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Line Transect Distance Sampling and Genetic Analyses Reveal a Small but Genetically Diverse Coastal Gopher Tortoise (*Gopherus polyphemus*) Population

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Abstract.—**Gopher Tortoises inhabit coastal systems, including barrier islands, across the southeastern U.S. St. Vincent National Wildlife Refuge is an uninhabited barrier island located off the coast of northwestern Florida. Although tortoises have been observed on the island, no information is available on the status of the population. We conducted a line transect distance sampling survey to evaluate the Gopher Tortoise population on St. Vincent Island. Additionally, we collected samples for genetic analyses from 11 individual tortoises captured opportunistically and via bucket traps on the island and 15 tortoises captured at nearby mainland sites. Surveys covered approximately 43% of the sampling frame and resulted in 55 burrows, 28 of which were occupied. The abundance estimate for the island was 52 tortoises (95% confidence interval [CI] = 27–100) and the density was relatively low at 0.071 tortoises/ha (95% CI = 0.037–0.136). Genetic analyses of two mtDNA markers identified a new haplotype unique to St. Vincent Island and another three haplotypes previously found across the southeastern U.S. The genetic composition of Gopher Tortoises on St. Vincent Island is representative of the entire southeastern U.S. but most closely aligns with tortoise populations east of the Apalachicola-Chattahoochee River system. Although the tortoise population on this island is small, the extent of seemingly appropriate habitat on the island and the genetic diversity of the population suggests the potential for growth with added management intervention.**

Key Words.— Apalachicola-Chattahoochee River system; conservation genetics; island colonization; reptile; riverine barrier to gene flow

Introduction

The Gopher Tortoise (*Gopherus polyphemus*) is one of the most studied species in the southeastern U.S; however, most research on this species has been conducted on populations in inland Upland Pine Forest habitats (Mushinsky and McCoy 1994; Dodd and Griffey 2002; McCoy et al. 2002; Goessling et al. 2021). Less is known about tortoise populations inhabiting coastal habitats (Waddle et al. 2006; Lau and Dodd 2015; Sinclair et al. 2010), particularly their population genetics (Winters et al. 2017). In fact, the most recent range-wide examination of genetic diversity and structure in Gopher Tortoise populations (Gaillard et al. 2017) included 47 sites, of which only seven were coastal sites, and none of which were located along the northern Gulf of America (known internationally as the Gulf of Mexico; hereafter referred to at the Gulf). Tortoises inhabiting coastal systems experience different habitat characteristics and threats than populations using inland systems. For example, habitats in coastal systems are often narrow and linear, which restricts available areas for burrows, while adjacent areas may be unsuitable for tortoises (Lau and Dodd 2015). Additionally, tortoise populations on barrier islands face isolation. Gopher Tortoises, like many turtle species, are particularly sensitive to population isolation because they have low annual reproductive potential, slow growth rates, specialized habitat requirements, and low dispersal

