SPECIES DELIMITATION OF SLIMY SALAMANDERS, *PLETHODON KISATCHIE* AND *PLETHODON MISSISSIPPI*, ACROSS THE LOWER MISSISSIPPI RIVER

by

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ABSTRACT

Species are fundamental units of biodiversity yet delimiting species can be challenging. Slimy Salamanders of the *Plethodon glutinosus* species complex are a classic example of cryptic species for which species boundaries and relationships have proved difficult to determine. Once thought to be a single species ranging across the eastern United States, protein analysis revealed high genetic divergences among geographically distinct groups of populations, leading to 16 species being recognized within the group. Two of these species, the Louisiana Slimy Salamander (*Plethodon kisatchie*) and the Mississippi Slimy Salamander (*Plethodon mississippi*), are closely related but occur on opposite sides of the Mississippi River, a strong barrier to gene flow in many organisms. Previous phylogenetic studies of *Plethodon* have only included 1–2 samples of each of these species, thus a rigorous test of their validity has never been conducted. To investigate the evolutionary relationships of *P. kisatchie* and *P. mississippi*, I obtained tissue samples from throughout their distributions, extracted DNA, and then amplified and sequenced the mitochondrial ND2 gene and three nuclear loci. Sequence data were then analyzed using coalescent-based species delimitation methods to test the hypothesis that *P. kisatchie* and *P. mississippi* are independently evolving and thus, valid species under the general lineage concept. Results supported *P. kisatchie* and *P. mississippi* are species distinct from one another. However, I also found evidence that *P. mississippi* is hybridizing with *P. glutinosus* in Alabama. Furthermore, little genetic

diversity occurs within *P. kisatchie*, likely due to recent separation from populations of *P. mississippi* (~520,000 years ago), which raises concern for the species' long-term conservation. Based on the results of this study, I recommend both *P. kisatchie* and *P. mississippi* continue to be recognized.

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CHAPTER 1

INTRODUCTION

One of the most contentious topics in modern evolutionary biology is the question of how to delimit species (Sites & Marshall 2003; Burbrink & Ruane 2021; Hillis et al. 2021). In addressing the issue of species delimitation, de Queiroz (2007) suggested that species be defined not by hard criteria of genetic distance, reproductive isolation, or geographic isolation, but by a holistic consideration of the factors involved in the formation of species. Through such consideration, species may be delimited on the premise that they are independently evolving metapopulations (lineages) and then supported by other lines of evidence (e.g., morphological differences, reproductive isolation, geographic isolation, niche divergence, etc.; de Queiroz 2007). No single method or criterion is sufficient to describe a species without artificially reducing the complexity inherent in the speciation process (Hey et al. 2003). Operating under this framework should then necessitate the use of multiple methods of analysis to provide several lines of evidence in support of a species delimitation. Today, there is a growing consensus that species limits should be investigated using varied methodologies to evaluate the hypothesis that they are independently evolving lineages (Marshall et al. 2006). However, such methods have yet to be applied toward the delimitation of many previously described species.

Slimy Salamanders (the *Plethodon glutinosus* complex) are lungless, terrestrial, direct-developing salamanders, occurring across most of eastern North America from New York to northern Florida and as far west as central Texas (Powell et al. 2016). Once thought to be a single wide-ranging species, as many as 16 species have been recognized within this group following allozyme and DNA analyses; however, this number is a subject of ongoing debate (Highton 1989; Frost & Hillis 1990; Highton 1995; Petranka 1998; Kozak et al. 2006; Wiens et al. 2006; Fisher-Reid & Wiens 2011; Highton et al. 2012; Joyce et al. 2019). As morphological differences between most species of the *P. glutinosus* complex are subtle or nonexistent (Carr 1996), genetic analysis has been the primary tool for describing and identifying species in this group (Highton 1989). However, such work is costly and time consuming. Consequently, many areas remain poorly sampled, leaving distribution limits unresolved and potentially additional cryptic species undiscovered. Complicating matters further, many Slimy Salamanders have been found to hybridize where their ranges overlap, leading to extensive introgression (Highton & Peabody 2000; Smith et al. 2018; Weins et al. 2006; Fisher-Reid & Wiens 2011). Nonetheless, phylogenetic investigations have shown that large rivers and areas of unsuitable habitat are strong barriers to gene flow (Shepard & Kuhns 2018; Smith et al. 2018).

The Louisiana Slimy Salamander (*Plethodon kisatchie*) and Mississippi Slimy Salamander (*Plethodon mississippi*) occur on opposite sides of the Mississippi River and its alluvial plain, both of which are strong geographic barriers in many organisms, including *Plethodon* (Soltis et al. 2006; Pyron & Burbrink 2010; Martin et al. 2016; Shepard & Kuhns 2018). *Plethodon kisatchie*, which occurs on the western side of the

Mississippi River from central Louisiana to southern Arkansas, is among the most understudied members of the *Plethodon glutinosus* complex. Only two distribution surveys and one morphological study comprise the research focused on this species (Boundy 2004; Warner 1971). *Plethodon mississippi*, which occurs on the eastern side of the Mississippi River from the Florida parishes of Louisiana to western Kentucky and across Mississippi to western Alabama, is better studied with recent research on range limits (Cunningham et al. 2009), phylogeography (Joyce et al. 2019), morphology and color pattern variation (Guyer et al. 2019), and landscape genetics (Burgess & Garrick 2020). Highton (1989) described the two species based on genetic distance calculated from allozyme variation, but the two are similar morphometrically (Warner 1971). Representatives of *P. kisatchie* and *P. mississippi* were included in several larger phylogenetic studies, but these studies only included samples from 1–2 populations of each species (Kozak et al. 2006, 2009; Wiens et al. 2006; Fisher-Reid & Wiens 2011; Highton et al. 2012). The two species are closely related and were even recovered as reciprocally monophyletic sister taxa in one study (Kozak et al. 2006), but intraspecific sampling has been inadequate to assess their evolutionary independence. Meacham (2023), with slightly expanded sampling of *P. mississippi* (7 populations), found that *P. kisatchie* is nested within *P. mississippi*, meaning that some *P. mississippi* are more closely related to *P. kisatchie* than they are to other *P. mississippi* (i.e., *P. mississippi* is paraphyletic). Reciprocal monophyly is not a requirement of species under the general lineage concept of species (de Queiroz 2007) and paraphyly is a common observation for several reasons (Funk & Omland 2003). For example, in the absence of gene flow, the time (number of generations) it takes for the gene lineages in two populations to sort after

the populations diverge depends on their effective population sizes and the mutation rate of the gene (Edwards & Beerli 2000). Thus, for recently diverged species, lineage sorting is expected to be incomplete, passing over time from polyphyly to paraphyly and eventually to monophyly (Funk & Omland 2003).

Incomplete lineage sorting between speciation events results in discordance among gene trees and mismatch between gene trees and the species tree (Maddison 1997; Edwards 2009). Many analytical approaches have been proposed for inferring phylogenetic relationships from multiple genes while accounting for gene tree discordance due to incomplete lineage sorting (Maddison & Knowles 2006; Rannala et al. 2020). The Multispecies Coalascent Model (Rannala & Yang 2003) is one such approach for inferring a species tree from multiple loci that can be extended to also delimit species (Yang & Rannala 2010; Fujita et al. 2012; Grummer et al. 2014). Here, I use coalescent-based species delimitation methods to test the hypothesis that *P. kisatchie* and *P. mississippi* are independently evolving and thus, valid species under the general lineage concept (de Queiroz 2007). My study will be just the second to use coalescent-based species delimitation methods on a member of the *Plethodon glutinosus* complex (see Kuchta et al. 2016 for *P. kentucki*) and the first such study to explicitly test a component of Highton's (1989) taxonomy for the group using these methods.

