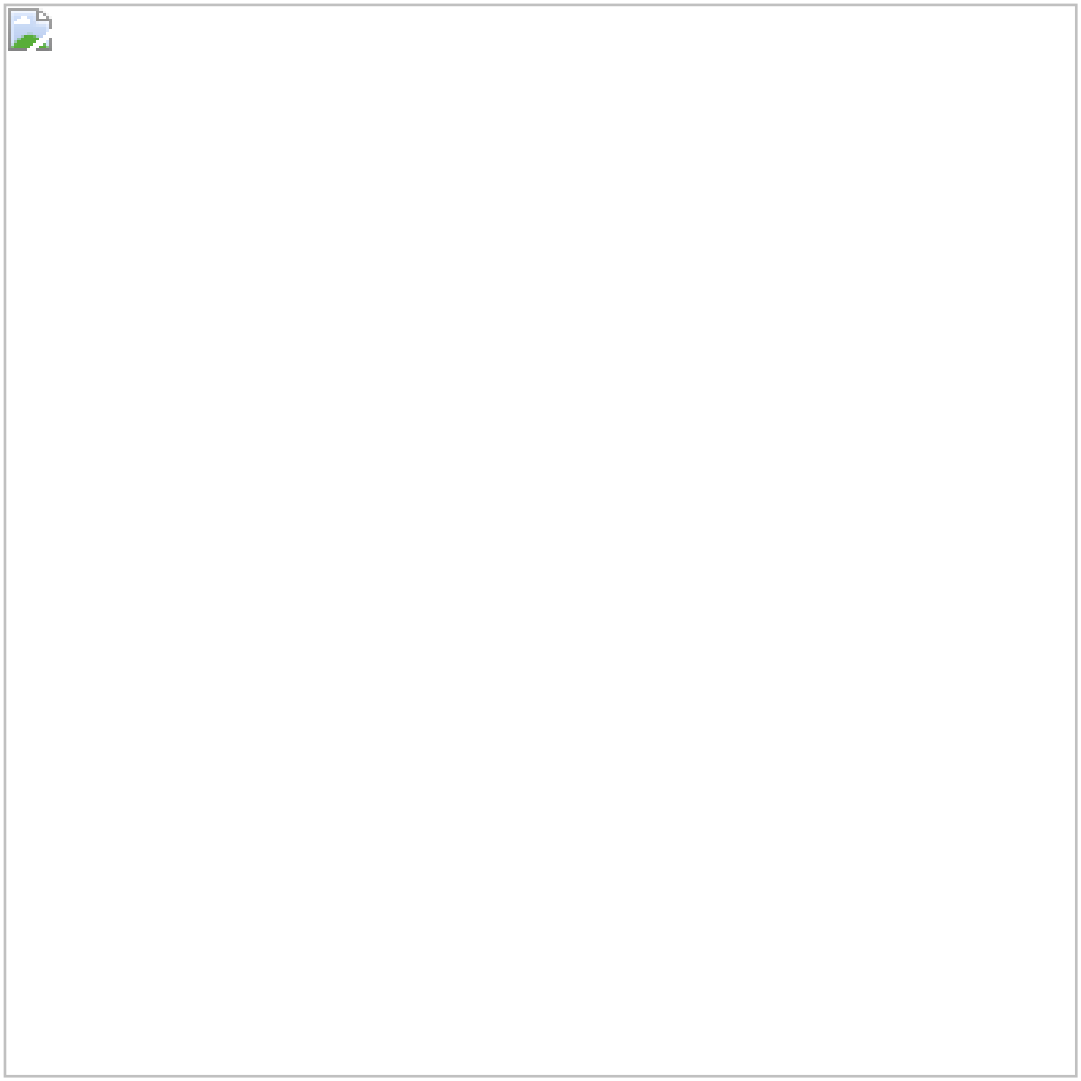

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Phylogeography of the Slimy Salamander Complex (*Plethodon*: Plethodontidae) in Alabama

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The *Plethodon glutinosus* complex is composed of 16 lineages that are thought to have conserved morphological characteristics and rapid rates of diversification. Typically, these lineages are recognized as species, but the monophyly of some has been questioned. Three lineages have distributions that converge in the state of Alabama: *Plethodon glutinosus*, *P. grobmani*, and *P. mississippi*. If these species are present in the state and are reproductively isolated, then we expected to recover three monophyletic lineages. If these species are present in the state, but exhibit extensive introgression, then we expected to recover sets of private haplotypes associated with each species and sets of shared haplotypes among species. We sampled 40 specimens of slimy salamanders from throughout the state. Samples were analyzed using two genes, cytochrome *b* ($n = 38$) and RPL12 ($n = 17$). Additionally, we added 47 cytochrome *b* sequences for Alabama specimens of the three species available on GenBank to examine relationships of this larger sample. We failed to recover three monophyletic lineages within any estimated gene tree and failed to recover sets of private haplotypes. Instead, haplotype-network structure revealed a single metapopulation. We conclude that Alabama contains a single species of slimy salamander, *Plethodon glutinosus*, with complex genetic connectivity throughout the state.

