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Cryptic Diversity and Conservation of Gopher Frogs across the Southeastern United States

Stephen C. Richter[1], Eric M. O'Neill[2,3], Schyler O. Nunziata[1,2], Andrew Rumments[1], Emily S. Gustin[1], Jeanne E. Young[4], and Brian I. Crother[5]

Identifying cryptic biodiversity is fundamental to evolutionary biology and to conservation efforts. This study investigated range-wide genetic diversity of Gopher Frogs, *Lithobates capito*, across the southeastern United States coastal plain to determine implications for taxonomy and conservation. We collected data for two mtDNA regions in 21 populations to identify genetic structure across the geographic distribution of the species. Based on population genetic, phylogenetic, and genealogical analyses, we recovered three reciprocally monophyletic mtDNA lineages corresponding to mainland coastal plain populations and two lineages within peninsular Florida. Breakpoints for these lineages did not occur in previously identified hotspots of amphibian phylogeographic breaks and did not follow currently recognized subspecies designations. We recommend these lineages be recognized as separate distinct population segments and be considered separately by the U.S. Fish and Wildlife Service for listing under the Endangered Species Act. Additionally, we propose an evolutionary hotspot for amphibians that deserves further attention.

An important aim in evolutionary biology and conservation is to identify cryptic biodiversity and understand how genetic variation within species is partitioned into populations and lineages and how historic geological, environmental, and biological processes influence genetic structure (Nelson, 1974; Avise, 1992; Kozak et al., 2008; Rissler and Smith, 2010). Comparative phylogeography has revealed geographic regions with phylogeographic breaks for multiple codistributed taxa (Avise, 1992; Swenson and Howard, 2005; Rissler and Smith, 2010). These breakpoints provide evidence that many taxa have similar evolutionary responses to biogeographic and environmental conditions and can be used to develop *a priori* hypotheses about predicted lineage breaks in unstudied species. Multiple breakpoints have been identified in the southeastern United States (Avise, 1992; Walker and Avise, 1998; Swenson and Howard, 2005), including hotspots for amphibian species (Rissler and Smith, 2010). In the southeastern coastal plain these areas include the Apalachicola basin in western Florida, the Mobile basin in Alabama, and northern peninsular Florida (Gilbert, 1987; Avise, 1992; Walker and Avise, 1998; Young and Crother, 2001; Pauly et al., 2007).

In this study, we investigated the phylogeographic patterns of gopher frogs, *Lithobates capito*, an endemic species of the Gulf and Atlantic Coastal Plains of the United States. Three subspecies of *L. capito* were historically recognized, the Carolina Gopher Frog, *L. capito capito*; the Florida Gopher Frog, *L. c. aesopus*; and the Dusky Gopher Frog, *L. c. sevosus*. However, current classification considers *L. capito* a single species, with no taxonomic breaks (Young and Crother, 2001; Frost et al., 2012), which might not account for cryptic genetic variation across the species' range.

Populations of *L. capito* have declined across much of its range at greater rates than other syntopic amphibian species because of habitat modification and destruction (Jensen and Richter, 2005). *Lithobates capito* is listed as IUCN near threatened and has a reduced distribution in North Carolina, South Carolina, Georgia, Florida (non-peninsular), and Alabama, and many populations are geographically isolated (Hammerson and Jensen, 2004; Jensen and Richter, 2005; Krysko et al., unpubl.). Peninsular Florida is the only portion of the range where the species' status appears stable (Jensen and Richter, 2005; Krysko et al., unpubl.). As a result, *L. capito* was recently petitioned for federal listing.

Species-level conservation risks loss of cryptic biodiversity in the form of distinct populations or genetic lineages (Purvis et al., 2005). The Endangered Species Act allows listing of distinct population segments of vertebrates that can be independently protected as threatened or endangered. Distinct genetic lineages that are geographically separate may be listed as distinct population segments, if warranted based on current status of the lineage (e.g., May et al., 2011). Because gopher frogs are in decline across much of their range, identification and protection of distinct population segments might be critical for preventing further range reduction.