CHAPTER 2

METHODS

2.1 Sample Collection

I obtained samples of *P. kisatchie* and *P. mississippi* through a combination of my own field collections and tissue loans/gifts from museum collections and other researchers. Due to a paucity of available tissue samples in museum collections, I focused my field sampling efforts on *P. kisatchie*. I compiled previously reported localities for *P. kisatchie* from museum collections (VertNet), the published literature (Warner 1971; Highton 1989), unpublished technical reports and theses (Warner 1969; Boundy 2004, 2007), citizen science databases (iNaturalist, HerpMapper), state agency databases (AGFC, LDWF, ANHC), the 2019 Arkansas Herp Atlas (Roberts 2020), and professional herpetologists. I surveyed historic localities as well as previously unsampled sites within the species' range that have suitable habitat (upland mixed hardwood forest; Boundy 2004, 2007) as determined by topographical maps and satellite images (Google Maps;2 Fig. 2-1). I searched for salamanders primarily between December and May 2021–2023, which are the months when *P. kisatchie* is reported to be most active above ground (Boundy 2007). I located *P. kisatchie* by clearing leaf litter and flipping cover objects, such as logs, rocks, and other debris. I captured individuals by hand and collected tissue samples either by clipping the tail tip (which salamanders can regrow) or extracting the liver following euthanasia (which yields the most genetic material). Euthanized

specimens were individually tagged and preserved in 10% formalin and all tissue samples were placed in individually labeled vials of >95% ethanol for DNA extraction in the lab. I recorded GPS coordinates (latitude, longitude) and weather conditions (humidity, temperature, and wind speed) at each locality.

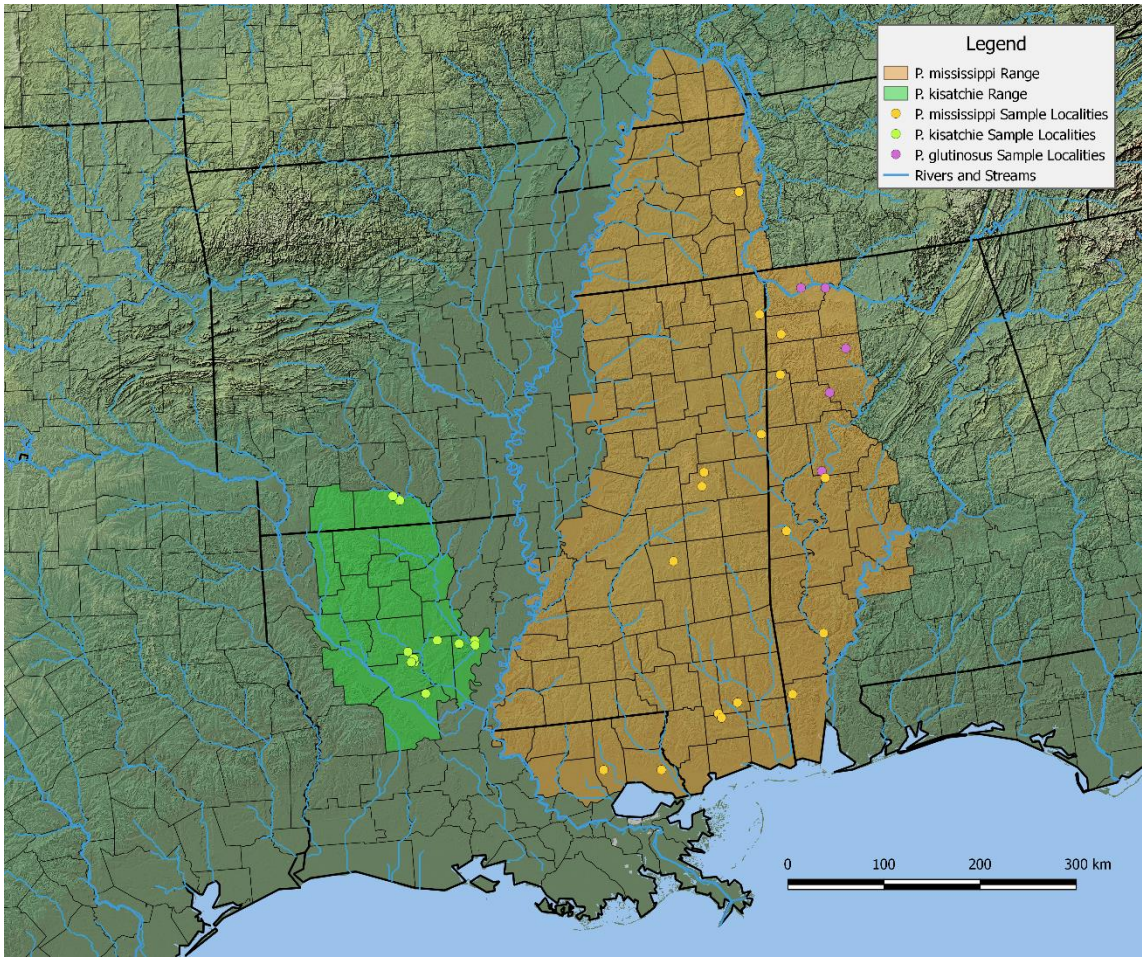

Figure 2-1: Map of sample locations for *P. kisatchie* (green) and *P. mississippi* (orange) with species distributions shown at county-scale based on Highton 1989, Powell et al. 2016, and Meacham 2023. Also shown are samples (magenta) that did not group with *P. mississippi* in phylogenetic analysis of the mitochondrial *ND2* gene and are likely *P. glutinosus*.

Tissue samples of *P. mississippi* were provided by the Auburn University Museum of Natural History as well as the Shepard Lab amphibian and reptile tissue

collection. To supplement sequence data collected from tissue samples, I also included DNA sequences of *P. kisatchie, P. mississippi*, and other closely related species of *Plethodon* generated previously by the Kozak Lab at the University of Minnesota or available on GenBank. The last included eight species of Slimy Salamanders (*P. albagula, P. aureolus, P. glutinosus, P. grobmani, P. kentucki, P. kiamichi, P. ocmulgee, P. savannah*) and *P. yonahlossee*, which served as the outgroup (Kozak et al. 2006, 2009). Locality information for all samples (tissues and sequences) is provided in the Appendix.

2.2 DNA Extraction and Sequencing

I extracted genomic DNA from tissue samples using the Qiagen DNEasy Animal Blood & Tissue Kit (Valencia, California). I used PCR to amplify the mitochondrial *ND2* gene, a portion of the nuclear *SLC8A3* gene, and two anonymous nuclear loci, Pglut16 and Pglut54. Locus and primer information is provided in Table 2-1. I chose these markers because they have been sequenced previously for all members of the *Plethodon glutinosus* complex and show moderate to high levels of sequence variation. I sent PCR products to Eurofins Genomics (Louisville, Kentucky) for purification and sequencing. To prepare sequence data for analysis, I visually examined and manually edited chromatograms in Geneious v.10 (Kearse et al. 2012). Length heterozygotes for Pglut16 and Pglut54 were resolved using Indelligent (Dmitriev & Rakitov 2008). I aligned sequences for each locus using the MUSCLE algorithm in Geneious (Kearse et al. 2012). Nuclear loci were phased to alleles using the program PHASE v.2.2.1 (Stephens et al. 2004).

Table 2-1: Information for loci sequenced in this study.

Locus	Location/ Type	Length (bp)	Primer Name	Primer Sequence (5'-3')	Source
ND2	mitochondrial/ protein-coding	1,041	Metf.6 Asnr.3	AAGCTTTC GGGCCCA TACC GCGTTTAG CTGTTAAC TAAA	Macey et al. 1997 Weisrock et al. 2001
SLC8A3	nuclear/ protein-coding	763	SLC8A3F SCL8A3R	AGCTTTCA ACATGTTC ATCATTCT ACCATCCC CTCTGTAA ACTCATAG	Roelants et al. 2007
Pglut16	nuclear/ anonymous non-coding	526	Pglut16F Pglut16R	GCAATAG AGCAGCC AGATAAA G TCAATAGC ACACTTGC AAAGAC	This study
Pglut54	nuclear/ anonymous non-coding	486	Pglut54F Pglut54R	AACATTGC AAACCAC TCTACTG AGCACGC TCTGTGAT ATTACTC	This study

2.3 Mitochondrial Phylogeography

I initially inferred phylogenetic relationships of *P. kisatchie*, *P. mississippi*, and other *P. glutinosus* complex species through analysis of the mitochondrial *ND2* gene.

Using the Bayesian information criterion in PartitionFinder v.2.1 (Lanfear et al. 2012), I determined the optimal partitioning strategy and substitution models were HKY + Γ for first codon positions, TN93 + Γ for second positions, and TN93 + Γ for third positions. I then inferred a phylogeny under the best scheme in the program BEAST v.1.8 (Drummond & Rambaut 2007; Bouckaert et al. 2019). I set a lognormally distributed age prior on the root node with mean=10.3 million years and SD=0.14 based on fossil-calibrated divergence time estimates of the most recent common ancestor (MRCA) of *P. yonahlossee* and the *P. glutinosus* complex (Thesing et al. 2016; Shepard & Kuhns 2018). I ran the analysis under a strict clock for 20 million generations, sampling every 2000th. I assigned a 10% burn-in and ensured ESS values were all >200 using Tracer v.1.6 (Rambaut & Drummond 2009). I generated a maximum clade credibility tree with median node ages using TreeAnnotator v.1.8 (Drummond & Rambaut 2007) and visualized the tree in FigTree v.1.4.3 (Rambaut 2016). I considered a posterior probability (PP) ≥0.95 as strong support for a node.

