Phylogenetic Relationships and Divergence Dating in the Glass Lizards (Anguinae)

by

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ABSTRACT

The Glass Lizards are a subfamily (Anguinae) of Anguid Lizards with an elongated limbless body plan that occur throughout the Northern Hemisphere primarily in North America, Europe, and Asia, but also have a presence in North Africa and Indonesia. We use twenty-five nuclear and mtDNA loci to generate a phylogeny to explore species relationships within the group as well as divergence dating. We also examine the group in the context of a coalescent species tree analysis and species delimitation. All major lineages were found to be monophyletic with potential cryptic diversity in some. The Anguinae are an old group first appearing in the Eocene and most lineages present by the beginning of the Miocene. The Anguinae likely did originate in Europe from an Anguidae ancestor that crossed the Thulean land Bridge, spreading to Asia after the drying of the Turgai Sea, then across Beringia as the climate permitted. Species delimitation did not support every accepted species grouping some closely related species together as units. A BAMM analysis found a steady rate of speciation.

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Table of Contents

Chapter	Page
I. Introduction.....	01-08
II. Methods.....	08-13
a. Sampling Protocols.....	08
b. Laboratory Protocols.....	08-09
c. Dataset Organization and Partition/Model Testing.....	09-10
d. Phylogenetic Analyses Protocols.....	10-11
e. Molecular Dating Protocols.....	11-12
f. Species Tree and Delimitation Analyses Protocols.....	12-13
g. BAMM protocols.....	13
III. Results.....	14-18
a. Alignment and Model Testing.....	14-15
b. Phylogenetic Analysis.....	15-17
c. Molecular Dating.....	17
d. Species Tree, Species Delimitation, and BAMM.....	17-18
IV. Discussion.....	19-32
a. Phylogeny of the Anguinae.....	19
b. Placement of the <i>Hyalosaurus</i> Lineage.....	19-20
c. Historical relationships of European Lineages.....	20-22
d. Relationships among Asian Lineages.....	22-23
e. Relationships among North American Lineages.....	23-24
f. Origin of Anguinae in Europe.....	24-25
g. Radiation of Anguinae in Europe.....	25-27
h. Radiation of the Anguinae in Asia.....	28-29
i. Radiation of the Anguinae in North America.....	29-31
j. Species Tree, Species Delimitation, and BAMM.....	31-32
V. Conclusion.....	33
VI. References.....	34-41
VII. Tables.....	42-55
VIII. Figures.....	56-62

List of Tables

Table 1. Voucher Numbers, Species Identification, General Locality, and Genbank Submission Numbers of Anguinae taxa used in this study.

Table 2. Locus Model Testing Results from Partition Finder.

Table 3. Locus Model Testing for Coalescent Analyses.

Table 4. MRCA of species and important Anguinae clades. Bold numbers indicate hard constraints under a uniform prior.

List of Figures

Figure 1. Map showing sampling localities of Anguinae samples or sequences used in this study.

Figure 2. Bayesian mtDNA phylogeny of Anguinae.

Figure 3. Bayesian nuclear phylogeny of Anguinae.

Figure 4. Bayesian concatenated phylogeny of Anguinae.

Figure 5. Chronogram of Anguinae denoting node dates and 95% credibility intervals.

Figure 6. Species Tree of Anguinae denoting consensus figure as well as a Densitree plot overlaying all post burn in trees.

1. Introduction

Climate Change has been one of the defining features of the Cenozoic with global greenhouse conditions of the Paleocene and Eocene eventually giving way to a cooling climate and the glacial cycles of the Pliocene and Pleistocene (Raymo and Ruddiman 1992, Zachos et al. 2001). This gradual climate change accompanied by both wetter and dryer periods produced dramatic habitat changes, particularly in forest habitat. During the Paleocene and Eocene a boreotropical forest existed into the arctic region of North America, Asia, and Europe. Gradually cooling climates caused the more boreal tropical elements to retreat and the forests to fracture with the appearance of grasslands and grassland dominated habitat. Grassland habitats are thought to first come into prominence with global cooling during the Oligocene. The actual spread of grassland habitat is complex, though, with the potential spread of C3 and C4 grasses and grassland dominated habitat occurring during two periods 20 million years apart (Stromberg 2011). The widespread appearance of grasslands and fragmentation of the forest habitat are thought to have promoted speciation and radiation of taxa into new forms to exploit this expanding niche. However, climatic fluctuations such as the Miocene Climatic Optima, with a temporary return to greenhouse conditions, and later glacial cycling would ensure a dynamic fluctuating forest/grassland environment.

While much of the explanation regarding the biogeography of the Gondwanaland continents and subcontinents (Antarctica, Africa, South America, Australia, and India) typically invoke vicariance due to the break-up of the supercontinent, the continents of the former Laurasian Supercontinent (North America, Asia, and Europe) have involved both continental scale breakup as well as intermittent re-connection by northern land bridges at various points

in the Cenozoic. North America and Europe were connected by both the Thulean and De Greers Land Bridges in the Eocene (Brikiatis 2014, Sanmartin et al. 2001). While both land bridges would be possible for warm adapted taxa when greenhouse conditions were present, the Thulean land bridge is suspected to have been a more important avenue for dispersal and the De Greers land bridge would have been a lesser-used route for these species due to longer seasonal nighttime conditions given its more northerly position (Tiffney 1985). Europe and Asia were separated by the Turgai Sea and later, by the Turgai Strait through the Paleocene and Eocene up until the Oligocene, when the drying of the Strait would allow a permanent connection to modern times (Briggs 1987) (Figure 1). Asia and North America would also be connected by the region known as Beringia. Although many biogeographic studies of this intercontinental interaction focus on more recent connections during cyclic periods during the Pleistocene, the link between North American and Asian continents is known to have been present throughout the earlier periods of the Cenozoic as well (Burbrink and Lawson 2007, Sanmartin et al 2001). The use of this intercontinental connection as a land bridge would primarily be limited by climate as the boreotropical forests of the Paleocene and Eocene degraded into more cold adapted forest, grasslands, and eventually the modern tundra.

The Neoanguimorphia are a group of squamate lizards with most of their diversity located in the New World. This group contains the venomous Gila Monsters and Beaded Lizards (Helodermidae), rock dwelling Xenosauridae, fossorial Annielidae, and the more generalist Dipsoglossidae and Anguidae (Conrad 2008, Conrad et al 2011, Pyron et al 2013, Vidal and Hedges 2009). The Anguidae, or Alligator Lizards, are fairly morphologically conserved in body type and are represented by both ground dwelling and arboreal forms.

Within the Anguidae, the subfamily Anguinae consist of a monophyletic assemblage of elongated legless grass-swimming ecomorphs. The Anguinae contains the only extant example of continental dispersal within the Neoamguimorphia out of the New World with the legless Glass Lizards occurring in Europe, North America, and Asia as well as North Africa and the previously land connected islands of Sumatra and Borneo. This extensive range is somewhat remarkable given a lower abundance of species seen in the Anguidae in general and extreme morphological conservation to a grass swimming body form, which may be indicative of limited niche exploitation. The Anguinae currently consist of 20 described species; 6 found in Europe, 1 in North Africa, 5 in Asia, 2 in Indonesia, and 6 in North America.

The Anguinae in Europe currently contain three genera which also contain the most distinct morphology within the subfamily. The generalist and monophyletic *Hyalosaurus koellikeri* is found in North Africa. The Slowworm species complex (*Anguis sp.*) is made up of five species which retain a number of paedomorphic traits such as small size as well as adaptations to colder environments, such as a viviparous birth and active at lower temperatures. Finally, there is the monotypic Sheltopusik (*Pseudopus apodus*), currently the largest member of the Anguinae with distinctive peg-like teeth. In Asia, the genus *Dopasia* occurs from eastern India to the island of Taiwan off the east coast of China. A morphological examination found at least two species groups and seven species, although many of the characters examined were internal (Nyugen et al 2011). In North America, Anguinae in the genus *Ophisaurus* are found primarily in the eastern US and east side of Mexico down to Veracruz with six species. Both the Asian and North American Anguinae are very similar in

morphology. This morphological similarity between species, even between different continents, has hindered a deeper understanding of the history of the Anguinae.

Previous work based on loci from the mitochondrial genome (mtDNA) hypothesized an out of North America then European origin for the Anguinae, a spread to Asia in the Oligocene after the drying of the Turgai Sea or Strait, then a dispersal back to North America through Beringia (Macey et al. 1999). Minimum dates were hypothesized, but it was noted these could be off if significant mtDNA saturation had occurred and the dating done was based on percent difference/rate calculation. Many taxa, and especially squamate reptiles, are suspected to have a center of origin in Asia due to a richness of species or basal forms in the region. A typical pattern, seen in many taxa, shows sister groups found in Asia and North America attributed to dispersal across Beringia followed by vicariance. In addition, during the Oligocene, with the drying of the Turgai Strait, Europe/Asian floral and faunal interchanges occurred as can be witnessed in the more Asian faunas of Europe (Hooker et al 2004). Other biogeographic patterns to consider include out of Europe and out of North America scenarios, although these appear much rarer than out of Asia scenarios. Both European and North American origin scenarios would explain a lack of species richness by extinction due to experiencing more extreme glaciation compared to Asia.

A series of molecular genetic studies of taxa within the Anguinae have continued to transform our understanding of the taxonomic units and relationships of the species within this subfamily. The mtDNA study by Macey et al. (1999) examined relationships within the Neoangimorphia and indicated that morphological based taxonomy and relationships may disagree with molecular data. *Pseudopus* and *Anguis*, the two most morphologically distinct

taxa were found to be sister taxa and the position of *Hyalosaurus* was uncertain, as were relationships between the Asian and North American species represented in the study. A more recent molecular genetic study of the Slowworm (*Anguis* sp.) found that the monotypic taxon *Anguis fragilis* was actually a species complex comprised of five species (Gvozdik et al. 2010, Gvozdik et al. 2013). In contrast, *Hyalosaurus koellikeri* from North Africa was found to be genetically very uniform and the only species studied with molecular methods throughout its range that did not display cryptic variation possibly due to bottlenecking and range reduction (De Pous et al. 2011). A molecular genetics study by Lin et al. (2003) showed *Ophisaurus harti* and *O. formenesis* in Taiwan formed only a single species. Finally, two localized studies examining *Anguis* and *Pseudopus* in Turkey may support the current subspecies arrangements for the species in the area (Keskin et al. 2013a, Keskin et al. 2013b) but extrapolation is difficult without range wide information.

The Anguinae are unique among many small taxa in that numerous fossils that represent the group have been found in both Europe and North America. In Europe the Anguinae first appear in multiple locations by 48.6 mya (Bolet and Evans 2013, Bolet and Auge 2014, Estravis 2000, Klembara and Green 2010, Meszoely and Haubold 1975, Rage and Auge 2003) and are known from the Oligocene (Cernansky and Auge 2012). In the Miocene taxa appear that are attributable to modern lineages. This is especially the case for the morphologically distinct *Pseudopus* lineage; *P. rugosus* (Klembara 2014) and *P. ahnikoviensis* (Klembara 2012) appear in the early Miocene, *Pseudopus laurillardii* (Klembara et al. 2010) occurs in the middle Miocene, *Pseudopus pannonicus* occurs in the late Miocene and Pliocene, and *Pseudopus apodus* is the sole surviving extant species.

In North America, the Anguinae are known in the Miocene from disarticulated fossils from Canada (Holman 1970) and Colorado (Holman 2003). More recent fossils occur in the Pliocene and Pleistocene and in some cases are attributed to modern species (Auffenburg 1955, Auffenburg 1956). The abundance of fossils generated soon after the group appears in Europe as well as for the *Pseudopus* lineage potentially make the Anguinae a good candidate group for a molecular dating approach. While numerous other fossils exist, the generalist forms are more difficult to tell apart and fossils alone may not be able to be attributed to the *Hyalosaurus*, *Dopasia*, *Ophisaurus*, or a stem lineage based on morphology alone, although molecular dating could potentially eliminate some possibilities.

