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A Genetic Analysis of the Impact of Generation Time and Road-Based Habitat Fragmentation on Eastern Box Turtles (*Terrapene c. carolina*)

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Historically, the Eastern Box Turtle (*Terrapene c. carolina*) was found in 31 counties in Michigan's Lower Peninsula, although it has been extirpated from 13 of those counties in the last ten years. One possible cause of the decline is road-based habitat fragmentation with resulting demographic and genetic consequences. Accurately identifying population structure is necessary to determine conservation units and aid in the recovery of *Terrapene c. carolina*. We genotyped 163 turtles at eight microsatellite loci from three locations in southwestern Michigan covering 360 km². We found high levels of genetic variation (H = 0.83; A = 16) and low levels of genetic differentiation (F_{ST} = 0.006) in the system. The three areas exist as a single population and there was a low rate (11%) of misassignment across the sites. There was initial evidence of a genetic bottleneck in two of the three populations and the system as a whole. However, additional analysis failed to find a mode-shift in allele frequencies and did not detect any further evidence of a bottleneck in any of the populations. We conclude that the conflicting genetic indication of a bottleneck, despite the geographic evidence, is due to the long generation time of *Terrapene c. carolina*. Further, our study suggests that the retention of genetic variation despite population declines allows managers flexibility in dealing with the conservation of long-lived species.

HABITAT fragmentation, a landscape-scale process that separates habitat (Fahrig, 2003), and its consequences on wildlife populations are of great concern (Couvet, 2001; Gibbs, 2001; Fahrig and Merriam, 2002). Habitat fragmentation negatively affects populations because it decreases interpatch dispersal (Vos and Chardon, 1998; Clark et al., 1999; Stow et al., 2001) and population size (MacNally and Brown, 2001; Driscoll, 2004; Kuo and Janzen, 2004). A decrease in interpopulation dispersal and population size leads to a reduction in the effective population size (N_e) followed by increased genetic drift, reduced genetic variation (Gray, 1995; Edenhamn et al., 2000) and increased inbreeding (Anderson et al., 2004). Inbreeding increases the number of deleterious alleles (Ralls et al., 1988) and fixed mutations (Lynch et al., 1995; Rowe and Beebee, 2003), while decreasing disease resistance (O'Brien and Evermann, 1988; Frankham, 1995) and the ability of a population to adapt (Lacy, 1993). These factors reduce fitness by decreasing survival and reproduction (Ryan et al., 2003) leading to further declines and erosion of genetic diversity, resulting in inbreeding depression (Hedrick and Kalinowski, 2000) and a decrease in the time to extinction (Lacy, 1993; Brook et al., 2002).

Habitat fragmentation and its associated negative consequences can be especially problematic for turtles such as *Terrapene c. carolina* (Harding, 1997; Klemens, 2000; Dodd, 2001). *Terrapene c. carolina* experiences low population growth rates due to small clutch sizes, low juvenile survival rates, and delayed maturity (Stickel, 1978; Doroff and Keith, 1990; Bowen et al., 2005; Wilson and Ernst, 2005). These factors are compounded in the decline of *Terrapene c. carolina* because turtles often remain in the same patches (Stickel, 1989; Claussen et al., 1991; Mitchell and Klemens, 2000) even after habitat degradation (Dodd, 2001). The species is listed as endangered in Maine, a species of special concern in Connecticut, Massachusetts, Michigan, New Hampshire, and New York, and declines have been reported throughout its range (Stickel, 1978; Williams and Parker, 1987; Stickel, 1989; Doroff and Keith, 1990; Schwartz and Schwartz, 1991; Dodd, 2001). In Michigan, *Terrapene c. carolina* was historically found in 31 counties in the Lower Peninsula, but within the past ten years has been found in only 18 counties (D. Hyde, unpubl.; Fig. 1).

