MOLECULAR ECOLOGY

Molecular Ecology (2010) **19**, 2501–2515 doi: 10.1111/j.1365-294X.2010.04661.x

Fat frogs, mobile genes: unexpected phylogeographic patterns for the ornate chorus frog (*Pseudacris ornata*)

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Abstract

The southeastern coastal plain of the United States is a region marked by extraordinary phylogeographic congruence that is frequently attributed to the changing sea levels that occurred during the glacial-interglacial cycles of the Pleistocene epoch. A phylogeographic break corresponding to the Apalachicola River has been suggested for many species studied to date that are endemic to this region. Here, we used this pattern of phylogeographic congruence to develop and test explicit hypotheses about the genetic structure in the ornate chorus frog (*Pseudacris ornata*). Using 1299 bp of mtDNA sequence and seven nuclear microsatellite markers in 13 natural populations of *P. ornata*, we found three clades corresponding to geographically distinct regions; one spans the Apalachicola River (Southern Clade), one encompasses Georgia and South Carolina (Central Clade) and a third comprises more northerly individuals (Northern Clade). However, it does not appear that typical phylogeographic barriers demarcate these clades. Instead, isolation by distance across the range of the entire species explained the pattern of genetic variation that we observed. We propose that *P. ornata* was historically widespread in the southeastern United States, and that a balance between genetic drift and migration was the root of the genetic divergence among populations. Additionally, we investigated fine-scale patterns of genetic structure and found the spatial scale at which there was significant genetic structure varied among the regions studied. Furthermore, we discuss our results in light of other phylogeographic studies of southeastern coastal plain organisms and in relation to amphibian conservation and management.

Keywords: anuran, comparative phylogeography, divergence times, genetic structure, historical demography, southeastern United States

Received 1 June 2009; revision received 12 March 2010; accepted 25 March 2010

Introduction

A central goal of population genetics is to illustrate how genetic variation is partitioned within and among natural populations of a species and to use these patterns of variation to better understand the historic processes that shape the species (Wright 1931; Templeton *et al.* 1995; Rosenberg *et al.* 2002). Genealogical concordance among co-distributed species might be expected if species share a common biogeographic history (Zink 1996; Arbogast & Kenagy 2001). Indeed, a number of studies have used phylogeographic agreement to characterize shared history of the biota of North America (e.g. Bernatchez & Wilson 1998), Europe (e.g. Taberlet *et al.* 1998; Hewitt 2000), tropical Australia (e.g. Schneider *et al.* 1998), and a number of other biogeographic regions (reviewed in Avise 2000). Although shared phylogeographic patterns provide a powerful tool to assess historical congruence, disagreements may also be informative (Avise 2000). When there are prior expectations based on regional geological history and genealogical concordance in other taxa, a lack of phylogeographic concordance may, for example, suggest a lack of shared history (e.g. a recent colonization event for one taxon vs. established lineages in a second) or it may indicate different gene flow boundaries or dispersal abilities

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among species. Given the potential for these patterns to illuminate regional evolutionary history, it is necessary to investigate phylogeographic structure with multiple co-distributed species.

The southeastern United States is a region of both an interesting geologic record and a thoroughly studied phylogeographic history. Although not glaciated per se, the southeastern United States was largely influenced by global glacial oscillations during the Quaternary Period. At the height of interglacial periods, sea levels in the southeastern United States were 5–30 m higher than contemporary levels (Cronin *et al.* 1981; Miller *et al.* 2005; Thompson & Goldstein 2005). Historically, these glacial–interglacial cycles caused major fluctuations in the sea levels during the late Pliocene and Pleistocene epochs that may have caused many of the phylogeographic patterns observable today. For example, it has been suggested that during these periods of highest sea levels, the Apalachicola River basin was a large saltwater embayment extending north through Alabama and Georgia to the piedmont of the Appalachian Mountains. This embayment may have created a saltwater channel that bisected the coastal plain and may have acted as a barrier to many species (Cooke 1945; Neill 1957). One would predict that such a striking geologic event would leave its mark on the regional biota. Indeed, a number of studies have explored the phylogeography of many freshwater, terrestrial and marine species from this region and have uncovered a general pattern of concordance (Avise *et al.* 1979, 1987; Bermingham & Avise 1986; Avise & Nelson 1989; Hayes & Harrison 1992; Vogler & DeSalle 1993; Ellsworth *et al.* 1994; Phillips 1994; Osentoski & Lamb 1995; Walker *et al.* 1995; Avise 2000; Donovan *et al.* 2000; Moriarty *et al.* 2007; Pauly *et al.* 2007; Burbrink *et al.* 2008).

In this study, we investigated the relative roles of historical geography, genetic drift and migration on the partitioning of genetic variation in the southeastern endemic ornate chorus frog (*Pseudacris ornata*), a member of the 'Fat Frogs' clade of chorus frogs (Moriarty & Cannatella 2004). Amphibians are generally characterized by limited dispersal abilities, high philopatry and are obligately associated with the areas around suitable habitats (e.g. breeding ponds). As a result, amphibians often occur naturally in fragmented populations and show a high degree of genetic structure (Beebee 1996, 2005; Newman & Squire 2001; Palo *et al.* 2004). Also following limited dispersal ability, major geographic barriers (e.g. large rivers or mountain ranges) known to restrict gene flow in other species are predicted to truncate the range of amphibians. Indeed, both the high genetic structure due to distance and the efficacy of geographic structures for restricting gene flow have been repeatedly demonstrated in previous studies involving amphibians (Shaffer *et al.* 2000; Newman & Squire 2001; Funk *et al.* 2005; Moriarty Lemmon *et al.* 2007; Pauly *et al.* 2007). Notably, two recent studies have examined patterns of population genetic structure in amphibians in the southeastern United States: Moriarty *et al.* (2007) investigated patterns of gene flow in *Pseudacris feriarum*, the upland chorus frog and Pauly *et al.* (2007) investigated population genetic structure in *Ambystoma cingulatum*, the flatwoods salamander. Both studies tested the hypothesis that the Apalachicola River served as a barrier to gene flow. Moriarty *et al.* (2007) found that *P. feriarum* exhibited reduced gene flow across the Apalachicola River and other rivers in the southeastern United States; Pauly *et al.* (2007) concluded that *A. cingulatum* should be split into two 'cryptic' species based on the level of differentiation among populations spanning the Apalachicola River. The genetic variation present in both of these species suggests that other co-distributed amphibians may harbour equal levels of differentiation.

Combining the expectation that amphibians have limited dispersal abilities with the well characterized biogeographic history of the southeastern United States, we tested specific hypotheses regarding the population genetic structure of *P. ornata*. First, we hypothesized that we would find phylogeographic concordance between *P. ornata* and other co-distributed species. Second, because we expected amphibians to be highly philopatric and exhibit limited dispersal abilities, we hypothesized that samples from distant geographic regions would be highly differentiated (e.g. show high $F_{ST,}$ complete mitochondrial lineage sorting and be separable based on genetic clustering algorithms). Furthermore, we expected to uncover patterns of population genetic structure in *P. ornata* on a relatively small geographic scale (i.e. tens of kilometers) congruent with similar studies on anurans (Newman & Squire 2001; Monsen & Blouin 2004). Lastly, we hypothesized that we would find an overall pattern of isolation by distance (IBD) and restricted gene flow. Such a pattern is common in other anuran species (e.g. Shaffer *et al.* 2000; Hoffman & Blouin 2004; Hoffman *et al.* 2004; Monsen & Blouin 2004) and is often attributed to the inability of individuals to disperse long distances (Wright 1943). The results found here are discussed in light of other phylogeographic studies of southeastern coastal plain organisms and in relation to amphibian conservation and management.