Currently, the study based solely on a single mtDNA fragment provides the only previously proposed biogeographic scenario for the Anguinae and includes a phylogeny containing a single species for most lineages. Due to a general lack of morphological differentiation among species within Anguinae, a larger sampling of species is needed to understand the species limits and biogeography of this group. This would allow for a more rigorous test of proposed species groups based on morphological differences and to better verify monophyly of continental groups and species relationships. Mitochondrial DNA is known to be highly informative at the species level due to a generally higher rate of mutation than nuclear loci, but its rapid mutation rate and increased rate of saturation can, in certain circumstances, be misleading for molecular dating, especially at the older time scales which have been theorized for the Anguinae and Anguidae lizards in general. Furthermore, the Anguinae provide a unique opportunity to examine historical biogeography between the Laurasian continents in species known to have limited dispersal capabilities.

In this study we generate the most complete species level phylogeny of the Anguinae to date using multiple nuclear loci and mtDNA loci. Multiple nuclear loci have advantages over using a single locus with a single history even if more phylogenetic information is present, such as with mtDNA alone, and may even be preferable if enough time has passed that the mtDNA has become saturated. We then combine the phylogenetic information with fossil data to generate a time calibrated phylogeny in order to examine possible causes and timing of speciation in the Anguinae. Finally, we explore the congruence between a super tree and coalescent species tree analyses and perform a species delimitation analysis to explore species delimitation in the Anguinae.

2. Methods

2.1 Sampling Protocols

Thirty-three tissue samples were selected in order to represent species level diversity within the Anguinae. Museum records were searched via online institutional databases or Herpnet (<http://herpnet.org>) and tissue samples requested from respective institutions with a goal of representing as many taxa at the species level as possible. Multiple samples were included where possible for wide-ranging taxa to account for the possibility of cryptic species or unclear identification. Additional samples and sequences were included from Genbank for the creation of a composite outgroup and to expand geographic sampling for the mtDNA dataset. Outgroup sequences were chosen to represent the Gerrhontinae. In a few cases the composite outgroup taxon was also supplemented with new generated sequence. Sequences were also used from a published Anguinae genome (Song et al. 2015) and transcriptome (Jing-jing et al. 2014). **Table 1** lists databases used, museum vouchers for tissue samples, and Genbank numbers for sequences used. **Figure 1** shows locality information of ingroup tissue samples and sequence data used in this study. Final datasets for mtDNA, nuclear DNA, and the concatenated dataset did differ due to exclusion or inclusion of Genbank data. Specifically, more sampling was available via Genbank for mtDNA, while the concatenated dataset involved exclusion of any sample lacking mtDNA even if represented with the nuclear dataset.

2.2 Laboratory Protocols

Twenty-five nuclear loci were selected based on both variability in the Anguinae at the species level and working in a high percentage of taxa. Twenty-four loci were exons while the

remaining locus was an EPIC (exon primed intron spanning) intron. Loci were selected that have been used in previous species level studies (Portik et al. 2012, Townsend et al. 2008, Vidal and Hedges 2005). Three regions of the mtDNA were also selected to generate a mtDNA phylogeny. Tissue samples were extracted with a standard salt extraction protocol (Sambrook and Russel 2001). Loci were amplified via the polymerase chain reaction (PCR) under standard conditions with a standard master mix. PCR amplification was verified via electrophoresis on 1% agarose gels. Successful PCR amplifications were cleaned with diluted Exo-Sap and cycle sequenced with either Big Dye 1.1 or Big Dye 3.1 chemistry. Cycle Sequencing reactions were performed in both the forward and reverse directions. Cycle sequencing product was cycle sequenced on both Abi 3100, 3730, and 3730X machines. Wet laboratory protocols followed standard and touch down PCR conditions.

2.3 Dataset Organization and Partition/Model Testing

Forward and reverse sequences generated in this study were assembled into contigs and ambiguities were examined by eye in Sequencher v 4.2 (Genecodes). Cleaned sequences were then assembled into alignments and gaps added manually.

The nuclear dataset was initially partitioned into 25 partitions, one for each nuclear gene fragment and mtDNA locus was partitioned by type of DNA (coding/codon position, tRNA, rDNA) then analyzed in Partition Finder v 2.1.1 (Lanfear et al. 2016) in Python 2.7.12 (<https://www.python.org/>) to determine optimal partitioning schemes for nuclear and mtDNA. The Bayesian Information Criteria (BIC) and greedy algorithm (Lanfear et al. 2012) was used limiting it to models in RAxML (Stamatakis 2014), Mr. Bayes (Ronquist et al. 2012), and

BEAST/BEAST2 (Bouckaert et al. 2014, Drummond et al. 2012, Drummond and Rambaut 2007) in different runs. For runs involving Mr. Bayes and BEAST/BEAST2, PhyML 3.0 was used as part of the Partition Finder package (Guindon et al. 2010). Mitochondrial data was also analyzed in Partition Finder with the nuclear dataset for the concatenated analysis. In this case the base partitions used were ones found in previous Partition Finder runs for the separate nuclear and mtDNA datasets. To determine optimal clock partitioning schemes for the molecular dating analysis the optimal nuclear partitioning scheme was examined in the R package ClockstaR2 (Duchene and Ho 2014, Duchene et al. 2014) under R version 3.1.3 (<https://www.r-project.org/>).

2.4 Phylogenetic Analyses Protocols

A phylogenetic tree was generated under both a Maximum-likelihood (ML) framework in RAxML v. 8.2.4 (Stamatakis 2014) and a Bayesian inference (BI) framework in Mr. Bayes v. 3.2.6 (Ronquist et al. 2012) for the mtDNA, nuclear DNA, and a concatenated dataset. The RAxML 8 analyses were partitioned using the partition schemes found in Partition Finder using the GTR model version with the best BIC score and ran under automatic settings for 1000 bootstraps. Separate RAxML analysis were run for mtDNA, nuclear DNA, and concatenated datasets. The Mr. Bayes analyses were partitioned according to the best Partition Finder scheme for the same three datasets. The Mr. Bayes analyses were run for 20 million generations with one cold chain and three hot chains. The Mr. Bayes analyses were examined in Tracer 1.6 (Rambaut et al. 2014) to determine that each analysis had reached stationarity and to determine burn-in. The analyses were repeated a total of four times to determine that different runs converged on the same answer and one run for 50 million generations to

examine if the analyses ran long enough. Nuclear and mtDNA were analyzed separately as well as concatenated together. Additionally, the analyses were run with additional outgroups present representing other families in the Neoanguimorphia to examine if outgroups affected the analyses. Analyses for RAxML and Mr. Bayes were run on the Cipres Science Gateway (Miller et al. 2010). Figtree v. 1.4.2 (<http://tree.bio.ed.ac.uk/software/figtree/>) was used to visualize the bootstrap values on the best tree from the Maximum-likelihood analysis and the posterior probabilities on the consensus tree from the Bayesian Inference analysis.

2.5 Molecular Dating Protocols

Molecular dating was performed with the BEAST v. 1.8.2 (Drummond and Rambaut 2007, Drummond et al. 2012) software. Partitioning for DNA data and clock partitioning/linkage was determined based on Partition Finder and ClockstaR results. For molecular dating highly supported clades from the phylogenetic analyses were constrained to monophyly in order to collect Most Recent Common Ancestor (MRCA) statistics. The BEAUTI program was used to generate an initial XML file then the file was modified by hand to include best fit phylogenetic models and a starting tree that did not violate initial starting conditions (clade monophyly and fossil calibrations). Dating constraints were added for the divergence of the Gerrhonotinae from the Anguinae as well as the *Pseudopus* lineage. The one outgroup (Gerrhonotinae) dataset was used for the molecular dating analysis to reflect the models of evolution in the Anguinae specifically and avoid complicating the molecular models of evolution. Only the nuclear DNA dataset was used to avoid issues with possible mtDNA saturation at deeper level relationships. The following fossil constraints and justifications follow;

1. *Pseudopus*- The MRCA of *Pseudopus* and *Anguis* was calibrated with a hard minimum of 16.9 mya with a soft maximum of 25.0 mya under a uniform distribution prior. The justification is the minimum date of the strata *P. ahnikoviensis* (Klembara 2012) and *P. rugosus* (Klembara 2014) was found in (land mammal age MN3). The maximum was set to 25.0 since no *Pseudopus* fossils are known before the Miocene. This interval is likely conservative.

2. Gerrhontinae/Anguinae- Due to several Eocene localities of fossil Anguinae in Europe the MRCA was set to a minimum of 48.6 mya based on minimum age of the strata (Bolet and Auge 2014, Bolet and Evans 2013, Estravis 2000, Klembara and Green 2010, Meszoely and Haubold 1975, and Rage and Auge 2003). A maximum age of 57.0 mya was set based on the first occurrence of the Thulean land bridge (Brikiatis 2014). Since it isn't clear when the land bridge connection was severed or when suitable habitat would be present to allow dispersal a uniform distribution was used. The molecular dating analyses were run for 50 million generations sampling every 1000 samples and repeated for four runs to determine that they converged on the same answer. TreeAnnotator (part of BEAST package) was used to remove 10,000 samples as burn in and combine different runs.

2.6 Species Tree and Delimitation Analyses Protocols

A species tree analysis was run in BEAST2 (Bouckaert et al. 2014). Species assignments were used placing “non-monophyletic” lineages into species for *Dopasia gracilis*, *Dopasia sokolovi*, and *Ophisaurus attenuatus longicaudus*. A second analysis was run placing nuclear gene tree lineages of *Dopasia* from Lao Cai, Vietnam and *Pseudopus sp.* from Israel as “species”.

Species tree analyses were ran for 250 million generations sampling every 10,000 generations. Burn in was verified in Tracer.

A species delimitation analysis was performed in BPP v 3.2 (Yang 2015) using the option to infer both the species tree and species delimitation during the same analysis. Species specified included both a “clumped” model hypothesis where some paraphyletic lineages (*D. gracilis* and *O. attenuates longicaudus*) were clumped into species as in the BEAST2 analysis and a “split” model where paraphyletic lineages were assigned to individual species. The analyses were run for 20000 generations sampling every 10 generations with 10000 generations discarded as burn in. The analysis was run with both species delimitation algorithms.

2.7 BAMM protocols

A fully bifurcating tree was taken from the BEAST analyses and R code was used from (<http://bamm-project.org/quickstart.html>) modified for the specific data file and analyses. The analysis was run to generate 5000 samples with a burn in of 1000 samples. In order to account for missing taxa the control file was modified due to three species missing from the *Anguis* lineage, three species missing from the *Ophisaurus* lineage, and four species missing from the *Dopasia* lineage.

3. Results

3.1 Alignment and Model Testing

The final concatenated alignment was 17281 bp and contained 1633 parsimony informative variable sites, 1608 parsimony uninformative variable sites. The nuclear loci dataset was 15191 bp in length and contained 529 parsimony informative sites and 701 parsimony uninformative sites. The mtDNA dataset was 2090 bp long and contained 691 parsimony informative variable sites and 140 parsimony uninformative sites.

For the mtDNA dataset Partitionfinder determined the best partitioning scheme contained four partitions; combining the first codon positions of Cytb and ND4 and tRNA with the GTR + G model, the second codon positions of Cytb and ND4 with HKY + I + G, the third codon positions of Cytb and ND4 with the GTR + I + G, and 16s rRNA with the HKY + I + G model. When limited to the GTR family of models for use in RAxML, the best partitioning scheme for mtDNA was the same, but with the GTR + I + G model with the highest BIC score.

For the nuclear loci dataset Partitionfinder determined the best partitioning scheme involved six partitioning schemes when models were limited to models available in Mrbayes and BEAST respectively although the actual partitions were not the same. When limited to the GTR family of models for use in RAxML, the partitioning scheme with the highest BIC score was GTR + I + G with three partitions. The concatenated dataset was found to have seven optimal partitions when constrained to the GTR family models with the GTR + I + G model scoring best under the BIC. Table 2 lists partitions and models for the nuclear and concatenated datasets found in Partitionfinder runs. Linking multiple genes was not explored for the concatenated

super tree analyses since the species tree analysis used unlinked gene fragments as independently evolving loci. Best fit models of evolution returned under both an examination of all available models and where analyses were done under a more limited model space are found in Table 3 for the species tree and coalescent analyses.

