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GENETIC STRUCTURING OF GOPHER TORTOISE (*GOPHERUS POLYPHEMUS*) POPULATIONS ON THE KENNEDY SPACE CENTER, FLORIDA, USA

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***Abstract.*—Gopher Tortoises (*Gopherus polyphemus*) located on the Kennedy Space Center (KSC) in east-central Florida have been the subject of a long-term population study but little is known about the genetic structure of the population. Other studies across the species' range have shown evidence that Gopher Tortoises have undergone population bottlenecks due to geographic isolation. We assessed the genetic diversity of Gopher Tortoises from five locations on KSC at six microsatellite loci in 96 individuals. Based on our analysis, the population does not subdivide into genetically distinct assemblages but appears to be one nearly continuous population (F_{ST} = 0.030). This suggests that natural movement and/or human relocation has maintained the population's gene flow across sites on the KSC. In terms of population genetics, we recommend that the tortoises on KSC be managed as a single population until further genetic analyses are completed.**

Key Words.—conservation; *Gopherus polyphemus;* Gopher Tortoise; management; population genetics

INTRODUCTION

Knowledge of the genetic structure of imperiled species may be essential to making informed management decisions (Allendorf and Luikart 2007; King 2009). This is especially true of populations with relatively limited ability for dispersal or for whom anthropogenic barriers such as roads may be difficult to cross. Population isolation can lead to a loss of genetic variation through the alteration of gene frequencies (Noss et al. 2006). On a larger scale a severe reduction in genetic variation can reduce a population's capacity to adapt to changing environmental conditions, make the individuals more susceptible to disease and in turn lead to disruption of the ecosystem (King 2009). Therefore, preservation of genetic variation in an imperiled species should be a priority of any management plan.

Gopher Tortoises (*Gopherus polyphemus*) are one of four native tortoise species in the United States and the only species found east of the Mississippi River (Ernst et al. 1994). Widely considered a keystone species in Florida and the southern reaches of Mississippi, Alabama, Georgia, South Carolina, and Louisiana (Auffenberg and Franz 1982; Kushlan and Mazzotti 1984; Diemer 1986, 1992; Breininger et al. 1994), they are threatened by habitat loss due to increased urban land use, agriculture, and phosphate/heavy metals mining (Auffenberg and Franz 1982; McCoy and Mushinsky 2005; Berish 2001). Gopher Tortoises in Florida experienced an 88% decline in longleaf pine forest habitat between 1936 and 1987 (Noss 1989; Kautz 1993). As a result of these threats, it is estimated that tortoise populations have declined by more than 80% since the 1880's (Auffenberg and Franz 1982).

With the rapid decline of suitable habitat due to increased construction and agriculture throughout the southeastern United States, Gopher Tortoises are protected at the international, federal, and state level. *Gopherus polyphemus* is listed as an Appendix II species under the Convention on International Trade in Endangered Species (Ipskipp and Gillett 2003) and tortoises west of the Tombigbee and Mobile Rivers are listed as threatened by the Endangered Species Act (U.S. Fish and Wildlife 1986). At the state level, Gopher Tortoises are listed as threatened in Georgia, Florida, and Louisiana, endangered in South Carolina and Mississippi, and protected as a non-game species in Alabama, USA.

In an attempt to protect tortoises in the pathway of construction, many individuals are relocated to new habitats. In Florida alone, > 25,000 tortoises were permitted for relocation in the 1990's (Enge et al. 2002). The actual number of relocations is probably much higher when considering the number of unpermitted relocations. However, until recently, the relocations were made with little or no regard to the genotype of the tortoises and the possible genetic impact on the population at their new home site. Schwartz and Karl (2005) used microsatellites to assess the genetic diversity of 300 Gopher Tortoises from locations across Florida and southern Georgia. They identified eight genetic subpopulations and evidence that several groups had undergone population bottlenecks due to isolation. Genetic variation in four groups from the Middle Florida