DELIMITATION of species boundaries remains variable among investigators despite general agreement that species are lineages (de Queiroz, 2005). This variation emerges because investigators differ in their focus on characteristics of metapopulations assumed to be necessary to discover new species (de Queiroz, 2007). Modern molecular tools have expanded our ability to estimate necessary conditions for speciation early in the process of divergence. In fact, in some models, speciation is possible even when gene flow continues between sister species (e.g., Burbrink and Guiher, 2015). However, the conclusion that two species exist, rather than one, is a hypothesis awaiting further evidence that supports or refutes it. Descriptions of current biological diversity (e.g., field guides or conservation legislation) frequently require acceptance of current taxonomic conclusions in the absence of additional data. Furthermore, taxonomies that remain unchallenged may become so entrenched in the literature that revision of them becomes difficult. For example, Burbrink's (2001) seminal work on ratsnakes (*Pantherophis obsoleta* complex) recognizes five species for a group previously considered to constitute a single, wide-ranging, and variable species. Burbrink's (2001) taxonomic conclusions dominate recent summaries of diversity (e.g., Crother et al., 2017) despite evidence of hybridization with no loss of fitness for the hybrids for some species pairs (Gibbs et al., 2006).

The *Plethodon glutinosus* complex represents a similar challenge to that of the ratsnake complex. These salamanders, originally thought to represent a single wide-ranging species, were divided into 16 species by Highton (1989), 14 of which now constitute the *P. glutinosus* complex (Highton et al., 2012) with the other two representing species described earlier (*P. aureolus*; Highton [1984]) or resurrected (*P. kentucki*; Highton and MacGregor [1983]). Unlike other members of the genus *Plethodon*, which typically are diagnosable based on unique color patterns, no such characters distinguish members of the *P. glutinosus* complex. Instead, species are defined based primarily on measures of allozyme dissimilarity among samples that Highton (1995) interpreted to result from rapid and recent diversification, but with rampant introgressive hybridization where species ranges abut. Using mitochondrial and nuclear sequence data, Wiens et al. (2006) and Fisher-Reid and Wiens (2011) reached a similar conclusion, retaining Highton's (1989) taxonomy for the group rather than an alternative one retaining most individuals in a single widespread species (e.g., Petranka, 1998). Hillis (2019) argued that the choice between such competing taxonomies rests on examination of patterns of gene flow across geographic boundaries between populations, with elevation of populations to species status being necessary only if gene flow is restricted due to reproductive isolation, reduced viability of hybrids, or similar factors associated with evolutionary divergence. Increased sampling of populations across their contact zones is vital in making such taxonomic judgments.

Here, we examine specimens of the *P. glutinosus* complex collected in the state of Alabama and use them to test the hypothesis that these conform to three species recognized by Highton (1995). These three putative species, *P. glutinosus*, *P. grobmani*, and *P. mississippi*, belong to Clade A of Wiens et al. (2006), a lineage of species that displays rampant introgressive hybridization. *Plethodon glutinosus* is thought to be centered on higher elevation sites of northeastern Alabama, *P. mississippi* on the Coastal Plain of western Alabama, and *P. grobmani* on the Coastal Plain of southeastern Alabama (Fig. 1; Cunningham, 2011). The Fall Line, separating Appalachian terranes from the Gulf Coastal Plain, generally divides the geographic ranges of *P. glutinosus* and *P. grobmani*. The Mobile River and watershed provide a natural border between the ranges of *P. grobmani* and *P. mississippi*. The border of the western extent of Sand Mountain and the Gulf Coastal Plain generally separates *P. glutinosus* from *P. mississippi*. However, evidence from Wiens et al. (2006) questions the monophyly of *P. glutinosus* (*sensu stricto*), and

