygosity and allelic diversity, and there was high gene flow between the two parental species. Ancestry values were sporadic and typical of species that have been hybridizing. Approximately 86% and 76% of *T. carolina* and *T. ornata*, respectively, were correctly assigned to their species. Seven of the hybrids had a high level of ancestry for *T. carolina* and three resembled *T. ornata*. Interestingly, all *T. carolina*, putative hybrids, and one *T. ornata* had haplotypes of *T. carolina*, whereas all but one *T. ornata* had haplotypes of *T. ornata*. These results suggest that hybridization has occurred for several generations within this population, and we discuss how such hybridization may influence population structure for such species of concern.

HYBRIDIZATION, or the breeding of individuals from genetically distinct lineages (Harrison, 1993), can play a constructive role in the evolutionary process. Hybridization is important for understanding population dynamics and for evaluating the conservation status of species (i.e., defining management units). For example, hybridization can result in increased genetic variation (Hartl and Clark, 2007), hybrid vigor (Rhymer and Simberloff, 1996), and niche expansion (Choler et al., 2004). More commonly, however, hybridization can threaten parental populations, especially those suffering low genetic diversity or increasing anthropogenic pressures (Rhymer and Simberloff, 1996; Frankham et al., 2002). Hybridization can lead to outbreeding depression and decreased offspring fitness via disruption of adaptive traits, chromosomal incompatibilities, and the breakdown of coadapted gene complexes (Allendorf and Waples, 1996). Moreover, backcrossing of hybrid individuals into the parental populations can threaten the genetic integrity of the two species (Frankham et al., 2002). In species with low reproductive rates, wasted reproductive effort (due to hybrid sterility or reduced fertility) can jeopardize a population (Nowak, 1991; Rhymer and Simberloff, 1996).

Terrapene carolina and *T. ornata* are of conservation concern throughout their range in the United States (Ceballos and Fitzgerald, 2004; Smith, 2004). Their cumulative range spans a large portion of the United States with sympatry occurring throughout the central United States (Dodd, 2001; Fig. 1). *Terrapene carolina* typically inhabit meadows and woodlands, whereas *T. ornata* tend to inhabit grasslands and pastures (St. Clair, 1998; Dodd, 2001). Despite differences in habitat preferences, box turtles displaying characteristics of both species have been observed in areas where the species are sympatric in distribution, including Illinois (Smith, 1955), Indiana (Clark, 1935), Louisiana (Blaney, 1968), Missouri (Shannon and Smith, 1949; Ward, 1968), and Texas (Lutterschmidt et al., 2007). Intermediate individuals have three toes on each hind foot, a solid yellow plastron (but see Clark, 1935; Ward, 1968), and a keeled carapace characteristic of *T. carolina*. However, carapace ornamentation resembles *T. ornata*, with light (typically yellow), radiating stripes overlaying dark pigmentation (Ward, 1968; Fig. 2). Morphometric analyses of the carapace shape of intermediate individuals collected from Walker County, Texas, indicate that carapace morphology (except ornamentation) of these putative hybrids is more similar to *T. carolina* than *T. ornata* (Lutterschmidt et al., 2007). It is possible that these two species are no longer reproductively isolated due to habitat modification and fragmentation (Rhymer and Simberloff, 1996). Because hybrids can be difficult to identify based solely on morphological features, molecular analyses are often needed to investigate hybridization (Rhymer and Simberloff, 1996).

Herein, we used eight nuclear microsatellite loci and one mitochondrial polymorphism to investigate potential hybridization of *T. carolina* and *T. ornata*. Our goal was to determine if these species are hybridizing and the extent of hybridization in southeast Texas. To our knowledge, this is the first published report investigating hybridization of these two species using molecular markers.

MATERIALS AND METHODS

From 7 April to 11 October 2004, we collected *T. carolina*, *T. ornata*, and putative hybrids by road sampling and incidental encounters in Walker County, Texas (Lutterschmidt et al., 2007; Buchman et al., 2010). All live-caught turtles were collected under a Texas Parks and Wildlife Scientific Research Permit and released after we obtained whole blood extractions from the femoral vein of 28 *T. carolina*, seven *T. ornata*, and ten putative hybrids. We included ten formalin-fixed samples (Lutterschmidt et al., 2010) taken from *T. ornata* specimens of the Texas Cooperative Wildlife Collection (TCWC) at Texas A&M University to increase our *T.*

