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Multilocus Phylogeography of Eastern Red-backed Salamanders (*Plethodon cinereus*): Cryptic Appalachian Diversity and Postglacial Range Expansion

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Abstract: Climatic and geological changes in eastern North America have shaped population history and genetic diversity in many taxa. A common finding of phylogeographic investigations is that southern populations exhibit relatively high levels of phylogeographic structure, whereas northern populations, especially those that have invaded postglacial landscapes, exhibit relatively little genetic differentiation. Here, we describe the results of a phylogeographic investigation of Eastern Red-backed Salamander (*Plethodon cinereus*), a species that is widely distributed throughout the northeastern United States and southeastern Canada, with roughly three quarters of its range north of the southernmost glacial extent during the last glacial maximum. To investigate patterns of genetic variation, we collected genetic samples from 202 individuals from 107 populations from across the range of *P. cinereus*, with denser sampling in the southern portion of the range. In total, 4486 base pairs (bp) of DNA were sequenced, including three mitochondrial DNA (mtDNA; 2239 bp) loci and three nuclear (2247 bp) loci. A mix of phylogenetic, population genetic, and clustering approaches were used to explore and summarize patterns of genetic variation. Bayesian phylogenetic analysis of mtDNA recovered six well-supported, geographically cohesive clades that increase in geographic range size from south to north, with a most recent common ancestor estimated at 1.49 million years (95% highest posterior density = 1.09–1.95). The northernmost clade possessed a horseshoe-shaped distribution, including the eastern seaboard, all or part of southeastern Canada, and Michigan, Indiana, and Ohio; thus, this clade was recovered south of the last glacial boundary in both the east and the west. Using simple population genetic tests, we showed evidence of range expansion and isolation by distance in most clades. Using the dispersal-extinction-cladogenesis biogeographic model in RevBayes, we inferred the ancestor of *P. cinereus* to occupy either the Blue Ridge or the Ridge and Valley physiographic province. In contrast to mtDNA, nuclear loci revealed little phylogeographic structure, and cluster analyses using the nuclear data were not well resolved. Finally, we compare our results with published and unpublished allozyme studies, and we identify several distributional and biogeographic questions that emerge from our findings.

Key words: BEAST; Dispersal-extinction-cladogenesis (DEC) model; Gene tree; Mitochondrial DNA; Nuclear DNA; Pleistocene; RevBayes; Species tree; Structure

Eastern North America has experienced a dynamic geological and climatic history, including the waxing and waning of glaciers during the Pleistocene, hydrological remodeling of river systems, and Miocene uplift of the Appalachian Mountains (Prince et al. 2010; Gallen et al. 2013). In the north, glacial retreat made new landscapes available that were rapidly invaded by many taxa (Davis and Shaw 2001; Herman and Bouzat 2016); to the south, geomorphological and climatic shifts altered dispersal routes, gene flow patterns, and species' distributions (Berendzen et al. 2003; Soltis et al. 2006). Such changes impacted the rates and patterns of vicariance and dispersal, arresting or promoting the evolution of phylogeographic structure and the formation of distinct evolutionary lineages (Kozak et al. 2006a; Kuchta et al. 2009). Perhaps as a consequence, extensive radiations in eastern North America by many taxa occurred, including crayfish (Crandall and Buhay 2008), darters (Jelks et al. 2008), and mussels (Haag 2012).

Eastern North America is also a biodiversity hotspot for salamanders (order Caudata; Highton 1995). Of the 10 extant families of salamanders in the world, seven are found in eastern North America; the Plethodontidae, which includes approximately 65% of all salamander species, has its greatest phylogenetic diversity in this region (Duellman and Sweet 1999; Wake 2017). Although adaptive differentiation characterizes some species complexes within the Plethodontidae (Kuchta and Wake 2016), many complexes are characterized by nonadaptive radiation, or species formation by fragmentation, whereby a species expands its range and then slowly disintegrates into a complex of divergent allopatric or parapatric groups (Kozak et al. 2006b; Rundell and Price 2009; Kuchta et al. 2018). Species complexes that evolve by nonadaptive radiation are typically characterized by high levels of phenotypic and ecological conservatism (Wake et al. 1983; Kozak and Wiens 2010) as well as isolation by distance and high levels of genetic structure, making them ideal for phylogeographic analyses (Martínez-Solano et al. 2007; Rovito et al. 2012; Reilly and Wake 2015; Tilley 2016) but problematic for species delimitation (Tilley et al. 2013; Kuchta et al. 2016a; Wake 2017).

Here, we provide a phylogeographic analysis of Eastern Red-backed Salamanders (*Plethodon cinereus*). This species has the largest geographic range of any species in the genus *Plethodon*. It is distributed from southern North Carolina, USA, northward through the Appalachian Mountains and the eastern seaboard into Nova Scotia and Quebec, Canada. To the west, it is found as far as eastern Illinois, northern Wisconsin, eastern Minnesota, and southern Ontario, Canada (Fig. 1). Roughly three quarters of its current distribution was covered by glaciers during the last glacial maximum (LGM; 18,000 years ago), suggesting that *P. cinereus* underwent a rapid and extraordinary postglacial range expansion (Highton and Webster 1976). This expansion occurred despite *P. cinereus* generally possessing territoriality, small home ranges, and limited vagility (Kleeberger and Werner 1982; Gergits and Jaeger 1990; Jaeger et al. 2016).

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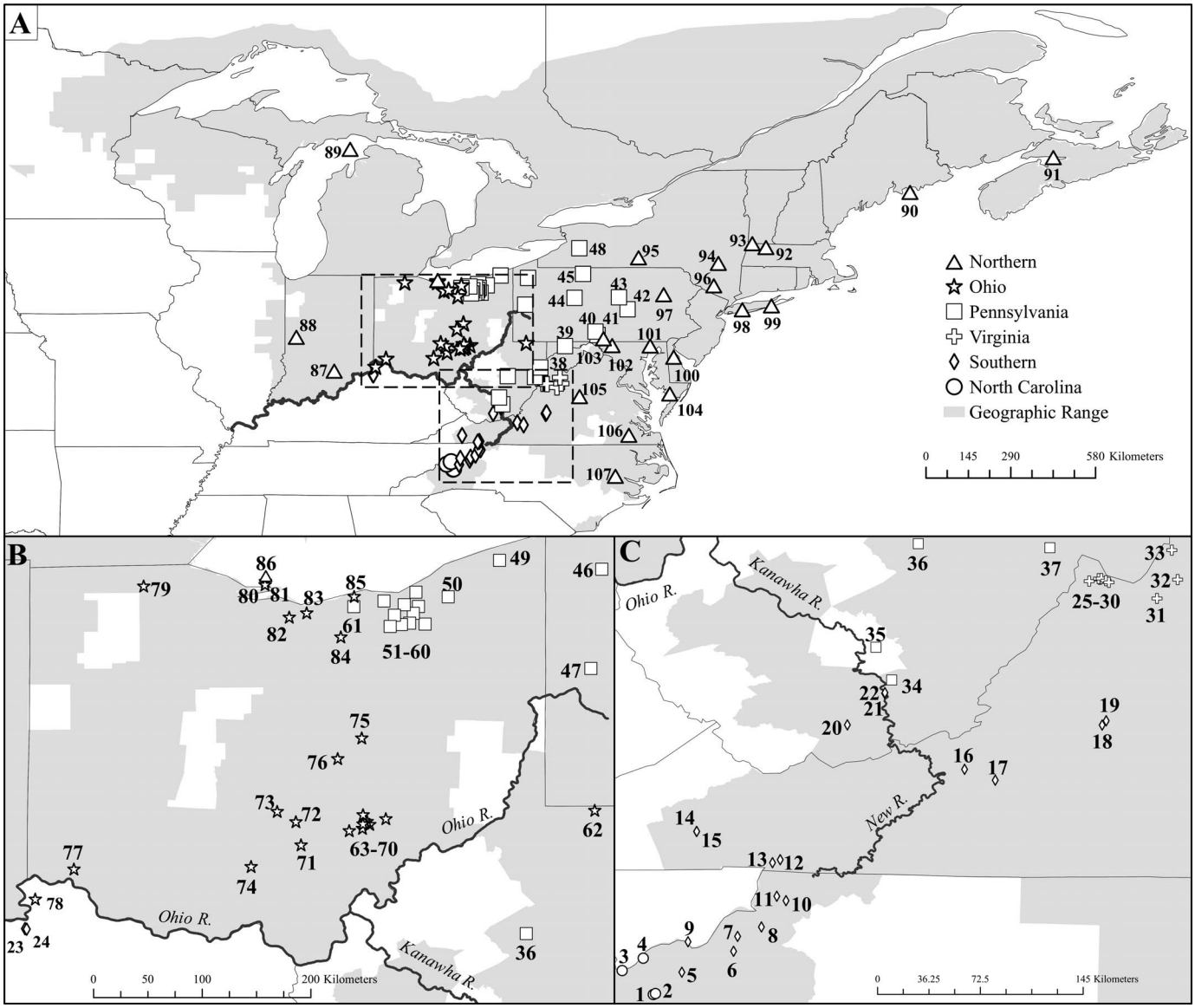