3.2 Phylogenetic Analysis

Both the Maximum likelihood and Bayesian inference phylogenetic analyses were highly congruent and agreed with high support on the presence of most of the clades found and showed agreement for nodes that lacked support. Typically most clades were supported with a bootstrap value of 100 or a posterior probability of 1. Figures 2-4 show phylogenetic relationships with unsupported branches (BS<70, PP<0.95) collapsed or denoted. The placement of the *Dopasia* genome is shown in the nuclear dataset (Figure 3), but the transcriptome is not shown due to a substantial lack of locus overlap with the dataset generated in this study.

The concatenated super tree analyses (Figure 4) found that the European Mediterranean Area (including the Near East and North African Regions), Asia, and North America all contained monophyletic clades with Asia and North America forming major sister lineages. Within Europe *Hyalosaurus*, *Anguis*, and *Pseudopus* formed a clade, although the position of *Hyalosaurus* as sister to the other lineages was not highly supported in the mtDNA analyses. In North America, *Ophisaurus* relationships found the *O. ventralis* was sister to the other sampled species, with *O. compressus* the next most basal taxon. Phylogenetic information regarding Asian species of *Dopasia* is made more difficult by lack of concrete

morphological characters or IDs on some specimens or dependence on internal morphological characters to ID species. In the concatenated analysis the most basal division occurs between the *D. gracilis* and *D. harti* species groups. (Nyugen et al. 2011) The *D. gracilis* group was divided into a southern and northern unit likely referring to *D. sokolovi* (south) and *D. gracilis* (north). MtDNA found that while both *D. sokolovi* and *D. gracilis* were monophyletic with this marker *D. gracilis* contained enough variability that cryptic lineages are a possibility (5.8-6.2 %).

The nuclear loci analyses (Figure 3) differed from the concatenated analysis in that within the North American *Ophisaurus*, *O. attenuatus longicaudus* failed to form a monophyletic lineage. This lack of monophyly received high support with both bootstrap and posterior probability values. Within Asia nuclear DNA still found support for a *D. gracilis* group and a *D. harti* species group, but things were less clear cut in the *D. gracilis* group. In the *D. gracilis* group the Lao Cai locality formed an independent lineage while the *D. sokolovi* clade formed within a polytomy of *D. gracilis* lineages.

The mtDNA only analysis agreed mainly with the concatenated analysis, which is not surprising since it likely heavily weighted the analyses due to the high informativeness compared to individual nuclear loci. The mtDNA analysis allowed the inclusion of data from previous studies deposited on Genbank (Table 1) and a more geographically complete picture of the Anguinae. Specifically, within *Pseudopus* the inclusion of a *P. a. thracius* indicates that the *Pseudopus* from Israel may belong to a third distinct lineage outside of the two described subspecies. Within the *D. harti* group the inclusion of samples from Eastern mainland China and Taiwan show a West-East genetic split within the *D. harti* group. With the Eastern unit the *D. harti* on Taiwan appear to form a distinct lineage from the mainland *D. harti*. Since with the

current level of geographic resolution the *D. harti* group appears to have lineages that correspond to *D. hainanensis* in the west as well as mainland *D. harti* and insular *D. formosensis*, while the *D. gracilis* species group seems to contain clades corresponding to *D. gracilis* and *D. sokolovi* phylogenetic trees (Figures 2-4) use these species names for clarity.

3.3 Molecular Dating

The molecular dating analysis found that the initial Anguinae divergence mean was 51.97 mya which was constrained by both a minimum and maximum date of the youngest strata date for the appearance of Anguinae fossils and the first appearance of the Thulean Landbridge. The initial divergence between the European lineages and the Asian and North American had a mean of 38.83 mya pre-Oligocene although the 95% credibility interval extends from the Eocene well into the Oligocene many of the dates are in agreement with a drying of the Turgai Sea. Most of the major lineages appear to be present by the Oligocene or beginning of the Miocene. Many of the species level divergences in major lineages occur in the Miocene. 95% credibility intervals are fairly broad likely due to a lack of constraints. A list of node means and 95% credibility intervals appear in Table 4 and are represented in Figure 5.

3.4 Species Tree, Species Delimitation, and BAMM

The species tree analyses found high support (PP = 0.99-1.00) for all species distinctions and for all deeper level relationships except those noted in Figure 6. The weakest relationship found was between the continental Asian and North American lineages with a PP = .80. In the analyses where specific lineages of *Dopasia gracilis* and *Ophisaurus attenuates longicaudus* that

failed to form a monophyletic unit were given species assignments did not differ in support from the analyses where they were combined with a single species. The main difference between the species tree analysis and the concatenated analyses was in the placement of *Pseudopus* and *Anguis* which were not found to be sister in the species tree analyses.

The species delimitation analysis found a high posterior probability (PP=1.000) for eight species within the Anguinae. Delimited species were primarily composed of species groups (often accepted species were combined) or major lineages which were also supported with a PP = 1. *Hyalosaurus*, *Anguis*, and *Pseudopus* were all delimited as single species. *Dopasia* was delimited into two species representing the two major species groups based on morphology (Nguyen et al. 2011). *Ophisaurus* was also placed into two delimited species consisting of *Ophisaurus ventralis* and then the three remaining *Ophisaurus* species represented in this study. The rate shift plot generated by BAMM showed no bursts in speciation rate in the Anguinae.

4. Discussion

4.1.1 Phylogeny of the Anguinae

Both the ML and BI analyses produced trees with similar topology and support for both the nuclear and mtDNA datasets. The Anguinae consists of three major lineages. One consists of the monotypic taxon *Hyalosaurus koellikeri* currently confined to North Africa and the remaining lineages in the European and Near Eastern geographic regions, *Anguis* and *Pseudopus*. The second and third major lineages consists of the Asian and North American species, respectively, which form sister continental lineages.

4.1.2 Placement of the *Hyalosaurus* Lineage

The mtDNA, nuclear, and concatenated datasets appear to agree on the placement of the *Hyalosaurus* lineage, placing the species sister to the European lineages *Anguis* and *Pseudopus* which is in close geographic proximity. In the case of mtDNA, placement of *Hyalosaurus* had weak support, essentially not contradicting the nuclear data set. Initial divergence among basal lineages within the Anguinae are characterized by short branches. If initial divergence happened fast in the Anguinae, deeper relationships may not be able to be determined with the molecular data. Fossil evidence does show a generalist form of Anguinae in mainland Europe since the Miocene (Klembara 2015). These fossils sometimes placed with *Ophisaurus* or *Dopasia* potentially could belong to the lineage leading to *Hyalosaurus* given the morphological similarity of *Hyalosaurus* and Asian and North American forms. The *Hyalosaurus* lineage was likely more widespread in mainland Europe. An older Oligocene fossil could also be attributed to *Hyalosaurus* (Cernansky and Auge 2012), especially given the divergence dates

occurring in the Oligocene in this study. This is especially true if *Dopasia* and *Ophisaurus* evolved in situ on their respective continents after an Anguinae dispersal out of Europe. However, the fossils could also represent another “generalist” Anguinae form, an extinct side branch, or belong to the lineage leading to the Asian-North American Clade. A lack of morphological diagnostic features between *Hyalosaurus*, *Ophisaurus*, and *Dopasia* makes the placement of fossils as well as the history of the *Hyalosaurus* lineage difficult with both morphological and molecular data. However, one morphological feature that does support the molecular topology of *Hyalosaurus* with other European taxa is the presence of extremely vestigial leg remnants. Based on the nuclear phylogeny *Hyalosaurus koellikeri* is a surviving relict that branched off from the other European lineages.

4.1.3 Historical relationships of European Lineages

Previous research has been inconclusive on the taxonomic identity of *Pseudopus* from the Israel, Jordan, Syria regions. While some distinctive characters have been noted, the animals appear to have more characters in common with *Pseudopus apodus thracius* from Greece and Western Turkey (Rifai et al. 2005). *Pseudopus a. thracius* was not available for the nuclear dataset, but was available via Genbank for the mtDNA dataset. The *Pseudopus* lineage from Israel appears to be a distinct lineage from both *P. a. apodus* and *P. a. thracius* in the mtDNA phylogeny and is fairly distinct from *P. a. apodus* in the nuclear dataset. If morphology is accurate, then the *P. apodus* lineage from Israel likely occurs in Jordan and Syria as well where the animals share a similar morphology according to Rifai et al. (2005). Further research is needed to examine the regional phylogenetic patterns within *Pseudopus apodus*, but it is likely that the species is composed of more than two subspecies level units based on the

position of the specimens from Israel. The subspecies biogeographic pattern could potentially be a refuge based pattern with distinct lineages found within Balkan Peninsula, the Eastern part of the range, and near the Mediterranean Sea.

The sister lineage, *Anguis*, appears to also have a refuge based pattern, but among species, with different species being found on the southern Mediterranean Peninsulas (Iberian, Apennine, and Balkan) as well as the Peloponnese region and mesic areas in the vicinity of the Black and Caspian Seas (Gvozdik et al. 2010, Gvozdik et al. 2013, Jablonski et al. 2016). Within the European lineages the sister relationship between the smaller cold tolerant *Anguis* and the larger more arid adapted *Pseudopus* found with mtDNA (Macey et al. 1999) was supported with the nuclear dataset.

The implication is that the extremely rudimentary hind limbs found in *Hyalosaurus* (Gunther 1873) and *Pseudopus* (Ananjeva et al. 2006) are an ancestral trait lost twice, once in *Anguis* as well as in the ancestor of the North American and Asian clade. The loss in *Anguis* may not be surprising given the adaptations in the lineage to colder, moister conditions such as viviparity and small size which has been assumed to be a paedomorphic trait. Remnants of hind limbs are one of the few morphological traits that might connect the *Hyalosaurus* lineage to the other European lineages since this trait is shared with *Pseudopus*. The implications of this would mean that mainland Europe until recently had three major lineages of Anguinae lizard, a generalist in *Hyalosaurus*, a larger arid adapted form in *Pseudopus*, and a smaller cold adapted form in *Anguis*. Instances in the sister group Gerrhonitinae are known where cold adapted and warmer adapted forms occupy the same geographic range although differ in habitat preference (Stebbins 1958), but three species sharing a range is currently not known. One method by

which the Anguinae niche might have been partitioned for three species is both by climate as well as by diet since all three lineages *Hyalosaurus*, *Pseudopus*, and *Anguis* have different tooth structure (Klembara et al. 2014). This may be significant because paleo reconstruction has found that the warmer more arid environment of the modern *Pseudopus apodus* may not have been the preferred climate of some of the ancestral species with the extinct *P. laurillardii* potentially occurring over broader habitats than modern *P. apodus*. (Klembara et al. 2010).

4.1.4 Relationships among Asian Lineages

Both the Asian and North American Clades were found to be monophyletic. Previous studies had been relatively taxon poor (Macey et al. 1999) making this conclusion previously unclear. While not every species level lineage was included in this study, the representation of most species and all major lineages makes it unlikely that future inclusion of additional species will break these monophyletic geographic units. Within the Asian clade the most basal split appears to take place between the *D. hainanensis* and the specimens belonging to the *D. gracilis* group (*D. gracilis* and *D. sokolovi*) in the nuclear dataset. The genetic data in this study support two groups within *Dopasia* that were proposed based on analysis of morphological traits (Nyugen et al. 2011). These groups consist primarily of a western *D. gracilis* group that ranges from eastern India to China and Vietnam and an eastern *D. harti* group from Western China and Vietnam to the island of Taiwan. It is unclear where the Indonesian species which occur on Sumatra and Borneo fallout within *Dopasia* due to lack of sampling in this or previous studies, although the *D. gracilis* group is the closest geographically to these islands and the Southern component that includes *D. sokolovi* is closest geographically. The split between the *D. gracilis*

and *D. harti* groups corresponds roughly to the high elevation Himalayan region which arcs downwards North of Vietnam.