Our objectives were to investigate the population genetic and phylogeographic pattern of *L. capito* across its range and determine implications for conservation. We analyzed two mtDNA regions and predicted to recover a phylogeographic breakpoint at the Apalachicola River basin based on congruence with other codistributed taxa (Avise, 1992; Rissler and Smith, 2010). Given the disparity of the species' status across the southeastern US, it is important to identify and protect all lineages of gopher frogs.

MATERIALS AND METHODS

Tissue samples were collected from localities throughout the range of *L. capito* (Fig. 1; Table 1). DNA was extracted from all tissues using the Qiagen DNEasy tissue protocol (Qiagen,

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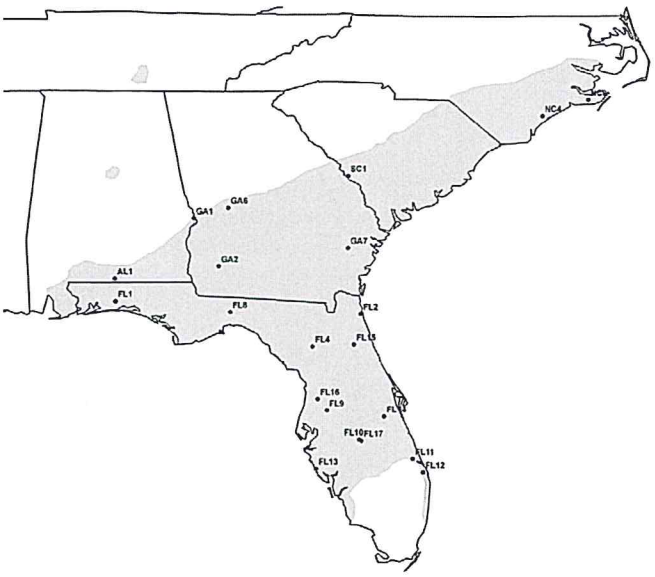

Fig. 1. Geographic distribution of gopher frogs (*Lithobates capito*) with sampling localities indicated. See Table 1 for site locations.

Inc., Valencia, CA, USA). We amplified the mitochondrial gene NADH dehydrogenase subunit 2 (ND2) using two primers developed for this study. The first was found in the methionine tRNA (5′–AGCTAAATAAGCTCTTGGGC) and the second was found in tryptophan tRNA (5′–CACTTAAGGCTTTGAAGGCC). We amplified a portion of the mitochondrial control region (CR) using CytbA-L (Goebel et al., 1999) and Hrana-1232 (Jaeger et al., 2001). The same primers were used to sequence the two mitochondrial regions along with two internal primers developed for this study in the ND2 gene (ND2for: 5′–TCTGRATACCTGAAGTCC; ND2rev: 5′–GTTTRGGGGCAAATCCTG) and an additional internal primer for the control region (CRINT1-U; Di Candia and Routman, 2007). PCR products were sequenced on an ABI 3730xl DNA Analyzer using a BigDye Terminator 3.1 Cycle Sequencing protocol (Applied Biosystems, Inc., Foster City, CA, USA). All sequence data were deposited in GenBank with accession numbers KJ566021–KJ566070 (control region) and KJ566071–KJ566120 (ND2).

We aligned regions of ND2 and CR separately using MUSCLE in GENEIOUS version R6 (created by Biomatters, available from: http://www.geneious.com/). We identified and removed a known repeat region found in the control region and concatenated the two regions for all analyses.

Table 1. Populations of *Lithobates capito* and *L. areolatus* sampled for mtDNA analysis.

