RESOLVING THE RELATIONSHIPS OF THE SQUAMATE TREE OF LIFE: AN ASSESSMENT OF NEW APPROACHES AND PROBLEMS

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April 30, 2014

A Senior Thesis presented to the faculty of the Department of Geology and Geophysics, Yale University, in partial fulfillment of the Bachelor's Degree.

Abstract

Since the division of The Deep Scaly Project into separate morphological and molecular teams, a truly integrated and wide scoped project has not been attempted. Much more can be done to understand how the members of Squamata are related to one another through an approach that combines the importance of both morphological and molecular evolution. Here we have developed a novel three-step methodological approach to squamate phylogenetics that incorporates the newest phylogeny-creating techniques and data from previous morphological and genetic analyses. First, we analyze a large squamate morphological dataset using Lewis's Mkv model under both a Bayesian and maximum likelihood framework. Second, we incorporate a previously constructed squamate DNA dataset and analyze the combined data within a "total evidence" framework. Finally, we adopt a methodology that treats genes, rather than nucleotides, as the character of interest.

We find that the separate analyses of the morphological and molecular datasets, even under Bayesian and maximum likelihood frameworks, still result in drastically different relationships between higher-order clades within Squamata. Additionally, we find that the combination of these two datasets results in a phylogeny with limited support for either topology, although it definitively leans in the direction of the molecular results. Finally, by reducing the molecular dataset to gene characters, we find significantly lower support for the higher-order relationships that are strongly supported in previous analyses. By combining this data with our morphological dataset, we discover that we have inversed the effect of the power in numbers problem.

We conclude that combining datasets, although possibly detrimental to results, should be treated as a source of understanding how the datasets may differ and how they may reflect different evolutionary histories.

I. Background

Assembling the Tree of Life

The Assembling the Tree of Life (AToL) project began as a nation-wide interdisciplinary research effort to reconstruct the evolutionary origin and history of all living things. Since its inception in 1999, under the funding of the National Science Foundation, the project has

addressed the evolutionary relationships of microbes, fungi, plants, numerous invertebrate groups, and all modern and extinct vertebrate groups. Additionally, the AToL project has produced numerous investigations of computational approaches and analysis and data management infrastructure for further phylogenetic analyses of systematic data. With these theoretical and technical advances, the development of a single Tree of Life is no longer a fantasy. Phylogenetic research such as this has many potential benefits to society, such as discovering new life forms, improving agriculture, identifying and tracking the transmission of emergent diseases, tracing developmental change, protecting ecosystems from invasive species, saving species on the brink of extinction, and developing new frameworks and infrastructure for searching for, sharing, and using data.

The Squamate section of the AToL project, titled "The Deep Scaly Project: Resolving Higher Level Squamate Phylogeny Using Genomic and Morphological Approaches", began in 2004 under the direction of Tod Reeder, John Wiens, Maureen Kearney, Jack Sites, Oliver Rieppel, Jessica Maisano, and Jacques Gauthier. Despite the title of the project, the group soon broke in two to separately use the morphological and genomic approaches. 30 peer-reviewed publications are attributed to this initial 6 year phase of the project; however, most of that literature is restricted to the molecular and genomic approaches performed under John Wiens (Brandley et al., 2008; Wiens, 2008; Wiens et al., 2006; Wiens et al., 2010a; Wiens et al., 2008). It was not until 2012 when Gauthier et al. were finally able to release the results from their morphological approaches (Gauthier et al., 2012). This landmark paper revealed the extreme incongruence between the histories that are inferred from the morphological and molecular data of Squamates. Further development of these two datasets have further produced incongruence, and new approaches to the systematics of Squamates are necessary to understand how the clade has evolved and why the genes and phenotypes do not agree.

Morphology vs. Molecules

Since the advent of modern computing, molecular phylogenetics has grown in size to the point where thousands of organisms can be analyzed, while taking into account the state changes of tens to hundreds of thousands of nucleotide sites. GenBank, a government-supported site for the hosting and distribution of genetic sequences, has made it possible to

access the sequences for more than 300,000 distinct organisms, making these molecular phylogenetic analyses easier than ever. Recently, a debate has developed between morphological and molecular phylogeneticists concerning the use of morphological data in phylogenetic systematics. While the use of isolated data of one kind or the other has its advantages and disadvantages, combining the data may also introduce other problems.

Many molecular systematists regard morphological data as inferior; however, there are many advantages to its use over molecular data. The use of morphological types of data allows for much more thorough sampling of taxa. This is mostly due to the ease of accessibility of specimens for morphological study such as museum specimens. Molecular studies can only use genetic material from fresh specimens, which may make it quite hard to sample particular species that are very rare in the wild or live in very remote areas of the world (Wiens, 2000). Thorough taxon sampling can subdivide long branches, which are commonly problematic in parsimony and likelihood analyses (Felsenstein, 1978; Huelsenbeck, 1997; Siddall, 1998). Additionally, each morphological character is often coded by different and multiple genes. Whereas genes can often evolve differently from one another over time, morphological data should be fairly impervious to this process (Doyle, 1992). Taxa in phylogenetic analyses are also generally described based on morphological characteristics. Therefore, morphology plays a leading role in determining which species are sampled, whether it is ultimately for a morphological or molecular analysis (Wiens, 2000).

Finally, fossils can only be included in systematic analyses through the use of morphological characters. Fossils can also be used to subdivide long branches and added to analyses as outgroups that are more closely related to the ingroup than the most closely related extant relative (Smith, 1998). Molecular analyses often fall short of determining the correct rooting and character polarity through the use of distantly related outgroups (Huelsenbeck, 1991; Huelsenbeck, 1994). While fossils may not provide complete data, they can often provide more information than extant taxa, especially when the extant taxa are extremely varied from each other (Gauthier et al., 1988). Fossils are also key tools for determining the rates and timing of macroevolutionary processes, including inferring divergence times of lineages (Smith, 1998; Wiens, 2004). Many criticisms with the use of fossils

in morphological and combined analyses stem from the large amount of missing data in the matrix. However, Gauthier et al. comment that "Perhaps the shortcomings of fossils have been emphasized while the effects of evolution and extinction on the information available in extant forms have been overlooked" (1988). In fact, the problem is not restricted to morphological matrices; new phylogenomic approaches often retain enormous amounts of missing data. Nonetheless, "It is the information content of the taxa coded what really matters, whether they are coded for 10% or 100% of their cells" (Giribet, 2010).

Unfortunately, the use of morphological data for phylogenetic analyses also has its disadvantages. Often morphological datasets are much smaller than those of molecular studies, often including fewer taxa and characters (Sanderson and Donoghue, 1989). Also, the genetic basis of morphological characters is generally unknown, although this is slowly changing. Therefore, there is no evidence for the independence of characters, and it must be assumed that all of the morphological characters have evolved independently (Emerson and Hastings, 1998). Additionally, this allows for the possibility of inferring relationships based on non-heritable variation. Often morphological selection can seem extremely arbitrary, and many researchers have called for explicit character selection criteria (Wiens, 2000). Molecular studies are able to include all of the nucleotides from the genes they are studying, but it would be impossible to account for every single minute detail that varies among the terminal taxa and unreasonable to account for all of the invariant characters within the taxa being studied. Especially with a group of organisms that have very little variation, it can often be difficult finding more characters to include to provide more phylogenetic resolution (Scotland et al., 2003).

Finally, many studies claim that there are greater amounts of convergence and homoplasy in morphological data than in molecular data because natural selection does not directly act on genetic material (Hedges and Maxson, 1996; Lamboy, 1994). Therefore, in an attempt to develop a larger morphological dataset, such data may actually accrue more homoplasy (Scotland et al., 2003). However, while it may be true that natural selection is stronger at the level of the organism and not at the level of the genes, "no generalities about

the phylogenetic utility of an entire class of characters, including morphology, are universally true" (Baker and Gatesy, 2002).

Meanwhile, using DNA for phylogenetic approaches also has advantages and disadvantages. Due to the nature of genetic information, genetic phylogenetic datasets tend to have large numbers of observable characters, which may produce more resolved and accurate phylogenetic results (based on simulations) (Hillis, 1987; Hillis et al., 1994). The morphological approach is limited in that it can only analyze morphologically distinct organisms but also cannot analyze the most distantly related lineages of life because it then becomes hard to pick characters for the analysis. However, molecular analyses can compare the genetic material from any two organisms that possess one or more of the same genes, whether they are conspecifics or very distantly related, because there is a wide range of substitution rates across nucleotide sites (Wiens, 2000). In this same vain, selection of molecular characters is quite objective through the inclusion of entire genes, and no characters are removed from the analysis due to invariability (Gift and Stevens, 1997; Wiens, 2000). Finally, several authors claim that molecular data contains less convergence and homoplasy because it is not directly acted on by natural selection (Hedges and Maxson, 1996; Lamboy, 1994).

However, using genetic material does have its shortcomings. While it has become less of a problem now, molecular sampling has generally been harden than morphological sampling due to the unavailability of fresh samples of species. Additionally, sequencing has traditionally been extremely expensive, although that is also quickly becoming irrelevant (Wiens, 2000). A large part of the argument against the use of molecular data stems from the incongruence of gene trees and species trees which has become quite prevalent (Doyle, 1992). However, including a number of different genes for an analysis has become the norm rather than the exception, and most scientific journals will no longer accept any analyses based solely on a single gene. Processes such as introgression, lineage sorting, and gene duplication may affect single genes but are unlikely to affect all of the genes that are included in modern molecular analyses (Doyle, 1992; Wiens, 2000).

Like the general feelings towards each of the separate approaches, a combined approach also has its advantages and disadvantages. Philosophically, many researchers believe

that all of the evidence should be used to get the most thorough analysis possible (De Queiroz et al., 1995). Additionally, combining all of this data then avoids any arbitrariness that could be inherent in either dataset or the methods used for consensus analyses (Kluge, 1989). Such consensus analyses are often inherently uninformed in the ways that they produce phylogenetic hypotheses and combined data has greater descriptive and explanatory power (De Queiroz et al., 1995; Kluge and Wolf, 1993). Finally, simulations have shown that combined data has a greater ability to uncover real phylogenetic groups that may otherwise be hidden by the use of separated data (De Queiroz et al., 1995; Hillis, 1987).

However, there are many reasons to keep data separate and perform analyses separately. For instance, it is very possible that poor resolution in one dataset may obscure the good resolution in another dataset (Bull et al., 1993; De Queiroz et al., 1995). Additionally, one dataset may overpower the other dataset merely by the virtue of having a larger number of characters. When combining datasets, it can often be hard to weight them in a manner that is not arbitrary when trying to account for different sizes of datasets (Bull et al., 1993; De Queiroz et al., 1995).