Materials and methods

Sampling

We collected samples from throughout the range of *P. ornata*. Individual frogs were located in the winter

and spring (November through May) of 2006–2009. Frogs were generally captured either by road cruising on rainy nights or by hand-capturing the calling males in breeding choruses. However, the samples from Barbour County, Alabama were captured in pitfall traps set along a drift fence surrounding an ephemeral pond. Special effort was made to sample populations in a continuum of pairwise geographic distances.

Overall, we obtained samples from 13 discrete geographic locations ranging from the Florida panhandle west of the Apalachicola River to east-central South Carolina and north into North Carolina (Table 1). Sample sizes at each collecting locality ranged from 1 to 85. We scored all specimens for sex and recorded the precise location of capture. For DNA analysis, we removed the longest toe on the right hind-foot with sterilized surgical scissors and placed toe clippings in vials filed with anhydrous calcium sulphate (a desiccant) in preparation for DNA extraction (see below). Additionally, we obtained 13 samples from the herpetological collections of the University of Texas, Austin (TNHC # 62178–62189).

DNA amplification and sequencing

We isolated DNA using DNeasy DNA extraction kits (Qiagen, Valencia, CA, USA) and followed the manufacture's 'Animal Tissue Protocol'. We used mitochondrial DNA (mtDNA) haplotypes and phylogenetic analyses to test the prediction of an overall pattern of large scale genetic structure as well as to assess whether the Apalachicola River acts as a consistent barrier to gene flow. We sequenced two disjointed sections of mtDNA in both directions for up to 14 samples per sampling locality (average of approximately seven samples per site; Table 1). Additionally, we sequenced a 707 bp section that spans partial 12S ribosomal RNA (rRNA; 484 bp), complete tRNA-Val (69 bp) and part of 16S rRNA (154 bp). We also sequenced a 592 bp section of the cytochrome-b (*cyt-b*) gene. We amplified and sequenced the fragment coding for RNA genes using primers 12L1 (5′-AAAAAGCTTCAAACTGGGATTAGATACCCCAC-TAT-3′) and 16Sh (5′-GCTAGACCATKATGCAAAA-GGTA-3′) which correspond to primer numbers 46 and 76, respectively, in Goebel *et al.* (1999). We amplified and sequenced the section of *cyt-b* using the primers MVZ 15 (5′- GAACTAATGGCCCACACWWTAC-GNAA-3′) and MVZ 18 (5′-GTCTTTGTATGAGAAG-TATG-3′) after Moritz *et al.* (1992). PCR reactions contained 5–10 ng template DNA, 1 unit Taq polymerase, 2.25 mM $MgCl_2$, 1X PCR buffer (10 mM Tris-HCL, 50 mM KCL and 0.1% Triton X-100), 0.2 μM each dNTP and 0.5 μM each primer. PCR thermocycling parameters were 95 °C for 4 min., 41 °C (cyt-b primers) or 46 °C (rRNA primers) for 45 s, 69 °C for 60 s, 35 × [95 °C for 45 s 45 °C (cyt-b primers) or 50 °C (rRNA primers) for 45 s, 72 °C for 45 s], 72 °C for 7 min.

We electrophoresed PCR amplicons in a 1% agarose-TAE gel, excised and purified positive bands using GENECLEAN III Kits according to manufacturer's protocols (Q-Biogen Inc., Irvine, CA, USA). Fragments were sequenced at Nevada Genomics Center (Reno, NV, USA). We aligned multiple fragments for each individual and reviewed base-calls for accuracy using SEQUENCHER v. 4.7 (Gene Codes Corp., Ann Arbor, MI, USA). We aligned complete, edited sequences using CLUSTAL

Table 1 General locality information and number of individuals used in mtDNA (N_{MIT}) and microsatellite (N_{NUC}) analyses

Location	Abbreviation	Latitude	Longitude	N_{MIT}	N_{NUC}
Cumberland Co., North Carolina	FORT	35.2208	−78.9941	8	8
Colleton Co., South Carolina A	COLA	33.0546	−80.4859	11	26
Colleton Co., South Carolina B‡	COLB	33.0422	−80.4277	–	26
Jasper Co., South Carolina	HARDY	32.4371	−81.0042	14	25
Barnwell Co., South Carolina†	BARN	33.2167	−81.7500	2	–
Aiken Co., South Carolina‡	SRS1	33.1574	−81.6763	–	16
Aiken Co., South Carolina†	SRS2	33.3177	−81.4769	5	52
Baker Co., Georgia	POND	31.2498	−84.4947	12	37
Telfair Co., Georgia†	TEL	31.8614	−82.8121	6	–
Clay Co., Florida	JEN	30.1519	−81.8787	7	29
Liberty Co., Florida	HWY	30.0861	−85.0404	8	85
Barbour Co., Alabama†	BAR	32.0371	−85.0839	5	–
Gulf Co., Florida†§	GULF	–	–	1	–

Location abbreviation, spatial coordinates and county were samples were collected are given.
†Localities that used only in mtDNA
‡Localities that used only in microsatellite analyses.
§No specific spatial coordinates are given.

W (Larkin *et al.* 2007). We concatenated disjoint alignments using BIOEDIT v. 7.07 (Ibis Biosciences, Carlsbad, CA, USA) creating a single alignment consisting of complete sequences from 79 samples (Table 1).

Statistical analysis of mtDNA data

To identify any deep divergence between existing populations and to determine the historic influence of the Apalachicola River (Hypothesis 1), we estimated phylogenetic relationships among unique haplotypes. We included all 47 complete sequences of unique haplotypes in this phylogenetic analysis along with three outgroup sequences of homologous regions in other *Pseudacris* species (*Pseudacris regilla*, GenBank accession numbers AY364542 and DQ195169 for RNA genes and *cyt-b*, respectively; *Pseudacris crucifer*, both genes sequenced in this study; and *Pseudacris illinoensis*, GenBank accession number AY291110 for RNA genes—*cyt-b* sequenced in this study). P. illinoensis, together with *P. streckeri*, form the sister clade to *P. ornata* and therefore represents an ideal outgroup species (Moriarty & Cannatella 2004; Moriarty *et al.* 2007).