The decline of *Terrapene c. carolina* in Michigan may be associated with road-based habitat fragmentation and the doubling of vehicular traffic in the U.S. in the last 20 years (U.S. Department of Transportation, 2006). Roads create unnatural dispersal corridors (Merriam et al., 1989) and increase predation risk (Temple, 1987; Marchand and Litvaitis, 2004), vehicle-related mortality (Gibbs and Shriver, 2002; Aresco, 2005), and human collection (Steen and Gibbs, 2003). These factors taken together reduce genetic variation (Gray, 1995; Kuo and Janzen, 2004) in *Terrapene c. carolina*. The effect of roads is of special concern for turtle conservation because turtle mortality increases substantially in areas with more than 1 km of roads per km² and traffic volume greater than 100 vehicles per lane per day (Gibbs and Shriver, 2002). Currently in southern Michigan there are almost 2 km of roads per km² (U.S. Census Bureau, Washington, D.C.; http://www.census.gov/geo/www/tiger). Road density in Michigan likely increases turtle mortality rates and isolation, potentially reducing genetic viability (Frankham, 1998).

Few studies have addressed the effects of fragmentation on terrestrial turtles. The lack of research may be attributed to several factors, such as the difficulty associated with estimating population size of *Terrapene c. carolina* (Schwartz, 2000; Converse et al., 2005; Dodd et al., 2006), their longevity (Nichols, 1939; Stickel, 1978; Schwartz, 2000), overlapping generations, and the difficulty in finding juveniles (Dodd, 2001; Converse et al., 2005). An accurate estimation of population structure and dispersal is necessary to determine conservation units and aid in the recovery of populations of *Terrapene c. carolina*. The purpose of our study was to examine population structure of *Terrapene c. carolina* in an area highly fragmented by roads. We sampled turtles from three locations in a 360 km² area in southwestern Michigan and determined the level of genetic variation, population structure, and rate of interpopulation dispersal. Also, we investigated whether the reduction in the range of

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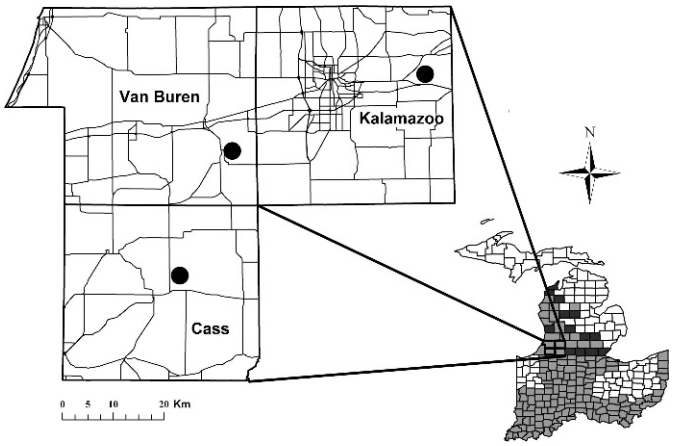

Fig. 1. Distribution of counties occupied by *Terrapene c. carolina* in Michigan, Ohio, and Indiana (MacGowan et al., 2004) are shaded. The counties in Michigan which have lost their *Terrapene c. carolina* populations in the last ten years are shown in black. Data on geographic declines in Ohio and Indiana were not available. The blow-up shows the 360 km² *Terrapene c. carolina* study area with sampled locations (black circles) in Van Buren, Kalamazoo, and Cass counties, Michigan. The black lines represent state and interstate highways.

Terrapene c. carolina in Michigan had produced a genetic bottleneck.

MATERIALS AND METHODS

Our study area consisted of three locations in southwestern Michigan: Fort Custer Military Base (Kalamazoo County), Edward Lowe Foundation (Cass County), and a private property located in Van Buren County (Fig. 2). Each site was separated by at least 2 km of roads per km², including several highways (U.S. Census Bureau, Washington, D.C.; http://www.census.gov/geo/www/tiger). Within each site we collected turtles by hand through haphazard visual searching from May to August 2005 and May to July 2006. Approximately 0.1–0.3 ml of blood was drawn from the femoral vein of each turtle using a 28-gauge heparinized needle, preserved in ACD Solution B (Qiagen, Valencia, CA), and stored at 4°C. Tissue samples also were collected from dead turtles found on roads within the three study areas. Although latitudinal impacts may alter the age of maturity, we assumed all animals to be adults if at least ten growth rings could be counted on their plastral scutes (Gibbons, 1998; Dodd, 2001). Every turtle was notched for individual identification (Gibbons, 1998) or a PIT tag was attached to the carapace with epoxy to prevent resampling and immediately released at the site of capture.