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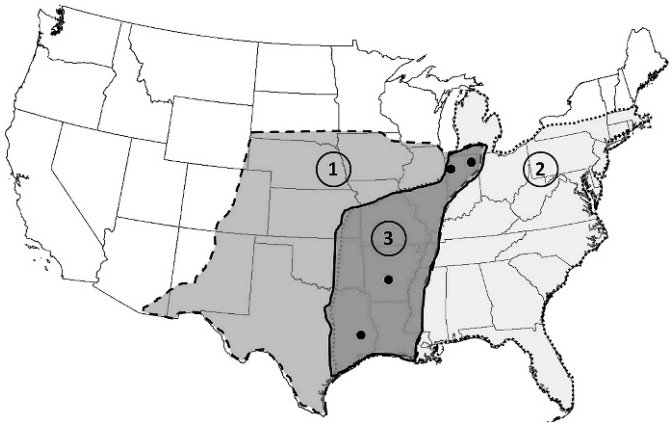

Fig. 1. Current geographic ranges of *Terrapene carolina* (1), *T. ornata* (2), and overlapping distributions (3) within the central U.S. (Dodd, 2001). Black dots indicate localities of reported hybridization events.

ornata sample size to 17 for genetic analyses. Because all hybrids are not intermediate in morphology, the low sample size of morphological intergrades ($n = 10$) can be attributed to the fact that they are rare, as evidenced by other reports also documenting low sample sizes (Clark, 1935; Shannon and Smith, 1949; Ward, 1968).

Genomic DNA was extracted from the blood of all live-caught turtles using a modified proteinase K method where samples were incubated in 500 µl of 20 mg/ml proteinase K at 60°C for 1 h. Two hundred fifty microliters of 5M NaCl were added to each sample and shaken vigorously followed by a 10 min ice incubation. Samples were then centrifuged for 10 min at 5000 RPM/g, the supernatant was transferred to a new tube, and 650 µl of isopropanol was added. Tubes were then incubated at room temperature for 15 min prior to maximum speed centrifugation for 15 min. The supernatant was discarded, tubes were allowed to air dry for 15 minutes, and 100 µl of TE (pH 7.5) was added to each sample prior to a final 10 min heating at 60°C. We extracted DNA from the TCWC preserved specimens (Lutterschmidt et al., 2010) using a QIAGEN DNEasy Kit.

Using polymerase chain reaction (PCR), we amplified all DNA samples across seven microsatellite loci developed for *T. carolina* (TCTB2, TCTG7, TCTO11, TCTP11, TCTQ17, TCTR7, TCTS2; Buchman et al., 2009) and one microsatellite locus for *T. ornata* (TO2T; Cureton et al., 2009). One primer from each primer pair (Table 1) contained a 5′–CAG tag (5′–CAGTCGGGCGTCATCA–3′) that allowed us to fluorescently label PCR products (Boutin-Ganache et al., 2001). Each 20 µl PCR reaction consisted of 1× PCR buffer (Promega, Sigma-Aldrich, Inc., Madison, WI), 0.6 mM dNTPs, 2.5 mM $MgCl_2$, 2.5 µM bovine serum albumin (BSA), 0.025 µM untagged primer, 0.5 µM CAG-tagged primer, 0.5 µM D4 WellRED fluorescent primer (Proligo, Sigma-Aldrich, Inc., St. Louis, MO), 0.5 U of DNA Taq polymerase (Promega, Sigma-Aldrich, Inc.), and 2 µl of genomic DNA. We used a touchdown PCR protocol to amplify all microsatellite loci: denaturation at 94°C for five minutes; 21 cycles of denaturation at 94°C for 30 seconds, amplification at 65°C for 30 seconds (the T_M dropped 0.5°C every cycle resulting in a decrease of 10°C over 21 cycles), and extension at 72° for one minute; 16 cycles of denaturation at 94°C for 30 seconds, amplification at 60°C for 30 seconds, and extension at 72°C for one minute; and final extension at 67°C for 45 minutes (Cureton et al., 2009). Following successful amplification, we electrophoresed PCR products on a Beckman Coulter CEQ™ 8000 sequencer (Beckman Coulter, Inc., Fullerton, CA) and scored all alleles using inclusive Beckman Coulter CEQ™ 8000 Genetic Analysis software (calibrated with DNA size standard—600).