Within the *D. gracilis* group several independent lineages (Myanmar, China, and Northern Vietnam) were found in the Northern part of the range while those in the south (Southern Vietnam and Laos) form a single monophyletic clade that may correspond to the species *D. sokolovi*, which previously has only been known from parts of Vietnam (Nyugen et al. 2011). Despite differing topologies in the nuclear and mtDNA datasets, *D. gracilis* appears to be paraphyletic with respect to *D. sokolovi*. This is not completely surprising given the vast geographic range of *D. gracilis*. While *D. hainanensis* is the only member of the *harti* group represented in the nuclear DNA dataset, the mtDNA dataset includes localities farther east in mainland China as well as Taiwan. In both cases the mtDNA appears to be potentially species level different, 7.3% and 8.0% different between the two Taiwanese localities and the nearest mainland locality in Eastern China and between 7.0% and 8.8% between the *Western D. hainanensis* samples and those further East. The implications of this are that *D. formosensis* from Taiwan may be a valid species. It was previously sunk due to a lack of genetic distinction from *D. harti* on Taiwan, but no mainland specimens were included in that study (Lin et al. 2003). *Dopasia harti* from the mainland was sampled with Genbank sequence from the Eastern mainland China (Pan et al. 2015). Due to the wide range of *D. harti* it is possible the *D. harti* group contains additional distinct species, as has been found in other wide-ranging herptofauna genera in China (Lu et al. 2004, Wu et al. 2010, Yuan et al. 2013).

4.1.5 Relationships among North American Lineages

Within the North American *Ophisaurus* clade the nuclear dataset placed *O. ventralis* as the most basal member with high support for the first time. Previous studies have found low support for the position of this species (Mohler et al., unpublished), or placed it outside other Anguinae (Pyron et al. 2013) due to both mtDNA molecular data or morphological differences found in *O. ventralis* compared to the other *Ophisaurus*. *Ophisaurus compressus* was then found as the next most basal lineage. Samples of *O. attenuatus* formed a single derived clade with two subclades associated with subspecies distinctions in the mtDNA and concatenated analyses. While *O. a. attenuatus* did form a monophyletic clade, the *O. a. longicaudus* samples did not form a monophyletic clade with the nuclear data contradicting mtDNA which did form distinct *longicaudus* and *attenuates* clades. Based on nuclear data alone *O. a. longicaudus* may form several distinct lineages, but a more range-wide multi-locus study is needed to determine patterns in the *Ophisaurus attenuatus*. While several species have overlapping geographic ranges in North America, it is suspected that they have different micro-habitat preferences and remain relatively reproductively isolated, although the latter has never been rigorously examined with genetics. Further data is needed to examine where the unsampled species *O. mimicus*, *O. incomptus*, and *O. ceroni* fit within the North American *Ophisaurus*.

4.2.1 Origin of Anguinae in Europe

The molecular dating analyses placed most of the outgroup divergences as pre-Cenozoic. The divergence between the Gerrhonitinae in North America and Anguinae in Europe is suspected to have occurred after a dispersal across the Thulean Land Bridge and before 48.6 million years when Anguinae fossils begin appearing at multiple sites in Europe (Bolet and Evans 2013, Bolet and Auge 2014, Klembara and Green 2010, Meszoely and Haubold 1975, and Rage and Auge

2003). The date of 48.6 mya is the lower limit of strata ages for when fossils of the Anguinae appear in Europe, and is likely conservative with the Thulean landbridge being broken around 50 mya. The mean date in this analysis for the divergence of the Anguinae was 51.97 mya which is a reasonable amount of time for the group's origin before fossils begin appearing in the fossil record (Drummond and Bouchaert 2015) and probably corresponds to degradation of the Thulean Landbridge before it was severed at 50 mya (Sanmartin et al 2001). While this particular node was restricted with hard constraints the Thulean Landbridge is seen as the most reasonable dispersal route. The De Greer's Land Bridge was not contemporary with the Thulean Bridge according to Brikiatas (2014) and would still be subject to month long darkness during Arctic Winters due to its high latitudinal position even in a hothouse climate. The other possibility, the Faroes Bridge, may have never been more than an island chain (Sanmartin et al. 2001). The date found in this study is in close agreement with dates found in larger surveys of squamata (Mulcahy et al. 2012, Vidal et al. 2012).

4.2.2 Radiation of Anguinae in Europe

Fossil Anguinae first appear in the Eocene at multiple localities in Europe, which was characterized primarily by greenhouse conditions, even at far Northern latitudes (Hubar and Sloan 2001, Sluijs et al. 2007). Europe additionally was hypothesized to have been a tropical/subtropical island archipelago during the Eocene (Prothero 1994).

Within Europe the lineage leading to the most derived forms, *Anguis* and *Pseudopus*, split off from *Hyalosaurus* in the late Oligocene (~27 mya) and separated from each other in the early Miocene. *Hyalosaurus* is currently confined to North Africa in Morocco, has both a relict

distribution in remnant oak forest, and appears to contain little genetic variation (De Pous et al. 2012). Based on the early divergence time estimated in this study for *Hyalosaurus* more generalist fossil forms from the Oligocene in Europe may belong to this lineage rather than *Ophisaurus* or *Dopasia* despite the difficulty distinguishing them from *Hyalosaurus* based on fragmentary fossils (Černaňský and Augé 2012). A generalist form existed in Europe until recently (Delfino et al. 2011), and it is likely that the *Hyalosaurus* lineage had a greater presence outside of North Africa if European “*Ophisaurus*” or “*Dopasia*” are actually *Hyalosaurus* and were eliminated in Europe, along with most of the boreotropical elements of the continent, as the climate progressively cooled and entered a series of glacial/interglacial cycles in the Pleistocene.

Pseudopus and *Anguis* diverge soon after the beginning of the Miocene and soon after the first appearance of *Pseudopus* in the fossil record. *Pseudopus* is relatively distinct in a number of morphological features and present in the fossil record from recent times back to near the beginning of the Miocene, where the earliest known species *P. rugosus* and *P. ahnikoviensis* begin to have characters that are less *Pseudopus*-like. Although currently *Pseudopus* and *Anguis* are distinct ecologically, with *Anguis* being more cold adapted and smaller with many suspected paedomorphic traits and *Pseudopus* as a larger more arid adapted member of the Anguinae, this may not have been the case throughout a considerable portion of the history of the lineages. The fossil form *Pseudopus pannonicus*, which is larger than modern *P. apodus*, is suspected of occurring in wetter forest conditions based on modeling (Klembara et al. 2010) than modern *P. apodus*. Thus, the modern ecological divergences between these lineages should be used with caution as justifications for initial divergence.

These two Anguinae genera do show morphological differences from each other as well as from the more generalist forms *Hyalosaurus*, *Dopasia*, and *Ophisaurus*. One of these differences occur in the teeth, with *Pseudopus* having distinctive peg like teeth whereas *Anguis* has sharper dagger-like teeth. Dietary differences could have played a role in lineage divergence or allowed the three different forms to coexist in the European region.

Intraspecies divergences within *Pseudopus* and *Anguis* occur towards the end of the period known as the Miocene climatic optimum (17-15 mya). During this period the progressive cooling in global climate since the Eocene was interrupted by a rapid return to warmer greenhouse conditions. Interestingly, *P. apodus* in Europe and the *P. apodus* in Israel were found to have a fairly deep divergence based on the nuclear dataset. In fact, the level of divergence was much deeper than would be expected for a subspecies level distinction. This is in contrast to relatively low divergence levels found with mtDNA data between *P. a. thracius* and *P. a. apodus* (Gvozdk et al. 2010). It is unclear why *Pseudopus* would have such low levels of mtDNA divergence with a large nuclear divergence although not without precedent and could potentially be due to bottleneck events that reduced mtDNA diversity, but not nuclear diversity (Canino et al. 2010, Grant and Leslie 1993). This would be in line with a refuge then expansion model for the species. Further fine scaled genetic work as well as morphological work is needed, but the *Pseudopus* from Israel and likely Jordon and Syria (Rifai et al. 2005) may represent a third evolutionary significant unit on par with the two known subspecies. Additional support for the impact of the Miocene Climatic Optimum comes from the divergence between the two species of *Anguis*, which was also found to have occurred around the end of the Miocene Climatic Optimum.

4.2.3 Radiation of the Anguinae in Asia

European taxa who could not disperse across water would have been unable to spread east to Asia due to the presence of the Turgai Sea/Straight (Figure 1) until the beginning of the Oligocene (~33.9 mya) which also corresponded to a cooling world-wide climate which involved the fracturing of forest habitat and the spread of grasslands. Anguinae are typically viewed as poor dispersers over water and not naturally found on any island that has not had a land connection in the past. Divergence of the surviving Anguinae lineages seems to have begun in the Oligocene with the dates found in this study being in agreement with a possible divergence between European and Asian/North American lineages after the drying of the Turgai Sea, although the 95% credibility interval contains much of the Eocene too. While this node could have been constrained by the drying of the Oligocene it was left unconstrained since it is unclear exactly when the area would become passible. The mean date of approximately 38 mya, if representative of the European/ Asian-North American split, means that soon after the drying of the Turgai Sea in the Oligocene the European and Asian-North American clades were on separate evolutionary trajectories. Most of the major lineages in Europe as well as the Asian and North American lineages diverge in the early Miocene (23-5 mya) with these lineages diverging into species in the later Miocene.

Within Asia the main divergence is between the *Dopasia harti* group and the *Dopasia gracilis* group. While it is unclear what caused the initial divergence whereas Europe and North America experienced ice ages and glaciation Asia experienced a more continental climate during this time (Guo et al. 2008). Dropping sea levels and a continental climate would result in fragmentation of forest and expanding grassland habitat. These cycles are a likely cause of

speciation in the Anguinae as well as a mechanism that would allow dispersal to Sumatra and Borneo during periods of low sea level. The *harti* group primarily occurs from Vietnam eastward to the island of Taiwan. The *gracilis* group occurs westward to eastern India. Most of the range of these groups is due to a single wide-ranging species (*D. gracilis* and *D. harti*, respectively). This study found evidence that these wide ranging species are likely made up of different lineages and likely contain several evolutionary distinct units. One possible explanation for this pattern, especially in light of the potential polytomy in the mtDNA for the *D. gracilis* group is range wide fragmentation of the forest habitat during climate cycling from forested to ecotone habitat (Smith et al. 1997). Within the *gracilis* group one more widespread lineage appears to occur in southern mainland Southeast Asia that may correspond to the species *D. sokolovi*. *Dopasia sokolovi* is morphologically diagnosable only by internal characters (vertebra number) so the identity of samples are speculative. This study found that if the samples do correspond to *D. sokolovi* based on known range then the species also occurs farther west into Laos. If it occurs much farther west, it may support a forest/grassland fracture model in the North with more widespread connectivity in the south.

4.2.4 Radiation of the Anguinae in North America

Between Asia and North America the main dispersal route would have been through Beringia and while the climate in that region would have progressively degraded from the Boreal Tropical forest of the Eocene to the tundra of the Pleistocene, numerous examples of both Amphibians and Reptiles (Brandley et al. 2011, Burbrink and Lawson 2007, Chen et al. 2013, Guo et al. 2013, Guo et al. 2012, Macey et al. 2006, Pramuk et al. 2008, Townsend et al. 2011, and Wuster et al. 2008) show that this is a viable route of dispersal for herpetofauna

between these continents. These dispersals include multiple examples of colubrid snakes, skinks, and vipers that show that dispersal events were unidirectional at least for squamate reptiles, from Asia to North America and were possible into the Miocene. The dispersal dates estimated for Anguinae lizards across Beringia fit within this time range seen for other squamate dispersals. The one way out of Asia dispersal into North America is a pattern seen in numerous squamate reptiles even though dispersals may not have been temporally concurrent. This supports the idea that Beringia may have been a one way landbridge connecting Asia and North America as far as squamate reptiles or that dispersal across Beringia may have required multiple smaller dispersal events through the Asia-Beringia-North America region. The two earliest fossils of the Anguinae in the New World occur in Miocene deposits of Canada (Holman 1970) and slightly younger deposits in Colorado (Holman 2003) both outside the modern range of *Ophisaurus*. Both of these dates occur after the dispersal dates found from molecular dating in this study. These fossil forms also occurred prior to the estimated divergence of modern species, suggesting that these fossils likely represent stem fossils or early offshoots of the *Ophisaurus* lineage. All of the species of *Ophisaurus* included in this study appear to have diverged before the late Pliocene and Pleistocene. Thus, making many of the species specific identification of fossils (Auffenberg 1955) from these periods likely to belong to surviving modern lineages rather than stem lineages and diagnostic characters likely have been present for some time. Unlike Europe where different ecological forms exist, or Asia where species do not seem to overlap over much of the range, within North America four species level lineages share overlapping geographical ranges in the Southeastern United States. Habitat specialization has been proposed as a segregating factor with *O. mimicus* being found in

wiregrass environments and *O. compressus* being more fossorial and found in sandy rosemary dominated habitat (Mohler et al., unpublished). The dates estimated for the distinction of *Ophisaurus* species level lineages suggest that this microhabitat specialization may have been in place for a relatively long time. McConkey (1954) proposed a biogeographic scenario for North American *Ophisaurus* which involved speciation due to different glaciation periods (mostly recent). While it is highly probable that North American *Ophisaurus* were affected by climate change as well as fracturing habitat the species level divergences in this study predate Pliocene/Pleistocene glaciation.