Also, a significant problem with combining data is the possible incongruence of the morphological and molecular data. Often morphological and molecular approaches have differences in assumptions, methods, and analyses; these differences can even preclude combining particular datasets (Bull et al., 1993). Finally, numerous studies have shown that gene evolution does not necessarily match the evolution of the species (De Queiroz, 1993; De Queiroz et al., 1995; Doyle, 1992; Wiens, 1998). Due to different levels of natural selection, differential rates of evolution, hybridization, horizontal transfer, or lineage sorting, it is possible that the genetic material and the morphological characters there may be recording different evolutionary histories (De Queiroz et al., 1995). “Combining the data implicitly assumes that all datasets are products of the same branching history; when this assumption does not hold, simple combination of all the data may not be the best approach” (De Queiroz et al., 1995).

Finding a Middleground

Is a pluralistic, “total evidence”, approach possible in phylogenetic systematics? Most certainly; a number of studies have already been performed using combined datasets (Wiens,

2009; Wiens et al., 2010b). Meanwhile, a simpler case of pluralism will most likely be maintained as long as morphological and molecular data remain opposed: separate morphological and molecular approaches, while inherently monistic, contribute to a pluralistic view within the field of phylogenetics. Unfortunately, with the new-found ease of molecular sequencing, the increased work required for morphological analyses may become a burden that many scientists are not willing to bear. Therefore, there should be a continued effort of characterizing species and recording morphological characters for future analyses.

Would a pluralistic approach be beneficial to phylogenetic systematics? Definitely; the field has grown quite a bit since the advent of genetic sequencing. The integration of morphological and molecular data brings together the importance of fossils and taxon naming (morphology) and the importance of supposedly-neutral and rapid evolution (molecules). However, in cases where separate analyses yield drastically different results, it may be more productive to maintain pluralism by undertaking both separate analyses and trying to understand why the different methods, data, and assumptions produce different results (Wiens, 2000). These underlying differences may ultimately lead to the broader understanding of macroevolutionary processes.

Since the division of The Deep Scaly Project into separate morphological and molecular teams, such an integrated approach has not been attempted until very recently (Wiens et al., 2010b). However, this analysis was extremely limited in its scope and approached the results with a particularly strong bias towards the previously resolved molecular results. While this analysis remains a key step in the production of a truly integrated and comprehensive Tree of Life, particularly for Squamates, much more can be done to understand how the members of Squamata are related to one another.

Therefore, the range of research put forth here is documented as the next step towards such an understanding. This research has been divided into three steps or sections, each approaching the problem of the Squamate Tree of Life from a progressively different angle. The first section covers the recent further development of the AToL Squamate morphology dataset, as first published by Gauthier et al. (2012). This section also discusses the new methods with which this dataset is now being tested, and the results from those analyses and how they

compare to those of Gauthier et al. (2012). The second section discusses the methodology and results of the analysis of a combined dataset consisting of the entirety of the morphology dataset of Gauthier et al. (2012) and the entirety of the molecular dataset of Wiens et al. (2012). The third section discusses a very new technique, here termed the Genes as Characters approach. This method is used to understand the true resolution of the molecular dataset of Wiens et al. (2012). This section explains the method, applies it to the 44 genes of that dataset, and describes the results of a combined data approach. Following this are some brief remarks on these methods as a whole, especially concerning the combination of these two datasets, and on some plausible next steps towards a unified theory of the evolution of squamates.

It should be noted that, although this research focuses solely on squamates, these methodologies, approaches, and remarks can be applied to any phylogenetic group. The combination of morphological and molecular data is slowly becoming common practice in the field of phylogenetics and systematics, and the application of these techniques to any and all taxa is crucial to the development of the field and of the evolutionary histories of these clades. Many other phylogenetic groups also possess phylogenetic inconsistencies between the analyses of various types of data, and the frame of mind and techniques put forth and applied here are of great importance to understand how and why datasets record different evolutionary histories and how to approach such situations.

II. Morphology with the Mkv Model

Introduction

In 1971, Walter Fitch developed a new method of cladistics that would revolutionize the field: maximum parsimony, or parsimony for short, took advantage of the morphological variation in species to infer how they were evolutionarily related. A parsimony analysis of discrete data (morphology, DNA, RNA, amino acids, etc.) employs Occam's razor by supposing the preferred evolutionary history is that which requires the least total change along its branches. Any trees with a greater number of total changes is considered inferior to this tree under maximum parsimony. While many other methods have also been developed since this time, parsimony remains a very common and useful tool in the field of phylogenetics. However, it is not without its flaws. Parsimony's simplicity makes it a very appealing application but also

leads to its downfall. This method assumes that all similarities must be homologous rather than homoplastic. Unfortunately, this often causes parsimony to infer that convergent taxa are actually closely related. This leads to a very similar problem that parsimony falls prey to: long branch attraction (Felsenstein, 1978; Felsenstein, 2004; Huelsenbeck, 1995; Huelsenbeck, 1997). Since long branches inherently possess more change than shorter branches, it is often likely that these branches will result in a high degree of convergent evolution. With this homoplasy, parsimony then infers that the long branches are closely related when this is in fact not true. Other methods of phylogenetic inference, such as Bayesian inference and maximum likelihood, are immune to long branch attraction, and may be more ideal for datasets that include long branches. Parsimony's simplistic approach has recently led to a final problem: that of the analysis of ever larger datasets. Maximum parsimony requires the assessment of all tree possibilities to determine the tree with the fewest changes. However, as more taxa are added to datasets, the number of possible trees increases remarkably—the number of possible trees for 65 taxa is larger than the number of elementary particles in the universe! Heuristic methods use hill-climbing algorithms to attempt to approach the best tree without analyzing all tree possibilities, but this computationally intensive process remains a problem for parsimony and other phylogeny inference methods.

While parsimony has remained a widely used method for phylogenetic inference, new methods, specifically those that use a likelihood function, have become very popular for the study of molecular data and the inference of evolutionary histories from DNA, RNA, and proteins. In particular, the paired use of Bayesian inference and maximum likelihood has become a new standard in molecular phylogenetics. Until recently, the use of these methods has been unfortunately limited to molecular datasets. Few effective and efficient methods were discussed until, in 2001, Paul O. Lewis published a breakthrough on this problem: a likelihood approach to morphological phylogenetics, termed the Mk model (Lewis, 2001). This model serves as a generalized Jukes-Cantor model (JC69). A lineage must always be in one of *k* possible states and that character is free to change to other states along branches with symmetrical probability of state changes. The number of changes along a branch is proportional to the branch length. However, change is not necessarily gradual, as the branch length only

represents the average amount of change over the entire branch. To correct for acquisition bias that is often prevalent in morphological datasets (no autapomorphic or invariable characters), Lewis also designed a subclass of his model, termed the Mkv model, where "v" stands for variable (as the datasets only contain variable characters) (Lewis, 2001). With this correction in place, the method correctly estimates branch lengths that would otherwise be largely overestimated. The Mkv model allows for rate heterogeneity between characters using a gamma distribution of rates. Additionally, the creation of likelihood methods for morphological data allows for the use of likelihood ratio tests, or likelihood hypothesis testing (AIC, BIC, DT, etc.). It should be noted here that, while the Mkv model attempts to model morphological evolution, it is merely just a set of assumptions like parsimony, which also attempts to model the evolution of morphological characters. In fact, the Mkv model is more persuasive in some ways as a model of morphological evolution than parsimony, as convergent evolution assuredly occurs and the tree of life is most definitely not the simplest answer, but the most *likely* one.

The Mkv model has been incorporated into both a Bayesian framework and a maximum likelihood framework (the software specifics for the analyses discussed in this paper are included below in Methods and Materials), both of which have been used with much success (Clarke and Middleton, 2008; Huelsenbeck and Ronquist, 2001; Lewis, 2001; Ronquist et al., 2012; Stamatakis, 2014; Wiens et al., 2010b). Due to the many advantages of the Mkv model (and the lack of other proper likelihood models for morphological phylogenetics), this section employs this likelihood method to an updated version of the AToL Squamate morphology dataset and compares those results to those of the original analyses of this dataset (Gauthier et al., 2012). Additionally, a novel partitioning of the morphology dataset is presented and the results of analyses of the partitioned dataset are compared to those of the unpartitioned dataset. Finally, this section discusses the use of the Mkv model in such a context.

Methods and Materials

The original squamate dataset was obtained from Jacques Gauthier following its publication (Gauthier et al., 2012). The specimen preparation, taxon sampling, character sampling, and initial data analyses of this preliminary dataset are described in that publication. Four snake taxa and a number of characters were added to the dataset by Nicholas Longrich

(Longrich et al., 2012). Since that publication, additional taxa have been added to the dataset to further cover taxonomic groups (see Figure 1), and additional characters have been scored for the entire set of taxa. The process of adding these taxa and characters will be discussed in a future manuscript. This brings the size of the current morphological dataset to 204 taxa (58 of which are extinct) and 779 morphological characters. 181 of the characters are ordered as in Gauthier et al. (2012). Due to the inclusion of fossil taxa and characters that are not applicable to all of the taxa, roughly 41% of the dataset is scored as missing data ("?") (according to RAxML v. 8 (Stamatakis, 2014)). The dataset contains at least two representatives of all squamate subfamilies and families. A hierarchical taxonomic listing can be found in Figure 1, according to both morphological (left) and molecular (right) systematists (Gauthier et al., 2012; Wiens et al., 2012). The taxonomic groups are arranged such that all groupings include all further indented groups below them (eg. Acrodonta includes Agaminae, Chamaelonidae, and Leiolepidinae). For this report, the morphological taxonomy is used for consistency when presenting phylogenies and referring to particular groups and their inclusive groups, except where noted. It is important to stress that the order is strictly alphabetical and does not imply any phylogenetic relationships besides the hierarchical ones that are shown by indentation. Additionally, the fossil taxa within the dataset represent both stem and crown taxa for most of the major taxonomic groups. Groups whose members are all extinct have been noted as such in Figure 1.