The distribution of *P. kisatchie* was resolved by Meacham (2023) whereas the eastern range limits of *P. mississippi* are unclear (Cunningham et al. 2009; Joyce et al. 2019). I used the *ND2* tree to identify samples labeled as *P. mississippi* that do not group with *P. mississippi*. Because these individuals would be species or potentially hybrids with other species outside my two taxa of interest, I excluded them from further analyses.

2.4 Species Delimitation

I used two coalescent-based species delimitation methods (Fujita et al. 2012) to test species hypotheses in *P. kisatchie* and *P. mississippi*. First, I used the Bayes Factor Delimitation (BFD) approach of Grummer et al. (2014) to evaluate two competing

hypotheses, one that considers *P. kisatchie* and *P. mississippi* to be two species and one that considers them to be a single species. For each hypothesis, I ran a species tree analysis under the Multispecies Coalescent Model (*BEAST) using an eight-species, four-locus dataset in BEAST v.1.8 (Heled & Drummond 2010). Using the Bayesian information criterion in PartitionFinder v.2.1 (Lanfear et al. 2012), I determined the optimal partitioning strategy and substitution models were: HKY + Γ for *ND2* first positions, TN93 + I for *ND2* second positions, TN93 for *ND2* third positions, HKY for *SLC8A3* first positions, HKY for *SLC8A3* second and third positions, K80 for Pglut16, and TN93 for Pglut54. Analyses were run under a strict clock with rates of nuclear loci scaled relative to *ND2* for 50 million generations, sampling every 5000th. Following each analysis, I calculated marginal likelihood estimates (MLE) by performing Path sampling and Stepping-stone sampling using 100 steps of 500,000 generations (Baele et al. 2012a, 2012b). I compared hypotheses by calculating the Bayes factor (2*ln*Bf), which is the difference in the MLEs of two competing models multiplied by two. A 2*ln*Bf = 0–2 means "not worth more than a bare mention", 2*ln*Bf = 2–6 means "positive" support, 2*ln*Bf = 6–10 provides "strong" support, and 2*ln*Bf > 10 means "decisive" support for choosing between two competing hypotheses (Kass & Raftery 1995). Support for *P. kisatchie* and *P. mississippi* being evolutionarily distinct would consist of the two-species model having a higher MLE and a 2*ln*Bf > 10 in comparison with the one species model.

Using the posterior set of trees from the analysis conducted under the preferred species hypothesis, I used TreeAnnotator v.1.8 (Drummond & Rambaut 2007) to construct a species tree and gene trees for each nuclear locus. For each, I calculated a maximum clade credibility tree with median node ages and visualized the tree in FigTree

v.1.4.3 (Rambaut 2016). I considered a posterior probability (PP) ≥0.95 as strong support for a node.

Secondly, I used BPP v.4.6.2 (Yang & Rannala 2010, 2014; Rannala & Yang 2013; Flouri et al. 2018) to delimit species and evaluate species hypotheses. This program uses the Multispecies Coalescent Model to compare species delimitation hypotheses using either a fixed guide tree or while simultaneously inferring a species tree. For all analyses, I used the phased four-locus dataset with heredity scalars of 1 for nuclear loci and 0.25 for the mitochondrial locus, and relative locus rates based on the median rates inferred in my *BEAST analysis. First, I ran a species delimitation analysis (A10) using the species tree from *BEAST as a guide tree. Species delimitations in BPP are sensitive to prior settings for θ (ancestral population size) and τ (root age of the species tree). I therefore tested species delimitation hypotheses across a wide range of prior scenarios including all combinations of small, medium, and large θ and young, moderate, and old τ (Table 3-1). I used algorithm 0 with a fine-tune parameter (ε) of 10. After a burn-in of 5000 generations, samples were collected every other generation until 50,000 samples were obtained. As recommended, I ran all analyses multiple times to ensure results were consistent. Next, I ran joint species delimitation and species tree analyses (A11) across a wide range of θ and τ prior scenarios (Table 3-2). Then, I trimmed my dataset to just *P. kisatchie* and *P. mississippi* (no outgroups) and ran delimitation analyses (A10) across a wide range of θ and τ prior scenarios, including under empirical estimates of these parameters (see below; Table 3-3). In all three sets of BPP analyses, I focused on the posterior probabilities that *P. kisatchie* and *P. mississippi* are two versus one species.

Lastly, I calculated the genealogical divergence index (*gdi*) of Jackson et al. (2017). The *gdi* quantifies the overall genetic divergence between two populations while considering their divergence time and population sizes (Jackson et al. 2017). For this analysis, I estimated θ and τ in BPP (A00) using the four-locus *P. kisatchie* and *P. mississippi* dataset (no outgroups). After several exploratory analyses, I used diffuse inverse-gamma priors for θ (3, 0.004) and τ (3, 0.0004). After a burn-in of 10,000 generations, samples were collected every 5th generation until 100,000 samples were obtained. Analyses were run four times and compared in Tracer v.1.6 (Rambaut & Drummond 2009) to ensure convergence and adequate ESS values. Results from the four analyses were combined and the mean values of θ and τ were calculated. The *gdi* is calculated as: $gdi = 1 - e^{-2\tau/\theta}$ (Jackson et al. 2017). Candidate species A is distinguished from candidate species B using $-2\tau_{AB}/\theta_A$ whereas candidate species B is distinguished from candidate species A using $-2\tau_{AB}/\theta_B$ (Jackson et al. 2017; Leache et al. 2018). Species are considered distinct when $gdi > 0.7$ whereas $gdi < 0.2$ indicates a single species. Values between 0.2–0.7 indicate ambiguous species status (Jackson et al. 2017).

CHAPTER 3

RESULTS

3.1 Sequence Data

My sequence dataset consisted of: 1) 1041 aligned base-pairs of *ND2* for 55 individuals (16 *P. kisatchie* from 11 localities, 24 putative *P. mississippi* from 22 localities, 14 individuals of eight *P. glutinosus* complex species, and one *P. yonahlossee*), 2) 763 aligned base-pairs of *SLC8A3* for 39 individuals (16 *P. kisatchie* from 11 localities, 17 *P. mississippi* from 15 localities, and 6 other *P. glutinosus* complex species), 3) 526 aligned base-pairs of Pglut16 for 38 individuals (16 *P. kisatchie* from 11 localities, 16 *P. mississippi* from 14 localities, and 6 other *P. glutinosus* complex species), and 4) 486 aligned base-pairs of Pglut54 for 37 individuals (16 *P. kisatchie* from 11 localities, 15 *P. mississippi* from 14 localities, and 6 other *P. glutinosus* complex species).

3.2 Mitochondrial Phylogeography

The *ND2* phylogeny shows that all samples identified as *P. kisatchie* formed a well-supported clade (PP=1.0) nested within *P. mississippi* (Fig. 3-1). Divergence of *P. kisatchie* from *P. mississippi* was estimated to be 520,000 years ago (95% HPD: 310,000–780,000; Fig. 3-1). Several samples putatively identified as *P. mississippi* grouped with other species, namely *P. glutinosus* and *P. kiamichi* (Fig. 3-1). These five samples all originated in Alabama on the eastern edge of the range of *P. mississippi* near the contact with *P. glutinosus* (Fig. 2-1). Because these individuals may be species other than *P. mississippi* or hybrids, I excluded them from further analyses. The remaining samples of *P. mississippi* formed a clade (PP=0.94) composed of a divergent sample from southwestern Alabama (Clarke County) and a clade comprising the other samples of *P. mississippi* and *P. kisatchie* (PP=1.0; Fig. 3-1).