For each population, we used GENEPOP 4.0 (Raymond and Rousset, 1995) to test for linkage disequilibrium between all pairs of loci and to calculate the observed heterozygosity (H_O), expected heterozygosity (H_E), and allelic diversity (A) for each locus. We also estimated F_{ST}, a measure of genetic differentiation, between all population pairs and estimated the number of migrants among the populations.

To identify the ancestry of the putative hybrids, we used a Bayesian algorithm implemented in STRUCTURE (Pritchard et al., 2000). This program estimates the proportion of each individual's genes that originated from each of the parental species. This program assigned all genotyped turtles to one of two species groups (k = 2; *T. carolina* or *T. ornata*) using 500,000 burn-in iterations and 1,000,000 repetitions. We assumed the admixed model (default parameters) and independent allele frequencies, and did not include prior population information about individuals across five iterations (following Schwartz and Beheregaray, 2008). This analysis resulted in a q-value for all individuals ($0 \leq$ q-value ≤ 1; the mean estimated proportion of the turtle's genome that had a specific ancestry). As a cut-off value for assigning each individual to a group (*T. carolina* or *T. ornata*), we classified individuals with a q-value ≥ 0.90 as a *T. carolina*, ≤ 0.10 as *T. ornata*, and $0.10 <$ q-values < 0.90 as a hybrid.

In addition to STRUCTURE, we used a least-log likelihood test implemented in GENALEX 6.3 to assign hybrid individuals to one of the two parental species (Peakall and Smouse, 2005). This test uses allele frequencies to calculate expected genotypic frequencies within and among populations. A DNA profile probability is calculated for each individual across all putative populations and used to assign an individual to the most likely population of origin. This analysis is different from STRUCTURE because it assigns individuals to populations based on expected genotypic frequencies rather than on a population's observed genotypic frequencies. Moreover, STRUCTURE uses repetitions and iterations to calculate the most probable population of the individual, whereas this test calculates the likelihood of the individual being assigned to all populations and then assigns to the most likely one. We ran the ''last population unknown'' option which does not include information about the population origin of the hybrid individuals in the analysis.

To further investigate hybridization, we amplified 1185 base pairs (bp) of the cytochrome *b* gene (cytb) using primers CYTBG (5′–AACCATCGTTGTWATCAACTAC–3′) and THR8 (5′–GGTTTACAAGACCAATGCTT–3′) in the aforementioned PCR reagent volumes (Spinks et al., 2004). All reactions were denatured for at 95°C for 5 minutes followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 58° for 30 seconds, and extension at 72°C for two minutes (Spinks et al., 2004). We then digested PCR products at all AG^CT sites in the cytb gene using restriction enzyme Alu1 (New England Biolabs, Ipswich, MA). This resulted in 155 and 1030 bp fragments for *T. carolina* and 53, 155, 300, and 677 bp fragments for *T. ornata*. Digestion products were visualized on 2% ethidium bromide stained

