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## Species Delimitation in Eastern Pine Snakes (*Pituophis melanoleucus*)

--Manuscript Draft--

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<b>Abstract:</b>	<p>The Eastern Pine Snake ( <i>Pituophis melanoleucus</i> ) is found throughout eastern North America. Taxonomy in this group has been controversial with several conflicting subspecific designations. Three subspecies of the Eastern Pine Snakes have been proposed according to their geographical locations: the northern Pine snake ( <i>P. m. melanoleucus</i> ), the Florida Pine snake ( <i>P. m. mugitus</i> ), and the Black Pine snake ( <i>P. m. lodingi</i> ). There are consistently unresolved relationships among these subspecific taxa in previous studies. We analyzed ultra-conserved elements (UCEs) to perform species tree estimation and species delimitation approaches implementing Bayesian inference methods. Species delimitation indicated that the plurality of datasets supported an ingroup of one species rather than three different subspecies. These results confirm prior findings of little divergence between the three putative subspecies and suggesting one single species.</p>

## Cover Letter

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October 25, 2021

To the Editorial Staff,  
American Society of Ichthyologists and Herpetologists,

I wish to submit an original manuscript entitled "Species Delimitation in Eastern Pine Snakes" for consideration by Ichthyology & Herpetology. In this study, we used ultra-conserved elements (UCEs) to estimate how many species of *Pituophis melanoleucus* were in our in-group sample. Using Bayesian phylogenetic methods, as well as the multispecies coalescent method *bpp*, we found no support for multiple species in this group.

I confirm that this work is original and has not been published elsewhere. We suggest that Richard Franz (University of Florida, [lrfranz08@gmail.com](mailto:lrfranz08@gmail.com)), Craig Rudolph (US Forest Service, [crudolph01@fs.fed.us](mailto:crudolph01@fs.fed.us)), and Richard. R Schaefer (US Forest Service, [rick.schaefer@usda.gov](mailto:rick.schaefer@usda.gov)) be considered as reviewers. All three of these authors have published on Pine Snakes in the southeastern United States.

Thank you for your consideration, and we look forward to hearing from you,

Basanta Khakurel	Zachary Nikolakis	Brian Crother	April Wright
			

*Title Page*

**Species Delimitation in Eastern Pine Snakes (*Pituophis  
melanoleucus*)**

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## ABSTRACT

The Eastern Pine Snake (*Pituophis melanoleucus*) is found throughout eastern North America. Taxonomy in this group has been controversial with several conflicting subspecific designations. Three subspecies of the Eastern Pine Snakes have been proposed according to their geographical locations: the northern Pine snake (*P. m. melanoleucus*), the Florida Pine snake (*P. m. mugitus*), and the Black Pine snake (*P. m. lodingi*). There are consistently unresolved relationships among these subspecific taxa in previous studies. We analyzed ultra-conserved elements (UCEs) to perform species tree estimation and species delimitation approaches implementing Bayesian inference methods. Species delimitation indicated that the plurality of datasets supported an ingroup of one species rather than three different subspecies. These results confirm prior findings of little divergence between the three putative subspecies and suggesting one single species.

## INTRODUCTION

Molecular phylogenetic data have a long history of application to species delimitation problems (Donoghue 1985). In the earliest forms, this took the form of the phylogenetic species concept (de Queiroz 2007), which posited that species were independent lineages on a phylogenetic tree. More recently, models that provide a more in-depth look at gene flow among taxa have been used for species delimitation from molecular data (Yang and Rannala 2010). In many ways, this is a return to the biological species concept, placing emphasis on genetic introgression and lineage separation. The multispecies coalescent (MSC) method uses both molecular phylogenetics and population genetics to counter problems, such as long-branch attraction and

the inherent subjectivity of interpreting the phylogeny, posed by delimiting species on the basis of phylogenetic trees (Yang and Rannala 2010, Yang 2015). Unlike the traditional phylogenetic methods which assume that the same tree underlies all gene loci, MSC accounts for coalescent processes in ancient and modern species and the resultant species-gene tree conflicts by allowing for multiple gene trees to underlie the data (Yang 2015). Different evolutionary processes operate at different geographical locations which would lead to population genetic reconstructing over time (Soltis et al. 2006).

