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Phylogeography and evolution of the Red Salamander (*Pseudotriton ruber*) ☆

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ABSTRACT

Phylogeographic studies frequently result in the elevation of subspecific taxa to species given monophyly, or the synonymy of subspecies that are not monophyletic. However, given limited or incongruent datasets, retention of subspecies can be useful to describe hypothesized incipient species or to illustrate interesting biological phenomena driving morphological diversity. Four subspecific taxa have been used to describe largely allopatric geographic variation within the species *Pseudotriton ruber*, a plethodontid salamander occupying stream and spring habitats across eastern North America: *P. r. vioscai* occurs in lowland Coastal Plain habitats, while *P. r. ruber*, *P. r. nitidus*, and *P. r. schencki* occupy upland regions in and around the Appalachian Mountains. *Pseudotriton ruber* co-occurs through its distribution with the aposematic newt *Notophthalmus viridescens*, and both species are hypothesized to be part of a Müllerian mimicry complex. In this study, we sequenced regions of two mitochondrial (cytochrome *b*, NADH dehydrogenase subunit 2) and one single copy nuclear protein-coding gene (pro-opiomelanocortin) from individuals sampled across much of the distribution of *P. ruber* and then used maximum-likelihood and Bayesian phylogenetic inference to test the monophyly of subspecies, reconstruct biogeographic history, and make inferences about morphological evolution. Phylogeographic hypotheses from mitochondrial and nuclear datasets described structure among populations of *P. ruber* which separated Coastal Plain and upland Appalachian populations, but subspecies were not monophyletic. Biogeographic reconstruction estimated the ancestor of all populations to have occupied and initially diverged in the Coastal Plain during the Pliocene (~3.6 mya), before one lineage subsequently invaded upland areas of Appalachia. Bold bright coloration of high elevation subspecies *P. r. nitidus* and *P. r. schencki* appears to have evolved twice. We hypothesize that the Müllerian mimicry complex with *N. viridescens* and *P. ruber* may provide a selective mechanism driving the co-evolution of striking bright and dull morphological variation among populations of both species. While *P. ruber* subspecies were not consistent with our criteria for diagnosing species (monophyly) and therefore could not be elevated to species, we advocate for the retention of subspecies because they describe hypotheses about an incipient species (*P. r. vioscai*) and how Müllerian mimicry may shape morphological diversity of species.

1. Introduction

Mayr (1963) defined subspecies as "an aggregate of local populations of a species, inhabiting a geographic subdivision of the range of the species, and differing taxonomically from other populations of the species", where 'differing taxonomically' means individuals within the subspecies have distinctive co-occurring characters (Mayr, 1963). Such populations may represent species awaiting a proper diagnosis, remnant non-random variation associated with incomplete species-forming processes, or paraphyletic populations which possess convergent morphology as a result of processes such as selection (Jorgensen et al., 2013). In the last three decades, however, a general consensus among systematic biologists has emphasized the use of phylogenetically-based taxonomy (e.g., Frost and Hillis, 1990), and application of this theory with molecular phylogenetic analyses to understand geographic variation of populations has frequently resulted in significant changes toward the use of subspecies. In many cases, phylogeographic studies result in the revision of wide-ranging species to be recognized as multiple, smaller-ranged species (e.g., Lemmon et al., 2007; Pauly et al., 2007; Gamble et al., 2008; Lamb and Beamer, 2012), often by elevating monophyletic subspecies to species

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(e.g., Glor and Laport, 2012). Alternatively, in cases where subspecies are not recovered as monophyletic in analyses, subspecific taxa are frequently placed in synonymy (e.g., Ennen et al., 2014; reviewed in Torstrom et al., 2014).

While our ability to diagnose and classify species as individuals is an inherently discrete procedure (de Queiroz, 1998), the process of speciation does not occur in such a clear fashion (Wake, 2006). Consequently, studies with limited genetic datasets (1–3 loci) are frequently challenged with the problem of revising subspecific taxonomies given results where subspecies are not monophyletic but do demonstrate phylogenetic structure. To remedy this issue, one approach has been to elevate basal and allopatric subspecies to be species, while maintaining subspecific taxonomy for populations that are not reciprocally monophyletic but exhibit distinctive morphological variation and strong phylogenetic signal (Mulcahy, 2008). Such a stance can serve to maintain awareness of putative incipient species awaiting a proper diagnosis given more data (e.g., Makowsky et al., 2010), or to emphasize interesting biological processes shaping morphological diversity, such as selection (Richmond and Reeder, 2002). Here we present a case study where subspecific taxonomy is useful to describe a hypothesized incipient species and also to illustrate an apparent case where Müllerian mimicry appears to be driving the parallel evolution of morphological variation of paraphyletic populations.

Pseudotriton ruber (Red Salamander) is a relatively large-bodied lungless salamander (family Plethodontidae; subfamily Hemidactylinae; tribe Spelerpini) occupying springs, lower-order streams, and terrestrial habitats in eastern North America, from southern New York, southwest through Appalachia, to Louisiana. While the majority of the species' range occurs in upland habitats in the Piedmont and Appalachian Mountains, the species also occupies Coastal Plain areas from South Carolina west to western Kentucky. *Pseudotriton ruber* is diagnosed morphologically by fused premaxillary nasal processes, relatively stout bodies with 16–18 costal grooves, and yellow or golden irises with a dark horizontal bar through each (Martof and Rose, 1963; Petranka, 1998). The species exhibits geographic variation in color pattern that has resulted in the description and recognition of four largely allopatric subspecies: the nominate *P. r. ruber* (Northern Red Salamander), *P. r. nitidus* (Blue Ridge Red Salamander), *P. r. schencki* (Black-chinned Red Salamander), and *P. r. vioscai* (Southern Red Salamander; Fig. 1; Appendix A). In particular, the greatest variation appears to exist between the brightly-colored body and iris in populations to the north and east (*P. r. ruber*, *P. r. nitidus*, *P. r. schencki*) relative to the drably-colored body and distinctive eye coloration of *P. r. vioscai* in the Coastal Plain (Appendix A; Petranka, 1998).

Some taxonomic uncertainty has been expressed about the appropriateness of subspecific designations for describing geographic variation of *P. ruber*. For example, Bruce (1968) did not recognize subspecific taxa in his detailed examination of the life history of *P. ruber* because he questioned the utilization of trinomials for the species (although his stance was not clarified). Next, Martof (1975) noted that larvae from the Appalachian Mountains are indistinguishable from those of populations in the Coastal Plain of Mississippi (Valentine and Dennis, 1964) and that little variation in life history exists among distant populations (Bruce, 1968), concluding that subspecific designations would not be appropriate until more comprehensive analysis of geographic variation in life history, morphology, and ontogenetic changes in pigmentation had been completed. On the other hand, Mount (1975) recognized two subspecies (*P. r. ruber* and *P. r. vioscai*) as occurring in Alabama; however, Mount noted that populations throughout a large region of central Alabama appeared to exhibit intermediate morphologies between the two subspecies, and he suggested that these populations represented intergrades (Mount, 1975). Despite Martof's (1975) recommendation, most authors thereafter recognized the subspecific designations and some form of Mount's intergrade hypothesis (e.g., Conant and Collins, 1998; Petranka, 1998).

To date, no study has attempted to examine geographic variation of *P. ruber* in a comprehensive fashion. This is striking to us because the species exhibits considerable morphological variation across its distribution, some of which corresponds to distinctive physiographic regions and/or biogeographic barriers (e.g., the Fall Line – the transition zone between upland Piedmont and Ridge and Valley habitats and the sandy Coastal Plain in southeastern North America), and each of these subspecies was described over 85 years ago (Appendix A). If morphological phenotypes within *P. ruber* correspond to allopatric ecoregions and are monophyletic in phylogeny, this would be consistent with speciation according to evolutionary, phylogenetic, and general lineage concepts (Wiley, 1978; Cracraft, 1983; de Queiroz, 1998, 2007). If morphological phenotypes do not exhibit phylogenetic signal and are distributed randomly across phylogeny, we consider this as a criterion allowing the rejection of subspecies taxonomy.

Alternatively, if morphological phenotypes are not monophyletic but exhibit phylogenetic structure, other interesting evolutionary processes may have generated morphological variation among populations. *Pseudotriton ruber* co-occurs with *Notophthalmus viridescens* (Eastern Newt), a salamander species with a terrestrial life-history stage ('eft') where individuals are brightly colored and produce a powerful neurotoxin in the skin that is a highly effective chemical defense against predators. Bright-red coloration of *N. viridescens* is thought to serve as an aposematic signal to would-be predators (reviewed in Petranka, 1998), and several lines of evidence suggest that *P. ruber* is part of a Müllerian mimicry complex with *N. viridescens*, within which both species are unpalatable and may benefit through co-evolution of similar aposematic coloration (Howard and Brodie, 1971, 1973; Brodie, 1977; Brandon and Huheey, 1981). This hypothesis is supported by the observation that morphology and coloration of *N. viridescens* efts vary across the species' distribution in ways that mirror those of *P. ruber*. Specifically, the distribution of the subspecies *N. v. louisianensis* (Central Newt), a population which is relatively dull in coloration during the eft stage and lacks red spots as adults, occurs in Coastal Plain habitats and overlaps greatly with the distribution of the dully-colored *P. r. vioscai* phenotype. Conversely, the brightly-colored *N. v. viridescens* (Red-spotted Newt) is distributed in upland Appalachia and is sympatric with *P. r. ruber*, *P. r. nitidus*, and *P. r. schencki*. Covariance with *Notophthalmus* morphology is also observed in two other hypothesized Müllerian mimics, *Pseudotriton montanus* (Mud Salamander) and *Gyrinophilus porphyriticus* (Spring Salamander). We find the geographic concordance and consistent shifts in color pattern between dull and brightly-colored populations of these four species to be particularly salient, and we suggest that the interplay between geographic variants of this Müllerian mimicry complex may be a selective mechanism influencing the distinct geographic coloration of *N. viridescens* and *P. ruber*. Because natural selection via a Müllerian mimicry complex may drive morphological variation among populations of *P. ruber*, a subspecific taxonomy could be a useful tool for highlighting the evolutionary diversity of the species (*sensu* Mayr, 1982).