4.3 Species Tree, Species Delimitation, and BAMM

Both the species tree with species assigned based on nuclear lineages and one based on clumping members of *D. gracilis*, *O. attenuatus longicaudus* found extremely high levels of support indicating that nuclear DNA may not be a good indicator of species determination for these lineages. This was also the case for the species delimitation analysis which clumped many well accepted species. This means that the analysis was likely very conservative in delimitating species (Carstens et al. 2013) and did not err in excessively delimitating species. In both of these cases analyses would be aided by further information such as morphology and ecological characters in order to assign species identification to potentially cryptic lineages or non-monophyletic species. The main disagreement between the species tree and concatenated analyses involves the relationships between the major lineages of *Anguis* and *Pseudopus* which have been assumed to be sister lineages. While all concatenated analyses (mtDNA, nuclear, mtDNA-nuclear) support this arrangement the coalescent based species tree finds three distinct European lineages with *Pseudopus* sister to the Asian and North American

lineages. This arrangement has not been supported previously with molecular or morphological data and may be unlikely, but not completely implausible. Further expansive multilocus or multi-evidence data may be needed to examine if this is potentially a real relationship or simply that of the 26 loci used in this study determined.

An examination of speciation rate did not show any increase in speciation rate. This may be evidence that the available niche space for the Anguinae may be fairly low and is filled fairly quickly. The evolution in the Anguinae as grass-swimmers may be tied to the rise of the grasslands with initial diversifications appearing when C3 grasslands appear in the Oligocene with further diversification occurring as C4 grasslands rise to prominence in the Miocene (Stromberg 2011). The Miocene Climatic Optimum could help explain some mid-Miocene diversification as a warmer wetter climate would change the distribution of grassland to forest habitat. Especially if drier fire prone conditions were related to the rise of C4 grasslands during the Miocene (Keeley and Rundel 2005). Other taxa associated with grassland habitat such as Falcons (Falconidae) show rapid diversification in the Miocene attributed to the appearance of prominent grassland habitat (Fuchs et al 2015). Global climate change tied to grassland habitat could be the main driver of speciation in the Anguinae through simple vicariance, especially if future ecological work supports niche limitation in the group.

5.0 Conclusion

The Anguinae represent a morphologically conserved, widespread, but not especially species rich group. The extensive range of the Anguinae may in part be due to being in the right place at the right time in order to disperse to Asia and across land bridges such as Beringia as well as to North Africa and Indonesia. The relatively low species count in the Anguinae when taken with both allopatric speciation as being the typical mode for speciation may mean that niche space for legless Anguid lizards is relatively low, potentially with room for only one species in one area typically. This is something that may actually be at play in other Anguinae groups as within the sister group the Gerrhonitinae it has been noted that typically only one species occurs in a given region, the few exceptions being where a live bearing cold tolerant species overlaps in range with a species adapted to warmer more arid habitat.

The Anguinae have a relatively low species count even when cryptic species are taken into account based on the size of the range of the group. Many of the species within the Anguinae are fairly wide-ranging and some of the species level lineages are fairly old. The Anguinae may represent an alternative survival strategy to groups with high speciation rates to guard against extinction. The Anguinae which have persisted since the Eocene and currently occupy a massive range in the Northern Hemisphere may represent a case of an alternative strategy where long lasting species with large ranges could offset a lower rate of speciation when compared to more specious groups.

References

- Ananjeva, N., 2006. The Reptiles of Northern Eurasia. Taxonomic Diversity, Distribution, Conservation Status. Sofia, Bulgaria: Zoological Institute, Russian Academy of Sciences.
- Auffenberg, W., 1955. Glass Lizards (*Ophisaurus*) in the Pleistocene and Pliocene of Florida. *Herpetologica*. 11(2), 133-136.
- Auffenberg, W., 1956. Additional records of Pleistocene lizards from Florida. *Quarterly Journal of the Florida Academy of Sciences*. 19, 157–167.
- Bolet, A., Evans, S.E., 2013. Lizards and amphisbaenians (Reptilia, Squamata) from the late Eocene of Sossís (Catalonia, Spain). *Palaeontol. Electron.* 16, 1–23.
- Bolet, A., Augé, M., 2014. A new miniaturized lizard from the late Eocene of France and Spain. *Anat. Rec.* 297, 505–515. doi:10.1002/ar.22855
- Bouckaert, R., Heled, J., Kühnert, D., Vaughan, T., Wu, C.-H., Xie, D., Suchard, M.A., Rambaut, A., and Drummond, A. J., 2014. BEAST 2: A Software Platform for Bayesian Evolutionary Analysis. *PLoS Computational Biology*. 10(4), e1003537. doi:10.1371/journal.pcbi.1003537
- Brandley, M.C., Wang Y., Guo X., de Oca A.N., Feria-Ortiz M., Hikida T., and Ota H., 2011. Accommodating heterogeneous rates of evolution in molecular divergence dating methods: an example using intercontinental dispersal of *Plestiodon* (Eumeces) lizards. *Systematic Biology*. 60: 3-15. DOI: 10.1093/sysbio/syq045
- Briggs, J.C., 1987. Biogeography and Plate tectonics. Amsterdam: Elsevier Science Publishers B.V. Developments in Palaeontology and Stratigraphy, 10.
- Brikiatis, L., 2014. The De Geer, Thulean and Beringia routes : key concepts for understanding early Cenozoic biogeography. *Journal of Biogeography*. 41(6), 1036–1054. doi:10.1111/jbi.12310
- Burbrink, F.T., Lawson, R., 2007. How and when did Old World rat snakes disperse into the New World ? *Molecular, Phylogenetics, and Evolution* 43(1), 173–189. doi:10.1016/j.ympev.2006.09.009
- Canino, M.F., Spies, I.B., Lowe, S.A., Grant, W.S., 2010. Highly discordant nuclear and mitochondrial DNA diversities in Atka mackerel. *Mar. Coast. Fish. Dyn. Manag. Ecosyst. Sci.* 2, 375–387. doi:10.1577/C09-024.1
- Černaňský, A., Augé, M.L., 2012. Additions to the lizard fauna (Squamata: Lacertilia) of the Upper Oligocene (MP 28) of Herrlingen 8, Southern Germany. *Neues Jahrb. für Geol. und Paläontologie - Abhandlungen*. 264, 11–19. doi:10.1127/0077-7749/2012/0228

- Conrad, J. L. 2008. Phylogeny and systematics of Squamata (Reptilia) based on morphology. *Bulletin of the American Museum of Natural History*. 310, 1–182.
- Conrad, J.L., Ast, J. C., Montanari, S., and Norell, M.A., 2011. A combined evidence phylogenetic analysis of Anguimorpha (Reptilia, Squamata). *Cladistics*. 27, 230–277.
- Carstens, B., Pelletier, T.A., Reid, N.M., and Satler, J.D., 2013. How to fail at species delimitation. *Mol. Ecol.* 22, 4369–4383. doi:10.1111/mec.12413
- Chen, X., Huang, S., Guo, P., Colli, G.R., Nieto, A., Oca, M. De, Vitt, L.J., Pyron, R.A., and Burbrink, F.T., 2013. Understanding the formation of ancient intertropical disjunct distributions using Asian and Neotropical hinged-teeth snakes (*Sibynophis* and *Scaphiodontophis*:Serpentes:Colubridae). *Molecular Phylogenetics and Evolution*. 66, 254–261.
- De Pous, P., Mora, E., Metallinou, M., Escoriza, D., Comas, M., Donaire, D., Pleguezuelos, J.M., Carranza, S., 2011. Elusive but widespread? the potential distribution and genetic variation of *Hyalosaurus koellikeri* (Günther, 1873) in the Maghreb. *Amphibi. Reptil.* 32, 385–397.
- Delfino, M., Bailon, S., and Pitruzzella, G., 2011. The late Pliocene amphibians and reptiles from "Capo Mannu D1 Local Fauna" (Mandriol, Sardinia, Italy). *Geodiversitas*. 33, 357–382
- Drummond, A.J., and Rambaut, A., 2007. "BEAST: Bayesian evolutionary analysis by sampling trees." *BMC Evolutionary Biology*. 7, 214.
- Drummond, A.J., Suchard, M.A., Xie, D., and Rambaut, A., 2012. Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Mol. Biol. Evol.* 29, 1969–1973. doi:10.1093/molbev/mss075
- Drummond A. J. and Bouckaert R. R., 2015. "Bayesian evolutionary analysis with BEAST", Cambridge University Press.
- Duchene, S., and Ho, S.Y., 2014. Using multiple relaxed-clock models to estimate evolutionary timescales from DNA sequence data. *Molecular Phylogenetics and Evolution*. 77, 65–70.
- Duchene, S., Molak, M., and Ho, S.Y., 2014. ClockstaR: choosing the number of relaxed-clock models in molecular phylogenetic analyses. *Bioinformatics*. 30(7), 1017–1019.
- Estravis, C., 2000. Nuevos mamíferos del Eoceno Inferior de Silveirinha (Baixo Mondego, Portugal). *Coloquios de Paleontologia*. 51, 281–31
- Fuchs, J., Johnson, J.A., and Mindell, D.P., 2015. Rapid diversification of falcons (Aves: Falconidae) due to expansion of open habitats in the Late Miocene. *Mol. Phylogenet. Evol.* 82, 166–182. doi:10.1016/j.ympev.2014.08.010

- Grant, W. S., and Leslie, R. W., 1993. Effect of metapopulation structure on nuclear and organellar DNA variability in semi-arid environments of southern Africa. *South African Journal of Science*. 89, 287–293.
- Guindon, S., Dufayard, J. F., Lefort, V., Anisimova, M., Hordijk, W., & Gascuel, O., 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Systematic Biology*. 59(3), 307–321.
- Guo, Z.T., Sun, B., Zhang, Z.S., Peng, S.Z., Xiao, G.Q., Ge, J.Y., Hao, Q.Z., Qiao, Y.S., Liang, M.Y., Liu, J.F., Yin, Q.Z., and Wei, J.J., 2008. A major reorganization of Asian climate by the early Miocene. *Clim. Past*. 4, 153–174. doi:10.5194/cpd-4-535-2008
- Guo, P., Liu, Q., Xu, Y., Jiang, K., Hou, M., Ding, L., Pyron, R.A., and Burbrink, F.T., 2012. Evolution Out of Asia : Natricine snakes support the Cenozoic Beringian Dispersal Hypothesis. *Mol. Phylogenet. Evol.* 63, 825–833. doi:10.1016/j.ympev.2012.02.021
- Guo, P., Zhang, L., Liu, Q., Li, C., Pyron, R.A., Jiang, K., and Burbrink, F.T., 2013. *Lycodon* and *Dinodon*: One genus or two? Evidence from molecular phylogenetics and morphological comparisons. *Mol. Phylogenet. Evol.* 68, 144–149. doi:10.1016/j.ympev.2013.03.008
- Günther, A., 1873. Description of a new Saurian (*Hyalosaurus*) allied to *Pseudopus*. *Ann. Mag. Nat. Hist.* 11, 351.
- Gvoždík, V., Jandžík, D., Lymberakis, P., Jablonski, D., and Moravec, J., 2010. Slow worm, *Anguis fragilis* (Reptilia: Anguidae) as a species complex: genetic structure reveals deep divergences. *Mol. Phylogenet. Evol.* 55(2), 460–472.
- Gvoždík, V., Benkovský, N., Crottini, A., Bellati, A., Moravec, J., Romano, A., Sacchi, R., and Jandžík, D., 2013. An ancient lineage of slow worms, genus *Anguis* (Squamata: Anguidae), survived in the Italian Peninsula. *Mol. Phylogenet. Evol.* 69, 1077–1092. doi:10.1016/j.ympev.2013.05.004
- Holman, A., 1970. Herpetofauna of the Wood Mountain Formation (Upper Miocene) of Saskatchewan. *Canadian Journal of Earth Sciences*. 7(5), 1317–1325. doi: 10.1139/e70-124
- Holman A., 2003. Early Hemingfordian (Early Miocene) Squamate Reptiles from the Quarry A Local Fauna, Logan County, Colorado. (Note). The Free Library. Michigan Academy of Science Arts & Letters
[https://www.thefreelibrary.com/Early+Hemingfordian+\(Early+Miocene\)+Squamate+Reptiles+from+the+Quarry...-a0103403524](https://www.thefreelibrary.com/Early+Hemingfordian+(Early+Miocene)+Squamate+Reptiles+from+the+Quarry...-a0103403524)
- Hooker, J.J., Collinson, M.E., and Sille, N.P., 2004. Eocene–Oligocene mammalian faunal turnover in the Hampshire Basin, UK: calibration to the global time scale and the major