The southeastern United States is an area with rich biodiversity consisting of almost half of the country's reptiles and amphibians (Graham et. al. 2010). The drainage of major rivers that flow south to the Gulf of Mexico (e.g., Mississippi, Apalachicola, Suwanee) have created differentiating barriers for biota in the east and the west (Soltis et. al. 2006). These complex barriers have provided opportunities for diversification of various flora and fauna in the region. About 20% of the total population of herpetofauna in the region is considered endemic (Graham et. al. 2010; Tuberville et. al. 2005). Longleaf pine, in particular, provides critical habitat for a number of endemic species (Guyer and Bailey 1993). One of such species is the eastern pine snakes, *Pituophis melanoleucus*. It has been hypothesized to have as many as three subspecies (Stull 1940; Crother 2012). In this manuscript, we use molecular species delimitation methods to examine the taxonomy in this group.

Morphological characters, and particularly synapomorphies, have typically been considered an important component of determining valid species (Assis and Rieppel 2011). However, pine snake subspecies are not observed to have synapomorphies and those that are potential synapomorphies are fairly labile, such as scale coloration. Individuals of different subspecies who appear to show signs of introgression have been observed in the wild (Scott 2008). In the

absence of traditional markers of species distinction according to the morphological or biological species concepts, we can make use of molecular data to identify cryptic species. There are multiple types of molecular data that can be brought to bear this question. Each type has different evolutionary properties that lead to the marker capturing different types of variation. Some of such molecular data are DNA barcode (Herbert et. a. 2003), UCEs (Bejerano et al. 2004; Winker et. al. 2018), ddRADseq (Peterson et al. 2012, Reitzel et. al. 2013), Sequence capture (Anderman et. al. 2020). In this study, we make use of a UCE dataset collected for phylogenetics in the pine snakes group (Nikolakis et al. 2021).

*Pituophis melanoleucus* occurs across a large range of southeastern United States where many geological barriers that may inhibit gene flow exist (Burbrink et al. 2000; Burbrink and Guiher 2015; McKelvy and Burbrink 2017; Myers et al. 2020). Examples of barriers are the Apalachicola and Mississippi river drainages which are believed to have created population differentiation among many groups of organisms (Pyron and Burbrink 2009; Soltis et. al. 2006). Prior studies have also supported population structure differences across these barriers. For example, some populations of tiger salamanders, rat snakes, musk turtles and snapping turtles exhibit different population structure in the eastern and western side of the Apalachicola barrier (Church et. al. 2003; Burbrink et. al. 2000; Burbrink 2002; Thomas et. al. 2014), but some populations of catfish show no genetic differences across the region (Avisé et. al. 1987). The eastern pine snakes have a wide range of habitats across the eastern United States and are thought to contain several distinct populations with high degrees of gene flow (Nikolakis et al. 2021). This species complex is currently classified with three geographic sub-specific taxa, the Northern Pine snake (*P. m. melanoleucus*), (2) the Florida Pine snake (*P. m. mugitus*), and (3) the Black Pine snake (*P. m. lodingi*) (Crother 2012). These snakes range widely in color from

uniformly black to having red/bronze patches (Guyer et al. 2019). The Black Pine snake has brown to black dorsal color, the Florida Pine snake has gray anterior color and rusty brown in the posterior with some brownish spots in the dorsal side, and the Northern Pine snake are creamy, yellowish ground in color with dark blotches in the entire body (Guyer et. al. 2019). This species complex is an ideal candidate for testing whether or not the current subspecific taxonomy represents evolutionary history through the use of species delimitation methods given the uncertainty of relationships from previous studies (Nikolakis et al. 2021, Rodríguez-Robles and De Jesus-Escobar 2000).

There are various tree-based and non-tree-based species delimitation methods that can be used to determine the species boundaries (Camargo and Sites 2013). *bpp* is a software that generates the Bayesian posterior distribution of species delimitation models using the multispecies coalescent framework (Yang and Rannala 2010). It uses reversible-jump MCMC to move between models of the number of populations present in the sample, while calculating the posterior probabilities associated with the model of population differentiation. *bpp* allows us to calculate the model likelihoods for different numbers of species. We used *bpp* to test how many species are present in our ingroup.

