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Kinship and Breeding Site Philopatry Drive Fine-Scale Genetic Structure in Fragmented Populations of the Gopher Frog (*Rana capito*) in North Carolina

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Abstract.—**The Gopher Frog (*Rana capito*) is a threatened species native to the southeastern longleaf pine ecosystem. Although once much more widespread across the southeastern United States, they now occur in North Carolina at only a handful of disjunct sites in the Coastal Plain and Sandhills regions of the state. The long-term persistence of these populations is thus a concern, as is a loss of genetic variation over time. We used mitochondrial and microsatellite markers to better understand the spatial structure of genetic variation and levels of genetic variability across these remaining populations in order to inform conservation and management decisions. Eight unique mitochondrial haplotypes were found, but these were all genetically similar to one another. Levels of genetic diversity based on the microsatellite analyses were similar across populations, but inbreeding coefficients in two populations were significant, suggesting a potential vulnerability to inbreeding depression. All disjunct populations showed significant genetic differentiation, which was not related to geographic distance. Conversely, within populations, the genetic relatedness of individuals between ponds decreased as distance between ponds increased. This kinship pattern is likely driven by strong breeding philopatry (individuals returning to the same ponds across years) and indicates that conservation actions at the scale of <1 km would primarily affect kin groups of Gopher Frogs, whereas conservation actions at scales ≥1.5 km would be needed to capture more distantly related individuals. Management efforts should thus focus on local metapopulation dynamics by maintaining multiple breeding ponds at each location, and by enhancing connectivity between these breeding ponds.**

The longleaf pine (*Pinus palustris*) ecosystem once covered the majority of the southeastern United States, stretching from Texas to the Carolinas and covering an area of approximately 36.5 million hectares. This ecosystem has suffered an incredible decline over the past two centuries, and now occurs in scattered pockets totaling <5% of its original expanse (USDA Forest Service, 2013). This dramatic loss has largely been due to direct anthropogenic effects, such as large-scale harvesting of pines and clearing of land for human development (Means and Grow, 1985; Means, 1996; Platt, 1999; USDA Forest Service, 2013). Furthermore, suppression of natural wildfires in remaining longleaf pine forests has, in many cases, led to the encroachment of hardwoods, which in turn modifies the canopy structure around lakes and ponds. This can have significant impacts upon the larval development of numerous aquatic species, including the Gopher Frog, *Rana capito* (= *Lithobates capito* of some authors), one of many species endemic to the longleaf pine forest ecosystem (Means and Grow, 1985; Bailey, 1991; Blaustein et al., 1999; Werner and Glennemeier, 1999; Skelly et al., 2002; Thurgate and Pechmann, 2007).

Like the longleaf pine ecosystem, Gopher Frogs were once much more widespread across the southeastern United States. Today, they are considered Endangered, Threatened, or of Special Concern in all of the states within their range (Florida to North Carolina; Jensen and Richter, 2005). In North Carolina, Gopher Frogs are listed as Endangered (North Carolina Wildlife Resources Commission; NCWRC, 2017) and currently occur at only a handful (seven) of disjunct sites in the Coastal Plain and Sandhills regions of the state (Fig. 1; NCWRC, 2018). Typically, Gopher Frogs breed in either temporary or semipermanent ponds that are shallow, have an open canopy and emergent herbaceous vegetation, and lack large, predatory fish (Moler and Franz, 1987; Bailey, 1991). Breeding usually occurs in the boreal spring, between February and April; however, in parts of their range, Gopher Frogs have also been documented to breed in the boreal autumn, between September and November (NCWRC, 2018). Males typically arrive at breeding sites before females and remain there longer (Bailey, 1991). Females deposit fist-sized egg masses, usually attached to vertical stems of upright vegetation (Bailey, 1990). In North Carolina, the mean number of eggs per mass has been documented to be 1,500–2,000 (Braswell, 1993). When not congregating to breed, adult Gopher Frogs remain in upland "refugia"; these refugia are typically stump cavities (a hole in the ground resulting from the decay of a tree's roots) or burrows made by the gopher tortoise (*Gopherus polyphemus*) or mammals found in longleaf pine forests (NCWRC, 2018). Although in North Carolina, Gopher Frogs have been documented to use refugia as far away as 3.5 km from their breeding pond (Humphries and Sisson, 2012), it is unclear whether this distance is typical. For example, Gopher Frogs in Georgia and Florida were found to move considerably smaller (maximal distances of 100–700 m) distances from breeding ponds to refugia (Roznik et al., 2009). Although it remains unclear to what degree Gopher Frogs might exhibit fidelity to a given breeding site and return to the same pond to breed year after year, individuals in North Carolina have been documented to return to the same refugial stump hole from year to year (Humphries and Sisson, 2012). This suggests that they may indeed exhibit some level of breeding site fidelity as well.