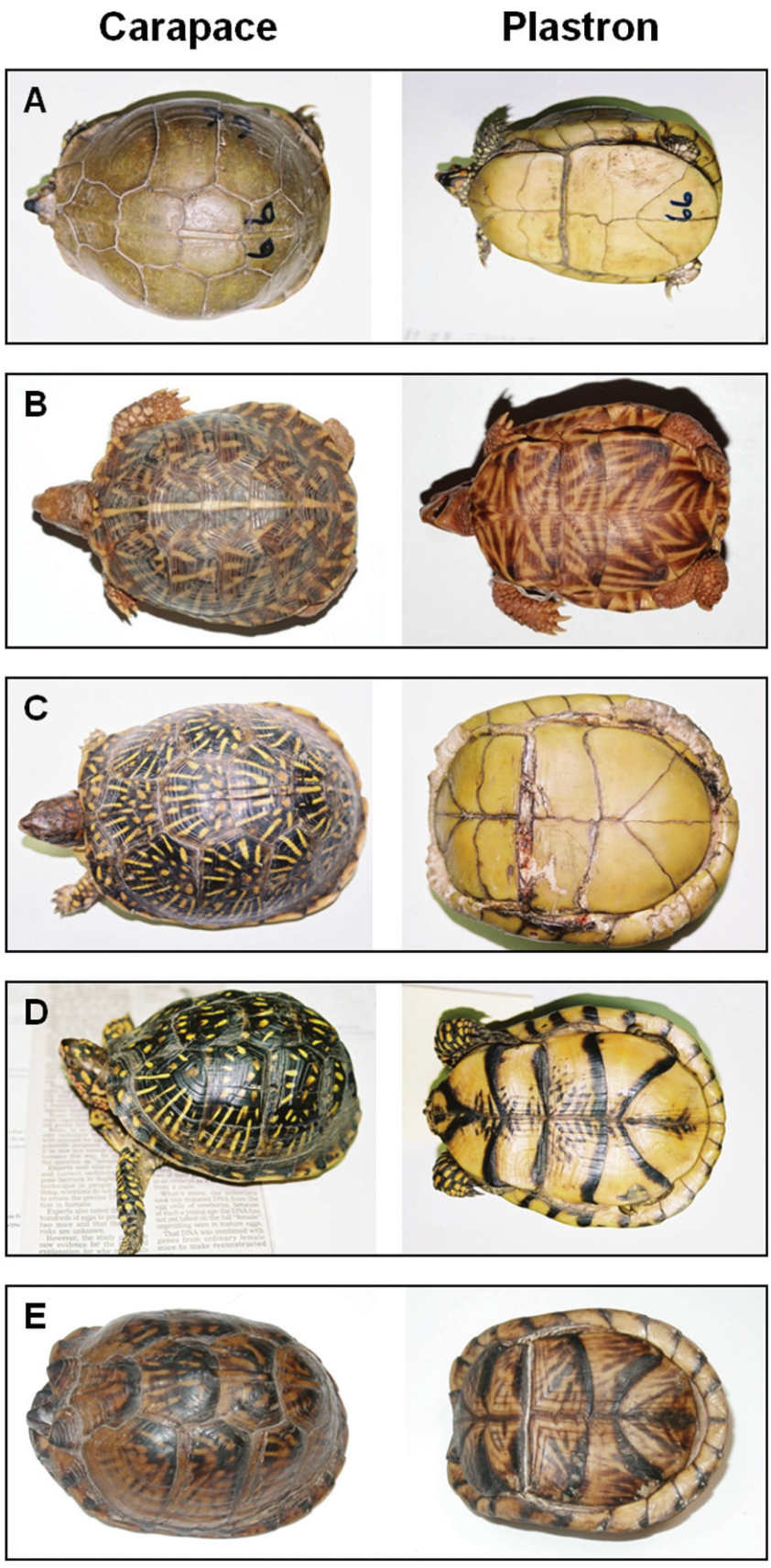

Fig. 2. Morphological characters of *Terrapene carolina* (A), *T. ornata* (B), and example putative hybrids (C, D, and E), showing an intermediate shell morphology and pigmentation. *Terrapene carolina*: olive colored, keeled, and high domed carapace; a solid yellow plastron; and three toes on hind feet. *Terrapene ornata*: black colored carapace with radiating yellow lines, an absent keel, and an absent high dome; a yellow with black mottling plastron; and four toes on hind feet. Putative hybrids: black colored carapace with radiating yellow lines with a keel and high dome; a yellow with or without black mottling plastron; and three toes on hind feet.

Table 1. Locus, Forward (F) and Reverse (R) Primer Sequence, Repeat Motif, and Expected Allele Size for Each Microsatellite Locus Used in This Study. Asterisks (*) indicate the primer in each pair that is tagged with a 5′–CAG tag (5′–CAGTCGGGCGTCATCA–3′).

Locus	5′–3′ Primer sequence	Repeat motif	Expected size
TCTR7	F: TTCTGGCCCTGTTCTCTCG* R: GGCAAACTAAACTCCACCACC	$(ATTT)_6$	183–211
TCTS2	F: TGGGAGCAGTATTGGACACC* R: AGCTTGTTGCTACCCCTTC	$(TC)_{11}TG(TA)_5$	202–230
TCTG7	F: GAAAGCCCTTGGGTGTGTG R: AGTGAACGATTTGCGTGTC*	$(CT)_2(GT)_{17}$	248–274
TCTQ17	F: ACTGGCCACTCCACTCATC* R: GGGGTGCGCTCTCTCTC	$(GT)_{12}(GA)_4$	167–215
TCTP11	F: GAAAATGTGTCCCAGGGCG* R: TGGTGGAAGGGTTGGAGAC	$(CA)_9$ ... $(CT)_5$	241–247
TCTO11	F: TAAAGGTGCCACAGGACCC* R: CCCCAAAAGCCTTCAGCAC	$(GA)_{14}$	245–255
TCTB2	F: ACAAATTAGTGAGTGGCACCTG* R: GGTCATTACCTACTTTGCCTTC	$(GA)_{10}$ ... $(GA)_2(GT)_5$	255–263
TO2T	F: TGCCGCCGAATTAATATGC R: GGCCGGTAGACGATATCCC*	$(CA)_{18}$	252–292

gels and all individuals were scored as having the cytb gene of *T. carolina* or *T. ornata* based on the observed fragment sizes.