The phylogenetic reconstruction used Bayesian Marcov chain Monte Carlo (MCMC) phylogenetic methods implemented in MRBAYES v. 3.2.1 (Ronquist & Huelsenbeck 2003). We used MRMODELTEST v. 2.2 (Nylander 2004) and the Akaike information criterion (AIC) to determine the model of DNA evolution most appropriate for different partitions of the entire dataset. These partitions were (i) the unpartitioned data set; (ii) *cyt-b* gene alone; (iii) RNA genes alone; (iv) first codon position of *cyt-b*; (v) second codon position of *cyt-b*; (vi) first and second codon position of *cyt-b*; and (vii) third codon position of *cyt-b*. We used the chosen models for each partition in four runs in MRBAYES. In these four runs, we modelled the entire dataset under a single model of sequence evolution (1X); allowing for separate models of evolution for partitions 2 and 3 (2X); separate models for partitions 3, 6 and 7 (3X); and separate models for partitions 3, 4, 5 and 7 (4X). We ran MRBAYES MCMC chains for four million generations and the first one million generations were discarded as burnin. Bayes Factors according to Kass & Raftery (1995) were used to choose an appropriate complex number of partitions and the phylogeny obtained with this partitioned dataset was reported. To further visualize the relationship among sequences, we created an intraspecific haplotype network of all haplotypes using the algorithm of Templeton *et al.* (1992) implemented in the program TCS v. 1.21 (Clement *et al.* 2000). Haplotypes were nested hierarchically into clades according to the rules of Templeton *et al.* (1987, 1992) and were mapped according to their sampling locality to enable the visualization of related clades and their geographic association.

Divergence times and historical demography

We used the program BEAST v1.5.2 (Drummond & Rambaut 2007) to understand divergence times in light of variable divergence rates and give an absolute temporal framework for *P. ornata* diversification. Using the GTR + G + I model, we initially ran two separate analyses to determine whether our data set conformed to a strict clock model or whether we needed to infer a more complex relaxed clock model. We calculated Bayes factors and compared the two models using TRACER v. 1.4.1 (Rambaut & Drummond 2007). This analysis indicated that the simpler strict clock model was appropriate (2LogBF = 2.84). Two studies have independently estimated the mutation rate for the 12S gene: Evans *et al.* (2004) estimated a rate of 0.00249 subs/site/year for Pipid frogs and Moriarty Lemon *et al.* (2007) estimated the rate to be 0.00277 subs/site/year for Hylid frogs. Because our dataset fits to a strict clock model and these two rate estimates from very distant lineages appear be consistent, our temporal analysis restricted the genetic dataset to the 12S gene and used the estimated mutation rate obtained for hylids. Thus, we used a normal prior for the clock.rate parameter with a mean = 0.00277 and SD = 2.9×10^{-4} and a Bayesian skyline coalescent for the tree prior. Four independents runs were initiated from random starting trees for 20 million generations and sampled every 1000. Based on the examination of preliminary runs, we conservatively eliminated the first four million generation as burnin and the remaining samples were combined to summarize the posterior distribution of dates on the maximum clade credibility tree.

We estimated changes in relative population size through time for the Central and Southern populations using Bayesian Skyline Plots (Drummond *et al.* 2005). This analysis infers past population dynamics without assuming a particular demographic scenario (i.e. population expansion, constant population). We ran four independent analyses for each population using the same model of sequence evolution utilized in the previous analysis (see above). Due to our moderate number of samples (less than 40 individuals per population), the skyline plots were inferred using five coalescent intervals (*m*) in BEAST v. 1.5.2 (Drummond & Rambaut 2007; Rambaut & Drummond 2007). Additionally, we tested for sudden population expansion via mismatch distributions (Rogers & Harpending 1992) and the Fu's *F* neutrality test (Fu 1997). Significance of test statistics were evaluated from 50 000 coalescent simulations in ARLEQUIN v. 3.11 (Excoffier & Schneider 2005).

Microsatellite genotyping

We further characterized the genetic structure in this species using seven unlinked microsatellite markers. Specifically, the microsatellite markers addressed Hypothesis 2 and 3, that due to limited dispersal abilities and high rates of philopatry, *P. ornata* would show high genetic divergence among sampling regions (high F_{ST}) and there would be detectable, fine-scale genetic structure over relatively short geographic distances with observable IBD. We genotyped all frogs (n = 304; nine populations; Tables 1 and 2) for seven unlinked microsatellite loci (two designed from *P. ornata*, five cross-amplified from other *Pseudacris* species). Primer and PCR amplification conditions for all seven loci followed the methods described in Degner *et al.* (2009). Amplified PCR products were sized using the CEQ 8000 genetic analysis system and software (Beckman-Coulter).

Statistical analysis of microsatellite data

To determine the variability and data-quality of the microsatellite dataset, we estimated basic information describing the characteristics of variation for 304 *P. ornata* at seven microsatellite loci among nine discrete sampling localities (average ~34 frogs per locality; Table 2). We estimated observed (H_O) and expected (H_E) heterozygosities, allelic richness (AR) and pairwise F_{ST} in FSTAT v. 2.9.3.2 (Goudet 2001). We tested for significant deviations from Hardy–Weinberg (HWE) and

Table 2 Population genetic information for microsatellite data used in this study

Locus	A_T		JEN	SRS 1	SRS 2	COL A	COL B	FORT	HARDY	HWY	POND
PCRU09	9	N	29	16	51	25	24	8	22	82	33
		A	4	4	6	3	5	3	4	6	6
		H_o	0.621	0.563	0.529	0.600	0.625	0.750	0.409	0.524	0.515
		H_E	0.696	0.573	0.502	0.635	0.665	0.580	0.500	0.736	0.580
PCRU14	6	N	28	16	50	26	24	8	25	82	35
		A	2	3	4	3	4	2	3	4	2
		H_o	0.107	0.500	0.360	0.231	0.708	0.250	**0.840**	0.268	0.200
		H_E	0.225	0.475	0.433	0.278	0.599	0.232	0.513	0.255	0.404
PCRU24	18	N	24	14	47	25	26	8	25	82	34
		A	7	3	3	6	2	3	2	9	5
		H_o	0.292	0.429	0.064	0.360	0.115	0.625	0.040	0.549	0.382
		H_E	0.341	0.368	0.063	0.322	0.111	0.536	0.040	0.555	0.456
POR105	41	N	16	14	49	9	20	8	23	63	27
		A	7	15	20	8	12	6	14	16	13
		H_o	**0.313**	0.929	0.959	1.000	0.800	1.000	0.870	**0.413**	**0.222**
		H_E	0.752	0.945	0.934	0.889	0.911	0.795	0.922	0.860	0.845
PTRI29	4	N	28	16	51	23	26	8	25	82	37
		A	1	2	3	1	1	3	3	3	3
		H_o	0.000	0.250	0.196	0.000	0.000	0.250	0.120	0.037	0.270
		H_E	0.000	0.317	0.242	0.000	0.000	0.241	0.190	0.036	0.240
POR165	45	N	16	11	42	22	25	5	25	74	37
		A	11	9	29	10	20	5	18	27	21
		H_o	0.750	0.545	0.833	0.682	0.800	1.000	0.800	**0.703**	0.865
		H_E	0.879	0.905	0.930	0.883	0.930	0.775	0.941	0.954	0.934
PCRU10	24	N	26	10	49	21	25	7	23	40	37
		A	7	2	6	5	4	3	4	10	5
		H_o	0.346	0.100	0.449	0.190	0.320	0.714	0.217	0.275	0.324
		H_E	0.426	0.411	0.571	0.405	0.284	0.548	0.316	0.503	0.331
Average across loci		H_E	0.474	0.571	0.525	0.487	0.500	0.530	0.489	0.557	0.541

Locality abbreviations cross reference with Table 1 and Fig. 3. For each locality and locus the number of diploid individuals (N) scored, the number of alleles (A) and observed and expected heterozygosities (H_O and H_E) are given. The total number of alleles for a given locus among all localities (A_T) is provided. Boldface indicates significant differences in observed vs. expected heterozygosities.

linkage equilibria (Fisher's exact test) in GENEPOP v. 4.0.7 (Raymond & Rousset 1995). Hypothesis 3 predicts we would uncover IBD in *P. ornata*. To test this hypothesis, we used the program IBDWS (Jensen *et al.* 2005) to estimate the correlation between log-transformed pairwise F_{ST} with log-transformed pairwise geographic distance between sampling localities. Slope and intercept for this relationship was estimated using Reduced Major Axis (RMA) regression to account for uncertainty in the independent variable (Jensen *et al.* 2005). IBD significance was accessed using a Mantel test with 9999 permutations.