Fig. 1.—Range of *Plethodon cinereus* in gray. Population numbers correspond with Table S1 in the Supplementary Materials, available online, and clade assignments reflect the mitochondrial phylogeny (Fig. 2). (A) Entire range of *P. cinereus*. (B) Sampling in Ohio. Note that 86 is on South Bass Island, whereas 80 and 81 are on the mainland. (C) Sampling in the southern Appalachians. Black lines indicate river drainages, whereas gray lines indicate state boundaries.

Because of its widespread distribution and high abundance, *P. cinereus* is among the most studied salamanders in the world, including detailed investigations of behavior (Jaeger et al. 2016), community ecology (Hairston 1987; Hickerson et al. 2017), population and landscape genetics (Cabe et al. 2007; Cameron et al. 2017; Hantak et al. 2019; Waldron et al. 2019), and color polymorphism (Anthony et al. 2008; Fisher-Reid et al. 2013; Reiter et al. 2014; Cosentino et al. 2017; Hantak and Kuchta 2018). However, range-wide phylogeographic work is limited. Previous research found elevated allozyme variation in the southern portions of the population range, whereas variation among northern populations was relatively limited (Highton and Webster 1976; Hass 1985). This difference was interpreted as evidence for a long history in southern montane areas with relatively recent postglacial range expansion into the north. However, relationships among genetic clusters were not well resolved, and the northern portion of the range was sparsely sampled.

The aims of this study were to document patterns of genetic diversity in *P. cinereus*, to infer a biogeographic history for the species, and to provide a historical scaffolding for ongoing studies. To this end, we sequenced three mitochondrial DNA (mtDNA) and three nuclear DNA (nDNA) loci from individuals from across the range of *P. cinereus* and analyzed our data using a combination of population genetic and phylogenetic approaches. We found moderate levels of phylogeographic structure, especially at the southern limits of the range, including novel phylogeographic units. Finally, we briefly discuss the relevance of our phylogeographic results for ongoing and future studies of *P. cinereus*.

MATERIALS AND METHODS

Sampling and Laboratory Techniques

We collected blood samples and tail tips from 202 individuals from 107 populations across the range of *P. cinereus*, with an emphasis on southern populations (Fig. 1). Our sampling was oriented toward geographic coverage and describing the limits of haplotype groups. Total genomic DNA was extracted using Qiagen DNeasy blood and tissue kits (Qiagen Corp., Valencia, California). We sequenced a total of 4486 base pairs (bp) of DNA: mtDNA sequences included most of the cytochrome-*b* gene (Cyt-*b*; 1133 bp), the complete NADH dehydrogenase subunit 2 gene (ND2; 1041 bp), and a portion of tryptophan transfer RNA ($tRNA^{trp}$; 65 bp). Nuclear sequences included the intron glyceraldehyde-3-phosphate dehydrogenase (GAPD; 564 bp), the intron myosin light chain 2a (MLC2A; 416 bp), and the exon recombination-activating gene 1 (RAG-1; 1267 bp). We sequenced most samples in both the forward and reverse directions; sampling details and primers are provided in the Supplementary Materials (see Supplementary Table S1 and S2, available online). Electropherograms for all sequences were examined using Geneious v7.1 (Kearse et al. 2012), and ambiguous base calls were manually corrected. We assembled forward and reverse reads using MUSCLE (Edgar 2004). The phase of heterozygous genotypes was estimated using PHASE v2.1.1 (Stephens et al. 2001). PHASE was run for 1000 iterations, with a thinning interval of two steps and a burn-in of 100 iterations. We phased polymerase chain reaction products exhibiting length heterogeneity because of indels using Champuru v1.0 (Flot 2007). Finally, we tested for the presence of intragenic recombination using the differences in the sum of squares test implemented in TOPALi (Milne et al. 2009), including a 10-bp increment, a window size of 100, and 500 parametric bootstraps. Recombination was not detected at any locus.

Phylogenetic Analyses

We inferred a time-calibrated mtDNA gene tree with BEAST v2.3.1 (Bouckaert et al. 2014) using our three loci under the linked trees model (concatenated analysis). For this analysis, we selected our partitioning scheme and models of evolution using PartitionFinder v2.1.1 (Lanfear et al. 2012), with the best model selected using the Akaike information criterion with a correction for small sample sizes (AICc). Cyt-*b* and ND2 were partitioned by codon, whereas $tRNA^{trp}$ was treated as a single locus (Supplementary Table S3, available online). We used multiple preliminary BEAST analyses to explore a diversity of priors and examined the results in Tracer v1.7.1 (Rambaut et al. 2018). An exponential tree prior showed a marginal posterior distribution in population growth rate that included zero, indicating a constant population size prior cannot be rejected. In addition, in runs with a relaxed lognormal clock the coefficients of variation in the clock rates abutted zero, indicating the data were compatible with a strict clock model. Accordingly, for our mtDNA tree we used a constant population size coalescent tree prior and a strict clock model. We did not include outgroup taxa, because BEAST samples the root position along with all other nodes in the tree (Drummond and Bouckaert 2015). We sampled substitution rate priors using lognormal distributions, with medians and 95% confidence intervals from Kuchta et al. (2016a) assuming a plethodontid crown age of 66 million years (myr; Shen et al. 2016). Analyses were run for 25–50 million generations, with samples saved every 5000 generations. We examined effective sample sizes (ESSs) and the stationarity of likelihood values using Tracer v1.7.1 (Rambaut et al. 2018). No ESS values were <200, and most were several thousand. Results were summarized using a maximum clade credibility tree in TreeAnnotator v2.5.2 (Heled and Bouckaert 2013). We discarded the first 25% of trees as burn-in, which was well beyond stationarity.