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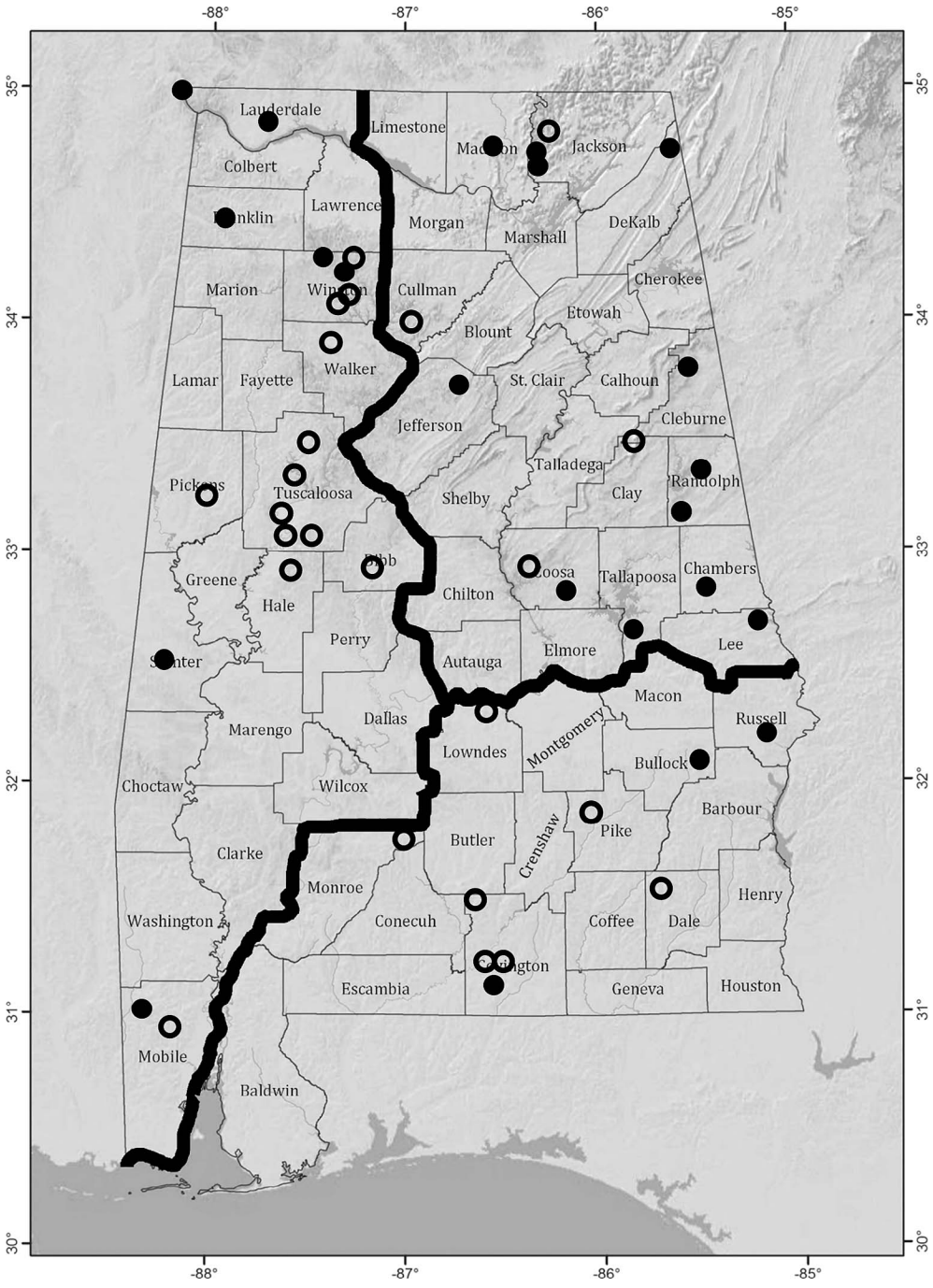

Fig. 1. Map of sample areas in Alabama. Solid symbols are new data generated during this study; open symbols are data from GenBank. Solid line, patterned after Cunningham et al. (2009), separates regions used to identify *P. glutinosus* (northeast portion of state), *P. grobmani* (southeastern portion of state), and *P. mississippi* (western portion of state).

data documenting the monophyly and geographic extent of *P. grobmani* and *P. mississippi* are lacking. Therefore, we sampled specimens of the *P. glutinosus* complex from the state of Alabama and supplemented these with similar data available from GenBank. A larger sample size from key locations across the state should give us a better understanding of what species are present within Alabama and where they are found. Using Highton's (1989) taxonomy as a hypothesis, we predict that, for each putative species studied here (*P. glutinosus, P. grobmani,* and *P. mississippi*), there will be a distinct monophyletic branch on phylogenetic trees estimated from both nuclear and mitochondrial data if speciation involves reproductive isolation. If speciation has been decoupled from reproductive isolation in these salamanders (Wiens et al., 2006), then three sets of private haplotypes, with some shared haplotypes mixed among contact zones, are expected from analysis of mitochondrial haplotypes. Failure to meet these expectations would allow rejection of the three-species hypothesis because of evidence that is consistent with gene flow within a single metapopulation lineage.