To test the prediction that genetic structure would be detectable even over small spatial scales, we used a genetic clustering algorithm (STRUCTURE v. 2.3; Pritchard *et al.* 2000). This method allows for both an estimate of the number of genetically distinct clusters (K) and for the assignment of individuals to these clusters based on genotypic data. Briefly, the algorithm in STRUCTURE probabilistically models the data as coming from K randomly-mating ancestral populations. Since K is specified by the user, many independent runs for several choices of K are usually computed. An *ad hoc* statistic that is based on the change in model Likelihood for increasing values of K, ΔK, has been shown to successfully identify the highest level of meaningful population structure under a wide variety of simulation scenarios (Evanno *et al.* 2005). A recent extension of the basic STRUCTURE model has been proposed for data sets with low information content (i.e. relatively few loci, little divergence and/or individuals; Hubisz *et al.* 2009). This new model makes use of the sampling locations by placing a higher prior weight on clustering outcomes when they are correlated with sampling locations (Hubisz *et al.* 2009). One of the benefits of this model is that it does not tend to uncover structure when none exist while still able to find subtle genetic structure (Hubisz *et al.* 2009).

We applied the STRUCTURE method to determine genetic structure at hierarchical spatial scales. The first analysis included all 304 individuals with microsatellite data (Table 1). Within each cluster that we identified at one hierarchy, we performed subsequent STRUCTURE analyses to test for evidence of substructure. This process was continued until no further substructure was uncovered. For each STRUCTURE run, the maximum value of ΔK was used to infer the number of clusters. However, because ΔK is undefined for $K = 1$ (i.e. completely admixed populations), we considered three criteria for subsequent STRUCTURE analyses to distinguish between $K = 1$ and $K = 2$ (i) the change in mean Likelihood; (ii) the mean Likelihood minus half the variance; and (iii) whether the assignments are biologically relevant (i.e. correspond to geography).

In STRUCTURE, we set most parameters to their default values except where noted below. For the highest level (i.e. the full dataset) we used the admixture model and correlated allele frequencies between populations. We let α, the degree of admixture, be inferred from the data. The parameter of the distribution of allele frequencies (k) was set to one. The first 100 000 generations of data were discarded as burn-in, and data were collected for 200 000 generations thereafter. Because all subsequent analyses had less information content (i.e. relatively fewer individuals than the full dataset), we chose to include location information to the above model (i.e. set LOCPRIOR = 1). A visual inspection of model Likelihood plotted against the number of generations and consistency across runs, supported these parameters as sufficient. For each value of K (1, 2, …, $l + 1$), where l equals the number of locations, 10 independent STRUCTURE runs were conducted to confirm convergence of parameter estimates across runs and to obtain estimates of the variance among runs.

Results

Phylogenetic analysis

Among the 79 in-group specimens examined, we obtained complete sequences (1299 bp of mtDNA) from all specimens and uncovered 47 unique *P. ornata* haplotypes (Fig. 1). The greatest pairwise uncorrected sequence divergence among these haplotypes was 2.8%. The Harmonic mean of Log likelihoods (LogL) of seven different partitions of the dataset obtained in Bayesian MCMC runs were compared for increasingly complex partitions of the dataset. Increased partitioning continued to increase the likelihood values as expected. Following the recommendations of Kass & Raftery (1995), we took increases in LogL values of 2LogBF >10 as evidence for using the more complex partition. Using this criterion, the final model chosen was the unpartitioned dataset under a GTR + I + G model of DNA evolution. Bayesian MCMC analysis using this overall model yielded a well resolved phylogeny of haplotypes (Fig. 1). Most notably, there were three well supported clades. Samples from North Carolina fell into their own 'Northern Clade' (Posterior probability = 1.0). The 'Central Clade' (Posterior probability = 1.0) contained haplotypes that originated from populations sampled from South Carolina to central Georgia. A 'Southern Clade' (Posterior probability = 1.0) included haplotypes from populations spanning Florida as well as southern Alabama and Georgia. Samples that spanned the Apalachicola River were not members of reciprocally monophyletic groups. In fact, the phylogenetic position of haplotypes sampled west of the Apalachicola River

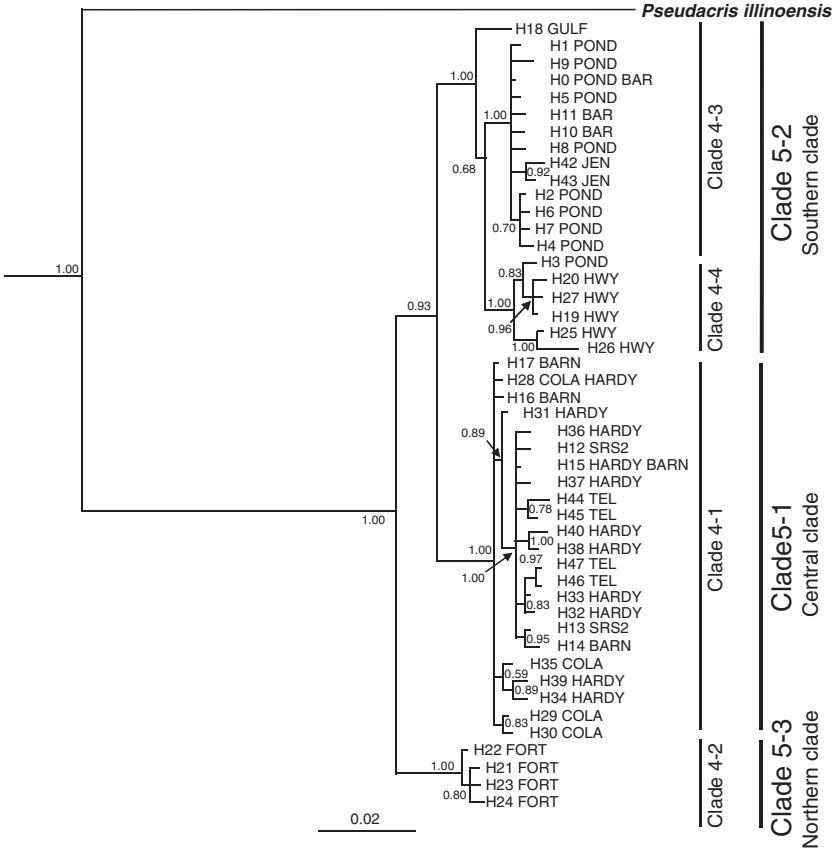

Fig. 1 Phylogenetic relationships among haplotypes sampled in *P. ornata*. Tips are labelled with both an alphanumeric designation representing unique haplotypes and the population where they occurred. Clades which correspond to nesting levels of the haplotype network (Fig. 2). Nodal support is displayed as Bayesian posterior probabilities. The phylogeny was rooted using homologous *P. regilla*, *P. illinoensis* and *P. crucifer* DNA sequences (only *P. illinoensis* shown).