The dataset was analyzed with a number of various programs that used parsimony and other methods. As with the initial analyses of Gauthier et al. (2012), PAUP* v. 4.0 Beta 10 was used to conduct parsimony bootstrap analyses of the morphological dataset using a combination of a Windows PC running Windows 8.1, a Mac Pro running OS X v. 10.5.8, and a Linux cluster (Swofford, 1989; Swofford, 2003). Of the 779 total characters, only 6 characters are parsimony-uninformative. 20 bootstrap analyses were run in parallel for 100 replicates each using stepwise addition, simple addition sequence (with *Sphenodon punctatus* as the reference taxon), TBR branch-swapping, and a time limit of 3600 seconds for each replicate. The majority rule consensus tree of the trees from these 2000 parsimony bootstrap replicates is presented in Figure 2. Additionally, a heuristic search with successive weighting was conducted also using PAUP* (Farris, 1969). However, this method has been considered circular, so the results are not

SQUAMATA (according to morphology)

- Iguania
  - Acrodonta
    - Agaminae
    - Chamaeleonidae
    - Leiolepidinae
  - Iguanidae
    - Corytophaninae
    - Crotaphytinae
    - Hoplocercinae
    - Iguaninae
    - Leiosaurinae
    - Liolaeminae
    - Oplurinae
    - Phrynosomatinae
    - Polychrotinae
    - Tropidurinae
  - Priscagaminae †
- Mosasauria †
- Polyglyphanodontia †
- Scleroglossa
  - Autarchoglossa
    - Amphisbaenia
      - Amphisbaenidae
      - Bipedidae
      - Rhineuridae
      - Trogonophidae
    - Anguimorpha
      - Anguidae
      - Xenosauridae
      - Varanoidea
        - Helodermatidae
        - Varanidae
    - Dibamidae
    - Scincomorpha
      - Lacertoidea
        - Gymnophthalmidae
        - Lacertidae
        - Teiidae
      - Scincoidea
        - Carusiidae †
        - Cordylidae
        - Gerrhosauridae
        - Globauridae †
        - Scincidae
          - Lygosominae
        - Xantusiidae
    - Serpentes*
  - Gekkota
    - Carphodactylidae
    - Diplodactylidae
    - Eublepharidae
    - Gekkonidae
    - Pygopodidae
    - Sphaerodactylidae

SQUAMATA (according to molecules)

- Dibamidae
- Gekkota
  - Carphodactylidae
  - Diplodactylidae
  - Eublepharidae
  - Gekkonidae
  - Pygopodidae
  - Sphaerodactylidae
- Lacertoidea
  - Amphisbaenidae
  - Bipedidae
  - Gymnophthalmidae
  - Lacertidae
  - Rhineuridae
  - Teiidae
  - Trogonophidae
- Scincoidea
  - Cordylidae
  - Gerrhosauridae
  - Scincidae
  - Xantusiidae
- Toxicofera
  - Anguimorpha
    - Anguidae
    - Helodermatidae
    - Lanthanotidae
    - Shinisauridae
    - Varanidae
    - Xenosauridae
  - Iguania
    - Acrodonta
      - Agamidae
      - Chamaeleonidae
    - Iguanidae
      - Corytophaninae
      - Crotaphytinae
      - Hoplocercinae
      - Iguaninae
      - Leiocephalinae
      - Leiosaurinae
      - Liolaeminae
      - Oplurinae
      - Phrynosomatinae
      - Polychrotinae
      - Tropidurinae
  - Serpentes*

Figure 1: Hierarchical taxonomic groupings within Squamata as determined by morphological (Gauthier et al. 2012, left) and molecular analyses (Wiens et al. 2012, right). Taxonomic groupings include all further indented taxonomic groups below them. Order is alphabetical within groups and does not imply phylogenetic relationships. *: Subgroupings not included here. †: Extinct group, not included in molecular taxonomic hierarchy. Modified from Townsend et al. 2004, Gauthier et al. 2012, and Wiens et al. 2012.

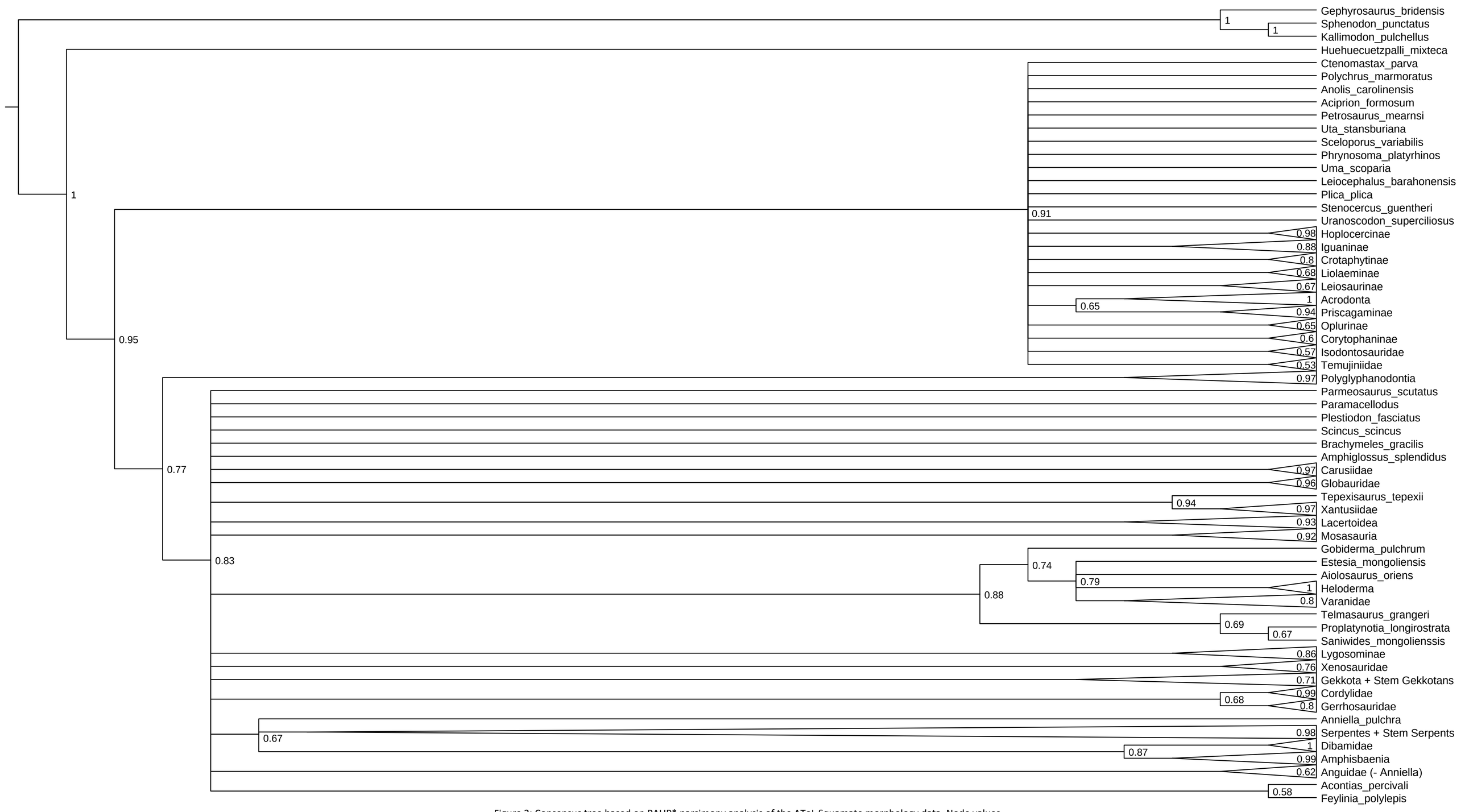

Figure 2: Consensus tree based on PAUP* parsimony analysis of the AToL Squamate morphology data. Node values represent support values from the bootstrap trees. Nodes with less than .50 bootstrap support have been collapsed.

included here. Finally, PAUP* v. 4.0 Beta 10 and PAUPRat were used to perform 200 iterations of the Parsimony Ratchet under its default settings (Nixon, 1999; Sikes and Lewis, 2001; Swofford, 2003). By searching a larger set of islands less thoroughly instead of searching a small subset of these islands more thoroughly, the Parsimony Ratchet produces a rapid parsimony analysis that finds the shortest tree much faster than standard parsimony analyses. The analysis found 64 trees with tree lengths of 6010, due to a degree of uncertainty in stem Iguanids and the Iguanid subfamily Phrynosomatinae. A majority rule consensus tree of those trees is presented in Figure 3.

Two Bayesian analyses were performed, for the partitioned and unpartitioned morphological datasets, using the Mkv likelihood model incorporated into a parallel (mpi) version of MrBayes v. 3.2.2 on a Linux cluster (Altekar et al., 2004; Huelsenbeck and Ronquist, 2001; Lewis, 2001; Ronquist and Huelsenbeck, 2003; Ronquist et al., 2012). The unpartitioned analysis performed 2 simultaneous MCMC runs with 4 chains each for 60,000,000 generations, taking samples every 500 generations (with the default burnin of 25%, or 12,000,000 generations). A majority rule consensus tree of the remaining 90,000 (x2) samples is presented in Figure 4. The final average standard deviation of split frequencies was .002. The partitioned analysis divided the data into 6 distinct character subsets based on the number of character states (2, 3, 4, 5, 8, and 9). A partition homogeneity test, or incongruence length difference test, performed by PAUP* produced a p-value of .27 (Farris et al., 1994). This shows that while the partitions may have some heterogeneity, there is no significant conflict between the partitions, but this also merits running them as separate partitions (Planet, 2006). The MCMC analysis of the partitioned dataset performed 4 simultaneous runs, each with 16 chains. Samples were taken every 500 generations over the course of 100,000,000 generations (with the default burnin of 25%, or 25,000,000 generations). A majority rule consensus tree of the remaining 150,000 (x4) samples is presented in Figure 5. The final average standard deviation of split frequencies was .02.

Finally, a parallel version of RAxML v. 8 was used to employ the Mk model, in the analysis of the unpartitioned dataset and a dataset consisting of 4 subsets (because RAxML requires subsets to consist of more than 1 character; so the subsets were 2, 3, 4, 5-9), on a

