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Phylogeographic analyses of the southern leopard frog: the impact of geography and climate on the distribution of genetic lineages vs. subspecies

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Abstract

The southeastern United States is a major phylogeographic break hotspot for amphibians, but the processes underlying this hotspot remain to be explicitly tested. We test the correlation of genetic lineages with subspecies breaks in the southeastern United States and the association of such breaks with climate, using *Rana sphenocephala* as a case study, and place our results in the broader context of the Alabama-Appalachian suture zone (AL-Appalachian SZ). We use genetic and ecological methods to (i) determine whether genetic lineages are coincident with the AL-Appalachian SZ or the subspecies and (ii) test the correlation of major climatic breaks with genetic structure and morphological variation in *R. sphenocephala*. Bayesian phylogenetic analyses of the ND1 mtDNA gene and microsatellite cluster analyses revealed two distinct lineages with over 4% sequence divergence. The geographic distributions of the two lineages are concordant with the AL-Appalachian SZ but do not correspond to the ranges of the subspecies based on morphology. Mantel tests revealed that isolation by distance and historical barriers to gene flow, rather than climate, are the major drivers of genetic divergence at neutral loci. Examination of climate breaks across the Southeast revealed a pattern incongruent with suture zone hotspots, suggesting that phylogenetic structure has been driven primarily by historical factors, such as isolation, the Appalachian Mountains and the Apalachicola/Chattahoochee/Flint River Basin. However, climate breaks are consistent with the geographic distribution of the subspecies of *R. sphenocephala*, suggesting that environmental pressures may be driving divergence in morphological traits that outpaces molecular evolution.

Keywords: *Lithobates sphenocephalus*, microsatellites, ND1, *Rana sphenocephala*, systematics

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Introduction

An increase in the application and integration of molecular techniques and geographic information science (GIS) has made it possible to explicitly test contrasting hypotheses about the processes impacting the distribution of populations through time (Hugall *et al.* 2002; Graham *et al.* 2004; Richards *et al.* 2007; Avise 2009; Buckley 2009; Knowles 2009; Hickerson *et al.* 2010; Oneal *et al.* 2010). Such a priori hypotheses have become increasingly complex and synthesize information and methodologies from a wide variety of disciplines (Hickerson *et al.* 2010). In particular, these methods have been used in species delimitation towards the goal of understanding the historical and ecological processes driving divergence, and ultimately speciation (e.g. Rissler & Apodaca 2007; Stockman & Bond 2007; Bond & Stockman 2008).

The southeastern United States has a complex geologic history, defined by the uplift and erosion of the Appalachian Mountains, sea level fluctuations and

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drainage basin fragmentation and fusion (Avise 1996; Kozak *et al.* 2006; Soltis *et al.* 2006). North American hotspots of suture zones [defined as clusters of contact zones, hybrid zones and phylogeographic breaks (Swenson & Howard 2005)] for trees, birds and mammals (Remington 1968; Swenson & Howard 2004, 2005) and amphibians (Rissler & Smith 2010) are commonly located in the southeast, particularly in Alabama and extending into the Appalachian Mountains (Fig. 1). These studies provide a backdrop for more detailed comparative phylogeographic analyses, as the processes underlying this hotspot pattern remain to be tested. Specifically, Rissler & Smith (2010) discuss contrasting hypotheses regarding the roles of vicariance vs. divergent selection in forming and maintaining the Alabama-Appalachian suture zone (hereafter AL-Appalachian SZ) hotspot. These hotspots have previously been explained as the result of post-glacial expansion from refugia in Texas, Louisiana and Florida (Remington 1968; Swenson & Howard 2005; and references cited therein). Deep phylogenetic breaks have also been attributed to isolation and the lack of gene flow enforced by the Appalachian Mountains and major river drainages (e.g. Church *et al.* 2003; Zamudio & Savage 2003; Austin *et al.* 2004; Moriarty & Cannatella 2004; Jones *et al.* 2006; Kozak *et al.* 2006). The Southeast is also climatically heterogeneous; the Florida peninsula in particular has a climate and ecology distinct from the rest of the region (Means & Simberloff 1987; Avise 2000). In addition, much of the region has been free of glacial advances and retreats, creating a region with high biodiversity that has been maintained for millions of years (Lydeard & Mayden 1995; Boschung & Mayden 2004). Determining the processes that have driven current patterns of diversity (either genetic or morphological) in any one organism can, however, be a challenge.

Amphibian species serve as excellent model taxa for investigating the effects of geography and climate on genetic structure. Many species have large geographic ranges that span multiple physiographic provinces, and studies have shown that species with widespread geographic ranges—especially those with low vagility—often consist of multiple evolutionary lineages or

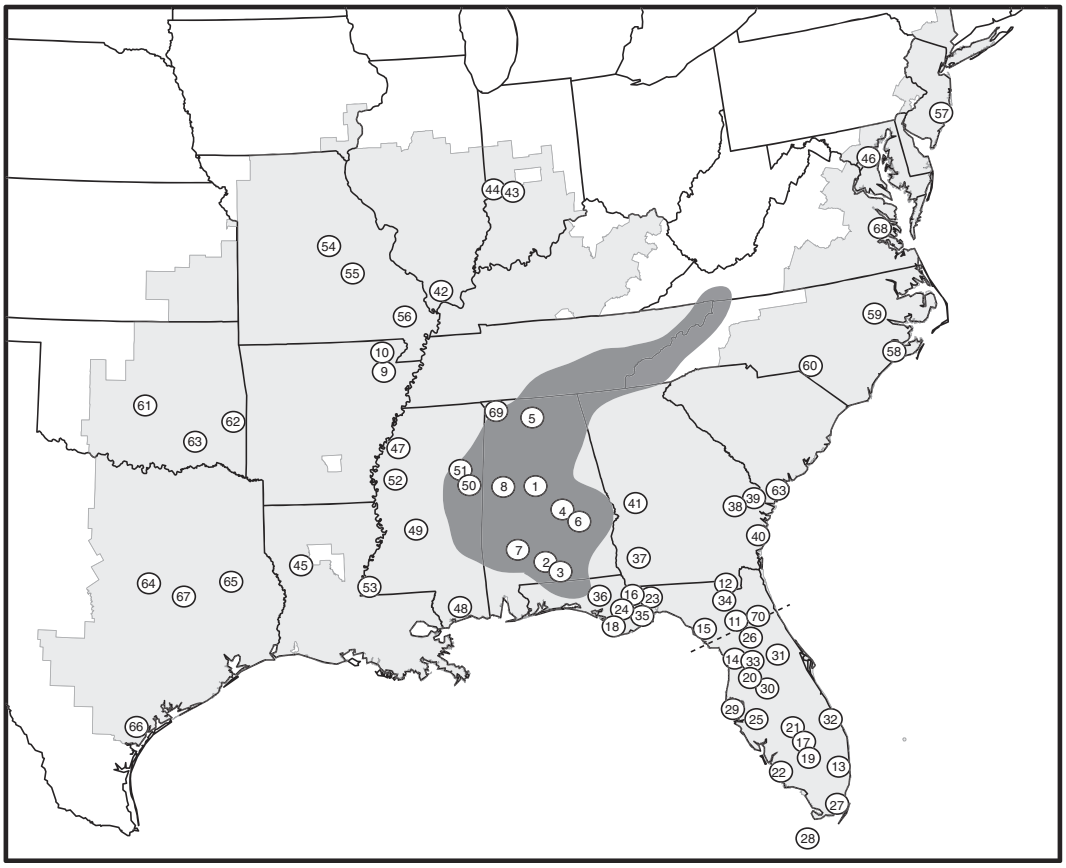

Fig. 1 Map of localities sampled for molecular analysis. Numbers correspond to Table 1. Light gray polygon depicts currently accepted species range (Natureserve 2008). Dark gray polygon depicts AL-Appalachian SZ (Rissler & Smith 2010). Dotted line indicates the boundary between the two subspecies (*Rana s. sphenocephala* south of the line and *Rana s. utricularia* north of the line).

cryptic species (e.g. Sites & Marshall 2003; Zamudio & Savage 2003; Hoffman & Blouin 2004; Sites & Marshall 2004; Lemmon *et al.* 2007; Pauly *et al.* 2007; Rissler & Apodaca 2007). Amphibians are also sensitive to small changes in climate; environmental differences have been shown to contribute to lineage divergence in some species (e.g. Graham *et al.* 2004; Kozak & Wiens 2006; Rissler & Apodaca 2007).

