PHYLOGEOGRAPHY AND POPULATION DEMOGRAPHY OF THE EASTERN WORM SNAKE *CARPHOPHIS AMOENUS*

by

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ABSTRACT

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Phylogeography and Population Demography of the Eastern Worm Snake *Carphophis amoenus*
(Under the direction of Dr. Frank Fontanella)

Climate has a profound influence on the distribution of species and changing climate conditions during the Pleistocene epoch, which displaced several species from their present day ranges. The Pleistocene epoch began about 1.8 million years ago and lasted until about 11,700 years ago. Of the three identified glaciation events in North America, the last one known as the Last Glacial Maximum (LGM) ended approximately 11 kya. Eastern North America contained the Laurentide Ice Sheet, which extended into a major portion of eastern North America, whereas southern United States were left unglaciated. During this time, the climate was drastically different, and much of the deciduous forests in Eastern North America changed to boreal forests. The changing climates, corresponding range shifts and geographic barriers can promote differentiation due to habitat fragmentation. The genus *Carphophis* dates back to the mid-late Pliocene and consists of two nominal species, *C. amoenus* (Eastern worm snake) and *C. vermis* (Western worm snake). The eastern worm snake further divides into two nominal subspecies, *C. a. amoenus* (Eastern worm snake) and *C. a. helenae* (Midwestern worm snake). This study utilizes the mitochondrial gene cytochrome b to investigate the phylogeographic structure and demographic patterns of the Eastern worm snake (*C. amoenus*). The results inferred a genetic pattern that does not correspond

to the current taxonomy, including five mitochondrial lineages, which may indicate the underestimation of species diversity. Of the five inferred lineages, only one lineage exhibits rapid population expansion, which is common in populations occurring at higher latitudes during an interglacial event. The remaining lineages are going through a gradual growth or are remaining relatively stable. Some of these genetic patterns do not match the patterns that are commonly observed in other organisms that are affected by the LGM and provides new insights on potential barriers throughout Eastern North America.

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I. INTRODUCTION

During the Pleistocene Epoch, which ranged from approximately 1.8 mya to about 11,700 years ago, there were a total of three identified glaciation events in North America (Berggren, 1972). The Last Glacial Maximum (LGM), also known as the Wisconsin glaciation event, extended from about 75 kya to about 11 kya. The Laurentide Ice Sheet reached its maximum at approximately 40° North (Hewitt, 1996), then from ~18 kya to ~11 kya it started to slowly recede from its maximum extent (Prentice et al. 1991). The expansion and contraction of glacial ice sheets are thought to have played an important role in shaping the distribution and biodiversity throughout the northern temperate regions, due to the changing climate and biotic factors (Hewitt, 1996). During glaciation events, populations at northern latitudes are driven into southern refugia as they track with suitable habitat. As the glacial ice sheet recedes, populations in higher latitudes would go through rapid expansion into previously glaciated areas, while populations lower in latitude, would be confined to more southern areas, a process known as the leading edge effect (Hewitt, 1996). The repeated patterns of isolation and expansion can promote genetic differentiation between populations and over time, results in the formation of distinct genetic lineages. The effects of glaciation on the distribution and genetic composition of species has been observed across several taxa, including the scorched mussel, *Brachidontes exustus* (Lee and Foighil, 2004), ring neck snakes, *Diadophis punctatus* (Fontanella et al. 2008), the Ozark minnow *Notropis nubilus* (Berendzen et al. 2010), the northern leopard frog *Rana pipiens* (Hoffman and Blouin, 2004) and white cedar *Chaemaecyparis thyoides* (Mylecraine et al. 2004). In addition to the effects of glacial cycles, there are several geographic barriers in Eastern North

America that further promote differentiations or isolation by restricting dispersal between populations. In Eastern US, these barriers include the Apalachicola River, which flows in Eastern Alabama and into the Florida panhandle, the Appalachian Mountains, which extends from Georgia to Canada, and the Mississippi River (Soltis et al. 2006). The combination of glacial cycles and the geographic barriers are expected to have greater effects on the genetic patterns in organisms that have a lower vagility and higher philopatry (Soltis et al. 2006; Fontanella et al. 2008).

The genus *Carphophis* currently consists of two nominal species, *C. amoenus* (eastern worm snake) and *C. vermis* (western worm snake). Currently *C. amoenus* divides into two subspecies *C. amoenus amoenus* (eastern worm snake) and *C. amoenus helenae* (midwestern worm snake). The two subspecies are currently distinguished through the morphological features associated with the prefrontal and internasal scales. These two scales are located between the nostrils and for *C. a. amoenus*, the two scales are separated, while *C. a. helenae*, the two scales are fused together (Fig. 1). These snakes are found throughout much of eastern and midwestern North America with limited overlapping distributions (Fig. 2) (Conant and Collins, 1998). *C.a. amoenus* is distributed throughout most of Eastern U.S., from southern New England to South Carolina and into central Georgia and central Alabama (Conant and Collins, 1998). The distribution of *C. a. helenae* ranges from southern Ohio, to southern Illinois and eastern Arkansas (Conant and Collins, 1998). They get their name from their worm like appearance, which is also their main food source; however, they will also prey on soft-bodied insects (Conant

and Collins, 1998). Their coloration of the dorsal side ranges from dark brown to a pink brown color, while the ventral side is usually a pink color. The *C. amoenus* complex are fossorial, and are most commonly found in damp leaf litter, rotting logs and under rocks (Ernst and Ernst, 2003). In a distribution study conducted in Alabama, Mount (1972) encountered worm snakes more frequently in upland mesic forests that were dominated by hardwood trees. Due to the fossorial nature of this colubrid snake, they are seldomly found above ground. Barbour et al. (1969) studied the home ranges and movement activity and conclude that *C. a. amoenus* have limited dispersal capabilities (~45m/day), short periods of activity (< 12 hrs/day), and periods of inactivity that can last up to 14 hrs. Their fossorial nature is likely due to prevent overheating in extreme temperatures (Ernst and Ernst, 2003), so they may have extended periods of inactivity.

Taxa with poor dispersal capabilities and small home ranges should retain the genetic patterns that develop during periods of isolation because mutations occurring in cytoplasmic genomes are transmitted maternally. Patterns of variation inferred from mitochondrial DNA should reflect the demographic history and historical processes responsible for the contemporary distribution (Avise, 2000). The small home range and limited dispersal abilities of *C. amoenus* fulfills a number of the conditions beneficial to the inference of historical patterns of migration and fragmentation. Therefore, the goals of this study are to 1) describe the lineage diversity and phylogeographic patterns of *C. amoenus*, 2) evaluate the subspecies designations, and 3) to examine the effects of Pleistocene glaciation events on population demography in glaciated and unglaciated regions using mitochondrial gene cytochrome b.

Fig. 1. The difference in head scales of the two subspecies of *C. amoenus*, modified from Conant and Collins reptiles and Amphibians: Eastern/Central North America, Third Edition, 1998.