For our nuclear loci, we used *BEAST v2.5.2 (Heled and Drummond 2010) to estimate topologies under the multispecies coalescent model (Drummond and Bouckaert 2015). Both alleles from each individual at each locus were used to provide information about the coalescent history of each tip. We conducted two analyses. For our first analysis, we designated the primary clades recovered in the mitochondrial tree as taxonomic units (Fig. 2; Supplementary Fig. S1, available online). Partitioning schemes and models of sequence evolution were chosen for each locus using AICc in PartitionFinder v2.1.1 (Lanfear et al. 2012; Supplementary Table S3). The species tree analysis was run for 50 million generations with samples saved every 5000 generations. For the second analysis, every individual was treated as a separate taxonomic unit for comparison with our mtDNA phylogeny. A key assumption of the multispecies coalescent model, which is violated when individuals in a phylogeographic study are treated as taxa, is that there is no gene flow between taxa; this can result in overestimation of population sizes, underestimation of divergence times, and low posterior probabilities (Leaché et al. 2013). However, we were only interested in obtaining a nuclear tree topology for comparison with our mtDNA topology and interpret the results of this second species tree analysis with caution. For this analysis, multiple preliminary runs failed to converge, suggesting our analyses were sensitive to overparameterization. Therefore, we ran our final analyses using the HKY+G model for each locus, analytical population size integration for the population model prior, strict clocks, and a Yule tree prior. Because our nuclear loci GAPD and MLC2A have similar rates of evolution (Supplementary Table S3), we linked their clock models, but RAG-1 was treated separately. We conducted two independent runs with Markov chain Monte Carlo (MCMC) lengths of 200 and 250 million generations, sampling every 5000 generations, and we assessed convergence by examining the sampled distributions from both runs using Tracer v1.7.1 (Rambaut et al. 2018). After discarding the first 50% of trees from each replicate as burn-in, we combined the runs using LogCombiner v2.5.2 (Bouckaert et al. 2014) and constructed the maximum clade credibility tree with TreeAnnotator v2.5.2 (Heled and Bouckaert 2013). We refer to this tree as our nDNA tree. Gene trees for each nuclear locus, inferred by *BEAST2 as part of our species tree analysis, are provided in Supplementary Fig. S2–S4.

Population Genetics

We used diversity indices to compare patterns of genetic differentiation among mitochondrial clades for both mtDNA and nDNA. The indices we used included gene diversity, the mean number of pairwise differences, and nucleotide

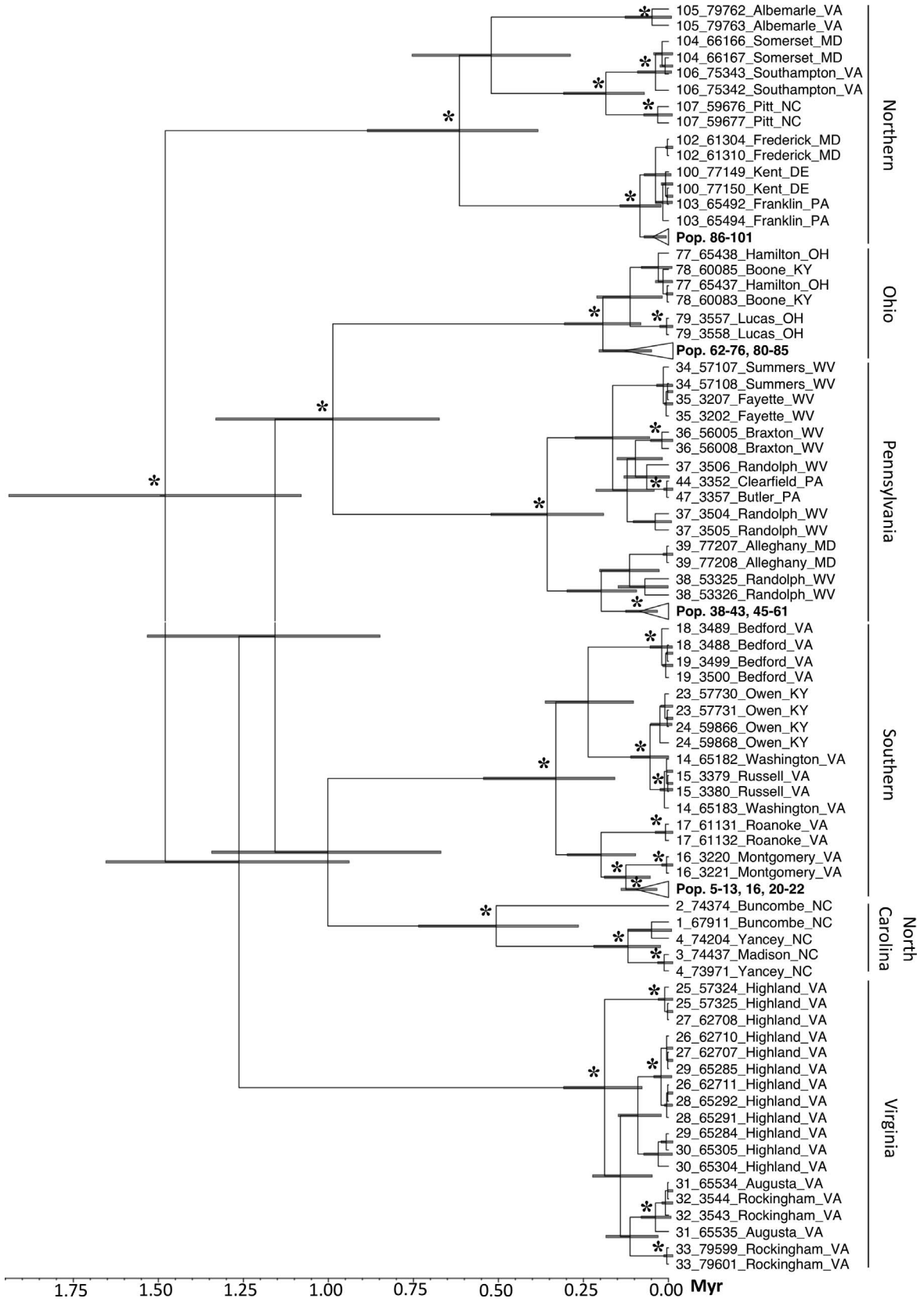

FIG. 2.—Bayesian mitochondrial DNA (mtDNA) phylogeny inferred using BEAST 2, with clade names provided on the right (Myr = millions of years before present). Tip labels include the population number followed by the individual's identification, county, and state. Population numbers correspond to Fig. 1 and Supplementary Table S1, available online. Asterisks above nodes denote nodes with ≥95% posterior support in our Bayesian analysis, and node bars illustrate 95% highest posterior density intervals. For brevity, some nodes with weak divergences have been collapsed; the full mtDNA gene tree is provided in Supplementary Fig. S1.

diversity (Nei 1987). We tested for demographic changes using Fu's F_S (Fu 1997) and mismatch distributions (Slatkin and Hudson 1991). Negative values of F_S indicate demographic expansion, with a null hypothesis of demographic stability. For mismatch distributions, we compared the distribution of each clade to the distribution expected under a null model of spatial expansion using the sum of square deviations statistic (Schneider and Excoffier 1999); significance was assessed via parametric bootstrapping of the dataset (1000 replicates). We calculated our diversity indices in Arlequin v3.5.2.2 (Excoffier and Lischer 2010). Finally, we used redundancy analysis (RDA) to test for isolation by distance (IBD) in our mtDNA sequence data using the R package vegan (Oksanen et al. 2018).