Genomic DNA was extracted from the blood and tissue samples using Qiagen DNeasy Kits (Qiagen, Valencia, CA) following manufacturer's instructions. Extracted DNA was amplified via PCR at eight microsatellite loci (*Gmu*B08, *Gmu*D16, *Gmu*A18, *Gmu*D40, *Gmu*D55, *Gmu*D87, *Gmu*D88, and *Gmu*D121; King and Julian, 2004). Polymerase chain reactions were carried out in an Eppendorf Mastercycler Gradient (Eppendorf, Westbury, NY) in 20 µl reactions consisting of 75 ng of template DNA, 250 µM dNTPs, 0.16 µM forward and reverse primer, 10× Eppendorf HotMaster Taq Buffer (Eppendorf, Westbury, NY), and 0.75 units of Eppendorf Hotmaster Taq Polymerase (Eppendorf, Westbury, NY). PCR temperature cycling was performed

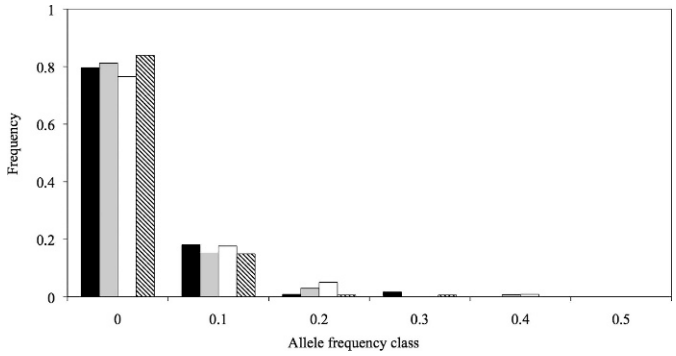

Fig. 2. The distribution of allele frequency classes across all loci for three populations of Michigan *Terrapene c. carolina*. Black bars = Kalamazoo County site, gray bars = Van Buren County site, white bars = Cass County site, and striped bars = all three sites combined.

under the following conditions: an initial denaturing at 94°C for 2 min, 35 cycles of 94°C denaturing for 45 sec, 58°C annealing for 45 sec, 72°C extension for 1 min 30 sec, and a final 5 min extension at 72°C (King and Julian, 2004). DNA fragments were measured using an Applied Biosystems 310 Automated DNA Sequencer and the programs Genescan Analysis 3.1.2 (Applied Biosystems, Foster City, CA) and Genotyper 2.0 Software (Applied Biosystems, Foster City, CA).

ARLEQUIN (Excoffier et al., 2005) was used to test for deviations from Hardy-Weinberg equilibrium, linkage disequilibrium, population differentiation, estimate allelic diversity (A), observed heterozygosity (H_o), and expected heterozygosity (H_e) at each locus. Bonferroni corrections were applied to deviations from Hardy-Weinberg equilibrium and assessments of linkage disequilibrium. We compared the number of alleles per locus at each site after adjusting for sample size by resampling the larger sample size down to the smaller sample size 1,000 times using the program Resampling Stats (http://www.resample.com/). Genetic differentiation was quantified using F_{ST} (Wright, 1965) as estimated in ARLEQUIN. STRUCTURE (Pritchard et al., 2000), a Bayesian based clustering program, was used to determine if population structure was present. STRUCTURE does not require the use of predefined population boundaries and creates genetic populations by grouping individuals into a predefined number of populations (K) to minimize deviations from Hardy-Weinberg equilibrium and linkage equilibrium. We evaluated the turtles for K = 1–6 using a burn-in period of 100,000 iterations of the Markov chain process followed by a run of 100,000 iterations; this process was repeated ten times at each K.