In this study, we assess how genetic diversity is partitioned geographically in a broad-ranging amphibian with two recognized subspecies and investigate the association of these distribution patterns with climate. We selected *Rana sphenocephala* for this case study for two reasons. In particular, the species' geographic range spans the AL-Appalachian SZ. In addition, the *Rana pipiens* species complex (*R. pipiens* Schreber *sensu lato*), of which *R. sphenocephala* is a member, consists of several species, delimited based on morphological, phylogenetic and allozyme data (Pace 1974; Case 1978; Hillis & Wilcox 2005). In contrast, the southern leopard frog, *R. sphenocephala* (Cope), currently contains only two subspecies, *R. s. sphenocephala* and *Rana s. utricularia*, that are geographically delimited based on morphological differences (Pace 1974). Studies at the species level within the complex and among other North American members of the genus *Rana* have reconstructed well-supported interspecific relationships (e.g. Case 1978; Hillis *et al.* 1983; Hillis & Wilcox 2005; Frost *et al.* 2006). However, few of these studies have investigated intraspecific relationships and evolutionary histories in the context of broader phylogeographic patterns (but see Hoffman & Blouin 2004; *R. pipiens*). Central to the understanding of biodiversity is the recognition and description of evolutionarily distinct lineages. However, traditional subspecies are frequently based on morphology (Mayr 1942), and these analyses can be confounded by convergent evolution or cryptic morphology (Agapow 2005). As such, subsequent genetic analyses often fail to corroborate these taxa (Ball & Avise 1992; Burbrink *et al.* 2000; Austin *et al.* 2002; Starkey *et al.* 2003; Moriarty & Cannatella 2004; Zink 2004; Gamble *et al.* 2008; Makowsky *et al.* 2010).

We address the following questions:

1 How is genetic diversity partitioned geographically in the widespread southern leopard frog (*R. sphenocephala*), and is it concordant with (i) the recognized subspecies' distributions or (ii) the AL-Appalachian SZ?
2 Are climate breaks within the *R. sphenocephala* species range concordant with (i) the subspecies break in peninsular Florida or (ii) the AL-Appalachian SZ, and are these climate breaks associated with genetic structure in *R. sphenocephala*?

Materials and methods

Species of interest

The leopard frog *R. sphenocephala* is widely distributed across the eastern United States, ranging from New Jersey, south to Florida and west to Oklahoma and eastern Texas. The species range spans 2 million km^2, including 23 states and the District of Columbia (Fig. 1). Two morphological subspecies are currently recognized: *R. s. sphenocephala*, found in peninsular Florida, and *R. s. utricularia*, found throughout the remainder of the species range (Pace 1974) (Fig. 1). We examined 186 specimens of *R. sphenocephala*, either collected or loaned from museums. Specimens were from 68 unique localities distributed throughout the species range (see Fig. 1 and see Table 1). Tissue consisted of toe clips or a portion of the liver stored in 95% ethanol. The ND1 sequence for one *R. sphenocephala* specimen (AY157645; Alachua Co., Florida) was downloaded from GenBank. Our outgroups consisted of one *R. blairi* sequence (AY157644) downloaded from GenBank and four *R. berlandieri* sequences generated in our study (GQ289048–50, GQ289071). It is worth noting that *R. sphenocephala* was recently proposed to be in the genus *Lithobates* (*Lithobates sphenocephalus*) (Frost *et al.* 2006). However, given that the name *Lithobates* was not necessitated to maintain monophyletic groups and that different herpetologists disagree with conclusions from that study, we follow Pauly *et al.* (2009) and continue to recognize the southern leopard frog in *Rana*.

Genetic analyses

DNA sequencing and microsatellite genotyping. DNA was extracted from tissue samples following the DNeasy blood and tissue protocol (Qiagen, Valencia, CA). Primers were created for the NADH dehydrogenase I (ND1) gene of the mtDNA genome, adapted from Hoffman & Blouin (2004): Rasp_ND1genefish_F (5′-CAA ATC CCC TTG CTA-3′) and Rasp_ND1genefish_R1 (5′-GCT CGA TTA GTC TCT GCA-3′), yielding an average of 590 bp of sequence data. This gene has been successfully used in reconstructing intraspecific relationships in a closely related species, *R. pipiens* (Hoffman & Blouin 2004).

We collected ND1 sequence data for 140 specimens (Table 1). A total PCR volume of 25 μL consisted of 1–20 ng of genomic DNA, 2.5 μL ThermoPol buffer (10X, New England BioLabs), 2.0 μL each primer (10 μM), 0.5 μL dNTPs (10 mM each dNTP), 0.2 μL Taq polymerase (New England BioLabs) and sterile distilled water. Amplification was carried out using the following protocol: an initial denaturation at 94 °C for 2 min, followed by 36 cycles of denaturation at 94 °C for 15 s,

Table 1 Individuals used in the genetic analyses. Subspecies designation is based strictly on the boundary depicted in Fig. 1. Associated voucher numbers are listed in Table S2 (Supporting information)

Genbank (ND1)	Subspecies	Haplotype	Map Code	State	Latitude	Longitude
GU182900	*Rana s. utricularia*	W16	1	Alabama	33.095	−87.057
GU182901*	*R. s. utricularia*	E6	2	Alabama	31.454	−86.787
GQ289112	*R. s. utricularia*	E2	3	Alabama	31.122	−86.649
GU182902	*R. s. utricularia*	W13	4	Alabama	32.505	−86.256
—†	*R. s. utricularia*		69	Alabama	34.900	−87.259
—†	*R. s. utricularia*		5	Alabama	34.585	−86.869
GQ289021	*R. s. utricularia*	W14	5	Alabama	34.585	−86.869
—†	*R. s. utricularia*		5	Alabama	34.585	−86.869
GQ289012	*R. s. utricularia*	W12	5	Alabama	34.585	−86.869
—†	*R. s. utricularia*		5	Alabama	34.585	−86.869
GQ289068	*R. s. utricularia*	W15	6	Alabama	32.341	−85.891
GQ289113	*R. s. utricularia*	W15	7	Alabama	31.720	−87.457
GQ289014	*R. s. utricularia*	W21	8	Alabama	33.090	−87.671
GQ289006	*R. s. utricularia*	W18	8	Alabama	33.090	−87.671
GQ289010	*R. s. utricularia*	W20	8	Alabama	33.115	−87.584
GQ289036	*R. s. utricularia*	W10	9	Arkansas	35.912	−90.780
GQ289034	*R. s. utricularia*	W10	10	Arkansas	36.187	−90.382
GQ289035	*R. s. utricularia*	W10	10	Arkansas	36.016	−90.595
GQ289081	*R. s. utricularia*	E13	11	Florida	29.652	−82.324
GQ289095*	*R. s. utricularia*	E26	11	Florida	29.652	−82.324
GQ289096	*R. s. utricularia*	E16	11	Florida	29.652	−82.324
GQ289097	*R. s. utricularia*	E18	11	Florida	29.652	−82.324
GQ289075	*R. s. utricularia*	E27	11	Florida	29.696	−82.345
—†	*R. s. utricularia*		11	Florida	29.730	−82.402
—†	*R. s. utricularia*		11	Florida	29.730	−82.402
—†	*R. s. utricularia*		11	Florida	29.730	−82.402
—†	*R. s. utricularia*		11	Florida	29.730	−82.402
—†	*R. s. utricularia*		11	Florida	29.730	−82.402
GQ289119	*R. s. utricularia*	E3	12	Florida	30.321	−82.411
GQ288997	*R. s. utricularia*	E27	15	Florida	29.688	−83.171
—†	*R. s. utricularia*		15	Florida	29.688	−83.171
GQ289000	*R. s. utricularia*	E16	15	Florida	29.688	−83.171
—†	*R. s. utricularia*		15	Florida	29.688	−83.171
GQ289067	*R. s. utricularia*	E25	16	Florida	30.478	−84.416
GQ289037	*R. s. utricularia*	E3	18	Florida	30.117	−85.189
GQ289039*	*R. s. utricularia*	E4	18	Florida	30.136	−85.200
GQ289057	*R. s. utricularia*	E3	23	Florida	30.007	−84.007
GQ289051	*R. s. utricularia*	E26	23	Florida	30.380	−84.311
GQ289041	*R. s. utricularia*	E3	24	Florida	30.260	−84.972
—†	*R. s. utricularia*		70	Florida	29.771	−81.867
GQ289121	*R. s. utricularia*	E7	34	Florida	30.058	−82.420
GQ288996	*R. s. utricularia*	E1	35	Florida	30.186	−84.418
JN185215	*R. s. utricularia*	E8	36	Florida	30.471	−85.586
JN185216	*R. s. utricularia*	E3	36	Florida	30.471	−85.586
JN185217	*R. s. utricularia*	E8	36	Florida	30.471	−85.586
JN185218	*R. s. utricularia*	E8	36	Florida	30.471	−85.586
GU182899	*R. s. utricularia*	E3	37	Georgia	31.271	−84.497
GQ289025	*R. s. utricularia*	E3	38	Georgia	31.992	−81.383
GQ289026	*R. s. utricularia*	E3	38	Georgia	31.992	−81.383
GQ289042	*R. s. utricularia*	E3	38	Georgia	31.992	−81.383
GQ289038	*R. s. utricularia*	E3	38	Georgia	31.992	−81.383
GQ289030	*R. s. utricularia*	E3	38	Georgia	31.992	−81.383
GQ289055	*R. s. utricularia*	E5	39	Georgia	32.104	−81.306
GQ289076	*R. s. utricularia*	E3	40	Georgia	31.398	−81.280
GQ289078	*R. s. utricularia*	E3	40	Georgia	31.398	−81.280