- cooling event. *Journal of the Geological Society*. 161, 161–172. doi:10.1144/0016-764903-091
- Huber, M. and Sloan, L.C., 2001. Heat transport, deep waters, and thermal gradients: Coupled simulation of an Eocene greenhouse climate. *Geophys. Res. Lett.* 28, 3481–3484. doi:10.1029/2001GL012943
- Jablonski, D., Jandzik, D., Mikulíček, P., Džukić, G., Ljubisavljević, K., Tzankov, N., Jelić, D., Thanou, E., Moravec, J., and Gvoždík, V., 2016. Contrasting evolutionary histories of the legless lizards slow worms (*Anguis*) shaped by the topography of the Balkan Peninsula. *BMC Evol. Biol.* 16, 99. doi:10.1186/s12862-016-0669-1
- Jing-jing, Z., Li W., Hong, G., Tong, X., Qiu-ying, B., Yong-xiang, Z., De-min, Z., and Gang, X, 2014. *De novo* transcriptome analysis and the phylogenetic position of glass lizards. *Journal of East China Normal University(Natural Science)*. 2014(4), 102-112.
- Keeley, J.E., and Rundel, P.W., 2005. Fire and the Miocene expansion of C4 grasslands. *Ecol. Lett.* 8, 683–690. doi:10.1111/j.1461-0248.2005.00767.x
- Keskin E., Hayretdag S., Cicek K., Ayaz D., and Tok C.V., 2013a. Genetic structuring of *Anguis fragilis* (L., 1758) inhabiting in the north of 40° north latitude in Turkey. *Mitochondrial DNA*. 24(5), 565-576. <http://dx.doi.org/10.3109/19401736.2013.770492>
- Keskin E. , Tok C.V., Hayretdag S., Cicek K., and Ayaz D., 2013b. Genetic structuring of *Pseudopus apodus* (Pallas, 1775) (Sauria: Anguidae) in north Anatolia, Turkey. *Biochemical Systematics and Ecology*. 50(October 2013), 411–418.
- Klembara, J., and Green, B., 2010. Anguimorph lizards (Squamata, Anguimorpha) from the Middle and Upper Eocene of the Hampshire Basin of Southern England. *Journal of Systematic Palaeontology*. 8, 97–129.
- Klembara, J., Böhme, M., and Rummel, M., 2010. Revision of the Anguine Lizard *Pseudopus laurillardii* (Squamata, Anguidae) from the Miocene of Europe, with Comments on Paleoecology. *J. Paleontol.* 84, 159–196. doi:10.1666/09-033R1.1
- Klembara, J. 2012. A new species of *Pseudopus* (Squamata, Anguidae) from the early Miocene of North-West Bohemia (Czech Republic). *Journal of Vertebrate Paleontology*. 32(4), 854–866.
- Klembara, J., Hain, M., Dobiašová, K., 2014. Comparative anatomy of the lower jaw and dentition of *Pseudopus apodus* and the interrelationships of species of subfamily Anguinae (Anguimorpha, Anguidae). *Anat. Rec.* 297, 516–544. doi:10.1002/ar.22854

- Klembara, J. 2015. New finds of anguines (Squamata, Anguidae) from the Early Miocene of Northwest Bohemia (Czech Republic). *Paläontologische Zeitschrift*. 89(2), 171–195. doi:10.1007/s12542-014-0226-4
- Lanfear, R., 2012. Manual, 29, 1695–1701. doi:10.1093/bioinformatics/btu033
- Lanfear, R., Frandsen, P. B., Wright, A. M., Senfeld, T., Calcott, B., 2016. PartitionFinder 2: new methods for selecting partitioned models of evolution formolecular and morphological phylogenetic analyses. *Molecular Biology and Evolution*. DOI: dx.doi.org/10.1093/molbev/msw260
- Lin, S., Chang, W., Chen, S., Shang, G., and Lue, K., 2003. Taxonomic Status of the Legless Lizard. *Zoological Studies*, 42, 411–419.
- Lu, S., Yuan, Z.-G., Pang, J., Yang, D., Yu, F., Mcguire, P., Xie, F., and Zhang, Y., 2004. Molecular phylogeny of the genus *Paramesotriton* (Caudata: Salamandridae). *Biochem. Genet.* 42, 139–48. doi:10.1023/B:BIGI.0000026630.78770.fb
- Macey, J.R., Schulte, J.A., Larson, A., Tuniyev, B.S., Orlov, N., and Papenfuss, T.J., 1999. Molecular phylogenetics, tRNA evolution, and historical biogeography in anguid lizards and related taxonomic families. *Mol. Phylogenet. Evol.* 12, 250–272.
- Macey, J.R., Schulte, J. a, Strasburg, J.L., Brisson, J. a, Larson, A., Ananjeva, N.B., Wang, Y., Parham, J.F., and Papenfuss, T.J., 2006. Assembly of the eastern North American herpetofauna: new evidence from lizards and frogs. *Biol. Lett.* 2, 388–392. doi:10.1098/rsbl.2006.0473
- McConkey, E. H. 1954. A systematic study of North American lizards of the genus *Ophisaurus*. *American Midland Nat.* 51(1), 133-171.
- Meszoely, C.A.M., and Haubold, H., 1975. The Status of the Middle Eocene Geiseltal Limbless Anguid Lizards. *Copeia*. 1975(1), 36-43.
- Miller, M.A., Pfeiffer, W., and Schwartz, T., 2010. "Creating the CIPRES Science Gateway for inference of large phylogenetic trees" in Proceedings of the Gateway Computing Environments Workshop (GCE), 14 Nov. 2010, New Orleans, LA pp 1 - 8.
- Mulcahy, D.G., Noonan, B.P., Moss, T., Townsend, T.M., Reeder, T.W., Sites, J.W., and Wiens, J.J., 2012. Molecular Phylogenetics and Evolution Estimating divergence dates and evaluating dating methods using phylogenomic and mitochondrial data in squamate reptiles. *Mol. Phylogenet. Evol.* 65, 974–991. doi:10.1016/j.ympev.2012.08.018

- Nguyen, T.Q., Bohme, W., Nguyen, T.T., Le, Q.K., Pahl, K.R., Haus, T., and Ziegler, T., 2011. Review of the genus *Dopasia* Gray, 1853 (Squamata: Anguidae) in the Indochina subregion. *Zootaxa* 68, 58–68.
- Pan, H.C., Liu, L., Li, P., Li, X.F., Liu, Z.L., 2015. The complete mitochondrial genome of Chinese glass lizard *Ophisaurus harti* (Squamata: Anguidae). *Mitochondrial DNA*, 26(2), 280-281.
- Portik, D.M., Wood, P.L., Grismer, J.L., Stanley, E.L., and Jackman, T.R., 2012. Identification of 104 rapidly-evolving nuclear protein-coding markers for amplification across scaled reptiles using genomic resources. *Conserv. Genet. Resour.* 4, 1–10. doi:10.1007/s12686-011-9460-1
- Pramuk, J.B., Robertson, T., Sites, J.W., and Noonan, B.P., 2008. Around the world in 10 million years: Biogeography of the nearly cosmopolitan true toads (Anura: Bufonidae). *Glob. Ecol. Biogeogr.* 17, 72–83. doi:10.1111/j.1466-8238.2007.00348.x
- Prothero, D.R., 1994. The late Eocene–Oligocene extinctions. *Annual Reviews of the Earth and Planetary Sciences*. 22, 145–165. doi:10.1146/annurev.ea.22.050194.001045
- Pyron, R.A., Burbrink, F.T., and Wiens, J.J., 2013. A phylogeny and revised classification of Squamata, including 4161 species of lizards and snakes. *BMC Evol. Biol.* 13, 93. doi:10.1186/1471-2148-13-93
- Rage, J.C. and Augé, M. 2003. Amphibians and squamate reptiles from the lower Eocene of Silveirinha (Portugal). *Ciências da Terra (UNL)*, 15, 103-116.
- Rambaut A, Suchard MA, Xie D & Drummond AJ (2014) Tracer v1.6, Available from <http://beast.bio.ed.ac.uk/Tracer>
- Raymo, M.E. & Ruddiman W.F. Tectonic forcing of late Cenozoic climate. *Nature* 359, 117 - 122 (10 September 1992); doi:10.1038/359117a
- Rifai, L., Baker, M.A.B.U., Shafei, D.A.L., Disi, A., Mahasneh, A., Amr, Z., 2005. *Pseudopus apodus* (Pallas, 1775) from Jordan, with notes on its ecology. *Herpetozoa*, 18, 133–140.
- Ronquist, F., Teslenko, M., Van Der Mark, P., Ayres, D.L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M.A., and Huelsenbeck, J.P., 2012. Mrbayes 3.2: Efficient bayesian phylogenetic inference and model choice across a large model space. *Syst. Biol.* 61, 539–542. doi:10.1093/sysbio/sys029
- Sambrook, J. & Russell, D.W., 2001. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Plainview, NY, USA.

- Sanmartín, I., Enghoff, H., Ronquist, F., 2001. Patterns of animal dispersal, vicariance and diversification in the Holarctic. *Biol. J. Linn. Soc.* 73, 345–390. doi:10.1006/bijl.2001.0542
- Sluijs, A., G. J. Bowen, H. Brinkhuis, L. J. Lourens, and E. Thomas (2007), The Palaeocene-Eocene thermal maximum super greenhouse: Biotic and geochemical signatures, age models and mechanisms of global change, *in* Deep Time Perspectives on Climate Change: Marrying the Signal from Computer Models and Biological Proxies, *edited by* M. Williams *et al.*, Geol. Soc. Spec. Publ., 347, 323–347.
- Smith, T.B., Wayne, R.K., Girman, D.J. and Bruford, M.W. 1997. A role for ecotones in generating rainforest biodiversity. *Science*. 276, 1855–1857.
- Stamatakis, A., 2014. RAxML version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics*. 30, 1312–1313. doi:10.1093/bioinformatics/btu033
- Stebbins, R.C., 1958. A new alligator lizard from the Panamint Mountains, Inyo County, California. *Am. Museum Novit.* 1883, 1–27.
- Strömberg, C. A. E., 2011. Evolution of Grasses and Grassland Ecosystems. *Annu. Rev. Earth Planet. Sci.* 39, 517–544. doi:10.1146/annurev-earth-040809-152402
- Song, B., Cheng, S., Sun, Y., Zhong, X., Jin, J., Guan, R., Murphy, R.W., Che, J., Zhang, Y., and Liu, X., 2015. A genome draft of the legless anguid lizard, *Ophisaurus gracilis*. *Gigascience*. 4, 17. doi:10.1186/s13742-015-0056-7
- Tiffney, B.H., 1985. The Eocene North Atlantic land bridge: its importance in Tertiary and modern phytogeography of the Northern Hemisphere. *J. Arnold Arbor.* 66, 243–273.
- Townsend, T.M., Alegre, R.E., Kelley, S.T., Wiens, J.J., and Reeder, T.W., 2008. Rapid development of multiple nuclear loci for phylogenetic analysis using genomic resources: An example from squamate reptiles. *Mol. Phylogenet. Evol.* 47, 129–142. doi:10.1016/j.ympev.2008.01.008
- Townsend, T.M., Leavitt, D.H., and Reeder, T.W., 2011. Intercontinental dispersal by a microendemic burrowing reptile (Dibamidae). *Proc. Biol. Sci.* 278, 2568–2574. doi:10.1098/rspb.2010.2598
- Vidal, N., and Hedges, S. B., 2005. The phylogeny of squamate reptiles (lizards, snakes, and amphisbaenians) inferred from nine nuclear protein-coding genes. *CR Biologies*. 328, 1000–1008.
- Vidal, N. and Hedges, S. B., 2009. The molecular evolutionary tree of lizards, snakes, and amphisbaenians. *CR Biologies*, 332, 129–139.