Population	County	Species
AL1	Covington	*Lithobates capito*
FL1	Okaloosa	*L. capito*
FL2	Duval	*L. capito*
FL4	Alachua	*L. capito*
FL8	Leon	*L. capito*
FL9	Pasco	*L. capito*
FL10	Polk	*L. capito*
FL11	St. Lucie	*L. capito*
FL12	Martin	*L. capito*
FL13	Sarasota	*L. capito*
FL14	Osceola	*L. capito*
FL15	Putnam	*L. capito*
FL16	Hernando	*L. capito*
FL17	Polk	*L. capito*
GA1	Chattahoochee	*L. capito*
GA2	Baker	*L. capito*
GA6	Taylor	*L. capito*
GA7	Long	*L. capito*
NC3	Carteret	*L. capito*
NC4	Pender	*L. capito*
SC1	Aiken	*L. capito*
MO1	Cass	*L. areolatus*
OK1	Tulsa	*L. areolatus*

Population genetic analyses.—The number of independent populations of *L. capito* was inferred with the mtDNA dataset using BAPS v.6 (Bayesian Analysis of Population Structure; Corander et al., 2008). Population genetic clustering of DNA sequences in a hierarchical manner can provide an increased resolution in the estimate of genetic population structure (Willems et al., 2012; Cheng et al., 2013); therefore, non-spatial genetic mixture analyses were performed hierarchically at the individual level. Specifically, data from each cluster was used as input for subsequent analyses until no further structure could be detected. We also generated a 95% parsimony haplotype network in TCS v. 1.21 (Clement et al., 2000). This allowed us to visualize genealogical relationships of haplotypes at the population level. We then overlaid haplotypes on populations across the range to determine the relationship between geography and genealogy.

Phylogenetic analyses.—We performed a partitioned Bayesian phylogenetic analysis on the concatenated mtDNA dataset including all individuals using MrBayes v3.2 (Ronquist et al., 2011). Evolutionary models for both mtDNA regions were chosen using the Bayesian Information Criterion in jModelTest 2.1.3 (Darriba et al., 2012). For the MrBayes analyses, two independent runs, each with four Markov chains, were used with the default temperature parameter of 0.2. Default priors were used with random trees to start each Markov chain. Chains were run for one million generations with topology and model parameter estimates sampled every 100 generations. The first 25% of the sampled trees from each of the two runs were discarded as burn-in, yielding a posterior distribution of 15,000 sampled trees. Convergence was assessed using the standard deviation of split frequencies and the potential scale reduction factors (see MrBayes v3.2 manual available from: http://mrbayes.sourceforge.net/mb3.2_manual.pdf).

Estimating divergence times.—To determine whether known geological events can explain the observed phylogeographic patterns, we used lineage divergence times estimated using MrBayes v3.2. First, we tested the strict molecular clock model against the non-clock model by comparing the harmonic means of the marginal likelihoods of these two models using a Bayes Factor comparison in MrBayes v3.2. The harmonic mean of the marginal likelihood of the strict clock model was 35 log likelihood units better than the non-clock model. A difference exceeding 5 log likelihood units is considered strong evidence in favor of the better model

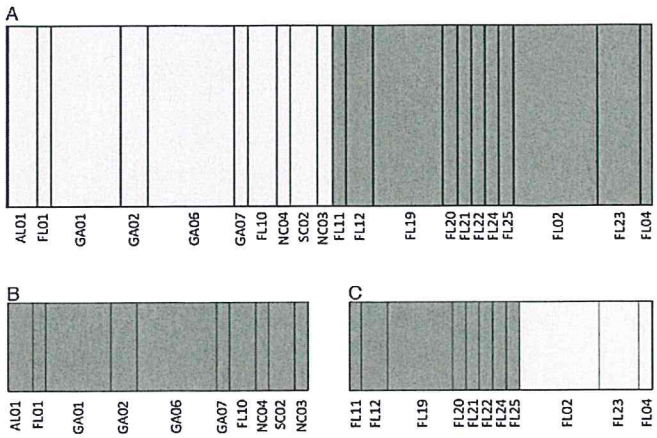

Fig. 2. Hierarchical clustering from Bayesian Analysis of Population Structure. (A) Initial mixture analysis including all *Lithobates capito* resulted in two groups in the optimal partition. (B) Hierarchical clustering of group one (Alabama, Georgia, South Carolina, North Carolina, and Florida panhandle) resulted in no further resolution. (C) Hierarchical clustering of group two (peninsular Florida) resulted in two additional groups (northern peninsular and southern peninsular groups).