We coded our nuclear sequences at each locus as alleles using GenAlEx v6 (Peakall and Smouse 2006), and we used STRUCTURE v2.3 (Pritchard et al. 2000) to infer the number of genotypic clusters in the nuclear data. STRUCTURE estimates the number of clusters in a sample by assigning individuals to K populations such that Hardy–Weinberg equilibrium is maximized and linkage disequilibrium is minimized. We evaluated $K = 1$–10 populations, with each K replicated 12 times with randomly generated starting seeds. For priors, we used the admixture model, a fixed value of $\lambda = 1$, an inferred α, and sampling localities. Each MCMC run consisted of 500,000 iterations, with the first 100,000 discarded as burn-in. We analyzed output from STRUCTURE using Structure Harvester v0.6.94 (Earl and vonHoldt 2012), CLUMPP v1.1.2 (Jakobsson and Rosenberg 2007), and DISTRUCT v1.1 (Rosenberg 2003). Finally, we used the statistics L(K) and ΔK (Evanno et al. 2005) to delineate the number of genotypic clusters. We used the same methods to test for substructure within each cluster (Converse et al. 2015).

Ancestral Area Inference

We reconstructed the distributions of ancestral lineages within *P. cinereus* using the dispersal-extinction-cladogenesis (DEC) model (Ree and Smith 2008; Smith 2009) in the RevBayes computational environment (Höhna et al. 2016). Along branches of a phylogeny (anagenetic change), the DEC model allows for dispersal (colonizing additional regions) and extinction (elimination from a region); at nodes in the phylogeny (cladogenetic changes), ranges can either be subdivided or shared. For this analysis, we randomly selected 1000 trees from the posterior distribution (excluding burn-in) of our mtDNA analysis and imported them into RevBayes, where we used MCMC methods to estimate ancestral ranges while accounting for phylogenetic uncertainty. We chose the DEC model because in preliminary analyses in the R package BioGeoBEARS (Matzke 2014) this model outperformed the DIVALIKE and BAYAREALIKE models (Supplementary Table S4). We did not include the jump dispersal parameter in our analysis because it has been criticized on statistical and conceptual grounds (Ree and Sanmartín 2018); moreover, range expansion and range fragmentation, rather than speciation associated with dispersal events, are thought to characterize species formation in *Plethodon* (Highton 1995). We included six physiographic provinces in our reconstruction: north of the glacial boundary, Appalachian Plateau, Ridge and Valley, Blue Ridge, Coastal Plain, and Piedmont. We chose these provinces because they represent coarse physiographic differences, and because they frequently demarcate ranges in *Plethodon* (Highton 1995). We allowed ancestral populations to occupy up to two regions simultaneously. In addition, we conducted time-stratified analyses with separate dispersal matrices for pre-LGM and post-LGM periods, which prevented populations from occupying locations north of the glacial boundary before glacial retreat. For the timing of glacial retreat, we used minimum and maximum values of 10,000 and 18,000 yr, respectively. We modeled range evolution for 5000 generations, with 25% discarded as burn-in.

RESULTS

Phylogenetic Relationships

Our Bayesian mitochondrial tree recovered six primary clades (posterior probabilities $\geq 95\%$) that were geographically cohesive (Figs. 1, 2; Supplementary Fig. S1). From north to south, we labeled these clades Northern, Ohio, Pennsylvania, Virginia, Southern, and North Carolina. The Northern Clade is found along the eastern seaboard from North Carolina to Nova Scotia. Populations belonging to this clade were also recovered in Indiana as well as on Beaver Island in Lake Michigan and on South Bass Island in Lake Erie. Along the eastern seaboard, southern Populations 104–107 are divergent from the rest of the Northern Clade, whereas Populations 86–100 share a single mtDNA haplotype. The Ohio and Pennsylvania clades are located south of the Northern Clade. The Ohio Clade is restricted to Ohio, except for a single population (78) south of the Ohio River in Boone County, Kentucky, and an apparently geographically isolated population (62) in Monongalia County in northern West Virginia. The Pennsylvania Clade is found in New York, Ohio, Pennsylvania, eastern Maryland, and West Virginia. Both the Ohio and Pennsylvania clades extend north of the glacial boundary, but to a lesser extent than the Northern Clade (Fig. 1). The Virginia Clade occupies a relatively small area in Highland, Augusta, and Rockingham counties in the Ridge and Valley physiographic province of Virginia. To the south, in the Blue Ridge Mountains, are the Southern and North Carolina clades. The Southern Clade has the larger distribution, including West Virginia south of the New River, western Virginia, and extreme northwestern North Carolina. In addition, two geographically disjunct populations (23 and 24) were recovered in Owen County, Kentucky, near the Ohio River. The North Carolina Clade occupies a relatively restricted distribution in extreme western North Carolina in Buncombe, Madison, and Yancey counties. In general, relationships among the six major mtDNA clades were not strongly supported (posterior probability [pp] < 0.95; Fig. 2). We recovered the Northern and Virginia clades as connecting toward the base of the tree, with the Northern Clade sister to the rest of *P. cinereus*. The Ohio and Pennsylvania clades were recovered as sister clades, as were the Southern and North Carolina clades. The most recent common ancestor of *P. cinereus* was estimated to have existed in the mid-Pleistocene 1.49 myr ago (95% highest posterior density [HPD] = 1.09–1.95; Fig. 2).

In contrast to our mtDNA tree, the topologies for our three nuclear gene trees were poorly resolved (Supplementary Fig. S2–S4). Statistical support was generally low, and the recovered clades did not circumscribe geographically