RESULTS

We did not detect linkage disequilibrium between any pair of loci across all populations (sequential Bonferroni-correction; 84 pair-wise comparisons). All loci were polymorphic and all populations had high allelic diversities of 8.75 (*T. carolina*), 10.50 (*T. ornata*), and 6.63 (putative hybrids; Table 2). Observed heterozygosities ranged from 0.185–0.800 with an average of 0.423, 0.553, and 0.550 in the *T. carolina, T. ornata*, and hybrid populations, respectively. In the population of *T. carolina*, four loci were heterozygote deficient (TCTQ17, TCTO11, TCTB2, and TO2T), while in the population of *T. ornata* six loci were heterozygote deficient (TCTR7, TCTS2, TCTG7, TCTQ17, TCTP11, and TCTO11) and two heterozygote excess (TCTB2 and TO2T). In the hybrid population, only one locus was heterozygote deficient (TCTG7). We detected significant genotypic differentiation between the population of *T. ornata* and putative hybrids ($F_{ST} = 0.0579$, $P < 0.001$), and the population of *T. ornata* and *T. carolina* ($F_{ST} = 0.1314$, $P < 0.001$), but not the population of *T. carolina* and putative hybrids ($F_{ST} = 0.0.0159$, $P > 0.05$). We estimated an average of 2.70 migrants among all populations per generation.

Based on our *a priori* classifications, STRUCTURE correctly assigned 86% (24/28) and 76% (13/17) of *T. carolina* and *T. ornata*, respectively. Q-values (Fig. 3) for correctly assigned *T. carolina* ranged from 0.911–0.993 with an average of 0.977, as opposed to 0.071–0.884 with an average of 0.656 for those incorrectly assigned. One of the *T. carolina* had a q-value typical of *T. ornata*, while the other three resembled putative hybrids ($0.10 <$ q-value < 0.90). Correctly assigned *T. ornata* had a mean q-value of 0.027 with a range of 0.010–0.079 compared to 0.530 and 0.112–0.981 for those incorrectly assigned. Three of the incorrectly assigned *T. ornata* had q-values characteristic of hybrids, while one resembled *T. carolina*. Seven of the putative hybrids had q-values ≥0.90 (*T. carolina*) against three hybrids with q-values of ≤0.10 (*T. ornata*). Hybrids characteristic of *T. carolina* had q-values ranging from 0.915–0.993 with a mean of 0.968, while those similar to *T. ornata* ranged from 0.025–0.038 with a mean of 0.032.

A least-log likelihood test using allele frequencies to calculate expected genotypic frequencies within and among populations was implemented in GENALEX 6.3 and assigned hybrid individuals to one of the two parental species (Peakall and Smouse, 2005). GENALEX v.6.3 correctly assigned only 46% (13/28) and 12% (2/17) of *T. carolina* and *T. ornata* to their respective populations. According to the statistical assignments, six of the putative hybrids were assigned to *T. carolina,* while the other four were assigned to *T. ornata*. Similar to the findings of Lutterschmidt et al. (2007), the *a priori* assignments were observed (i.e., except for putative hybrids), two distinct clusters partitioned (*T.*

Table 2. Observed Heterozygosity (H_O) per Individual Locus, Allelic Diversity (A), and F_{ST} Values for *T. carolina*, *T. ornata*, and Hybrid Populations with the Calculated Number of Migrants (N_M). Deviations from Hardy-Weinberg equilibrium are indicated by an asterisk (*); two asterisks (**) indicate significant population differentiation.