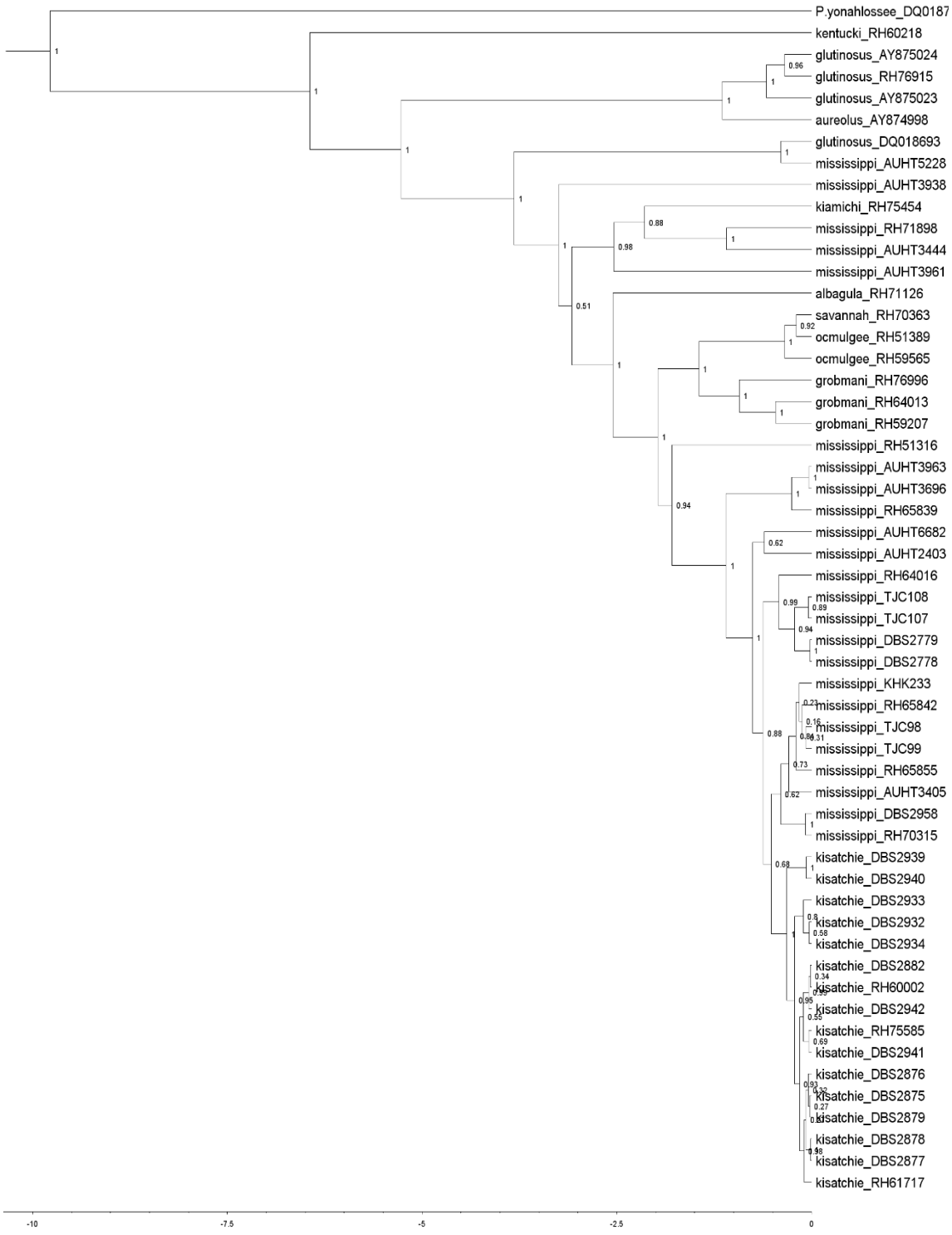

Figure 3-1: *ND2* chronogram for 55 samples of 11 species of Slimy Salamanders (*Plethodon glutinosus* complex) inferred by BEAST. Values on nodes are posterior probabilities. The X axis is Millions of Years Ago.

3.3 Species Delimitation

Bayes Factor Delimitation strongly supported the hypothesis that *P. kisatchie* and *P. mississippi* are distinct species. The MLE for the two-species model based on Path sampling was -6684.6 compared to -6786.7 for the one species model. The MLE for the two-species model based on Stepping-stone sampling was -6686.7 compared to -6788.8 for the one species model. Both sampling methods yield Bayes factors (2*ln*Bf) of 204.2, which is decisive support for the two-species model. The species tree from the two-species analysis shows relationships among the eight species analyzed (Fig. 3-2). Support values are generally low (0.53–0.63), but it shows *P. kisatchie* and *P. mississippi* are sister taxa and together form a clade with *P. glutinosus* (Fig. 3-2). That clade is sister to a clade comprising *P. grobmani* and *P. ocmulgee* and then the western species, *P. kiamichi* and *P. albagula*, form a clade that is sister to all other species excluding *P. kentucki* (Fig. 3-2). Gene trees for each nuclear locus are shown in Figs. 3-3, 3-4, and 3-5.

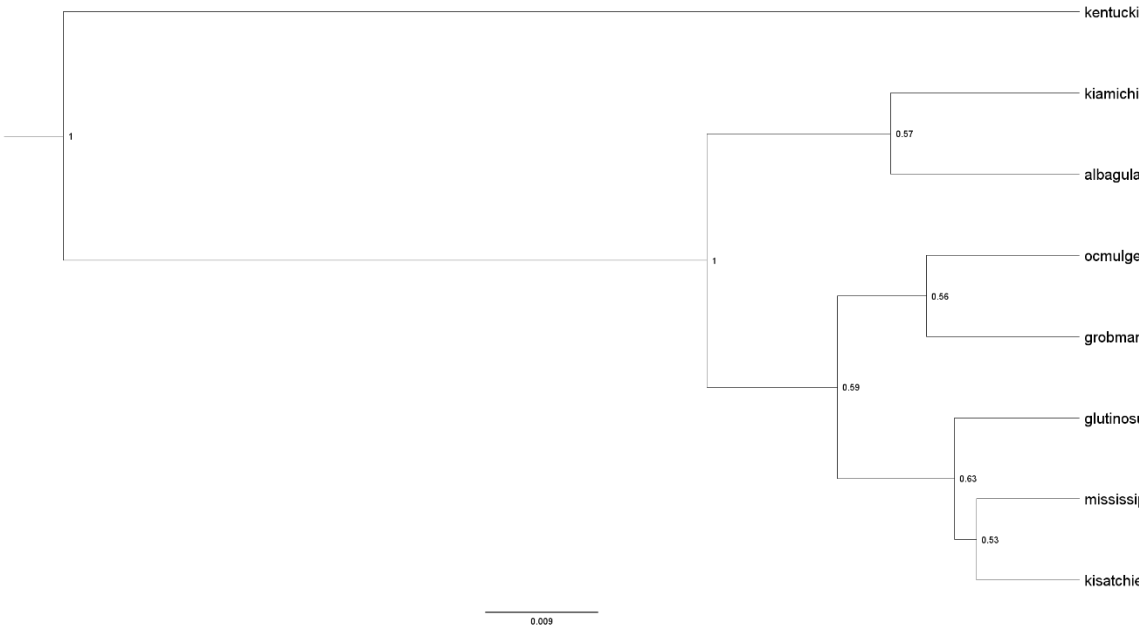

Figure 3-2: *BEAST species tree showing relationships among eight species of Slimy Salamanders (*Plethodon glutinosus* complex) inferred using four loci. Values on nodes are posterior probabilities.