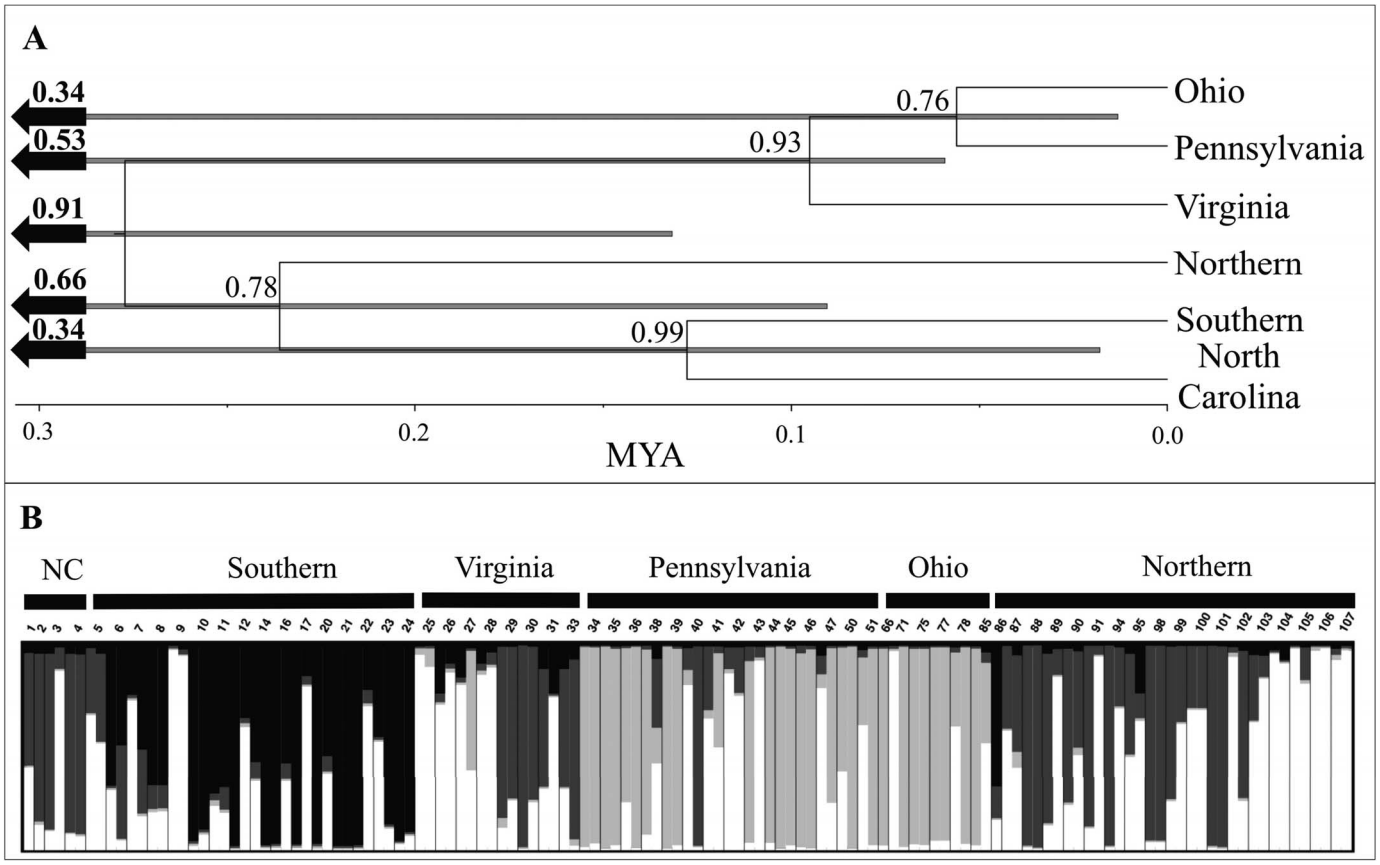

FIG. 3.—(A) Inferred species tree using nuclear DNA sequence data, with mtDNA clades of *Plethodon cinereus* treated as species (MYA = millions of years before present). Bars illustrate 95% highest posterior density (HPD) intervals for node age, and numbers at nodes represent posterior probabilities. Bold numbers indicate the uppermost value of the 95% HPD interval. (B) Population clusters identified by STRUCTURE for $K = 4$ with mtDNA clade membership labeled above. NC = North Carolina. In both panels, clade names correspond to collection localities indicated in Fig. 1.

cohesive groups or recover any mtDNA clade. However, some clades reminiscent of patterns in the mtDNA data were recovered. For example, in the MLC2A gene tree one clade ($pp = 0.20$) included many (but not all) of the alleles from Populations 104–107, which in the mtDNA tree was a coastal subclade of the Northern Clade. GAPD and RAG-1 also recovered clades that included some (but not all) of the alleles from Populations 104–107 ($pp = 0.93$ and 1, respectively). Similarly, in MLC2A another clade ($pp = 0.71$) included alleles from Populations 1–4 (North Carolina Clade) as well as alleles from Populations 5 and 9 (Southern Clade), which are the geographically closest populations to the North Carolina Clade. Clades including some (but not all) of the alleles from Populations 1–5 and 9 were also recovered in the GAPD gene tree ($pp = 0.80$) and the RAG-1 gene tree ($pp = 0.79$).

Our nDNA tree, which was a species tree inferred using all the nuclear loci with individuals designated as species, was, like our nuclear gene trees, discordant from our mtDNA gene tree and did not form geographically cohesive groups (Supplementary Fig. S5). It was also weakly supported, with the most strongly supported node $pp = 0.55$. However, some relationships hinted at the mtDNA gene tree. Populations 104–107, which are the coastal populations in the Northern Clade, formed a clade. In addition, the populations in the North Carolina Clade (1–4), together with one individual from nearby Population 5, formed a clade, and subsets of individuals from the Virginia Clade formed two different clades (Populations 26–30, 26–28). Lastly, in many instances, individuals from the same population, or nearby populations, were recovered as sister to each other.

Finally, our species tree that was inferred using nuclear loci with the mtDNA clades designated as species did not match our mtDNA gene tree (Fig. 3A), although statistical support for relationships was not robust. As with our mtDNA tree, we recovered the Ohio and Pennsylvania clades as sister clades ($pp = 0.76$). Also, we recovered the Southern and North Carolina Clades as sister clades ($pp = 0.99$). In contrast to our mtDNA gene tree, a clade that included the Northern, Southern, and North Carolina clades was recovered ($pp = 0.78$). Also conflicting with the mtDNA gene tree, a clade was recovered including the Ohio, Pennsylvania, and Virginia clades ($pp = 0.93$). Finally, the species tree had more recent divergence dates, with a most recent common ancestor estimated at 0.28 myr (95% HPD = 0.13–0.91 myr).

Population Genetic Structure

Patterns of genetic diversity reflected our phylogenetic results, with mtDNA substantially more diverse than nuclear loci (Table 1; Supplementary Tables S5–S7). The mean number of pairwise differences for mtDNA was 34.13 but ranged from 2.92 to 8.15 for nuclear loci. When we tested for population expansion using Fu's F_S on the mtDNA data, the

TABLE 1.—Genetic diversity for mitochondrial DNA (concatenated cytochrome-*b* gene, tryptophan transfer RNA, and NADH dehydrogenase subunit 2 gene) of *Plethodon cinereus* (clade names correspond to collection localities indicated in Fig. 1).

Clade	No. of populations	No. of individuals	No. of segregating sites	Gene diversity	Mean no. of pairwise differences	Nucleotide diversity	Fu's F_S	Sum of square deviations	Redundancy analysis (adjusted R^2)
All collection localities	64	122	181	0.99	34.13	0.0162	−23.92*	0.005	0.2353**
Northern	17	33	36	0.91	7.26	0.0036	−5.28	0.013	0.3591**
Populations 86–103	14	27	2	0.86	0.5	0.0002	−23.11*	NA	NA
Populations 104–107	3	6	23	1	11.47	0.0057	−0.75	0.11	0.5074**
Ohio	5	9	8	1	3.39	0.0017	−6.39*	0.202**	0.0774
Pennsylvania	14	29	30	0.99	6.38	0.003	−13.03*	0.038	0.3484**
Virginia	8	15	14	0.97	3.92	0.0019	−6.06*	0.145**	0.3706**
Southern	16	31	23	0.98	5.49	0.0026	−20.13*	0.009	0.4339**
North Carolina	4	5	22	1	9.4	0.0045	−0.42	0.051	−0.2600

* $P < 0.02$ (significance level recommended by Fu 1997); ** $P < 0.05$.

Ohio, Pennsylvania, Virginia, and Southern clades showed evidence of expansion, as did the subclade within the Northern Clade that includes populations north of the glacial boundary (Populations 86–103). Despite their wide distribution from Indiana to Maryland, these populations share a single mitochondrial haplotype, consistent with recent, rapid range expansion. Using mismatch distributions, only the Virginia and Ohio clades had distributions consistent with range stability. Our demographic test results varied among the nuclear loci, perhaps because of their relatively low levels of variation. Finally, RDA indicated IBD throughout the range of *P. cinereus* and within every clade except the North Carolina and Ohio Clades (Table 1; Supplementary Fig. S6). When we divided the Northern Clade into unglaciated versus glaciated areas, populations from unglaciated areas exhibited IBD.