MATERIALS AND METHODS

New tissue samples of the focal taxa were taken from 40 specimens collected throughout the state of Alabama. Because the three putative species cannot be distinguished by external features, county of collection was used to identify each specimen ($n = 22$ for *P. glutinosus*, $n = 6$ for *P. grobmani*, $n = 12$ for *P. mississippi*; Fig. 1). Tissues and vouchers were deposited in the herpetology collection of the Auburn University Natural History Museum (AUM; Supplemental Appendix; see Data Accessibility) and then used for DNA

isolation. Whole genomic DNA was extracted from toe or tail clips using an E.Z.N.A. Tissue DNA Kit (Omega Bio-tek, Norcross, GA). A mitochondrial marker, cytochrome *b* (cyt *b*), was chosen because it has high mutation rates compared to single copy nuclear DNA (Wan et al., 2004). A nuclear marker, RPL12, was chosen because it is an intron (Fisher-Reid and Wiens, 2011) and therefore has a high rate of substitution, making it a good marker for population genetic analysis (Zhang and Hewitt, 2003). The combination of these two allowed us to use an integrated approach to understanding genetic change within sample populations (Rubinoff, 2006; Edwards, 2009).

Genes were isolated and amplified by polymerase chain reaction (PCR) using primers and protocols from Wiens et al. (2006) and Fisher-Reid and Wiens (2011). Final PCR products were size-verified on a 1% agarose gel through electrophoresis. A total of 38 samples from 17 counties in Alabama was amplified for cyt *b*. Of these samples, 15 were amplified successfully and generated complete Sanger reads for RPL12, as did two additional samples not included in the cyt *b* samples. These samples included specimens from the geographic ranges of all three putative species and covered the north–south and east–west extent of the state. PCR purification, sample preparation, and sequencing were performed at Beckman Coulter (Danvers, MA). Chromatographs from forward and reverse runs were assembled, and contiguous sequences were aligned and edited by eye using Geneious version 6.0.6 (https://www.geneious.com; Kearse et al., 2012). Aligned sequences of 605 bp were generated for cyt *b* and of 453 bp were generated for RPL12.

Best-fit models of evolution for each gene were tested using PartitionFinder (Lanfear et al., 2012). Bayesian Inference (BI) was performed in MrBayes 3.2.2 on CIPRES Science Gateway (Miller et al., 2010; Ronquist et al., 2012), using *Ensatina eschscholtzii* as an outgroup. Each BI had two runs with four chains each for 10,000,000 generations and was sampled every 1,000 generations. A 25% burnin was calculated using the sump option and a 50% consensus tree was created. These analyses generated independent phylogenetic trees for each gene. Posterior probabilities for node support of greater than or equal to 90% were used to identify significant clusters of specimens. As a final Bayesian analysis, we added cyt *b* sequences of 12 *P. glutinosus,* 13 *P. grobmani*, and 22 *P. mississippi* available from GenBank to our samples. Due to inconsistencies among sequences, a final cyt *b* alignment was trimmed to 256 bp consistent with the additional samples. We used BI, as described above, to generate a phylogenetic tree for this larger sample and to interpret significant clustering within the tree. For all three BI analyses, we expected recovery of three monophyletic branches, each representing a separate species, under the hypothesis that Highton (1989) correctly characterized diversity within Alabama and that the species are reproductively isolated. Because a hypothesis predicting three distinct branches was tested with each BI analysis, we assume the combined cyt *b* data are sufficient despite their shortened alignment.

To assess the hypothesis that Alabama contains three species exhibiting extensive hybridization (Wiens et al., 2006), a TCS network was generated for our new cyt *b* dataset and for the combined dataset, with individuals colorized by species based on their geographic location within the state (Fig. 1). The networks were created using the program PopART (Leigh and Bryant, 2015). This program inferred gene genealogies of the haplotypes present in the dataset through statistical parsimony and produced interconnected networks that included putative missing haplotypes. Under the hypothesis of three species with introgression, we expected to recover three sets of private haplotypes with some shared haplotypes mixed among contact zones. Again, we assume the shortened cyt *b* alignment for the combined data is sufficient to reveal the expected sets of private haplotypes.