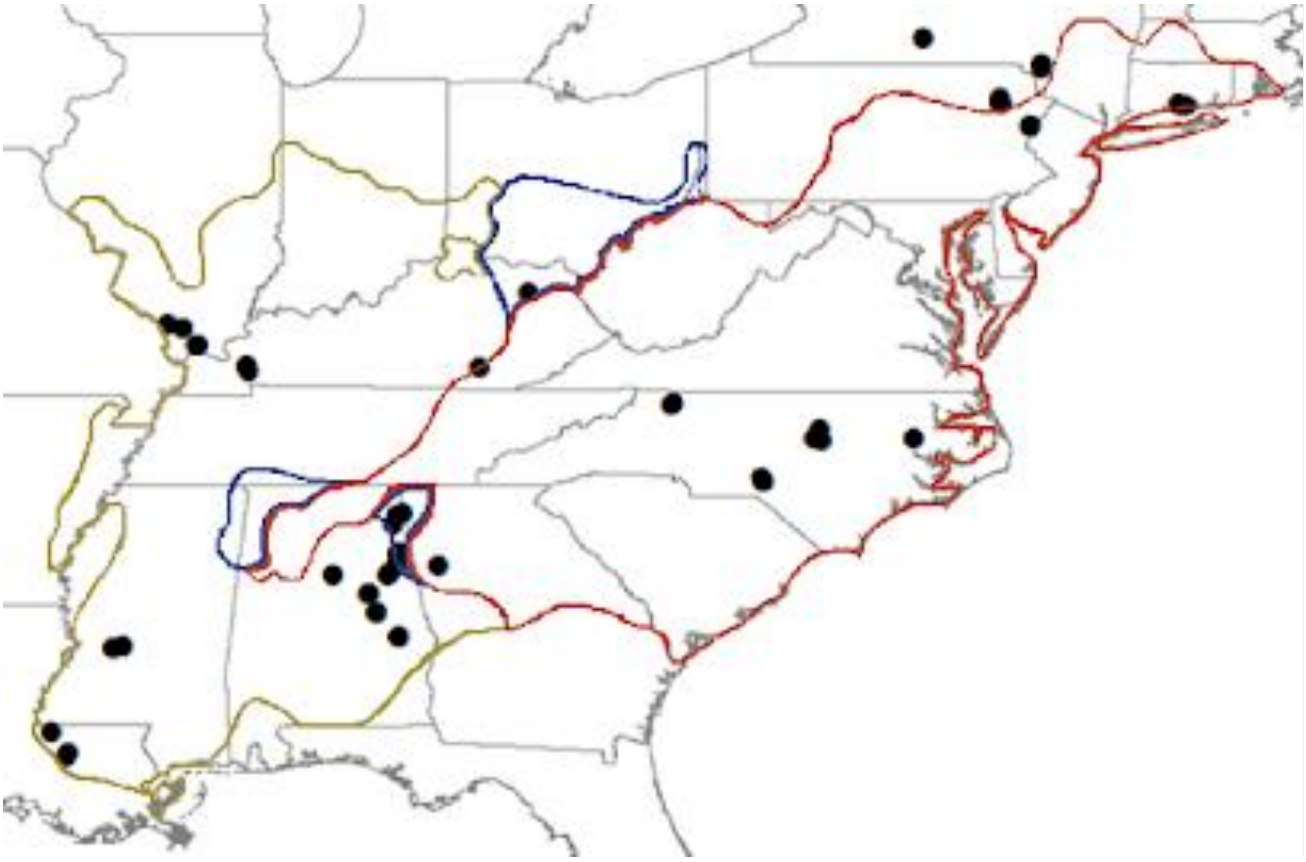


Fig. 2. Distribution of *Carphophis amoenus*. Black dots indicate where each samples were obtained. Outlined areas show distribution of the subspecies of C. amoenus, red in *Carphophis amoenus amoenus*, yellow is *Carphophis amoenus helenae*, and blue is where their distributions overlap.

II. MATERIALS AND METHODS

We obtained 85 specimens of *Carphophis amoenus* through either field collections or tissues obtained from museum loans (Fig. 2) (Appendix). Each tissue sample was preserved in 95% ethanol.

Molecular Analyses:

Genomic DNA was extracted from liver, muscle or shed skins using Qiagen DNA extraction kit, following the manufacturer's protocols. The mitochondrial gene cytochrome b (1117bp) was amplified via Polymerase chain reactions (PCR) using previously published primers and protocols (Burbrink et al. 2000; Arevalo et al. 1994; Fontanella et al. 2008). The cytochrome b gene region has been used successfully to examine intraspecific variation in snakes (Parson et al. 2000; Burbrink et al. 2000; Fontanella et al. 2008).

Reactions consisted of one µl of DNA 10 µl of Master Mix (Pro Mega), 0.75 µl of the forward and reverse primers and 12.5 µl of water. To determine if the primers and mtDNA from each sample annealed properly, gel electrophoresis was performed and 3.5 µl of ethidium bromide was injected into the gel. Only the DNA fragments that fluoresce under the uv light were used, since it indicates that the primers and DNA sequences annealed properly. To purify the amplified fragments, 5 µl ExoSap-it (USP Corp) was added to each PCR tube and heated to 37°C for 45 min. to activate the enzyme, then the temperature was raised to 80°C to denature the enzyme. Purified PCR products were sequenced in both directions using BigDye v. 3.1 Cycle Sequencing

Ready Reaction (Applied Biosystems, Perkin-Elmer, CA, USA). Reactions were purified with the CleanSeq Dye-terminator removal kit (Agencourt) and analysed on the ABI Prism 3730 sequencer (Applied Biosystems, Perkin-Elmer, CA, USA). Sequences were assembled, edited and aligned using SEQUENCHER 4.7 (Gene Codes, Corp.). All sequences were translated into amino acid sequences, then were checked and confirmed of their species through Blast-N.

Phylogenetic Analyses:

The outgroup taxa consisted of *Diadophis punctatus* (ringneck snake), *Farancia abacura* (mud snake), *Farancia erytrogramma* (rainbow snake), and *C. vermis* (Western worm snake), all of which belong to the subfamily of Dipsadidae (Zaher et al. 2009). ModelTest v. 2.1.10 was used to obtain the best fit model of nucleotide substitutions based on the Akaike information criteria (Akaike, 1973). Bayesian analysis was conducted using Mrbayes v. 3.2.7 in which the HKY+G (Hasegawa-Kishino-Yano plus Gamma) model was applied to each codon position. Six markov chains were started from a random tree with the analyses run for 1.0×10^7 generations, sampling with every 1000th generation for 3,000,000 generations. The 'burn-in' (the sample points prior to reaching the plateau phase) were discarded, while the remaining trees were combined to find a posterior probability estimate of the phylogeny. The nodes/tree topology were estimated as means of the posterior probability density. Figtree v. 1.1.2 (available at http://tree.bio.ed.ac.uk/software/figtree/) was used to produce a majority rule consensus tree from the post burn-in trees.

Population Demography

The average number of pairwise differences (π) or number of segregating sites (S) can be used to calculate the nucleotide diversity (Fontanella et al. 2008). Since S is dependent on the sample size, it alone is not a good measure for calculating the DNA polymorphism (Tajima, 1989). Each population (lineage) inferred from the phylogeny (Fig. 3), was assessed to determine if it is growing, in decline or if it is stable. Neutrality tests can be conducted in DNAsp (Rozas and Rozas, 1999) to indicate the growth rate of each population or lineage based on the null hypothesis (Rarírez-Soriano et al. 2008; Fu, 1997; Ramos-Onsins and Rozas, 2002).

Neutrality tests were conducted to test the null hypothesis of each population evolving neutrally. Tajima's D and Fu's Fs test statistics were all calculated using DNAsp 5.10.01 with a confidence interval of 95%. Coalescent simulations were done for 10,000 generations to provide the statistical significance ($p < 0.05$) and confidence intervals. Out of the neutrality tests used, Fu's Fs is the more powerful test (Fu, 1997; Ramírez-Soriano et al. 2008), especially when dealing with larger sample sizes, while R_2 statistical test is better for smaller sample sizes (Ramos-Onsins and Rozas, 2002). A negative Tajima's D and Fu's Fs can be a result of recent mutations, due to there being an excess of rare alleles (Tajima, 1989; Fu, 1997). However, it can also indicate that the population has undergone a recent bottleneck, due to the effects on DNA polymorphisms (Tajima, 1989). Negative values of D or Fs that are not significant, are likely results for populations going through a gradual expansion (Fontanella et al. 2008). For populations that are stable or contracting, there will be an elimination in rare alleles resulting in a

positive Tajima's D (Tajima, 1989) and Fu's Fs (Fu, 1997). Mismatch distribution of pairwise differences was another statistic calculated in DNAsp to test the null hypothesis of population size changes. Unimodal distribution would indicate there is a growth in the population, while multimodal distribution would represent populations that are relatively stable or that are contracting (Harpending et al. 1998).

III. RESULTS

Phylogenetic Reconstruction

The aligned data set consisted of 1117 base pairs from 115 individuals, 85 *C. amoenus*, 23 *C. vermis* and four *Diadophis punctatus*, two *Farancia abacura* and one *Farancia erytrogramma*. Twenty five percent of 3,000,000 generations were discarded as 'burn-in, with the remaining generations used to conduct the inferred phylogenetic tree. The Bayesian analysis inferred *Carphophis amoenus* as a monophyletic group consisting of five well-supported lineages, and three major clades (Fig 3).

The first major phylogenetic split within the *C. amoenus* species is the result of the oldest inferred lineage for this species and forms an Appalachian clade. This lineage is confined to regions east of the Appalachian Mountains and extends from North Carolina, north across Pennsylvania, into southern New York and into southern Connecticut. Lineage 1 is also likely distributed at higher latitudes than the remaining inferred lineages.

The next major phylogenetic split forms the next clade (Mississippian River clade), which contains Lineage 2. This lineage is largely distributed along the eastern side of the Mississippi River and appears to be restricted to the eastern portion of the Mississippi Flood Plain. This lineage ranges from the southern portions of Louisiana, north across Mississippi and into southern Illinois and western Kentucky.