For the STRUCTURE analysis using nuclear loci, the Evanno et al. (2005) method showed a peak at $K = 4$ (Supplementary Fig. S7). Similarly, Ln Pr(X|K) increased until $K = 4$, with a plateau and an increase in variance for larger values of K. In general, evidence of structure is weak, with most individuals displaying membership in two or more clusters (Fig. 3B). Cluster 1 corresponds to individuals from throughout the range of *P. cinereus*, but it especially includes individuals belonging to the Northern Clade and the Virginia Clade. Cluster 2 largely corresponds to the Southern Clade but also includes a population on South Bass Island in Lake Erie (86). Cluster 3 corresponds to Populations 34–39 in the Pennsylvania Clade (in West Virginia and western Maryland) and Populations 43–85 in the Pennsylvania and Ohio Clades (central Pennsylvania west through Ohio). Cluster 4 includes the North Carolina Clade, individuals in the Virginia Clade, and all of the Northern Clade except the southeastern populations. There was no evidence of substructure in any cluster (data not shown).

Ancestral Area Inference

We modeled range evolution in *P. cinereus* using the DEC model (Ree and Smith 2008) on our posterior distribution of mtDNA gene trees in the program RevBayes (Höhna et al. 2016). In this reconstruction, the ancestor of extant *P. cinereus* populations occupied either the Blue Ridge or the Ridge and Valley physiographic province with nearly equal probability (Fig. 4). The ancestor of the Northern Clade was inferred to have originated in the Piedmont and then dispersed into the Blue Ridge and Appalachian Plateau physiographic provinces before rapidly expanding northward following the retreat of the Pleistocene glaciers. The ancestor of all populations exclusive of the Northern Clade was inferred to have occupied the Appalachian Plateau. Populations that dispersed to the Ridge and Valley province are ancestral to the Virginia Clade. The ancestor of the North Carolina Clade is inferred to have occupied the Coastal Plain before dispersing into the Blue Ridge, whereas the ancestor of the Southern Clade may have occupied the Ridge and Valley before dispersing into the Blue Ridge and Appalachian Plateau provinces. Finally, the ancestor of the Ohio Clade mostly likely originated in the Ridge and Valley before expanding into the Appalachian Plateau, whereas the Pennsylvania Clade originated in the Appalachian Plateau. As with the Northern Clade, following glacial retreat several populations in the Ohio and Pennsylvania Clades expanded northward into formerly glaciated regions in Ohio, Pennsylvania, and New York. Whether members of these clades are also found in Canada, or whether the Northern Clade occupies the entire range of *P. cinereus* in Canada, awaits further study.

DISCUSSION

Phylogeographic Diversity

In our phylogeographic analysis of *P. cinereus*, we recovered high levels of genetic structure using mtDNA, whereas our nuclear loci were far less variable. Consistent with phylogeographic analyses of other plant and animal taxa in the region, much of the diversity was in the southern portion of the range (Soltis et al. 2006; Herman and Bouzat 2016; Kuchta et al. 2016b, 2018). In our mtDNA gene tree, we recovered six major clades (Figs. 1, 2). The Northern Clade has the most extensive distribution, including the eastern seaboard, Beaver Island in Lake Michigan, South Bass Island in Lake Erie, and Indiana. It is likely that this clade extends across southern Canada, although this needs to be verified through denser geographic sampling. Although populations of this clade along the eastern seaboard (North Carolina, Maryland, Virginia) exhibit phylogeographic structure, the northern populations (86–103) share a single mtDNA haplotype. South of the Northern Clade are the Ohio and Pennsylvania Clades. The Ohio Clade is largely distributed north and west of the Ohio River; however, a single population was found south of the Ohio River in Owen County, Kentucky, and a geographically isolated population was recovered in Monongalia County, West Virginia, east of the Ohio River. The clades with the smallest geographic distributions—the North Carolina, Southern, and Virginia

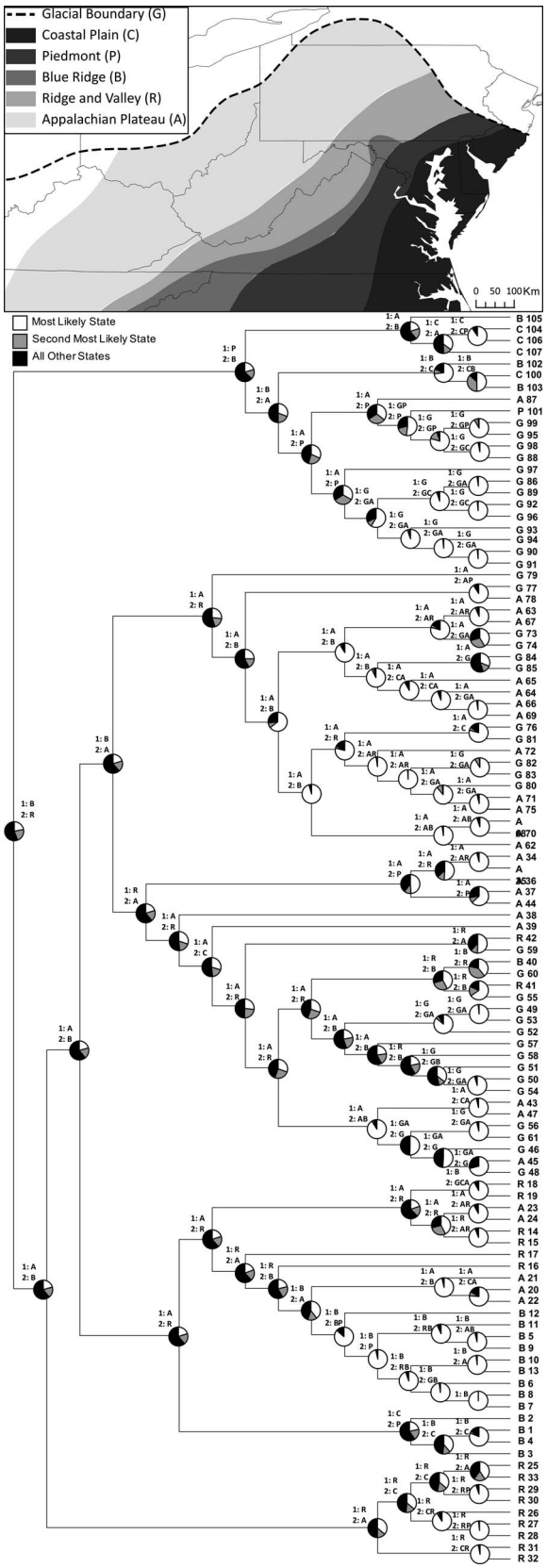

FIG. 4.—Ancestral range inference reconstructed using the dispersal-extinction-cladogenesis model on a posterior distribution of mitochondrial DNA gene trees in RevBayes. The map at the top illustrates the physiographic provinces, including abbreviations in the legend. To improve readability, branch lengths are not proportional to time. Pie charts at the nodes present the probability of ancestral geographical ranges, with white and gray indicating the first and second most likely ancestral ranges, respectively, and black representing the cumulative probability of all other possible ancestral ranges. The identities of the most likely ranges are provided to the top left of each pie chart. The bold letter to the left of each taxon label indicates the province from which each individual was sampled. Taxon labels provide the population number.

Clades—are in the south. The North Carolina Clade is the southernmost clade. It is restricted to the southern Blue Ridge Mountains and has the smallest distribution of any clade. The Virginia Clade also has a small distribution and is limited to three counties in northwestern Virginia; increased sampling could extend the distribution into West Virginia.