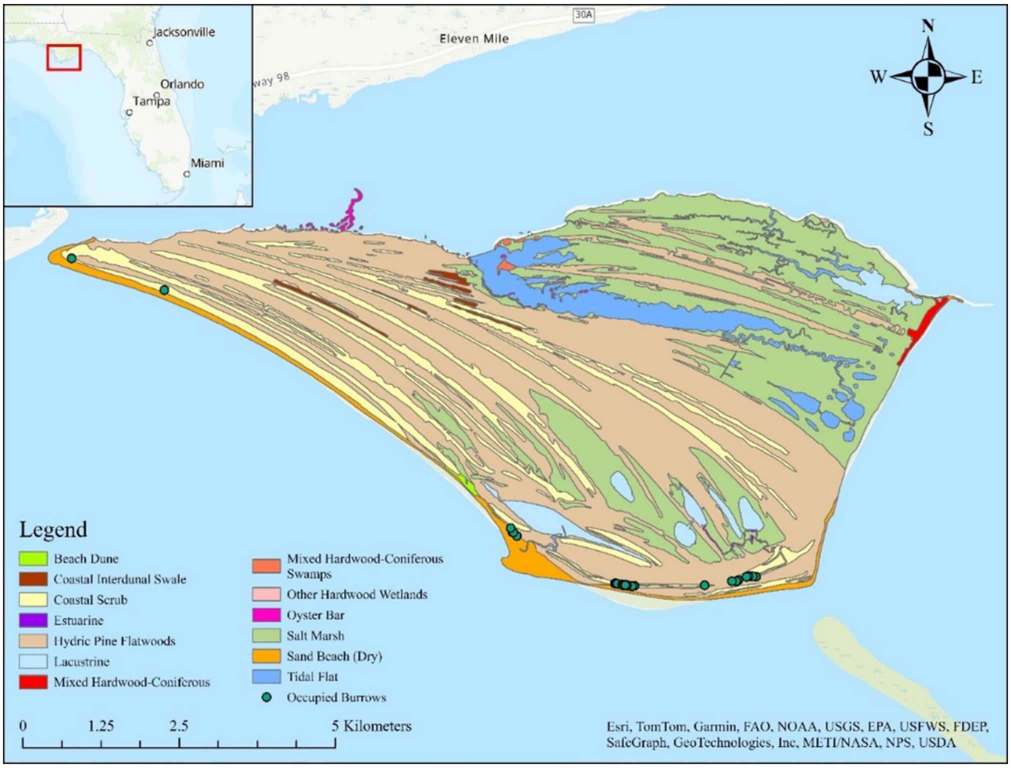

Figure 1. St. Vincent Island, Florida**, USA** (red rectangle on inset map), showing locations of occupied Gopher Tortoise **(***Gopherus polyphemus*) burrows and habitat types based on the Florida Cooperative Land Cover map (https://www.fnai.org/).

ability (Eubanks et al. 2003; Ernst and Lovich 2009; Ennen et al. 2011). Island populations may be distinct from mainland populations and experience a higher degree of inbreeding and reduction in genetic diversity due to reduced gene flow and amplified effects of drift and local adaptation, with consequences to their long-term health and stability (Saccheri et al. 1998; Crnokrak and Roff 1999; Hedrick and Kalinowski 2000; Nieminen et al. 2001; Whiteley et al. 2010).

Population declines due to habitat fragmentation, poor hatching success (Noel et al. 2012), and low recruitment (Epperson and Heise 2003) led to the Federal listing of Gopher Tortoise populations west of the Mobile and Tombigbee rivers in Alabama as Threatened under the U.S. Endangered Species Act (hereafter referred to as western populations; U.S. Fish and Wildlife Service 1986). Populations east of those rivers (hereafter referred to as eastern populations) are not federally listed (U.S. Fish and Wildlife Service 2022) but are state listed as Threatened in Georgia and Florida. The eastern populations have been divided into five genetically distinct regions (Clostio et al. 2012; Gaillard et al. 2017). An accurate assessment of tortoise populations located along the boundaries separating these regions will help managers enact measures to benefit these populations (Tuberville et al. 2009).

St. Vincent Island, part of the St. Vincent National Wildlife Refuge, is located immediately west of the mouth of the Apalachicola River in northwestern Florida and forms the western boundary of Apalachicola Bay. It is one of the largest barrier islands along the northern Gulf coast (14.5 km long and 6.5 km wide; Fig. 1). This approximately 5,000-ha barrier island has been uninhabited since 1968 and includes varied habitats, with dune ridges, freshwater lakes and sloughs on the east end, and dry upland pine forests on the west end (U.S. Fish and Wildlife Service 2012). Although Gopher Tortoises have been known to inhabit the island since 1979, no formal studies have been conducted to assess population size, evaluate genetic diversity, or to understand historical relationships with mainland populations, including the potential origin of this population (U.S. Fish and Wildlife Service 2012). Surveys have been conducted sporadically in areas known to support gopher tortoise populations; however, prior surveys only covered approximately 200 ha and were conducted haphazardly, rather than using standardized methods such as Line Transect Distance Sampling (LTDS; Thomas et al. 2010). Opportunistic sightings have confirmed tortoise nesting on St. Vincent Island (U.S.

Figure 2. The sampling frame for Gopher Tortoise (*Gopherus polyphemus*) surveys using line transect distance sampling on St. Vincent Island, Florida, USA, in 2019 and locations of occupied tortoise burrows. The sampling frame consisted of soils ranked as highly and moderately suitable for Gopher Tortoises (https://websoilsurvey.nrcs.usda.gov/app/).

Fish and Wildlife Service 2012).

Gopher tortoises are known to occur throughout the southeastern U.S. with two major river systems acting as barriers to gene flow: the Mobile-Tombigbee rivers and the Apalachicola-Chattahoochee River system (Osentoski and Lamb 1995; Clostio et al. 2012; Ennen et al. 2012; Gaillard et al. 2017). These large rivers, particularly the Apalachicola-Chattahoochee River system, represent distinct barriers to gene flow for many species (Avise 2000; Austin et al. 2004; Burbrink et al. 2008; Jackson and Austin 2009), including turtles (Avise 2000; Pauly et al. 2007; Clostio et al. 2012). The location of St. Vincent Island immediately (about 10 km) west of the mouth of the Apalachicola River (i.e., barrier to gene flow among tortoise populations) may influence the genetic composition of the tortoise population on the island. The island formed approximately 4,000 y ago and does not appear to have ever been connected to the mainland (see Stapor 1971; Otvos 1984; Kimble 2012), suggesting tortoises were never able to expand onto St. Vincent via land. Our objectives were to: (1) estimate the population size of gopher tortoises on St. Vincent Island to assess population viability; (2) inform management decisions; and (3) compare the haplotype diversity of this population to that of tortoises across the range of the species. To address these objectives, we conducted a LTDS survey in potential tortoise habitat on St. Vincent Island and sequenced mitochondrial DNA from tortoises on the island and at nearby mainland sites.