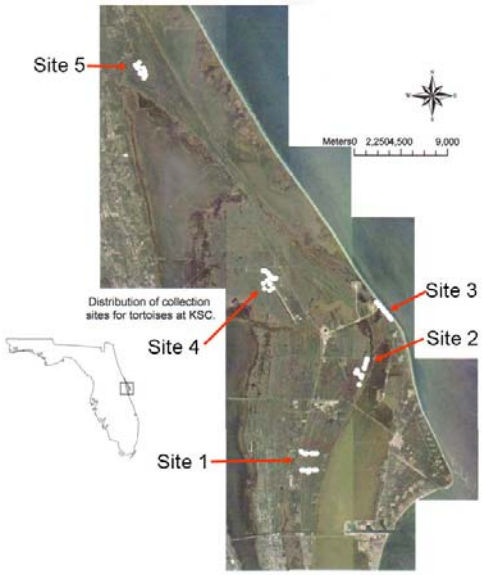

FIGURE 1. Location of Gopher Tortoises (*Gopherus polyphemus*) sampled from Kennedy Space Center, Florida, USA.

assemblage (EA-USF Eco Area, Hillsborough Co. [EA], Oldenburg Mitigation Park, Hernando Co. [OM], Lake Louisa State Park, Lake Co. [LL], and Wekiwa Springs State Park, Orange Co. [WS]) suggested that several individuals had been introduced into the populations, most likely through human intervention.

A primary management concern for Gopher Tortoises is to prevent complete isolation of populations by facilitating gene flow through well-conceived relocations while working to maintain the unique identity of the subpopulations that currently exist. However, this requires additional data on the genetic structure and diversity of extant, natural populations, information which is very limited.

The Kennedy Space Center (KSC) is a 56,000 ha protected site on the east coast of Florida. KSC is managed jointly by the National Aeronautics and Space Administration (NASA), the U.S. Fish and Wildlife Service, and the National Park Service. Gopher Tortoises on KSC have been the focus of a long-term population study and are thought to exist in very large numbers (Smith et al. 1997; Pike et al. 2005), but the genetic structure of the population has not been analyzed. These tortoises have largely been protected from habitat loss and have been, due to the security restrictions of the site, protected from human collecting for at least 50 years. During past construction on the site, Gopher Tortoises have been relocated on KSC, usually for short distances (Rebecca Bolt, pers. comm.). Manmade structures such as roadways and railroad tracks may lessen/inhibit gene flow by restricting movement, fragmenting populations and increasing mortality through vehicle strikes and predation. Baldwin et al. (2004) reported that roads separating female Painted Turtles (*Chrysemys picta*) from their preferred nesting sites resulted in higher mortality rates as the animals attempted to cross the road and lower survival for the eggs/hatchlings due to increased predation. Boarman et al. (1997) and Guyot and Clobert (1997) reported that mortality of Desert Tortoise (*Gopherus agassizii*) and Hermann's Tortoise (*Testudo hermanni*) populations, respectively, along roadways declined when barrier fences and culverts/tunnels were constructed. The KSC site has roadways and railroad tracks in several locations and these structures may act as barriers to gene flow between the collection sites.

In this study, we analyzed the genetic diversity of Gopher Tortoises located on KSC to determine if the tortoises are genetically structured into distinct populations or if they form one genetic population. A second goal of the study was to determine if manmade barriers (e.g., roads, canals), hindered gene flow among sites.

TABLE 1. Intrapopulation genetic diversity at six microsatellite loci for five *Gopherus polyphemus* sampling sites from the Kennedy Space Center, Florida, USA. For each population we provide the sample size (N), average number of alleles (N_a), the observed heterozygosity (H_o), the expected heterozygosity (H_e), F_{IS} which indicates deviation from random mating, and allelic richness (R_S). *Indicates significant values ($p < 0.05$).