(Kass and Raftery, 1995). We therefore used the strict clock model to estimate divergence times between the major clades of interest. No fossil calibrated mutation rate for ND2 is available for *Lithobates*; therefore, we followed Macey et al. (1998) and Schoville et al. (2011) by using two substitution rates, 0.57% and 0.69% per million years, which represent minimum and maximum estimates from a wide range of vertebrate ectotherms. We only used the ND2 data for estimating divergence times because similar estimates of mutation rates were not available for the control region.

RESULTS

The final alignment included 50 sequences and was 1305 bp in length (ND2 = 830 bp; CR = 475 bp). Because of missing data for some sequences, the mean ungapped sequence length was 1249 bp (Min = 1140; Max = 1303). Uncorrected sequence divergence between *L. areolata* and *L. capito* was 10.3–11.1%.

The initial mixture analysis from BAPS v.6 (Corander et al., 2008), including all *L. capito,* resulted in two groups in the optimal partition (marginal likelihood = −2316.0613; Fig. 2). Group one included Alabama, Georgia, South Carolina, North Carolina, and the panhandle of Florida. Hierarchical clustering of this group resulted in no further resolution. Group two included all populations in peninsular Florida. Hierarchical clustering of this group resulted in two additional groups: a northern peninsular group (FL2, FL4, FL23) and a southern peninsular group (FL11, FL12, FL24, FL19, FL20, FL21, FL22, FL25; marginal likelihood = −908.998).

The best-fit models, chosen by jModelTest 2.1.1, for ND2 and CR were HKY and TrN+I, respectively. After one million generations, the average standard deviation of the split frequencies between the two MrBayes runs was <0.01 and the potential scale reduction factors for all parameters were ≥1.00, indicating that the two runs had converged onto a stationary distribution.

The phylogenetic analysis of mtDNA resulted in a monophyletic *L. capito* containing three highly supported (PP = 1.0) allopatric clades, which match completely with the three groups identified in the hierarchical BAPS analysis (Fig. 3). Because these clades/groups are well supported by both population genetic and phylogeographic analyses, these likely represent independent lineages that have been genetically isolated from one another for a considerable length of time. One clade occurs in the coastal plain of