Materials and Methods

Survey design.—Following Smith et al. (2009a), we used soil type to identify suitable tortoise habitat in which to conduct our LTDS on St. Vincent Island. We used the Web Soil Survey of the U.S. Department of Agriculture to identify highly and moderately suitable soils (https://websoilsurvey.nrcs.usda.gov/app/) for Gopher Tortoise burrows to determine our survey sampling frame. The sampling frame included pine flatwoods, scrub, hardwood hammock, live oak hammock, beach dune, and coastal grasslands. The total area of the sampling frame was 734 ha (Fig. 2).

We used ArcGIS Pro (Esri, Redlands, California, USA) and program Distance (version 7.3; Thomas et al. 2010) to design the survey following Smith and Howze (2016). We used a systematic random survey design with transects (40 m width) running north to south that were perpendicular to known elevation gradients in the sampling frame. Transects varied widely in length and we spaced them 85 m apart. We used ArcGIS Collector (Esri) to collect location data for the actual transects walked, using a GPS unit (model TDC150; Trimble, Westminster, Colorado, USA) with sub-meter accuracy.

Surveys.—We conducted surveys in 2019, employing a three-observer method as described in Smith et al. (2009b) in which one observer navigated along the transect center line, while the other two observers surveyed parallel transects approximately 3–5 m on each side. We programmed the GPS unit with pre-determined transect lines, which we used to navigate the study transects. We excluded unsuitable habitats (e.g., wetlands, impassable vegetation) from the survey. We documented start and end locations of each transect, along with all tortoises and observed tortoise burrows, using the GPS. We used a video camera scope with an above-ground monitor to inspect burrows and determine occupancy, and we classified burrows as occupied, unoccupied, or undetermined.

Survey data analysis.—We processed survey data in ArcGIS Pro, using the NEAR tool to generate perpendicular distances between occupied tortoise burrows and the transect line from which the burrow was observed. We then imported the processed data into program Distance 7.3 (Thomas et al. 2010). We truncated data by removing the 5% of burrows farthest from transect center lines to remove outliers. We ran two models with a key function and series adjustment using burrow width as a covariate, including a half-normal key function with cosine series expansion and a hazard rate key function with cosine series expansion.

Tortoise captures.—After the LTDS was completed, we initiated capture efforts at known, occupied burrows on St. Vincent Island. We primarily used pitfall/bucket traps and opportunistic capture; however, we also deployed flap traps and cage traps (Enge et al. 2012). Trapping efforts occurred from March through September 2020 and 2021, which corresponds to the time period when tortoises are most active (Douglass and Layne 1978). Pitfall/bucket traps consisted of 5-gallon (about 19 L) plastic buckets with holes drilled in the bottom for drainage and filled with several inches of sand to provide cushion and minimize distance to bottom of the bucket. We buried the buckets in the apron of each occupied burrow until the tops of the buckets were level with the surrounding soil, covered them with a thin material such as newspaper or cloth, and then camouflaged them with surrounding soil. We also designed and deployed flap traps according to Enge et al. (2012). We deployed cage traps, so the opening was directly in front of the burrow opening. We checked each trap every 2 h from sunrise to sunset and then closed traps overnight by either securing a lid to the buckets, closing the cage traps, or removing the flap traps. We monitored traps on St. Vincent Island daily over 10 d in 2020 and 19 d in 2021.

We weighed and measured all captured tortoises and then inserted an 8 mm Passive Integrated Transponder (PIT; Biomark, Boise, Idaho, USA) tag in their left rear leg. Additionally, we notched tortoises on their marginal scutes using a modified pattern and methods described by Cagle (1939). Finally, we collected biological samples including blood, carapace cores, and/or toenails for genetic analyses. We collected blood samples from the brachial vein using a 22 gauge, 5/8” needle with a 3 m1 syringe, kept on ice while in the field, and then frozen for storage.