In contrast to our mtDNA data, our three nuclear loci provided little phylogeographic resolution, and Bayesian cluster analyses did not recover any strongly divergent groups. The loci used in this study have been useful in other contexts, however: they were informative in a study of species delimitation in the *Plethodon wehrlei* species complex (Kuchta et al. 2018), and they were also useful in a phylogenetic analysis of relationships within *Plethodon* (Fisher-Reid and Wiens 2011). Accordingly, the low phylogeographic resolution of the nuclear loci in this study likely reflects relatively limited phylogeographic differentiation within *P. cinereus* (Kuchta et al. 2016a).

Biogeography

According to our molecular clock analyses of mtDNA variation, the ancestor of *P. cinereus* was inferred to have existed 1.49 myr ago (95% HPD = 1.09–1.95 myr). This is after the start of the Pleistocene (2.588 myr), an epoch marked by repeated glacial cycles. During the LGM, the Laurentide Ice Sheet extended south to ~39°N, with zones of permafrost extending hundreds of kilometers southward beyond the ice sheet (Hewitt 1999; Jackson et al. 2000). In other studies, populations currently occupying previously glaciated areas tend to be characterized by low levels of genetic diversity, with dispersal routes and the location of refugia during glaciation strongly influenced by regional geomorphology (Hewitt 1996; Kuchta and Tan 2005; Soltis et al. 2006). For example, in Europe the east–west orientation of major mountain ranges resulted in many shared migration routes and refugia during the LGMs (Hewitt 1999), whereas in eastern North America the north–south alignment of the Appalachian Mountains, which are surrounded by low-relief landscapes that extend even into subtropical regions, did not so directly constrain migration patterns (Soltis et al. 2006).

In our phylogeographic analyses, we found that populations of *P. cinereus* north of the glacial boundary belong to three different clades (Ohio, Pennsylvania, and Northern), suggesting multiple glacial refugia. Population genetic tests are consistent with range expansion in most clades. In particular, one haplotype in the Northern Clade is distributed from Maryland and Delaware northward into Nova Scotia; moreover, it was found on two islands in the Great Lakes and in Indiana south of the glacial boundary. Assuming this haplotype is also found in the intervening region in Canada, the Northern Clade possesses a horseshoe-shaped distribution with two widely separated parts located south of the glacial boundary. Given that the

remainder of the Northern Clade is located in the mid-Atlantic states and that glaciers near coastal areas receded sooner than inland areas (Dyke 2004), we suggest that the Northern Clade originated along the eastern seaboard; following glacial retreat, it expanded its range northward, westward across Canada, and then southward back into unglaciated regions west of the Great Lakes. Northern populations of Spring Peepers (*Pseudacris crucifer*) may have followed a similar route, although the southward expansion is not known to extend into unglaciated areas (Austin et al. 2002).

This is an astonishing range expansion for an organism with no breeding migrations, small home ranges, and limited dispersal ability (Jaeger et al. 2016). How was such an extensive, rapid postglacial colonization possible? Colonization events that require expansion rates to exceed contemporary dispersal rates has been termed Reid's paradox (Clark et al. 1998). It may be that during postglacial expansion the absence of competitors facilitates long-distance dispersal. Alternatively, studies of contemporary movement may largely miss long-distance dispersal. For example, although Gergits and Jaeger (1990) found that individuals of *P. cinereus* were usually recovered within a meter of their original capture site (see also Liebgold et al. 2011), Marsh et al. (2004) showed that dispersing individuals were able to colonize islands of forest-like habitat in open fields. Finally, during postglacial range expansion, exploratory members may come to dominate the range edge and produce offspring who are more exploratory, leading to the evolution of enhanced dispersal rates (Canestrelli et al. 2016).

We lack the sampling to statistically assess the role that rivers have played in the phylogeographic divergence of *P. cinereus*, but there are suggestive patterns. The New and Kanawha rivers, which were formerly part of the ancient Teays River, have been identified as an important biogeographic barrier for plethodontid salamanders (Highton 1999; Kozak et al. 2006a; Kuchta et al. 2016b). For example, the Kanawha River largely separates *P. electromorphus* from its sister species *Plethodon richmondi* (Highton 1999). In West Virginia, the Southern and Pennsylvania clades of *P. cinereus* are found on the west and east side the New River (Populations 20–22 and 34–35, respectively; <10 km). However, the separation is not complete, because Populations 16–19 of the Southern Clade are also located on the east side of the New River (Fig. 1). In addition to differentiation across the New River, the Ohio River seems to largely exclude *P. cinereus* from Kentucky and Indiana (Fig. 1). The sole exception to this pattern is a small area in Boone, Owen, and Kenton counties in north-central Kentucky (Hass 1985). In our mtDNA data, the northernmost sample in Boone County, Kentucky, is most closely related to a population in Hamilton County, Ohio, just across the Ohio River. Thus, it appears that at this one point *P. cinereus* managed to disperse across the Ohio River, because the close relationship between the populations suggests their divergence does not predate the origin of the Ohio River (Melhorn and Kempton 1991). By contrast, our samples from nearby Owen County, Kentucky (Populations 23 and 24), belong to the Southern Clade, from which they are geographically disjunct, being most closely related to Populations 14 and 15 in Washington County, Virginia, ~317 km to the southeast. Populations 23 and 24 suggest that the Southern Clade may have recently been distributed across eastern Kentucky, much as *P. richmondi* is today (Highton 1999). An alternative hypothesis is that *P. cinereus* in Owen County, Kentucky. are the result of a human-mediated introduction. To date, the mtDNA haplotypes in Owen County have not been recovered in any other population in the Southern Clade, as one would expect if the populations were due to a recent introduction. Denser sampling in western Virginia is needed to resolve this issue. In addition, whether the Ohio and Southern clades in Kentucky meet in a secondary contact is unclear.

An intriguing pattern in our mtDNA data is that the geographic range size of mitochondrial clades increases with latitude. This parallels Rapoport's rule, the poleward increase in species' geographic ranges. Stevens (1989) hypothesized that species at greater latitudes experience dramatic seasonality and therefore must tolerate a wider range of environmental conditions, resulting in poleward species having larger geographic ranges. Our study suggests that postglacial colonization also leads to larger poleward distributions, an idea raised by previous researchers in other systems (Price et al. 1997).

Comparison with Allozyme Studies

The first study of phylogeographic diversity in *P. cinereus* was conducted by Highton and Webster (1976), who used 18 allozyme markers to quantify variation among 15 populations. This was followed by an unpublished master's thesis by Hass (1985), who used 24 allozyme loci to examine variation within and among 52 populations. The geographic spread of the sampling by Hass (1985) was concentrated on the central and southern portion of the range of *P. cinereus* and was not as geographically extensive as Highton and Webster (1976) or the current study. Hass (1985) identified four genetic clusters that she termed Groups I–IV. In Fig. 5, we illustrate how our mtDNA correlates with these genetic groups.

In general, our phylogeographic results are broadly concordant with the prior allozyme studies. For example, all three studies documented higher levels of phylogeographic diversity in the south and less diversity in the north. In addition, our mtDNA clades roughly correspond to the groups recovered by Hass (1985; North Carolina Clade = Group I; Southern Clade = Group II; Northern Clade = Group III; Pennsylvania Clade = Group IV), although there are discordances (see below). Moreover, although Hass (1985) did not sample in Ohio, she did collect two samples that may be members of the Ohio Clade, from Boone County, Kentucky, and from Harrison County, West Virginia. Her Boone County population is the same as our Population 78, whereas the latter population is ~60 km from our Population 62 (the only population we sampled in West Virginia with an Ohio Clade haplotype). Hass (1985) even suggested that her population from Harrison County, West Virginia, might be sufficiently divergent as to constitute a new group, which could be the Ohio Clade. Denser geographic sampling is needed to identify the distribution of the Ohio Clade and determine whether populations in West Virginia are geographically contiguous with Ohio Clade populations in Ohio.