Population	N	N_a	H_o	H_e	F_{IS}	R_S
Site1	20	3.5[1]	0.468	0.515	0.0999	3.302
Site2	24	4.5	0.431	0.541	0.2244*	3.912
Site3	13	3.5	0.333	0.449	0.2941*	3.500
Site4	18	3.8	0.445	0.476	0.0943	3.693
Site5	21	4.5[1]	0.409	0.511	0.2166*	4.084

[1] Locus GP96 had only 1 allele present among the individuals analyzed in these populations.

MATERIALS AND METHODS

Sample collection.—We collected 96 tortoises from five areas adjacent to roadways on KSC (Fig. 1). The five areas were separated by distances of 7 km to over 45 km and sample sizes ranged from 13 to 24 per site (Table 1). We recorded GPS positions and physical measurements for each individual including body mass, straight midline carapace length (SCL), maximum plastron length (MPL), midline plastron length (MinPL), and gender. We permanently marked each individual by drilling through the scute with a 3.2 mm drill bit. We captured drill shavings in filter paper and placed samples in 50 ml sterile tubes for storage and transport for DNA extraction (Dawes et al. 2008). We remarked individuals that had been previously marked in the same pattern with a larger drill bit and we recorded the identification number of the animal. Individuals were used only once in this study.

DNA extraction and amplification.—We extracted DNA using the protocol described by Dawes et al. (2008). Briefly, we froze drill bit shavings in liquid nitrogen and then pulverized samples between two flat ended bolts. We incubated the powdered shell material for 3–5 d in 0.5 M ethylenediaminetetraacetic acid pH 8.0 (EDTA) at 37°C, while shaking at 225 rpm to decalcify. Following decalcification, we incubated the samples in buffer ATL from the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Valencia, California, USA) with Proteinase K at 56ºC, and agitated at 225 rpm for 2 d until lysed. The purified DNA was eluted following the manufacturer's protocol.

We obtained individual genotypes using six polymorphic microsatellite loci (Schwartz et al. 2003). We conducted polymerase chain reaction (PCR) in 15 µl volumes under the following conditions: 1.5 µl 10X PCR buffer with 15 mM $MgCl_2$ (Gene Choice, Continental Lab Products, San Diego, California, USA), 0.2 mM dNTPs (Roche, Indianapolis, Indiana, USA), 0.2 µm of each primer (GP15, GP26, GP30, GP61, GP81, GP96) (Schwartz et al. 2003), 1.25 units of *Taq* polymerase (Gene Choice) and 30 ng of template DNA. The PCR thermocycling program was 94ºC for 3 min, 35 cycles of 94ºC for 30 s, 60ºC for 30 s, and 72ºC for 45 s followed by a final extension of 72ºC for 5 min. We verified PCR products by gel electrophoresis on a 1.5% agarose gel with ethidium bromide. We labeled the forward primer for each locus with a fluorescent dye (Sigma Proligo, St. Louis, Missouri, USA) to facilitate fragment analysis on a CEQ8000 Genetic Analysis System (Beckman Coulter, Fullerton, California, USA).