To compare genetic diversity and infer historical relationships of populations, including the origin of individuals on St. Vincent Island, we also collected samples from tortoises at nearby areas on the mainland. Using the same capture and sampling methods used on St. Vincent Island, we placed bucket traps at occupied burrows on Cape San Blas (n = 2) and Tyndall Air Force Base (n = 13; Fig. 1). Trapping effort occurred on Cape San Blas over 8 d in 2020 and 3 d in 2021, and on Tyndall Air Force Base over 5 d in 2020 and 11 d in 2021.

Genetic analyses.—We extracted DNA from 11 individuals from St. Vincent Island and 15 from the mainland sites (two samples from Cape San Blas and 13 from Tyndall Air Force Base), for a total of 26 samples. We used DNeasy Blood and Tissue kit (Qiagen, Inc., Valencia, California, USA) following the manufacturer instructions to extract DNA from blood samples. We amplified two mitochondrial DNA markers by PCR using the primers ND4G and ND4R (Ennen et al. 2012) for NADH dehydrogenase 4 (ND4) and primers CytbF2 and CRR3 for cytochrome b (Cytb; Clostio et al. 2012). All PCR was conducted with a total reaction volume of 25 µL comprising 12.5 µL of GoTaq Green Master Mix 2X (Promega, Madison, Wisconsin, USA; Cat. No. M7122), 1.0 µL

Table 1. Program Distance model output for Line Transect Distance Sampling for Gopher Tortoises (*Gopherus polyphemus*) on St. Vincent Island, Florida, USA with the selected model indicated by the asterisk (*). Heading abbreviations are AIC = Akaike's Information Criterion, K = number of parameters, D = density of individuals, LCL = 95% lower confidence limit, UCL = 95% upper confidence limit, CV = coefficient of variation, N = number of individuals, and GOF Chi-p = Goodness-of-fit Chi-square Test probability.

Model	ΔAIC	AIC	K	D	D LCL	D UCL	D CV	N	N LCL	N UCL	N CV	GOF Chi-p
Half-Normal Cosine*	0.00	189.11	2	0.071	0.037	0.136	0.342	52	27	100	0.342	0.207
Hazard Rate Cosine	1.44	190.55	3	0.070	0.037	0.131	0.329	51	27	96	0.329	0.231

of each 10µM primer, 1.5 µL of 20–30 ng/µL DNA template, 0.5 µL of 0.1% bovine serum albumin, and nuclease-free water to final volume. We performed PCR amplification conditions for ND4 and Cytb as reported in Ennen et al. (2012) and Clostio et al. (2012), respectively.

We checked PCR products on a 1% agarose gel, purified products using ExoSAP-IT (Applied Biosystems, Foster City, California, USA; Cat. No. 78200.200 UL) following manufacturer instructions and sent them for sequencing to the Keck DNA Sequencing Facility at Yale University. We sequenced samples using the forward primers for each marker, except in one case where we found a new haplotype (see Results). In this case, we re-amplified the sample and re-sequenced in both directions to confirm the polymorphic sites. We checked electropherograms by eye using FinchTV 1.4.0 (Geopiza, Inc; Seattle, Washington, USA) and used BioEdit (Hall 1999) to trim the sequence and make a separate alignment for each marker. Although ND4 and Cytb are both mitochondrial DNA markers and are linked, we used both markers in this study because the two previous genetic studies (Ennen et al. 2012; Clostio et al. 2012), including samples from the southeastern U.S., differed in which markers they used and in their geographic sampling. To compare haplotype diversity found on St. Vincent Island and place these haplotypes into a larger geographic context, we downloaded ND4 and Cytb sequences from previously published studies from the National Center for Biotechnology Information (NCBI) GenBank archive to include representative haplotypes from Florida, Georgia, Alabama, Mississippi, Louisiana, and South Carolina (Ennen et al. 2012; GenBank accession numbers JF298788–JF298800; Clostio et al. 2012; GenBank accession numbers JQ920348–JQ920353). We built haplotype networks and haplotype maps separately for each marker including our samples and the sequences downloaded from GenBank, and then with both markers together for our samples only using the TCS method (Clement et al. 2000) in PopART software (Leigh and Bryant 2015). We calculated haplotype diversity (h) and nucleotide (π) diversity in DnaSP (Rozas et al. 2017) for each marker for two locations: St. Vincent Island and Tyndall Air Force Base, as they had a sufficient number of samples for these analyses. While we excluded samples from Cape San Blas from the individual locality calculations, we included them in the overall across-location measure of diversity analyses (see Results section).