We used UCEs to obtain the evolutionary relationships within *Pituophis melanoleucus*, to determine if UCEs are best suited for this type of analyses, and to determine if there is support for multiple subspecies in *Pituophis melanoleucus*. We used *bpp* (Flouri et. al. 2018) for delimiting species under the multispecies coalescent. We also used *RevBayes* (Höhna et. al. 2016) to determine if there is any existing phylogenetic structure within the group, as the phylogenetic species concept defines species as a distinct group of conspecific individuals (Woodruff 2001). This study builds on the recent work of Nikolakis et al. (2021) and will help to

provide a better understanding of diversity patterns of pine snakes in the eastern United States and may provide additional insight on the utility of UCEs to study phylogenetic relationships in recently diverged clades. In this manuscript, we use both the phylogenetic tree and the MSC to delimit species in the genus *Pituophis*.

## **MATERIALS AND METHODS**

### **Sample collection, DNA extraction and Bioinformatics**

Tissue Samples were collected from forty-three specimens of *Pituophis melanoleucus* from their geographical distribution (Fig. 1, Table 1). The samples were de-multiplexed, filtered, and processed by removing adapter sequences and ambiguous bases using the program *Illumiprocessor* which is incorporated in the software *Phyluce* v.1.5 (Faircloth 2015) according to protocol outlines in Nikolakis et. al. (2021).

### **Phylogenetic Analyses**

We conducted phylogenetic analyses using a nucleotide substitution model from *RevBayes* software, v.1.1.1 (Höhna et. al. 2016). We used the general time reversible (GTR) model (Tavaré 1986) of sequence evolution, which allows six exchangeability rates between nucleotide states. The exchangeabilities are drawn from a Dirichlet distribution with an uninformative prior and allows the data to determine the value of the exchange abilities. We also used Gamma-distributed among site rate variation to allow different sites to evolve at different rates (Yang 1994). The MCMC was run to replicate 50,000 generations and the resulting log files were



viewed in *Tracer* v.1.7.1 (Rambaut et. al. 2018) to check for convergence. The output files were then summarized into maximum clade credibility trees (Helfrich et. al. 2018) and majority-rule consensus trees using *RevBayes*.

To delimit species, we used Bayesian Phylogenetics and Evolution, *bpp* (Yang & Rannala 2010), a genealogical method that estimates the time of origin, time of diversification, and the effective population multiplied by the mutation rate, for each species. We used the model A11 which estimates the species delimitation and the species tree (Fluori et. al. 2020). In this analysis the species delimitation model and the species phylogeny both change in the MCMC. The results showing posterior probability distributions indicate whether the lineages can be differentiated from each other. In the analysis, the subspecies of pine snakes were labelled according to their geographical distribution (FE – Far East; ME – Mid East; TN – Tennessee Valley and OG – Outgroup). The burnin was specified at 8000 and each dataset ran for 100000 generations. The output file contained posterior probabilities of the best fit models and the arrangement of species labelled per their geographic location. The line containing all the probabilities for the best fit was extracted from the output files using a UNIX script and a histogram was created to visualize the number of species of pine snakes.

To create a concatenated tree for comparing consensus trees across UCEs, we then used the summarized trees from *RevBayes* (Höhna et. al. 2016) and built a consensus network in *R* (R core team) using the packages *ape* (Paradis & Schliep 2019), *phangorn* (Schliep 2011) and *phytools* (Revell 2012). The code and data for all the analyses are stored in GitHub (<https://github.com/basanta33/Pituophis>).

## 135   **RESULTS**

### 136   **Sequence Data**

137   The majority of the variation recovered was observed in the extreme regions of each UCE and  
138   there was little correlation between the variable sites and locus lengths (see Nikolakis et. al. 2021  
139   for further details).

### 140   **Species Delimitation with BPP**

141   We carried out a study to examine whether there is any support for the recognition of subspecies  
142   within *Pituophis melanoleucus*. The model A11 accommodated for the gene tree uncertainty and  
143   variable population sizes over time to explore different species delimitation models and different  
144   species phylogenies. We processed the output from the datafiles with variable sites to obtain the  
145   posterior probabilities of different species groupings of pine snakes. When we combined the  
146   probabilities obtained from all the datasets to a single file and made a histogram, the plurality of  
147   the datasets indicated that there is an ingroup of one species of *Pituophis melanoleucus* and the  
148   outgroup of *P. ruthveni*, *P. catenifer*, and *Pantherophis obsoletus* (Figure 2). The posterior  
149   probabilities of delimitation were obtained based on a guide tree which accounts for the  
150   phylogenetic uncertainty.