Recent gene flow was estimated using an assignment test (Paetkau et al., 1997) as implemented in GENECLASS2 (Piry et al., 2004). We used the likelihood computation

$$L = \frac{L_{\text{home}}}{L_{\text{max}}},$$

where L_{home} is the likelihood of the individual arising in the population where it was sampled and L_{max} is the maximum likelihood associated with the individual arising in any population including the population from which the individual was sampled. We used a frequency of 0.01 for missing alleles (Paetkau et al., 2004) and a re-sampling algorithm with 1,000 as the minimum number of simulated individuals, with a Type 1 error value of $\alpha = 0.01$. The effective population size for each area was estimated by

calculating the evolutionary effective population size using the step-wise mutation model of Ohta and Kimura (1973)

$$N_e = \left(\frac{1}{8\mu}\right)\left\{\left(\frac{1}{1-H}\right)^2 - 1\right\},$$

where H is the average heterozygosity across all loci and μ is the microsatellite mutation rate (5×10^{-4}; Garza and Williamson, 2001).

The programs BOTTLENECK (Luikart and Cornuet, 1996; http://www.ensam.inra.fr/URLB) and M-RATIO (Garza and Williamson, 2001) were used to determine if the turtles experienced a recent bottleneck. BOTTLENECK calculates the heterozygosity from the observed number of alleles at each locus assuming mutation-drift equilibrium. In a bottleneck, alleles are lost more rapidly than heterozygosity. If the observed heterozygosity is greater than the heterozygosity expected at drift-mutation equilibrium, a bottleneck has occurred (Cornuet and Luikart, 1996). We ran 1,000 iterations using the two-phased mutation model with a 90% proportion of single step mutations (Garza and Williamson, 2001) and a variance of 12 (Piry et al., 1999).

We also used the M-ratio to determine if a bottleneck has occurred. M-ratio concludes that a bottleneck occurred if a low percentage of allelic states are filled at each microsatellite locus. Specifically, the M-ratio compares the number of alleles (k) to the range of alleles (r) found at a locus (Garza and Williamson, 2001). The M P Val and Critical M (available at http://swfsc.noaa.gov/textblock.aspx?Division=FED&id=3298) were used to estimate the M-ratio and determine significance respectively. The M P Val calculates the M value based on $\theta = 4N_e\mu$, P_S (percentage of one-step mutations), and Δ_g (mean size of non one-step mutations; Garza and Williamson, 2001). We calculated the M value for each site and for all the sites combined using values (P_S = 0.90, $\Delta_g = 3.5$, and $\mu = 5 \times 10^{-4}$) recommended by Garza and Williamson (2001).

RESULTS

We captured a total of 163 turtles (Kalamazoo n = 53, Van Buren n = 70, and Cass n = 40) including 23 juveniles (<10 growth rings per plastral scute). No significant deviations from Hardy-Weinberg equilibrium (Bonferroni corrected α = 0.002; all $P > 0.012$) or linkage disequilibrium (Bonferroni corrected α = 0.006; all $P > 0.056$) were found at any loci following Bonferroni correction. While there were no significant differences in allelic diversity (Fisher's Exact Test, all $P > 0.10$) or heterozygosity (Kurskal-Wallis P = 0.69; Table 1), the three sites were found to have significant genetic differentiation between all pairs (all $P < 0.001$). All pair-wise F_{ST} values were low, but significant (all $P < 0.001$), and the F_{ST} estimates increased with distance between sites (Table 2). STRUCTURE showed no evidence of population substructure, with the most probable number of populations for the observed genotypes being one (P = 1.00). GENECLASS2 revealed an 11% dispersal rate between the three sites with a total of 18 individuals mis-assigned (all $P < 0.05$; Table 3).