Results

The final LTDS resulted in 1,079 transects totaling 79.6 km in length and covered 318.4 ha, which was approximately 43% of the sampling frame (Fig. 2; U.S. Geological Survey data release, https://doi.org/10.5066/P13JYY4G). During the survey, we located 55 tortoise burrows. Of those, 28 were occupied, 22 were unoccupied, and five were undetermined. The occupied burrows were located in hydric pine flatwoods (48%), coastal scrub (44%), and sand beach (8%). The Distance sampling output for the two top competing models resulted in selection of the half-normal cosine model (Table 1). This model estimated an abundance of 52 tortoises (95% confidence interval [CI] = 27–100) with a density of 0.071 tortoises/ha (95% CI = 0.037–0.136; Table 1).

Tortoise captures.—On St. Vincent Island, 17 captures of 16 tortoises occurred during trapping efforts. We captured 10 of those tortoises in bucket traps while seven were opportunistically captured by hand while traversing the island during trapping efforts. Of those 16 tortoises captured on the island, eight (50%) were adult males, seven (43.8%) were adult females, and one was a hatchling of indeterminate sex. We captured two adult male tortoises in bucket traps on Cape San Blas, and 20 individuals (eight adult males, six adult females, six unknown) were captured in bucket traps and opportunistically sampled on Tyndall Air Force Base.

Genetics.—Sequences of 735 bp of Cytb and 709 bp of ND4 were obtained for 26 samples from St. Vincent Island and nearby mainland localities (Table 2). The haplotype networks reconstructed using our