Table 1 *Continued*

Genbank (ND1)	Subspecies	Haplotype	Map Code	State	Latitude	Longitude
GQ289077	*R. s. utricularia*	E3	40	Georgia	31.398	−81.280
GQ289031	*R. s. utricularia*	E3	41	Georgia	32.578	−84.269
GQ289032	*R. s. utricularia*	W17	41	Georgia	32.578	−84.269
GQ289033	*R. s. utricularia*	E3	41	Georgia	32.578	−84.269
GQ289043	*R. s. utricularia*	W23	42	Illinois	37.108	−89.327
GQ289044	*R. s. utricularia*	W9	42	Illinois	37.108	−89.327
GQ289047	*R. s. utricularia*	W9	42	Illinois	37.108	−89.327
GQ289046	*R. s. utricularia*	W20	42	Illinois	37.108	−89.327
GQ289045	*R. s. utricularia*	W9	42	Illinois	37.108	−89.327
GQ289018	*R. s. utricularia*	W19	43	Indiana	39.509	−87.059
GQ288992*	*R. s. utricularia*	W20	44	Indiana	39.538	−87.427
GQ289022*	*R. s. utricularia*	W20	44	Indiana	39.538	−87.427
GQ289004	*R. s. utricularia*	W9	45	Louisiana	31.753	−92.917
GQ289005	*R. s. utricularia*	W10	45	Louisiana	31.753	−92.917
—†	*R. s. utricularia*		45	Louisiana	31.753	−92.917
GQ289115*	*R. s. utricularia*	E22	46	Maryland	39.049	−76.806
—†	*R. s. utricularia*		47	Mississippi	34.132	−90.530
GQ289003	*R. s. utricularia*	W11	47	Mississippi	34.132	−90.530
—†	*R. s. utricularia*		47	Mississippi	34.132	−90.530
—†	*R. s. utricularia*		47	Mississippi	34.132	−90.530
GQ289104	*R. s. utricularia*	W24	48	Mississippi	30.554	−89.084
GQ289105	*R. s. utricularia*	W25	48	Mississippi	30.554	−89.084
GQ289101	*R. s. utricularia*	W20	49	Mississippi	32.325	−90.151
GQ289102	*R. s. utricularia*	W18	49	Mississippi	32.325	−90.151
GQ288991*	*R. s. utricularia*	W20	50	Mississippi	33.302	−88.615
—†	*R. s. utricularia*		50	Mississippi	33.302	−88.615
GQ288990	*R. s. utricularia*	W20	50	Mississippi	33.302	−88.615
—†	*R. s. utricularia*		50	Mississippi	33.302	−88.615
—†	*R. s. utricularia*		50	Mississippi	33.302	−88.615
—†	*R. s. utricularia*		50	Mississippi	33.302	−88.615
—†	*R. s. utricularia*		50	Mississippi	33.302	−88.615
—†	*R. s. utricularia*		50	Mississippi	33.302	−88.615
GQ288989	*R. s. utricularia*	W20	51	Mississippi	33.525	−88.763
GQ289103	*R. s. utricularia*	W22	52	Mississippi	33.485	−90.621
—†	*R. s. utricularia*		53	Mississippi	31.071	−91.506
—†	*R. s. utricularia*		53	Mississippi	31.071	−91.506
—†	*R. s. utricularia*		53	Mississippi	31.071	−91.506
GQ289008	*R. s. utricularia*	W24	53	Mississippi	31.071	−91.506
GQ289016	*R. s. utricularia*	W24	53	Mississippi	31.071	−91.506
—†	*R. s. utricularia*		53	Mississippi	31.071	−91.506
—†	*R. s. utricularia*		53	Mississippi	31.071	−91.506
GQ289063	*R. s. utricularia*	W10	54	Missouri	38.582	−92.102
GQ289059	*R. s. utricularia*	W10	54	Missouri	38.586	−92.121
GQ289060	*R. s. utricularia*	W10	54	Missouri	38.586	−92.121
GQ289061	*R. s. utricularia*	W10	54	Missouri	38.586	−92.121
GQ289062	*R. s. utricularia*	W9	54	Missouri	38.586	−92.121
GQ289058	*R. s. utricularia*	W9	54	Missouri	38.590	−92.132
GQ289056	*R. s. utricularia*	W10	54	Missouri	38.594	−92.056
GQ289064	*R. s. utricularia*	W7	55	Missouri	37.951	−91.511
GQ289065	*R. s. utricularia*	W10	56	Missouri	36.988	−90.148
GU182895	*R. s. utricularia*	E23	57	New Jersey	39.649	−74.443
GU182896	*R. s. utricularia*	E23	57	New Jersey	39.649	−74.443
GU182897	*R. s. utricularia*	E24	57	New Jersey	39.649	−74.443
GU182898	*R. s. utricularia*	E24	57	New Jersey	39.649	−74.443
—†	*R. s. utricularia*		57	New Jersey	39.649	−74.443
JN185211*	*R. s. utricularia*	E24	57	New Jersey	39.649	−74.443