Statistical analysis.—We calculated the number of alleles (N_a), expected heterozygosity (H_e), observed heterozygosity (H_o), and allelic richness (R_s) for each population using GeneAlEx6 (Peakall and Smouse 2006). The presence of null alleles was tested for by Micro-Checker using the Oosterhout, Chakraborty, Brookfield 1 and Brookfield 2 methods of null allele estimation (Oosterhout et al. 2004). We used an analysis of molecular variance (AMOVA; Excoffier et al. 1992) to determine partitioning of genetic variation among the populations and among individuals within the populations from microsatellite allele frequencies according to Weir and Cockerham (1984) using Arlequin ver. 3.01 (Company, City, State, USA; Excoffier et al. 2005). We calculated global and pairwise F_{ST} (Weir and Cockerham 1984) values using FSTAT v2.9.3 (Goudet 2001) to measure population differentiation. Corrected F_{ST} values were calculated using FreeNA (Company, City, State, USA) with 50,000 replicates for calculation of the 95% confidence interval (Chapuis and Estoup 2007). We calculated Wright's F_{IS} for each population using the Weir and Cockerham estimator θ to test for population-level deviations from Hardy-Weinberg equilibrium (Weir and Cockerham 1984) in Genetix v4.05 (Belkhir et al. 1996–2004). We tested significance of F_{IS} with 1,000 permutations. We tested population structure using Structure v2.3.3 Pritchard et al. 2000; Hubisz et al. 2009) with a burnin of 5,000 and 50,000 MCMC reps after burnin, admixture model with independent allele frequencies and parameters of k = 2 to 10 with three iterations at each k value. We also analyzed population structure with Bayesian analysis using Partition with 10,000 observations of the Markov chain, k = 2 to 10 and a burnin of 1,000 (Belkhir and Dawson 2001). Two samples (one each from Site 1 and Site 5) were removed from the Partition analysis due to missing allele data. We analyzed individual genotypes for population assignment (Paetkau 1995) using Doh (Brzustowski 2008) and GeneClass2 (Piry et al. 2004). We tested isolation by distance using a Mantel test of the association between genetic distance and the logarithm of geographic distance in the IBD (Isolation by Distance) program (Bohonak 2002). We tested genetic distance as $F_{ST}/(1-F_{ST})$ and geographic distances (km) were based on GPS coordinates. The Mantel test was based on 10,000 permutations of spatial locations among the sample populations. We tested recent reductions in effective population size using Bottleneck 1.2.02 (Cornuet and Luikart 1997).

RESULTS

Levels of polymorphism for the tortoises at each collection site (hereafter referred to as populations) varied, with the average number of alleles ranging from 3.6 to 5.2 (Table 1). Site 1 and Site 5 populations had only one allele present for locus GP96. Allelic richness (R_S), which accounts for sample size bias, showed little difference among the five populations with values ranging from 3.3 to 4.1. Average observed (H_o) and expected (H_e) heterozygosities ranged from 0.35 to 0.47 and 0.45 to 0.54, respectively. Three of the five populations had significantly positive multilocus F_{IS} values indicating overall heterozygote deficiency. Micro-checker results suggested the presence of null alleles for loci GP15 and GP30; however there was no evidence of large allelic dropout in the dataset for either locus. Analysis by Bottleneck using the Wilcoxon test suggests that the population at Site 1 may have undergone a bottleneck in the past.

The estimated global F_{ST} value was statistically significant at 0.0300. AMOVA found that only 3.0% of the variation occurred among populations. Pairwise analysis of F_{ST} estimates between the five populations (Table 2) resulted in values ranging from 0.0046 to 0.0813 and corrected values ranged from 0.0058 to 0.0695. Uncorrected F_{ST} values were significant between populations at Sites 1 and 3 and between Sites 4 and 5.

TABLE 2. Pairwise F_{ST} estimates of *Gopherus polyphemus* populations based on six microsatellite loci. Bold values indicate significant differences at $p < 0.05$. Corrected values calculated with FreeNA software are given below the diagonal and uncorrected values calculated with FSTAT are given above the diagonal.

	Site1	Site2	Site3	Site4	Site5
Site1		0.0046	**0.0751**	**0.0467**	0.0089
Site2	0.0058		0.0081	0.0234	0.0203
Site3	0.0537	0.0063		0.0305	**0.0797**
Site4	0.0448	0.0213	0.0304		0.0331
Site5	0.0111	0.0201	0.0695	0.0344	

TABLE 3. Population assignment of *Gopherus polyphemus* individuals by Doh (first number) and by GeneClass2 (second number) software based on genetic background from six microsatellite loci. Bold values indicate the animals assigned to the population from which they were sampled.