(H18, H0, H10 and H11) seemed relatively random within this clade. Furthermore, haplotype H0 was sampled on both sides of the Apalachicola River. This analysis did not support the hypothesis that the Apalachicola River has acted as a consistent historic partition for this species.

Figure 2 shows the intraspecific network and nesting design obtained using the nesting algorithm in Templeton *et al*. (1987). This network consisted of two reticulations (H17-H28, H31- inferred haplotype) that could not be removed based on *a priori* knowledge and so were nested according to the nesting algorithm. The 95% confidence limit of validity of parsimony estimated in TCS was 15 steps; however, several of the connections between haplotypes required more than 15 steps and these specific connections should be interpreted in light of this. Higher level hierarchical nesting of haplotypes yielded four 4-step clades in three 5-step clades (Fig. 2) which were grouped together in a single 6-step clade. All 4- and 5-step clades were supported in the phylogenetic analysis except for the placement of haplotype H18 outside clade 4–3 in the phylogenetic reconstruction (Fig. 2). The geographic distribution of the higher order (4- and 5-step) clades is displayed in Fig. 3.

Divergence times and historical demography

The strict clock phylogenetic tree obtained via BEAST matched the tree obtained with MRBAYES (Fig. 1). The divergence time between *Pseudacris illinoensis* and *P. ornata* was estimated to be 16.3 Ma (95% CI = 9.7–24.2). The three main lineages within *P. ornata* appeared to have originated during late Pliocene and early Pleistocene. Thus, the first diversification event that led to the Northern population and the Southern + Central populations was inferred to have occurred during the late Pliocene around 2.8 Ma (95% CI = 1.5–4.4). The time of divergence between the Southern and Central populations was estimated to be 2.0 Ma (95% CI = 1.0–3.2).

Bayesian skyline plots indicated that the Central and Southern populations underwent demographic expansion around 200 000 years ago (Fig. S1, Supporting Information). Similarly, the mismatch distribution analyses, of the observed pair-wise genetic differences among individuals did not differ from the model expected under a sudden expansion (Central, $P = 0.56$; Southern, $P = 0.33$). Lastly, the Fu's F shows significant evidence for demographic expansion in the Central population ($Fs = -6.13$, $P = 0.02$) and is consistent with

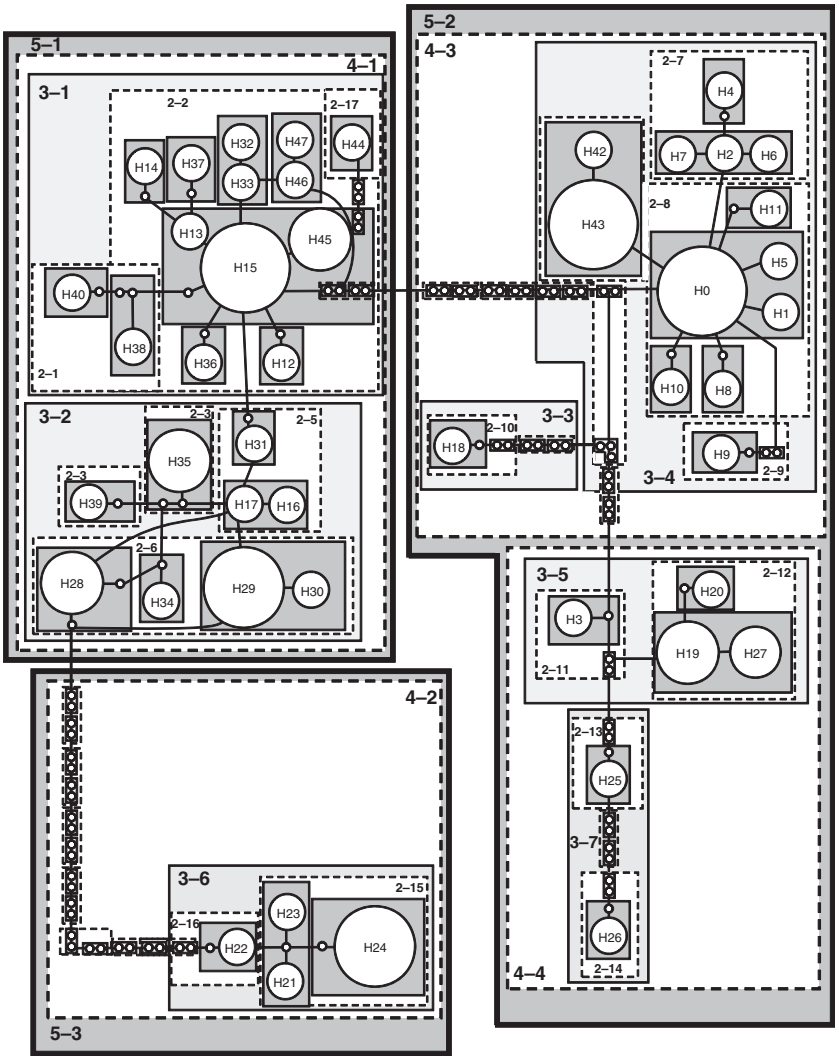

Fig. 2 Haplotype network of 47 unique haplotypes created using the algorithm of Templeton *et al.* (1992) implemented in the computer program TCS. Individual haplotypes are represented by circles. The size of each circle represents the relative frequency of that haplotype in the total sample (n = 79). The smallest circles represent unsampled inferred haplotypes. Neighbouring haplotypes are distinguished by one mutation in 1299 bp of mtDNA. Hierarchically nested clades as determined by the method of Templeton *et al.* (1987, 1992) are indicated. Note that haplotypes that compose Clade 5-1 fall within the Central region, haplotypes that compose Clade 5-2 fall within the Southern region and haplotypes that compose 5-3 fall within the Northern region.

a population expansion in direction for the Southern population (*Fs* = −3.15, P = 0.13).

Microsatellite analysis

After sequential Bonferroni correction for multiple comparisons, there remained five out of 63 significant deviations from HWE (corrected α = 0.00079). HWY had two loci significantly out of HWE while JEN, HARDY and POND each had a single locus that was out of HWE (Table 2). Locus Por 105 was the only locus that was out of HWE in multiple populations. All significant deviations from HWE were due to heterozygote deficiencies and thus may be due to the presence of null alleles in some populations. Because no locus or population was consistently out of HWE, all loci and populations with microsatellite data were utilized in all microsatellite analyses. Per-sampling-locality expected heterozygosity (H_E) ranged from 0.00 to 1.00 with an average multi-locus H_E estimate of 0.52 (SD = 0.29). Within-population allelic richness averaged 4.37 alleles per population (based on the smallest sample size of five diploid individuals) with little variation among geographic regions.