Table 1 *Continued*

Genbank (ND1)	Subspecies	Haplotype	Map Code	State	Latitude	Longitude
JN185212*	*R. s. utricularia*	E23	57	New Jersey	39.649	−74.443
JN185213	*R. s. utricularia*	E23	57	New Jersey	39.647	−74.443
JN185214	*R. s. utricularia*	E23	57	New Jersey	39.647	−74.443
—†	*R. s. utricularia*		58	North Carolina	34.915	−76.892
GQ289094	*R. s. utricularia*	E3	58	North Carolina	34.915	−76.892
GQ289082	*R. s. utricularia*	E32	58	North Carolina	34.915	−76.892
GQ289114*	*R. s. utricularia*	E21	59	North Carolina	35.673	−77.234
GQ289052*	*R. s. utricularia*	E20	60	North Carolina	34.828	−79.397
GQ288993	*R. s. utricularia*	W1	61	Oklahoma	35.222	−97.269
GQ288994	*R. s. utricularia*	W9	61	Oklahoma	35.222	−97.269
—†	*R. s. utricularia*		61	Oklahoma	35.222	−97.269
GQ288995	*R. s. utricularia*	W4	61	Oklahoma	35.222	−97.269
GQ289001	*R. s. utricularia*	W4	62	Oklahoma	34.877	−94.706
GQ289002	*R. s. utricularia*	W2	63	Oklahoma	34.408	−95.917
GQ289066	*R. s. utricularia*	W4	64	Texas	31.404	−96.983
—†	*R. s. utricularia*		65	Texas	31.388	−94.975
GQ289019	*R. s. utricularia*	W3	65	Texas	31.388	−94.975
—†	*R. s. utricularia*		65	Texas	31.388	−94.975
GQ289020	*R. s. utricularia*	W6	65	Texas	31.388	−94.975
—†	*R. s. utricularia*		65	Texas	31.388	−94.975
GQ289011	*R. s. utricularia*	W5	65	Texas	31.388	−94.975
GQ289118	*R. s. utricularia*	W8	66	Texas	28.267	−97.353
GQ289120	*R. s. utricularia*	W2	67	Texas	31.119	−96.355
GQ289093	*R. s. utricularia*	E3	68	Virginia	37.407	−76.714
GQ289108	*Rana s. sphenocephala*	E29	13	Florida	26.210	−80.362
—†	*R. s. sphenocephala*		13	Florida	26.259	−80.856
GQ289040	*R. s. sphenocephala*	E10	14	Florida	28.851	−82.219
GQ289013	*R. s. sphenocephala*	E13	14	Florida	28.870	−82.413
GQ289111	*R. s. sphenocephala*	E17	17	Florida	26.877	−81.173
GQ289079	*R. s. sphenocephala*	E17	19	Florida	26.624	−80.935
GQ289074	*R. s. sphenocephala*	E17	19	Florida	26.624	−80.919
GQ289072*	*R. s. sphenocephala*	E17	19	Florida	26.624	−80.919
GQ289073	*R. s. sphenocephala*	E16	19	Florida	26.646	−81.420
—†	*R. s. sphenocephala*		19	Florida	26.448	−80.980
GQ288998	*R. s. sphenocephala*	E16	20	Florida	28.472	−82.067
—†	*R. s. sphenocephala*		20	Florida	28.647	−82.336
GQ289117	*R. s. sphenocephala*	E13	21	Florida	27.209	−81.242
GQ289086	*R. s. sphenocephala*	E15	22	Florida	26.333	−81.717
GQ289083	*R. s. sphenocephala*	E17	22	Florida	26.333	−81.717
GQ289084	*R. s. sphenocephala*	E13	22	Florida	26.333	−81.717
GQ289085	*R. s. sphenocephala*	E28	22	Florida	26.333	−81.717
GQ289087	*R. s. sphenocephala*	E17	22	Florida	26.333	−81.717
GQ289088*	*R. s. sphenocephala*	E17	22	Florida	26.333	−81.717
GQ289089	*R. s. sphenocephala*	E17	22	Florida	26.333	−81.717
GQ289090	*R. s. sphenocephala*	E17	22	Florida	26.333	−81.717
GQ289091	*R. s. sphenocephala*	E13	22	Florida	26.333	−81.717
GQ289092	*R. s. sphenocephala*	E30	22	Florida	26.683	−81.733
—†	*R. s. sphenocephala*		22	Florida	26.333	−81.717
—†	*R. s. sphenocephala*		22	Florida	26.333	−81.717
—†	*R. s. sphenocephala*		22	Florida	26.683	−81.733
GQ289110	*R. s. sphenocephala*	E11	25	Florida	27.523	−82.161
GQ289015	*R. s. sphenocephala*	E13	26	Florida	29.336	−82.017
—†	*R. s. sphenocephala*		26	Florida	29.336	−82.017
—†	*R. s. sphenocephala*		26	Florida	29.336	−82.017
GQ289007	*R. s. sphenocephala*	E13	26	Florida	29.336	−82.017
—†	*R. s. sphenocephala*		26	Florida	29.108	−81.841

Table 1 *Continued*

Genbank (ND1)	Subspecies	Haplotype	Map Code	State	Latitude	Longitude
GQ289116	*R. s. sphenocephala*	E29	27	Florida	25.331	−80.493
GQ289098	*R. s. sphenocephala*	E17	27	Florida	25.399	−80.572
GQ289099	*R. s. sphenocephala*	E31	27	Florida	25.399	−80.572
GQ289100	*R. s. sphenocephala*	E17	27	Florida	25.399	−80.572
—†	*R. s. sphenocephala*		27	Florida	25.399	−80.572
GQ289109	*R. s. sphenocephala*	E19	28	Florida	24.706	−81.383
GQ289106	*R. s. sphenocephala*	E14	29	Florida	27.715	−82.686
GQ289107	*R. s. sphenocephala*	E14	29	Florida	27.715	−82.686
GQ289080	*R. s. sphenocephala*	E17	30	Florida	28.166	−81.721
—†	*R. s. sphenocephala*		30	Florida	27.844	−81.531
—†	*R. s. sphenocephala*		30	Florida	27.844	−81.531
GU182903	*R. s. sphenocephala*	E13	31	Florida	28.825	−81.312
GU182904	*R. s. sphenocephala*	E12	31	Florida	28.825	−81.312
GQ289053	*R. s. sphenocephala*	E17	32	Florida	27.252	−80.313
GQ289054	*R. s. sphenocephala*	E29	32	Florida	27.252	−80.313
GQ289069	*R. s. sphenocephala*	E17	32	Florida	27.305	−80.342
GQ289070	*R. s. sphenocephala*	E17	32	Florida	27.305	−80.342
GQ288999	*R. s. sphenocephala*	E9	33	Florida	28.607	−82.021
—†	*R. s. sphenocephala*		33	Florida	28.823	−81.971

*Samples used in ND1 sequence analysis only.
†Samples used in microsatellite analysis only.

annealing at 48 °C for 15 s, extension at 72 °C for 30 s and a final extension at 72 °C for 1 min.

PCR products were cleaned with a QIAquick PCR purification kit (Qiagen) and sequenced in both directions at Macrogen (ABI 3730XL, Seoul, South Korea) or the University of Alabama Molecular Laboratory (ABI 3100, Tuscaloosa, AL). Sequences were combined in SEQUENCHER v. 4.6 (Gene Codes Corporation). All sequences were deposited in GenBank (http://www.ncbi.nlm.nih.gov) (see Table 1 for accession numbers).

A total of 166 specimens (Table 1) were genotyped for six microsatellite loci that were developed for *R. sphenocephala* (McKee *et al*. 2011): Rasp03, Rasp07, Rasp09, Rasp10, Rasp42 and Rasp67. Based on the PCR protocol used by McKee *et al.* (2011), a 12.5-μL reaction was used, containing 1.0 μL PCR Buffer II (10X, Applied Biosystems), 1.3 μL $MgCl_2$ (25 mM), 0.76 μL labelled primer (10 μM), 0.76 μL unlabelled primer (10 μM), 1.7 μL dNTP solution (5 mM), 1 U AmpliTaq (Applied Biosystems) and 2–5 ng of genomic DNA. Amplification was carried out according to the specifications in McKee *et al.* (2011), using either touchdown protocols or constant annealing temperatures. Fluorescently labelled primers were added to 10 μL Hi-Di formamide and 0.5 μL Genescan 500 ROX size standard (Applied Biosystems). Amplified products were genotyped on an ABI 3730 DNA Analyzer (Applied Biosystems) at the University of Maine, and peaks were scored automatically and confirmed manually using STRAND (University of California-Davis, Veterinary Genetics Lab).

Phylogenetic reconstruction: mtDNA sequence data. Sequences were aligned and manually edited using the ClustalW method in MACVECTOR v. 9.0 (MacVector, Inc.). The GTR+G model of evolution was selected by JMODELTEST (Posada 2008) under the AICc criterion. To minimize the problems associated with running a Bayesian analysis with identical nucleotide sequences, sequences were collapsed to unique haplotypes [TCS v.1.21 (Clement *et al.* 2000)], and only unique haplotypes were used in the phylogenetic analysis. We conducted a Bayesian analysis using MRBAYES 3.1 (Ronquist & Huelsenbeck 2003) with a GTR model of evolution and gamma-distributed rate variation across sites, random starting trees, two simultaneous runs of 10 million generations and sampling every 5000 generations. TRACER v.1.4.1 (Rambaut & Drummond 2007) was used to assess convergence and determine appropriate burn-in. The first 100 samples (500 000 generations) were omitted as burn-in. In addition to *R. sphenocephala*, four *R. berlandieri* specimens were sequenced and one *R. blairi* (AY157644; Hoffman & Blouin 2004) sequence was downloaded from GenBank to be used as outgroups based on previous molecular work within the genus *Rana* (Hillis & Wilcox 2005).

To test monophyly of the peninsular Florida subspecies *R. s. sphenocephala*, we conducted a Bayesian test of monophyly in PAUP* v.4.0b10 (Swofford 2000). We constructed a constraint tree [MACCLADE v.4.08 (Maddison & Maddison 2005)], constraining all peninsular Florida haplotypes to a monophyletic clade that

excluded all other haplotypes. The constraint tree and the set of post-burn-in Bayesian trees were imported into PAUP*, all outgroups were removed and all Bayesian trees that were not compatible with the constraint were filtered out. At the statistical significance level of $\alpha = 0.05$, the hypothesis of monophyly would be rejected if fewer than 5% of the Bayesian trees remained after filtering (Miller *et al.* 2002, Buschbom & Barker 2006, Weisrock *et al.* 2006, Linnen & Farrell 2007, Wüster *et al.* 2007; Spinks & Shaffer 2009; Fujita & Papenfuss 2010). Because three haplotypes are found both north and south of the subspecies boundary (Fig. 2), we conducted two separate tests of monophyly. The first constraint included all haplotypes occurring south of the subspecies boundary, and the second constraint included only haplotypes found south of the boundary but not north.