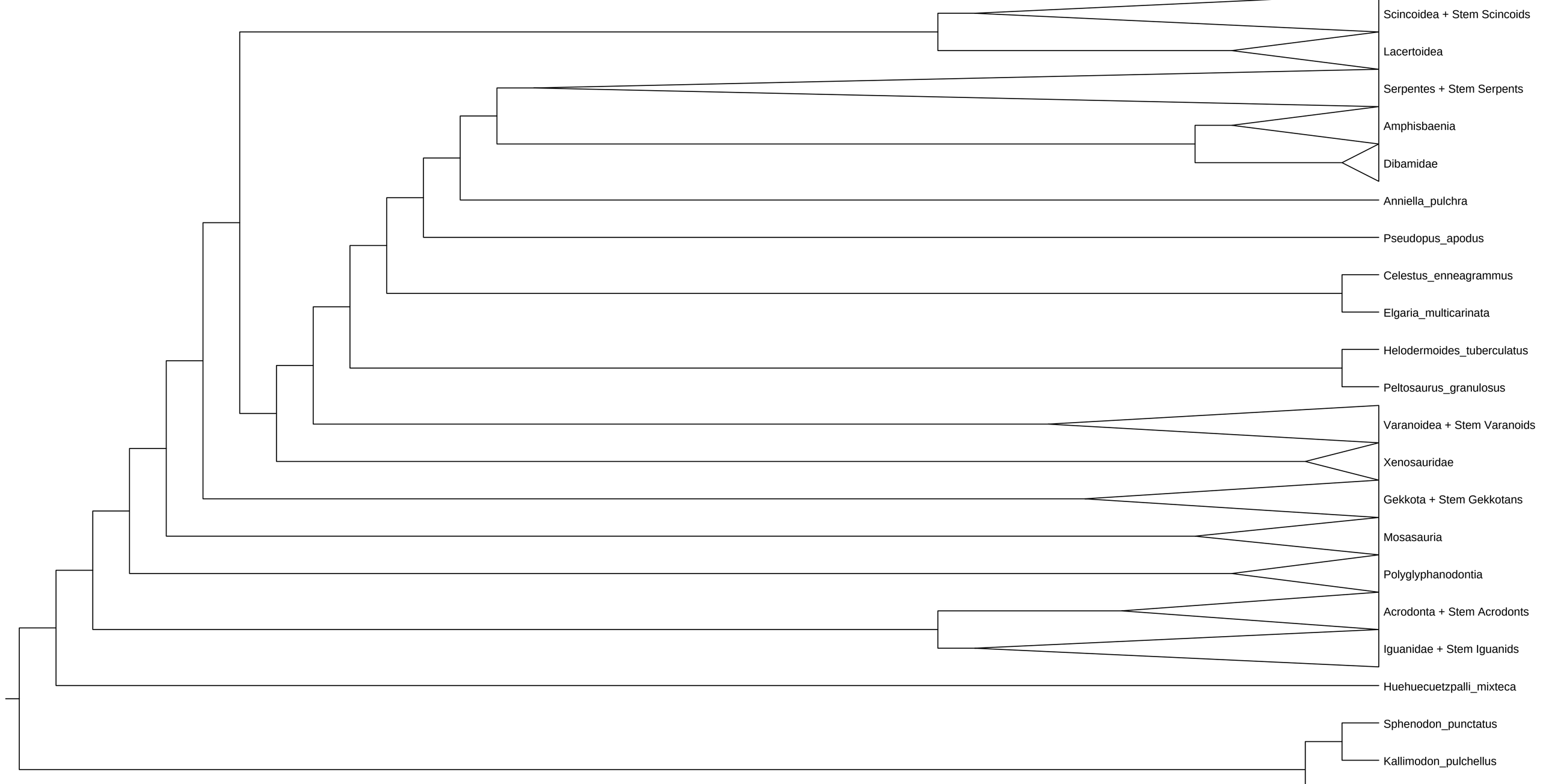

Figure 3: The most parsimonious tree as produced by the PAUP* Ratchet (PaupRat) parsimony analysis of the AToL Squamate morphology dataset.

Figure 4

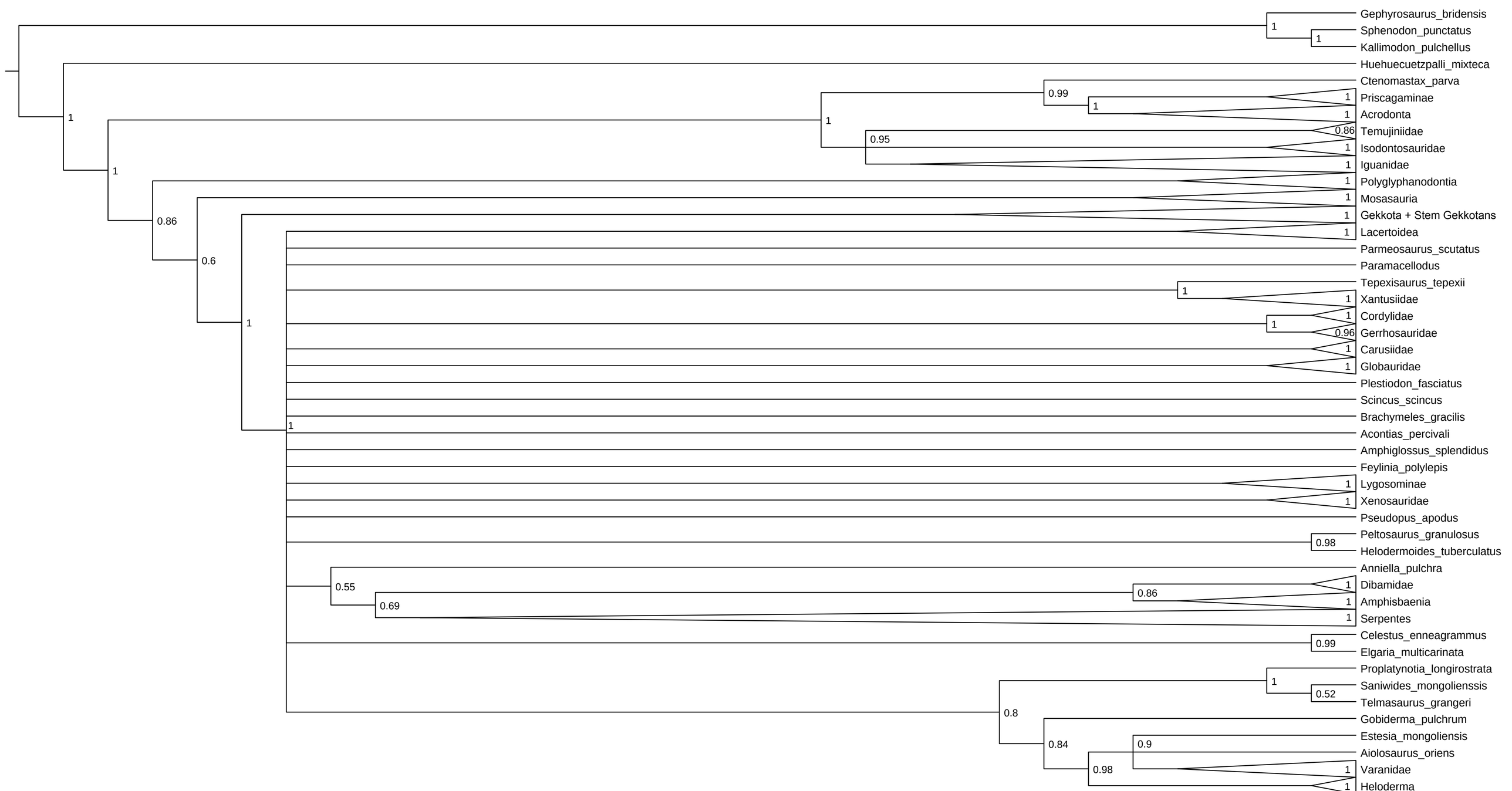

Figure 5: Consensus tree based on MrBayes bayesian inference of the partitionedAToL Squamate morphology data. Node values represent support values from the posterior trees. Nodes with less than .50 posterior support have been collapsed.

Linux cluster (using the –m MULTIGAMMA and –K MK options) (Lewis, 2001; Ott et al., 2010; Stamatakis, 2006; Stamatakis, 2014). 10,240 standard maximum likelihood bootstrap replicates were performed on the partitioned dataset. A majority rule consensus tree of the bootstrap replicates is presented in Figure 6. For comparison, the unpartitioned dataset was analyzed with RAxML's rapid bootstrap algorithm and the extended majority-rule consensus tree bootstopping criterion (using the -x and -# autoMRE options) (Pattengale et al., 2009; Stamatakis, 2014; Stamatakis et al., 2008). The rapid bootstrap algorithm has been shown to be 8 to 20 times faster than RAxML's standard bootstrapping algorithm, and both the bootstrapping algorithm and the bootstopping criterion have been shown to be experimentally robust. 208 bootstrap replicates were performed on a Linux cluster before the bootstopping criterion was achieved. The majority rule consensus tree of these bootstrap replicates is presented in Figure 7. As part of the rapid bootstrapping, RAxML also found the best-scoring maximum likelihood tree through a thorough tree search (using the -f a option). This tree is presented in Figure 8.

Majority rule consensus trees and bootstrap values for the parsimony and RAxML analyses were computed using SumTrees v. 3.3.1, part of the DendroPy v. 3.12.1 Python package (Sukumaran and Holder, 2010). Tracer v. 1.5 was used to confirm convergence of the Bayesian inference analyses (Rambaut and Drummond, 2007). Clades in Figures 2-8 have been collapsed and annotated for a display that is easier to read, and bootstrap and posterior values have been added to the branches using FigTree v. 1.4 and Adobe Acrobat (Rambaut, 2012). The groups within the collapsed clades can be found in Figure 1 (morphological definitions) and the specific taxa within the collapsed clades can be found in Appendix 1 of Gauthier et al. (2012) and also in a future manuscript.

Results

The parsimony analysis of the AToL Squamate morphology dataset (Figure 2) supports most of the subfamily and lower order relationships that were proposed by Gauthier et al. (2012) (see Figure 1). Additionally, some higher order groups are supported as monophyletic, such as Acrodonta, Mosasauria, Polyglyphanodontia, Amphisbaenia, Lacertoidea, Gekkota, and Serpentes. Most of these groups are strongly supported (more than .9 bootstrap values). While