- Vidal, N., Marin, J., Sassi, J., Battistuzzi, F.U., Donnellan, S., Rodriguez, R.C., and Vega, D., 2012. Molecular evidence for an Asian origin of monitor lizards followed by Tertiary dispersals to Africa and Australasia. *Biol Lett.* 8(5), 853–855.
- Wu, Y., Wang, Y., Jiang, K., Chen, X., and Hanken, J., 2010. Homoplastic evolution of external colouration in Asian stout newts (*Pachytriton*) inferred from molecular phylogeny. *Zool. Scr.* 39, 9–22. doi:10.1111/j.1463-6409.2009.00400.x
- Wüster, W., Peppin, L., Pook, C.E., and Walker, D.E., 2008. A nesting of vipers: Phylogeny and historical biogeography of the Viperidae (Squamata: Serpentes). 49, 445–459. doi:10.1016/j.ympev.2008.08.019
- Yang, Z. 2015. A tutorial of BPP for species tree estimation and species delimitation. *Current Zoology.* 61, 854–865.
- Yuan, Z., Jiang, K., Ding, L., Zhang, L., and Che, J., 2013. A new newt of the genus *Cynops* (Caudata: Salamandridae) from Guangdong, China. *Asian Herpetol. Res.* 4, 116–123. doi:10.3724/SP.J.1245.2013.00116
- Zachos, J., Pagani, M., Sloan, L., Thomas, E., and Billups, K., 2001. Trends, rhythms, and aberrations in global climate 65 Ma to present. *Science* 292(5517), 686–693.

Table 1. Voucher Numbers, Species Identification, General Locality, and Genbank Submission Numbers of Anguinae taxa used in this study. More detailed information may be available from specific museum databases AMNH = American Museum of Natural History, CAS = California Academy of Sciences, FMNH = Field Museum of Natural History, Chicago, INHS = Illinois Natural History Survey, KU = Biodiversity Institute and Natural History Museum, University of Kansas, MVZ = Museum of Vertebrate Zoology, UC Berkeley, NCSM = North Carolina Museum of Natural Science, ROM = Royal Ontario Museum, TAUM = Tel Aviv University Natural History Museum, UF = Florida Museum of Natural History.

Voucher	Species	Locality	ADNP	AHR	AMEL	BDNF	BMP2
MVZ178120	Hyalosaurus koellikeri	Morocco	XX#####	XX#####	XX#####	XX#####	XX#####
MVZ218728	Anguis colchica	Georgia					
MVZ238522	Anguis colchica	Iran					
MVZ238523	Anguis colchica	Iran					
MVZ197610	Anguis fragilis	Germany					
MVZ218775	Pseudopus apodus	Georgia					
MVZ233250	Pseudopus apodus	Turkmenistan					
MVZ246008	Pseudopus apodus	Iran					
TAUM16223	Pseudopus apodus	Israel					
TAUM16544	Pseudopus apodus	Israel					
ROM30852	Dopasia hainanensis	Tuyan Quang, Vietnam					
MVZ224111	Dopasia hainanensis	Vinh Phuc, Vietnam					
MVZ230055	Dopasia hainanensis	Vinh Phuc, Vietnam					
CAS233231	Dopasia gracilis	Chin State, Myanmar					
AMNH15377	Dopasia gracilis	Ha Gaing, Vietnam					
ROM40319	Dopasia gracilis	Lao Cai, Vietnam					
ROM40320	Dopasia gracilis	Lao Cai, Vietnam					
AMNH14722	Dopasia sokolovi	Quang Nam, Vietnam					
NCSM79751	Dopasia sokolovi	Kom Tum, Vietnam					
NCSM77336	Dopasia sokolovi	Lam Dong, Vietnam					
FMNH258689	Dopasia sokolovi	Champasak, Laos					
MVZ137540	Ophisaurus ventralis	North Carolina, USA					
MVZ137541	Ophisaurus ventralis	North Carolina, USA					
UF151479	Ophisaurus ventralis	Florida, USA					
UF151363	Ophisaurus ventralis	Florida, USA					
UF150363	Ophisaurus compressus	Florida, USA					
UF150374	Ophisaurus compressus	Florida, USA					
INHS23264	Ophisaurus a. attenuates	Illinois, USA					

KU307850	Ophisaurus a. attenuates	Kansas, USA					
KU307860	Ophisaurus a. attenuates	Kansas, USA					
NCSM79286	Ophisaurus a. longicaudus	South Carolina, USA					
UF151424	Ophisaurus a. longicaudus	Florida, USA					
Voucher	Species	Locality	CMOS	DNAH3	FSTL5	Kiaa1217	Kiaa1549
MVZ178120	Hyalosaurus koellikeri	Morocco	XX#####	XX#####	XX#####	XX#####	XX#####
MVZ218728	Anguis colchica	Georgia					
MVZ238522	Anguis colchica	Iran					
MVZ238523	Anguis colchica	Iran					
MVZ197610	Anguis fragilis	Germany					
MVZ218775	Pseudopus apodus	Georgia					
MVZ233250	Pseudopus apodus	Turkmenistan					
MVZ246008	Pseudopus apodus	Iran					
TAUM16223	Pseudopus apodus	Israel					
TAUM16544	Pseudopus apodus	Israel					
ROM30852	Dopasia hainanensis	Tuyen Quang, Vietnam					
MVZ224111	Dopasia hainanensis	Vinh Phuc, Vietnam					
MVZ230055	Dopasia hainanensis	Vinh Phuc, Vietnam					
CAS233231	Dopasia gracilis	Chin State, Myanmar					
AMNH15377	Dopasia gracilis	Ha Gaing, Vietnam					
ROM40319	Dopasia gracilis	Lao Cai, Vietnam					
ROM40320	Dopasia gracilis	Lao Cai, Vietnam					
AMNH14722	Dopasia sokolovi	Quang Nam, Vietnam					
NCSM79751	Dopasia sokolovi	Kom Tum, Vietnam					
NCSM77336	Dopasia sokolovi	Lam Dong, Vietnam					
FMNH258689	Dopasia sokolovi	Champasak, Laos					
MVZ137540	Ophisaurus ventralis	North Carolina, USA					
MVZ137541	Ophisaurus ventralis	North Carolina, USA					
UF151479	Ophisaurus ventralis	Florida, USA					
UF151363	Ophisaurus ventralis	Florida, USA					

UF150363	Ophisaurus compressus	Florida, USA					
UF150374	Ophisaurus compressus	Florida, USA					
INHS23264	Ophisaurus a. attenuates	Illinois, USA					
KU307850	Ophisaurus a. attenuates	Kansas, USA					
KU307860	Ophisaurus a. attenuates	Kansas, USA					
NCSM79286	Ophisaurus a. longicaudus	South Carolina, USA					
UF151424	Ophisaurus a. longicaudus	Florida, USA					
Voucher	Species	Locality	Kiaa1549	Kif24	MKL1	NGFB	NTF3
MVZ178120	Hyalosaurus koellikeri	Morocco	XX#####	XX#####	XX#####	XX#####	XX#####
MVZ218728	Anguis colchica	Georgia					
MVZ238522	Anguis colchica	Iran					
MVZ238523	Anguis colchica	Iran					
MVZ197610	Anguis fragilis	Germany					
MVZ218775	Pseudopus apodus	Georgia					
MVZ233250	Pseudopus apodus	Turkmenistan					
MVZ246008	Pseudopus apodus	Iran					
TAUM16223	Pseudopus apodus	Israel					
TAUM16544	Pseudopus apodus	Israel					
ROM30852	Dopasia hainanensis	Tuyen Quang, Vietnam					
MVZ224111	Dopasia hainanensis	Vinh Phuc, Vietnam					
MVZ230055	Dopasia hainanensis	Vinh Phuc, Vietnam					
CAS233231	Dopasia gracilis	Chin State, Myanmar					
AMNH15377	Dopasia gracilis	Ha Gaing, Vietnam					
ROM40319	Dopasia gracilis	Lao Cai, Vietnam					
ROM40320	Dopasia gracilis	Lao Cai, Vietnam					
AMNH14722	Dopasia sokolovi	Quang Nam, Vietnam					
NCSM79751	Dopasia sokolovi	Kom Tum, Vietnam					
NCSM77336	Dopasia sokolovi	Lam Dong, Vietnam					
FMNH258689	Dopasia sokolovi	Champasak, Laos					
MVZ137540	Ophisaurus ventralis	North Carolina, USA					

MVZ137541	Ophisaurus ventralis	North Carolina, USA					
UF151479	Ophisaurus ventralis	Florida, USA					
UF151363	Ophisaurus ventralis	Florida, USA					
UF150363	Ophisaurus compressus	Florida, USA					
UF150374	Ophisaurus compressus	Florida, USA					
INHS23264	Ophisaurus a. attenuates	Illinois, USA					
KU307850	Ophisaurus a. attenuates	Kansas, USA					
KU307860	Ophisaurus a. attenuates	Kansas, USA					
NCSM79286	Ophisaurus a. longicaudus	South Carolina, USA					
UF151424	Ophisaurus a. longicaudus	Florida, USA					
Voucher	Species	Locality	PRLR	PTGER4	RAG2	SCNAIP	ZEB2
MVZ178120	Hyalosaurus koellikeri	Morocco	XX#####	XX#####	XX#####	XX#####	XX#####
MVZ218728	Anguis colchica	Georgia					
MVZ238522	Anguis colchica	Iran					
MVZ238523	Anguis colchica	Iran					
MVZ197610	Anguis fragilis	Germany					
MVZ218775	Pseudopus apodus	Georgia					
MVZ233250	Pseudopus apodus	Turkmenistan					
MVZ246008	Pseudopus apodus	Iran					
TAUM16223	Pseudopus apodus	Israel					
TAUM16544	Pseudopus apodus	Israel					
ROM30852	Dopasia hainanensis	Tuyen Quang, Vietnam					
MVZ224111	Dopasia hainanensis	Vinh Phuc, Vietnam					
MVZ230055	Dopasia hainanensis	Vinh Phuc, Vietnam					
CAS233231	Dopasia gracilis	Chin State, Myanmar					
AMNH15377	Dopasia gracilis	Ha Gaing, Vietnam					
ROM40319	Dopasia gracilis	Lao Cai, Vietnam					
ROM40320	Dopasia gracilis	Lao Cai, Vietnam					
AMNH14722	Dopasia sokolovi	Quang Nam, Vietnam					
NCSM79751	Dopasia sokolovi	Kom Tum, Vietnam					