The last major phylogenetic split forms the Mid-Eastern clade, which consists of Lineages 3, 4, and 5. Lineage 3, which consists of a single individual, is located in Central Alabama. Lineages 4 and 5 have overlapping distributions across western Alabama, with Lineage 5 extending further south into southeastern Alabama. Lineage 4 extends from their area of overlap in eastern Alabama, north into eastern Kentucky.

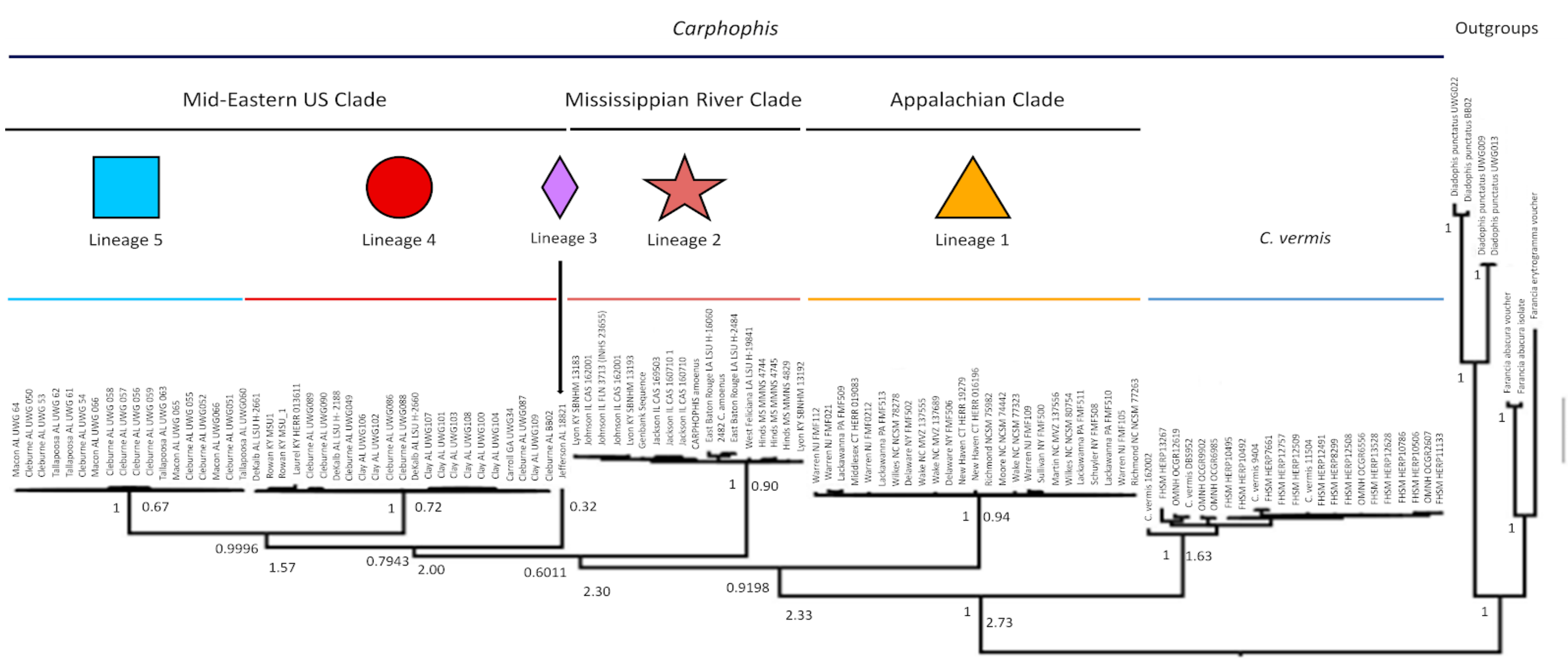


Fig. 3. Constructed phylogenetic tree consisting of *Carphophis amoenus*, *Carphophis vermis*, *Diadophis punctatus*, *Farancia abacura* and *Farancia erytrogramma*. Numbers to the left of each node indicates the posterior probability (support) for each lineage and the numbers to the right of each node represents the time to the most recent common ancestor.

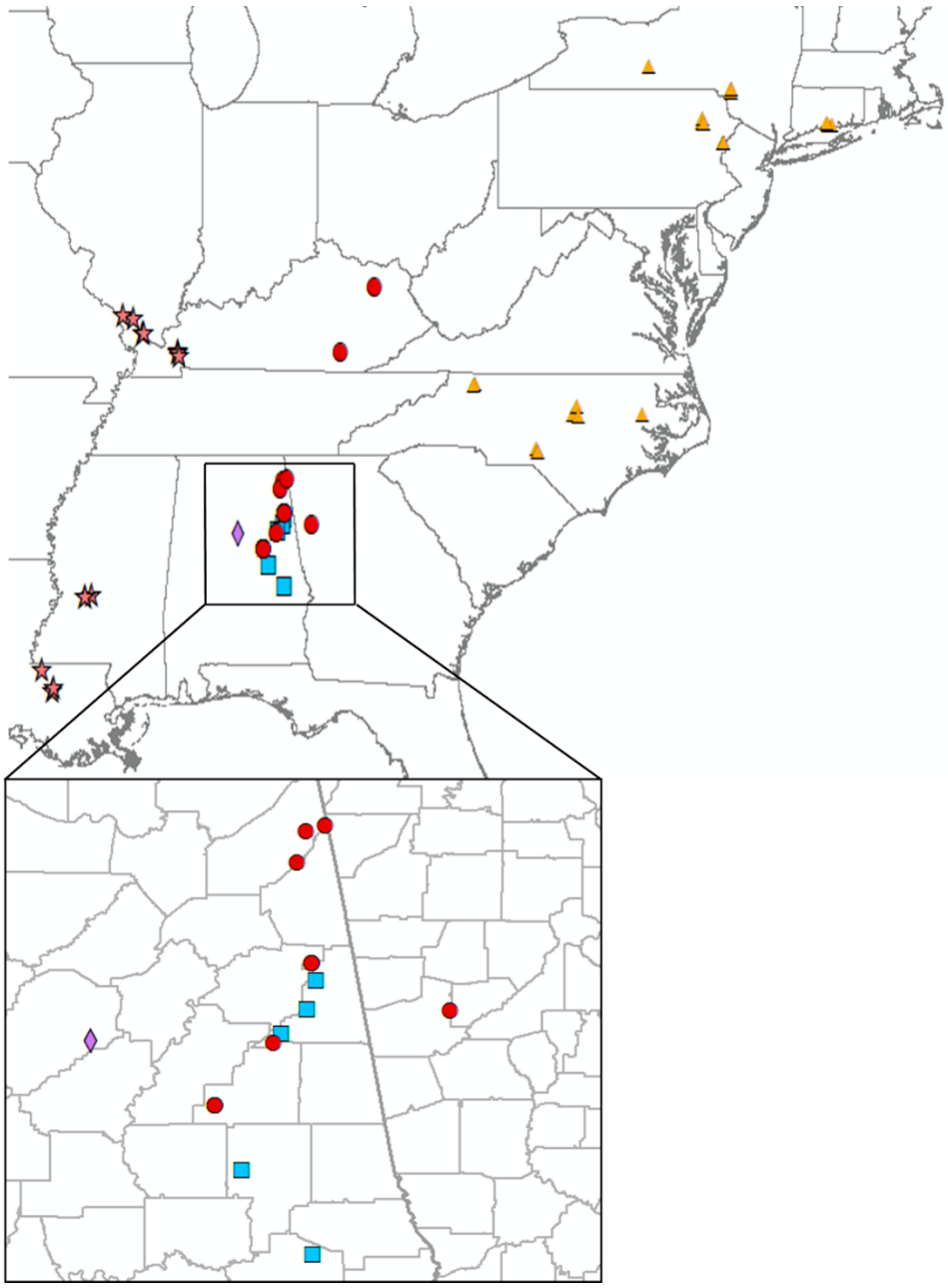


Fig. 4. Map showing the distribution of each *C. amoenus* population lineage diagnosed by mtDNA variation. Symbols correspond to Fig. 3 and indicate the collection locality.