A recent phylogenetic analysis of spelerpine salamanders suggested that *P. ruber* subspecific taxa are not monophyletic (Bonett et al., 2014). Specifically, this study found that *P. r. ruber* and *P. r. vioscai* were not monophyletic but that a Coastal Plain population exhibited a distinctive haplotype and was sister to other populations, suggesting divergence and potentially undiagnosed taxonomic diversity among populations of *P. ruber*. In this study, we collected mitochondrial and nuclear DNA sequence data from a large sample of *P. ruber* populations. We then used

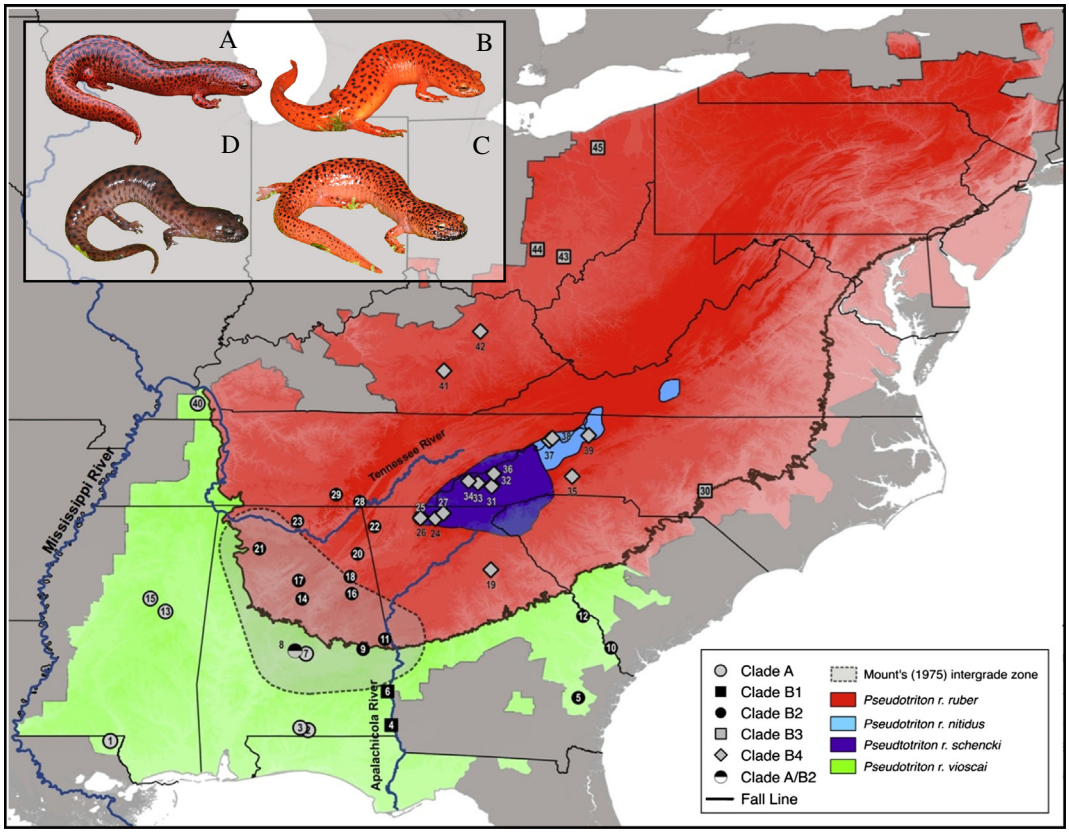

Fig. 1. Geographic distribution and representative photographs of the four *Pseudotriton ruber* subspecies: *P. r. ruber* (Northern Red Salamander; A), *P. r. nitidus* (Blue Ridge Red Salamander; B), *P. r. schencki* (Black-chinned Red Salamander; C), and *P. r. vioscai* (Southern Red Salamander; D). The map was adapted from Mount (1975), Petranka (1998), and Niemiller and Reynolds (2013). Symbols indicate the sampled populations, are labeled numerically by population (Appendix B), and are shaped according to the major mitochondrial clades (legend) to which they belong in Fig. 2. The Fall Line is indicated by a bold black line; this line separates the distinctive sandy physiography of the Coastal Plain to the south and west from the rockier physiography of the upland Appalachian region (see Fig. 4). The Apalachicola, Tennessee, and Mississippi rivers are labeled by blue lines. High-elevation relief of the Appalachian Mountains is indicated by darkened topographic coloration within range polygons. A disjunct population of *P. r. ruber* from northern New York is not shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

maximum-likelihood and Bayesian phylogenetic analyses to estimate the genealogical history of populations and test whether or not *P. ruber* subspecies are monophyletic lineages in phylogeny. We generated phylogenetic hypotheses which describe strong genetic structuring among populations of *P. ruber*. Lastly, we used a time-calibrated phylogeny and ancestral range estimation analysis to generate hypotheses about the historical biogeography and evolution of geographic color variation of *P. ruber*.

2. Materials and methods

2.1. Taxon sampling

We collected *P. ruber* by visiting low-order creeks, springs, and seepages from February–October 2013. We searched for salamanders by flipping cover objects (e.g., logs, rocks) or raking leaf-litter in hydric microhabitats and by scooping submerged leaf-litter detritus and muddy seepages by hand or dipnet. Tissues were acquired by clipping the posterior end of the tail; samples were immediately placed in vials containing RNAlater (Ambion) or 95% Et-OH. Adults were collected whenever possible to verify subspecific identity of individuals based on morphology. However, because adults are difficult to detect given their fossorial habits, the majority of our samples were larvae collected by dipnet. Because we were unable to verify the subspecies of larvae, we used recent range maps (Conant and Collins, 1998; Petranka, 1998; Niemiller and Reynolds, 2013) to infer a subspecific category for each sample.

We attempted to collect samples evenly throughout the species' range, including populations attributable to each subspecies. We augmented our sample by soliciting tissues from natural history museums. Our final sample included 20 individuals from 14 localities within the range of *P. r. ruber*, eight individuals from three localities within the range of *P. r. nitidus*, 22 individuals from nine localities within the range of *P. r. schencki*, 18 individuals from 11 localities within the range of *P. r. vioscai*, and 15 individuals from eight localities within the putative *ruber* × *vioscai* intergrade zone (Fig. 1; Appendix B). Our samples are spread relatively evenly across the species' range, except for the northeastern-most populations, from which we were unable to obtain samples. Tissue samples were sequenced for multiple individuals from some populations ($N = 21$), but only one individual was sequenced for many populations ($N = 25$).

2.2. Molecular methods

DNA was extracted from tissue samples using the DNeasy Blood and Tissue kit (Qiagen) using the animal tissue protocol. Genomic DNA quantity and quality were assessed using a NanoDrop 2000 Spectrophotometer (Thermo Scientific), and extracted DNA was then stored at −20 °C. Extracted DNA and tissue samples are currently housed at the Auburn University Museum of Natural History.

Primer sequences for two mitochondrial genes encoding the proteins cytochrome *b* (CytB) and NADH dehydrogenase subunit 2 (NAD2) and one nuclear gene encoding proopiomelanocortin

(POMC) were obtained from Lamb and Beamer (2012). Polymerase chain reaction (PCR) was performed for each sample in a total reaction volume of 25 μL comprising 16.9 μL of distilled water, 2.5 μL of *Taq* (TaKaRa ExTaq) buffer, 2.0 μL dNTP (with $MgCl_2$), 1.25 μL of each primer, 0.125 μL of *Taq* DNA polymerase, and 2 μL of template DNA. Cycling parameters were also taken from Lamb and Beamer (2012). PCR products were run on a 2% agarose gel with 1 μL of loading dye (EZvision) and 4 μL of PCR product in each well, and were visually checked for quality on a transilluminator (Benchtop 2UV). The PCR products were purified with ExoSap-IT (Affymetrix) before standard sequencing reactions were carried out by Macrogen (Rockville, Maryland).

Sequences were aligned and edited using Geneious v. 5.6.3 (Biomatters, Auckland, New Zealand), resulting in 866 base pairs (bp) for CytB, 474 bp for NAD2, and 477 bp for POMC. Our alignments for each mitochondrial gene (CytB, NAD2) contained 88 terminal taxa, including 82 *P. ruber* and six out-group samples (*Pseudotriton montanus*, *Gyrinophilus porphyriticus*, *Stereochilus marginatus*, *Eurycea cirrigera*, *Urspelerpes brucei*). The nuclear gene (POMC) was sequenced for a subset of *P. ruber* individuals ($N = 25$) and one sequence from Lamb and Beamer (2012) was included from GenBank; thus, the nuclear dataset (see below) was more limited from a sampling perspective.