RESULTS

BI analysis of our new cyt *b* data recovered a tree with weak support for most interior nodes and no evidence of the three expected primary lineages (Fig. 2A). Of five specimens of *P. grobmani*, three from Covington County clustered significantly. The other two samples of *P. grobmani* (Bullock and Russell counties) clustered with no other specimens. Of 12 specimens of *P. mississippi*, a significant cluster of five specimens from western Alabama (Franklin, Mobile, and Sumter counties) was recovered, but this cluster included a specimen of *P. glutinosus* from eastern Alabama (Cleburne County). A second significant cluster of five specimens of *P. mississippi* from Winston County was recovered, but this cluster was not significantly associated with the cluster from western Alabama. The other specimens of *P. mississippi* included two separate specimens from northeastern Alabama (Lauderdale County), one of which clustered significantly with a specimen of *P. glutinosus* from central Alabama (Jefferson County) and the other of which clustered significantly with specimens of *P. glutinosus* from northeastern Alabama (Jackson County). Finally, of 22 specimens of *P. glutinosus*, six occurred in a significant cluster of Jackson County specimens that included a specimen of *P. mississippi* from Lauderdale County, three occurred in a significant cluster of DeKalb County specimens that included no other specimens, ten occurred in a poorly supported cluster of eastern Alabama specimens (Chambers, Cleburne, Coosa, Lee, Randolph, and Tallapoosa counties) that included no other specimens, and one Cleburne County specimen occurred in the significant cluster of specimens of *P. mississippi* from western Alabama.

BI analysis of RPL12 revealed only a single significant cluster of two specimens of *P. grobmani* from Bullock and Covington counties and one specimen of *P. mississippi* from Mobile County (Fig. 2B). The third specimen of *P. grobmani* was excluded from this cluster, and none of the 13 specimens of *P. glutinosus* showed significant clustering. Addition of cyt *b* sequences for specimens from GenBank failed to improve clustering of specimens into the three expected lineages (Fig. 3). In fact, increasing replication within species yielded a reduced number of significant clusters overall. Only one small cluster of two *P. grobmani* from Monroe County, one small cluster of four *P. mississippi* from Hale, Tuscaloosa, and Walker counties, and one large cluster of 17 specimens of *P. mississippi* from Lauderdale, Tuscaloosa, and Winston counties and 14 specimens of *P. glutinosus* from Cullman, Jackson, and Jefferson counties were significant.

Haplotypes of cyt *b* sequences were variable across the three focal species (Fig. 4). Despite extensive variation, no sets of private haplotypes emerged associated with the identified species, either when based on the new cyt *b* dataset or when based on the combined dataset. Instead, specimens identified as *P. glutinosus* separated individuals identified as *P. grobmani* and *P. mississippi* via shared haplotypes, a pattern that became more complex and reticulated when our new data were combined with previous