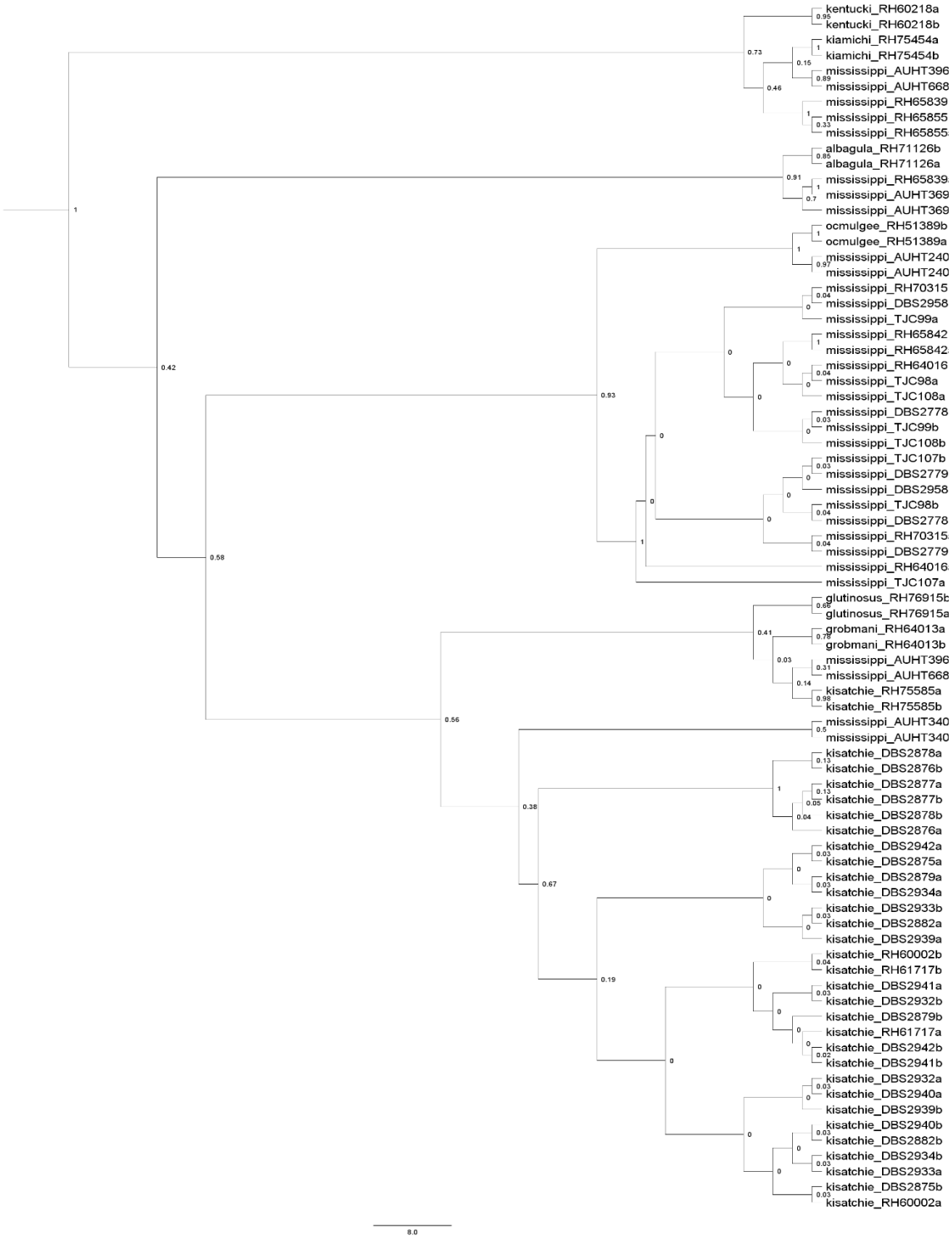

Figure 3-3: *SLC8A3* gene tree for 39 samples of eight species of Slimy Salamanders (*Plethodon glutinosus* complex) inferred by *BEAST. Values on nodes are posterior probabilities.

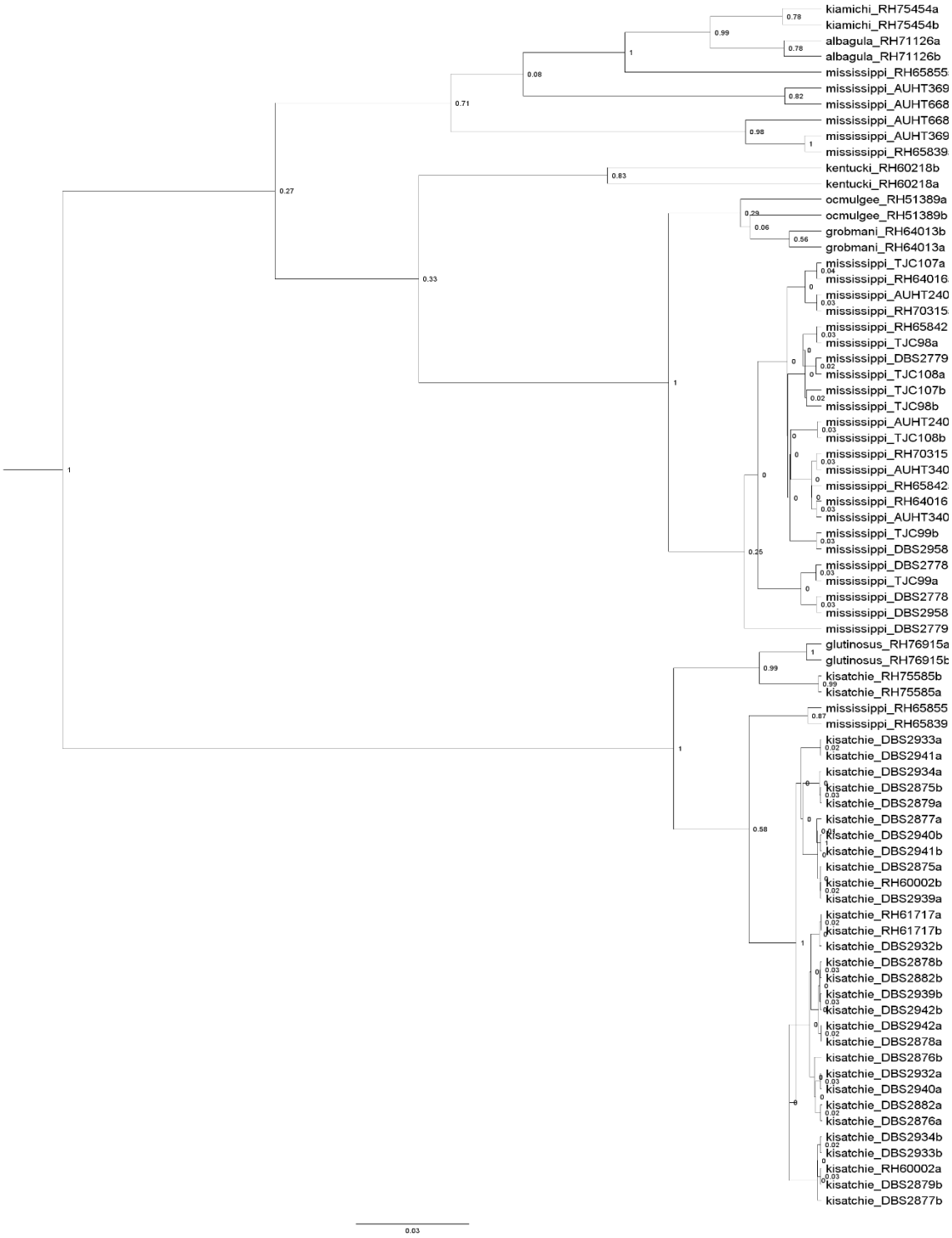

Figure 3-4: Pglut16 gene tree for 38 samples of eight species of Slimy Salamanders (*Plethodon glutinosus* complex) inferred by *BEAST. Values on nodes are posterior probabilities.