2.3. Phylogenetic analyses

We generated phylogenetic trees of *P. ruber* and outgroup taxa by analyzing four datasets – (1) two concatenated mitochondrial genes (CytB, NAD2), (2) a single gene alignment of the nuclear gene POMC, (3) a subset mitochondrial dataset which included mitochondrial data for the same samples analyzed for the nuclear gene POMC, and (4) a concatenated mitochondrial and nuclear dataset. The program PartitionFinder (Lanfear et al., 2012) was used to select optimal partitioning schemes and substitution models using maximum likelihood (ML) analyses in RAxML v. 7.2.8 (RAxML; Stamatakis, 2006). From a fully partitioned analysis (each codon position as a separate partition for each gene), PartitionFinder selected an optimal partitioning scheme for each analysis using the 'greedy search' option with BIC in RAxML. Three partitions were recovered for the mitochondrial genes across all analyses – one for each codon position (e.g., partition 1 = position 1 of CytB + position 1 of NAD2). For POMC, codon positions 1 and 2 were consistently recovered as a partition to the exclusion of codon position 3. Partitions shared the same model and were jointly parameterized. Partitioning schemes for all analyses and substitution models for all partitions are in Appendix C. Of the two partitioning schemes and substitution models allowed by RAxML, the GTR + G substitution model was selected for all partitions of both mitochondrial genes in all four analyses and for all partitions of POMC in the single gene analysis, but the GTR + I + G model was selected for all partitions of POMC in the fourth analysis (Appendix C). Each analysis employed 1000 random addition sequence replicates, and bootstrap support values were generated by 1000 non-parametric bootstrap replicates.

We inferred an additional phylogeny from the two concatenated mitochondrial genes dataset using BEAST (v1.8.1, Drummond et al., 2012). We approximated node ages within the inferred phylogeny with a strict molecular clock calibrating nodes with age estimates from Bonett et al. (2014). We assigned lognormal priors (mean in real space) to the node representing the most recent common ancestor (MRCA) of the *P. ruber* ingroup as 5.5 mya (log SD = 0.35), the *P. ruber* + (*P. montanus* + *G. porphyriticus*) ingroup node as 27 mya (log SD = 0.15, offset = 1), the *S. marginatus* + (*P. ruber* + (*P. montanus* + *G. porphyriticus*)) node as 29 mya (SD = 0.2, offset = 4), and the (*E. cirrigera* + *U. brucei*) node as 44 mya (SD = 0.1). A Yule (speciation) model was used as the tree prior. The node age and 95% C.I. was used to reconstruct a second chronogram using all ingroup taxa under an uncorrelated lognormal clock analysis with a coalescent model (constant population size) as the tree prior; we report this second chronogram with all ingroup specimens in our paper. For both analyses, two separate Markov Chain Monte Carlo (MCMC) searches were performed for 100,000,000 generations with sampling every 10,000. Effective sample size (ESS) and graphical examination of chain convergence were assessed in Tracer (v. 1.6; Rambaut et al., 2014). Topology convergence was graphically assessed using AWTY (Are We There Yet, Wilgenbusch et al., 2004). Log files and trees from separate runs were assembled with LogCombiner (v1.8.1, Drummond et al., 2012) and 10% of generations were discarded as burn-in. A maximum clade credibility consensus tree was produced using TreeAnnotator (v1.8.1, Drummond et al., 2012) with median node heights.

Individuals from five closely-related plethodontid species from each representative genus within the plethodontid tribe Spelerpini were used as outgroup taxa for the full mitochondrial phylogenetic analysis; the same outgroups were used for the other three analyses except for *P. montanus*. Data for outgroups were obtained by (1) collecting tissue samples and generating sequence data for two individuals of *P. montanus* (for the full mitochondrial analysis), and (2) downloading sequence data for *G. porphyriticus*, *S. marginatus*, *U. brucei*, and *E. cirrigera* from GenBank (Appendix B). All trees were rooted with the node comprising *E. brucei* and *E. cirrigera*.

We sought to sequence the mitochondrial markers for a large number of samples because mitochondrial loci are characterized by a relatively high mutation rate and can be informative for understanding fine-scale phylogeographic structure. However, because the mitochondrial genome is maternally inherited, inferences from these data are limited to describing phylogeographic structure of females and may mask true gene flow mediated by male dispersal. This may be particularly important because an emerging body of literature from terrestrial tetrapods has demonstrated that females frequently experience limited dispersal relative to that of males (Dobson, 1982), including for salamanders (Liebgold et al., 2011; Helfer et al., 2012). Such differential dispersal may cause patterns of mitochondrial evolution to strongly reflect maternal lineages and overestimate population structure within hypothesized species. Consequently, we used the major phylogeographic structure inferred from the mitochondrial analysis to guide which samples we sequenced for the nuclear gene POMC; thus, the POMC dataset was a subset of populations representing each clade identified in the mitochondrial analysis.

2.4. Ancestral range estimation

We estimated the ancestral range of *P. ruber* populations using a likelihood-based inference approach implemented in the software package *BioGeoBEARS* (Matzke, 2014) in the statistical program R (R; R Core Team, 2015). The analysis was performed on the dated Bayesian phylogeny inferred from the mitochondrial dataset comprising all *P. ruber* specimens (described above). Specimens were collapsed into 15 population-groups of comparable branch length (Appendix D) to estimate the ancestral ranges of monophyletic populations rather than specimens (Matzke, 2013). The geographical areas used in the reconstruction were the Coastal Plain (C), Piedmont (P), Ridge and Valley (R), Blue Ridge (B), and the Appalachian Plateau (A) physiographic regions (Fenneman, 1928). We scored each specimen in the phylogeny ($N = 83$) as occurring within one of the above geographic areas, and then scored each population-group to geographic areas based on the localities of its constituent specimens (Appendix D). Two populations (23, 45)

Table 1
Summary statistics describing variation within major phylogenetic clades of the Red Salamander (*Pseudotriton ruber*), as inferred from maximum-likelihood phylogenetic analyses of mitochondrial (concatenated CytB and NAD2) and nuclear (POMC) sequence data.

Locus	Clade	Sample size	Haplotypes	Polymorphic sites	π (nucleotide diversity)
CytB + NAD2	A	12	9	62	0.0133
	B1	3	2	5	0.0025
	B2	27	25	87	0.0120
	B3	6	1	0	0.0000
	B4	35	25	52	0.0075
POMC	A	10	3	3	0.0022
	B	16	14	10	0.0016

occurred in the Interior Low Plateau adjacent to the Appalachian Plateau, but we scored these as occurring in the latter region to simplify the number of parameters included in the model-building process. Because *BioGeoBEARS* allows populations to be distributed across multiple regions, some population-groups were scored as occurring in more than one area.

We built six different models estimating the biogeography of *P. ruber*: the Dispersal-Extinction Cladogenesis Model (DEC), the Dispersal-Extinction Cladogenesis Model with the founder parameter *j* (DEC + J), the Dispersal-Vicariance Analysis (DIVA), the Dispersal-Vicariance Analysis with the founder parameter *j* (DIVA + J), Bayesian inference of historical biogeography for discrete areas (BAYAREA), and Bayesian inference of historical biogeography for discrete areas with the founder parameter *j* (BAYAREA + J; Matzke, 2013, 2014). Each model allows for the estimation of dispersal, vicariance, and extinction using maximum-likelihood (DEC, DEC + J, DIVA, DIVA + J) or Bayesian (BAYAREA, BAYAREA + J) approaches (Matzke, 2014). We compared and ranked each of the six models using Akaike's Information Criterion (ΔAIC; Akaike, 1974) and calculated model weight (the probability of each model being the true best model). We used the model with the lowest AIC (ΔAIC = 0.00) for inference, but we also gave consideration to other models within the top-model set of ΔAIC < 2.00 (Burnham and Anderson, 2002).

3. Results

We recovered 62 haplotypes among 83 individuals from 46 localities of *P. ruber* based on the full mitochondrial dataset (Table 1), from which we identified five clades in our ML phylogenetic analysis (Clades A, B1–B4; Fig. 2). The topology of *P. ruber* samples in this phylogeny consisted of two well-supported sister groups (support value = 100), Clade A and Clade B, where Clade B comprised two sister groups (81) including Clades B1 + B2 (81) and Clades B3 + B4 (63), respectively.

In the mitochondrial ML analysis, the four subspecific taxa of *P. ruber* did not form monophyletic groups to the exclusion of one another. Specifically, Clade A was composed of *P. r. vioscai* samples from the Coastal Plain of western Kentucky, Mississippi, Louisiana, and south-central Alabama, along with samples from two localities within Mount's *P. r. ruber* × *vioscai* intergrade zone (Mount, 1975). Sister to Clade A was a monophyletic Clade B with two sister groups. One interior group comprised the sister Clades B1 and B2: Clade B1 included *P. r. vioscai* from southeast Alabama and southwest Georgia, and Clade B2 included *P. r. vioscai* from southeast Georgia and *P. r. ruber* and hypothetical intergrade localities from upland Piedmont habitats of Georgia, Alabama, and Tennessee. The other interior group of the mitochondrial phylogeny contained the sister Clades B3 and B4: Clade B3 included upland *P. r. ruber* from Ohio and eastern North Carolina, and Clade B4 included *P. r. ruber* from northeast Georgia, central Kentucky, and central North Carolina, and all samples of *P. r. nitidus* and *P. r. schencki*. Clade B4 was the most taxonomically diverse group, from a subspecific perspective, but no subspecies were recovered as monophyletic within this clade.