The combination of their highly fragmented distribution in North Carolina (Fig. 1), a lack of suitable intervening habitat to serve as dispersal corridors, and their potential to exhibit breeding pond site fidelity, could have multiscale impacts on the population genetic structure of Gopher Frogs. For example, on a relatively broad geographic scale, the interpopulation distances involved (Fig. 1), coupled with the lack of suitable dispersal corridors, suggests that natural gene flow between each of the state's seven remaining populations of *R. capito* is unlikely. As such, each of the seven remaining populations may represent a

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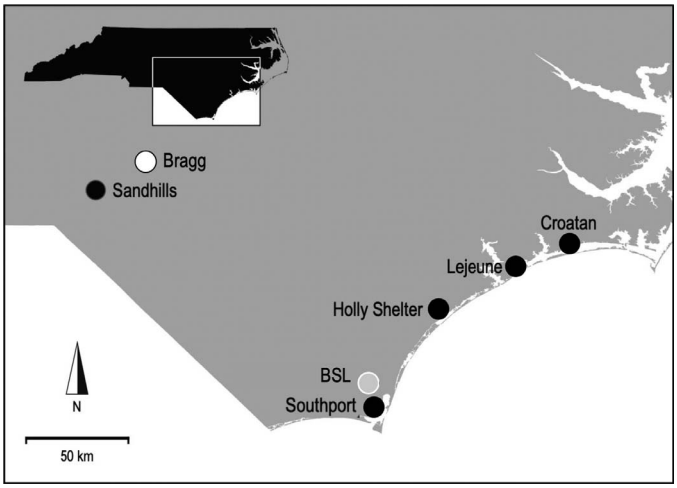

FIG. 1. Map displaying the locations (circles) of known Gopher Frog (*Rana capito*) populations in North Carolina. These populations are located at Boiling Spring Lakes (BSL), Holly Shelter Game Lands (Holly Shelter), Military Ocean Terminal Sunny Point-Southport (Southport), Sandhills Game Lands (Sandhills), Croatan Game Lands (Croatan), Fort Bragg (Bragg), and Camp Lejeune (Lejeune). BSL is designated with a gray circle because only two individuals were analyzed (with mtDNA only) for this site and Bragg is designated with a white circle because no samples from this location were analyzed in this study (see Methods).

distinct "nascent" evolutionary lineage (Chavez et al., 2014). At a finer geographic scale, relatively small effective population sizes, a limited number of suitable breeding ponds, and the potential that individuals may tend to return to the same breeding pond year after year, suggest that levels of genetic variation might be low, and levels of inbreeding might be high within populations. This is potentially problematic for the long-term persistence of these populations, as loss of genetic variability and inbreeding depression are two genetic problems that have contributed to, or have been suspected to contribute to, the endangerment of many species in the United States (Czech et al., 2000).

Previous genetic work used mitochondrial DNA (mtDNA) sequence data to examine large-scale patterns of genetic variation in *R. capito* across the geographic range of the species (Richter et al., 2014); Gopher Frogs from 21 sites were examined and three reciprocally monophyletic mtDNA lineages were described: a broadly distributed southeastern Coastal Plain lineage and two peninsular Florida lineages. However, the Richter et al. (2014) study only included samples from two (Croatan National Forest and Holly Shelter Game Land in Carteret and Pender counties, respectively) of the seven currently recognized populations of Gopher Frogs in North Carolina, which both fell into the broadly distributed southeastern Coastal Plain lineage of *R. capito*. As such, much of the population genetic structure and within-population levels of genetic variability of Gopher Frogs in North Carolina remain unknown.

The primary goal of this study was to provide a more complete picture of the geographic patterns of genetic variation and levels of genetic variability in North Carolina Gopher Frogs by analyzing multilocus (mtDNA and microsatellite) genetic data. Whereas the mtDNA sequence data provide information on relatively broad geographic and deep (i.e., Pleistocene–Holocene) temporal scales (Blois and Arbogast, 2006; Arbogast et al., 2017), the microsatellite genotype data can be used to evaluate levels of recent and ongoing gene flow at relatively fine geographic and temporal scales (Adrian et al., 2017). Furthermore, the latter can be used to examine kin structure, providing important insights into levels of relatedness among individuals (Kamel et al., 2012; Stachowicz et al., 2013). Specifically, we tested several hypotheses: 1) that the phylogeographic structure of *R. capito* in North Carolina will provide evidence of relatively deep divergences and nascent evolutionary lineages; 2) that genetic diversity will vary substantially among populations due to the effects of drift, and that inbreeding will be significant; and 3) that kin structure within ponds should be low given the relatively long dispersal distances documented in North Carolina Gopher Frogs. Analyses such as these can then be used to develop an initial genetic framework to help inform conservation and management decisions.

MATERIALS AND METHODS

Sampling and DNA Extraction.—We received approximately 200 samples purportedly from *R. capito* from the NCWRC representing six of the seven known populations in North Carolina: Boiling Spring Lakes, Croatan Game Lands, Holly Shelter Game Land, Camp Lejeune, Sandhills Game Land, and Military Ocean Terminal Sunny Point-Southport (Fig. 1). Sampling access to two sites was either not possible (Fort Bragg) or extremely limited (Boiling Spring Lakes) because Gopher Frog breeding ponds at these sites are on an active military base and primarily on privately owned land, respectively. Sampling effort and success varied between sites due to natural variation in Gopher Frog breeding effort and difficulty of access to some sites. Sampling occurred between 1 January 2016 and 31 December 2018 and was opportunistic, with samples taken from every putative *R. capito* egg mass found in each pond surveyed. Although egg masses were sometimes "clustered" in one part of a pond, they were not overlapping; typically, each egg mass was >0.3 m away from the next nearest mass, and in most cases, was at least 1 m away (and often substantially more). The samples provided by NCWRC primarily consisted of 1–2 eggs per egg mass preserved in EtOH, which we stored in 95% EtOH at −80°C at the University of North Carolina Wilmington (UNCW). We extracted genomic DNA from a single egg from each egg mass sample using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Venlo, Netherlands).