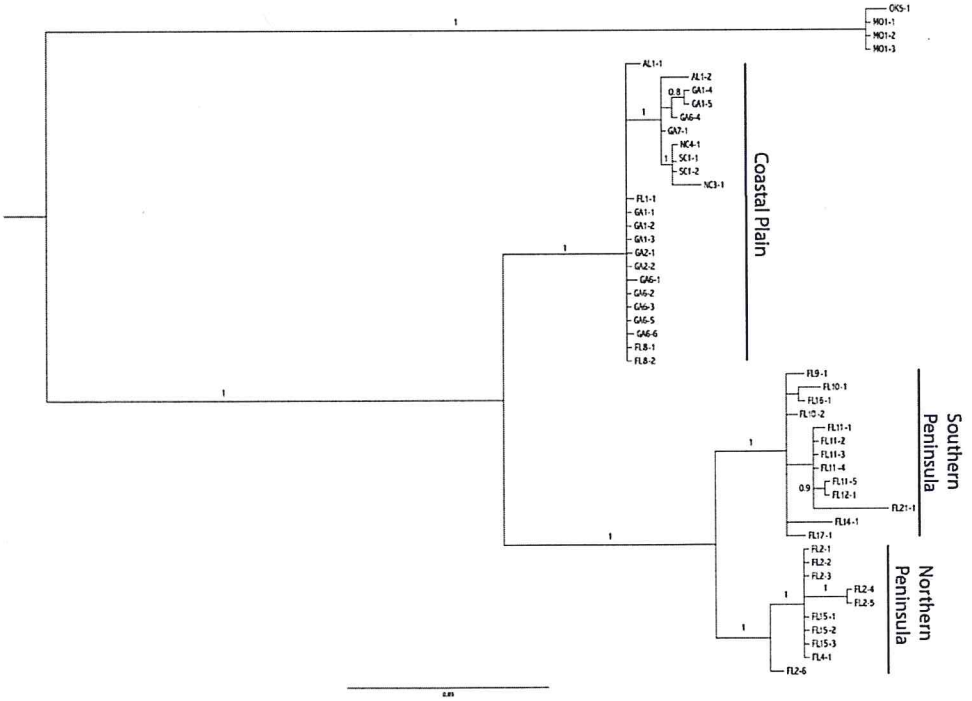

Fig. 3. Phylogenetic tree resulting from the partitioned Bayesian phylogenetic analysis of mtDNA data. Three highly supported clades (PP = 1.0) match exactly the groups defined in the Bayesian Analysis of Population Structure (see Fig. 2).

Table 2. Divergence time in millions of years using ND2 data only. Top values are for mutation rate/ma of 0.69% and bottom values are for 0.57%. The 95% Highest Posterior Density (HPD) is presented as a credibility interval for each analysis.

Lineages	Mean	Median	95% HPD
Coastal Plain vs. two Peninsular Lineages	1.9 2.3	1.9 2.3	1.3–2.4 1.4–3.1
Northern Peninsula vs. Southern Peninsula Lineages	1.1 1.3	1.0 1.2	0.7–1.5 0.8–1.8

Mississippi, Alabama, Georgia, South Carolina, North Carolina, and the panhandle of Florida and is hereafter referred to as the Coastal Plain Lineage. The second clade is located in northeastern Florida, while the third clade occurs in southern peninsular Florida referred to hereafter as the Northern Peninsular Lineage and Southern Peninsular Lineage, respectively. Several additional well-supported clades exist within these major clades; however, these were either not allopatric with respect to other samples, or not consistent with the results from BAPS, suggesting that these clades do not represent independent organismal lineages.

Divergence time between the Coastal Plain and the two Peninsular Lineages was estimated to be 1.9–2.3 mya, and between Northern Peninsular and Southern Peninsular Lineages was estimated to be 1.1–1.3 mya depending on the mutation rate used (Table 2). Corrected pairwise sequence divergence between *L. areolata* and the ingroup was 22.3–26.1%. Corrected sequence divergence between the Coastal Plain and Peninsular Lineages was 6.3–8.9%. Corrected sequence divergence between the Peninsular Lineages was 2.3–4.7%.

Genealogical patterns recovered with the 95% parsimony haplotype network corroborated phylogenetic and BAPS analyses with two networks that correspond to the Coastal Plain Lineage and the two Peninsular Lineages (Fig. 4). Nineteen of the 24 haplotypes found were from single populations (Fig. 5). The most likely ancestral haplotypes were haplotype C in the Coastal Plain Lineage (in populations FL8, GA2, and GA6; Fig. 5) and haplotype M in the Northern Peninsular Lineage (in populations FL2, FL15, and FL4). Within the Coastal Plain Lineage, haplotypes A–F were primarily found in the west and G–K primarily in the east (Fig. 5); the haplotype network depicts their genealogical affinities (Fig. 4). However, haplotypes E, F, and H were found in eastern populations AL1, GA1, and GA6 but shared a genealogical affinity with western haplotypes (Figs. 4, 5). This supports the hypothesis of southern Alabama and southwestern Georgia as a contact zone for two coastal plain lineages.