Divergence Dating

The times to the most recent common ancestor for each lineage were estimated in BEAST v. 1.6.1 using the relaxed uncorrelated lognormal clock in order to account for the uncertainty in branch lengths and tree topology (Drummond and Rambaut, 2007). The HKY+G model was used for phylogenetic estimates. Since the fossil record of *C. amoenus* dates back to the early Pleistocene (Holman, 2000), the prior for the fossil node was set to an average of 1.8 my, with a lognormal standard deviation of 0.57. This produced a 95% confidence interval from 0.9851 to 4.1054. This time frame in North America spans from the Blancan (4.75 to 1.806 my), late Pliocene, and the Irvingtonian (1.806 to 0.3 my), Pleistocene. The analysis ran for 30 million generations and sampled every 1000 generations, following a pre-burn in of 3000.

Due to the oldest fossil being found in Florida (Holman, 2000), it is likely the origin of the *Carphophis* species originated in the eastern US. The divergence between *C. ameoneus* and *C. vermis* (~2.73 mya) occurred during the Blancean time frame in the late Pliocene (Table 1). The first divergence event for *C. amoenus* occurred about 2.33 mya during the early Pleistocene in the Blancan time frame. The ages of each inferred lineage occurred prior to the LGM which occurred about 75 kya, with the youngest lineage being roughly 0.32 my.

Origin (Mya)			
Node	Mean Age	SD	CI (95%)
1	2.7315	1.0942	1.0430 to 4.8791
2	2.3287	0.8775	0.9851 to 4.1054
3	2.2957	0.9294	0.9016 to 4.1646
4	1.9998	0.8081	0.7215 to 3.5565
5	1.5695	0.6332	0.5944 to 2.8079
MtDNA lineage	Mean Age	SD	CI (95%)
Lineage 1	0.9424	0.5304	0.0405 to 1.9436
Lineage 2	0.9027	0.5852	0.0165 to 2.0021
Lineage 3	0.3239	0.3728	2.5169×10^{-5} to 1.0779
Lineage 4	0.7225	0.4641	3.0888×10^{-4} to 1.5707
Lineage 5	0.6754	0.4326	2.4589×10^{-5} to 1.4658
vermis	1.6322	0.7047	0.5929 to 3.0272

Table 1. The divergence dates and the mean date of origin (Mya) for each lineage of *Carphophis amoenus*, and *Carphophis vermis*. Standard deviation (SD) and Confidence interval (95%) were calculated using uncorrelated lognormal Bayesian relaxed molecular clock in BEAST v. 1.6.1.

Population Growth and Diversity

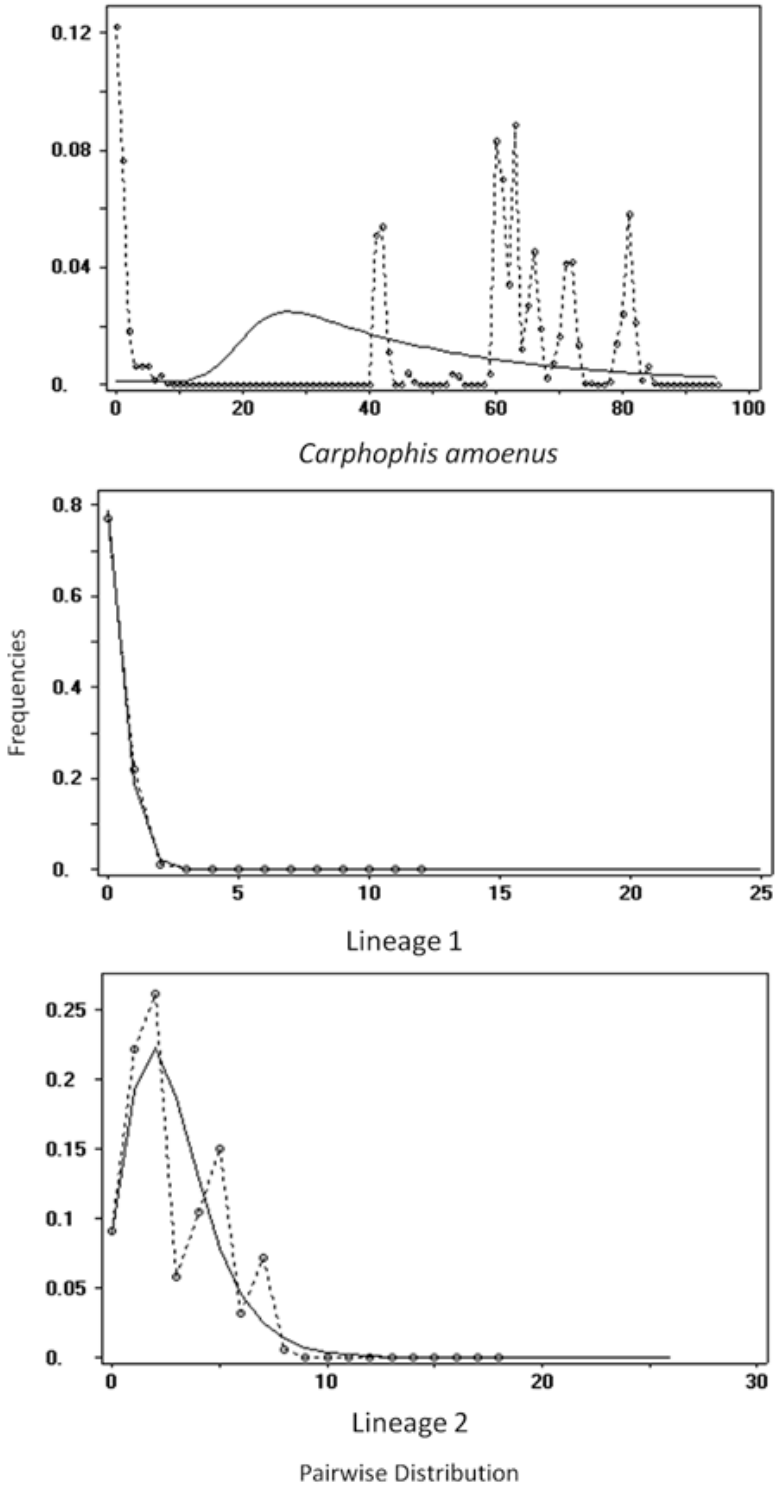
Different neutrality tests were conducted for each lineage, with the exception of the outgroups (*D. punctatus, F. erytrogramma, F. abacura* and *C. vermis*) and lineage 3, since it only contains one individual. However, this sample was grouped into the *C. amoenus* lineage, to calculate the DNA polymorphisms and to conduct each of the neutrality tests for the whole species. These tests are used to examine growth rates of populations based on the null hypothesis of it evolving neutrally. If any of the tests prove to be non-significant ($p \geq 0.05$), the null hypothesis that the populations are evolving neutrally can be accepted (Fontanella et al. 2008). Inconsistent values between Tajima's D and Fu's Fs are likely a result of the Fu's Fs being a more powerful test than Tajima's D (Fu, 1997; Ramos-Onsins and Rozas, 2002). Fu's Fs show the population growth based on the number of haplotypes (Fu, 1997), while Tajima's D shows the population growth based on the number of segregating sites and the number of pairwise differences between sequences (Tajima, 1989).

The DNA polymorphisms, number of haplotypes (H), Haplotype diversity (Hd), nucleotide diversity (π) and pairwise differences (K) of each lineage, along with Tajima's D, Fu's Fs, Ramos-Onsins and Rozas R_2 and the raggedness (r) statistics were all recorded in Table 2. The significance of Tajima's D and Fu's are indicated by an asterisk which are also shown in Table 2. The only lineage in which both the Tajima's D (-1.73; p=0.002) and Fu's Fs (-3.08; p=0.002) were significant, was Lineage 1.

The results of mismatch distribution of pairwise differences (Fig. 5) were all unimodal with the exception of the whole species of *Carphophis amoenus*. Unimodal indicates there is a growth in the population, while multimodal represents populations that are either relatively stable or that are contracting (Harpending et al. 1998). However, the statistical strength of mismatch distributions are relatively weak (Ramos-Onsins and Rozas, 2002). Both the raggedness (r) Ramos-Onsins and Rozas R_2 statistics were obtained from conducting the mismatch distributions and are also reported in Table 2.