Mitochondrial DNA Analyses.—We amplified the mtDNA ND2 gene using the primers and protocols described in Richter et al. (2014). We used Genewiz LLC (www.genewiz.com) for cleanup and sequencing of polymerase chain reaction (PCR) products. We successfully amplified and sequenced the mtDNA ND2 gene for 182 samples from North Carolina (Supplemental Data, Table S1). We visually inspected for errors, trimmed, and assembled chromatograms from all mtDNA sequences using Sequencher v4.9 (Gene Codes Corporation, Ann Arbor, Michigan, USA). We performed a nucleotide blast search for each sequence on GenBank in order to determine if a sample was a strong (>99%) or identical match with an existing sequence from *R. capito*, or potentially from another species. We determined that 21 of the samples we received were actually a very close or identical match to the co-occurring Southern Leopard Frog, *Rana sphenocephala*. These 21 samples were thus excluded from all subsequent analyses, leaving a total of 161 samples that we were confident were from individuals of *R. capito*.

We used the "merge identical sequences" command in PAUP (Version 4.0a, build 167; Swofford, 2003) to identify the number of unique mtDNA ND2 haplotypes found among

the 161 North Carolina samples of *R. capito* from which we obtained new sequence data, and to assign each individual to a haplotype group. We then calculated the frequency of each haplotype at each of the six North Carolina localities from which we had samples of *R. capito*. To evaluate levels of genetic variation in the mtDNA sequence data, we used ARLEQUIN ver. 3.0 (Excoffier et al., 2005) to estimate gene diversity (the probability that two randomly chosen haplotypes are different in the sample) and nucleotide diversity (π) (the average number of nucleotide differences per site between two DNA sequences in all possible pairs in the sample population) for each of the five localities in North Carolina, for which we had more than two samples.

To examine the phylogenetic relationships of the North Carolina samples of *R. capito* with those found elsewhere in the geographic range of the species, we first downloaded 50 mtDNA ND2 sequences (46 of *R. capito* and 4 of *R. aureolata*) from Richter et al. (2014) from GenBank. The *R. aureolata* (Crawfish Frog) sequences were used as outgroups in subsequent analyses. The Richter et al. (2014) sequences represented individuals from each of the three mtDNA clades they identified: one from the Coastal Plain of the southeastern United States and two clades found in peninsular Florida. In total, our phylogenetic analysis matrix contained 211 individuals (207 *R. capito* and 4 *R. aureolata*) and 1,035 base pairs of ND2 sequence data. We then used the "merge identical sequences" command in PAUP (Version 4.0a, build 167; Swofford, 2003) to reduce the data set to just the unique mtDNA ND2 haplotypes. This resulted in 16 ND2 haplotypes for *R. capito* (8 distinct haplotypes observed among the 161 individuals of *R. capito* from North Carolina and 8 additional haplotypes observed in the ND2 portion of the 46 sequences of *R. capito* deposited on Genbank by Richter et al. [2014], the latter of which represented individuals from throughout the range of *R. capito*; Supplemental Data, Table S2) and the 4 *R. aureolata* outgroup sequences. To infer phylogenetic relationships among the 16 resulting ND2 haplotypes, we performed a full heuristic maximum-likelihood search under the GTR + gamma model (with the shape parameter of the gamma distribution estimated during the search) in PAUP. The four *R. aureolata* sequences from Richter et al. (2014) were designated as outgroups. To assess nodal support, we performed 1,000 bootstrap replicates using the fast stepwise addition option.

Microsatellite Analyses.—We genotyped individuals at 11 microsatellite markers developed specifically for *R. capito* and amplified them via PCR following Nunziata et al. (2012). Each well on a PCR plate contained 2X Qiagen Multiplex Mix (5.0 μL), ddH_2O (0.5 μL), either primer soup A or B (1.5 μL), and 15–25 ng/μL DNA (3.0 μL). We multiplexed primers as follows: soup A was composed of equal concentrations of primers LICA 15, 25, 40, 41, and 43 and soup B was composed of equal concentrations of primers LICA 5, 7, 11, 14, 18, and 47. We prepared PCR products for fragment analysis on the ABI Prism 3130XL Genetic Analyzer at the UNCW's Center for Marine Science DNA Sequencing Core Facility. We combined 0.5 μL of each multiplex PCR product with 9 μL of Hi-Di Formamide and 0.3 μL of GeneScan-600 (LIZ) size standard (Applied Biosystems, Foster City, California, USA) prior to being run on the analyzer. We scored fragments using the software STRand version 2.3.69 (Toonen and Hughes, 2011).

We calculated standard diversity indices, tests for linkage disequilibrium (LD), inbreeding coefficients (F_{IS}) according to Weir and Cockerham (1984), and observed and expected heterozygosity (H_O and H_E) using ARLEQUIN ver. 3.0 (Excoffier et al., 2005). We calculated allelic richness (A) and rarefied allelic richness (A_R) using FSTAT version 2.9.3.2 (Goudet, 1995). We used GENEPOP 4.0 (Raymond and Rousset, 1995) to test for conformation to Hardy–Weinberg expectations within each population (HWE). We used ML-Relate (Kalinowski et al., 2006) to estimate mean relatedness (r) among individuals within a population. In the absence of inbreeding, the expected value of r for 1) unrelated individuals, 2) parent–offspring or full siblings, and 3) half siblings is 0, 0.5, and 0.25, respectively (Queller and Goodnight, 1989).

To assess the degree of genetic differentiation among populations, we calculated all pairwise values of population differentiation using Weir and Cockerham's F-statistics (Weir and Cockerham, 1984) and tested for significance with 10,000 permutations of the data. We performed Mantel tests to test for isolation by distance among populations and among ponds using Isolation by Distance Web Service, version 3.2.3 (Jensen et al., 2005), and tested for significance of correlations with 10,000 matrix randomizations. The geographic distance (in km) between sites was calculated as the shortest straight-line path connecting those points.