For the nuclear gene POMC, we recovered 17 haplotypes among 26 individuals from 21 localities (Table 1), from which we identified two well supported clades in the ML analysis (support value = 89; Fig. 3). The nuclear tree was characterized by a clade comprising samples from the Coastal Plain of western Kentucky south to Louisiana and east to southeastern Georgia (nuclear Clade A), which was sister to a clade including other Coastal Plain samples from southeast Georgia and central Alabama and all other upland samples (nuclear Clade B). Support values were generally low except for the node describing a split between the two clades (89/100). Overall, the POMC tree described *P. r. vioscai* as paraphyletic and other subspecific taxa as non-monophyletic within nuclear Clade B, although support values within Clade B were low. The third and fourth RAxML analyses (3: subsetted mitochondrial; 4: concatenated mitochondrial and nuclear) generated trees with the same structure as that of the first analysis (1: full mitochondrial dataset), so we only described the full mitochondrial analysis here.

Although the mitochondrial and nuclear trees were similar in some aspects, discordance between phylogenies was observed in three cases. First, *P. r. vioscai* samples from the lower Chattahoochee River drainage aligned with samples from south Alabama west and north to western Kentucky in the nuclear tree (nuclear Clade A; Fig. 3), but were sister to samples from upland Piedmont/Ridge and Valley habitats (mitochondrial Clade B2) in the mitochondrial phylogeny (Fig. 2). Second, samples from southeastern Coastal Plain of Georgia (Effingham, Burke, Wayne counties) were recovered within a clade including Piedmont/Ridge and Valley localities in the mitochondrial phylogeny; however, the nuclear tree recovered Burke County specimens as sister to all samples from the lower Chattahoochee west and north to western Kentucky (within nuclear Clade A), whereas Effingham and Wayne specimens were within a group including all upland samples (nuclear Clade B). Thirdly, two individuals from Autauga County, Alabama possessed mitochondrial Clade A haplotypes, whereas a third individual had a mitochondrial Clade B2 haplotype; however, each of these three individuals possessed nuclear Clade B haplotype.

The Bayesian phylogenetic analysis of the full mitochondrial dataset recovered phylogenetic structure generally concordant with the structure of the ML analyses (Fig. 4); this phylogeny described a well-supported deep split between Clades A and B, and interior structure within Clade B as consistent with the ML phylogeny from the full mitochondrial dataset. Posterior probability was generally high for each of the major clades identified in the ML analysis, except for the split between mitochondrial Clades B1 and B2 (0.96). The time-calibrated BEAST phylogeny described the deepest split within *P. ruber* as occurring 3.6 mya (2.8–4.6, 95% CI), with diversification of mitochondrial Clades A and B1–B4 occurring between 1.4 and 1.9 mya (Fig. 4); relative to the estimates

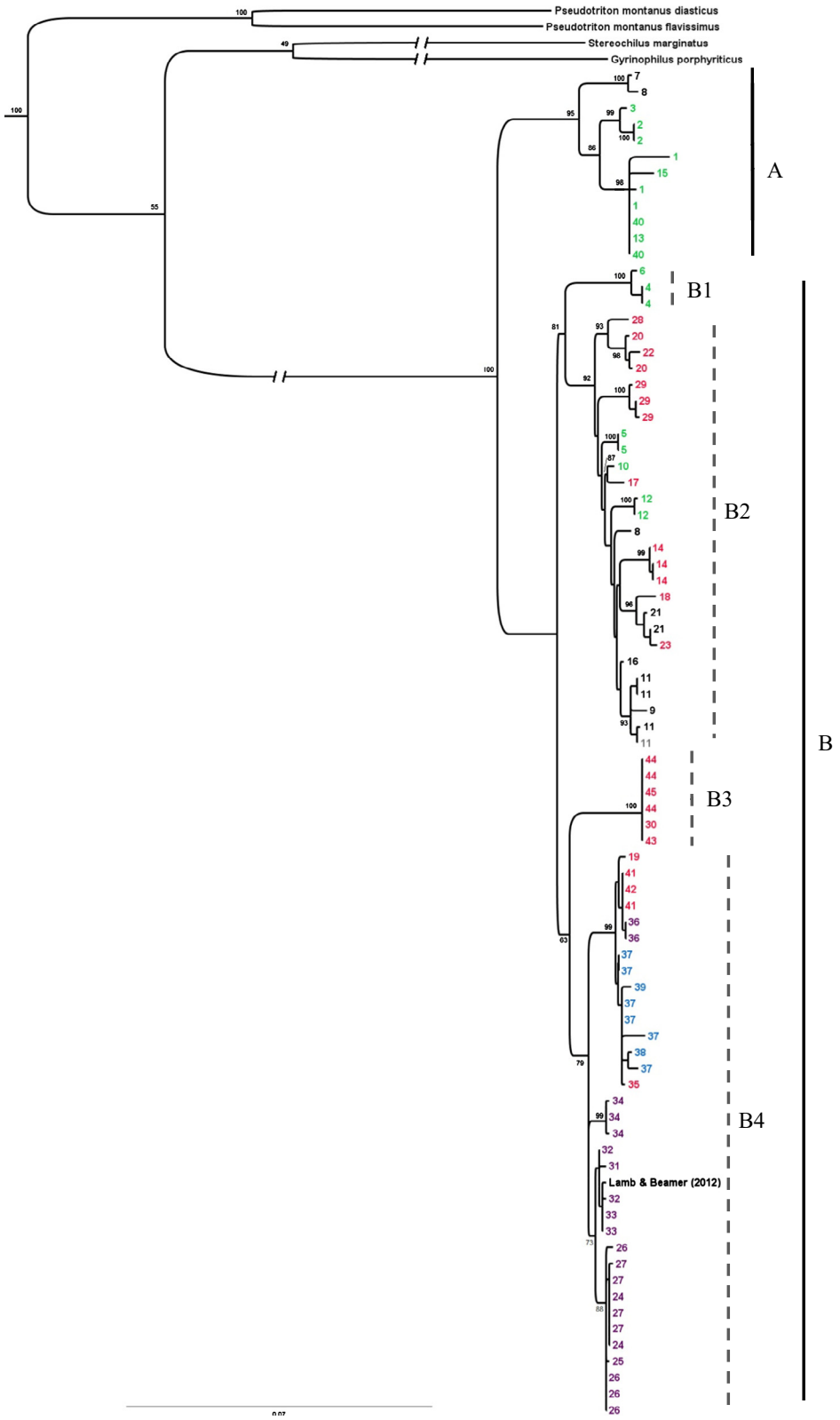

Fig. 2. Maximum-likelihood phylogeny of the Red Salamander (*Pseudotriton ruber*) generated from concatenated mitochondrial genes (CytB, NAD2) generated in RAxML. Specimen tip-labels designate populations from Fig. 1 and are colored by subspecific taxa (red – *P. r. ruber*; blue – *P. r. nitidus*; purple – *P. r. schencki*; green – *P. r. vioscai*; gray – Mount's intergrade zone) as inferred from Fig. 1. Bootstrap support is provided for values >60% support; nodes without values received support values <60%. Two outgroup species (*Urspelerpes brucei*, *Eurycea cirrigera*) are not shown, and the branch lengths for *G. porphyriticus*, *S. marginatus*, and the root of *P. ruber* are not to scale. No locality and subspecific category was known for one *P. ruber* sample from GenBank (Lamb and Beamer, 2012); this specimen is labeled with black. Clades A and B describe the backbone phylogenetic structure and are indicated by solid black lines adjacent to terminal taxa; Clades B1, B2, B3, and B4 are well supported clades of comparable branch length within Clade B and are indicated by dotted lines adjacent to taxa. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