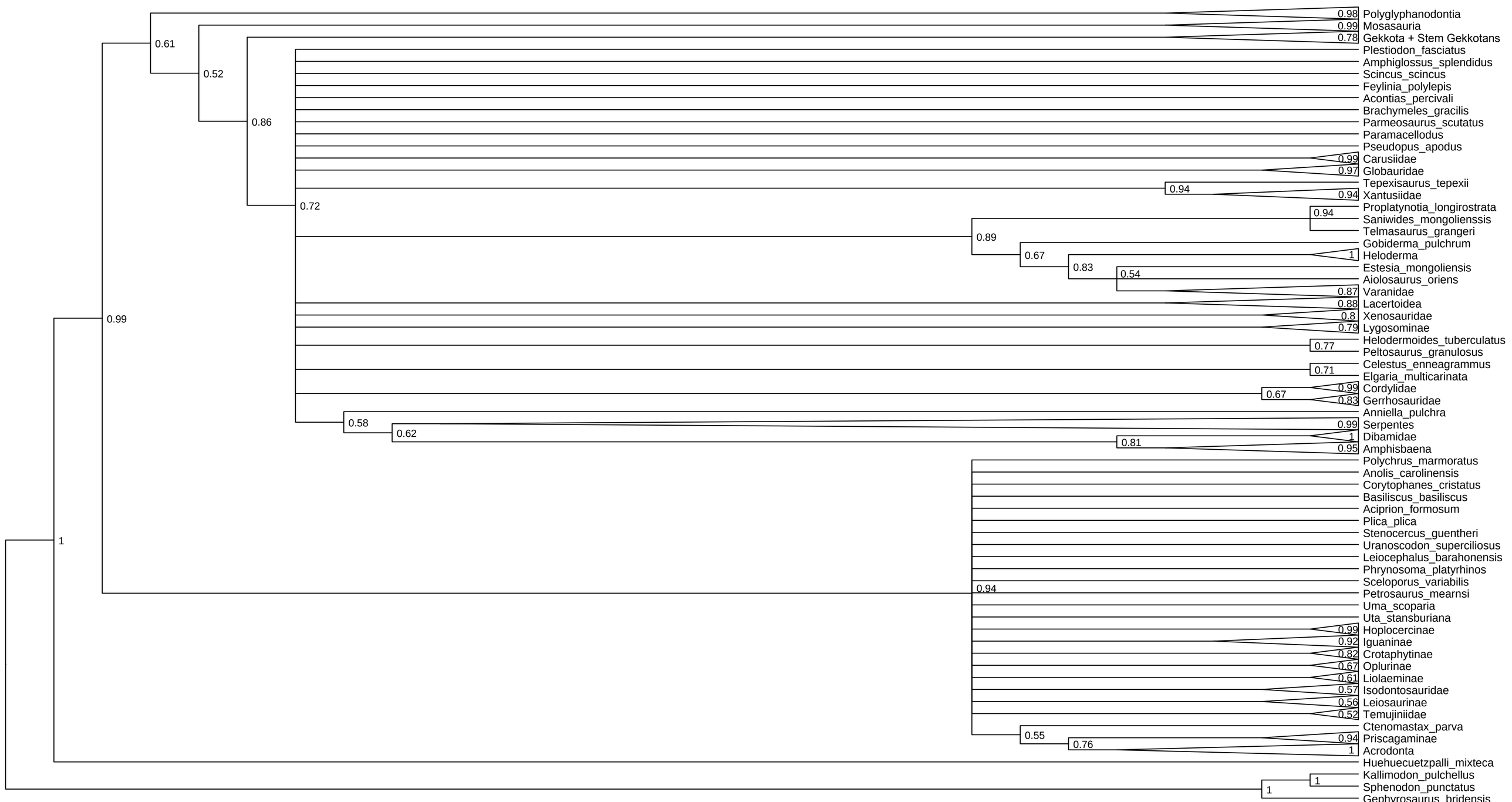

Figure 6: Consensus tree based on RAxML maximum likelihood analysis of the partitioned AToL Squamate morphology data.
Node values represent support values from the bootstrap trees. Nodes with less than .50 bootstrap support have been collapsed.

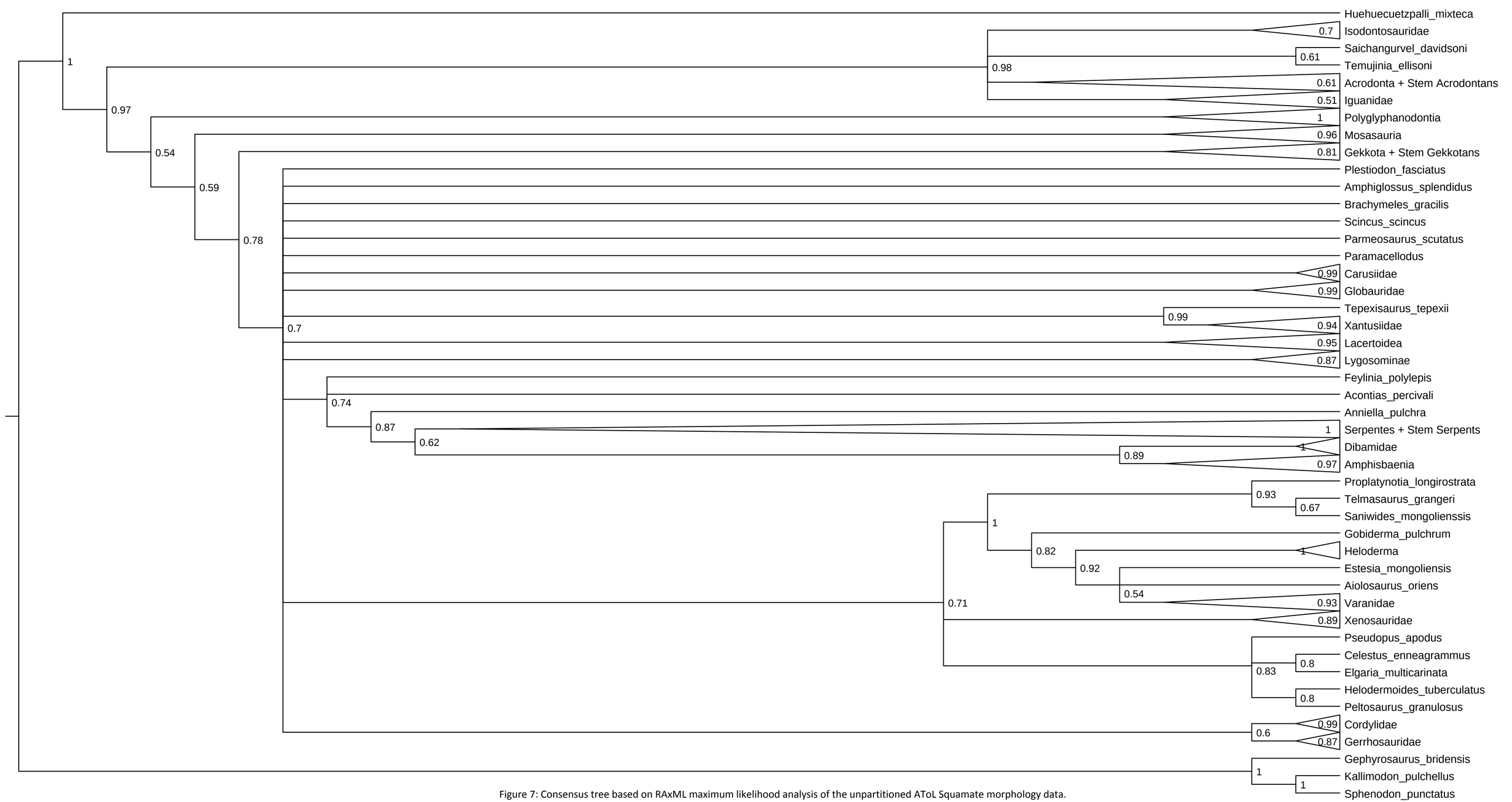

Figure 7: Consensus tree based on RAxML maximum likelihood analysis of the unpartitioned AToL Squamate morphology data.
Node values represent support values from the bootstrap trees. Nodes with less than .50 bootstrap support have been collapsed.

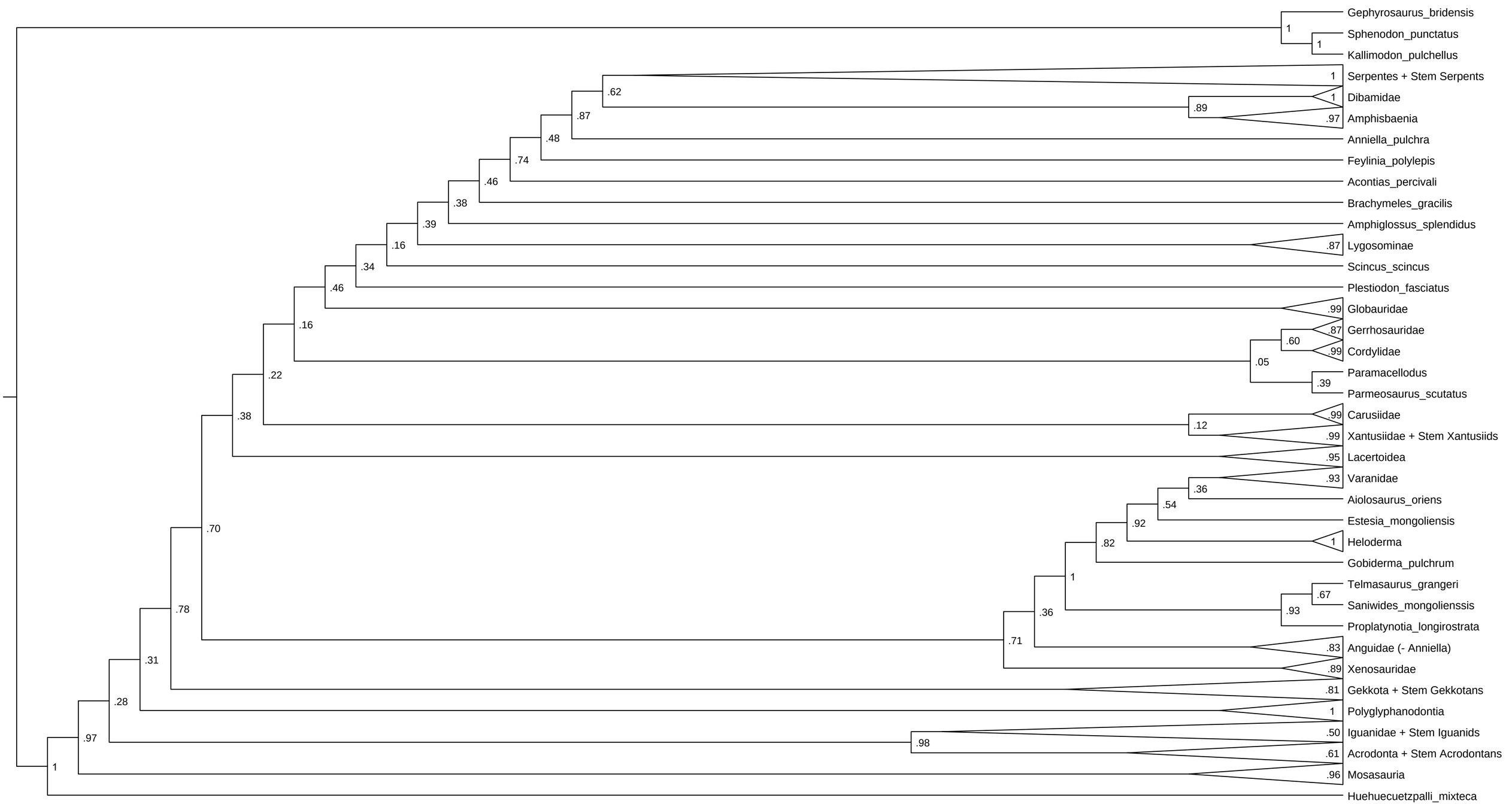

Figure 8: Maximum likelihood tree based on RAxML rapid maximum likelihood and bootstrap analysis of the AToL Squamate morphology dataset. Node values represent support values from the bootstrap trees. No nodes have been collapsed to polytomies.

the relationships within Iguanidae and between Iguanidae and Acrodonta are not resolved, Iguania is strongly supported as monophyletic and as the most basal of the extant groups. Dibamidae, Amphisbaenia, and *Anniella* resolve as sisters to Serpentes. Scleroglossa is not supported, and Mosasauria and all of Scleroglossa make up a large polytomy.