To investigate spatial patterns of family structure, we calculated kinship coefficients (Loiselle et al., 1995), which are based on the relative probability of two alleles being identical by descent between each pair of individuals, using GENODIVE (Meirmans and Van Tienderen, 2004). To determine whether kin were more likely to be clustered in the same population and the same pond, we used the PERMANOVA+ 1.0.2 software add-on in PRIMER7 (Clarke and Gorley, 2006) to conduct a 1-way ANOVA on kinship coefficients following the approach of Iacchei et al. (2013). To further investigate kin relationships among frogs, we binned individuals according to the following levels of kinship (k): "nearly identical," $0.57 > k > 0.375$; "full siblings," $0.375 > k > 0.1875$; "half siblings," $0.1875 > \kappa > 0.09375$; and "quarter siblings," $0.09375 > k > 0.047$. Here the bounds represent the midpoints between coancestry coefficients (Loiselle et al., 1995; Iacchei et al., 2013). We also conducted a permutation test where the observed number of closely related individuals within populations was compared with a null distribution of kinship coefficients generated by randomly assigning frogs to populations (Iacchei et al., 2013; Adrian et al., 2017).

Results

MtDNA Analyses.—We deposited the 161 mtDNA ND2 sequences obtained for *R. capito* on GenBank under accession numbers MT576144–MT576249 and MT576251–MT576305. Copies of the following files are available from the corresponding author upon request: 1) a nexus file consisting of a matrix of 1,035 base pairs of ND2 sequence data and 211 individuals (207 *R. capito* and 4 *R. aureolata*); 2) the nexus file we used in our phylogenetic analysis, which contained only the unique ND2 haplotypes of *R. capito* and *R. aureolata*; and 3) the ARLEQUIN input file we used to estimate haplotype and nucleotide diversity. Within North Carolina, we identified eight distinct mtDNA ND2 haplotypes (Table 1). When compared with those from Richter et al. (2014), six of the eight North Carolina haplotypes we identified (haplotypes B, C, E, F, G, and H) were new, and two (haplotypes A and D) matched ND2 sequences from Richter et al. (2014; Supplemental Data, Table S2). Estimates of mtDNA ND2 gene diversity for the five localities in North Carolina for which

TABLE 1. Summary of the number of individuals from each locality in North Carolina that exhibited each of the eight mtDNA ND2 haplotypes (designated as A–H) that we identified in the state. Note that we did not receive any samples from Fort Bragg and only two samples from Boiling Spring Lakes.

	Haplotypes								
Localities	A	B	C	D	E	F	G	H	Total
Holly Shelter	53	1	0	0	0	0	0	0	54
Southport	10	2	0	0	0	1	0	0	13
Sandhills	1	62	0	0	0	0	1	0	64
Croatan	4	0	0	6	0	0	0	1	11
Lejeune	4	2	10	0	1	0	0	0	17
Boiling Springs Lake	2	0	0	0	0	0	0	0	2
Total	74	67	10	6	1	1	1	1	161

we had more than two samples were 0.0370, 0.0620, 0.4103, 0.6176, and 0.6182 for Holly Shelter, Sandhills, Southport, Lejeune, and Croatan, respectively. Estimates of nucleotide diversity (π) were 0.000036, 0.000060, 0.000824, 0.000874, and 0.002073 for Holly Shelter, Sandhills, Lejeune, Southport, and Croatan, respectively.

Our maximum-likelihood analysis of the mtDNA ND2 sequence data set recovered four nearly identical trees, each with a likelihood score of 1954.064 (Fig. 2). The estimated value of the shape parameter of the gamma distribution was 0.715130. All of the trees contained the same three major mtDNA lineages within *R. capito* as those originally identified by Richter et al. (2014) based on concatenated partial ND2 and control region mtDNA sequences. Both of the peninsular Florida lineages were strongly supported in our analyses; however, the "southeastern coastal clade" received relatively weak bootstrap support in our analysis (Fig. 2). All eight of the haplotypes that we found in North Carolina fell into this latter clade, along with all non-Florida ND2 reference sequences for this species from Richter et al. (2014) that we downloaded from GenBank.

Microsatellite Analyses.—We successfully genotyped 154 individual Gopher Frogs at 11 microsatellite loci from five of the North Carolina populations (Table 2, Supplemental Data, Table S1); Boiling Spring Lakes was not included because it only contained 2 individuals. A spreadsheet of all genotypes is available from the corresponding author on request. The mean number of alleles per locus ranged from 3.7 (Croatan) to 7.5 (Sandhills) across populations; similarly, mean allelic richness, rarefied to 11 individuals, ranged from 3.1 to 4.3 across populations (Table 2). H_O (range: 0.41 to 0.64) was lower than H_E (range: 0.48 to 0.69) for all populations. There were significant deviations from HWE in the Sandhills population. Two populations (Croatan and Lejeune) had significant F_{IS} estimates