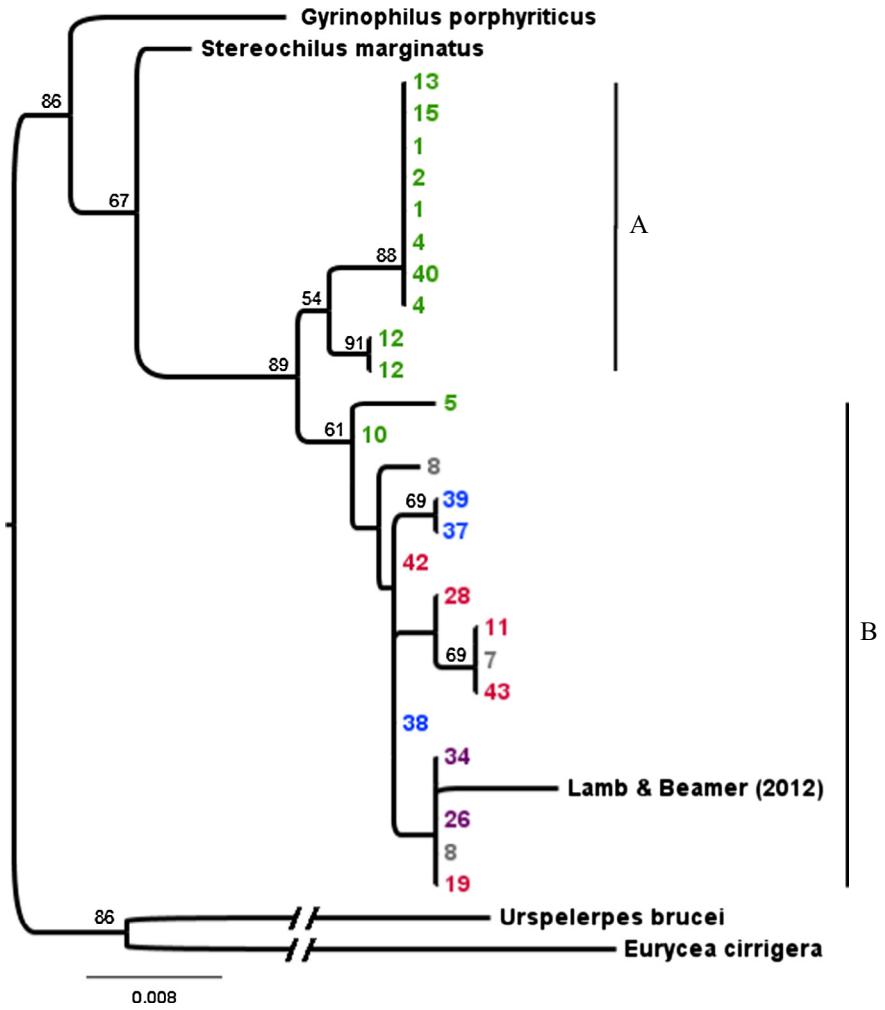

Fig. 3. Maximum-likelihood phylogeny of the Red Salamander (*Pseudotriton ruber*) generated from nuclear DNA sequences (POMC) using RAxML. Specimen tip-labels designate populations from Fig. 1 and are colored as in Fig. 2. Bootstrap support values are as in Fig. 2. No locality and subspecific category was known for one *P. ruber* sample from GenBank (Lamb and Beamer, 2012); this specimen is labeled with black. The tree was characterized by two well-supported clades (A and B), which are indicated by solid lines adjacent to terminal taxa. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

presented by Bonett et al. (2014), our dates are slightly more recent but are within the confidence intervals of the previous study.

Of the six biogeographic models built and ranked using *BioGeoBEARS*, the DEC + J model provided the most well-supported estimation of ancestral ranges for *P. ruber* populations (model weight = 0.39; Table 2). This model suggested that the ancestor of all *P. ruber* populations occupied areas in the Gulf Coastal Plain, until populations of mitochondrial Clade B subsequently dispersed ~3.6 mya, expanding the species' range out of the Coastal Plain to occupy additional upland areas (Fig. 4). Specifically, the common ancestors of the mitochondrial Clades B1 and B2 were estimated to have occupied Coastal Plain habitat, but the common ancestor of Clades B3 and B4 was estimated to have occupied upland areas of the Piedmont, Blue Ridge, and Appalachian Plateau.

The DEC and DIVA + J models also were well supported (ΔAIC = 0.94, 1.02, respectively). The DIVA + J model made similar predictions as the DEC + J model but differed by estimating the ancestor of Clade B2 to have occupied Piedmont habitat rather than Coastal Plain. The DEC model was most distinctive in the top-model set and predicted that (1) the ancestors of Clades B3 and B4 occupied Piedmont habitat, and (2) the ancestor of Clades B1 and B2 may have also occupied the Ridge and Valley and Appalachian Plateau.

4. Discussion

4.1. Phylogeography and historical biogeography

For both the ML and Bayesian mitochondrial phylogenies, Clade A included Coastal Plain samples from western Kentucky south to Louisiana and east to south-central Alabama. This population appears to be primarily bounded to the north by the Fall Line, a well recognized biogeographic barrier between Coastal Plain and upland organisms. The Fall Line separates the distinctive sandy soils of the Coastal Plain from the rocky physiography of the Appalachian uplands, and the contrast of these habitats may serve as a barrier to dispersal across the Fall Line for locally-adapted individuals. The eastern boundary of mitochondrial Clade A is unclear, but it may extend to include the areas around the Choctawhatchee River drainage in Alabama and Florida.

Phylogeographic analyses of diverse taxa from southeastern North America have demonstrated a general east-west split in geographic variation centered on or adjacent to the Apalachicola drainage (i.e., the Apalachicola discontinuity, Soltis et al., 2006), including terrestrial *Ambystoma* and aquatic *Pseudobranchus* salamanders (Donovan et al., 2000; Church et al., 2003; Liu et al., 2006). Ancestral range estimation of the time-calibrated mitochondrial phylogeny (Fig. 4) suggested that the common ancestor of

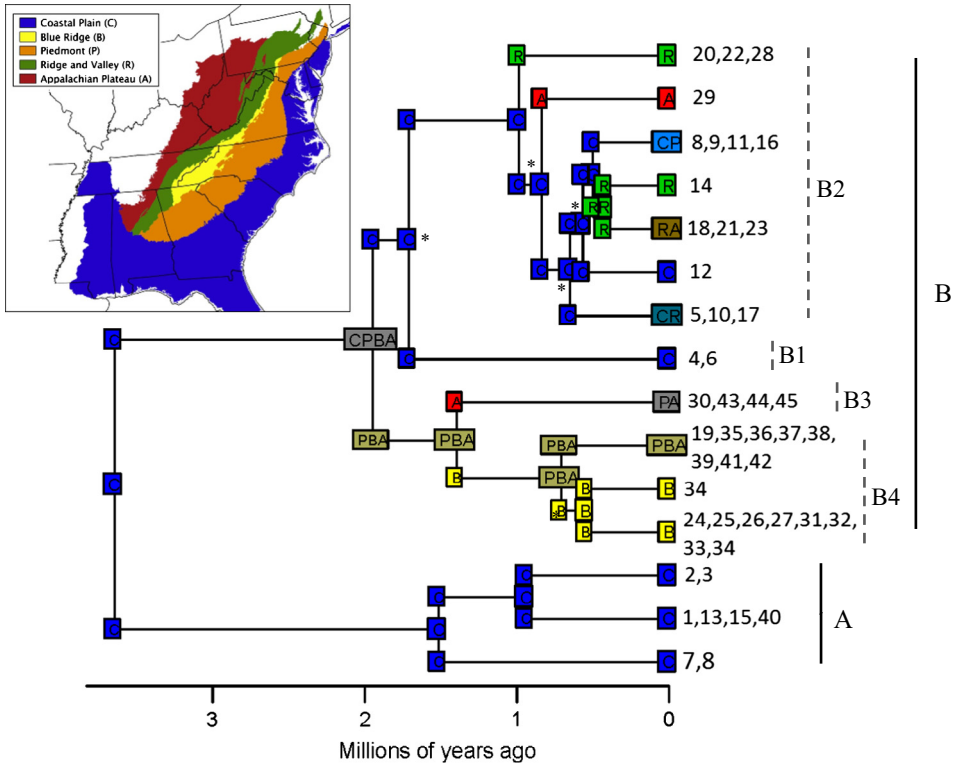

Fig. 4. Bayesian chronogram of the Red Salamander (*Pseudotriton ruber*) as inferred by BEAST (Drummond et al., 2012). Ancestral range estimation is overlayed on each node (C = Coastal Plain; P = Piedmont; R = Ridge and Valley; B = Blue Ridge; A = Appalachia Plateau; and combinations of those areas; following Fenneman (1928) and the inset map) based on the DEC + J model constructed *BioGeoBEARS* (Matzke, 2014). Tip labels represent populations (as in Fig. 1; Appendices B and D). Major clade structure is indicated by solid black lines (Clades A and B) and dotted gray lines (Clades B1, B2, B3, B4; as in Fig. 2). All nodes received posterior probabilities ⩾0.95, except those indicated by ∗. Because the ancestral range of outgroups was not of interest for this analysis, they are not shown here.

Table 2
Comparison of biogeographic models of ancestral range estimation for Red Salamander (*Pseudotriton ruber*) populations constructed using *BioGeoBEARS* (Matzke, 2014). The DEC + J model was most well supported, but the DEC and DIVA + J models also received considerable support within the top-model set of ΔAIC < 2.

Model	Log-likelihood	No. of parameters	*d*	*e*	*j*	AIC	ΔAIC	Model weight
DEC + J	−35.55	3	0.074	0.000	0.060	77.09	0.00	0.39
DEC	−37.01	2	0.088	0.000	0.000	78.03	0.94	0.24
DIVA + J	−36.05	3	0.095	0.000	0.055	78.11	1.02	0.23
DIVA	−38.20	2	0.128	0.000	0.000	80.40	3.31	0.07
BAYAREA + J	−37.48	3	0.052	0.000	0.118	80.95	3.86	0.06
BAYAREA	−43.97	2	0.064	0.553	0.000	91.94	14.85	0.00

P. ruber occupied the Coastal Plain at the start of the Pliocene. Approximately 3.6 mya (2.8–4.6, 95% CI), this population is hypothesized to have split into Clades A and B, which were both estimated to have occupied the Coastal Plain. During the late Miocene, the divide between the Gulf and Atlantic coastal drainages was located in southeastern Alabama and Florida, east of the Chattahoochee River and the Apalachicola River basin (Galloway et al., 2011), and the position of this drainage divide is consistent with the present-day geographic split between mitochondrial Clades A and B. Dating of this divergence also coincides with a major glacial episode at the start of the Pliocene (Riggs, 1984), during which populations may have been isolated into two warm, low-elevation refugia east and west of the Gulf and Atlantic coastal divide (Galloway et al., 2011), thus possibly explaining the deepest phylogeographic structure in *P. ruber*. Alternatively, the majority of nuclear DNA variation indicates a general split between two present-day populations, one in the Gulf Coastal Plain (except for Burke, GA specimens) and one spread widely across Atlantic Coast Plain and upland Appalachian areas. More recently, the Gulf and Atlantic coastal divide moved eastward to the current position in central Georgia and Florida during the Pliocene (Galloway et al., 2011), and present-day phylogeographic structure of nuclear DNA appears to be best explained by the location of the coastal divide in the Pliocene. However, regardless of what historic event(s) drove genetic structuring of *P. ruber*, phylogeography of *P. ruber* is best described by geographic splits west (mitochondrial) or east (nuclear) of the Apalachicola drainage, and is thus consistent with an Apalachicola discontinuity (Soltis et al., 2006).