Bayesian analyses are known to overestimate probabilities and return very high posterior values even for data sets with little variation (Douady *et al.* 2003; Erixon *et al.* 2003). We therefore supplemented our primary phylogenetic Bayesian analysis with likelihood and parsimony measures of statistical support. Bootstrap analyses were run under the maximum-likelihood (ML) criterion in RAXML v.7.0.3 (Stamatakis 2006; Stamatakis *et al.* 2008) and maximum-parsimony (MP) in PAUP*, with each run consisting of 1000 iterations, using the tree bisection and reconstruction algorithm. The same model of sequence evolution was used in the Bayesian and ML analyses.

Average within- and between-lineage nucleotide diversity and sequence divergence were calculated in DNASP v.4.90.1 (Rozas *et al.* 2003). For this study, lineages were defined as those monophyletic clades with strong nodal support (≥0.95 Bayesian PP, ≥0.70 MP or ML) and high inter-clade genetic divergence relative to within-clade variation.

Genotype analyses: microsatellites. The number of alleles per locus was determined in ARLEQUIN v.3.5.1.2 (Excoffier & Lischer 2010). The program STRUCTURE v.2.3.3 (Pritchard *et al.* 2000; Falush *et al.* 2003), which implements a Bayesian clustering algorithm, was used to determine the number of genetically distinct clusters (*K*) of samples. STRUCTURE was run using the admixture model (Pritchard *et al.* 2000) and assuming correlation

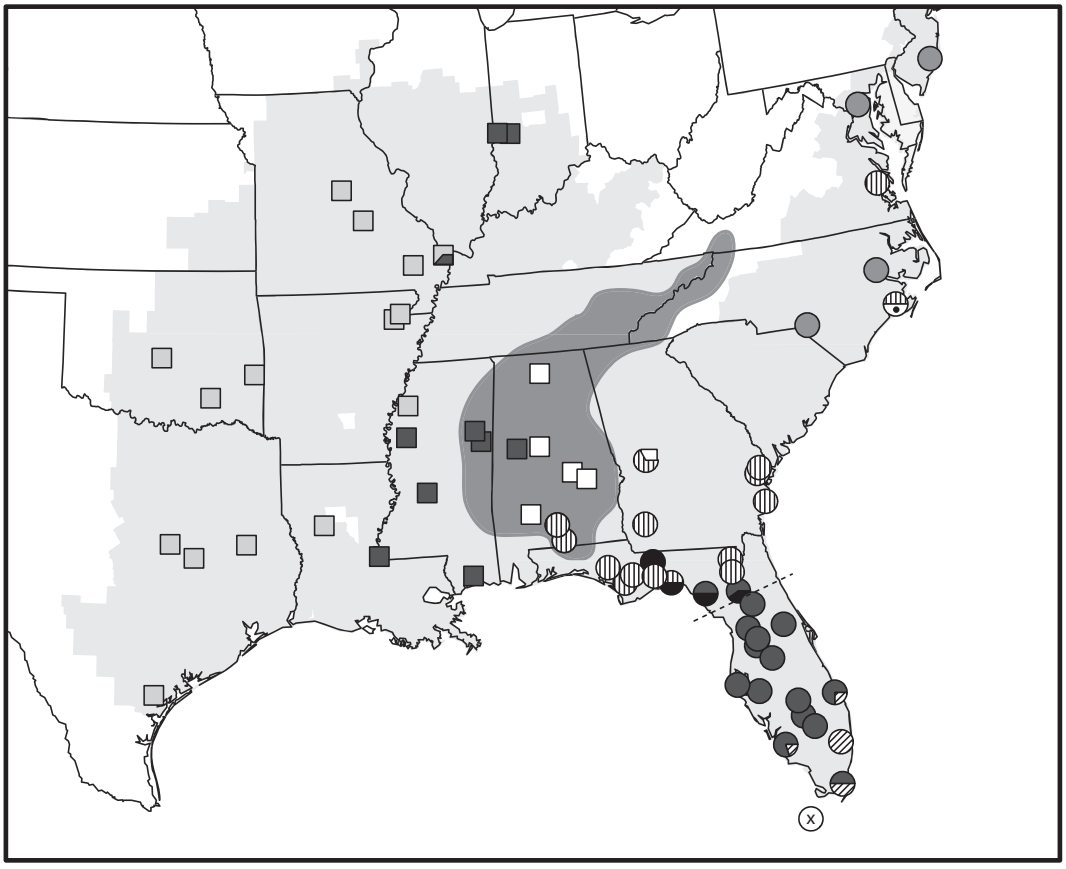

Fig. 2 Map of strongly supported ND1 haplotype clades. Shapes and shading correspond to Fig. 3. Squares, western lineage; circles, eastern lineage.

of allele frequencies among clusters (Falush *et al.* 2003). No other a priori population information was used. We tested the range of possible numbers of clusters from $K = 1$ to 10. For each K, a total of 20 trials were run, each consisting of 500 000 iterations with a burn-in of 50 000. The appropriate number of genetically distinct clusters was identified by selecting the K with the highest posterior probability (Pritchard *et al.* 2000) and confirmed using the ad hoc statistic ΔK described by Evanno *et al.* (2005). STRUCTURE assigns individuals to clusters by analysing allele frequency data and assigning probability values q_{k1}, q_{k2}, …, q_{kn}, where q_k is the proportion of the individual's ancestry from cluster k. Individuals of mixed ancestry were identified as those with values of q between 0.10 and 0.90 (Vähä & Primmer 2005).

Climate analyses

Climate has been shown to affect genetic and morphological evolution across many different taxa (e.g. Mayr 1942, 1982; Phillimore & Owens 2006). To test the correlation between genetic structure in *R. sphenocephala* and the spatial patterning of climate variation within the species range, a partial Mantel test was run in the R PACKAGE v.3.01 (Casgrain & Legendre 2001) using a Euclidian distance matrix of the climate variables extracted for each locality associated with a genetic sample (see below for elaboration), compared to the mtDNA genetic distance matrix (pairwise uncorrected p), and controlling for geographic distance. This test allowed us to determine whether genetic divergence in *R. sphenocephala* is associated with environmental divergence. Alternatively, because of low vagility, another major factor in lineage divergence among anurans is isolation by distance (e.g. Hoffman & Blouin 2004). To test this hypothesis for *R. sphenocephala*, we conducted a Mantel test comparing the Euclidian geographic distance matrix constructed from the latitude and longitude information for each specimen and the mtDNA genetic distance matrix, using 999 permutations.

We then investigated where major climatic breaks occur within the *R. sphenocephala* geographic range. First, an ecological niche model was generated for *R. sphenocephala* by MAXENT v.3.2.19 (Phillips *et al.* 2006), using natural history collection species occurrence data downloaded from the online databases HerpNET (herpnet.org) and GBIF (gbif.org), and 19 WorldClim bioclimatic layers (Hijmans *et al.* 2005) for temperature and precipitation. The spatial resolution of the climate layers was 1 km^2, and the climate data used to create the layers were restricted to records from 1950 to 2000 (Hijmans *et al.* 2005). The resulting niche model was used as a probability distribution to weight the locations of random points so that the random points were concentrated in areas with a higher probability of species occurrence. A total of 10 000 random points were generated using the Hawth's Tools extension in ARCGIS v.9.1 (Environmental Systems Research Institute, Redlands, CA, USA), and climate data were extracted from each layer for each of these localities in DIVA v.5.4 (Hijmans *et al.* 2001). The data for each variable were standardized in STATISTICA by subtracting each value from the sample mean and dividing the result by the sample standard deviation. A k-means cluster analysis was run, classifying the random points into two clusters. Because the k-means analyses were run to determine the correlation between environmental breaks and either the geographic distributions of the *R. sphenocephala* subspecies or the major southeastern US phylogenetic break, the number of clusters was set a priori to two.

The geographic boundary between the two subspecies of *R. sphenocephala* separates peninsular Florida from the rest of the species' geographic range (Pace 1974; and see Fig. 1), and the Florida peninsula is distinct in climate and habitat from the more temperate regions that make up the rest of the species range (Means & Simberloff 1987; Avise 2000). To further examine environmental breaks occurring in Florida, a second k-means analysis used only the points that fell out in the coastal cluster of the first k-means analysis (see Results). This analysis also classified the random points into two clusters.

A principal components analysis (PCA) was run using the climate data extracted from the 10 000 random points to determine the relative contribution of each climate variable to environmental variation between the clusters, and a MANOVA was used to assess statistical significance. These analyses were performed twice: first using all 10 000 random points, with the peninsular Florida cluster separated and the remainder of the points grouped into a second single cluster; and the second analysis using only the points in the coastal cluster, with the peninsular Florida cluster separated from the remainder of the coastal points.