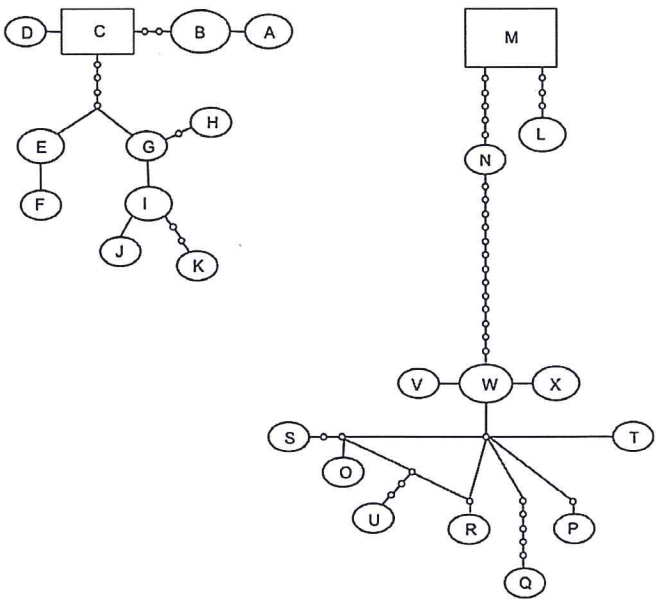

Fig. 4. Genealogical patterns represented by a 95% parsimony haplotype network for the Coastal Plain Lineage (left network) and the Northern and Southern Peninsular lineages (right network). Geographic distribution of haplotypes are depicted in Figure 5.

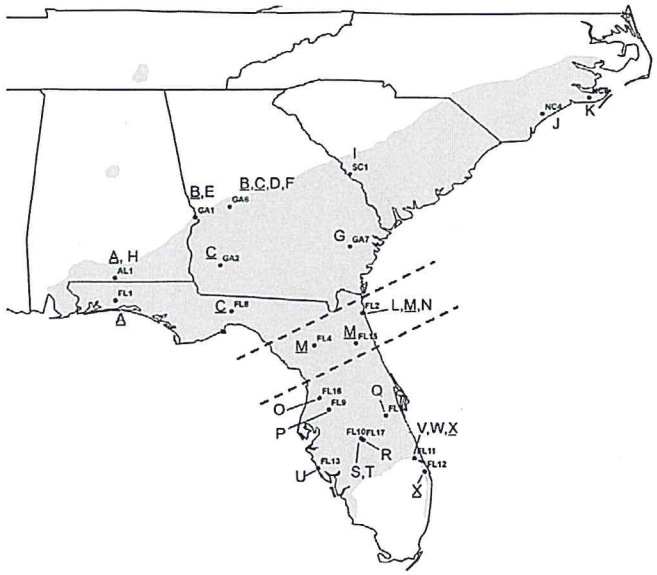

Fig. 5. Distribution of haplotypes (letters) with hypothesized genetic lineage boundaries (dotted lines) overlaid on geographic distribution and sampling localities of *Lithobates capito*. See Table 1 for site locations and Figure 4 for haplotype network. Nineteen haplotypes are unique to a single population. Five haplotypes are shared among multiple populations (represented by underlined letters).

DISCUSSION

Historical biogeographic factors have influenced gopher frog distribution and genetic connectivity across the geographic range. The mitochondrial DNA delineated three allopatric lineages within the range of *L. capito*: the Coastal Plain Lineage in the mainland US coastal plain, and the Northern and Southern Peninsular Lineages in peninsular Florida, which make up a monophyletic group. The lineages we recovered do not follow geographic boundaries of previously recognized subspecies of *L. capito*, thus we concur with Young and Crother (2001) that subspecific designations should be disregarded.