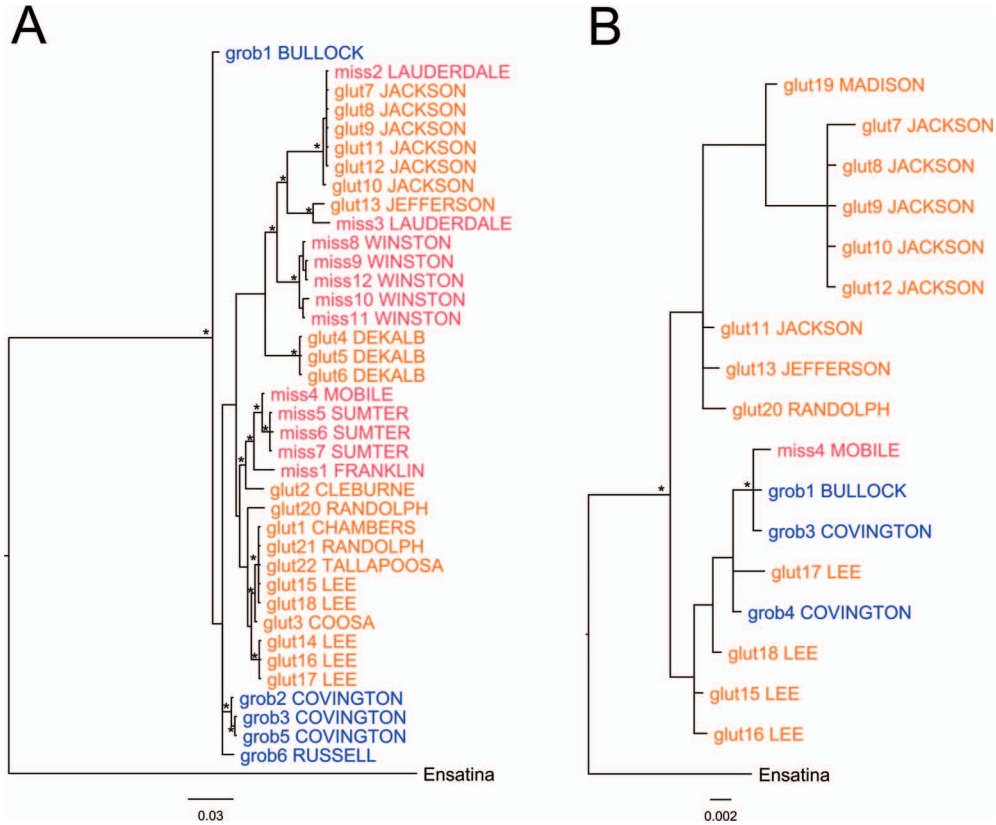

Fig. 2. Bayesian analysis of (A) cyt *b* data and (B) RPL12 data from new samples. Nodes with probabilities greater than 95% are indicated. See Data Accessibility for tree files.

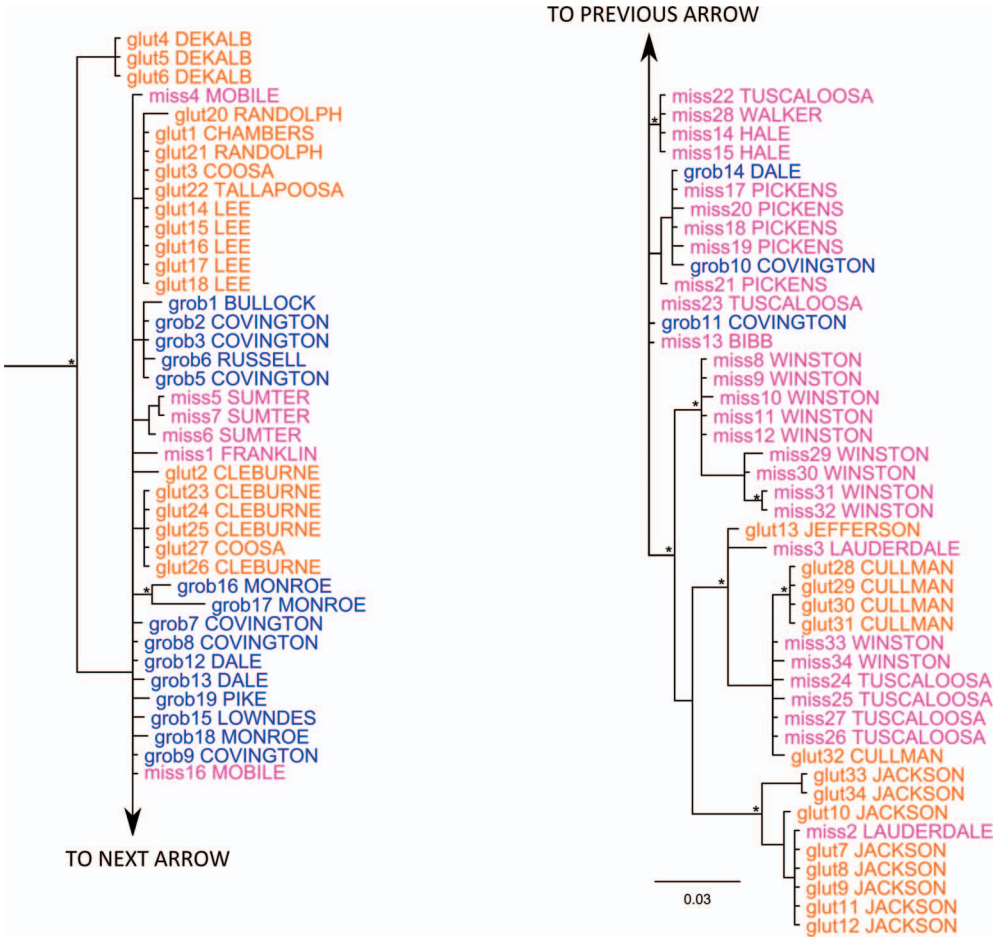

Fig. 3. Bayesian analysis of cyt *b* data from combined samples. Nodes with probabilities greater than 95% are indicated. See Data Accessibility for tree file.