Despite the largely congruent results, there are some important differences between the allozyme and the mtDNA datasets (Fig. 5). First, Hass (1985) recognized a population

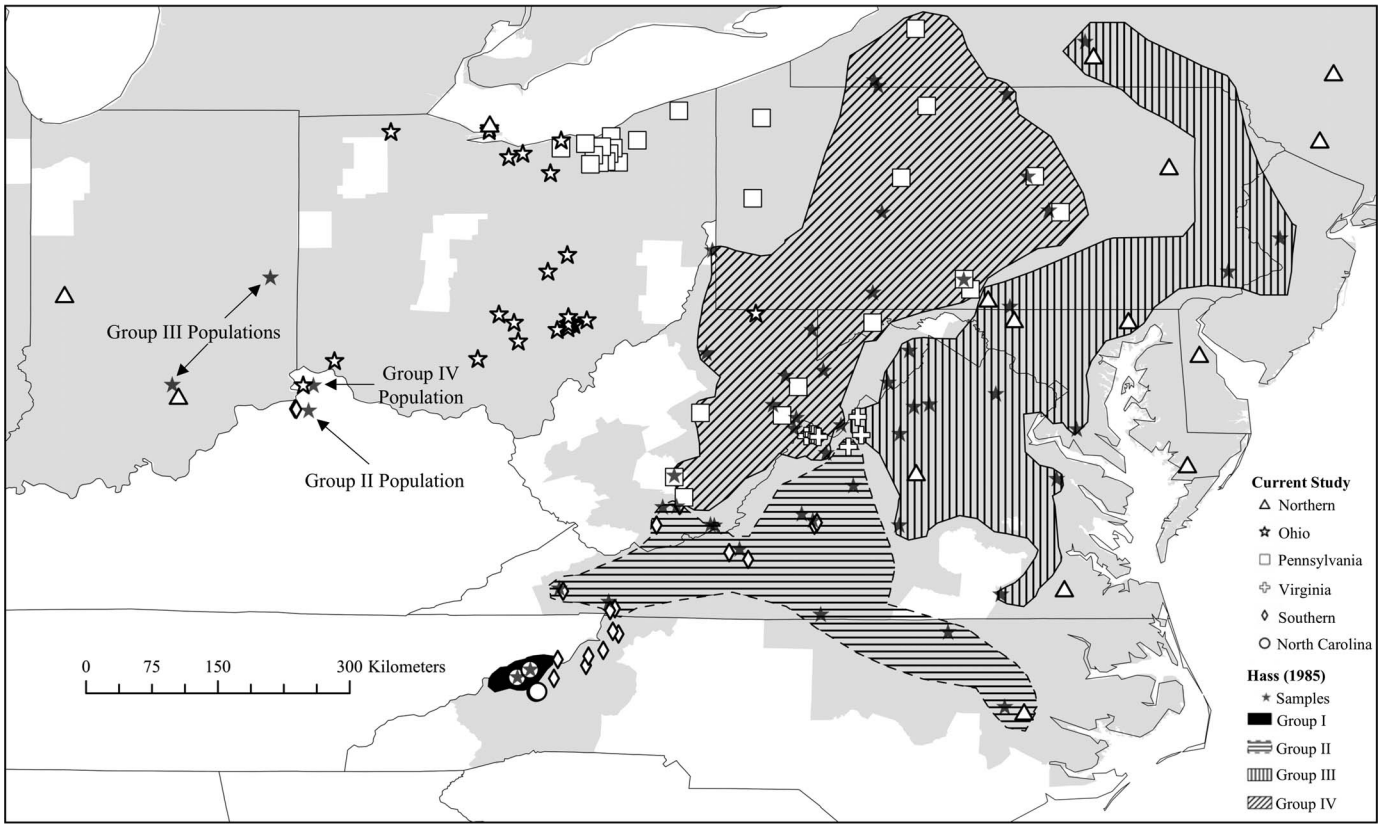

Fig. 5.—Comparison of the mitochondrial clades recovered in this study to the genetic clusters identified by Hass using allozyme data (1985).

in Pitt, North Carolina (our Population 107), as a member of her Group II (similar in distribution to our Southern Clade). By contrast, using mtDNA we recovered haplotypes from this population as belonging to the Northern Clade. The Bayesian clustering analysis of our nuclear loci was not informative in distinguishing between these two possible group assignments. It is possible that the southernmost populations in eastern North Carolina have nDNA that associates them with populations in the southern Blue Ridge Mountains, whereas mtDNA identifies them as a member of the Northern Clade. Although the distribution of *P. cinereus* in the southern Piedmont of Virginia is patchy, denser population sampling and more informative nuclear markers are needed to address the evolutionary history of the southeasternmost populations of *P. cinereus*.

Second, Hass (1985) did not recover the Virginia Clade as a genetic cluster but instead interpreted patterns of variation in this area (our Populations 25–30) as a hybrid zone between divergent groups (Fig. 5). By contrast, our recovery of a distinct mitochondrial clade in this area instead suggests that these populations constitute a distinct historical unit. It is conceivable that this region is a broad zone of admixture between the Southern Clade, Pennsylvania Clade, and remnants of a former, admixed Virginia Clade, of which mtDNA haplotypes remain. Again, studies with denser geographic sampling and more informative nuclear loci are needed to better understand the history and nature of secondary contacts in this region (Waldron et al. 2019).

Finally, as discussed previously, although we discovered a mtDNA clade centered in Ohio that we call the Ohio Clade, neither allozyme study clearly identified this clade.

Future Research

This study has implications for future and past research on *P. cinereus*, which has long been a model organism in ecology, evolution, and behavior (Petranka 1998; Anthony and Pfingsten 2013; Jaeger et al. 2016). Our study provides a framework for research on patterns of differentiation in *P. cinereus*; indeed, comparative studies of territoriality, mate recognition, and landscape genetics are ongoing (Hantak et al. 2019; Kunkel et al. 2019; Waldron et al. 2019). In addition, we have identified several novel patterns in our phylogeographic data that can inform future research efforts. Is the Northern Clade distributed across Canada, and if so, was an initial northward postglacial range expansion followed by a southern expansion into Indiana and Ohio? How did Southern Clade populations come to be found in northern Kentucky? Is the Virginia Clade a valid historical unit, or are those populations genetically admixed? What is the nature of secondary contacts between phylogeographic units in *P. cinereus* (Waldron et al. 2019) and are any of them characterized by reproductive isolation? What is the relationship between phylogeographic variation and patterns of phenotypic variation, especially with respect to the well-known striped/unstriped polymorphism (Fisher-Reid et al. 2013; Cosentino et al. 2017; Hantak et al. 2019)? Given the low resolution of our nuclear data, future work on patterns of genetic variation should use a larger number of nuclear markers, such as single-nucleotide polymorphisms or sequence data obtained using next generation sequencing methods.

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Supplemental Material

Supplemental material associated with this article can be found online at https://doi.org/10.1655/Herpetologica-D-19-00045.S1

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