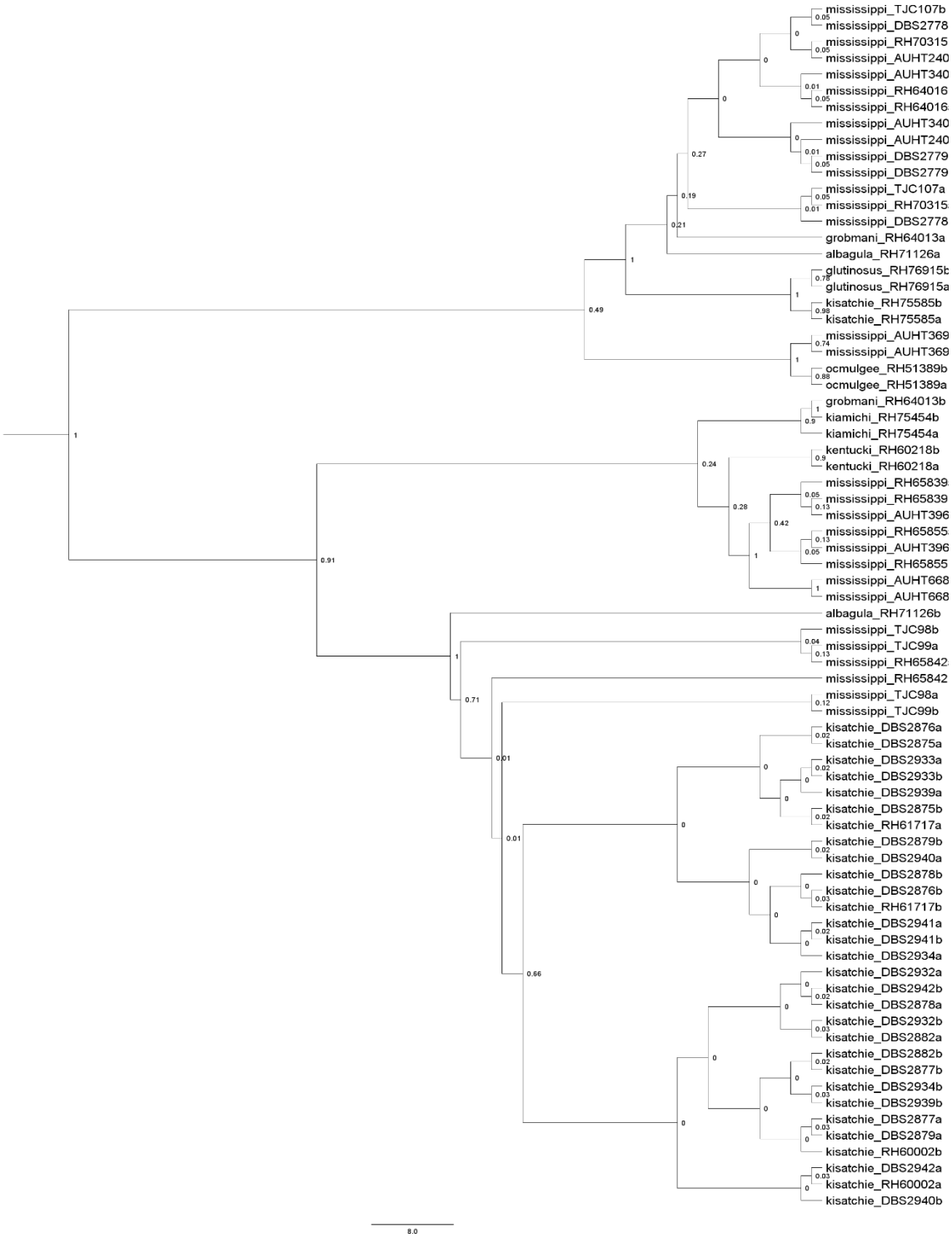

Figure 3-5: Pglut54 gene tree for 37 samples of eight species of Slimy Salamanders (*Plethodon glutinosus* complex) inferred by *BEAST. Values on nodes are posterior probabilities.

Species delimitation in BPP using the *BEAST species tree as a guide tree supported *P. kisatchie* and *P. mississippi* as distinct species across a broad range of θ and τ values (Table 3-1). Joint (unguided) species tree and species delimitation also supported *P. kisatchie* and *P. mississippi* as distinct species under small and medium values of θ, but favored collapsing them into a single species under large values of θ (Table 3-2). Species delimitation using the *P. kisatchie* and *P. mississippi* dataset (no outgroups) also consistently supported two species across a wide range of θ and τ values, including under empirical estimates of these parameters (see below; Table 3-3).

Posterior estimates of τ for the root age of the MRCA of *P. kisatchie* and *P. mississippi* averaged 0.000196. Estimates of θ averaged 0.00201 for the ancestor of *P. kisatchie* and *P. mississippi*, 0.00199 for *P. kisatchie*, and 0.00207 for *P. mississippi*. Values of *gdi* were 0.180 for *P. kisatchie* from *P. mississippi* and 0.173 for *P. mississippi* from *P. kisatchie*, supporting the hypothesis they are a single species.

Table 3-1: Results of BPP using the *BEAST species tree (A10) for different values of θ and τ. The posterior probability (PP) that *P. kisatchie* and *P. mississippi* are distinct species is shown in the last column.

θ (α, β)	τ (α, β)	General expectation of prior distribution	Relative level of gene tree discordance	PP of two species
2, 0.001	2, 0.1	Small pop. size, deep divergence	lowest	1.00
2, 0.001	2, 0.01	Small pop. size, moderate divergence	low	1.00
2, 0.001	2, 0.001	Small pop. size, shallow divergence	low-moderate	1.00
2, 0.01	2, 0.1	Medium pop. size, deep divergence	low-moderate	1.00
2, 0.01	2, 0.01	Medium pop. size, moderate divergence	moderate	1.00
2, 0.01	2, 0.001	Medium pop. size, shallow divergence	moderate-high	1.00
2, 0.1	2, 0.1	Large pop. size, deep divergence	moderate-high	1.00
2, 0.1	2, 0.01	Large pop. size, moderate divergence	high	1.00
2, 0.1	2, 0.001	Large pop. size, shallow divergence	highest	1.00

Table 3-2: Results of BPP using no guide tree (A11) for different values of θ and τ. The posterior probability (PP) that *P. kisatchie* and *P. mississippi* are distinct species is shown in the last column.

θ (α, β)	τ (α, β)	General expectation of prior distribution	Relative level of gene tree discordance	PP of two species
2, 0.001	2, 0.1	Small pop. size, deep divergence	lowest	1.00
2, 0.001	2, 0.01	Small pop. size, moderate divergence	low	1.00
2, 0.001	2, 0.001	Small pop. size, shallow divergence	low-moderate	1.00
2, 0.01	2, 0.1	Medium pop. size, deep divergence	low-moderate	0.999600
2, 0.01	2, 0.01	Medium pop. size, moderate divergence	moderate	1.00
2, 0.01	2, 0.001	Medium pop. size, shallow divergence	moderate-high	0.999940
2, 0.1	2, 0.1	Large pop. size, deep divergence	moderate-high	0.479220
2, 0.1	2, 0.01	Large pop. size, moderate divergence	high	0.367060
2, 0.1	2, 0.001	Large pop. size, shallow divergence	highest	0.293520

Table 3-3: Results of BPP for different values of θ and τ using only samples of *P. kisatchie* and *P. mississippi* (A10). The posterior probability (PP) that *P. kisatchie* and *P. mississippi* are distinct species is shown in the last column.

θ (α, β)	**τ (α, β)**	**General expectation of prior distribution**	**Relative level of gene tree discordance**	**PP of two species**
2, 0.001	2, 0.1	Small pop. size, deep divergence	lowest	0.968340
2, 0.001	2, 0.01	Small pop. size, moderate divergence	low	1.00
2, 0.001	2, 0.001	Small pop. size, shallow divergence	low-moderate	1.00
2, 0.01	2, 0.1	Medium pop. size, deep divergence	low-moderate	1.00
2, 0.01	2, 0.01	Medium pop. size, moderate divergence	moderate	1.00
2, 0.01	2, 0.001	Medium pop. size, shallow divergence	moderate-high	1.00
2, 0.1	2, 0.1	Large pop. size, deep divergence	moderate-high	1.00
2, 0.1	2, 0.01	Large pop. size, moderate divergence	high	1.00
2, 0.1	2, 0.001	Large pop. size, shallow divergence	highest	1.00
2, 0.002	2, 0.0002	Empirical	Empirical	1.00

CHAPTER 4

DISCUSSION

Despite the controversy surrounding Highton's (1989) delimitation of species in the *Plethodon glutinosus* complex (Frost & Hillis 1990), his taxonomy has seldom been tested. I found strong but not unanimous support for the hypothesis that *P. kisatchie* and *P. mississippi* are independently evolving lineages. Speciation is a complex process and conflict among methods or criteria is common, especially in recently diverged species (Hey et al. 2003; de Queiroz 2007). Based on the results presented here, there is sufficient evidence to continue recognizing *P. kisatchie* and *P. mississippi* as distinct species.

4.1 Mitochondrial Phylogeography

All samples of *P. kisatchie* formed a monophyletic group in the *ND2* gene tree, but this clade was nested within *P. mississippi*, rendering *P. mississippi* paraphyletic. The distribution of *P. kisatchie* in central Louisiana and southern Arkansas is seemingly isolated from other *P. glutinosus* complex species (Meacham 2023), whereas the distribution of *P. mississippi* appears to overlap with the distributions of the closely related *P. glutinosus* and *P. grobmani* on the eastern edge of the species' range (Highton 1989; Powell et al. 2016). Five samples from the eastern edge of the range of *P. mississippi* in Alabama grouped with species other than *P. mississippi* and *P. kisatchie*. Joyce et al. (2019) found that populations from within each of the currently delineated ranges of *P. mississippi*, *P. grobmani*, and *P. glutinosus* in Alabama do not form

reciprocally monophyletic groups. The five samples I identified as not *P. mississippi* are likely *P. glutinosus,* although *P. glutinosus* is polyphyletic (Kozak et al. 2006; Wiens et al. 2006; Highton et al. 2012). Previous phylogenetic studies of *Plethodon* had limited intraspecific sampling with just 1–2 samples of *P. mississippi* and 4–5 samples of *P. glutinosus* (Kozak et al. 2006; Wiens et al. 2006; Highton et al. 2012). Given my results and those of Joyce et al. (2019), it is possible some *P. mississippi* included in previous phylogenetic studies were misidentified, which would explain why *P. mississippi* was sometimes inferred to be the sister taxon of *P. kiamichi* (Wiens et al. 2006; Martin et al. 2016) rather than *P. kisatchie* (Kozak et al. 2006; Meacham 2023; this study).