All three sites had large effective population sizes (Kalamazoo N_e = 9,516; Cass N_e = 8,401; Van Buren N_e = 6,675). Despite this, BOTTLENECK found that both Kalamazoo and Van Buren had significant deviations from drift-mutation equilibrium due to excess heterozygosity ($P <$ 0.01, $P < 0.04$, respectively); Cass County showed no significant deviations from drift-mutation equilibrium (P = 0.125). When we combined all three sites, a significant excess of heterozygosity was found ($P < 0.02$). Neither the individual sites nor the combined sites showed a shift in the modal allele frequency distribution away from the rarest allele class (Fig. 2). The M-ratios were not greater than expected at any of our sites singly (Kalamazoo: M = 0.92, P = 0.99; Van Buren: M = 0.92, P = 0.98; Cass: M = 0.83, P = 0.85) or when all three sites were combined (M = 0.92, P = 0.98).

DISCUSSION

Our results indicated that sites of *Terrapene c. carolina* occurrence in southwestern Michigan have a high level of connectivity and a low level of genetic population structure despite being separated by up to 60 km of highly fragmented habitat. The high level of connectivity we found was unexpected because the long distances and high road density between sites make it unlikely that a turtle could successfully migrate between our study locations. Even though transient individuals are found in populations of *Terrapene* (Kiester et al., 1982; Williams and Parker, 1987; Schwartz and Schwartz, 1991), and individuals have been recorded traveling up to 10 km (Dodd, 2001), the distances and fragmentation between our sites make it unlikely that these sites are directly exchanging migrants. It is more likely that the current dispersal found by the assignment test is due to unidentified intermediate populations (Slatkin, 2005) giving the appearance of direct migration (Howeth et al., 2008). While we did not search for turtles outside of our three sites, it is likely that turtle populations exist between our sites. Additionally, the long generation time of *Terrapene c. carolina* also likely contributes to the observed connectivity. Given the time period when the majority of these roads were constructed, these sites have been separated for only 50–75 years, which is equivalent to two to five generations of *Terrapene c. carolina*. The short number of generations may not be enough time to observe significant genetic changes.

High levels of connectivity between populations separated by great distances of highly fragmented habitat also have been found in other species with long generation times. Kuo and Janzen (2004) reported an F_{ST} of 0.099 between populations of Ornate Box Turtle (*Terrapene ornata*) in Nebraska and Illinois. Cunningham et al. (2002) found low F_{ST} values ranging from 0.018–0.030 between three populations of Geometric Tortoise (*Psammobates geometricus*) located approximately 40 km apart in the West Cape Province in South Africa. A Φ value (an estimate of population structure based on segregating polymorphisms; Tajima, 1989) of 0.037 was found between nine southern populations of Arizona Desert Tortoise (*Gopherus agassizii*) ranging from 16–186 km apart (Edwards et al., 2004). These results suggest that our findings are more likely to represent the levels of historic population structure and gene flow prior to habitat fragmentation (Richtsmeier et al., 2008). If this is the case, then our Michigan sites were not isolated before fragmentation occurred, and the genetic effects of fragmentation have not yet come into effect.

Generation times of *Terrapene c. carolina* likely also are responsible for the large N_e estimates, being more reflective of the past, before habitat fragmentation occurred. Thus, the high N_e estimates are similar because they each represent an independent sample from a larger population of *Terrapene c.*

Table 1. Allelic Diversity (A), Observed Heterozygosity (H_O), and Expected Heterozygosity (H_e) for *Terrapene c. carolina* from Three Counties in Michigan.

	Kalamazoo			Van Buren			Cass		
Locus	A	H_O	H_e	A	H_O	H_e	A	H_O	H_e
GMUD08	6	0.72	0.74	6	0.73	0.72	5	0.78	0.72
GMUD16	19	0.89	0.93	20	0.83	0.93	18	0.88	0.90
GMUA18	34	0.79	0.96	38	0.69	0.96	30	0.73	0.96
GMUD40	16	0.87	0.92	17	0.89	0.92	13	0.98	0.91
GMUD55	11	0.87	0.88	14	0.89	0.89	12	0.78	0.88
GMUD87	16	0.85	0.91	18	0.94	0.91	13	0.83	0.88
GMUD88	13	0.85	0.87	13	0.77	0.88	14	0.80	0.89
GMUD121	13	0.91	0.90	12	0.77	0.86	14	0.85	0.89

carolina, as shown by our STRUCTURE results, and are applicable to a larger geographic area than our study area alone. Our N_e estimates also are based on an evolutionary time frame which may be orders of magnitude greater than the current N_e (Michel et al., 2006).