Based on historical biogeography of Florida, we estimate that the separation between the Coastal Plain Lineage and Peninsular Lineages formed when gopher frog habitat in peninsular Florida was isolated from the rest of the coastal plain, which occurred from the late Pliocene to early Pleistocene (i.e., 2.5–3 Mya; MacNeil, 1950; Neill, 1957; Gilbert, 1987; reviewed in Webb, 1990). This corresponds

roughly to our maximum estimate of divergence time between the Coastal Plain Lineage and the Peninsular Lineages (2.3 Mya; Table 2). Additionally, during this same time period ridges in the peninsula were disconnected as an archipelago by unsuitable lowland or coastal habitat (MacNeil, 1950; Neill, 1957; Webb, 1990; Marshall et al., 2000), which likely led to the separation of the Northern Peninsular and Southern Peninsular Lineages.

The current distribution of gopher frogs in Florida and historic biogeography of the region supports the Suwannee River basin as the breakpoint between the Coastal Plain and Northern Peninsular ESU, which corresponds to the Wicomico Shoreline and Suwannee Strait of the Miocene to late Pleistocene (reviewed in Webb, 1990) and currently is composed primarily of lowland terrestrial and wetland habitat. The exact breakpoint between the Northern and Southern Peninsular Lineages is not as clear, but our genetic analyses combined with historic biogeography of the region (MacNeil, 1950; Webb, 1990) and concordant patterns of mtDNA divergence in co-distributed taxa (McDonald and Hamrick, 1996; Branch et al., 2003; Mulvaney et al., 2005) suggest that refugial populations were concentrated on the Lake Wales Ridge for the southern lineage and in the Central Highlands for the northern lineage.

Although Remington (1968) identified northern Florida as a major zone of hybridization between peninsular and continental species and races, recent studies that reanalyzed Remington's data and extended analyses to include phylogeographic breaks and contact zones of closely related species did not identify this region as a hotspot for trees, birds, mammals (Swenson and Howard, 2005), or amphibians (Rissler and Smith, 2010). This region is evolutionarily important and we feel that additional research will reveal it as a hotspot for amphibians for the following reasons. (1) Only five of the 40 amphibian species included in Rissler and Smith (2010) are distributed into mid-peninsular Florida, and only two of these were originally studied across the peninsula to mainland. Neither species had a breakpoint in this region, but for one species with insufficient sampling, southern cricket frogs (*Acris gryllus*), the one population sampled from central Florida was a sister taxon to all other populations of the species (Gamble et al., 2008). (2) Although flatwoods salamanders, *A. cingulatum*, are not distributed into central Florida, the Suwanee River is a phylogeographic break (Pauly et al., 2007). (3) Seven of the 28 amphibian species currently distributed from the central peninsula into the mainland coastal plain have been studied with sufficient sampling. Four of these seven species were not included in the analyses of Rissler and Smith (2010) but have lineage breakpoints or contact zones in northern peninsular Florida: striped newts, *Notophthalmus perstriatus* (May et al., 2011); gopher frogs (this study); eastern newts, *N. viridescens* (Takahashi et al., unpubl.); and northern and southern dwarf sirens, *Pseudobranchus striatus* and *P. axanthus* (Liu et al., 2006). Additionally, other co-distributed taxa have a similar genetic break at the Suwannee River in northern Florida, including plants (Sewell et al., 1996; Maskas and Cruzan, 2000), turtles (Walker and Avise, 1998; Roman et al., 1999; Clostio et al., 2012), and mammals (Avise et al., 1983; Ellsworth et al., 1994).

The two genetic breaks we recovered did not correspond to our predicted location, the Apalachicola River basin, but this region is evolutionarily important for the species. The Apalachicola River extends north into the Flint River basin in southern Alabama and southwestern Georgia and corresponds to a contact-zone hotspot for amphibians (Rissler and Smith, 2010; Newman and Rissler, 2011) and other organisms (Remington, 1968; Swenson and Howard, 2005). This area represents a contact zone for gopher frogs as demonstrated by Alabama and western Georgia populations containing the most common haplotypes and haplotypes from this region clustering with eastern and western coastal plain populations on the phylogenetic tree.