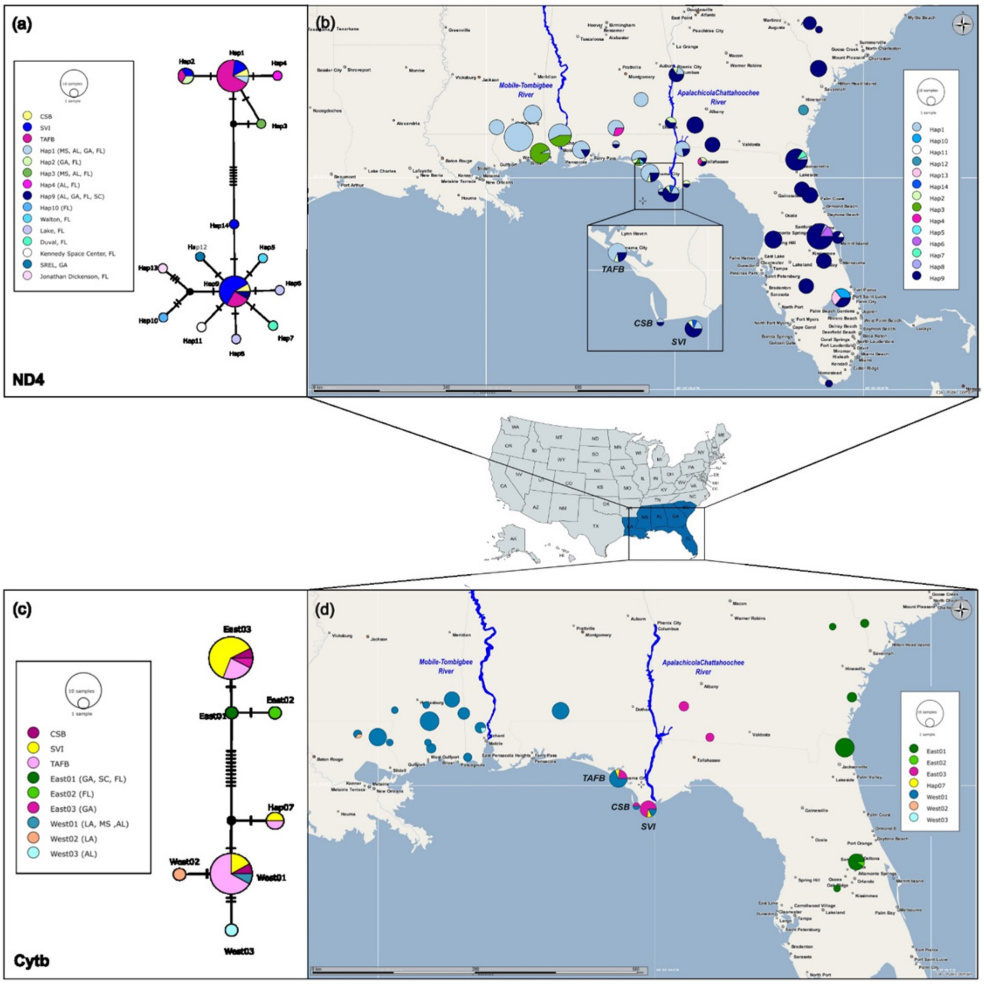

Figure 3. Haplotype networks and geographical distributions of Gopher Tortoise (*Gopherus polyphemus*) mitochondrial haplotypes in the southeastern U.S. (a) TCS network for ND4 haplotypes. (b) Sample sites and distribution of ND4 haplotypes. (c) TCS network for Cytb haplotypes. (d) Sample sites and distributions of Cytb haplotypes. Colors in the haplotype networks (a and c) represent the geographic origin of the specimen and the size of each circle is proportional to the frequency of the haplotype. Dashes across lines indicate one mutational step. Small dark nodes represent median vectors. In (b) and (d), the size of each pie is proportional to the sample size and the pie slice within the circle corresponds to the frequency of a haplotype at each locality. The colors in (b) and (d) represent different haplotypes as indicated in the legend. The Mobile-Tombigee River and the Apalachicola-Chattahoochee River are shown in blue.

data only (ND4 and Cytb) or our data plus available GenBank data for ND4 and Cytb identified two major groups for each marker (Fig. 3, Appendix Figure). The two groups largely represent localities to the east and west of the Apalachicola-Chattahoochee rivers (Fig. 3, Appendix Figure). Haplotype designations follow Ennen et al. (2012) for ND4 and Clostio et al. (2012) for Cytb. Independently of the marker, our sampling sites, Cape San Blas, Tyndall Air Force Base, and St. Vincent Island, include representative haplotypes belonging to both groups. The 709 bp of ND4 yielded four haplotypes across our sampling localities, including a new haplotype in St. Vincent Island (Hap14 in Fig. 3; G14 in Appendix Figure). For ND4, Hap1 was the most common haplotype across our sampled populations, primarily found in Tyndall Air Force Base (n = 9; 69.2%), with additional occurrences in St. Vincent Island (n = 2; 18.18%), and Cape San Blas (n = 1; 50%). Hap9 was the second most frequent haplotype, predominantly detected in St. Vincent Island (n = 7; 63.6%), with smaller representations in Tyndall Air Force Base (n = 3; 23%), and Cape San Blas (n = 1; 50%). Overall, St. Vincent Island was largely represented by haplotype Hap9, which is common in Florida, Georgia, and northern Alabama, while Tyndall Air Force Base was largely represented by haplotype Hap1, common to populations west of the Apalachicola-Chattahoochee rivers. The two samples from Cape San Blas, a locality in between Tyndall Air Force Base and St. Vincent

Table 2. Sampled locality, number of sequences (n), number of haplotypes found in each locality (H), haplotype diversity (h), and nucleotide diversity (π) for the ND4 and Cytb markers for Gopher Tortoises (*Gopherus polyphemus*) inhabiting coastal sites in northwestern Florida, USA. Cape San Blas was not included in within-locality measures as only two samples were available for this locality (na in the Table). Nonetheless, Cape San Blas samples were included in the overall analyses (Total).

	n	H	h	π
Locality				
St. Vincent Island	11	4	0.600	0.006
Cape San Blas	2	2	na	na
Tyndall Air Force Base	13	3	0.500	0.005
Total	26	4	0.634	0.007
Cytb				
St. Vincent Island	11	3	0.500	0.009
Cape San Blas	2	2	na	na
Tyndall Air Force Base	13	3	0.500	0.008
Total	26	3	0.590	0.011

Island, correspond to each of these haplotypes (Fig. 3, Appendix Figure).

The 735 bp of Cytb revealed three haplotypes across our sampling locations, including a new haplotype shared between a sample from St. Vincent Island and one sample from Tyndall Air Force Base (Hap07 in Fig. 3; samples G18 and G5, respectively, in Appendix Figure). The most common haplotype found in our samples was West01 in Tyndall Air Force Base (n = 9; 69.2%), while East03 was the most frequent in St. Vincent Island (n = 8; 72.7%). Similarly to what was observed with ND4, St. Vincent Island is mostly represented by a haplotype common in Georgia, east of the Apalachicola-Chattahoochee rivers, while Tyndall Air Force Base is mostly represented by a haplotype common to the west of these rivers and Cape San Blas is represented by one sample of each haplotype (Fig. 3, Appendix Figure). Analyses revealed lower haplotype diversity in Cytb than ND4 (Table 2).