Our bottleneck results are ambiguous and do not provide sufficient evidence to conclude that the *Terrapene c. carolina* in Michigan have undergone a genetic bottleneck, an unexpected result given the known population decline. Our results suggest that these turtles are in the early stages of a genetic bottleneck and, given the long generation time of *Terrapene c. carolina*, the genetic changes have not reached a detectable level yet. The discrepancy in the results from BOTTLENECK and the M-ratio test is due to the differences in how each program tests for the presence of a bottleneck. Both programs are based on the loss of alleles, but only the M-ratio is influenced by where the lost alleles occurred in the distribution of allele sizes. For example, if the rare alleles are more common on the extremes of a range and a bottleneck occurs, the result would be a decrease in both the range of alleles and the number of alleles per locus. Therefore, the M-ratio would still be large, even though a bottleneck has taken place. However, the observed levels of heterozygosity would still be high relative to the number of alleles found at those loci and BOTTLENECK would indicate that a bottleneck occurred.

Even though BOTTLENECK found that *Terrapene c. carolina* at two of the three sites had been bottlenecked, and the turtles at the three sites combined displayed a bottleneck, a mode-shift in allele frequencies, which would be expected in a population that has undergone a bottleneck, did not occur. We probably failed to observe a mode-shift in this system because the population reduction was not severe, or the bottleneck was too recent to detect. Luikart et al. (1998) indicated it may take 5–10N_e generations for bottlenecks to create a mode-shift and, assuming a generation time of 20 years, it would take 100–200 years for *Terrapene c. carolina* to display a mode-shift in their allele frequencies.

Despite the lack of definitive evidence of a genetic bottleneck in Michigan's *Terrapene c. carolina*, the population decline is not under question. The genetic implications of these demographic effects are unknown given the large time scale differences between population declines and the loss of genetic variation in species with long generation times. Population declines occur over years, whereas genetic variation is lost over generations. Thus, while we have seen a decline in the number of *Terrapene c. carolina* in Michigan, an additional 100 or more years may be needed to detect the genetic effects of this decline. The scenario of the maintenance of high genetic diversity in species with long generation times despite severe population declines has been found by others (Ciofi et al., 2002; Cunningham et al., 2002; Kuo and Janzen, 2004). Even though our study did not indicate any negative effects of habitat fragmentation on the population genetics of *Terrapene c. carolina*, it seems certain that the high road density in Michigan will eventually cause *Terrapene c. carolina* to display reduced genetic variation resulting from decreased dispersal and reduced population size.

The delay in loss of genetic variation relative to the decline in population size in long-lived species provides a unique conservation opportunity in species with long lives. While the decline in numbers is unambiguous, the long life span of *Terrapene c. carolina* allows managers more time to offset the negative genetic effects of a bottleneck. In essence, the bottleneck is occurring in slow motion relative to humans, providing managers an opportunity to have conservation biology not be a crisis science in species with long generations.

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Table 2. Population Pair-wise F_{ST} Values (below Diagonal) and Geographic Distances in km (above Diagonal) for Three Populations of Eastern Box Turtles (*Terrapene carolina*) in Michigan.

	Kalamazoo	Van Buren	Cass
Kalamazoo	—	38	63
Van Buren	0.007	—	24
Cass	0.008	0.006	—

Table 3. Assignment Test Results for Three Populations of Eastern Box Turtles (*Terrapene carolina*) in Michigan. The rows indicate sites of capture and the columns indicate sites to which the animals were assigned.

	Kalamazoo	Van Buren	Cass
Kalamazoo	38	8	0
Van Buren	3	64	3
Cass	0	4	36

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