	Observed heterozygosity (H_O)									F_{ST}	
Population	TCTR7	TCTS2	TCTG7	TCTQ17	TCTP11	TCTO11	TCTB2	TO2T	A	*T. carolina*	*T. ornata*
T. carolina	0.259	0.667	0.750	0.500*	0.333	0.360*	0.185*	0.333*	8.75	-	-
T. ornata	0.313*	0.765*	0.647*	0.529*	0.706*	0.438*	0.500	0.529*	10.50	0.1314**	-
Hybrids	0.400	0.800	0.500*	0.700	0.600	0.400	0.200	0.800	6.63	0.0159	0.0579**
Averages	0.302*	0.722*	0.667*	0.551*	0.510*	0.392*	0.283*	0.432*	8.63	-	-
$N_M = 2.70$											

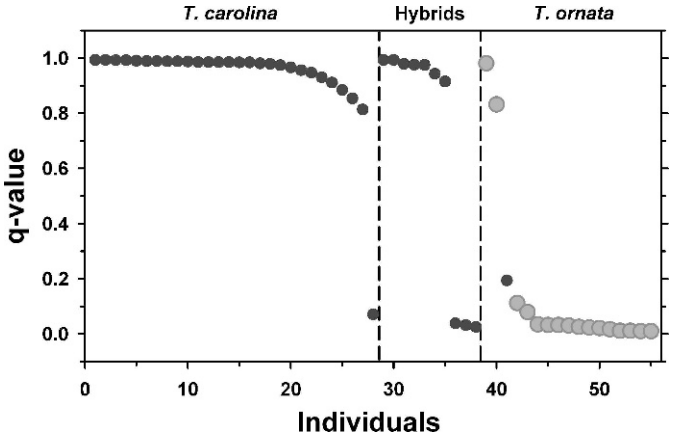

Fig. 3. STRUCTURE analysis using *a priori* classification of all individuals ordered by decreasing q-values. Individuals with *Terrapene carolina* cytb haplotype are indicated with solid dark-gray circles; individuals with *T. ornata* cytb haplotype are indicated with light gray circles.

carolina and *T. ornata*) with hybrids in both cluster (Fig. 4). The *T. carolina* cluster had one *T. ornata* and seven putative hybrids, while the *T. ornata* cluster had one *T. carolina* individual and three putative hybrids.

All 28 *T. carolina* and ten putative hybrids possessed a cytb gene characteristic of *T. carolina*. Alternatively, 16 of the 17 *T. ornata* had cytb genes typical of *T. ornata* with one having a *T. carolina* cytb gene (Fig. 3).

DISCUSSION

In this study, we used microsatellite markers to determine if hybridization is occurring between the box turtles *T. carolina* and *T. ornata*. Our analyses suggest that introgression is likely occurring in this population and that gene flow is unidirectional in which hybridization occurs between female *T. carolina* and male *T. ornata*. Our results from GENALEX were less accurate than those from STRUCTURE based on our *a priori* classifications. This is likely due to GENALEX calculating expected genotypic frequencies for each population based upon the present alleles. If the populations are not in Hardy-Weinberg equilibrium at the loci tested, then the genotypes expected to be in the population would not be present, thus skewing accuracy.

Although relatively high observed heterozygosity and allelic diversity would suggest that both parental populations are healthy, Zhang et al. (2007) cautions that these estimates of diversity may be poor indicators of a species' conservation status. For example, four and seven loci in the populations of *T. carolina* and *T. ornata*, respectively, were heterozygote deficient relative to Hardy-Weinberg equilibrium. This is not surprising, considering populations in hybrid contact zones commonly deviate from Hardy-Weinberg equilibrium (Randi and Bernard-Laurent, 1999). Interestingly, only one locus deviated from Hardy-Weinberg equilibrium in the putative hybrid population. This single deviant locus may be an artifact of the putative hybrid's small sample size. F_{ST} values for all the *T. carolina* × *T. ornata* and hybrid × *T. ornata* are moderate, but indicate significant population deviation. The non-significant differentiation, and low F_{ST} value, between *T. carolina* and putative hybrids indicates that the hybrids sampled genetically resemble *T. carolina* more than *T. ornata*. This could be due to either