### 151   **Consensus Network**

152   The output trees obtained from *RevBayes* were summarized to obtain maximum credibility trees  
153   and consensus trees. The consensus trees obtained were composed into a network of  
154   phylogenetic trees which reflected little to no variation amongst the subspecies of *P.*  
155   *melanoleucus*. The tree obtained from the *phangorn* (Schliep 2011) package enabled us to  
156   evaluate the conflicting phylogenetic signals from the collected datasets. The tree (Figure 4)

indicates that there is little variation among the topologies estimated from different pine snakes over the eastern side of the United States. If different loci had supported different phylogenetic trees, we would have expected the nodes of the trees to connect to the tips in a web-like structure.

## DISCUSSION

### Corroboration of a single species of Eastern Pine Snakes

Our results show that *Pituophis melanoleucus* is not composed of various distinct geographic lineages within the eastern United States. The Bayesian consensus tree (Figure 4) indicates that there is little to no variation among the subspecies of *P. melanoleucus*. Figure 3 also indicates that there is little to no phylogenetic structure among the samples collected from different parts of the eastern United States. This result agrees with Nikolakis's study (2021) which used sequence-capture based approach to understand the factors that correlate with genetic diversity and distance in the lineages of *P. melanoleucus*. The eastern pine snake diverged within the *Pituophis* about 6 to 3 million years ago (Pyron and Burbrink 2009). The complex of *P. melanoleucus* appears to be of a single species with very little genetic differentiation, as indicated by the posterior probabilities of the best fit model for the species tree generated by *bpp*. In addition to the results from *bpp*, the majority rule consensus tree obtained from *RevBayes* also indicated that we get the same trees across the sites, a near-polytomy with little geographic structure (Figure 3). The consensus network compiled using all the 4600 data files show that

there is no connection between multiple tips and each node thus indicating little topology variation among the subspecies of pine snakes in the eastern region (Figure 4).

### **Gene flow across a geographic barrier**

The three different geographical lineages: far-eastern, mid-eastern and Tennessee clades are separated by the Apalachicola/Chattahoochee River Basin acting as a geographical barrier (Nikolakis 2021). Previous squamate phylogeographic studies across that region (Burbrink et al., 2000, 2008; Soltis et. al. 2006; Weinell and Austin, 2017; Myers et. al. 2020) indicated that there is significant genetic variation among the clades existing across the barrier. Despite the geographical barrier, molecular species delimitation indicates that the three lineages of pine snakes are not distinct from each other. *bpp* should detect if there is gene flow across the barrier. In our analysis, *bpp* indicated that there was only one population 4650 times and two populations (one being the outgroup) 4654 times during the two runs of the datasets (Fig. 2). The geographic barrier did not seem to have much effect on the isolation of the population of pine snakes across the mid-eastern and far-eastern sides. Due to the indication of little genetic differentiation, it can be concluded that gene flow has been maintained in the population of eastern pine snakes across that region. This disunity in the previous phylogeographic analyses (Burbrink et al. 2008, 2000; Weinell and Austin 2017) of the snakes and our study could be because of the distribution of the species of the pine snakes across the eastern United States. Previous movement studies indicate that *P. melanoleucus* is a very mobile species with their home ranges spanning from approximately 35 hectares to over 105 hectares (Nikolakis et. al. 2021) which could contribute to the gene flow among the different populations.