Comprehensive population sampling is paramount for determining species-level diversity and distributions in Slimy Salamanders (Highton 1989; Meacham 2023). My sampling revealed new information about the relationship between *P. kisatchie* and *P. mississippi*, which had been represented in previous phylogenetic studies by only 1–2 populations of each species (Kozak et al. 2006, 2009; Wiens et al. 2006; Fisher-Reid & Wiens 2011; Highton et al. 2012). Depending on which 1–2 samples are chosen to represent *P. kisatchie* and *P. mississippi*, one could find the species are reciprocally monophyletic or that *P. mississippi* is paraphyletic, and divergence time estimates could be up to ~3.44x older than what I found. My sampling also provided new insight into genetic diversity within each species. Unexpectedly, I found little genetic diversity within *P. kisatchie*, which is likely due to the recent (~520,000 years ago) divergence from *P. mississippi*. In contrast, diversity within *P. mississippi* traces back to a common ancestor ~1.79 million years ago. Genetic diversity is thought to be related to a species' ability to adapt to changing environments (Barrett & Schluter 2007; Markert et al. 2010; Rousselle

et al. 2020) so the low diversity within *P. kisatchie* may be a concern for the species' long-term conservation.

4.2 Species Delimitation

Bayes Factor Delimitation strongly supported the hypothesis that *P. kisatchie* and *P. mississippi* are distinct species. The species tree generated using *BEAST inferred *P. kisatchie* and *P. mississippi* are sister taxa with *P. glutinosus* sister to that clade. The species tree also inferred sister taxa relationships between *P. albagula* and *P. kiamichi* as well as *P. ocmulgee* and *P. grobmani*. Support values were low (PP=0.53–0.63) for all relationships, which is expected given the recent divergences and small number of loci. Although I did not include all of the same taxa, my species tree topology is similar to that of the mitochondrial gene trees inferred by Kozak et al. (2006) and Meacham (2023) except for the placement of *P. glutinosus*. My species tree is also nearly identical in topology to the nuclear DNA only concatenated tree of Fisher-Reid & Wiens (2011). In contrast, Wiens et al. (2006) recovered *P. kiamichi* as sister to *P. mississippi* and *P. grobmani* as sister to *P. kisatchie* whereas Highton et al. (2012) placed *P. grobmani* as sister to *P. kisatchie* with *P. savannah* and *P. ocmulgee* forming a clade that is sister to *P. mississippi*.

Under most scenarios, BPP supported that *P. kisatchie* and *P. mississippi* are distinct species. Posterior probabilities for their independence were consistently high across a broad range of θ and τ values when using a guide tree (A10) with the eight-species dataset and when analyzing *P. kisatchie* and *P. mississippi* only. The two species were collapsed into a single species only under large values of θ in the unguided BPP analyses (A11) of the eight-species dataset. Sukumaran & Knowles (2017) found BPP

may oversplit taxa because it detects population structure rather than species divergences. Inconsistency in support for a species delimitation across a range of θ and τ values may indicate that population structure is being detected rather than species. However, Leache et al. (2019) pointed out the simulations performed by Sukumaran & Knowles (2017) were unrealistic of the speciation process. Jackson et al. (2017) similarly argued that while BPP is a reliable method of detecting genetic isolation, it may oversplit taxa even where gene flow is present and developed the *gdi* as an empirical measure to aid in coalescent-based species delimitation. In my study, *gdi* estimates between *P. kisatchie* and *P. mississippi* were small (<0.2), supporting the hypothesis they are a single species. The *gdi* attempts to estimate the overall divergence between two taxa by considering genetic isolation and gene flow (Jackson et al. 2017). It tends to be more conservative than BPP, but the parameter space within which it is useful remains to be determined (Jackson et al. 2017; Leache et al. 2019). Empirical values of θ for *P. kisatchie* and *P. mississippi* were small, but the value for τ was especially small due to the recent divergence of these species. It is possible that *gdi* is not useful under such circumstances (e.g., when $2\tau/\theta < 1$) and more work needs to be done to determine when it is an appropriate metric to use (Leache et al. 2019).

Plethodon kisatchie and *P. mississippi* are allopatric with a strong geographic barrier (the Mississippi River) between them. Their divergence is relatively recent, but a high degree of lineage sorting is already evident in the gene trees as all but one individual of *P. kisatchie* consistently grouped into a single clade. In contrast, individuals of *P. mississippi* grouped into multiple clades in the nuclear loci gene trees. A complete lack of gene flow and smaller population size of *P. kisatchie*, such as may occur via founder-

effect speciation (Templeton 2008; Matzke 2014), could explain this observation. Martin et al. (2016) found east-to-west dispersals across the Mississippi River occurred multiple times in plethodontid salamanders, including twice in Slimy Salamanders. Although I omitted samples of *P. mississippi* that placed outside the *P. mississippi* + *P. kisatchie* clade in my *ND2* tree, it is possible that some *P. mississippi* included in my nuclear dataset were hybrids between *P. mississippi* and *P. glutinosus*. If that was the case, then some alleles of *P. glutinosus* may have been incorrectly assigned to *P. mississippi*. Correct taxon assignment and no gene flow are assumptions of the Multispecies Coalascent Model implemented in *BEAST and BPP. Inclusion of hybrid individuals and incorrect taxon assignment would act to increase estimates of θ and τ. Given the decisiveness of BFD results, the consistency of BPP results across most analyses, and my small empirical estimates of θ and τ, I do not think these potential issues had an effect on species delimitation. However, the close relationship of *P. glutinosus* to *P. kisatchie* and *P. mississippi* inferred in my species tree may be a consequence.

4.3 Conclusions

Based on the results of this study, I recommend both *P. kisatchie* and *P. mississippi* continue to be recognized. Although *P. kisatchie* is recently diverged from *P. mississippi*, the two are geographically isolated by a strong physical barrier (the Mississippi River) and appear to be evolving independently (de Queiroz 2007). However, further investigation of *P. mississippi* is warranted. In finding polyphyly of *P. mississippi*, *P. grobmani*, and *P. glutinosus* in Alabama, Joyce et al. (2019) argued for a taxonomy that consolidates the *P. glutinosus* complex into three species: *P. aureolus*, *P. kentucki*, and a wide-ranging *P. glutinosus*. Although *P. mississippi* may be hybridizing to some

extent with other *P. glutinosus* complex species, I argue that such a broad change in taxonomy is premature and would likely result in some independent lineages not being recognized. A phylogenomic investigation of all *P. glutinosus* complex species with comprehensive sampling across their respective distributions and contact zones is necessary to support such an extensive consolidation of this complex. Because several Slimy Salamander species are of conservation concern, resolving species limits and stabilizing the taxonomy of the group should be prioritized.

SPECIES DELIMITATION OF SLIMY SALAMANDERS, *PLETHODON MISSISSIOOI* AND *PLETHODON KISATCHIE*, ACROSS THE LOWER MISSISSIPPI RIVER:

APPENDIX A

Table A-1: Sample and locality information for individuals used in this study.