Global F_{ST} was highly significant, but low (F_{ST} = 0.126, 95% CI 0.05–0.24). Thirty-one of the 36 (~86%) of the pairwise F_{ST} estimates were significantly different than zero after Bonforonni correction (Table 3). The highest values of F_{ST} were generally in comparison to FORT or JEN (average F_{ST} = 0.25 and 0.17, respectively). All other pairwise comparisons were low to moderate (F_{ST} = 0.02–0.28). Thus, the large-scale patterns of structure observed in the microsatellite data were congruent with the overall pattern of structure seen in the mtDNA data. A Mantel test for correlation between log-transformed genetic and log-transformed geographic distance showed a significant association (P = 0.0001, slope = 0.78, Mantel r = 0.79) indicating an

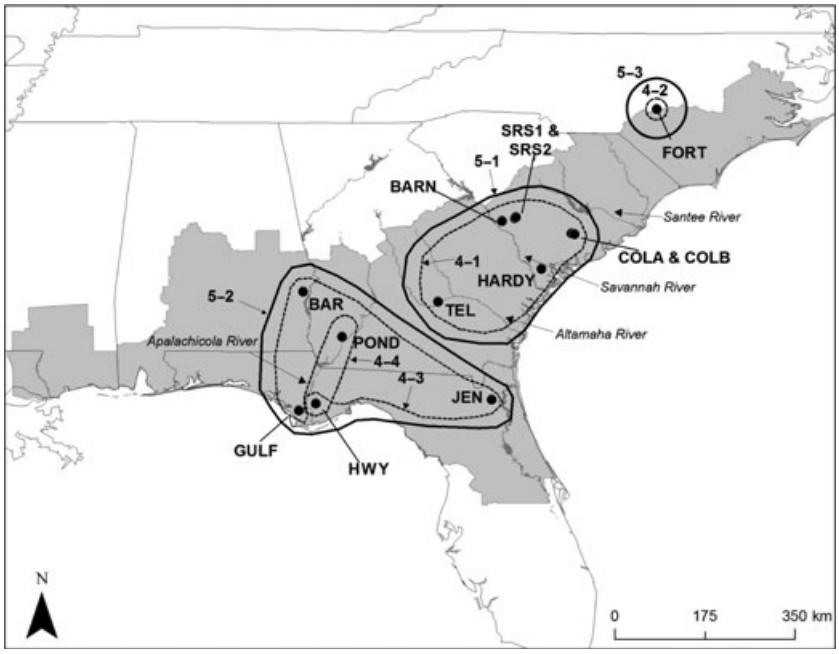

Fig. 3 Current range of *P. ornata*, sampling localities and geographic distribution of higher order clades in nested cladogram. The natural range of *P. ornata* is shaded in grey. The location of sampling sites is indicated by black dots labelled by site name (abbreviations cross reference to Table 1). Thick dark lines denote regional differentiation recovered at five-step clades. Dotted lines denote four-step clades, indicating further division of haplotype relationships within each region.

Table 3 Pairwise F_{ST} (Weir & Cockerham1984; above diagonal) and spatial distance (km; below diagonal) among populations of *Pseudacris ornata* used in Mantel test (see text)

	Northern			Central			Southern		
Population	FORT	SRS1	SRS2	COLA	COLB	HARDY	POND	JEN	HWY
FORT	–	0.2266	**0.2513**	0.2721	**0.2149**	**0.2711**	**0.2391**	0.2775	**0.2271**
SRS1	337	–	**0.0167**	**0.0604**	**0.0489**	**0.0321**	**0.0881**	0.1643	**0.1464**
SRS2	314	23	–	**0.0451**	**0.0446**	**0.0351**	**0.1149**	**0.1956**	**0.1798**
COLA	280	112	96	–	**0.0334**	**0.0627**	**0.1455**	0.2357	**0.1935**
COLB	275	117	102	6	–	**0.0472**	**0.1181**	**0.1868**	**0.1560**
HARDY	360	102	105	84	86	–	**0.0681**	**0.1544**	**0.1278**
POND	676	340	363	428	433	356	–	**0.0587**	**0.0513**
JEN	624	334	350	348	349	267	279	–	**0.0593**
HWY	803	467	490	543	547	464	139	305	–

Boldface F_{ST} denote significant associations (adjusted nominal level of 5% for multiple comparisons is 0.0014). Population abbreviations cross reference to Tables 1 and 2 and Fig. 3.

overall trend of IBD (Fig. 4). SE estimates for IBD slope and intercept were 0.08 and 0.20, respectively.

We used a genetic clustering method implemented in STRUCTURE to identify discrete genetic clusters within the dataset and to determine the geographic scale in which significant genetic structure occurred. This analysis was conducted in several hierarchical scales. At the broadest scale (Fig. 5A) the greatest ΔK occurred at $K = 2$. Other methods we used to infer the value of K [i.e. change in mean $\Pr(X \mid K)$ and mean $\Pr(X \mid K)$ minus half of the variance] corroborated with the ΔK criterion. At this level, one cluster (all the central samples) corresponded well with geography; the other cluster grouped southern localities POND, JEN and HWY with North Carolina's FORT. The unexpected assignments from the northern and southern clade are likely due to the small sample size at some sampling localities (e.g. FORT) as mtDNA analyses and F_{ST} statistics based on microsatellite data did not support the close relationship between Northern Clade and Southern Clade populations (Figs 1–3; Table 3). Due to the misassignment of these individuals, we discarded FORT from all downstream STRUCTURE analyses.

For each individual cluster identified in the first STRUCTURE analysis, we ran STRUCTURE again to look for evidence of substructure. For the central localities

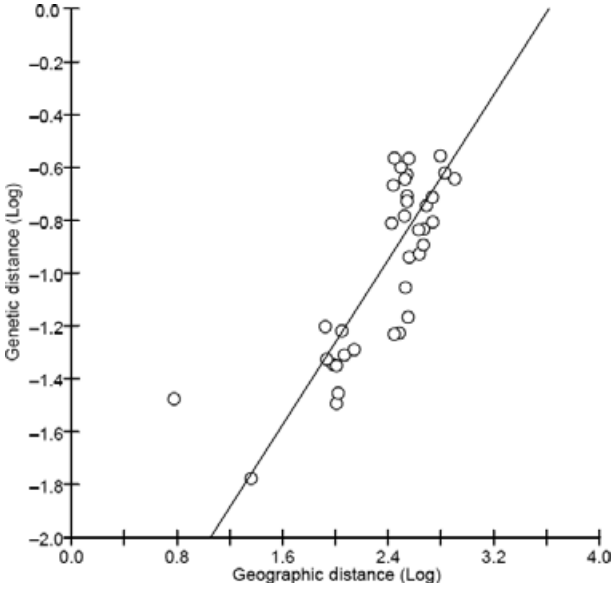

Fig. 4 Plot of log-transformed F_{ST} values (based on microsatellite data) vs. log-transformed geographic distance (km) among all sampling localities indicating evidence for isolation by distance. F_{ST} values are plotted against corresponding log linear Euclidean distances (d) between sites. The equation of the best fit line (shown) is $F_{ST} = 0.78d - 2.82$ (Mantel Test, one-sided $P = 0.0001$ from 9999 randomizations).