Although we did not find reciprocal monophyly of groups within the Coastal Plain Lineage, presence of unique east and west haplotypes suggests there were potentially two lineages, presumably separated during the last glacial maximum (100,000–20,000 ya; Jackson et al., 2000). The biogeographic history of the Coastal Plain Lineage is further informed by disjunct populations: the northernmost-recorded populations of *L. capito* are just west of the Appalachian Mountains in the Cumberland Plateau of central Tennessee (Miller and Campbell, 1996) and Ridge and Valley Province of Alabama (Mount, 1975; Fig. 1). The Plateau has isolated areas of animal and plant species characteristic of the southeastern US coastal plain that represent relicts of interglacial periods and high sea levels of the Pleistocene (Jones, 1989; Corser, 2008). Although we were unable to obtain samples from these disjunct populations (none have been captured since the 1990s), we predict that they would strengthen support for a western coastal plain lineage.

The uncorrected sequence divergence between crawfish frogs and gopher frogs (10.3–11.1%) was at a level expected for different species. Within gopher frogs, the maximum uncorrected sequence divergence (4.3%) was relatively high but not as high as others have found for delineating new species. For example, Pauly et al. (2007) described *Ambystoma bishopi* as a distinct species from *A. cingulatum* based on morphological, nuclear, and mtDNA data with an mtDNA uncorrected sequence divergence of 5.6–6.2%. We recommend future research assess nuclear and morphological characters to address species-level questions within gopher frogs, especially given that the genealogical analyses resulted in two separate 95% parsimony networks. Additionally, although our study strongly delineates three lineages, all of which occur in Florida, further genetic data are required to determine the actual breakpoints among them. A large number of extant populations in the putative contact zone provide an opportunity for study, and we predict this zone will be in the southern Central Highlands because these ridges and existing, unsampled populations connect sampled populations from our Northern and Southern Peninsular Lineages.

Conservation and management.—Our study has direct implications for conservation and management. Our data support the recognition of coastal plain populations as a genetically distinct evolutionarily significant unit (ESU) from the two Peninsular Florida ESUs. *Lithobates capito* has been petitioned for federal listing under the Endangered Species Act, and the Act allows for the listing of distinct population segments (DPS) of vertebrate species. We recommend that the US Fish and Wildlife Service consider the Coastal Plain, Northern Peninsular, and Southern Peninsular ESUs as DPSs and evaluate their status individually if it determines that the entire species does not warrant federal protection. Peninsular Florida is the only region where the status of *L. capito* is stable, and it has more populations (>100 known;

K. Enge, pers. comm.) than the rest of the range combined. Based on current status of the species in Alabama (three known populations; M. Bailey, pers. comm.), Georgia (16 known populations; J. Jensen, pers. comm.), panhandle Florida (23 known populations; K. Enge, pers. comm.), South Carolina (<10 known populations; S. Bennett, pers. comm.), and North Carolina (seven known populations; M. Sisson, pers. comm.), the Coastal Plain DPS warrants immediate listing. We agree with Pennock and Dimmick (1997) that delineation of DPSs should not be limited to determination of ESUs based on genetic data, but sufficient evidence exists to delineate the coastal plain populations as a DPS based on a geographical break that corresponds to our genetic data.

This study also has implications for management of gopher frogs, specifically to informing translocation practices. A Florida Fish and Wildlife Conservation Commission (FWC) policy has allowed statewide translocation of gopher frogs as commensals of gopher tortoises (*Gopherus polyphemus*) when tortoises are moved from areas to be developed. The FWC has temporarily stopped this practice until effects of translocation are studied (Anna Farmer, pers. comm.). Because the translocations have a risk of disease transfer with no benefit to recipient populations, we do not support this policy of moving gopher frogs (or other commensals of gopher tortoises) to new natural areas. If the FWC considers reinstating the policy, the decision should be delayed until boundaries of ESUs are better defined with further genetic analyses, and individual gopher frogs should only be translocated to populations within their ESU, preferably only to populations within natural migration distance (1–5 km; reviewed in USFWS, 2012).

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