Sample ID	Species	Locality	Lat., Long.	ND2	SLC 8A3	Pglut 16	Pglut 54
RH71126	*Plethodon albagula*	Tip Top Roadside Park, Iron Co., Missouri	37.56028, -90.67111	X	X	X	X
AY874998	*Plethodon aureolus*	Farr Gap, Unicoi Mtns, Monroe Co., Tennessee	35.46250, -84.02694	X			
AY875023	*Plethodon glutinosus*	I.C. King Park, Knox Co., Tennessee	35.89167, -83.95250	X			
AY875024	*Plethodon glutinosus*	Terra Alta Lake, along road on north shore of lake, Preston Co.,	39.45667, -79.51889	X			

		West Virginia					
DQ018693	*Plethodon glutinosus*	Henderson Creek at Jct. Henderson Creek/Graham Rd, Sequatchie Co., Tennessee	35.36750, -85.34033	X			
RH76915	*Plethodon glutinosus*	Turkey Run SP, Parke Co., Indiana	39.88722, -87.18889	X	X	X	X
RH59207	*Plethodon grobmani*	Lakeland, Lanier Co., Georgia	31.04250, -83.09833	X			
RH64013	*Plethodon grobmani*	Half-mile Creek Swamp, 0.5 miles NE of Silver Springs,	29.21740, -82.04380	X	X	X	X

		Marion Co., Florida					
RH76996	*Plethodon grobmani*	Marianna, Jackson Co., Florida	30.82361, -85.30417	X			
RH60218	*Plethodon kentucki*	Breaks Interstate Park, Dickenson Co., Virginia	37.29370, -82.30310	X	X	X	X
RH75454	*Plethodon kiamichi*	Round Mtn, LeFlore Co., Oklahoma	34.61528, -94.49722	X	X	X	X
DBS2875	*Plethodon kisatchie*	Just N of Indian Creek on W side of FR168/Edwards Rd, Grant Par., Louisiana	31.71743, -92.43559	X	X	X	X
DBS2876	*Plethodon kisatchie*	N of Zion on Hwy 472, W side of	31.82128, -92.49548	X	X	X	X

		Pardon Hill Rd just N of PR648/Little Mail Rt Rd, Winn Par., Louisiana					
DBS2877	*Plethodon kisatchie*	Bear Creek, ~4.9 km W of Hwy 165 in Georgetown on Hwy 500, SE of intersection of Bear Creek Rd and Barrett H Corley Rd, Grant Par., Louisiana	31.75187, -92.43807	X	X	X	X
DBS2878	*Plethodon kisatchie*	Bear Creek, ~4.9 km W	31.75187, -92.43807	X	X	X	X

		of Hwy 165 in Georgetown on Hwy 500, SE of intersection of Bear Creek Rd and Barrett H Corley Rd, Grant Par., Louisiana					
DBS2879	*Plethodon kisatchie*	Indian Creek on FR131, just S of creek, Grant Par., Louisiana	31.71722, -92.46558	X	X	X	X
DBS2882	*Plethodon kisatchie*	Cypress Creek Field Rd, Union	33.25381, -92.45592	X	X	X	X

		Co., Arkansas					
DBS2932	*Plethodon kisatchie*	N of Green Gables, Millcreek Rd (FS 132) at Beaver Creek on W side of creek, Rapides Par., Louisiana	31.41207, -92.33634	X	X	X	X
DBS2933	*Plethodon kisatchie*	N of Green Gables, Millcreek Rd (FS 132) at Beaver Creek on W side of creek, Rapides	31.41207, -92.33634	X	X	X	X

		Par., Louisiana					
DBS2934	*Plethodon kisatchie*	N of Green Gables, Millcreek Rd (FS 132) at Beaver Creek on W side of creek, Rapides Par., Louisiana	31.41207, -92.33634	X	X	X	X
DBS2939	*Plethodon kisatchie*	St. Mary Falls, Sicily Island WMA, Catahoula Par., Louisiana	31.86760, -91.75318	X	X	X	X
DBS2940	*Plethodon kisatchie*	Rock Falls, Sicily Island WMA,	31.82550, -91.75307	X	X	X	X

		Catahoula Par., Louisiana					
DBS2941	*Plethodon kisatchie*	Hooter Creek/Haggerty Creek on Catahoula Church Rd, Catahoula Par., Louisiana	31.85369, -91.92647	X	X	X	X
DBS2942	*Plethodon kisatchie*	Cypress Creek Field Rd, Union Co., Arkansas	33.25381, -92.45592	X	X	X	X
RH60002	*Plethodon kisatchie*	New Calion, site 88, Union Co., Arkansas	33.29944, -92.52889	X	X	X	X
RH61717	*Plethodon kisatchie*	Indian Creek, site	31.72083, -92.46722	X	X	X	X

		90 (Type locality), Grant Par., Louisiana					
RH75585	*Plethodon kisatchie*	4 mi E Olla, La Salle Par., Louisiana	31.90470, -92.16250	X	X	X	X
AUHT2403	*Plethodon mississippi*	Hwy 217, at Puppy Creek Crossing, Mobile Co., Alabama	31.01766, -88.34782	X	X	X	X
AUHT3405	*Plethodon mississippi*	Sumter County Recreation Association Park, Sumter Co., Alabama	32.55340, -88.19995	X	X	X	X
AUHT3444	*Plethodon mississippi*	Wildwood Park, Lauderdale	34.80308, -87.69204	X			

		Co., Alabama					
AUHT3696	*Plethodon mississippi*	Piney Point Park, Franklin Co., Alabama	34.39596, -87.98480	X	X	X	X
AUHT3938	*Plethodon mississippi*	Bankhead National Forest, Winston Co., Alabama	34.17625, -87.27745	X			
AUHT3961	*Plethodon mississippi*	0.16 road km south of AL 102 on Frozen Hollow Rd., Walker Co., Alabama	33.78701, -87.52527	X			
AUHT3963	*Plethodon mississippi*	Buttahatchee River at CR 16,	34.02066, -88.05236	X	X		X

		floodplain on east bank, Lamar Co., Alabama					
AUHT5228	*Plethodon mississippi*	Town Creek at Foster Mill Rd., south of road and east of creek, Lawrence Co., Alabama	34.76775, -87.41591	X			
AUHT6682	*Plethodon mississippi*	Allen Acres, 50 m south of Lock 9 Road, 800 m east Black Warrior River , Hale Co., Alabama	32.99753, -87.69711	X	X	X	X

DBS2778	*Plethodon mississippi*	~24 km S of New Augusta, Hickory Creek at Jimmy Breland Rd (FS 309) crossing, Perry Co., Mississippi	31.00821, -88.95478	X	X	X	X
DBS2779	*Plethodon mississippi*	~24 km S of New Augusta, Hickory Creek at Jimmy Breland Rd (FS 309) crossing, Perry Co., Mississippi	31.00821, -88.95478	X	X	X	X

DBS2958	*Plethodon mississippi*	Hammond, North Oak Park, Tangipahoa Par., Louisiana	30.52710, -90.47761	X	X	X	
KHK233	*Plethodon mississippi*	Natchez Trace State Park, Henderson Co., Tennessee	35.77867, -88.25600	X			
RH51316	*Plethodon mississippi*	Stave Creek, site 87, Clarke Co., Alabama	31.54861, -87.93000	X			
RH64016	*Plethodon mississippi*	Maxie, site 86, Forrest Co., Mississippi	30.92778, -89.17667	X	X	X	X
RH65839	*Plethodon mississippi*	Tishomingo State Park, site	34.61056, -88.19889	X	X	X	X

		79 (Type locality), Tishomingo Co., Mississippi					
RH65842	*Plethodon mississippi*	Near Forest, site 83, Scott Co., Mississippi	32.41028, -89.48389	X	X	X	X
RH65855	*Plethodon mississippi*	Columbus, site 84, Lowndes Co., Mississippi	33.49028, -88.34778	X	X	X	X
RH70315	*Plethodon mississippi*	5 mi S Talisheek, St. Tammany Par., Louisiana	30.46500, -89.85390	X	X	X	X
RH71898	*Plethodon mississippi*	Between Fosters and Ralph, site	33.06500, -87.72639	X			

		77, Tuscaloosa Co., Alabama					
TJC107	*Plethodon mississippi*	Flint Creek WP, Stone Co., Mississippi	30.88481, -89.14417	X	X	X	X
TJC108	*Plethodon mississippi*	Flint Creek WP, Stone Co., Mississippi	30.88481, -89.14417	X	X	X	
TJC98	*Plethodon mississippi*	Hawthorn Rd., Winston Co., Mississippi	33.07634, -89.07710	X	X	X	X
TJC99	*Plethodon mississippi*	Sturgis Rd., Winston Co., Mississippi	33.20495, -89.03650	X	X	X	X

RH51389	*Plethodon ocmulgee*	S Alma, Bacon Co., Georgia	31.44194, -82.43139	X	X	X	X
RH59565	*Plethodon ocmulgee*	Near Thomaston, Upson Co., Georgia	32.79389, -84.25833	X			
RH70363	*Plethodon savannah*	Near Hephzibah, Richmond Co., Georgia	33.33000, -82.06361	X			
DQ018706	*Plethodon yonahlossee*	Surry Co., North Carolina		X			

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