### **Use of UCE for recently diverged lineages**

Ultra-conserved Elements (UCEs) are highly conserved regions within the genome that are shared among evolutionarily distant taxa (Bejerano et al. 2004). The use of UCEs has been increasing in phylogeny inference across many vertebrate taxa (Gustafson et. al. 2019). Although UCE is an important molecular marker, for this complex the use of molecular markers other than UCE would be a better option. Some UCEs in this study did not even distinguish between the outgroup and the ingroup taxa, so we had very little information in those sites. Of the sites with enough information to discriminate in- from the outgroup, 83% supported one in-group population (Figure 3). This is because of hypothesized recent evolution of the pine snakes from other *Pituophis* (Pyron and Burbrink 2009). The oldest fossils of *P. melanoleucus* have been found in Florida dating 0.8 to 2.5 mya and more northern fossils from Pennsylvania have been dated from 0.1 mya (Holman 2000). These data could indicate that the lineage diverged during the late Pleistocene when there were environmental fluctuations leading to periods of isolation and connection. These periods led to the maintenance of gene flow through the contacts of the different populations. As UCEs are conserved sequences, they evolve very slowly, thus decreasing the power to detect variation among the organisms that have recently diverged from its common ancestor (Winker et. al. 2018). Thus, further study with larger sample sizes and broader genomic coverage will be needed to determine the species limits between these taxa (Sukumaran and Knowles 2017).

## CONCLUSION

Using a dataset of ultra-conserved elements, we find no evidence for multiple subspecies in *Pituophis melanoleucus*. Using the multispecies coalescent and the phylogenetic species concept, we substantiate *Pituophis melanoleucus* as a single species across its whole range, a hypothesis

suggested by previous authors. However, given low informativity of many UCEs, we suggest that other data types would be preferable for a study on this time scale.

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**Figure and Table Captions:**

- Figure 1: Sampling sites across southeastern United States. The different colored regions represent habitat of the different subspecies of pine snakes. Red: Black Pine Snake; Yellow: North Pine Snake; Green: Florida Pine Snake.
- Figure 2: Number of times the posterior probabilities of the number of populations supported appear in the *bpp* output.
- Figure 3. Consensus Phylogeny. We see no consistent phylogenetic structure to the tree.
- Figure 4: Consensus Network of the pine snakes samples. A lack of web-like structure indicates that there is little to no variation amongst the samples collected.
- Table 1: A table containing all the subspecies sample and their collection locations.

<b>Taxon</b>	<b>Sampling Region</b>
<i>Pantherophis obsoletus</i>	Jefferson LA
<i>Pituophis catenifer</i>	Sioux NE
<i>Pituophis catenifer</i>	Wheeler TX
<i>Pituophis ruthveni</i>	Bienville LA_1
<i>Pituophis ruthveni</i>	Jackson MS_1
<i>Pituophis m. lodingi</i>	Bienville LA_2
<i>Pituophis m. lodingi</i>	Jackson MS_2
<i>Pituophis m. lodingi</i>	Jackson MS_3
<i>Pituophis m. lodingi</i>	Mobile AL
<i>Pituophis m. lodingi</i>	Perry MS_1
<i>Pituophis m. lodingi</i>	Perry MS_2
<i>Pituophis m. lodingi</i>	Perry MS_3
<i>Pituophis m. melanoleucus</i>	Autauga AL
<i>Pituophis m. melanoleucus</i>	Burlington NJ_1
<i>Pituophis m. melanoleucus</i>	Burlington NJ_2
<i>Pituophis m. melanoleucus</i>	Humphreys TN
<i>Pituophis m. melanoleucus</i>	Huntingdon TN
<i>Pituophis m. melanoleucus</i>	Marion GA_1
<i>Pituophis m. melanoleucus</i>	Marion GA_2
<i>Pituophis m. melanoleucus</i>	Ocean NJ
<i>Pituophis m. melanoleucus</i>	Richmond NC
<i>Pituophis m. melanoleucus</i>	Rockingham NC
<i>Pituophis m. melanoleucus</i>	Scotland NC
<i>Pituophis m. melanoleucus</i>	Winston AL
<i>Pituophis m. melanoleucus</i>	Hoke NC
<i>Pituophis m. mugitus</i>	Baker GA
<i>Pituophis m. mugitus</i>	Covington AL_1
<i>Pituophis m. mugitus</i>	Covington AL_2
<i>Pituophis m. mugitus</i>	Pierce GA
<i>Pituophis m. mugitus</i>	Turner GA
<i>Pituophis m. mugitus</i>	Alachua FL
<i>Pituophis m. mugitus</i>	Bristol FL
<i>Pituophis m. mugitus</i>	Brooksville FL
<i>Pituophis m. mugitus</i>	Cantonment FL
<i>Pituophis m. mugitus</i>	Hamilton FL
<i>Pituophis m. mugitus</i>	Hernando FL
<i>Pituophis m. mugitus</i>	Highlands FL
<i>Pituophis m. mugitus</i>	Lafayette FL
<i>Pituophis m. mugitus</i>	Liberty FL
<i>Pituophis m. mugitus</i>	Marion FL
<i>Pituophis m. mugitus</i>	Okaloosa FL
<i>Pituophis m. mugitus</i>	Okeechobee FL_1
<i>Pituophis m. mugitus</i>	Okeechobee FL_2