The parsimony ratchet analysis (Figure 3) resulted in a best tree that shared many similarities with the taxonomy as put forth in Gauthier et al. (2012). The topology supports a monophyletic and basal Iguania which consists of monophyletic Acrodonta and Iguanidae clades. Polyglyphanodontia and Mosasauria are resolved as stem Scleroglossan clades. Gekkota is resolved as the most basal Scleroglossan group, followed by Autarchoglossa, which then consists of monophyletic Amphisbaenia, Dibamidae, Scincomorpha, and Serpentes, and a paraphyletic Anguimorpha. Amphisbaenia and Dibamidae serve as a sister clade to Serpentes, followed by *Anniella* and the rest of Anguidae as a sister group to that resolved clade.

The Bayesian inference analysis of the unpartitioned AToL Squamate morphology dataset (Figure 4) has yet to finish.

The Bayesian inference analysis of the partitioned morphology dataset (Figure 5) resulted in slightly more resolution than the parsimony analysis. Again, most subfamily and lower order relationships are resolved as in Gauthier et al. (2012). Acrodonta, Iguanidae, Dibamidae, Amphisbaenia, Serpentes, and some other family level groups are resolved with bootstrap support values of 1. Amphisbaenia and Dibamidae still make up a clade that is the sister group to Serpentes, with *Anniella* still the sister to this clade, although with less support than in the parsimony analysis. There is total support for the monophyly of Scleroglossa and for the monophyly of Gekkota as the sister clade to the monophyletic Autarchoglossa.

The maximum likelihood analysis of the partitioned morphology dataset (Figure 6) shares many similarities with the parsimony analysis (Figure 2). Iguanidae is not resolved as monophyletic, although there is strong support for the monophyly of Acrodonta and Iguania (> .90). Unlike the unpartitioned ML analysis, Xenosauridae is no longer resolved within Anguimorpha and is part of the large polytomy that makes up Autarchoglossa. Gekkota is fairly strongly supported as the sister of Autarchoglossa, but there is very weak support for the monophyly of Scleroglossa.

The maximum likelihood analysis of the unpartitioned morphology dataset (Figure 7) resulted in a fairly similar topology to that of the Bayesian inference (Figure 5). The same lower order and higher order relationships are apparent in both results, although the maximum likelihood results have lower support in general relative to the Bayesian inference results. There is stronger support for the *Anniella* + Serpentes + Dibamidae + Amphisbaenia clade than in the Bayesian inference and partitioned ML analysis results. *Acontias* and *Feylinia* are supported (.74) as the sisters to this clade. Xenosauridae and the remainder of Anguidae are fairly supported (.71) as forming a clade (Anguimorpha) with Varanoidea (except for *Anniella*). There is less support for Scleroglossa and Autarchoglossa than in the Bayesian inference results, although Gekkota is still supported as the sister to Autarchoglossa.

The maximum likelihood tree for the unpartitioned morphology dataset (Figure 8) provides slightly more information than the bootstrap analysis, although it also provides some information that should not be trusted completely. For instance, the ML tree has Mosasauria as the most basal clade of Squamata, but there are very few morphological characters that would support such a topology. Scincoidea and Scincomorpha are both resolved as paraphyletic, with very little support for either clade.

Discussion

The use of the Mkv model under both Bayesian inference and maximum likelihood frameworks increases the resolution of the higher order relationships within Squamata. Most, if not all, nodes have higher support values in these frameworks (especially Bayesian inference) than under parsimony. This may be due to the nature of Bayesian inference. First of all, the giant polytomies inferred by the other methods may reveal that the Bayesian inference is falling prey to the star tree paradox (Lewis et al., 2005). While the true phylogeny is very unlikely to represent a “star”, the convergence and high degree of similarity between clades and the potential for erased evolutionary past may cause difficulties in terms of resolution. In this case, Bayesian inference may find strong support for particular trees, even when multiple trees are expected to be equally supported. A true assessment of this problem could be performed by taking into account the inferred branch lengths of the phylogeny and the low bootstrap support. This could be fixed by modifying the prior distribution of trees. Another possible

problem with the Bayesian inference is the lack of various models for morphological evolution. The analysis was run under the assumption of a gamma distribution of rates and a discrete morphology model that is based on the model described by Lewis (2001). If this model is not suitable for the dataset and is underspecified, it is very likely that the posterior probabilities are biased and may be too liberal in their confidence (Huelsenbeck and Rannala, 2004; Lemmon and Moriarty, 2004). Even with a proper model of morphological evolution, posterior values may still be high when compared to bootstraps (Cummings et al., 2003; Douady et al., 2003). The relationships between the taxa may not be resolved due to convergence or processes that have erased character states that may have eluded to particular relationships. The inclusion of more fossil taxa may improve this problem, although including more fossil taxa may also significantly increase the problem of missing data in the dataset (which is already at ~41%). Overall, we support the use of the Mkv model and Bayesian and likelihood frameworks for the study of morphological data in addition to parsimony analyses.

Interestingly, partitioning the morphology introduces more uncertainty under maximum likelihood. Unfortunately, the Bayesian or likelihood frameworks did not adequately deal with the supposed convergence of snakes, dibamids, and amphisbaenids (Brandley et al., 2008; Townsend et al., 2004; Wiens et al., 2006). The loss of limbs includes a large set of character changes that would be significant in a parsimony analysis. It is possible that the parsimony analysis is trapped in the Felsenstein zone due to the long branches of these groups (and consequent convergence) (Felsenstein, 2004; Huelsenbeck, 1995; Huelsenbeck, 1997). Maximum likelihood and Bayesian inference should be immune to long-branch attraction, but unfortunately the monophyly of this group is still supported under these frameworks. Furthermore, the maximum likelihood analysis of the unpartitioned morphology dataset supported the inclusion of the limbless skinks *Feylinia* and *Acontias*, further supporting this grouping of convergent limbless squamates.

The results of these analyses are very similar to those of the previous analyses performed by Gauthier et al. (2012). There is generally more support for clades in all of the results than in those of Gauthier et al. (2012), likely due to the inclusion of more than 150 more phenotypic characters and a number of new taxa, both extant and extinct. The addition of the

maximum likelihood framework adds another layer of support for the topology proposed by Gauthier et al. (2012) and generally supported here.

III. Combined Morphology and DNA Approach

Introduction

The combination of different types of data has become extremely popular in phylogenetic systematics under the "total evidence" approach (Baker et al., 1998; Bull et al., 1993; Chippindale and Wiens, 1994; Eernisse and Kluge, 1993; Farias et al., 2000; Giribet, 2010; Huelsenbeck et al., 1996; Menard et al., 2013; Perrard et al., 2013; Wiens, 2009; Wiens et al., 2010b). However, data and/or signal heterogeneity between and within the combined datasets may ultimately result in the loss of phylogenetic information and/or competition between varying signals (Bull et al., 1993; De Queiroz et al., 1995; Donoghue and Sanderson, 1992; Eernisse and Kluge, 1993; Felsenstein, 2004; Hillis, 1987; Huelsenbeck et al., 1996; Planet, 2006; Rieppel, 2009; Wiens, 1998; Wiens and Hollingsworth, 2000). While there may be drawbacks to combining data to produce "total evidence" datasets, the combination of data can also produce extremely informative results and may provide insight into the signals of both original datasets and the methods with which they are analyzed (Baker et al., 1998; Donoghue and Sanderson, 1992; Eernisse and Kluge, 1993; Hillis, 1987; Huelsenbeck et al., 1996; Planet, 2006). Even with extremely incongruent datasets, performing combined analyses may provide information that would otherwise be unattainable. This section discusses a "total evidence" approach to squamate systematics.

Methods and Materials

The combined dataset consists of 199 taxa, 779 morphological characters, and 33,717 nucleotide base pairs (Gauthier et al., 2012; Wiens et al., 2012). The datasets were combined using Mesquite v. 2.75 (Maddison and Maddison, 2011). Parallel partition homogeneity tests, or incongruence length difference tests, were performed by PAUP* with the morphology and molecular data treated as separate partitions, producing a p-value of .04 for all of the separate runs (Farris et al., 1994). These incongruence test results should be treated as preliminary, but they do imply that these two partitions may reflect different evolutionary histories (Barker and

Lutzoni, 2002; Darlu and Lecointre, 2002; Planet, 2006). Nonetheless, this combined dataset is used for the analyses of this section.

The methods are similar to those in Section II: parsimony, maximum likelihood, and Bayesian inference analyses were performed on the combined dataset. PAUP* v. 4.0 Beta 10 was used to conduct parsimony bootstrap analyses on a combination of a Windows PC running Windows 8.1, a Mac Pro running OS X v. 10.5.8, and a Linux cluster (Swofford, 1989; Swofford, 2003). Of the 34,496 total characters, 12,038 are constant and only 19,188 of the remaining characters are parsimony-informative. 16 bootstrap analyses were run in parallel for 100 replicates each using stepwise addition, simple addition sequence (with *Acontias sp.* as the reference taxon), TBR branch-swapping, and a time limit of 3600 seconds for each replicate. The majority rule consensus tree of the trees from these 1600 parsimony bootstrap replicates is presented in Figure 9. PAUP* v. 4.0 Beta 10 and PAUPRat were used to perform 200 iterations of the Parsimony Ratchet under its default settings (Nixon, 1999; Sikes and Lewis, 2001; Swofford, 2003). The analysis found 122 trees with a total tree length of 153,680. A majority rule consensus tree of those trees is presented in Figure 10.