Results

Genetic analyses

Sequence lengths for the ND1 region of the mtDNA genome averaged around 590 bp. The 140 *R. sphenocephala* specimens consisted of 57 unique haplotypes with 70 polymorphic sites (i.e. singletons). Bayesian analyses identified two strongly supported major lineages: an eastern lineage (PP = 1.0) and a western lineage (PP = 0.99), distributed in distinct geographic space

Table 2 Population genetics results from DNASP for the eastern and western lineages of *Rana sphenocephala*, from ND1 sequence data

Clade	*h*	*k*	pi (π)	% SD
Western	25	7.132	0.01372	1.37
Eastern	32	5.19	0.00998	0.97
Between clades	57	23.116	0.02704	4.45

h is the number of haplotypes, *k* is the average number of nucleotide differences, pi (π) is the nucleotide diversity per site and % SD is the percent sequence divergence.

(Figs 2 and 3). This phylogenetic break for *R. sphenocephala* is not congruent with the geographic distribution of the subspecies in peninsular Florida. Instead, the break occurs in eastern Alabama and western Georgia, congruent with the AL-Appalachian SZ (Rissler & Smith 2010). Haplotypes between lineages differed by an average of 23.12 characters, and average pairwise sequence divergence between lineages was 4.45%. Average sequence divergence within the western lineage was 1.37%, and average sequence divergence within the eastern lineage was 0.97% (Table 2). The Bayesian test of monophyly retained 1.6% of post-burn-in trees when all peninsular Florida haplotypes were constrained to a monophyletic clade and retained no trees under the constraint omitting haplotypes found north of the subspecies boundary, thus rejecting the hypothesis of monophyly of peninsular Florida.

Haplotypes within the western lineage were partitioned geographically west-east, and basal resolution of the three subclades was not strongly supported (Fig. 3).

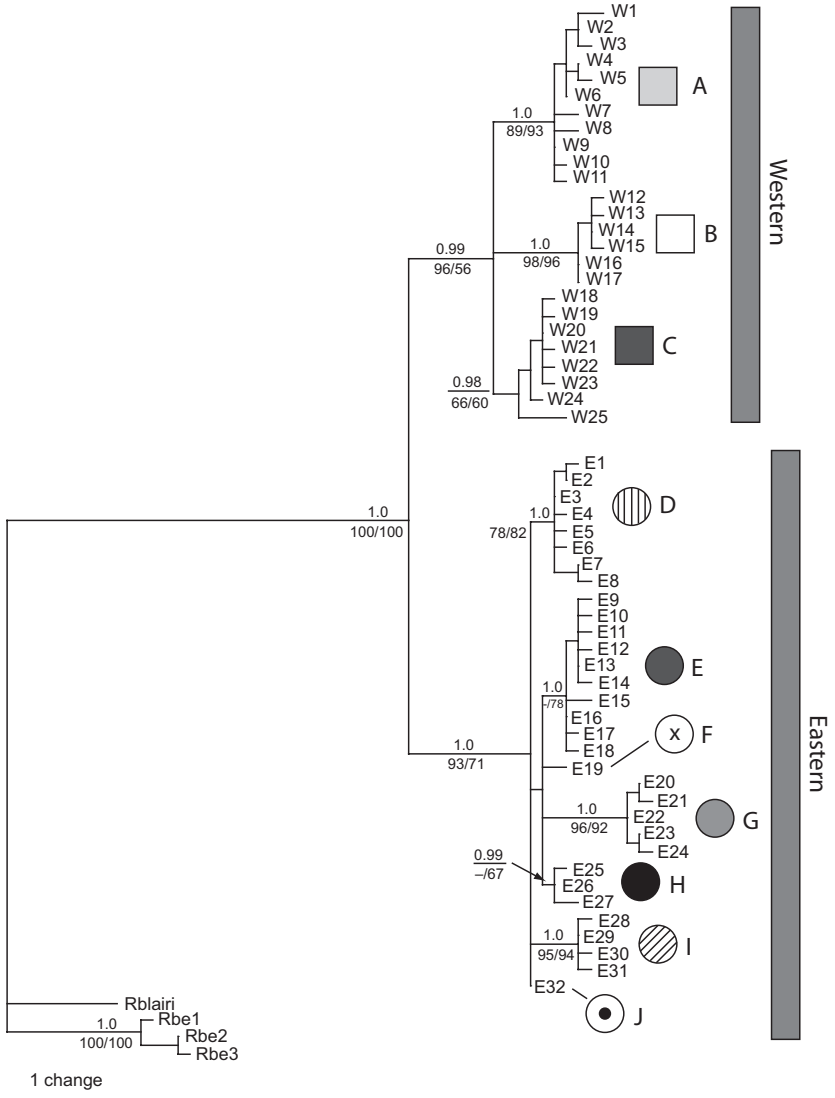

Fig. 3 Bayesian gene tree for the ND1 gene, with Bayesian posterior probabilities above nodes and maximum-parsimony/maximum-likelihood bootstraps ≥50 below nodes. Shapes and shading correspond to Fig. 2. Tip labels correspond to Table 1.

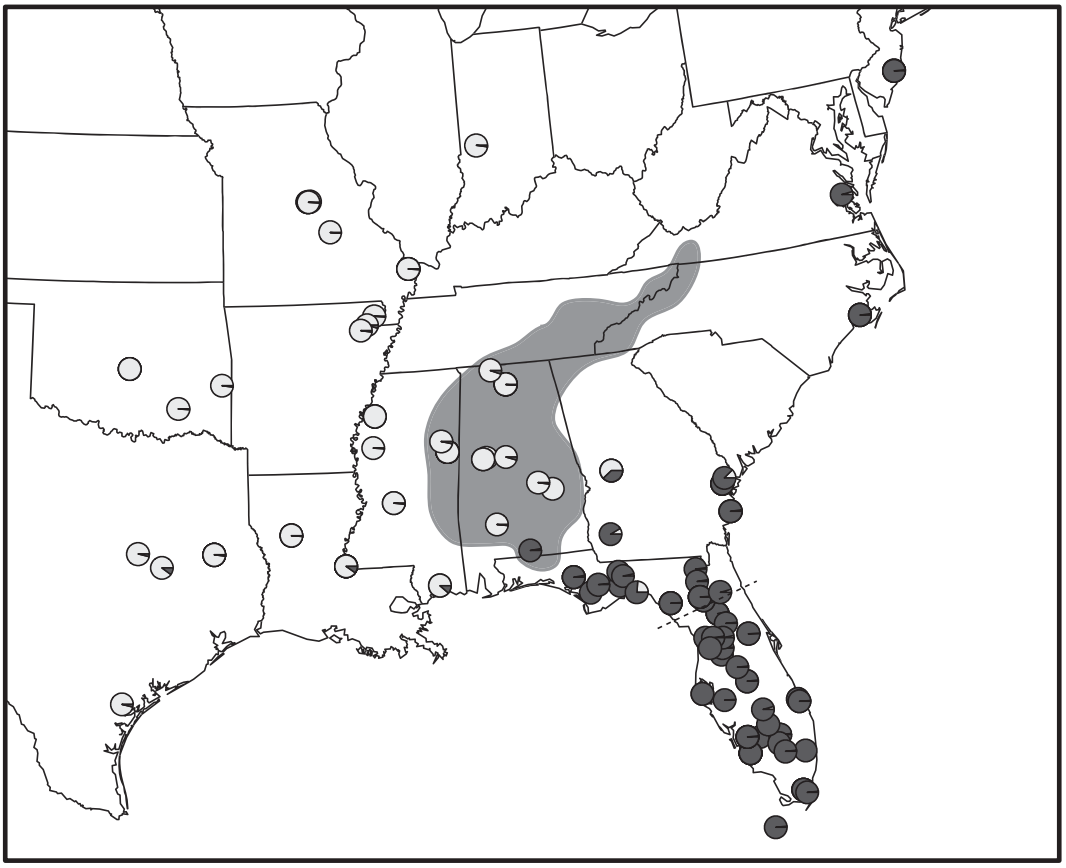

Fig. 4 Map of clusters from microsatellite analysis in STRUCTURE. Pie chart partitions correspond to cluster membership probability, *q*, per locality. Gray polygon depicts AL-Appalachian SZ. Dotted line represents subspecies break.