*Pituophis m. mugitus*

*Pituophis m. mugitus*

PonceDe Leon FL

Santa Rosa FL

Figure 1

[Click here to access/download;Figure;Figure 1.png](#)

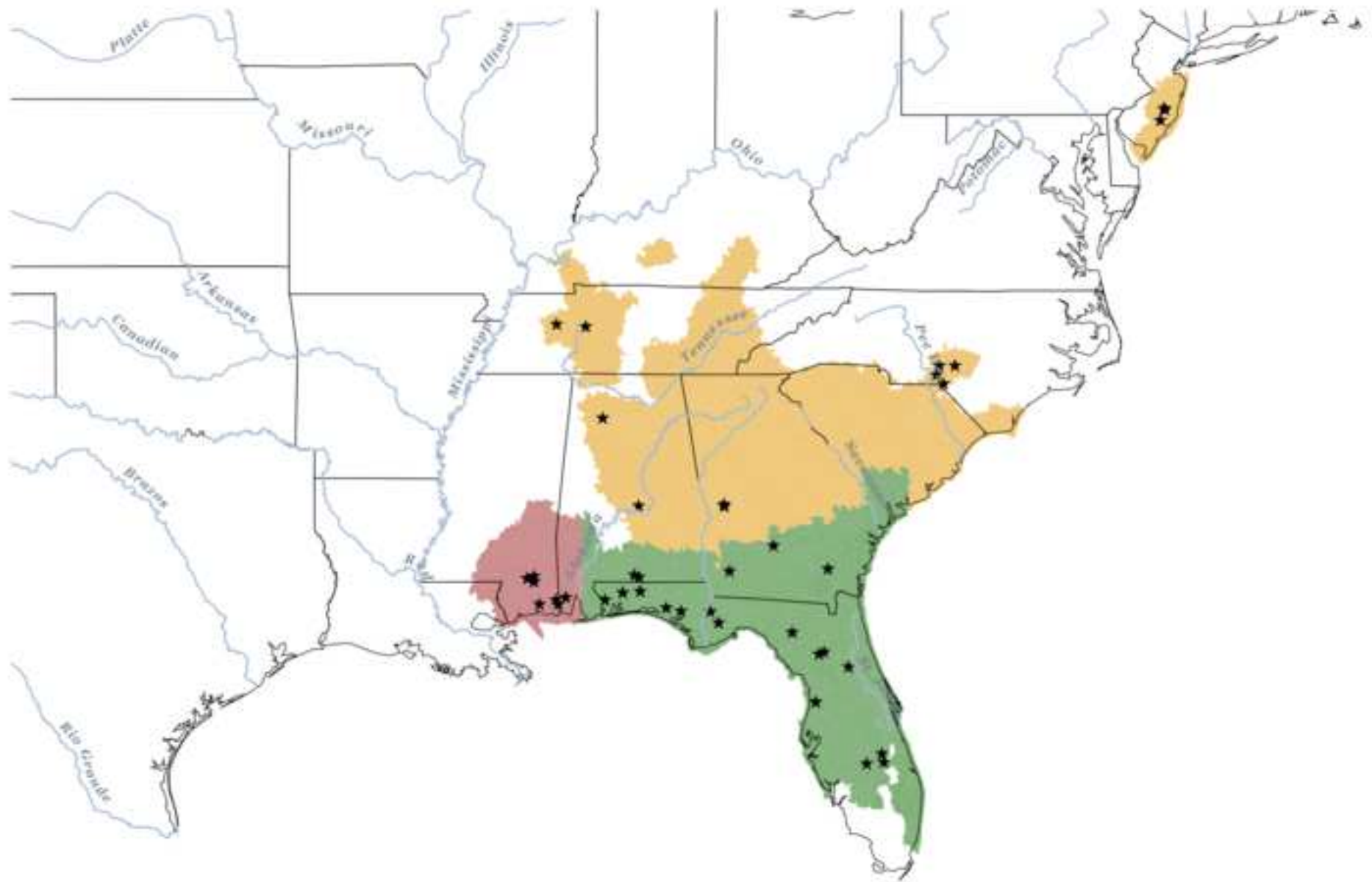
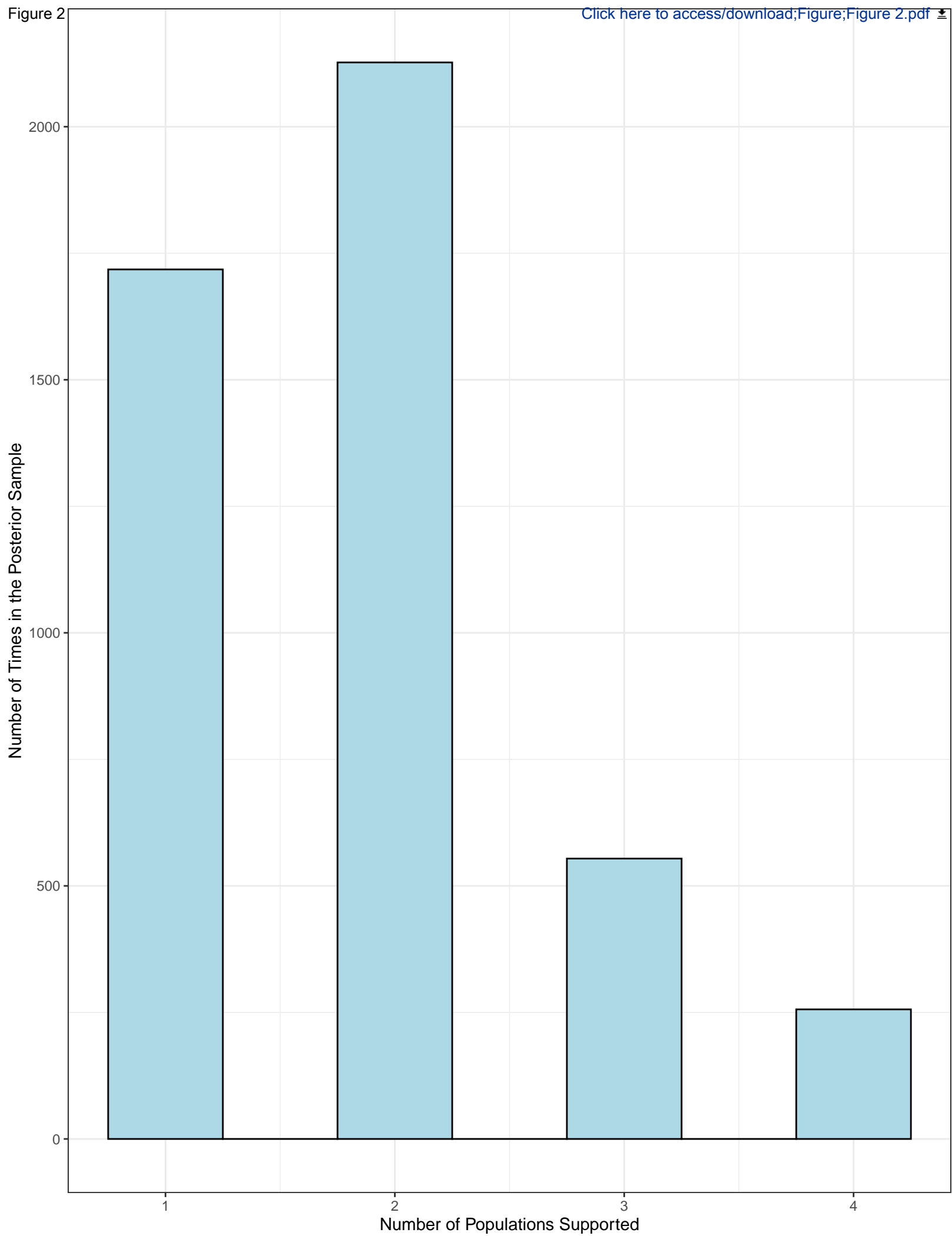