A Bayesian inference analysis was performed on the combined dataset using a parallel (mpi) version of MrBayes v. 3.2.2 on a Linux cluster (Altekar et al., 2004; Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003; Ronquist et al., 2012). The analysis was partitioned into 133 subsets: the morphology was treated as a single partition, and each codon position of each gene was treated as a single partition (132 partitions). The Mkv model was applied to the morphology partition, and a GTR+Γ model was applied to each codon position partition (the makers of RAxML advise against using the GTR+I+Γ model). All partition models were unlinked and the ordering of morphological characters was also applied as in Gauthier et al. (2012). The MCMC analysis performed 8 simultaneous runs, each with 4 chains. Samples were taken every 1000 generations over the course of 50,000,000 generations (with the default burnin of 25%, or 12,500,000 generations). A majority rule consensus tree of the remaining 37,500 (x8) samples is presented in Figure 11. The final average standard deviation of split frequencies was .01. Another Bayesian inference analysis has begun treating only the separate

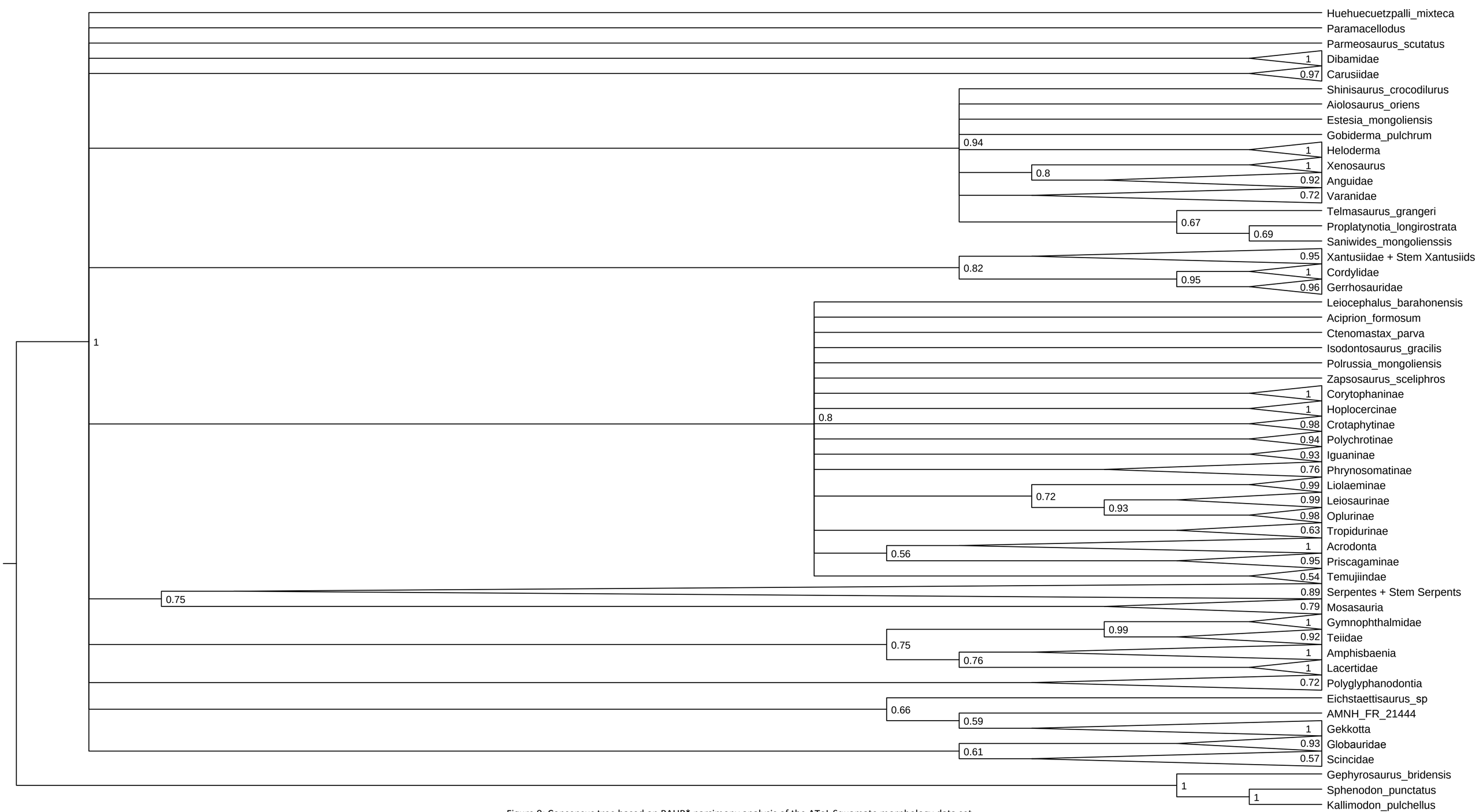

Figure 9: Consensus tree based on PAUP* parsimony analysis of the AToL Squamate morphology data set combined with the Wiens et al. 2012 DNA data set. Node values represent support values from the bootstrap trees. Nodes with less than .50 bootstrap support have been collapsed.

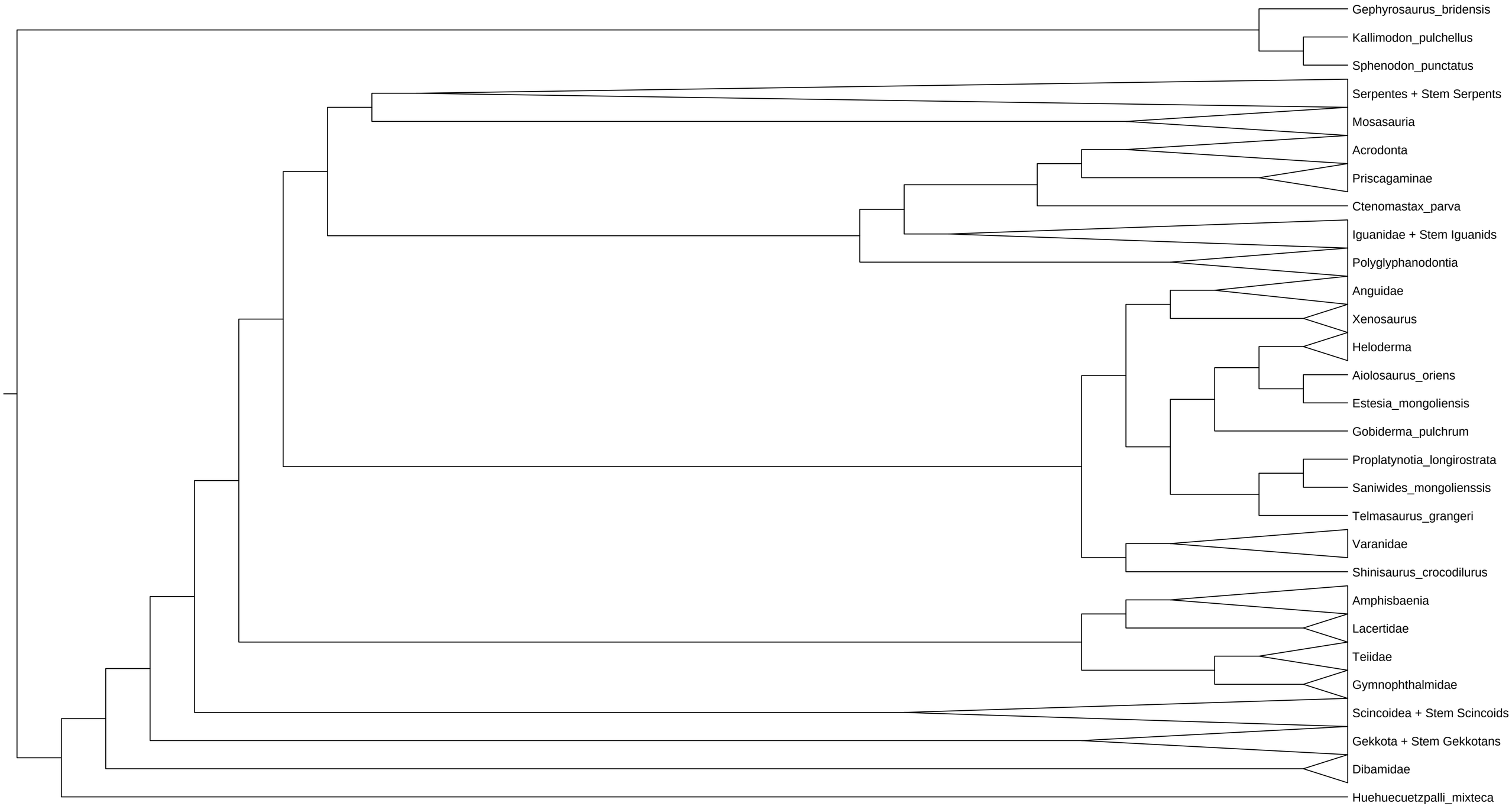

Figure 10: The most parsimonious tree as produced by the PAUP* Ratchet (PaupRat) parsimony analysis of the AToL Squamate morphology data set combined with the Wiens et al. 2012 DNA data set.

Figure 11

genes as separate partitions (45 total partitions). This analysis has not concluded yet and likely will not be done before submission of this report.

Finally, a parallel version of RAxML v. 8 was used to analyze the combined dataset on a Linux cluster (Lewis, 2001; Ott et al., 2010; Stamatakis, 2006; Stamatakis, 2014). The same partitioning scheme as the Bayesian inference analysis, described above, was applied to the maximum likelihood analysis (using the –m GTRGAMMA and –K MK options). 185 standard maximum likelihood bootstrap replicates were performed on the partitioned dataset. A majority rule consensus tree of the bootstrap replicates is presented in Figure 12. To determine that enough bootstrap replicates were performed and the result correctly represented the signal of the data, the partitioned dataset was also analyzed with RAxML's rapid bootstrap algorithm and the extended majority-rule consensus tree bootstopping criterion (using the -x and -# autoMRE options) (Pattengale et al., 2009; Stamatakis, 2014; Stamatakis et al., 2008). 160 bootstrap replicates were performed on a Linux cluster before the bootstopping criterion was achieved. As part of the rapid bootstrapping, RAxML also found the best-scoring maximum likelihood tree through a thorough tree search (using the -f a option). This tree is presented in Figure 13, including support values as determined by the rapid bootstrapping.

Majority rule consensus trees and bootstrap values for the parsimony and RAxML analyses were again computed using SumTrees v. 3.3.1, part of the DendroPy v. 3.12.1 Python package (Sukumaran and Holder, 2010). Tracer v. 1.5 was once more used to confirm convergence of the Bayesian inference analysis (Rambaut and Drummond, 2007). Clades in Figures 9-13 have been collapsed and annotated for a display that is easier to read, and bootstrap and posterior values have been added to the branches using FigTree v. 1.4 and Adobe Acrobat (Rambaut, 2012). The groups within the collapsed clades can be found in Figure 1 (morphological definitions) and the specific taxa within the collapsed clades can be found in Appendix 1 of Gauthier et al. (2012) and also in a future manuscript.