Within the eastern lineage, haplotypes were partitioned north-south, and haplotype diversity was concentrated in Florida (Fig. 2; 22 haplotypes in FL vs. 11 haplotypes along the Atlantic Coast). While subclades within the western and eastern lineages had high posterior probabilities and bootstrap values, the genetic variation within each lineage was very small relative to the overall divergence between the west and east.

In the microsatellite analysis, the number of alleles per locus ranged from 6 to 22. Bayesian cluster analyses of the microsatellite data in STRUCTURE identified two genetically distinct clusters that correspond to the two mtDNA lineages (Figs 4 and 5). Pairwise F_{ST} between the two clusters was calculated in ARLEQUIN (F_{ST} = 0.0542, $P < 0.001$). However, because F_{ST} has been shown to be inappropriate for highly variable loci (Merimans & Hedrick 2011), we used an additional statistic, G''_{ST} (GENODIVE v.2.0b20, Merimans & Van Tienderen 2004), which standardizes G_{ST} by the maximum possible value given the amount of intra-cluster allelic variation (Merimans & Hedrick 2011). G''_{ST} between the two clusters was 0.363 ($P < 0.001$). Eleven of the 166 individuals were admixed (q between 0.10 and 0.90), but the power to detect admixed individuals was likely low, given the low number of loci used and relatively low F_{ST} (Vähä & Primmer 2005). The admixed individuals were not clustered in any one particular geographic region, but instead were scattered throughout the entire *R. sphenocephala* species range (Fig. 4). Subsequent Structure analyses of the two clusters individually did not reveal any substructure. Because of this pattern and the relatively small amount of genetic variation within the two major lineages, we do not recognize any of the mtDNA subclades as distinct evolutionary lineages and recommend instead that the western and eastern lineages each be elevated to species. However, we also note the possibility that additional microsatellite loci and larger population sample sizes could potentially reveal more fine-scaled patterns.

Climate analyses

The partial Mantel test between the genetic and climate matrices was not significant ($P = 0.311$), indicating that climate is not a major driver of lineage divergence in these frogs, at least at this phylogenetic scale. These

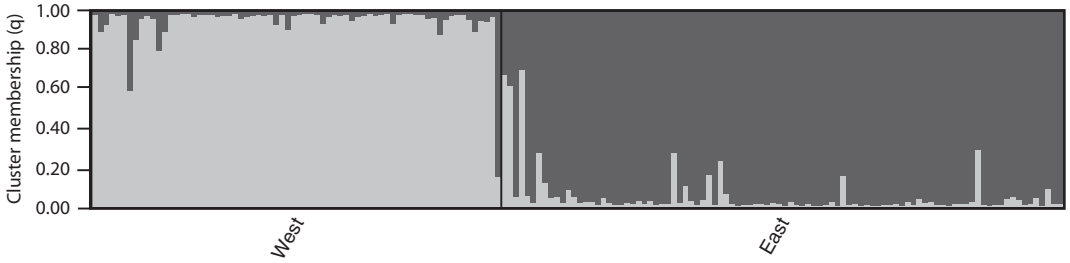

Fig. 5 Assignment of individuals to clusters based on microsatellite analysis in STRUCTURE. 'East' and 'west' labels correspond to mtDNA lineages in Fig. 3.

data instead suggest that genetic structure in *R. sphenocephala* is driven primarily by isolation by distance (Mantel statistic reference value = 0.697, Mantel's *t* approximation = 32.91, $P < 0.001$). While additional statistical tests with higher power are available (see Raufaste & Rousset 2001; Legendre & Fortin 2010) and could be used with finer resolution data, results from the Mantel and partial Mantel tests are consistent with our other data described later in showing no evidence of a correlation between climate and phylogenetic structure at this spatial scale.

The first *k*-means analysis classified the 10 000 random points into a coastal cluster—spanning the entire Gulf and Atlantic Coasts and peninsular Florida—and an inland cluster (Fig. 6a,b). This environmental break does not correspond to the AL-Appalachian SZ hotspot or the *R. sphenocephala* genetic break. However, the *k*-means analysis of the coastal cluster classified the points into a peninsular Florida cluster and a cluster spanning the remainder of the Gulf and Atlantic Coasts (Fig. 6b). This environmental break corresponds to the traditional subspecies based on morphology, suggesting that divergent selection may be driving morphological variation in *R. sphenocephala*. The climate of the peninsular Florida cluster is significantly different from both the remainder of the coastal range (Wilks lambda = 0.11691, $P < 0.001$) and the rest of the entire species range (Wilks lambda = 0.15107, $P < 0.001$).

Discussion

Patterns of genetic structure in the southern leopard frog

The geographic patterning of genetic variation in the wide-ranging *R. sphenocephala* revealed by mitochondrial and microsatellite analyses indicates that two unique lineages meet in the general area of the AL-Appalachian SZ (Figs 2 and 4; also see Fig. 1 in Rissler & Smith 2010). Although this pattern is common among some North American taxa (e.g. Austin *et al.* 2002; Masta *et al.* 2002; Church *et al.* 2003; Kozak *et al.* 2006; Lemmon *et al.* 2007; Beamer & Lamb 2008; Jackson & Austin 2010; Butler *et al.* 2011), other studies of southeastern taxa show phylogenetic breaks that are not specific to the suture zone (e.g. Avise *et al.* 1979, 1987; Donovan *et al.* 2000; Zamudio & Savage 2003; Gamble *et al.* 2008; Degner *et al.* 2010; Makowsky *et al.* 2010). In some cases, this discrepancy can perhaps be attributed to the lack of fine-scaled sampling so that it is unclear where lineages actually meet. The Mississippi River (Fig. 2) may be another biogeographic break for *R. sphenocephala*, a pattern consistent with other codistributed taxa (Austin *et al.* 2004; Moriarty & Cannatella 2004; Jackson & Austin 2010; Makowsky *et al.* 2010), although the genetic differentiation between *R. sphenocephala* collected west and east of the river is relatively low (Table 2).

The AL-Appalachian SZ hotspot is in a region where multiple physiographic provinces meet, providing opportunities for post-glacial divergent selection and ultimately speciation (Rissler & Smith 2010). In particular, the Appalachian Mountains have been shown to be a barrier to dispersal and, hence, a phylogeographic break for several widespread amphibian taxa, including *Pseudacris crucifer* (Austin *et al.* 2002, 2004), *Ambystoma tigrinum* (Church *et al.* 2003), *Ambystoma maculatum* (Zamudio & Savage 2003), *Desmognathus marmoratus* and *D. quadramaculatus* (Jones *et al.* 2006). The Appalachians are a barrier to dispersal for *R. sphenocephala*, as well, although the lack of sampling immediately east and west of the mountains precludes any definitive conclusion.

In the same region, the general area of the Apalachicola—Chattahoochee—Flint River Basin (ACF) is the site of congruent phylogeographic breaks for many taxa (see Avise 2000; Burbrink *et al.* 2000; Zamudio & Savage 2003; Soltis *et al.* 2006). The ACF begins with the headwaters of the Chattahoochee River in the Appalachian Mountains of northeastern Georgia and also contains the Flint River that begins in northwestern Georgia. These two rivers converge at the Georgia/Florida state line into the Apalachicola River, which