Figure 2



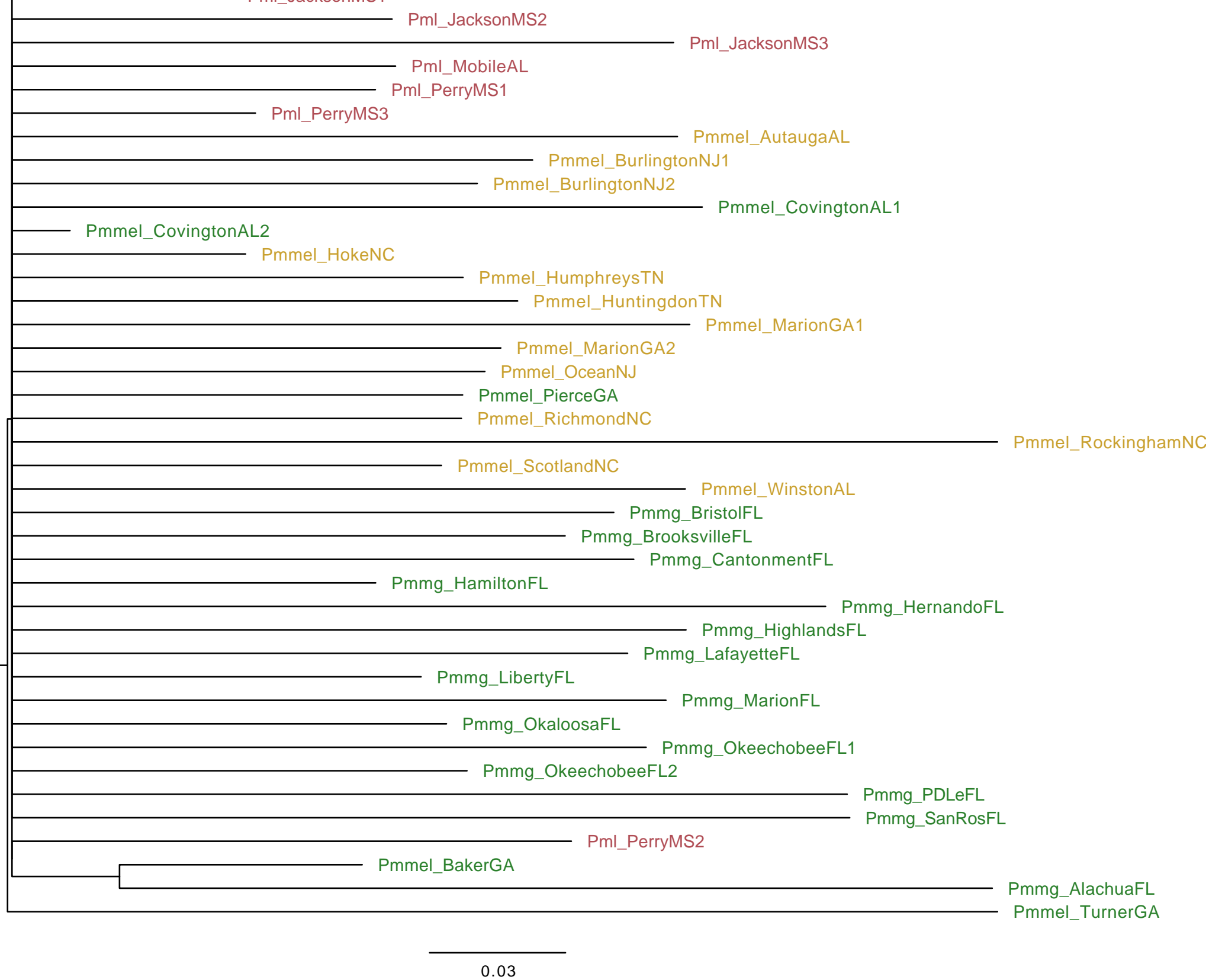


Figure 4