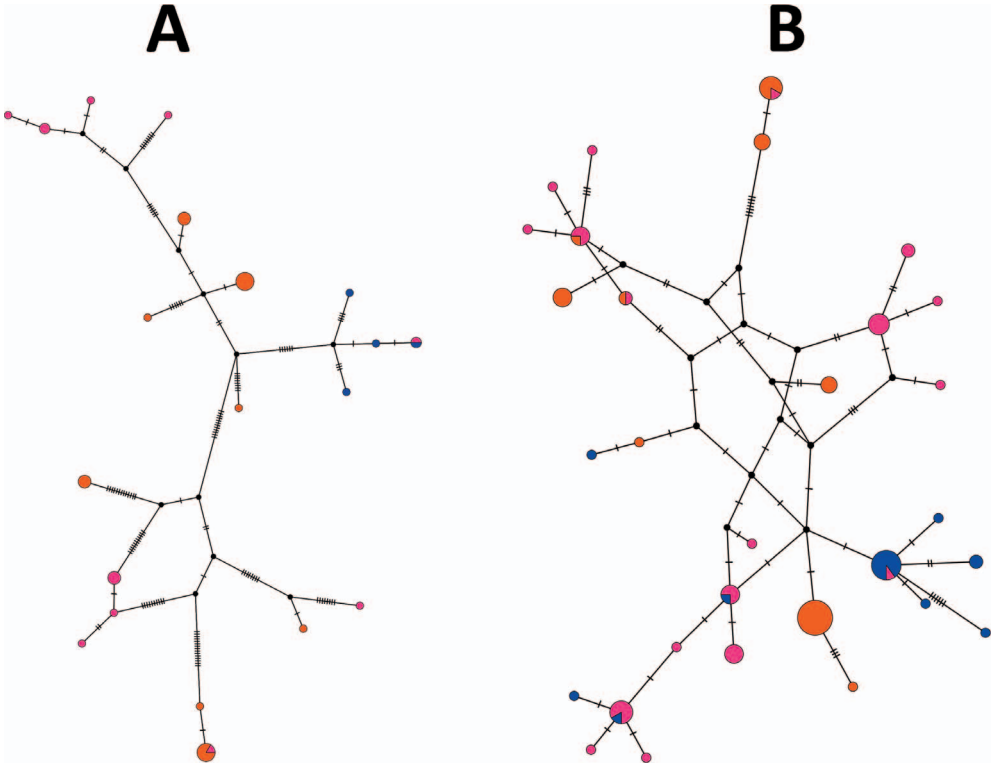

Fig. 4. Haplotype network based on (A) new cyt *b* samples and (B) combined cyt *b* samples. Size of circle indicates number of individuals possessing a haplotype. Blue = *P. glutinosus*; pink = *P. grobmani*; orange = *P. mississippi*.

data. Members of *P. grobmani* and *P. mississippi* shared three haplotypes, and three others were shared by members of *P. glutinosus* and *P. mississippi*. Haplotypes of *P. grobmani* and *P. glutinosus* were separated by as few as one step (Fig. 4).

DISCUSSION

The hypothesis that Alabama contains three monophyletic, reproductively isolated species of slimy salamander, *Plethodon glutinosus, P. grobmani,* and *P. mississippi*, is not supported. Both the mitochondrial and nuclear markers from our data produce weak phylogenetic structure with haphazard participation of the three putative species in those significant lineages that are revealed. Our phylogenetic approach for revealing independent lineages found support for only a single lineage. Similarly, our data reject the hypothesis that three species of slimy salamander are present in Alabama with extensive introgression because three sets of private haplotypes are not evident. Instead, haplotypes were shared between putative *P. grobmani* and *P. mississippi* and between putative *P. glutinosus* and *P. mississippi,* with some haplotypes of *P. grobmani* and *P. glutinosus* being separated by a single step. Thus, contemporary patterns of gene flow for slimy salamanders from Alabama show genetic structure expected of a single metapopulation lineage. That metapopulation structure reveals significant phylogenetic clustering of putative *P. glutinosus* across the Tennessee River. This occurs via clustering of specimens that are putative *P. mississippi* from Lauderdale County with specimens that are putative *P. glutinosus* from Jackson County north of the Tennessee River and via clustering of other specimens that are putative *P. mississippi* from Lauderdale County with other specimens that are putative *P. glutinosus* from Jefferson County south of the Tennessee River and near the center of the state. This suggests gene flow across a major river barrier in the northern part of the state. Similarly, specimens showing significant clustering along the western portion of the state cluster significantly with a specimen from Cleburne County, along the eastern portion of the state. This suggests significant east–west gene flow within slimy salamanders and that this gene flow crosses the Fall Line, the proposed barrier between putative *P. mississippi* and *P. glutinosus*. Finally, clustering of a specimen from Mobile County, on the western side of Mobile Bay, with specimens from Cleburne, Covington, and Bullock counties east of Mobile Bay indicate gene flow across this proposed barrier between putative *P. grobmani* and *P. mississippi*.