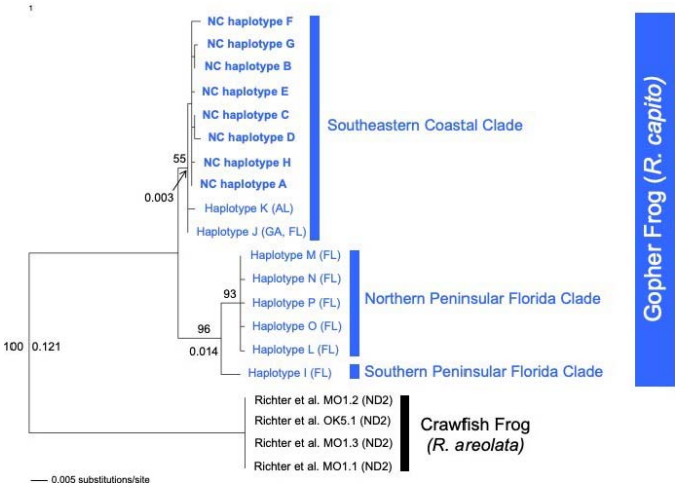

FIG. 2. Maximum-likelihood phylogenetic tree of Gopher Frog ND2 haplotypes (blue). Included are the eight ND2 mtDNA haplotypes we observed in newly sequenced individuals of *R. capito* sampled from throughout North Carolina (bold) and eight ND2 reference haplotypes taken from Richter et al. (2014). Four ND2 sequences of the Crawfish Frog (*R. aureolata*; black) from Richter et al. (2014) were used as outgroup taxa in the phylogenetic analysis. All individuals of North Carolina *R. capito* that we sequenced belong to the Southeastern Coastal Clade, one of three lineages of *R. capito* originally identified by Richter et al. (2014). A full summary of all of the samples we examined and the haplotype group to which they were assigned is presented in Supplemental Data, Tables S1, S2.

(Table 2). Overall, LD was significantly nonzero in all populations, with Lejeune having the highest percentage of loci in LD (20%). Mean *r* ranged from 0.17 to 0.44, with global mean of 0.27, which broadly corresponds to half-sibling relationships. A global mean *k* of 0.19 also corresponds to this level of relatedness (range: 0.09 to 0.34). There was a significant positive correlation between F_{IS} and *k* across populations ($r^2 = 0.34$, $P = 0.04$), and a significant negative correlation between F_{IS} and population size ($r^2 = 0.71$, $P = 0.02$).

All pairwise population comparisons of genetic differentiation were significant (Table 3); however, there was no correlation between geographic distance and F_{ST} ($r^2 = 0.03$, $P = 0.64$). This pattern was similar when evaluating the relationship between geographic distance and mean relatedness among individuals between populations ($r^2 = 0.03$, $P = 0.51$). However, when considering within-population comparisons (that is, among ponds within a location), we found a significant negative correlation between geographic distance and relatedness ($r^2 = 0.24$, $P = 0.03$). Individuals from nearby ponds were more closely related to one another than individuals from more distant ones, suggesting that movement between ponds might shape kin structure at local scales (Figs. 3a, 3b).

TABLE 2. Summary statistics for each Gopher Frog population averaged over all 11 microsatellites. Observations include: number of frogs sampled (n_f), mean number of alleles per locus (*A*), mean allelic richness (A_R), observed heterozygosity (H_O), expected heterozygosity (H_E), mean inbreeding coefficient (F_{IS}), mean relatedness (*r*), mean kinship coefficient (*k*), percentage of loci pairs in linkage disequilibrium (LD), and percentage of loci out of HWE. * Significant at $P < 0.05$.

Population	n_f	*A*	A_R	H_O	H_E	F_{IS}	*r*	*k*	% LD	HWE
Croatan	11	3.7	3.1	0.41	0.48	0.15*	0.44	0.34	7.2	18.1%
Holly Shelter	54	5.5	3.3	0.53	0.55	0.03	0.26	0.12	10.9	36.3%
Lejeune	17	6.1	4.3	0.56	0.69	0.18*	0.17	0.16	20.0	9.1%
Sandhills	60	7.5	4.1	0.64	0.66	0.03	0.17	0.09	16.3	36.3%
Southport	12	4.7	3.8	0.60	0.66	0.09	0.29	0.25	14.5	9.1%
Mean		5.5	3.7	0.55	0.61	0.096	0.27	0.19	13.8	21.8%

TABLE 3. Matrix of geographic distance (km) above diagonal, and pairwise genetic distance (F_{ST}) below diagonal. * Significant at $P < 0.05$.

	Croatan	Holly Shelter	Lejeune	Sandhills	Southport
Croatan	—	126	85	283	204
Holly Shelter	0.360*	—	48	214	105
Lejeune	0.233*	0.190*	—	277	127
Sandhills	0.255*	0.187*	0.168*	—	226
Southport	0.265*	0.244*	0.132*	0.223*	—

The mean kinship among frogs within a population was 0.19 ± 0.10, which was higher than the mean kinship among frogs located in different populations (−0.05 ± 0.09). Indeed, kinship coefficients were significantly greater for within-population than among-population comparisons (*pseudo*-$F_{4,150} = 1.4$, $P = 0.0008$), supporting the observation that the majority of kin comparisons were between Gopher Frogs located in the same population. Furthermore, there were significantly more kin groupings than expected by chance in all populations (Fig. 4), and significantly fewer unrelated individuals than would be expected by chance. Similarly, mean relatedness among frogs within a population was 0.27 ± 0.11, which was higher than the mean relatedness among frogs located in different populations (0.03 ± 0.06).

This kin structure was evident on an even finer scale. The mean relatedness values for frogs within a pond was usually higher than those of frogs located in different ponds (Table 4) even though ponds were located at most 2.5 km apart (Fig. 3b). The two populations where values for within- vs. among-pond kinship were most similar (Croatan and Southport) both had mean between-pond distances of only approximately 0.5 km. The other three populations all contained ponds separated by greater distances. Moreover, for the Holly Shelter and Sandhills populations where multiyear samples from individual ponds were available, kinship coefficients were almost identical within and across years (Table 5), with no significant difference between within- and among-year k values (T-test$_{5,8} = 0.88$, $P = 0.43$). These results strongly suggest that in North Carolina, individual Gopher Frogs have fidelity to their natal breeding pond and/or tend to return to a specific breeding pond repeatedly in successive years.