Lineage	Sample Size	S	H	Hd	π	K	Tajima's D	Fu's Fs	R_2	r
Carphophis amoenus	85	141	21	0.878	0.05006	48.5577	2.0738	26.9347	0.1708	0.0333
Lineage 1	25	3	4	0.230	0.00023	0.24000	-1.7333*	-3.0845*	0.1083	0.3467
Lineage 2	18	13	9	0.908	0.00293	2.84314	-0.9241	-2.2951	0.0982	0.0836
Lineage 4	23	6	6	0.715	0.00100	1.06719	-1.0513	-1.8360	0.1004	0.1545
Lineage 5	18	1	2	0.366	0.00033	0.36601	0.4881	0.7961	0.1830	0.2058

Table 2. Sample size, segregating sites (S), number of haplotypes (H), haplotype diversity (Hd), nucleotide diversity (π) and pairwise differences (K). The significance ($p \leq 0.05$) of the statistics Tajima's D and Fu's Fs, are indicated by an *. Romos-Onsins and Rozas R_2 and the raggedness statistic r were calculated with Mismatch Distribution.


0.12
0.08
0.04
0.
0
20
40
60
80
100
Carphophis amoenus
0.8
0.6
0.4
0.2
0.
Frequencies
0
5
10
15
20
25
Lineage 1
0.25
0.2
0.15
0.1
0.05
0.
0
10
20
30
Lineage 2
Pairwise Distribution

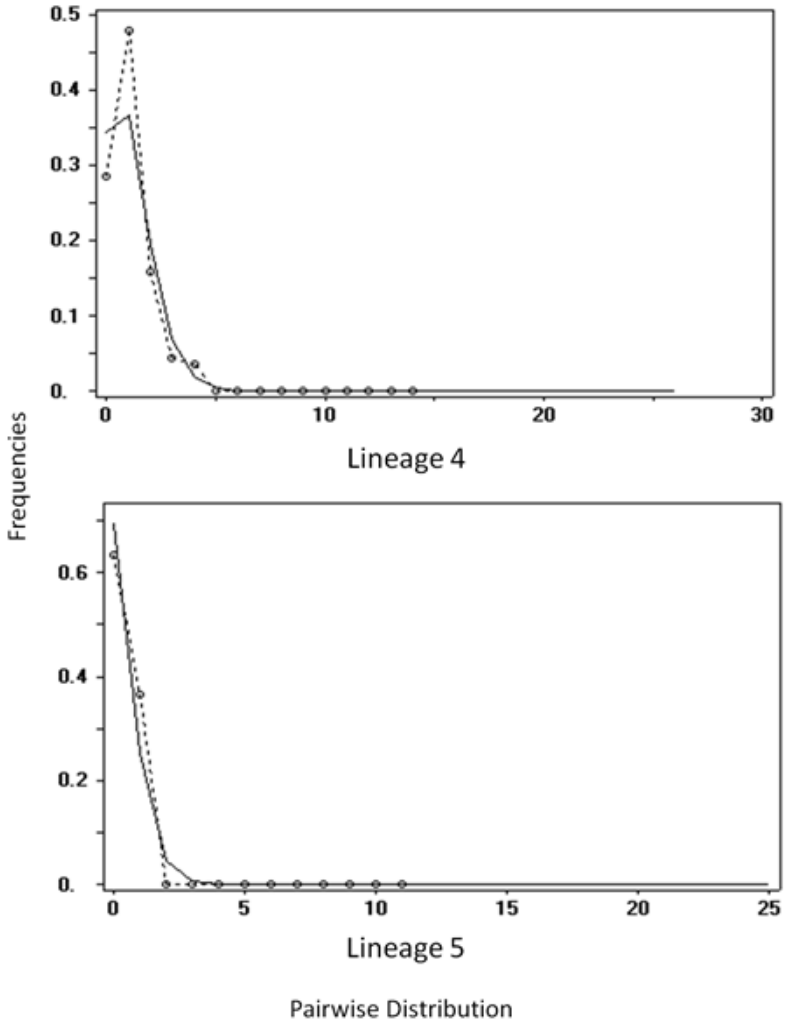


Fig. 5. Mismatch distribution of pairwise differences for all inferred lineages and both species (*C. amoenus* and *C. vermis*), with the exception of Lineage 3. Open circles represent observed distribution for each pairwise difference. Solid lines show the expected distributions for each pairwise difference.

IV. DISCUSSION

Phylogeographic Patterns of *Carphophis amoenus*

There were five inferred lineages within the species *Carphophis amoenus*, three of which contain potential geographic ranges that overlap. The extent of the geographic ranges for lineages 3, 4 and 5 are still unknown and more sampling in the area will need to be done. The known geographic range of Lineage 3 is in central Alabama, where this one individual was obtained from Bibb county Alabama. Lineage 4 is distributed in eastern Kentucky, likely in Tennessee, although samples were not obtained from this state, and throughout the Eastern side of Alabama and was found in Western Georgia. Lineage 5 consists of geographic range that is located in Eastern Alabama and the only samples that were grouped in this inferred lineage appear to be restricted to this area. The complex geography in Eastern US promotes potential genetic patterns in closely related species (Soltis et al. 2006). With the low dispersibility of the *C. amoenus* (Barbour et al. 1969), the effects of geographic barriers on the genetic patterns becomes more pronounced. The Appalachian Mountains may be limiting dispersal and gene flow between Lineage 1 and the remaining lineages to the west of the mountain range. The Mississippi River is likely a barrier which limits the dispersal of *C. amoenus* to the western side of the Mississippi River. With the geographic range of *C. vermis* occurring to the west of the Mississippi River and the geographic range of *C. amoenus* occurring primarily to the east of the Mississippi River, this geographic range is likely a barrier between the two species. The North American Coastal Plain (NACP) is considered a biodiversity hotspot, where it ranges from Massachusetts to Florida, into Georgia, Alabama, throughout Mississippi, up through southern Illinois, southeastern Missouri,

down into Arkansas, southeastern Oklahoma, eastern and northeastern Mexico (Noss et al. 2015). Although this region of the US contains many flat habitats, which is generally coincided with having low endemism, the NACP is a region that contains many endemic species, due to the displacement through glaciation events (Noss et al. 2015). Lineages 1 and 2 contain portions of their distribution that fall into this region. Lineage 5 appears to consist of distributions that also fall into this region, with the potential for the distribution of Lineage 3 expand into the NACP. Lineages 4 is adjacent to this biodiversity hotspot, whereas Lineage 2 is located primarily in the NACP

The Appalachian Mountains and the Mississippi River are two geographic barriers which have an effect on the genetic structuring in *C. amoenus*, and are also found to have an effect on the genetic breakpoints across different taxa including: American rat snake *Elaphe obsoleta*, (Burbrink et al. 2000), painted turtle *Chrysemys picta*, (Starkey et al. 2003), Virginia pine *Pinus virginia*, (Parker et al. 1997) and spring peeper *Pseudacris crucifer*, (Austin et al. 2004). There are however, three remaining genetic breakpoints associated with *C. amoenus* located in Alabama. This genetic structuring is not unique to *C. amoenus* and similar structures can be observed in horned passalus *Odontotaenius disjunctus* (Garrick et al. 2019) and ground skink *Scincella lateralis* (Jackson and Austin, 2010).

The divergence event that gave rise to the *C. amoenus* species occurred during the early Pleistocene (Blancan time frame in North America). During this time, the northern hemisphere was going through a cooling event, which eventually led to the Kansan glaciation event (about 1.6 my) in North America (Berggren, 1972). This division resulted in Lineage 1, which dates back to the Irvingtonian time frame in the Pleistocene. The next split occurred about 2.30 mya during the early Pleistocene, giving rise to Lineage 2. Lineages 1 (0.94 my) and 2 (0.90 my) are the oldest of the five inferred lineages in *C. amoenus* (Fig. 6). This coincides with the Kansan glaciation event, which changed the climate and ecology in unglaciated areas, which may have shifted current populations of *C. amoenus* to remain in southern refugia. Once the glacier receded about 0.9 mya, the populations which consisted of the haplotypes in the two inferred lineages, eventually split off during the expansion to higher latitudes and created two inferred lineages. Currently, *C. amoenus* does not extend past the fault line in Georgia however; some of the oldest fossils were found in Florida and have been dated back to the early Pleistocene during the Irvingtonian time frame (Holman, 1976; 2000). Due to the oldest known fossils of *C. amoenus* being found in Florida, it is likely that this species originated from the Eastern U.S.