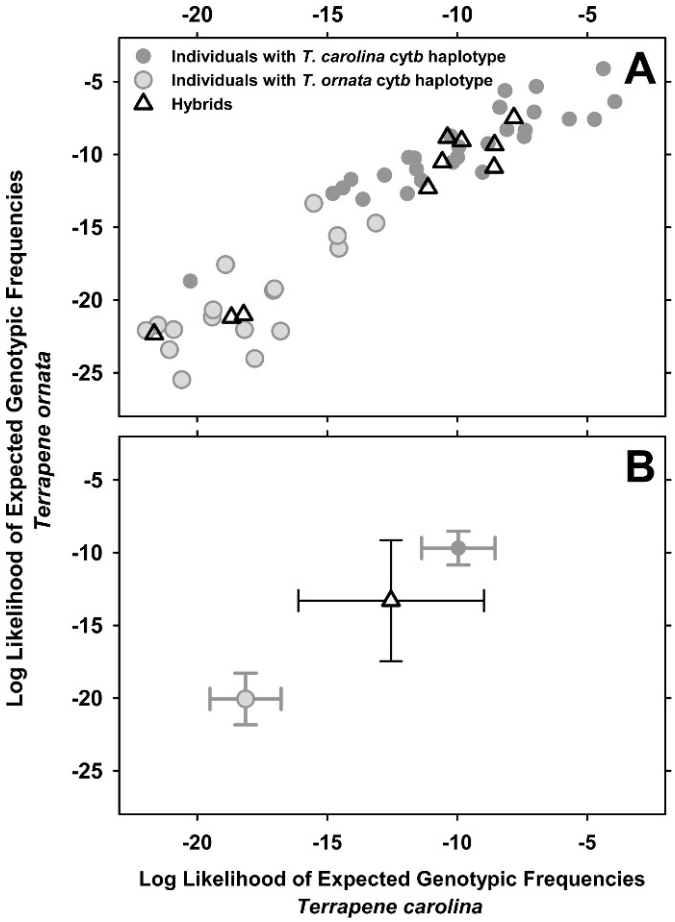

Fig. 4. Results of the least-log likelihood test using allele frequencies implemented in GENALEX 6.3 to calculate expected genotypic frequencies within and among populations with *Terrapene carolina* versus *T. ornata* (A) and the resulting 95% confidence intervals of these frequencies (B). Classified *T. carolina* are indicated by small dark-gray circles, *T. ornata* by large light-gray circles, and hybrids by open triangles.

recent or more frequent gene flow between hybrids and the parental *T. carolina* as Lutterschmidt et al. (2007) also demonstrated with putative hybrid carapace morphology more closely resembling that of *T. carolina* than *T. ornata*. The relatively high number of estimated migrants, and F_{ST} values, indicates that there is likely enough gene flow to negate complete differentiation between the two species (or at least *T. carolina*) and putative hybrids.

Based on a q-value cut-off of 0.10, STRUCTURE assigned the majority of *T. carolina* (86%) and *T. ornata* (76%) correctly, although three and four were misidentified (when compared to our *a priori* classifications), respectively. The most likely explanations for the ''incorrect'' classification of parental individuals are long-time hybridization and historical introgression. If we collected first-generation hybrids, then we would expect q-values of 0.5. The q-values of successive hybrid generations would vary depending on with whom hybrids are mating (i.e., backcrossing and introgression). If long-time hybridization has been occurring, this could explain why we get unexpected q-values for these six ''parental individuals'' (Duvernell et al., 2007). These six individuals could posses diluted characters that were previously thought to be species-specific (i.e., shell ornamentation). Long-term hybridization may also explain

why the hybrids we observed are slightly different from previously reported hybrids. For example, hybrids previously reported (Clark, 1935; Ward, 1968) had radiating lines on their carapace and plastron, a characteristic we did not observe in classified hybrids. It is possible that three turtles classified as *T. ornata* box turtles were truly hybrids, yet we misidentified them because they resembled *T. ornata* (i.e., carapace and plastron ornamentation and four toes). Similarly, this would explain why we classified one individual as a *T. carolina*, yet it was ''truly a hybrid.''

STRUCTURE assigned seven hybrids to *T. carolina* and three to *T. ornata*, suggesting that none of the putative hybrids were hybrids based strictly on q-values. Although the majority of individuals were misclassified in GENALEX, the population assignment tests yielded overall results similar to those from STRUCTURE, as seven of the putative hybrids were assigned to *T. carolina* while three were assigned to *T. ornata*. We suspect the slight difference in the results from the two tests is due to the sample size as the least-likelihood test is more sensitive to small sample sizes than the Bayesian algorithm in STRUCTURE. Interestingly, all hybrids had q-values <0.10 or >0.90, which were our two cut-off values for assigning individuals to the parental species. If one imposes a more strict cut-off q-value of 0.05, 75%, 29%, and 30% of *T. carolina, T. ornata*, and putative hybrids are assigned correctly; thus, as a more strict cut-off value is imposed, the accuracy of the results decreases (based on *a priori* classifications). Future investigations that include ''pure'' *T. carolina* and *T. ornata* from outside of their zone of sympatry maybe helpful in such analyses where there is much genetic admixture.