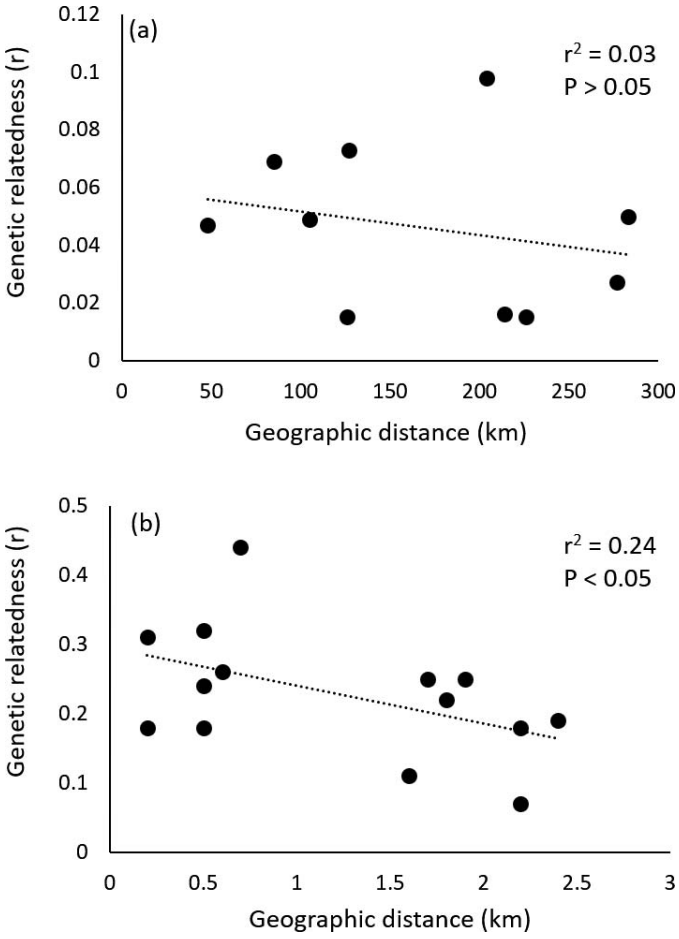

FIG. 3. Isolation by distance: regression of genetic distance (mean r) vs. geographic distance (km) for (a) population pairs (regional scale) and (b) pond pairs within a population (local scale).

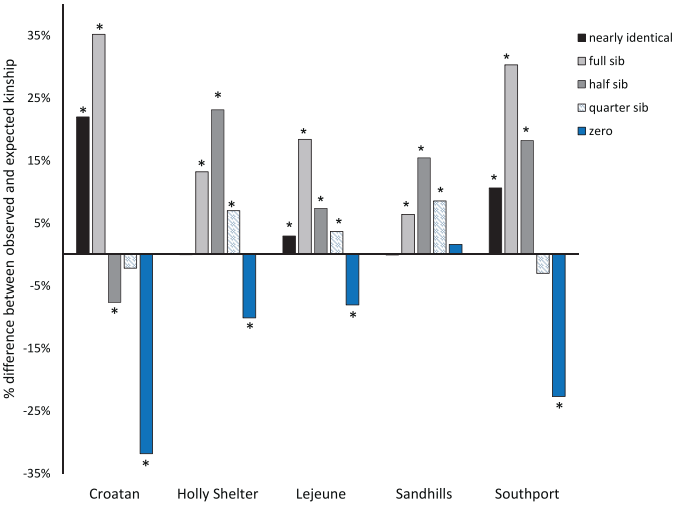

FIG. 4. Proportion of observed kinship comparisons for each population that were more or less than expected for each of four kinship categories (nearly identical: $0.375 < k < 0.57$; full sibling: $0.1875 < k < 0.375$; half sibling: $0.09375 < k < 0.1875$; and quarter sibling: $0.047 < k < 0.09375$). Zero refers to those individuals who are unrelated. Expected numbers of kinship comparisons for each category based on pairwise kinship coefficients that were calculated after randomly shuffling individuals among populations. * Significant differences at $P < 0.05$.

DISCUSSION

The longleaf pine ecosystem once covered the majority of the southeastern United States, but it has largely disappeared over

TABLE 4. Kinship values for ponds located within the five sampled populations. Observations include: number of ponds sampled (n_p), the mean distance between all ponds within a location, the mean relatedness (r) and kinship coefficients (k) for individuals located within the same ponds in a given population, and the mean r and k for individuals located in different ponds within the same population.

		Distance	Within ponds		Among ponds	
Population	n_p	(km)	r	k	r	k
Croatan	2	0.7	0.43	0.42	0.44	0.43
Holly Shelter	3	1.4	0.27	0.17	0.25	0.12
Lejeune	4	1.5	0.20	0.21	0.18	0.18
Sandhills	2	2.2	0.19	0.11	0.18	0.09
Southport	3	0.4	0.34	0.19	0.24	0.20

TABLE 5. Kinship coefficients calculated in two ponds over time. Pond 1 in Holly Shelter was sampled in 2016, 2017, and 2018, while Pond 1 in Sandhills was sampled twice in 2016 and once in 2017. Observations include: number of individuals sampled (*n*), and kinship coefficients (*k*) for within-year and among-year comparisons.