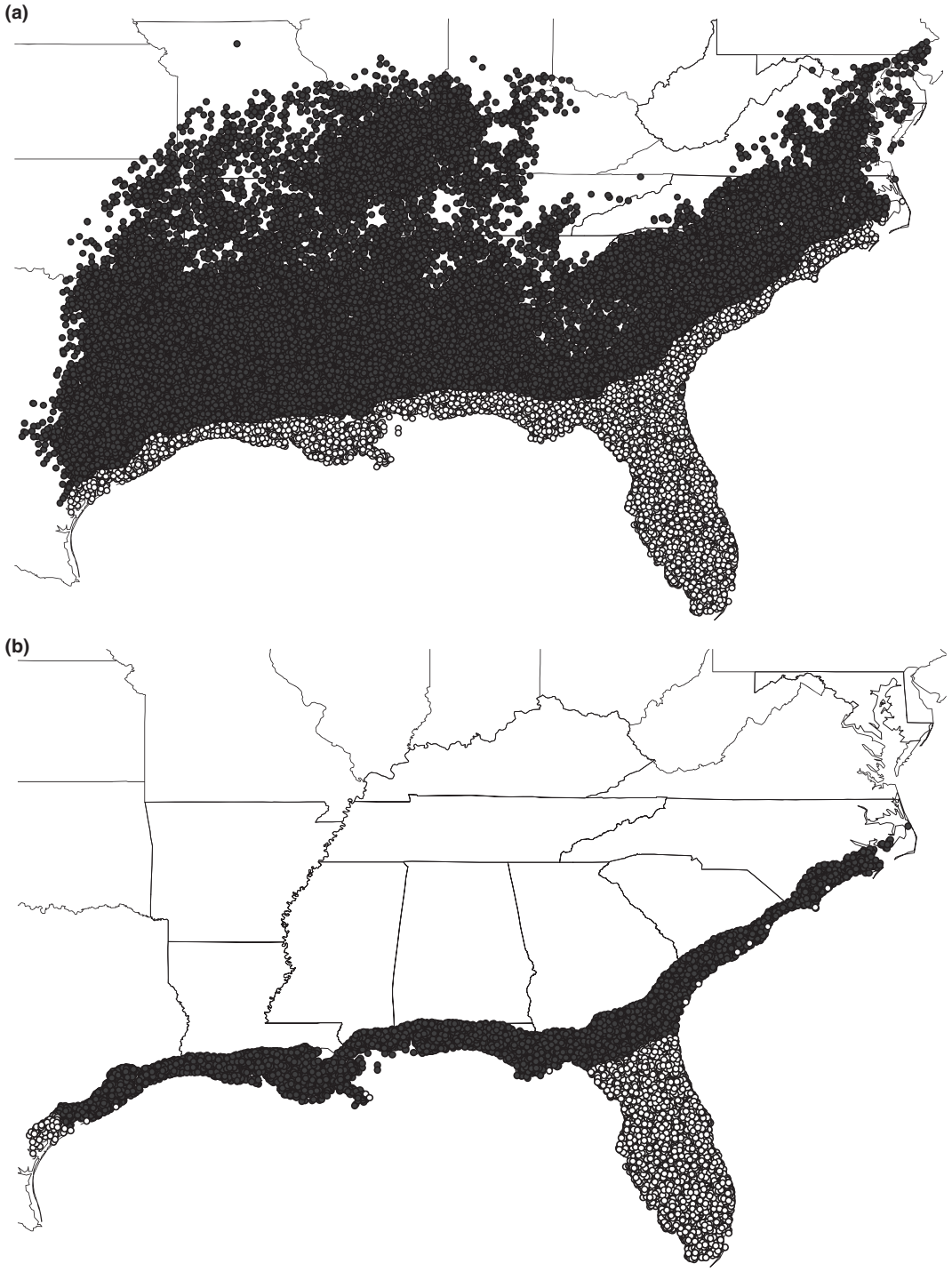

Fig. 6 (a) *K*-means analysis of 10 000 random points, location weighted according to the probability distribution of the ecological niche model for *Rana sphenocephala*. White dots indicate the coastal cluster and dark gray dots indicate the inland cluster. (b) *K*-means analysis of the coastal cluster only. White dots indicate the peninsular Florida cluster.

then continues to the Gulf of Mexico. The Alabama—Coosa—Tallapoosa River Basin (ACT) is a less well-studied drainage system in Alabama that begins in northwestern Georgia with the headwaters of the Coosa and Tallapoosa Rivers. The two rivers converge into the Alabama River, which runs southwest across Alabama. For *R. sphenocephala*, the major phylogeographic break corresponds to both the ACF (Georgia) and the ACT

(south Alabama) and suggests that during the Pleistocene when species were restricted to glacial refugia, *R. sphenocephala* was split into two refugia—one on the Atlantic Coastal Plain and another on the Gulf Coastal Plain [as hypothesized by Swenson & Howard (2005)]. Subsequent spread of the Atlantic group was likely limited to the west by the Appalachian Mountains and to the south by the Apalachicola River. While our mtDNA haplotype data for the eastern lineage support the hypothesis of isolation in peninsular Florida and postglacial expansion northward, the microsatellite analyses did not reveal any population substructure within the eastern or western lineages. We therefore caution that explicit testing of this and other hypotheses about patterns of population demographics and migration will require intensive population-based sampling and additional nuclear loci.

Interestingly, two individuals in the eastern lineage are from localities in Alabama west of the ACF, suggesting that the modern-day Apalachicola River is not an absolute barrier to gene flow in *R. sphenocephala*. However, ancient drainage basins have been shown to play a major role in intraspecific genetic structure, particularly in amphibians [e.g. semiaquatic plethodontid salamanders (Kozak *et al.* 2006)] and freshwater fishes (Strange & Burr 1997; Kreiser *et al.* 2001; Near *et al.* 2001; Hardy *et al.* 2002; Berendzen *et al.* 2003). The specimens that form clade B (Figs 2 and 3) in Alabama and west Georgia were collected from sites located directly on or very close to the historic Appalachian drainage system (see Kozak *et al.* 2006), and the historic drainage system—as opposed to the modern ACF drainage system—does in fact correspond to the phylogenetic break between the eastern and western lineages in south Alabama. We anticipate that more intensive sampling in south Alabama and the Florida panhandle will further elucidate the phylogeographic history of *R. sphenocephala* in this region and clarify the effects of historical vs. contemporary gene flow on the genetic structure of this species.

Climate and patterns of genetic vs. morphological structure

While the genetic structure within *R. sphenocephala* is consistent with the AL-Appalachian SZ hotspot as identified by Swenson & Howard (2005) and Rissler & Smith (2010), our data show that broad-scale climatic breaks in the southeastern United States are not congruent with this hotspot. Environmental breaks do not correspond to the genetic patterns in *R. sphenocephala*, but they are consistent with the recognized subspecies (Fig. 6b). This suggests that the environment could be affecting morphology through some form of selection driven by unique temperature or precipitation in peninsular Florida. According to Pace (1974), *R. sphenocephala* in peninsular Florida (*R. s. sphenocephala*) have vestigial oviducts, whereas those from the remainder of the species range (*R. s. utricularia*) do not. Pace (1974) also noted that frogs of the subspecies *R. s. sphenocephala* in peninsular Florida were generally larger than frogs of the subspecies *R. s. utricularia*. Other vertebrate species also have subspecies confined to Florida, including the eastern king snake *Lampropeltis getula*, cricket frog *Acris gryllus*, chorus frog *Pseudacris nigrita*, siren *Pseudobranchus striatus*, eastern newt *Notophthalmus viridescens* and others. This result is consistent with longstanding ideas about the reality of subspecies, especially that they are simply evidence of the adaptive response of species to particular climatic conditions (Mayr 1942, 1982; Phillimore & Owens 2006). Future research could further investigate this correlation between climate and morphology by incorporating analyses of physiological differences between populations. We also note that analyses of palaeoclimatic data could reveal different patterns from the recent climate data used in this study and could further illuminate the historical impacts of climate on patterns of genetic variation.

Ecological patterns of divergence and future directions

The role of ecology, and specifically climate, in driving genetic divergence has often been overlooked in phylogeographic and systematic studies (but see Lapointe & Rissler 2005; Rissler & Apodaca 2007; Stockman & Bond 2007; Kozak *et al.* 2008). For the broad-ranging *R. sphenocephala*, climate did not significantly help to explain patterns of genetic structure. Based on our data, and following the 'unified' species concept which defines species as separately evolving lineages (De Queiroz 2007), *R. sphenocephala* is clearly a complex of two separate species, but neither of them corresponds to the recognized subspecies. The subspecies were named according to morphological traits that differ in peninsular Florida—an area with unique climate (Fig. 6b). Whether and how climate impacts morphology through effects on fitness remains to be explicitly tested.

We believe it is important to examine both geographic and climatic impacts on patterns of biodiversity, especially how they may differentially impact genetic vs. morphological patterns. The increasing use of multiple spatially explicit biogeographic tools is making this integration ever more possible. Vital to the study of biodiversity is an understanding of the relationships among taxa and the criteria used to delimit species. With the rapid growth of the fields of genomics and bioinformatics, the persistence of typological subspecies is contentious (Mayr 1982; Burbrink *et al.*

2000; Haig *et al*. 2006; Phillimore & Owens 2006), but we argue that investigating recognized subspecies can enhance our understanding of evolutionary processes, such as climate-driven divergent selection. This study revealed that while phylogeographic patterns in the southeastern United States are historical rather than driven by climate, environmental factors may differentially affect isolated populations and drive morphological evolution.

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Data accessibility

DNA sequences: GenBank accession nos: GQ288989–GQ289121; GU182895–182904; JN185211–185218.

Phylogenetic data: TreeBASE Submission ID: 11850.

Microsatellite data uploaded as Supporting information.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Each sample is listed in two rows, one row for each allele.

Table S2 GenBank Accession numbers for mtDNA sequences and voucher IDs for all samples used in genetic analyses.

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