While the vast majority of the present-day distribution of *P. ruber* is distributed in upland areas of Appalachia, ancestral area estimation suggested that the common ancestor of all populations occupied the southeastern Coastal Plain, and initial diversification occurred in this landscape. It was not until ∼1.4–1.9 mya when

populations underwent subsequent lineage diversification and dispersal, resulting in the expansion of the species' range to include Appalachia (Fig. 4). Specifically, ancestral range estimation suggested that dispersal of Clade B expanded the species' range to also include upland areas of the Piedmont, Blue Ridge, and Appalachian Plateau; heuristic examination of the nuclear phylogeny is consistent with this interpretation.

Clade B3 included one sample from central North Carolina and the northern-most localities in the study from Ohio. The clade exhibited no variation; all specimens shared the same haplotype. Although we have a limited sample from the northern range, that samples from eastern North Carolina exhibited the same haplotype as samples from multiple localities in Ohio suggests that this clade may encompass much of the northern distribution of *P. ruber*. Previous phylogeographic studies of plethodontid salamanders have found low genetic richness of northern lineages (e.g., Kozak et al., 2006), possibly as a result of population growth and range expansion following recession of glaciers during climactic periods of warming. We suggest that post-glacial population expansion provides a reasonable explanation for the low diversity and pattern of 'northern purity' (*sensu* Provan and Bennett, 2008) for mitochondrial Clade B3, but greater sampling from this region is needed to more thoroughly support this hypothesis for *P. ruber*.

4.2. Taxonomy of Pseudotriton ruber

The primary goal of this study was to evaluate the monophyly of *P. ruber* subspecies, with particular interest in the *P. r. vioscai* population characterized by the most distinctive phenotype. None of the four morphological subspecies of *P. ruber* formed reciprocally monophyletic clades in our mitochondrial phylogeny, and the nuclear gene tree similarly failed to recover monophyly of any subspecies. Given the results of Bonett et al. (2014) and the more comprehensive sample provided here, the available phylogenetic data suggest that the four morphological subspecies of *P. ruber*, as they have been historically recognized, do not represent independently evolving monophyletic lineages to the exclusion of one another, and we do not suggest that alpha-taxonomic changes should be made at this time. Instead, the results here describe a morphologically variable species with genetic structure corresponding to distinctive physiogeographic regions, biogeographic barriers, and historical events in eastern North America.

Despite failure to demonstrate monophyly, our results do show significant historical structure among *P. ruber* specimens. The greatest phylogeographic structure was observed between samples from the Coastal Plain and upland Appalachian areas, supporting a divergence of *P. r. vioscai* in the Coastal Plains from all other subspecific taxa. However, monophyly of the Coastal Plain populations was disrupted by samples of *P. r. vioscai* from Effingham and Wayne counties that were recovered within nuclear Clade B along with upland specimens of *P. r. ruber*, *P. r. nitidus*, and *P. r. schencki*. Additionally, information from the mitochondrial genome conflicted with that of the nuclear genome by documenting a divergence within the Coastal Plain at the Apalachicola River drainage. Many other studies have documented that mitochondrial and single nuclear genes can provide misleading results about phylogeny and speciation (e.g., Wiens et al., 2010). Our mitochondrial and nuclear phylogenies were incongruent in three primary ways, and three hypotheses are proposed here as potential explanations for these patterns of discordance. First, if the *P. r. vioscai* specimens examined represent a single species, discordance between phylogenies may indicate intraspecific gene flow and introgression between upland and Coastal Plain populations. Second, the non-monophyly of Coastal Plain specimens might represent an example(s) where one or both of the gene trees differs from the species tree, possibly resulting from incomplete lineage sorting between two emerging species. Because the effective population size of mitochondrial DNA is reduced relative to that of nuclear loci (Rand et al., 2004), mitochondrial DNA lineages are expected to sort faster than nuclear DNA lineages. Consequently, incomplete lineage sorting is more likely a factor contributing to patterns seen in nuclear data. If the POMC gene tree is inaccurate regarding the true evolutionary trajectory of *P. r. vioscai*, our methods may have failed to detect that population as a lineage distinct from the population attributable to *P. ruber*. Lastly, a third explanation for discordance is that one or more of the phylogenies could suffer from insufficient character or taxon sampling (Wiens et al., 2010). Given the morphological and ecological distinctiveness of *P. r. vioscai*, we favor the second hypothesis as the most likely, and predict that a future study with additional nuclear loci may recover a monophyletic *P. r. vioscai* in species-tree analyses, either across the entire Coastal Plain, as suggested by our sample of the nuclear genome, or as a lineage located west of the Apalachicola drainage, as suggested by the mitochondrial genome. Either case would allow a diagnosis and necessitate recognition of *P. r vioscai* as a distinct species. For now, however, we find the nuclear phylogenetic structure, morphological distinctiveness, and peripheral distribution of *P. r. vioscai* samples to be features consistent with past and present definitions of subspecies (Jorgensen et al., 2013). Therefore, we recommend the retention of the taxon *vioscai* to describe the morphologically and ecologically distinctive Coastal Plain populations of *P. ruber*.

While the structure of our phylogenies did not support the recognition of *P. r. nitidus* and *P. r. schencki* as distinct lineages, we similarly do not recommend dismissing those taxa. These high elevation taxa are distinctive in morphology and approach monophyly; neither of these taxa are distributed randomly across mitochondrial Clade B. Specifically, Clade B4 in the mitochondrial phylogeny (Fig. 2) is defined by a node which encompasses all populations of *P. r. nitidus* and *P. r. schencki*; the topology of this clade suggests that the *schencki* phenotype evolved twice and the *P. r. nitidus* phenotype evolved once within this node. The relatedness and phylogenetic signal of these populations is expected by a null hypothesis of isolation by distance, but we view the consistent bright coloration and distinctive phenotypes of high elevation populations within mitochondrial Clade B4 as striking and similarly recommend the retention of the taxa *P. r. nitidus* and *P. r. schencki* for describing high elevation populations in the southern Blue Ridge Mountains. Indeed, we think the retention of trinomials for *P. ruber* highlights an interesting evolutionary situation, where Müllerian mimicry appears to provide a selective mechanism generating phenotypic variation within the species. While the use of subspecies may not reflect true biological entities and much sentiment exists against its use (Wilson and Brown, 1953), we think the retention of subspecific trinomials here is a useful tool (*sensu* Mayr, 1982) for (1) diagnosing ecotypes of *P. ruber*, (2) illustrating interesting evolutionary processes which appear to be generating morphological variation (*P. r. nitidus*, *P. r. schencki*), potentially via convergent evolution and a Müllerian mimicry complex, and (3) maintaining awareness for a taxon which may ultimately be diagnosed as a distinct species (*P. r. vioscai*).

Range maps to date have mostly described the distributions of *P. ruber* subspecies as having clearly delineated ranges (e.g., Petranka, 1998), but we do not believe these morphologies are distributed so neatly in reality. Our experiences in the field and with museum specimens suggest that the species is highly variable and

that subspecific characteristics vary greatly through ontogeny of individuals, within populations, and even within subspecies; these observations echo previous authors who noted such variation and suggested the need for a thorough investigation of color pattern and pigmentation through ontogeny and among subspecies of *P. ruber* (Bruce, 1968; Martof, 1975; Mount, 1975). We predict that such a study would find some subspecific characters (e.g., the black chin of *schencki*, white face freckles of *vioscai*) to be distributed more widely than previous distribution maps of subspecies have indicated. Similarly, the interspersion of some Georgia, Kentucky, and North Carolina specimens of *P. r. ruber* within the lineage containing all *P. r. nitidus* and *P. r. schencki* specimens suggests intergradation or a need for improved understanding of subspecific distribution patterns.

Mount (1975) described populations in a large swath of central Alabama as containing intergradient individuals between *P. r. ruber* and *P. r. vioscai*, suggesting that this represents an expansive zone of gene flow between Coastal Plain and upland populations. Mount's hypothesis predicts that localities within the intergrade zone would contain salamanders showing admixture between northern (*P. r. ruber*) and southern (*P. r. vioscai*) populations. Our data were inconsistent with Mount's hypothesis, as the majority of our samples from this region aligned with specimens from northern localities in our phylogenetic analyses and showed no genetic influence of *P. r. vioscai*. Therefore, we suggest that the drab red coloration of Piedmont, Ridge and Valley, and Appalachian Plateau individuals in Alabama represents a consistent feature of animals in this region, rather than indicating broad gene flow between subspecies. Our specimens from within the range of *P. r. ruber* were distributed broadly across nuclear and mitochondrial Clades B, indicating that the drab color pattern might result from convergent evolution.