Discussion

We found that the Gopher Tortoise population on St. Vincent Island, which has a relatively long history of inhabitance (since 1979; U.S. Fish and Wildlife Service 2012), is small but has a balanced sex ratio and high haplotype diversity (Hohenlohe et al. 2021). Tortoises on the island are representative of populations west and east of the Apalachicola-Chattahoochee River system, and while they share genetic similarities to nearby mainland populations, a unique haplotype was documented on the island. Previous studies suggest the Apalachicola River represents a biogeographic barrier that separates Gopher Tortoises into two populations east and west of the river, with some overlap between the two groups particularly near the river (Osentoski and Lamb 1995; Ennen et al. 2012). Ennen et al. (2012) suggested that although the river inhibited gene flow historically (i.e., since the Pleistocene), it may not continue to block gene flow in recent times and that perhaps both tortoise populations have experienced a period of expansion. Along with tortoise translocation, this dispersal may contribute to haplotype diversity in northwestern Florida (Ennen et al. 2012). Our study highlights the importance of considering coastal Gopher Tortoise populations across northwestern Florida, regardless of population size, particularly as they may contribute to overall genetic diversity of the species.

The characteristics of a Minimum Viable Population of Gopher Tortoises include at least 0.4 ha of habitat per tortoise and a minimum site size of 100 ha (Gopher Tortoise Council 2014). At its current density of 0.07 tortoises/ha, the St. Vincent Island tortoise population is below the accepted threshold for a viable population. We identified 318.4 ha of appropriate tortoise habitat on St. Vincent Island, however, suggesting there is sufficient habitat to support population expansion. The current state of knowledge, including our findings here, suggest that the tortoise population on the island could expand and grow. In fact, during our surveys and in opportunistic observations, we documented tortoise hatchlings, juveniles, mating activity, and one tortoise nest on the island, providing evidence of tortoise reproduction on St. Vincent Island (Palandri and Lamont 2021).

Historically, Gopher Tortoise were associated with Longleaf Pine (*Pinus palustris*) forests, which provided tortoises with the habitat characteristics optimal for burrowing and foraging: widely spaced canopy trees, a limited midstory, and an understory that includes pyrogenic grasses (Fill et al. 2017; Pudner et al. 2021). Ideal habitat for tortoise burrows was defined by Tuberville et al. (2007) as having 30–50% canopy cover, 20% shrub cover, and lower basal area than surrounding habitat. At our site, burrow distributions were approximately evenly divided between hydric pine flatwoods and coastal scrub habitats, with a comparatively smaller proportion observed within sand beach habitat. Additionally, all but one of the burrows we located on St. Vincent Island were found along the coast, approximately 350 m inland of the Gulf. This could suggest that tortoises are restricted to coastal habitats on St. Vincent Island by lack of appropriate habitat in the interior portion of the island, even though this area on the island is dominated by pine flatwoods. Upland habitats on St. Vincent Island, however, have historically not been managed for tortoises (e.g., prescribed fires) and as such, these areas are typically dense and nearly impenetrable by humans. Although these flatwood habitats are classified as hydric and may be considered unsuitable for tortoises (Castellón et al. 2020), tortoises have been documented burrowing and foraging in hydric pine flatwoods (Beever and Dryden 1993; Voves and Rothermel 2024). In general, however, tortoise use of upland habitats can be improved through thinning of trees and conducting prescribed burns (Darracq et al. 2016; Hunter and Rostal 2021). Increasing use and frequency of these management actions within upland habitats on the island may help maintain optimal canopy cover with a diverse understory that is ideal for tortoises and could encourage the tortoise population on St. Vincent Island to expand.

The sex ratio of tortoises we documented on St. Vincent Island was 1.1M:1F, which is similar to ratios reported elsewhere (Berish and Moore 1993; Tuberville et al. 2014; Greene et al. 2020; Howell et al. 2020). Stable Gopher Tortoise populations are characterized by high female survival and balanced adult sex ratios (Folt et al. 2021). Nesting female tortoises that inhabit developed areas are more susceptible to road mortality but in protected areas, such as on St. Vincent Island, differential sex-based mortality has not been demonstrated (Tuberville et al. 2009). Both tortoises we found dead on St. Vincent Island were males. One appeared to be killed by a Cottonmouth (*Agkistrodon piscivorus*; Palandri and Lamont 2021) while the cause of mortality for the second male was unknown.