We observed cytb haplotypes of *T. carolina* in all *T. carolina* and putative hybrids as well as one *T. ornata*. Alternatively, all except one *T. ornata* possessed cytb haplotypes of *T. ornata*. This suggests that gene flow between the two species is unidirectional, with female *T. carolina* and male *T. ornata*, and that introgression is occurring between the hybrids and parental species. There is no documented evidence that male *T. ornata* prefer to mate with female *T. carolina*, although this hypothesis would be supported by our results. Because of the one *T. ornata* possessing a cytb haplotype of *T. carolina*, it seems likely that introgression of hybrids with the parental lineages is occurring (Lara-Ruiz et al., 2006). For example, the *T. ornata* with a cytb haplotype of *T. carolina* is likely the result of a male *T. ornata* and female *T. carolina* × *T. ornata* hybrid mating event. The low frequency of observed *T. ornata* with haplotypes of *T. carolina* may further suggest male *T. ornata* preferring *T. carolina*, as mitochondrial haplotypes are only passed maternally. One alternative explanation for the observed cytb patterns is incomplete lineage splitting. However, this seems unlikely given that most phylogenies do not place these two taxa as sister species within *Terrapene* (Feldman and Parham, 2002; Stephens and Wiens, 2004; but see Stephens and Wiens, 2003; Spinks et al., 2009). Introgression, as we observed in this study, seems to be frequent in known turtle hybridization events (Vilaca et al., 2008; Reis et al., 2009). In particular, introgression has been independently reported in the Asian box turtles *Cuora trifasciata* and *C. serrata* by Spinks and Shaffer (2007) and Stuart and Parham (2004), respectively. Thus, introgression seems to occur rather frequently in most turtle hybridization events.

Hybridization and introgression have likely occurred in our sampled population for several generations, as evidenced by the sporadic q-values of some individuals and observed pattern of cytb haplotypes. Multiple reports of observed hybrids (Clark, 1935; Shannon and Smith, 1949; Smith, 1955; Ward, 1968; Lutterschmidt et al., 2007) suggest that hybridization has occurred throughout the sympatric range of these two species. However, only one study has investigated and proposed the occurrence of hybridization in *Terrapene* based upon a more detailed analysis of shell morphology (Lutterschmidt et al., 2007). It is still unclear what effect hybridization may have on this population. Understanding the degree of hybridization within this population and the directionality of gene flow is essential to better understand the hybridization dynamics in other turtle populations. Data presented here are very much parallel to systems that suffer from similar conservation concerns. Upon outside review of this manuscript, we were made aware of the potential significance of such research on *Terrapene* to research surrounding the Asian turtle crisis (Parham et al., 2001; Stuart and Parham, 2004; Shi et al., 2005). There is a significant lack of known samples, and it is impossible to study this crisis in genetic detail within the wild. Researchers are therefore limited as many specimens are from captive and hybridizing populations. Thus, this study of *Terrapene* in southwest Texas was reviewed as ''special and interesting'' in that it provides a unique opportunity to understand the dynamics of hybridization in the wild. We sincerely appreciate such praise and humbly hope the results reported herein aid future researchers and potential management units in successful conservation measures for all turtle species.

MATERIAL EXAMINED

Terrapene carolina (TC), *T. ornata* (TO), and putative hybrids (PH) used in genetic analyses referenced by collector's log numbers (Everett D. Wilson) with photographic reference deposited in the Sam Houston State University Vertebrate Museum (SHSVM): EDW-TC01 through EDW-TC25, EDW-TC27, EDW-TC31, EDW-TC39, EDW-TO01 through EDW-TO07, EDW-PH01 through EDW-PH10. TCWC specimens of *Terrapene ornata* examined for quick tissue (Lutterschmidt et al., 2010) samples and genetic analysis include: TCWC 13979, 18770, 20093, 20099, 33110 (Brazos Co.); TCWC 30692, 30694 (Grimes Co.); TCWC 20096 (Leon Co.); TAM 297, 298 (Walker Co.).

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