Although our data fail to support broad gene flow between *P. r. ruber* and *P. r. vioscai*, some gene flow clearly exists and appears to be mediated by female dispersal across the Fall Line Hills (i.e., a female *vioscai* x male *ruber* hybrid), along the lower Chattahoochee River (i.e., female *ruber* × male *vioscai* hybrid), and below the Fall Line in southeastern Georgia (i.e., female *ruber* × male *vioscai* hybrid). These results indicate historical gene flow across the Fall Line in Alabama, but in a limited geographic area than the broad area hypothesized by Mount (1975). Because all three specimens from Autauga County, AL exhibited nuclear Clade B haplotypes, we suggest that this evidence supports extending the spatial distribution of *P. r. ruber* south to encompass the Fall Line Hills area in south-central Alabama. In general, however, mitochondrial DNA loci provide poor markers for studying hybridization, and future work with nuclear loci could seek to evaluate the presence and magnitude of gene flow among *P. ruber* subspecies.

5. Conclusions

We have provided a first comprehensive test of the monophyly of four subspecies of the Red Salamander (*P. r. ruber*, *P. r. nitidus*, *P. r. schencki*, *P. r. vioscai*) using phylogenetic analyses of mitochondrial and nuclear markers. Our results found that these taxa, as historically delineated, do not represent monophyletic lineages and thus should not be considered as distinct evolutionary species at this time. Instead, we described genetic variation and phylogenetic structure among populations of *P. ruber* which generally separated Coastal Plain and upland Appalachian populations. Ancestral range estimation suggests that *P. ruber* evolved in the Coastal Plain in the early Pliocene, before the ancestor expanded the species' distribution to occupy upland areas of Appalachia during the late Pliocene or early Pleistocene. Within upland populations, those occupying the highest elevations are notably bright in color and occupy the same sites where the subspecies of Eastern Newt (*N. v. viridescens*) is bright and abundant. This pattern is consistent with that expected from Müllerian mimicry, in which selective pressure yields differentiation of aposematic upland subspecies (*P. r. schencki* and *P. r. nitidus*) in concert with other aposematic models and yields differentiation of drab subspecies in areas where other model prey also are drab (*P. r. vioscai*) or are uncommon (*P. r. ruber*). Because the subspecific phenotypes within *P. ruber* show substantial phylogenetic signal and can be mapped to distinct geographic regions, they are consistent with past and modern definitions of what subspecies should be (Jorgensen et al., 2013) and we recommend that they be retained. Retention of trinomials in this case highlights what we consider to be an interesting evolutionary problem given the possibility that Müllerian mimicry plays a role in generating phenotypic variation within *P. ruber*.

Alternatively, our finding of significant phylogenetic signal among subspecies might be used to invoke speciation models allowing for substantial gene flow (e.g., Burbrink and Guiher, 2015). In this case, the subspecies of *P. ruber* might be viewed as separate species for which zones of hybridization would require evaluation with larger datasets. However, our trees resolved *P. r. ruber* as strongly polyphyletic, and we suggest this is strong reason to avoid elevating subspecific taxa to species at this time. Regardless of whether these taxa are viewed as species or subspecies, our data do not support Mount's (1975) hypothesis of a large zone of gene flow between *P. r. ruber* and *P. r. vioscai* in central Alabama. Instead, we found limited evidence of overlap between these two taxa, a feature that we argue further supports retention of *P. r. vioscai* as a distinct taxon. Lastly, we suggest that our study provides an example of how subspecies can be useful to describe hypotheses about incipient species and to illustrate how important biological processes shape morphological diversity of species.

Acknowledgments

Specimen and sample collection was performed under the specifications of the following permits and protocols: Alabama Scientific Permit 7587, Georgia scientific collecting permit (29-WJH-13-124), Kentucky scientific collecting permit (#SC1311294), North Carolina Wildlife Collection License (#13-SC00790), Ohio Division of Wildlife Wild Animal Permit (#14-383), Tennessee Scientific Collecting Permit (#378), Great Smoky Mountains and Cuyahoga Valley National Park Scientific Research and Collecting permits (GRSM-2013-SCI-1154, CUVA-2013-SCI-0012, respectively), and Auburn University IACUC #2010-1827. We graciously thank the following for providing tissues, helping collect samples, or hospitality while in the field: the Louisiana State University Museum of Natural Science Collection of Genetic Resources, University of Alabama Herpetology Collection (P. Scott, L. Rissler), North Carolina Museum of Natural Sciences (J. Beane, B. Stuart), Cincinnati Museum Center (H. Mays), D. Stevenson, T. Pierson, A. Jenkins, C. Brune, J. MacGregor, B. Skinner, J. Jensen, S. Sterrett, J. Goessling, C. Painter, M. Painter, R. Warmenhoven, K. Pilarski, S. Koebly, and D. Carter. Photographs were graciously provided by C. Brune, J. Folt, M. Niemiller, T. Pierson, and J. Williams. We thank D. Cotoras for providing encouraging analytical assistance, and J. Goessling and C. Brune provided helpful comments on an earlier manuscript. This paper is contribution no. 727 of the Auburn University Museum of Natural History.

Appendix A

Common name, etymology, defining characteristics, type locality, and citation for each subspecies of *Pseudotriton ruber*.

Taxon	Common name	Etymology	Defining characters	Type locality	Description
Pseudotriton r. ruber	Northern Red Salamander	*ruber* – red (Latin)	Red dorsal ground color with a heavily spotted dorsum	"Les Etats Unis"; restricted to "vicinity of Philadelphia"	Sonnini de Manoncourt and Latreille (1801) and Dunn (1918)
Pseudotriton r. nitidus	Blue Ridge Red Salamander	*nitidus* – bright (Latin)	Light or bright red color; small maximum body size; few dorsal spots on tail; limited chin spotting	White Top Mountain, Virginia	Dunn (1920)
Pseudotriton r. schencki	Black-chinned Red Salamander	*schencki* – patronym honoring Carl Schenck, founder of the Biltmore School of Forestry, the first of its kind in North America	Bold, dark spotting on chin; large maximum body size	"Sunburst, Haywood County, North Carolina"	Brimley (1912)
Pseudotriton r. vioscai	Southern Red Salamander	*vioscai* – patronym honoring Percy Viosca Jr., who collected the type specimen of this taxon	Dark, brownish ground color; "herring-boned" dorsal pattern; white-speckled chin; dark spots on undersurfaces of hind legs; gray/white perforations across yellow iris; less defined black eye bar	"10 miles west of Bogalusa, Louisiana"	Bishop (1928)

Appendix B

Sample number, a priori taxon, population, locality information, and GenBank accession numbers for sequences encoding the CytB, NAD2, and POMC genes of each specimen included in the study. Sample numbers refer to field collection numbers or museum numbers, and abbreviations are as follows: FOLT (Brian Folt field series), LSUMZ (Louisiana State University, Museum of Natural Sciences Genetic Resources Collection), CMC-H (Cincinnati Museum Collection – Herpetology unit), NCSM (North Carolina Sciences Museum), UAHC (University of Alabama – Herpetology Collection), DAB (David Beamer field series; Lamb and Beamer, 2012), USNM (United States National Museum; from Lamb and Beamer, 2012). Locality names are of counties or parishes in each state.

Collector number	Taxon	Population	Locality	CytB	NAD2	POMC
LSUMZ-H1579	*P. r. vioscai*	1	Washington, LA	KR054911	KR054829	–
LSUMZ-H2853	*P. r. vioscai*	1	Washington, LA	KR054920	KR054838	KR054949
LSUMZ-H20597	*P. r. vioscai*	1	Washington, LA	KR054921	KR054839	KR054950
FOLT-262	*P. r. vioscai*	2	Covington, AL	KR054883	KR054801	KR054936
FOLT-263	*P. r. vioscai*	2	Covington, AL	KR054884	KR054802	–
FOLT-336	*P. r. vioscai*	3	Covington, AL	KR054903	KR054821	–
FOLT-328	*P. r. vioscai*	4	Early, GA	KR054901	KR054819	KR054945
FOLT-329	*P. r. vioscai*	4	Early, GA	KR054902	KR054820	KR054946
FOLT-188	*P. r. vioscai*	5	Wayne, GA	KR054856	KR054774	KR054929
FOLT-190	*P. r. vioscai*	5	Wayne, GA	KR054857	KR054775	–
FOLT-321	*P. r. vioscai*	6	Barbour, AL	KR054900	KR054818	–
FOLT-283	*P. r. ruber* × *vioscai*	7	Autauga, AL	KR054885	KR054803	KR054937
FOLT-284	*P. r. ruber* × *vioscai*	8	Autauga, AL	KR054886	KR054804	KR054938
FOLT-285	*P. r. ruber* × *vioscai*	8	Autauga, AL	KR054887	KR054805	KR054939
FOLT-105	*P. r. ruber* × *vioscai*	9	Macon, AL	KR054844	KR054762	–
FOLT-300	*P. r. vioscai*	10	Effingham, GA	KR054888	KR054806	KR054940
FOLT-306	*P. r. ruber* × *vioscai*	11	Lee, AL	KR054891	KR054809	KR054942
FOLT-307	*P. r. ruber* × *vioscai*	11	Lee, AL	KR054892	KR054810	–
FOLT-308	*P. r. ruber* × *vioscai*	11	Lee, AL	KR054893	KR054811	–
FOLT-310	*P. r. ruber* × *vioscai*	11	Lee, AL	KR054894	KR054812	–
FOLT-311	*P. r. vioscai*	12	Burke, GA	KR054895	KR054813	KR054943
FOLT-312	*P. r. vioscai*	12	Burke, GA	KR054896	KR054814	KR054944
LSUMZ-H1933	*P. r. vioscai*	13	Winston, MS	KR054913	KR054831	KR054948
FOLT-339	*P. r. ruber* × *vioscai*	14	Shelby, AL	KR054905	KR054823	–
FOLT-340	*P. r. ruber* × *vioscai*	14	Shelby, AL	KR054906	KR054824	–