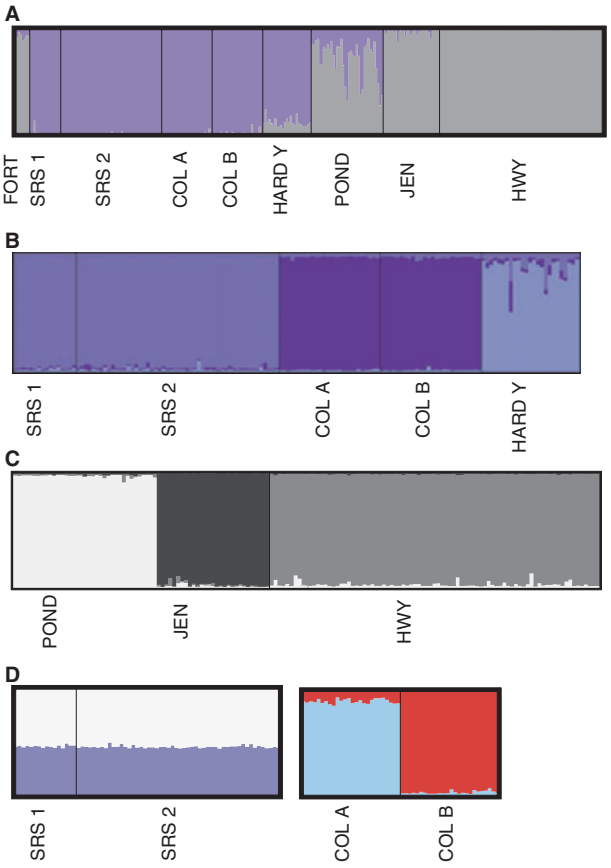

Fig. 5 Results of hierarchical clustering analysis as implemented in STRUCTURE. For A–D the population abbreviations are shown below the structure output. Each individual is shown as a column; the membership coefficient for each *K* is noted as the relative proportion of each colour. (A) The highest level of structure estimated using the Evanno *et al.* (2005) criterion is $K = 2$. For each *K* in (A), a subsequent STRUCTURE analysis revealed substructure among central (B) and southern (C) localities. (D) Two final analyses within (B) show the degree of ancestry between SRS 1 and SRS 2 (left) and between COLA and COLB (right).

(SRS1, SRS2, COLA, COLB and HARDY) we ran STRUCTURE considering three discrete locations in the prior corresponding to geography: (i) SRS1 and SRS2; (ii) COLA and COLB; and (iii) HARDY. STRUCTURE identified three genetic clusters (Fig. 5B). STRUCTURE analysis within the southern localities (JEN, HWY and POND) revealed three distinct clusters when considering location information (Fig. 5C).

We preformed two third-order STRUCTURE analyses to test for further substructure between: (i) SRS1 and SRS2; and (ii) COLA and COLB. For each of these analyses, we chose to incorporate location information. No further substructure was uncovered between SRS1 and SRS2 (Fig. 5D; $r = 8.81$). On the other hand, STRUCTURE identified two clusters separating COLA and COLB ($r = 0.25$). These data suggest that the while Liberty County samples (HWY) or Aiken County samples (SRS1 and SRS2) exist as single breeding populations, Colleton County samples are not panmictic.

Discussion

The broad utility of comparative phylogeography is the ability to characterize biogeographically important features that influence the evolutionary history of co-distributed species. Moreover, even when a species does not match patterns found among other species cohabitating a specific area, the mechanisms driving the divergent pattern can be resolved. A recent literature review of the biogeography of unglaciated eastern North America (Soltis *et al.* 2006) suggests that the complexity of the biogeography of unglaciated North America seems to have been underappreciated. Indeed, there are a number of distinct biogeographic features in eastern North America which contribute to the genetic structure of species in this region. With regard to our data, we found that *P. ornata* splits into three genetic clades that occur across geographically distinct areas (i.e. Northern, Central and Southern) of eastern North America. The analysis of microsatellite data was generally in agreement with the pattern of genetic structure inferred from mtDNA. The detection of three geographically discrete

groups begs the question of whether there are loose biogeographic features that are coincident with the genetic breaks and may have caused their divergence. We address this question by comparing *P. ornata* structure to that of species found across eastern North America.

In the Southern clade, the Apalachicola River and historical features associated with it (e.g. periodic saltwater embayment) may be particularly important in determining the phylogeography of southeastern coastal plain endemics (Pauly *et al.* 2007). Although not all southeastern species that show phylogenetic breaks exhibit those breaks at the same location (e.g. the Apalachicola River; see review in Soltis *et al.* 2006), but at least nine southeastern coastal plains taxa belonging to a broad array of phylogenetic groups show a discontinuity at the Apalachicola River indicating high concordance across many taxa including amphibians (*Pseudobranchus striatus*; Liu *et al.* 2006), reptiles (*Sternotherus minor*, *Gopherus polyphemus*; Osentoski & Lamb 1995; Walker & Avise 1998), mammals (*Geomys pinetis*, *Peromyscus polionotus*; Avise *et al.* 1979, 1983), gymnosperms (*Pinus clausa*; Parker *et al.* 1997), and angiosperms (*Dicerandra linearifolia*, *Polygonella gracilis/P. macrophylla*; Oliveira *et al.* 2007; Lewis & Crawford 1995). Thus, when considering only southeastern coastal plain endemic organisms, the phylogeographic concordance is extraordinarily consistent. Our data suggests that the Apalachicola River has not significantly impeded gene flow in *P. ornata*. Alternatively, substantial *P. ornata* migration (or range expansion) has occurred across this river. A more thorough sampling from west of the Apalachicola River will be required to formally distinguish between these hypotheses. Nevertheless, testing this hypothesis may prove difficult as recent surveys from west of the Apalachicola River indicate that populations of *P. ornata* have recently become extirpated (S Richter, pers. comm.). The Central clade also did not correspond to other common geographic barriers. For example, the Central clade included populations spanning both the northern branch of the Altamaha River system and the Savannah River. Both of these rivers have been found to be biogeographic barriers for freshwater-dependent aquatic and terrestrial species including fishes (e.g. Gilbert *et al.* 1992; Wirgin *et al.* 2001; Wooten *et al.* 1988) and other amphibians (e.g. *Pseudobranchus striatus*—Liu *et al.* 2006; *Rana capito*—Conant & Collins 1991).

Divergence points in *P. ornata* do not appear coincident with other salient geographic features corresponding to genetic structuring in other terrestrial co-distributed taxa. Phylogeographic structure may be better explained by the particular life history of these frogs. Irwin (2002) suggested that deep phylogeographic structure could be detected in absence of biogeographic barriers and in species with low dispersal rates and small population sizes. Other studies have found that *Pseudacris* species usually travel infrequently. For example, Kramer (1973) studied movement *P. triseriata* for a 5-month period and found that 76% of frogs stayed within 100 m of breeding ponds while only 2% moved more than 200 m. A recent study by Lemmon & Moriarty (2008) indicated that within the trilling chorus frog clade, per generation dispersal distance ranged from 57.38 m–373.37 m depending on species studied. The inability to move long distances could have severed the gene flow among contiguous populations generating the deep genetic divergence among nuclear loci and monophyletic mitochondrial haplogroups. These data are in agreement with previous theoretical and empirical studies that stress the need to account for dispersal capabilities and variance in coalescent times before making evolutionary inferences based only on phylogeographic patterns and genetic distances (Edwards & Beerli 2000; Irwin 2002). The most parsimonious explanation for these observations suggests that IBD is responsible for the geographic structuring of mtDNA haplotypes in this species. The Mantel test, detecting an overall pattern of isolation by distance, supports this conclusion.