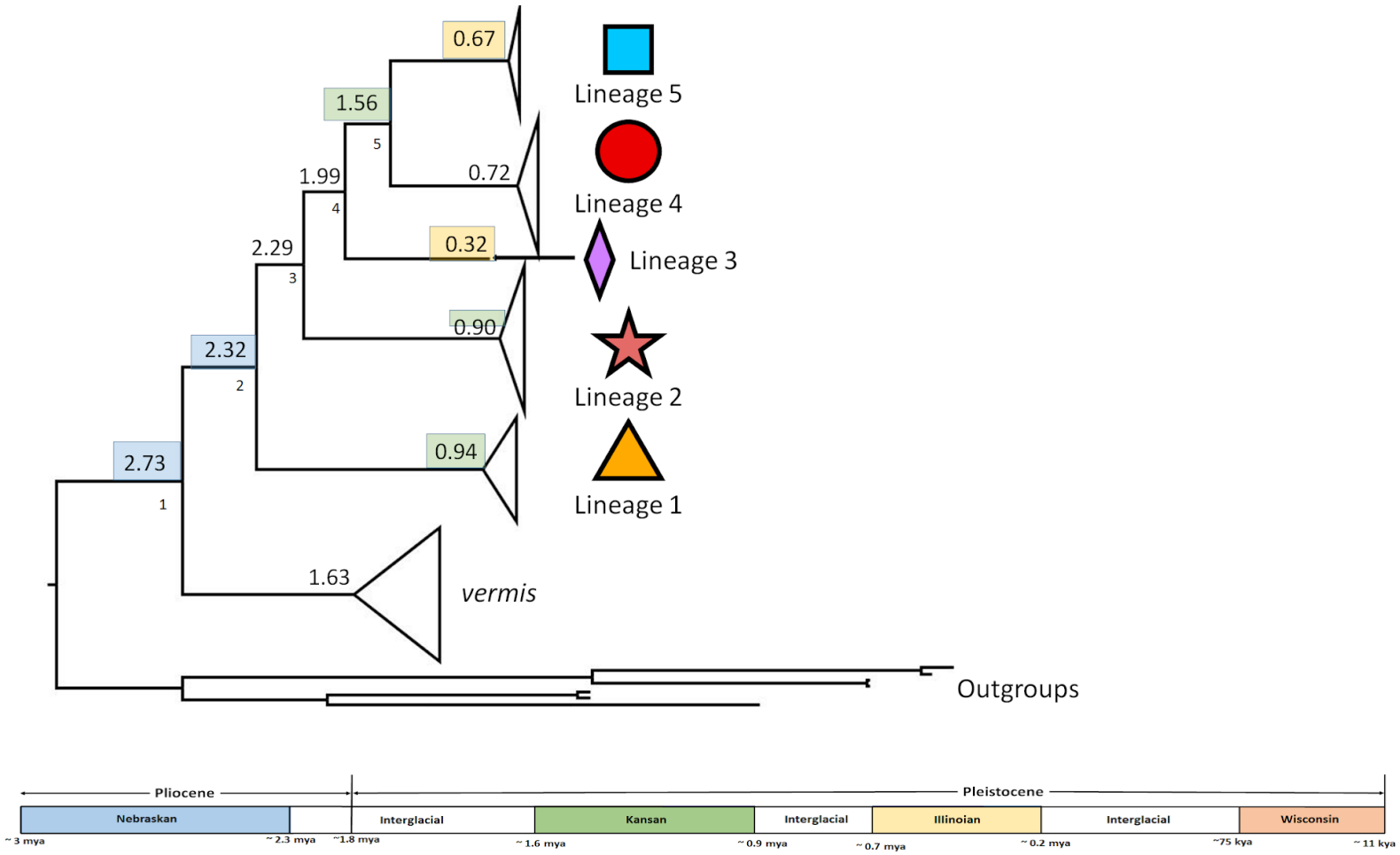


Fig. 6: Glaciation events and Phylogenetic tree. Colored numbers are associated with certain colored glaciation events.

Demographic History

During the Pleistocene, the glaciation events have become more pronounced (Hewitt, 1996). In eastern North America, the Laurentide glacier extend south to ~40°N, which caused a shift in the climate driving organisms into lower latitudes (Hewitt, 1996). During the Pleistocene there were a series of glacial and interglacial events, in which interglacial is a warmer event that occurred in between glacial events. During interglacial events, the glacial ice sheets would recede to higher latitudes, temporarily changing the climate along the glacial margin (Hewitt, 1996). As glaciers recede, species living closer to the glacial margin would experience rapid growth out of southern refugia into more northern latitudes, a process is known as the leading edge effect (Hewitt, 1996). The effects of glaciation events combined with barriers to dispersal, can promote genetic differentiation between populations isolated in southern refugia, due to increased genetic drift associated with decreased population size (genetic bottlenecks) and by limiting dispersal between different populations.

Appalachian Clade

Lineage 1 is distributed throughout the eastern coast of the US, and is likely found at higher latitudes than the other inferred lineages. Both the Tajima's D (-1.73) and Fu's Fs (-3.08) (Table 2) are significantly negative for this lineage, which are shown in populations that are not evolving neutrally and that this population is going through a rapid expansion. The unimodal distribution (Fig. 5), along with a low haplotype and nucleotide diversity, further support the

evidence that this population is going through a rapid growth. Rapid expansion during an interglacial event is a common characteristic to species, which contain a geographic range higher in latitude than those of closely related species living in more southern refugia. This lineage has been largely shaped by the Appalachian Mountains, creating a barrier between the inferred lineages found in the east and west of the mountain range.

The Appalachian Mountains discontinuity are commonly observed in many species, some of which include tiger salamander *Ambystoma tigrinum tigrinum*, (Church et al. 2003), slider turtle *Trachemys scripta*, (Avise et al. 1992), and groundnut *Apios americana,* (Joly and Bruneau, 2004). This genetic discontinuity is likely a result of glacial advances to lower latitudes driving the current populations to recede further south in latitude, and as the glacier would recede to higher latitudes, populations would expand to previously glaciated areas (Soltis et al. 2006; Hewitt, 1996). The Appalachian Mountains range from Canada to Georgia creating a geographic barrier, which can potentially limit the dispersibility of many species. This is observed in *C. amoenus*, where the eastern and western parts of the Appalachian Mountains contain different genetic structuring in this species.

The current geographic range of Lineage 1 expands into previously glaciated areas, which would be unsuitable for this species to survive. In order for this population to survive, they would have

to persist in more southern refugia until an interglacial event occurs. The age of this lineage is 0.94 my, while the age of the first glaciation event in the Pleistocene (Kansan) is approximately 1.6 to about 0.9 my (Berggren, 1972) (Fig. 6). This lineage is older than the interglacial event following the Kansan glaciation event, which indicates the formation of this population was likely affected by the Laurentide glacier. The result of the interglacial event after 0.90 my would allow this population to expand into higher latitudes.

Mississippian River Clade

Lineage 2 consists of the geographic range, which is distributed along the eastern side of the Mississippi River. Extends as far north as southern Illinois and as far south as southern Louisiana. This lineage contains non-significant negative values for Tajima's D (-0.9241) and Fu's Fs (-2.2951) (Table 2), which can indicate a slow or more gradual growth. This lineage also exhibits a unimodal distribution, which indicates a population growth. The nonsignificant negative values, along with the high number of haplotypes, further supports the evidence that this population is expanding at a slow or gradual rate.

C. amoenus is known to extend into the eastern most location of Arkansas (Fig. 2). However, since samples were not obtained from Arkansas, the extent of the geographic range for Lineage 2 are unknown. This is the only lineage distributed along the eastern side of the Mississippi River.

More data will be beneficial in the phylogeographic study of *C. amoenus*, since it will provide more insight on the extent of each of the genetic breakpoints/patterns. The Mississippi River is another common genetic breakpoint (Soltis et al. 2006). The Mississippi River discontinuity is commonly seen all throughout the taxa, where there are genetically different clades and some examples consist of eastern fence lizard *Sceloporus undulates*, (Leaché and Reeder, 2002), loblolly pine *Pinus taeda*, (Al-Rabab'ah and Williams, 2002) and northern leopard frog *Rana pipiens,* (Hoffman and Blouin, 2004). The geographic distribution of *C. amoenus* extends to the very east of Arkansas, which is west of the Mississippi River. However, samples of *C. amoenus* were not obtained from this area, so we cannot be sure what the genetic structure is for this species in that area.

Mid-Eastern US Clade

The statistics determining the change in population size or DNA polymorphisms were not collected from Lineage 3, due to only one individual being grouped into this inferred lineage. So, the effects of the Pleistocene glaciation are not yet known and more sampling needs to be done with this lineage to analyze if the LGM had an effect on the demography of this population. Lineage 4 contains non-significant negative Tajima's D (-1.0513) and Fu's Fs (-1.836) (Table 2), along with unimodal distribution, and a high number of haplotypes, which are results that are concordant to that of a population going through a gradual growth. However, Lineage 5 contains non-significant positive values for Tajima's D (0.4881) and Fu's Fs (0.7961) (Table 2). These

results are observed in populations which are either stable or contracting. Although the mismatch distribution is unimodal, all the other results suggest a stable population. The reason for the discrepancy between the statistical tests could be that the mismatch distribution is a weaker statistic (Ramos-Onsins and Rozas 2002).