Population	Year	*n*	*k*
Holly Shelter	2016	16	0.161
	2017	5	0.086
	2018	23	0.114
	2016 vs. 2017	—	0.114
	2016 vs. 2018	—	0.117
	2017 vs. 2018	—	0.098
Sandhills	2016a	33	0.084
	2016b	11	0.095
	2017	9	0.110
	2016a vs. 2016b	—	0.089
	2016a vs. 2017	—	0.098
	2016b vs. 2017	—	0.096

the last two centuries (Means and Grow, 1985; Means, 1996; Platt, 1999). Due to a combination of both human destruction and degradation, and suppression of natural annual forest fire regimes, populations of *R. capito*, one of many species endemic to this ecosystem, have been considerably impacted (Means and Grow, 1985; Bailey, 1991; Thurgate and Pechmann, 2007). Our work, which uses multilocus molecular markers to evaluate the population genetics of Gopher Frogs in North Carolina at multiple spatial and temporal scales, provides important insights into the consequences of such impacts.

MtDNA Phylogeographic Structure of North Carolina Populations of R. capito.—The eight mtDNA ND2 haplotypes that we found in North Carolina Gopher Frogs all fell into the geographically widespread southeastern Coastal Plain lineage previously identified by Richter et al. (2014), as did all non-Florida ND2 reference sequences for this species from Richter et al. (2014) that we downloaded from GenBank (Fig. 2). Although six of the eight mtDNA ND2 haplotypes we observed in North Carolina Gopher Frogs were novel (Supplemental Data, Table S2), all were quite closely related to one another from a phylogenetic perspective (Fig. 2). As such, we did not find any evidence to support the presence of highly divergent "cryptic" evolutionary lineages (Arbogast et al., 2017) in the Gopher Frogs we examined from North Carolina. Interestingly, we also did not find the populations from the Sandhills (in the interior of North Carolina) to be particularly distinct from the more coastal populations we examined. Overall, the phylogeographic structure of North Carolina Gopher Frogs is consistent with relatively recent (within the last few hundred years) anthropogenically caused fragmentation that isolated populations in the state from each other and from more southerly populations, comprising the southeastern coastal lineage of *R. capito*.

Levels of Genetic Diversity in North Carolina Populations of R. capito.—Gene diversity is the haploid equivalent to expected heterozygosity for diploid data and is defined as the probability that two randomly chosen haplotypes are different in the sample (Excoffier et al., 2005). In contrast, nucleotide diversity is the probability that two randomly chosen homologous nucleotide sites are different, and it is therefore equivalent to gene diversity at the nucleotide level for DNA sequence data. As such, values of nucleotide diversity are generally quite small in populations whose haplotypes tend to differ by one or a few nucleotide substitutions, which is what we observed in this study. Therefore, gene diversity is a more useful measure for evaluating relative levels of mtDNA genetic diversity within North Carolina populations of the Gopher Frog. For the five populations in which we had more than two samples, our results indicate that mtDNA gene diversity is highest in Croatan (0.6182), Lejeune (0.6176), and Southport (0.4103), and lowest in Sandhills (0.0611) and Holly Shelter (0.0374). These results differ from the patterns of nuclear genetic diversity based on the microsatellite analyses (see Table 2), where, for example, values such as rarified mean allelic richness (A_R) and observed and expected heterozygosity (H_O and H_E, respectively) did not vary significantly among the five populations, and inbreeding (F_{IS}) was actually highest at Croatan and Lejeune.

Several factors could be contributing to the different patterns observed in the mtDNA and microsatellite diversity estimates. First, given the relatively small number of individuals examined from Croatan (11), Southport (12), and Lejeune (17), this could be, in part, a sampling-size effect. Second, the ND2 mtDNA gene is a single genetic locus, whereas the microsatellites were used to assay variation at 11 loci. Third, the mtDNA ND2 and microsatellite markers are assessing genetic variation in different parts of the genome (mitochondrial vs. nuclear), which can have distinctly different population genetic histories (Blois and Arbogast, 2006). Finally, and probably most importantly, the mtDNA gene diversity values appear to reflect the presence of a few relatively rare ancestral haplotypes that have not yet gone extinct (Table 1); in contrast, the microsatellite-based parameters reflect patterns of population structure, relatedness among individuals, and levels of genetic diversity established much more recently and on a substantially finer spatial scale. Thus, although both types of molecular markers provide important information, they do so for different parts of the genome and on different spatial and temporal scales (Arbogast et al., 2017).

Nuclear Patterns of Genetic Variation Across Space.—Given their relative geographic isolation from one another, it is not surprising that all pairwise comparisons of F_{ST} between the five North Carolina populations we examined were significant (Table 3). Mantel tests revealed that the relationship between genetic differentiation and geographic distance was nonsignificant at this regional scale (Fig. 3a). This lack of spatial genetic structure is exemplified in the lower F_{ST} values between the inland Sandhills population and the four coastal populations vs. the comparisons between coastal populations, such as Holly Shelter and Croatan (Table 3). Population differentiation likely reflects the effects of genetic drift, which occurs more quickly in smaller populations. Gene flow among populations is extremely limited (possibly nonexistent) and each population is undergoing independent ecological and evolutionary trajectories. This is supported by estimates of relatedness and kinship among individuals located in different populations that are effectively zero. In contrast, the relationship between geographic and genetic distance was significant at the local scale, when pond pairs within a population were analyzed (Fig. 3b). An examination of Fig. 3b suggests that at the scale of approximately 0–1 km, conservation actions would primarily affect close family groups of Gopher Frogs (parent–offspring and full siblings), and that conservation actions at the scale of 1.5–2.5 km (or greater) would be needed to affect more distantly and/or unrelated individuals.