Our estimates of divergence times and historical demography indicate that divergence times between clades originated long before the occurrence of Pleistocene glacial fluctuations that are believed to be important in other eastern species (e.g. Ellsworth *et al.* 1994). Indeed, *P. ornata* and its contemporaneous sister clade (*P. illinois* + *P. streckeri*) diverged during the Miocene, a period that was important for the diversification of chorus frogs (Moriarty *et al.* 2007). Furthermore, the diversification within *P. ornata* started at the end of the Pliocene. During this period continuous and dramatic changes in vegetation cover and temperature may have severed gene flow across the range of *P. ornata* generating the observed phylogenetic pattern. Similar temporal patterns have been observed in other ectotherms (Moriarty *et al.* 2007; Fontanella *et al.* 2008; Guiher & Burbrink 2008). However, Pleistocene glacial fluctuations did influence population demography of *P. ornata*. The Bayesian skyline plots and the mismatch distribution analysis accordingly suggest a historical expansion in both Southern and Central populations. This expansion is probably the result of reduction of range size due to aridification during Pleistocene climatic fluctuations and the subsequent increase in population size due to improving habitat for this pond-breeding species (cf. Hoffman & Blouin 2004; Moriarty *et al.* 2007). Overall, our data suggests that phylogeographic patterns observed for *P. ornata* do not correspond to patterns found in other species because phylogeographic breaks

in *P. ornata* predated the Pleistocene and are largely influenced by IBD since their original divergence.

Our second hypothesis predicted high allelic differentiation among populations and detectable genetic structure on small spatial scales. Indeed, the most geographically distant populations showed moderate to high allelic differentiation; however, genetic distance between some populations separated by fairly large geographic distances were very low to moderate (e.g. F_{ST} between POND and HWY was 0.05 despite 139 km separation). Our second hypothesis also predicted that genetic structure would be significant even on microgeographic scales of several kilometres. There was evidence for fine scale population genetic structure in this species; however, this fine scale genetic structure was only present in the more fragmented and disturbed of three widespread sampling localities (i.e. SRS1 and SRS2; COLA and COLB and within HWY). In Colleton Co., SC where the landscape was largely agricultural with a relatively higher density of roads, two samples separated by 6 km and on opposite sides of a state highway were distinguishable based on assignment to different genetic clusters in the STRUCTURE analysis and these showed significant values of F_{ST} between discrete sampling sites ($F_{ST} = 0.03$, $P < 0.01$ for null hypothesis $F_{ST} = 0$). However, samples from Liberty Co., FL (i.e. HWY) collected over an even larger geographic range (~14 km) but in a continuous forest landscape (within the Apalachicola National Forest), were not assignable to discrete genetic clusters and appeared to be from a single population (Fig. 5C). Similarly, STRUCTURE failed to distinguish among individuals between SRS1 and SRS2 located within the US Department of Energy's Savannah River Site (separated by 23 km of relatively unfragmented landscape). It is worth noting, however, that pairwise F_{ST} data between SRS1 and SRS2 did reveal significant structure, albeit low ($F_{ST} = 0.0167$; Table 3).

Previous studies involving amphibians have found differences in genetic structure, migration and dispersal or density and abundance in rural vs. urban habitats (Rubbo & Kiesecker 2005), developed vs. undeveloped habitats (Carr 1940; Vos *et al.* 2001) and fragmented vs. non-fragmented habitats (Gibbs 1998). Thus, we find it reasonable to hypothesize that the differences seen among these three sampling sites are due at least in part to development and habitat fragmentation. Further research explicitly addressing these landscape genetic questions may shed light on the differences in gene flow among these sampling sites.

Do these patterns fit our expectations? We had three a priori predictions based on previous data. Hypothesis 1 predicted a phylogeographic concordance between *P. ornata* and other co-distributed species. In general, we did not observe this pattern. A subsequent review of the phylogeographic literature on eastern North American species suggested that our findings were indeed out of the ordinary in that known biogeographic barriers were not reflected by *P. ornata*. Our results may stem partly from the fact that genealogical divergence in *P. ornata* pre-dates the Pleistocene. Hypothesis 2 predicted substantial allelic differentiation among geographically proximate populations. We found that only the populations in a more developed landscape were significantly diverged at small spatial scales. This suggested the possibility that recent habitat fragmentation could be a significant determinate of fine-scale genetic structure in this species. Hypothesis 3 predicted an overall pattern of IBD based on the presumed limitations of amphibian dispersal. We did observe this overall pattern of IBD; however, we were surprised by the large geographic scale at which this pattern was evident. Using microsatellite data, we found that F_{ST} for samples separated by as much as 150 km was low ($F_{ST} < 0.1$, Table 3).

Amphibians are often thought to naturally occur in highly structured metapopulations (Beebee 1996, 2005; Newman & Squire 2001; Palo *et al.* 2004; but see Hoffman *et al.* 2004). In *P. ornata*, genetic structure was less pronounced than we predicted based on this supposition. With regard to management strategies for endangered amphibian populations, this species highlights the complexities that can be uncovered even when strong phylogeographic concordance of similar species suggests the occurrence of a specific phylogeographic pattern. Our results suggest that a species-specific understanding of historical isolation would be required to management strategies that require knowledge of genetic structure (e.g. translocations).

Acknowledgements

We would like to thank Emily Moriarty Lemmon, Aubrey Heupel, Joyce Brown and Nicole Thurgate for help collecting the tissue samples used in this study and to David Cannatella for loaning tissues under his care at the Texas Natural History Collections, Texas Memorial Museum. Thanks also to Jan Williams who provided help with various aspects of the molecular laboratory work in this study. Todd Castoe and Josh Reece provided much appreciated help with various aspects of the molecular data analyses. We thank Lisa McCauley for help with map design and Dr. Thorpe and three anonymous reviewers for insightful comments which improved the manuscript. This study is funded in part through the University of Central Florida Biology Graduate Research Enhancement Award. The animal use protocol was approved by the University of Central Florida IACUC Committee (Animal Project Number 06-01W). All unique mtDNA sequences reported in this paper were deposited in GenBank under accession numbers GU985281–GU985438.

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This study was conducted at the University of Central Florida as part of J.F.D.'s thesis on the evolutionary history of *P. ornata*. He is currently working on his PhD at U. Chicago in the Committee on Genetics, Genomics and Systems Biology. D.M.S was an undergraduate in the lab and is currently a masters student at UNF in the Department of Biology researching salt marsh ecology. T.D.H was a masters student in the Hoffman lab researching the landscape genetics of anurans. He is currently working on his PhD at U. Idaho in the Department of Biology. J.M.D was a PhD student at UCF and his research focuses on biogeography and historical demography of snakes. E.A.H is an Assistant Professor at UCF with research that focuses on population genetics, conservation genetics and invasion genetics of numerous taxa.

Supporting Information

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

Fig. S1 Bayesian skyline plots depicting relative population sizes through time in two population of *Pseudacris ornata*. Demographic parameters were obtained in Beast 1.5.2 with four independent MCMC coalescent simulations of 20 million generations each and using the GTR + G + I model. Dotted vertical lines represent the mean, lower and upper 95% credibility intervals for the tMRCA for each population (the upper limit for the Southern Clade is not shown). Coloured shade represents the 95% credibility region for the relative population size.

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