(*continued on next page*)

Appendix B (*continued*)

Collector number	Taxon	Population	Locality	CytB	NAD2	POMC
FOLT-341	*P. r. ruber* × *vioscai*	14	Shelby, AL	KR054907	KR054825	–
LSUMZ-H1932	*P. r. vioscai*	15	Choctaw, MS	KR054912	KR054830	KR054947
LSUMZ-H2058	*P. r. ruber* × *vioscai*	16	Clay, AL	KR054914	KR054832	–
FOLT-338	*P. r. ruber* × *vioscai*	17	Jefferson, AL	KR054904	KR054822	–
UAHC-15543	*P. r. ruber*	18	Calhoun, AL	KR054923	KR054841	–
FOLT-196	*P. r. ruber*	19	Clarke, GA	KR054858	KR054776	KR054930
LSUMZ-H2193	*P. r. ruber*	20	Cherokee, AL	KR054915	KR054833	–
LSUMZ-H2627	*P. r. ruber*	20	Cherokee, AL	KR054919	KR054837	–
FOLT-316	*P. r. ruber* × *vioscai*	21	Winston, AL	KR054898	KR054816	–
FOLT-317	*P. r. ruber* × *vioscai*	21	Winston, AL	KR054899	KR054817	–
FOLT-138	*P. r. ruber*	22	Walker, GA	KR054850	KR054768	–
FOLT-114	*P. r. ruber*	23	Madison, AL	KR054845	KR054763	–
FOLT-199	*P. r. schencki*	24	Gilmer, GA	KR054859	KR054777	–
FOLT-200	*P. r. schencki*	24	Gilmer, GA	KR054860	KR054778	–
FOLT-178	*P. r. schencki*	25	Gilmer, GA	KR054855	KR054773	–
FOLT-118	*P. r. schencki*	26	Gilmer, GA	KR054846	KR054764	–
FOLT-122	*P. r. schencki*	26	Gilmer, GA	KR054847	KR054765	KR054926
FOLT-124	*P. r. schencki*	26	Gilmer, GA	KR054848	KR054766	–
FOLT-127	*P. r. schencki*	26	Gilmer, GA	KR054849	KR054767	–
FOLT-201	*P. r. schencki*	27	Fannin, GA	KR054861	KR054779	–
FOLT-203	*P. r. schencki*	27	Fannin, GA	KR054862	KR054780	–
FOLT-204	*P. r. schencki*	27	Fannin, GA	KR054863	KR054781	–
FOLT-205	*P. r. schencki*	27	Fannin, GA	KR054864	KR054782	–
FOLT-144	*P. r. ruber*	28	Marion, TN	KR054851	KR054769	KR054927
LSUMZ-H2202	*P. r. ruber*	29	Franklin, TN	KR054916	KR054834	–
LSUMZ-H2203	*P. r. ruber*	29	Franklin, TN	KR054917	KR054835	–
LSUMZ-H2204	*P. r. ruber*	29	Franklin, TN	KR054918	KR054836	–
NCSM-79029	*P. r. ruber*	30	Moore, NC	KR054924	KR054842	–
FOLT-237	*P. r. schencki*	31	Swain, NC	KR054874	KR054792	–
FOLT-232	*P. r. schencki*	32	Swain, NC	KR054872	KR054790	–
FOLT-235	*P. r. schencki*	32	Swain, NC	KR054873	KR054791	–
FOLT-238	*P. r. schencki*	33	Graham, NC	KR054875	KR054793	–
FOLT-242	*P. r. schencki*	33	Graham, NC	KR054876	KR054794	–
FOLT-243	*P. r. schencki*	34	Graham, NC	KR054877	KR054795	–
FOLT-244	*P. r. schencki*	34	Graham, NC	KR054878	KR054796	–
FOLT-245	*P. r. schencki*	34	Graham, NC	KR054879	KR054797	KR054934
NCSM-79519	*P. r. ruber*	35	Rutherford, NC	KR054925	KR054843	–
FOLT-247	*P. r. schencki*	36	Swain, NC	KR054880	KR054798	–
FOLT-249	*P. r. schencki*	36	Swain, NC	KR054881	KR054799	–
FOLT-212	*P. r. nitidus*	37	Unicoi, TN	KR054865	KR054783	KR054932
FOLT-213	*P. r. nitidus*	37	Unicoi, TN	KR054866	KR054784	–
FOLT-214	*P. r. nitidus*	37	Unicoi, TN	KR054867	KR054785	–
FOLT-215	*P. r. nitidus*	37	Unicoi, TN	KR054868	KR054786	–
FOLT-217	*P. r. nitidus*	37	Unicoi, TN	KR054869	KR054787	–
FOLT-219	*P. r. nitidus*	37	Unicoi, TN	KR054870	KR054788	–
FOLT-225	*P. r. nitidus*	38	Unicoi, TN	KR054871	KR054789	KR054933
FOLT-250	*P. r. nitidus*	39	Watauga, NC	KR054882	KR054800	KR054935
FOLT-301	*P. r. vioscai*	40	Marshall, KY	KR054889	KR054807	–
FOLT-303	*P. r. vioscai*	40	Marshall, KY	KR054890	KR054808	KR054941
FOLT-147	*P. r. ruber*	41	Rockcastle, KY	KR054852	KR054770	–
FOLT-148	*P. r. ruber*	41	Rockcastle, KY	KR054853	KR054771	–
FOLT-158	*P. r. ruber*	42	Menifee, KY	KR054854	KR054772	KR054928
CMC-H12099	*P. r. ruber*	43	Athens, OH	KR054922	KR054840	KR054931
FOLT-349	*P. r. ruber*	44	Hocking, OH	KR054908	KR054826	–
FOLT-351	*P. r. ruber*	44	Hocking, OH	KR054909	KR054827	–
FOLT-352	*P. r. ruber*	44	Hocking, OH	KR054910	KR054828	–
FOLT-315	*P. r. ruber*	45	Summit, OH	KR054897	KR054815	–
DAB-9997	*Pseudotriton ruber*	–	–	JQ920615	JQ920799	JQ920721
FOLT-171	*P. montanus diasticus*	–	Menifee, KY	KR054760	KR054758	–
FOLT-175	*P. montanus flavissimus*	–	Macon, AL	KR054761	KR054759	–
DAB 9998	*Stereochilus marginatus*	–	–	JQ920617	JQ920801	JQ920723

Appendix B (*continued*)

Collector number	Taxon	Population	Locality	CytB	NAD2	POMC
DAB 9999	*Gyrinophilus porphyriticus*	–	–	JQ920616	JQ920800	JQ920722
DAB 3260	*Eurcea cirrigera*	–	–	JQ920622	JQ920806	JQ920728
USNM 55823	*Urspelerpes brucei*	–	–	JQ920618	JQ920802	JQ920724

Appendix C

Partition schemes and models selected for RAxML (Stamatakis, 2006) after employing a 'greedy' search strategy and ranking models using Bayesian Information Criterion (BIC) in PartitionFinder (Lanfear et al., 2012).

Maximum-likelihood analysis	Partition	Partition content	Model
1 – Mitochondrial	1	CYTB_pos2, ND2_pos2	GTR + G
	2	CYTB_pos3, ND2_pos3	GTR + G
	3	CYTB_pos1, ND2_pos1	GTR + G
2 – POMC	1	POMC_pos1, POMC_pos2	GTR + G
	2	POMC_pos3	GTR + G
3 – Concatenated mitochondrial	1	CYTB_pos2, ND2_pos2	GTR + G
	2	CYTB_pos3, ND2_pos3	GTR + G
	3	CYTB_pos1, ND2_pos1	GTR + G
4 – Concatenated mitochondrial + POMC	1	CYTB_pos2, ND2_pos2	GTR + G
	2	CYTB_pos3, ND2_pos3	GTR + G
	3	CYTB_pos1, ND2_pos1	GTR + G
	4	POMC_pos1, POMC_pos2	GTR + I + G
	5	POMC_pos3	GTR + I + G

Appendix D

Geography table input for the ancestral range estimation analysis using *BioGeoBEARS* (Matzke, 2014). The Bayesian chronogram was pruned to only describe 15 clades of comparable branch length (Clade column), and each clade comprised multiple specimens from one or more populations (Populations; same as in Appendix B). Geography was coded by the distribution of each clade and described whether clades occurred in the Coastal Plain (C), Piedmont (P), Ridge and Valley (R), Blue Ridge (B), and/or the Appalachian Plateau (A), following Fenneman (1928).

Clade	Populations	Geography (C, P, R, B, A)
1	7, 8	10000
2	2, 3	10000
3	1, 13, 15, 40	10000
4	4, 6	10000
5	20, 22, 28	00100
6	29	00001
7	5, 10, 17	10100
8	12	10000
9	18, 21, 23	00101
10	14	00100
11	8, 9, 11, 16	11000
12	30, 43, 44, 45	01001
13	19, 35, 36, 37, 38, 39, 41, 42	01011
14	34	00010
15	24, 25, 26, 27, 31, 32, 33, 34	00010

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