There are three different mitochondrial groups located in Alabama, which could be due to the genetic barriers associated with fragmentation. The genetic barriers which attribute to the coalescent events between these three lineages, are still unknown and more sampling in the area would gain a better insight. However, due to the fossorial nature of the worm snake, limited dispersibility is the likely cause giving rise to additional lineages. The different geographic landforms in these regions can help prevent gene flow between the different populations. Lineage 5 is the only lineage which had a positive Tajima's D and Fu F's values, which are indicative to populations that are stable. The leading edge hypothesis is being displayed in Lineages 4 and 5 with Lineage 4 persisting in higher latitudes (Fig. 4) it is able to expand into more northern latitudes without the limitations of another population of closely related species affecting its dispersibility. Where Lineage 5 is more confined to southern latitudes, due to Lineage 4 restricting their expansion north.

Lineage 4 (0.72 my) is estimated to be older than each of the three inferred lineages within this geographic range, while Lineage 5 is estimated to be about 0.67 my. Lineages 4 and 5 are

estimated to have originated from the Irvingtonian time frame in North America, while Lineage 3 is estimated to have originated from the Rancholabrean time frame in North America. The youngest estimated date of each of the inferred lineages is Lineage 3, which is about 0.32 my. This however, could be likely due to only being calculated from one individual and more samples which belong to this inferred lineage would provide a better and closer estimate to the actual age of this lineage. This age is still older than the LGM which took place about 0.18 my, and the result of this glacial advance was not the result of the formation of this lineage.

V. CONCLUSION

Since all the divergence events and the formation of each of the inferred lineages occurred prior to the LGM, this glaciation event would have affected the geographic ranges and/or the demography of each lineage. Although the glaciation events were not a direct cause of lineage formation or divergence events of the five inferred lineages, Lineages 1, 2 and 4 contain a larger geographic range than the remaining inferred lineages. Lineage 1 is the only lineage, which consists of a population that has experienced a rapid expansion (Fig. 7), while Lineages 2 and 4 show patterns consistent with slower growth (Table 2). The pattern associated with Lineage 1 is commonly observed in populations, which consists of geographic ranges along the glacial margin (Hewitt, 1996). During the interglacial event, after the LGM, Lineage 1 was able to go through a rapid expansion; whereas, Lineage 5 with nonsignificant positive values and its geographic range this population is likely restricted to more southern latitudes, resulting in a smaller geographic range, preventing an expansion.

Currently the *C. amoenus* species consists of two recognized subspecies *C. a. amoenus* (Eastern worm snake) and *C. a. helenae* (Midwestern worm snake). If the current taxonomy represented the evolutionary history, then we would expect to see main clades with haplotype distribution consistent with the geographic distributions of the nominal subspecies. The lineage diversity inferred from this study does not correspond to the current taxonomy and suggests that in its current state, the taxonomy underestimates the genetic diversity and potential species diversity

across the range. There are often inconsistencies between the mtDNA, which measures historical divisions in many species, and the relationship with subspecies boundaries (Zink, 2004), which is observed in this species. Identifying subspecies based on a single morphological characteristic, can end up eliminating the name of many subspecies when compared with mtDNA (Zink, 2004). The American rat snake, *Elaphe obsoleta* is an example of subspecies being identified based on the color pattern (Burbrink et al. 2000). The cactus wren, *Campylorhynchus brunneicapillus* is another example where there were six identified subspecies; however, there were only two mtDNA groups (Zink et al. 2001). There are three genetically different mtDNA groups of the *C. amoenus* species in Alabama and this species consists of more genetic diversity than previously thought. Implementing conservation efforts would greatly influence the preservation of the genetic diversity of this species and help reduce the effects that humans have on their habitat.

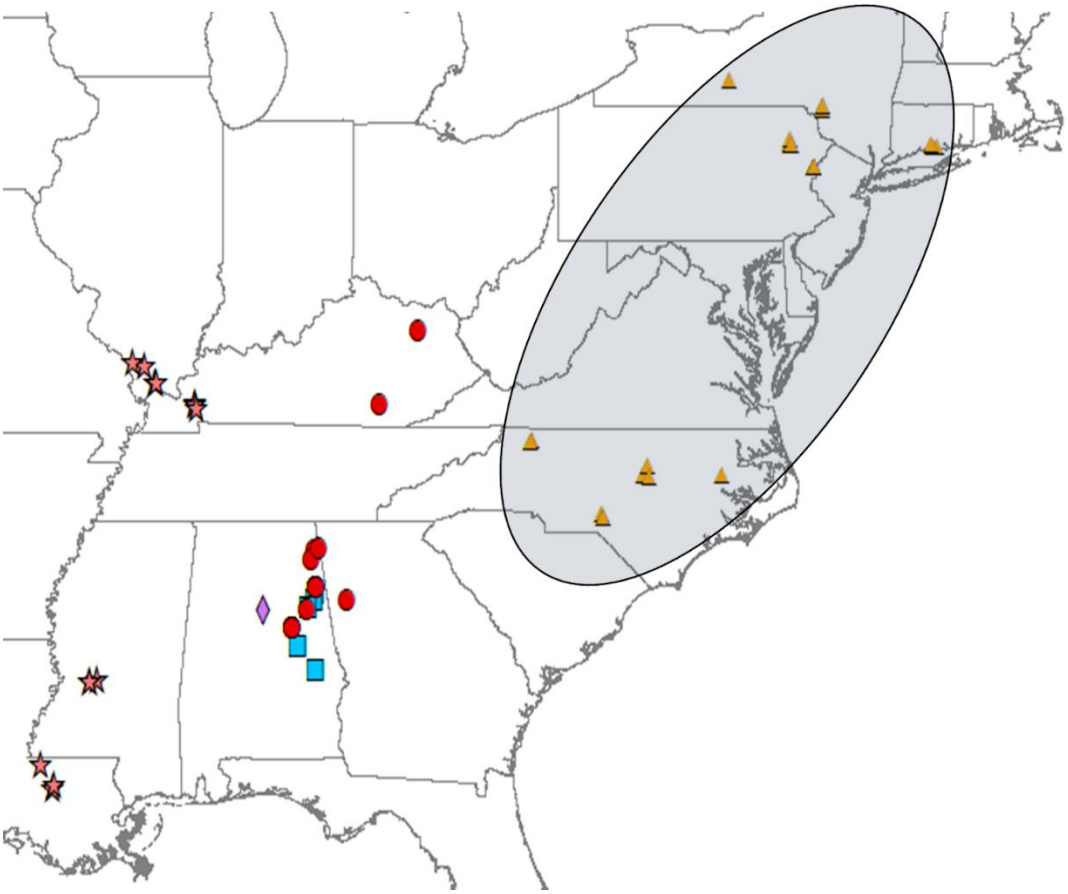


Fig 7. Outlined lineage indicates rapid expansion.

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Appendix

Genus	Species	County	State	Accession Number	Museum
Carphophis	amoenus	Carroll	GA	UWG 034	University of West Georgia
Carphophis	amoenus	TNF	AL	UWG 049	University of West Georgia
Carphophis	amoenus	TNF	AL	UWG 050	University of West Georgia
Carphophis	amoenus	TNF	AL	UWG 051	University of West Georgia
Carphophis	amoenus	TNF	AL	UWG 052	University of West Georgia
Carphophis	amoenus	TNF	AL	UWG 053	University of West Georgia
Carphophis	amoenus	TNF	AL	UWG 054	University of West Georgia
Carphophis	amoenus	TNF	AL	UWG 055	University of West Georgia
Carphophis	amoenus	TNF	AL	UWG 056	University of West Georgia
Carphophis	amoenus	TNF	AL	UWG 057	University of West Georgia

Carphophis	amoenus	TNF	AL	UWG 058	University of West Georgia
Carphophis	amoenus	TNF	AL	UWG 059	University of West Georgia
Carphophis	amoenus	TNF	AL	UWG 060	University of West Georgia
Carphophis	amoenus	TNF	AL	UWG 061	University of West Georgia
Carphophis	amoenus	TNF	AL	UWG 062	University of West Georgia
Carphophis	amoenus	TNF	AL	UWG 063	University of West Georgia
Carphophis	amoenus	TNF	AL	UWG 064	University of West Georgia
Carphophis	amoenus	TNF	AL	UWG 065	University of West Georgia
Carphophis	amoenus	TNF	AL	UWG 066	University of West Georgia
Carphophis	amoenus	TNF	AL	UWG 086	University of West Georgia
Carphophis	amoenus	TNF	AL	UWG 087	University of West Georgia
Carphophis	amoenus	TNF	AL	UWG 088	University of West Georgia
Carphophis	amoenus	TNF	AL	UWG 089	University of West Georgia