	Site1	Site2	Site3	Site4	Site5	Total
Site1	**11/8**	4/6	2/2	1/0	2/4	20
Site2	7/8	**2/10**	10/2	2/0	3/4	24
Site3	2/2	3/6	**5/3**	2/1	1/1	13
Site4	2/2	1/6	5/0	**9/3**	1/7	18
Site5	5/6	3/1	0/0	4/3	**9/11**	21

Analysis by Structure and Partition revealed no genetic partitioning among the populations (data not shown). The probability that all samples formed one continuous population was 97.3% in Partition. Mantel test results suggest there is no genetic partitioning due to distance (data not shown). Assignment tests in GeneClass2 and Doh software assigned 35 and 36, respectively, out of 96 tortoises as native to their collection site (Table 3).

DISCUSSION

We analyzed the genetic background of Gopher Tortoises located on a large protected habitat to determine if the tortoises form genetically distinct populations or a single continuous population. Analysis of six microsatellite loci showed no partitioning of genetic variation between the five collection site populations. The global F_{ST} estimate among the five populations was very low, which suggests a lack of genetic partitioning and that gene flow is or has recently occurred among the five populations. These F_{ST} estimates were similar to those seen in studies of Geometric Tortoises (*Psammobates geometricus*) $F_{ST} \geq 0.042$ (Cunningham et al. 2002), Desert Tortoises $F_{ST} = 0.037$ (Edwards et al. 2004), Ornate Box Turtles (*Terrapene ornata*) $F_{ST} = 0.099$ (Kuo and Janzen 2004), and other populations of Gopher Tortoises $F_{ST} = 0.023$ (Schwartz and Karl 2005). It is important to note that our collection sites were 7–45 km apart and those in the studies mentioned above ranged from 15-90 km (Cunningham et al. 2002) to over 850 km (Kuo and Janzen 2004). The smaller distances could facilitate gene flow among the sites thereby minimizing inbreeding. However, the low F_{ST} values seen in the studies above with more distant sites suggest that terrestrial chelonians may tend to have weak genetic structure overall.

Schwartz and Karl (2005) reported pairwise FST values of 0.060 to 0.424 for the populations sampled across the Florida peninsula. Our pairwise values are much lower, 0.0058 to 0.0695 (corrected FST). Further comparison of the KSC population to the assemblages reported by Schwartz and Karl (2005) may show that the KSC population is part of one of these assemblages or that it is indeed isolated and forms a unique assemblage.

It is important to note that Schwartz and Karl (2005) used nine microsatellite loci in their analysis and although we used only six of the nine, we selected the most polymorphic loci (Schwartz et al. 2003). The low levels of genetic structuring found at KSC are consistent with three non-exclusive explanations. First, despite distance and manmade barriers to dispersal (e.g., roads, large canals), gene flow may be occurring among locations, especially because tortoises at KSC are known to use multiple seasonal habitats (Smith et al. 1997). Secondly, low F_{ST} levels may reflect the fact that human barriers to dispersal have occurred only within the past 50 (paved roads) to 120 years (manmade canals), which represents only a few generations of isolation for long-lived species such as tortoises. In addition, while mortality from road traffic and entrapment on railroad tracks occurs at KSC, it does not appear to be sufficient to prevent gene flow from occurring. Third, humans may be enhancing dispersal via translocation both by federal resource managers and by local citizens seeking to release pet tortoises on a protected habitat. Although the number of individuals needed to maintain gene flow may be more than the oft-cited one per generation (see discussion in King 2009), the large number of tortoises at KSC, coupled with the human propensity to move these animals from roadsides where they appear to be in jeopardy, could easily result in human-aided dispersal at this site. Here we report the lack of genetic partitioning and the low level of genetic variation present in the Gopher Tortoise populations on KSC. From the perspective of population genetics, we suggest that the resource managers at KSC manage Gopher Tortoises on the various federal subdivisions of the site as one population pending additional genetic analysis.

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PATRICK J. DAWES was a master student at Towson University when he conducted this research. (Photographed by Colleen Sinclair).

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