Despite St. Vincent Island being a relatively small island (14.5 km long and 6.5 km wide) and located only approximately 0.3 km from the mainland coast, it appears to harbor a small persistent population of tortoises. These tortoises are represented by at least four distinct mitochondrial haplotypes. Although we found a lower haplotype diversity in Cytb than ND4, supporting the lower genetic variability of this marker in turtles (Chiari et al. 2005; see also Lourenço et al. 2013), both markers supported the same finding. On St. Vincent Island, we discovered that the majority of the sampled tortoises were genetically similar to tortoises located east of the Apalachicola and Chattahoochee rivers, although genetic representation of samples from west of these rivers also occurred on the island, which is not uncommon for populations in close proximity to the river (Osentoski and Lamb 1995; Ennen et al. 2012). Interestingly, another sampled location in proximity (about 40 km) to St. Vincent Island, Tyndall Air Force Base, was genetically represented by haplotypes from west of these rivers. Additionally, St. Vincent Island has a rich and varied history with periods of time that may have facilitated tortoise translocations to the island (see U.S. Fish and Wildlife Service 2012, for a summary of the history of the island). It was privately owned from the mid-1800s until 1968, when it was purchased by The Nature Conservancy. In 1908, the owner developed the land as a productive cattle ranch and imported species such as Sambar Deer (*Rusa unicolor;* U.S. Fish and Wildlife Service 2012). In 1948, after sale to another private owner, species such as zebra (genus *Equus*), Elands (*Taurotragus oryx*), and various exotic birds were brought to the island to develop an exotic game preserve (U.S. Fish and Wildlife Service 2012). During this period of private ownership, Gopher Tortoises from sites throughout the southeastern U.S. may have been brought to the island. Although the island became part of the National Wildlife Refuge system in 1968 and remained uninhabited since that time, public access is available, and as such, it is possible that visitors to the refuge brought tortoises from the mainland to the island. Release of captive tortoises by citizens has been documented throughout the southeastern U.S. and can impact inferences of genetic structuring of tortoise populations (Diemer 1986; McKee et al. 2021; Sinclair et al. 2010).

In addition to the potential for human-mediated translocation of tortoises, it has been proposed that tortoises may have naturally colonized islands, such as St. Vincent, by swimming or floating from the mainland (Krause 2010; Samonds et al. 2012). Tortoises, including Gopher Tortoises, have been observed floating and actively swimming (Carr 1952; Brode 1959; Gaymer 1968) including in the Gulf (Beyer 2020), which supports the plausibility of overwater dispersal to St. Vincent Island from the mainland, a distance of about 0.3 km. On both St. Vincent Island and Tyndall Air Force Base, samples were representative of the genetic diversity occurring across the southeastern U.S. (Clostio et al. 2012; Ennen et al. 2012), which suggests some admixture among genetically isolated lineages. With each genetic marker, we found a low frequency of unique haplotypes on St. Vincent Island, supporting the idea of a locally established interbreeding population on the island. This is not completely surprising as we documented evidence of breeding on the island, and some haplotypes have been shown to have narrow distributions (Ennen et al. 2012). A more fine-scale sampling of the coastal area on and near the island could help to further confirm that these haplotypes are unique to St. Vincent Island and better understand relationships of these tortoises with coastal populations.

Currently, land management on St. Vincent Island, including prescribed burns, are not conducted consistently. Fire suppression is often considered one of the greatest threats to Gopher Tortoise persistence (Mushinsky et al. 2006), as it results in closed canopy forests (Auffenberg and Franz 1982; Diemer 1989; Frost 1993). Prescribed fire can be used to reduce mid-story vegetation, increase space between trees, promote production of groundcover, and increase thermoregulatory opportunities for tortoises (Diemer 1986; Yager et al. 2007; Howell et al. 2020). To encourage expansion of the tortoise population on St. Vincent Island, refuge managers may consider altering their prescribed burn schedule and spatial extent, and thinning trees, specifically to improve habitat for tortoises, which could promote full use of the habitats we identified as being appropriate for tortoises in our surveys.

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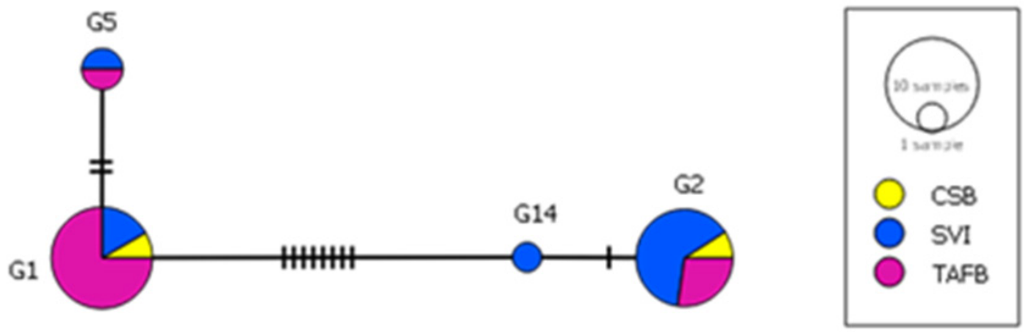

Appendix Figure. Haplotype network of concatenated ND4 and Cytb internal sequences of the Gopher Tortoise (*Gopherus polyphemus*). The size of the node is proportional to the frequency of the haplotype. Dashes across lines indicate one mutational step.