Relatedness and Kinship Analyses.—Our results indicate high levels of relatedness and kinship within populations and within

ponds, with values consistent with individuals being related, on average, on the order of that expected for siblings or parent–offspring. This suggests that Gopher Frog populations at local sites are comprised of closely related individuals, such that individual Gopher Frogs would commonly return to their natal ponds or a to pond in which they had bred recently. This is further strengthened by the observation of similar kinship values across years. We would not expect to see this pattern if Gopher Frogs were frequently dispersing to a new or different breeding pond in successive years.

These kinship analysis results are notable given the substantial distances (0.5–3.5 km; mean = 1.3 km) Gopher Frogs have been documented to move from their breeding ponds to summer refugia (Humphries and Sisson, 2012). Thus, despite these long-distance movements, it would appear that individuals return to their natal or recent breeding pond to breed. Given the ability of Gopher Frogs to navigate long distances to the same tree stump as a summer refugium in successive years (Humphries and Sisson, 2012), perhaps it should not be surprising that the genetic data suggest they are also adept at returning to the same breeding pond in successive years.

Management Implications.—While it would be beneficial to preserve the diversity of mtDNA ND2 haplotypes we recovered in this study (Table 1), especially the six haplotypes we found exclusively in North Carolina (Supplemental Data, Table S2), it is worth emphasizing that these haplotypes are all quite closely related to one another phylogenetically; none of the North Carolina haplotypes stand out as being strongly differentiated from an evolutionary perspective (Fig. 2). As such, we argue that the microsatellite results, which provide a more detailed and finer-scale perspective on the population genetics of Gopher Frogs in North Carolina than do the mtDNA data, should play a stronger role in terms of informing management. Although they contain some sample size limitations, the microsatellite analyses suggest that for the five populations for which we had $\geq$11 individuals, the populations have comparable levels of genetic variation as measured by parameters such as A, A_R, and H_O (Table 2). Although translocations have been successful in other species to alleviate detrimental genetic effects such as genetic load, inbreeding depression, and reduced genetic variation, that arise in small fragmented populations (Weeks et al., 2011), our microsatellite data indicate that there does not appear to be a particularly high diversity population of Gopher Frogs in North Carolina that might serve as a source for translocating individuals to other, lower diversity, populations in the state. Moreover, translocation carries with it several challenges including the potential for outbreeding depression and the loss of local adaptation following genetic mixing (Moritz, 1999); though the predicted probability of outbreeding depression in crosses between two populations of the same species is low for populations with the same karyotype, isolated for <500 yr, and that occupy similar environments (Frankham et al., 2011). Finally, disease spread among amphibians has been shown to be exacerbated by human activities, and the potential for translocations of a pathogen or infected host should also be considered (Price et al., 2016).

Two populations (Croatan and Lejeune) did show significant homozygote excesses and, as a result, significant inbreeding coefficients (F_{IS}; Table 2). F_{IS} was also positively correlated with kinship and negatively correlated with sample size. Therefore, while the overall level of genetic diversity might be similar among the five populations, the microsatellite data suggest that mating is occurring primarily among kin at Croatan and Lejeune (at least based on the samples from those locations that we were able to analyze). If these data are representative of the Croatan and Lejeune populations in general, it would mean that they are particularly vulnerable to inbreeding depression. Inbreeding depression is a decline in the fitness of the offspring that is a direct consequence of higher relatedness between mates. Inbreeding results in a reduction in the average heterozygosity of the offspring, which can reduce survival and fecundity either because decreased heterozygosity by itself decreases fitness, as has been shown for the major histocompatibility complex, or because such individuals are more likely to be homozygous for deleterious recessive alleles (Halverson et al., 2006). Halverson et al. (2006) found that inbreeding negatively affected the survival of Wood Frog (*Rana sylvatica*) larvae in the wild, suggesting that Gopher Frogs might also be sensitive to the effects of inbreeding depression. If relative sample size reflects relative population size at Croatan and Lejeune, introduction of individuals from other populations might be useful to alleviate this potential problem. However, before considering this action, it would be valuable to collect microsatellite data from additional samples from both of these populations if possible, and to evaluate the degree to which the sample sizes in this study most likely reflect 1) the overall relative population size at a site, 2) only a subpopulation at a site due to an inability to access all areas that likely have breeding individuals, or a combination of 1 and 2.

The relationship between dispersal and kin structure suggests that individuals may commonly return to the same breeding ponds year after year. As such, immediate family groups (parents, siblings) tend to be present within the same pond. Mean kinship among frogs within a pond is higher than mean kinship among frogs from different ponds, a pattern reflected in all five populations. Conserving not only genetic diversity, but reducing the likelihood of inbreeding, would likely be enhanced by maintaining multiple breeding ponds within a site at distances between 1 and 3 km. This may promote some dispersal between ponds while also preserving distinct familial lineages of frogs.

Management efforts should focus on facilitating metapopulation dynamics within each of the Gopher Frog sites in North Carolina by maintaining and establishing multiple breeding ponds at each site, and by enhancing connectivity between these breeding ponds. This could be accomplished by restoring long leaf pine savannah habitat between locations to enable corridors for animal movement (Taylor et al., 1993). Although translocation of individuals between the known Gopher Frog populations is feasible (NCWRC, 2018), we would not recommend doing that at this time based on the genetic data gathered to date. However, the significant inbreeding coefficients and high kinship estimates seen in the Lejeune and Croatan populations are potentially concerning because it could make them vulnerable to the effects of low genetic diversity and inbreeding depression. As such, further study of these two populations is clearly warranted.

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