Based on broad taxon sampling of the taxa proposed by Highton (1989) and broad gene sampling, Wiens et al. (2006) and Fisher-Reid and Wiens (2011) concluded that the *P. glutinosus* complex consists of many species, each constrained geographically but hybridizing extensively with neighboring species. Under the model implied by this taxonomy, our results for Alabama specimens might indicate that the state is an extensive zone of hybridization with the expected sets of private haplotypes occurring elsewhere. Such a model would require restriction of the core areas of each putative species to geographic regions much smaller than those implied by current maps (e.g., Powell et al., 2016). In fact, based on those maps, an Alabama-wide hybrid zone would represent 25% of the ca. 225,700 km^2 geographic range of putative *P. mississippi* and 21% of the ca. 170,350 km^2 geographic range of putative *P. grobmani*. Presumably, these percentages would increase if similar patterns of hybridization occur in association with other states and putative species. A similar estimate for the percentage of area associated with an Alabama zone of hybridization for *P. glutinosus* cannot be estimated because all previously published phylogenies of this species have recovered at least two independent lineages for it (Weisrock et al., 2006; Wiens et al., 2006; Fisher-Reid and Wiens, 2011; Highton et al., 2012) and the geographic extent of neither lineage has been

hypothesized. Therefore, it is unclear what proportion of the range of this taxon is represented by its proposed presence in Alabama.

Published phylogenetic studies reveal problems of monophyly in other species of the *P. glutinosus* complex; these problems mirror those we reveal for putative *P. glutinosus*, *P. grobmani*, and *P. mississippi* when replicate specimens of each species are evaluated. For combined mitochondrial data of Wiens et al. (2006; cyt *b* and ND4), 12 species of the *P. glutinosus* complex were represented by multiple individuals, seven of which are non-monophyletic on their gene tree. Similarly, their nuclear gene tree (RAG-1) contained three species for which multiple individuals were sampled, all of which are non-monophyletic. Kozak et al. (2006), Weisrock et al. (2006), and Highton et al. (2012) also presented phylogenetic analyses that included members of the *P. glutinosus* complex, with Kozak et al. (2006) showing paraphyly for four of nine putative species represented by multiple samples, Weisrock et al. (2006) showing paraphyly for six of nine multiply sampled species, and Highton et al. (2012) showing paraphyly for two of three such species. Two species, *P. savannah* and *P. sequoyah,* have only been sampled once in gene trees and two others, *P. kiamichi* and *P. kisatchie*, have been sampled only twice. So, the problem of non-monophyly in replicate samples has not been adequately assessed in these four putative taxa. However, each of them causes paraphyly in other putative species within gene trees, leading Wiens et al. (2006) to suggest that *P. savannah* is conspecific with *P. ocmulgee*, *P. sequoyah* is conspecific with *P. albagula*, and *P. chlorobryonis* is conspecific with *P. variolatus*.

Highton et al. (2012) argued for retention of the species of the *P. glutinosus* complex because of strong support from morphological and allozyme evidence. We argue for use of the taxonomy of Petranka (1998), in which the *P. glutinosus* complex consists of three species, *P. aureolus*, *P. kentucki*, and a single, widespread metapopulation lineage for *P. glutinosus*. Our analytical approach implements a decision tree advocated by Hillis (2019) for assessing taxonomy based on evaluation of reproductive isolation or restriction of gene flow across putative species boundaries. Within Alabama, we find no evidence of color differences (e.g., Guyer et al., 2019, in this volume) and no unique alleles (Highton, 1989) or haplotypes that diagnose the three taxa proposed for the state by Highton et al. (2012). Based upon rules of taxonomic priority, we recommend use of *P. glutinosus* as the species identification for all slimy salamanders within Alabama and recommend that museum archives and conservation plans be based on this taxonomy. We find no value in considering individuals from Alabama to represent hybrids because the putative zone of hybridization is so broad and haplotype variation so consistent with metapopulation structure as to question whether these three species can be individuated elsewhere. We suspect that similar analyses of most of the rest of the *P. glutinosus* complex will reveal similar difficulties in demonstrating monophyly of the species generated by Highton (1989). We note that the choice of taxonomy for the *P. glutinosus* complex is not trivial because this choice affects the design and interpretation of ecological and evolutionary studies. If slimy salamanders represent a single species within Alabama, then this reduces the number of instances of niche conservatism that need to be invoked for the group (Kozak et al., 2006), reduces the need to invoke speciation without reproductive isolation (Wiens et al., 2006), and questions the validity of some niche models and cases of interspecific competition (Cunningham et al., 2009).

DATA ACCESSIBILITY

Supplemental material is available at https://www.copeiajournal.org/ch-18-170.

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