NCSM77336	Dopasia sokolovi	Lam Dong, Vietnam					
FMNH258689	Dopasia sokolovi	Champasak, Laos					
MVZ137540	Ophisaurus ventralis	North Carolina, USA					
MVZ137541	Ophisaurus ventralis	North Carolina, USA					
UF151479	Ophisaurus ventralis	Florida, USA					
UF151363	Ophisaurus ventralis	Florida, USA					
UF150363	Ophisaurus compressus	Florida, USA					
UF150374	Ophisaurus compressus	Florida, USA					
INHS23264	Ophisaurus a. attenuates	Illinois, USA					
KU307850	Ophisaurus a. attenuates	Kansas, USA					
KU307860	Ophisaurus a. attenuates	Kansas, USA					
NCSM79286	Ophisaurus a. longicaudus	South Carolina, USA					
UF151424	Ophisaurus a. longicaudus	Florida, USA					
Voucher	Species	Locality	ZFP36L1	a-Enol	Cand1	DLL	LRRN
MVZ178120	Hyalosaurus koellikeri	Morocco	XX#####	XX#####	XX#####	XX#####	XX#####
MVZ218728	Anguis colchica	Georgia					
MVZ238522	Anguis colchica	Iran					
MVZ238523	Anguis colchica	Iran					
MVZ197610	Anguis fragilis	Germany					
MVZ218775	Pseudopus apodus	Georgia					
MVZ233250	Pseudopus apodus	Turkmenistan					
MVZ246008	Pseudopus apodus	Iran					
TAUM16223	Pseudopus apodus	Israel					
TAUM16544	Pseudopus apodus	Israel					
ROM30852	Dopasia hainanensis	Tuyan Quang, Vietnam					
MVZ224111	Dopasia hainanensis	Vinh Phuc, Vietnam					
MVZ230055	Dopasia hainanensis	Vinh Phuc, Vietnam					
CAS233231	Dopasia gracilis	Chin State, Myanmar					
AMNH15377	Dopasia gracilis	Ha Gaing, Vietnam					
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NCSM79751	Dopasia sokolovi	Kom Tum, Vietnam					
NCSM77336	Dopasia sokolovi	Lam Dong, Vietnam					
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MVZ137540	Ophisaurus ventralis	North Carolina, USA					
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INHS23264	Ophisaurus a. attenuates	Illinois, USA					
KU307850	Ophisaurus a. attenuates	Kansas, USA					
KU307860	Ophisaurus a. attenuates	Kansas, USA					
NCSM79286	Ophisaurus a. longicaudus	South Carolina, USA					
UF151424	Ophisaurus a. longicaudus	Florida, USA					
Voucher	Species	Locality	16s	Cytb	ND4		
MVZ178120	Hyalosaurus koellikeri	Morocco	XX#####	XX#####	XX#####		
MVZ218728	Anguis colchica	Georgia					
MVZ238522	Anguis colchica	Iran					
MVZ238523	Anguis colchica	Iran					
MVZ197610	Anguis fragilis	Germany					
MVZ218775	Pseudopus apodus	Georgia					
MVZ233250	Pseudopus apodus	Turkmenistan					
MVZ246008	Pseudopus apodus	Iran					
TAUM16223	Pseudopus apodus	Israel					
TAUM16544	Pseudopus apodus	Israel					
ROM30852	Dopasia hainanensis	Tuyan Quang, Vietnam					
MVZ224111	Dopasia hainanensis	Vinh Phuc, Vietnam					
MVZ230055	Dopasia hainanensis	Vinh Phuc, Vietnam					

CAS233231	Dopasia gracilis	Chin State, Myanmar					
AMNH15377	Dopasia gracilis	Ha Gaing, Vietnam					
ROM40319	Dopasia gracilis	Lao Cai, Vietnam					
ROM40320	Dopasia gracilis	Lao Cai, Vietnam					
AMNH14722	Dopasia sokolovi	Quang Nam, Vietnam					
NCSM79751	Dopasia sokolovi	Kom Tum, Vietnam					
NCSM77336	Dopasia sokolovi	Lam Dong, Vietnam					
FMNH258689	Dopasia sokolovi	Champasak, Laos					
MVZ137540	Ophisaurus ventralis	North Carolina, USA					
MVZ137541	Ophisaurus ventralis	North Carolina, USA					
UF151479	Ophisaurus ventralis	Florida, USA					
UF151363	Ophisaurus ventralis	Florida, USA					
UF150363	Ophisaurus compressus	Florida, USA					
UF150374	Ophisaurus compressus	Florida, USA					
INHS23264	Ophisaurus a. attenuates	Illinois, USA					
KU307850	Ophisaurus a. attenuates	Kansas, USA					
KU307860	Ophisaurus a. attenuates	Kansas, USA					
NCSM79286	Ophisaurus a. longicaudus	South Carolina, USA					
UF151424	Ophisaurus a. longicaudus	Florida, USA					
Gene	Species	Genbank #					
a - Enol	-----	XX#####					
ADNP	Elgaria multicarinata	GU432443					
AHR	Elgaria multicarinata	JF818270					
AMEL	-----	-----					
BDNF	Elgaria multicarinata	GU457854					
BMP2	Elgaria multicarinata	GU457887					
CAND1	Elgaria multicarinata	GU432602					
CMOS	Elgaria multicarinata	AF039479					
DLL	Elgaria multicarinata	GU432645					
DNAH3	Elgaria multicarinata	GU457916					

FSTL5	Elgaria multicarinata	GU457947		
GPR37	Elgaria multicarinata	GU432752		
Kiaa1217	Elgaria multicarinata	-----		
Kiaa1549	Elgaria multicarinata	-----		
Kif24	Elgaria multicarinata	-----		
LRRN	Elgaria multicarinata	GU432560		
MKL1	Elgaria multicarinata	JF804445		
NGFB	Elgaria multicarinata	GU432720		
NTF3	Elgaria multicarinata	GU456010		
PRLR	Elgaria multicarinata	JN880830		
PTGER4	Elgaria multicarinata	JN662841		
RAG2	Elgaria kingie	HQ426514		
SCNAIP	Elgaria multicarinata	GU432680		
ZEB2	Elgaria multicarinata	GU456232		
ZFP36L1	Elgaria multicarinata	JN654892		
16s	Abronia graminea	AB080273		
Cytb	Abronia graminea	AB080273		
ND4	Abronia graminea	AB080273		

Table 2. Models and Partitions from Partitionfinder under the BIC for maximum likelihood and Bayesian analyses.

	RAxML (mtDNA Dataset)	MrBayes (mtDNA Dataset)	RAxML (Nuclear Dataset)	MrBayes (Nuclear Dataset)	BEAST (Nuclear Dataset)	RAxML (Concatenated Dataset)	MrBayes (Concatenated Dataset)
Partition 1	Codon Position 1, tRNA (GTR+I+G)	Codon Position 1, tRNA (GTR+G)	CMOS, RAG2, ZEB2, FSTL5, ADNP, CAND1, DLL, GPR37, alphaenolase, ZFP36L1 (GTR+I+G)	CMOS, FSTL5, CAND1, ADNP, AHR, RAG2, ZEB2 (HKY+G)	BDNF, CMOS, FSTL5, CAND1, ADNP, AHR, RAG2, ZEB2 (HKY+G+X)	CMOS, RAG2, ZEB2, FSTL5, ADNP, CAND1, DLL, GPR37, alphaenolase, ZFP36L1, Kif24, AMEL, KIAA1549, LRRN (GTR+I+G)	CMOS, FSTL5, CAND1, ADNP, AHR, RAG2, ZEB2 (HKY+G)
Partition 2	Codon Position 2 (GTR+I+G)	Codon Position 2 (HKY+I+G)	PRLR, AHR, NTF3, BDNF, SCNAIP, KIAA1217, DNAH3, PTGER4, MKL1, BMP2, NGFB (GTR+I+G)	AMEL (F81+G)	AMEL (HKY+G+X)	PRLR, AHR, NTF3, BDNF, SCNAIP, KIAA1217, DNAH3, PTGER4, MKL1, BMP2, NGFB, LRRN (GTR+I+G)	AMEL (F81)
Partition 3	Codon Position 3 (GTR+I+G)	Codon Position 3 (GTR+I+G)	Kif24, AMEL, KIAA1549, LRRN (GTR+I+G)	BDNF, ZFP36L1, DLL, GPR37 (K80+I)	ZFP36L1, MKL1, BMP2, GPR37, DLL (K80+G)	SCNAIP, PTGER4, NGFB, DNAH3, KIAA1217, NTF3, PRLR (GTR+I+G)	BDNF, ZFP36L1, DLL, GPR37 (K80+I)
Partition 4	16s (GTR+I+G)	16s (HKY+I+G)	NA	MKL1, BMP2, SCNAIP, NGFB, PTGER4, DNAH3, KIAA1217, PRLR, NTF3 (HKY+I+G)	SCNAIP, PTGER4, NGFB, DNAH3, KIAA1217, NTF3, PRLR (HKY+I+G+X)	alphaenolase, KIAA1549, Kif24, mtDNA Codon Position 2 (GTR+I+G)	MKL1, BMP2, SCNAIP, NGFB, PTGER4, DNAH3, KIAA1217, PRLR, NTF3 (HKY+I+G)
Partition 5	NA	NA	NA	alphaenolase, KIAA1549, Kif24	alphaenolase, KIAA1549, Kif24	mtDNA Codon Position 1, 16s	alphaenolase, KIAA1549, Kif24,

				(K80+I+G)	(K80+I+G)	(GTR+I+G)	mtDNA Codon Position 2 (HKY+I+G)
Partition 6	NA	NA	NA	LRRN (HKY+G)	LRRN (HKY+G+X)	mtDNA Codon Position 3, tRNA (GTR+I+G)	LRRN (HKY+G)
Partition 7	NA	NA	NA	NA	NA	NA	mtDNA Codon Position 1, 16s (SYM+G)
Partition 8	NA	NA	NA	NA	NA	NA	mtDNA Codon Position 3 (GTR+I)

Table 3. Locus Model Testing Results from Partition Finder. RAXML not shown since it is limited to GTR models.

Locus	Mr. Bayes Model	BEAST Model
Alpha Enolase	K80	--
ADNP	HKY	--
AHR	HKY	--
AMEL	HKY	--
BDNF	K80	--
BMP2	K80 + G	TrNef + G
CAND1	K80	--
CMOS	K80	--
DLL	JC + G	--
DNAH3	K80 + I	--
FSTL5	K80 + I	--
GPR37	K80	--
Kiaa1217	HKY	--
Kiaa1549	HKY + G	--
Kif24	HKY + G	--
LRRN	HKY + I	--
MKL1	K80	--
NGFB	K80 + I	--
NTF3	K80 + G	--
PRLR	HKY + G	--
PTGER4	K80	--
RAG2	HKY	--
SCNAIP	K80	--
ZEB2	HKY + G	TrN + G
ZFP38L1	HKY	--
mtDNA	GTR + G + I	--

Table 4. MRCA of species and important Anguinae clades. Bold numbers indicate hard constraints.

MCRA	Min 95% Cred. Interval	Mean	Max 95% Cred. Interval
D. hainanensis	1.06	9.72	24.97
D. sokolovi	.83	5.52	12.37
D. gracilis/D. sokolovi	5.6	16.74	30.51
Dopasia	9.2	22.88	36.47
O. ventralis	1.61	11.57	25.92
O. compressus	.11	5.13	17.59
O. attenuatus attenuatus	.4	4.81	12.57
O. a. attenuatus/O. a longicaudus	3.27	11.61	24.87
O. compressus/O. attenuatus	6.73	16.86	30.82
Ophisaurus	10.5	23.07	39.34
Dopasia/Ophisaurus	16.98	32.08	46.63
Pseudopus apodus apodus	.26	5.78	14.58
Pseudopus apodus spp.	.11	3.88	12.7
Pseudopus	3.69	12.98	20.92
Anguis colchica	.43	5.87	14.22
Anguis	3.52	12.36	20.92
Anguis/Pseudopus	16.9	20.49	24.43
Anguis/Pseudopus/Hyalosaurus	18.17	27.74	41.68
Anguinae	25.81	39.39	52.92
Anguinae/ <i>Gerrhonotinae</i>	48.6	51.86	57.0

Figure 1. Map showing sampling localities of Anguinae samples or sequences used in this study. Circles with black dots represent mtDNA localities taken from Genbank. 1 = Approximate location of Thulean Land bridge, 2 = Approximate location of Turgei Strait/Sea, 3 = Beringia/Bering Land bridge.

Figure 2. Bayesian mtDNA phylogeny of Anguinae. Samples marked with (G) were included from Genbank and not generated in this study. Asterisks denote $pp < .95$ and $BS < 70$.

Figure 3. Bayesian nuclear phylogeny of Anguinae. Asterisks denote $pp < .95$ and $BS < 70$.

Figure 4. Bayesian concatenated phylogeny of Anguinae. Asterisks denote $pp < .95$ and $BS < 70$.

Figure 5. Chronogram of Anguinae denoting node dates and 95% credibility intervals.

Figure 6. Species Tree of Anguinae denoting consensus figure as well as a Densitree plot overlaying all post burn in trees.