Carphophis	amoenus	TNF	AL	UWG 090	University of West Georgia
Carphophis	amoenus	DeKalb	AL	LSU H-2188	Louisiana State University
Carphophis	amoenus	East Baton Rouge	LA	LSU H-2484	Louisiana State University
Carphophis	amoenus	DeKalb	AL	LSU H-2660	Louisiana State University
Carphophis	amoenus	DeKalb	AL	LSU H-2661	Louisiana State University
Carphophis	amoenus	East Baton Rouge	LA	LSU H-16060	Louisiana State University
Carphophis	amoenus	Jefferson	AL	LSU H-18821	Louisiana State University
Carphophis	amoenus	West Feliciana	LA	LSU H-19841	Louisiana State University
Carphophis	amoenus	Jackson	IL	CAS 169503	California Academy of Sciences
Carphophis	amoenus	Jackson	IL	CAS 160710	California Academy of Sciences
Carphophis	amoenus	Johnson	IL	CAS 162001	California Academy of Sciences

Carphophis	amoenus	Moore	NC	NCSM 74442	North Carolina State Museum
Carphophis	amoenus	Richmond	NC	NCSM 75982	North Carolina State Museum
Carphophis	amoenus	Richmond	NC	NCSM 77263	North Carolina State Museum
Carphophis	amoenus	Wake	NC	NCSM 77323	North Carolina State Museum
Carphophis	amoenus	Wilkes	NC	NCSM 78278	North Carolina State Museum
Carphophis	amoenus	Wilkes	NC	NCSM 80754	North Carolina State Museum
Carphophis	amoenus	Laurel	KY	HERR 013611	Yale Peabody Museum
Carphophis	amoenus	New Haven	CT	HERR 016196	Yale Peabody Museum
Carphophis	amoenus	Middlesex	CT	HERR 019083	Yale Peabody Museum
Carphophis	amoenus	New Haven	CT	HERR 019279	Yale Peabody Museum

Carphophis	amoenus	Johnson	IL	FLN 3713 (INHS 23655)	Illinois Natural History Survey
Carphophis	amoenus	Rowan	KY	MSU 1	University of West Georgia
Carphophis	amoenus	Wake	NC	MVZ 137689	Museum of Vertebrate Zoology, UC Berkeley
Carphophis	amoenus	Martin	NC	MVZ 137556	Museum of Vertebrate Zoology, UC Berkeley
Carphophis	amoenus	Wake	NC	MVZ 137555	Museum of Vertebrate Zoology, UC Berkeley
Carphophis	amoenus	Lyon	KY	SBNHM 13183	Fort Hays Sternberg Museum of Natural History
Carphophis	amoenus	Lyon	KY	SBNHM 13192	Fort Hays Sternberg Museum of Natural History
Carphophis	amoenus	Lyon	KY	SBNHM 13193	Fort Hays Sternberg Museum of Natural History
Carphophis	amoenus		NJ	FMF 021	Frank M. Fontanella
Carphophis	amoenus		NJ	FMF 0212	Frank M. Fontanella
Carphophis	amoenus		NJ	FMF 105	Frank M. Fontanella
Carphophis	amoenus		NJ	FMF 109	Frank M. Fontanella

Carphophis	amoenus		NJ	FMF 112	Frank M. Fontanella
Carphophis	amoenus		NY	FMF 500	Frank M. Fontanella
Carphophis	amoenus		NY	FMF 502	Frank M. Fontanella
Carphophis	amoenus		NY	FMF 506	Frank M. Fontanella
Carphophis	amoenus		NY	FMF 508	Frank M. Fontanella
Carphophis	amoenus		PA	FMF 509	Frank M. Fontanella
Carphophis	amoenus		PA	FMF 510	Frank M. Fontanella
Carphophis	amoenus		PA	FMF 511	Frank M. Fontanella
Carphophis	amoenus		PA	FMF 513	Frank M. Fontanella
Carphophis	amoenus	Hinds	MS	MMNS 4829	Mississippi Museum of Natural Science
Carphophis	amoenus	Hinds	MS	MMNS 4744	Mississippi Museum of Natural Science
Carphophis	amoenus	Hinds	MS	MMNS 4745	Mississippi Museum of Natural Science
Carphophis	amoenus	Clay	AL	UWG 100	University of West Georgia
Carphophis	amoenus	Clay	AL	UWG 101	University of West Georgia

Carphophis	amoenus	Clay	AL	UWG 102	University of West Georgia
Carphophis	amoenus	Clay	AL	UWG 103	University of West Georgia
Carphophis	amoenus	Clay	AL	UWG 104	University of West Georgia
Carphophis	amoenus	Clay	AL	UWG 106	University of West Georgia
Carphophis	amoenus	Clay	AL	UWG 107	University of West Georgia
Carphophis	amoenus	Clay	AL	UWG 108	University of West Georgia
Carphophis	amoenus	Clay	AL	UWG 109	University of West Georgia
Carphophis	amoenus		AL	BB_05	University of West Georgia
Carphophis	amoenus			Genbank Sequence	Genbank
Carphophis	amoenus			CARPHOPHIS amoenus	Genbank
Carphophis	amoenus			2482 C. amoenus	
Carphophis	vermis			162002	

Carphophis	vermis	Crawford	KS	FHSM HERP13267	Fort Hays Sternberg Museum of Natural History
Carphophis	vermis	Le Flore	OK	OMNH OCGR12619	Sam Noble Oklahoma Museum of Natural History
Carphophis	vermis			DBS952	
Carphophis	vermis	Le Flore	OK	OMNH OCGR9902	Sam Noble Oklahoma Museum of Natural History
Carphophis	vermis	Polk	AR	OMNH OCGR6985	Sam Noble Oklahoma Museum of Natural History
Carphophis	vermis	Bourbon	KS	FHSM HERP10495	Fort Hays Sternberg Museum of Natural History
Carphophis	vermis	Bourbon	KS	FHSM HERP10492	Fort Hays Sternberg Museum of Natural History
Carphophis	vermis			9404	Fort Hays Sternberg Museum of Natural History
Carphophis	vermis	Wilson	KS	FHSM HERP7661	Fort Hays Sternberg Museum of Natural History
Carphophis	vermis	Elk	KS	FHSM HERP12757	Fort Hays Sternberg Museum of Natural History

Carphophis	vermis	Wyandotte	KS	FHSM HERP12509	Fort Hays Sternberg Museum of Natural History
Carphophis	vermis			11504	
Carphophis	vermis	Jefferson	KS	FHSM HERP12491	Fort Hays Sternberg Museum of Natural History
Carphophis	vermis	Linn	KS	FHSM HERP8299	Fort Hays Sternberg Museum of Natural History
Carphophis	vermis	Jefferson	KS	FHSM HERP12508	Fort Hays Sternberg Museum of Natural History
Carphophis	vermis	Atoka	OK	OMNH OCGR6556	Sam Noble Oklahoma Museum of Natural History
Carphophis	vermis	Lyon	KS	FHSM HERP13528	Fort Hays Sternberg Museum of Natural History
Carphophis	vermis	Wyandotte	KS	FHSM HERP12628	Fort Hays Sternberg Museum of Natural History
Carphophis	vermis	Cherokee	OK	HFSM HERP10786	Fort Hays Sternberg Museum of Natural History
Carphophis	vermis	Bourbon	KS	FHSM HERP10506	Fort Hays Sternberg Museum of Natural History

Carphophis	vermis	Latimer	OK	OMNH OCGR2607	Sam Noble Oklahoma Museum of Natural History
Carphophis	vermis	Allen	KS	FHSM HERP11133	Fort Hays Sternberg Museum of Natural History
Diadophis	punctatus			UWG022	
Diadophis	punctatus	Oxford	AL	BB02	
Diadophis	punctatus			UWG009	
Diadophis	punctatus			UWG013	
Farancia	abacura			Farancia abacura voucher	Genbank
Farancia	abacura			Farancia abacura isolate	Genbank
Farancia	erytrogramma			Farancia erytrogramma voucher	Genbank