Results

The parsimony analysis of the combined dataset (Figure 9) resulted in very low resolution of most of the higher order relationships with Squamata. Most subfamilies and some families as defined by Gauthier et al. (2012) and in Figure 1 (left) are supported to varying

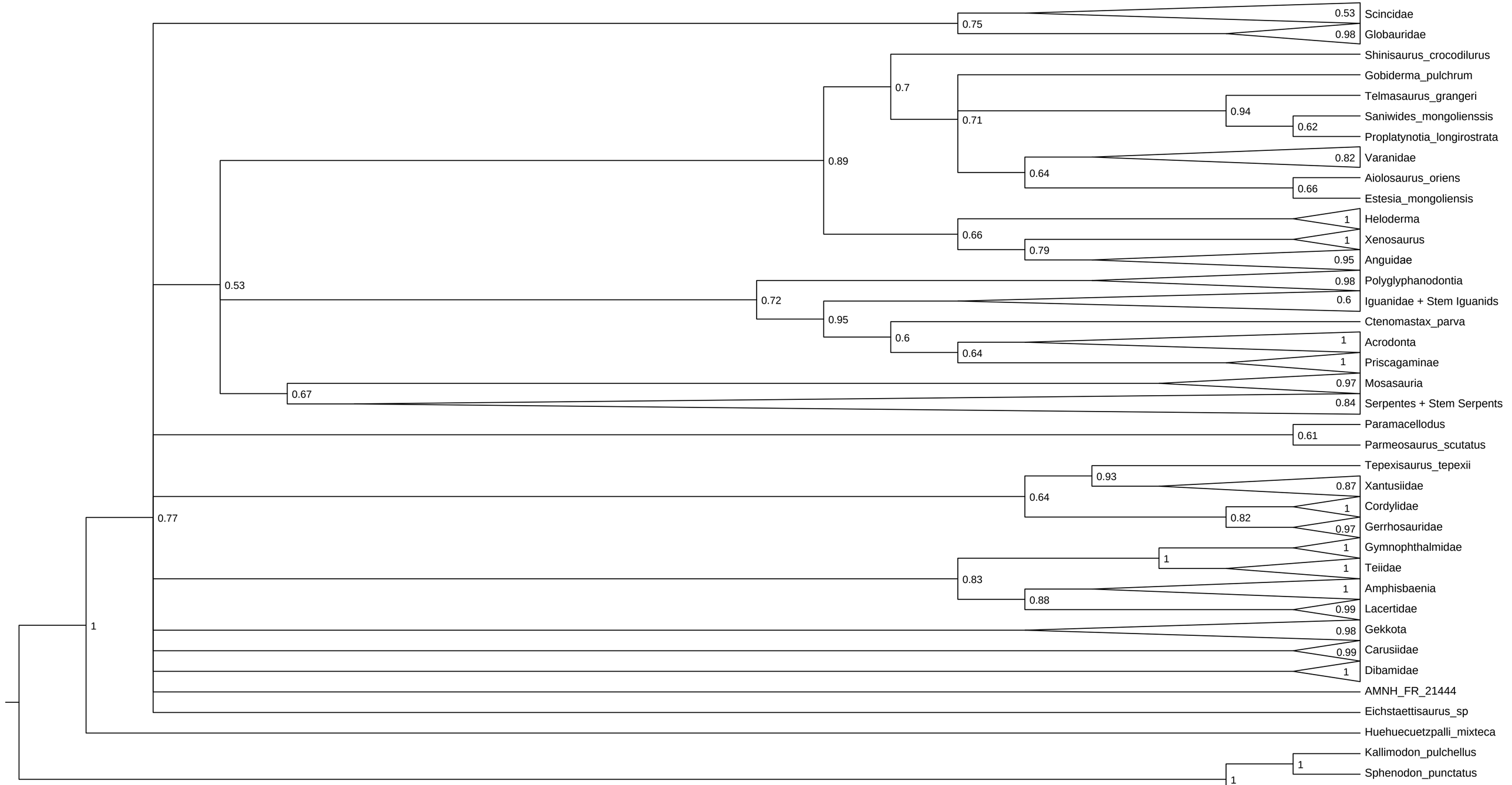

Figure 12: Consensusree based on RAxML maximum likelihood bootstrap analysis of the AToL Squamate morphology data set combined with the Wiens et al. 2012 DNA data set. Node values represent support values from the bootstrap trees. Nodes with less than .50 bootstrap support have been collapsed.

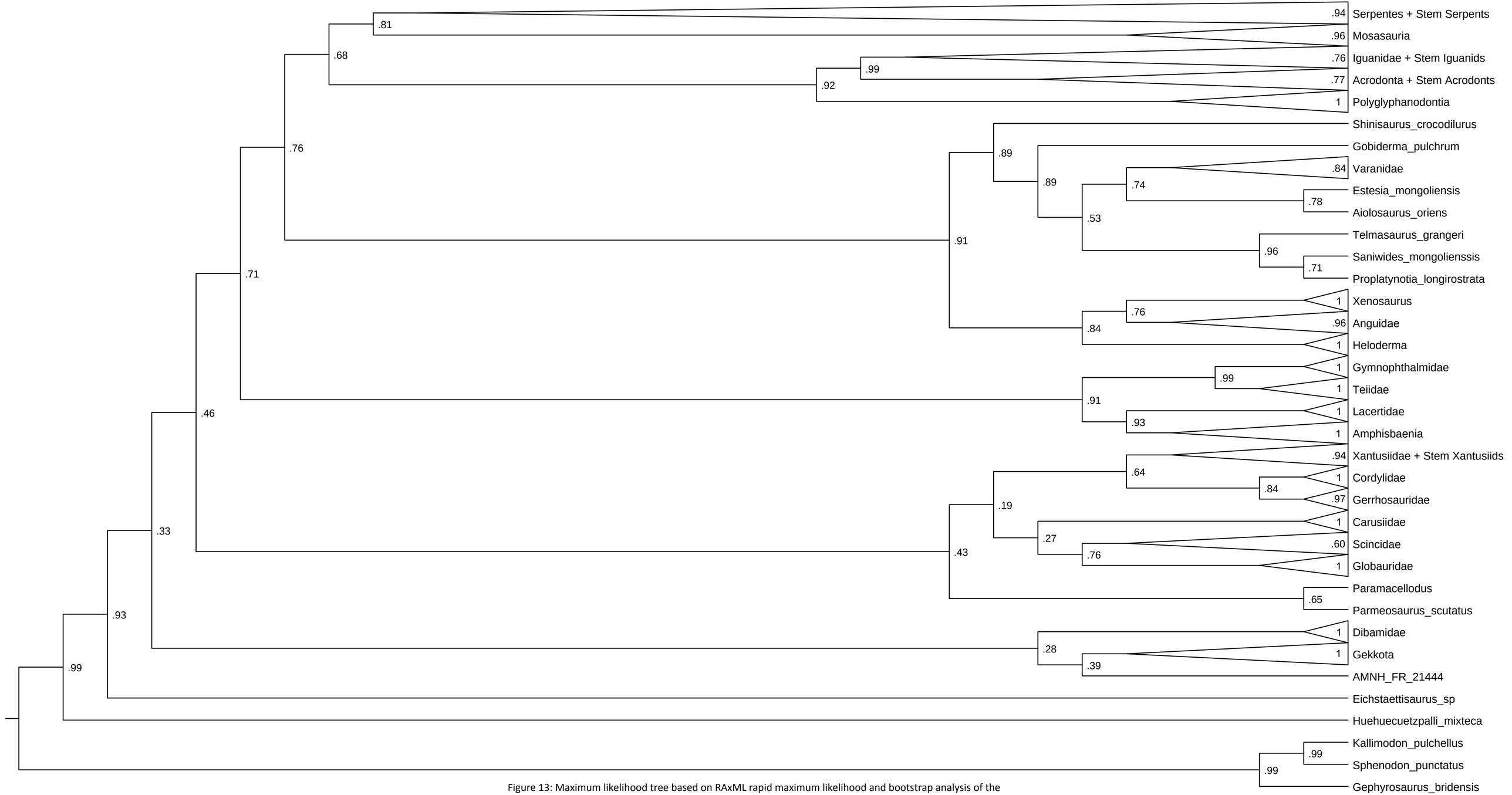

Figure 13: Maximum likelihood tree based on RAxML rapid maximum likelihood and bootstrap analysis of the AToL Squamate morphology data set combined with the Wiens et al. 2012 DNA data set. Node values represent support values from the bootstrap trees. No nodes have been collapsed to polytomies.

degrees as monophyletic, notably Scincidae, Anguidae, Varanidae, and Lacertidae. Additionally, the monophyly of Acrodonta, Amphisbaenia, and Gekkota are supported by almost all bootstraps. Iguania is supported as monophyletic (.8 bootstrap support), although Iguanidae is not resolved. Anguimorpha is supported by 94% of all bootstraps, but Scleroglossa is not resolved nor are Autarchoglossa or Scincomorpha. Mosasauria is resolved as the sister to Serpentes, Amphisbaenia as the sister to Lacertidae, and Anguidae as the sister to *Xenosaurus*.

The parsimony ratchet of the combined dataset (Figure 10) has much more resolution, although the true support for some of the resolved clades is certainly questionable. All of the supported clades of the parsimony analysis (Figure 9) are supported by the parsimony ratchet. Dibamidae is resolved as the most basal extant squamate clade, while Iguania is resolved as one of the most derived clades and as sister to Serpentes + Mosasauria. Scincoidea is resolved as monophyletic, Lacertoidea is resolved as paraphyletic, and Scincomorpha, Autarchoglossa, and Scleroglossa are resolved as polyphyletic.

The Bayesian inference analysis of the combined dataset (Figure 11) has not finished.

The maximum likelihood analysis of the combined dataset (Figure 12) has much more resolution and support for clades than the parsimony analysis (Figure 9), although much of the tree remains highly unresolved. All family-level clades are resolved as monophyletic with varying degrees of support. Anguimorpha is strongly supported (.89 bootstrap value), although *Heloderma* is not resolved as part of Varanoidea. Mosasauria is again resolved as the sister of Serpentes, although the support (.67) is lower than that from the parsimony analysis (.75). There is a supported (.53) clade consisting of Anguimorpha, Iguania, Mosasauria, and Serpentes (see Toxicofera in Figure 1 [right]). Scincoidea is not resolved as monophyletic, but Lacertoidea is supported as a clade that also includes Amphisbaenia (see Figure 1 [right]). Finally, Polyglyphanodontia is resolved (.72 bootstrap value) as a stem Iguanian clade.

The rapid maximum likelihood and bootstrap analysis of the combined dataset (Figure 13) gives some insight into the higher-order relationships within Squamata, although, as in the parsimony ratchet, this is merely the best tree. The nodes with low bootstrap support values should be treated as questionable. Scincoidea is supported as monophyletic, although with poor support (.43). The Anguimorpha + Iguania + Mosasauria + Serpentes clade is more

supported (.71) after this analysis than from the general maximum likelihood analysis (.53). Rather than supported as the most basal clade as in the parsimony ratchet, Dibamidae is resolved (with very low support) as the sister to Gekkota, which together make up the oldest extant clade of Squamata. Polyglyphanodontia is strongly support (.92) as the sister group of Iguania.

Discussion

Once again, using the Mkv model under the maximum likelihood framework resulted in higher resolution of the higher-order relationships within Squamata. There is high support for family level clades, which are consistent between Gauthier et al. (2012) and Wiens et al. (2012). However, many of the higher-order clades and relationships proposed by Gauthier et al. (2012) are resolved as paraphyletic or polyphyletic, such as Scincomorpha, Lacertoidea, Autarchoglossa, and Scleroglossa (see Figure 1 [left]). Rather, there is much greater support for the clades that are proposed by Wiens et al. (2012), such as Lacertoidea (with Amphisbaenia) and Toxicofera (see Figure 1 [right]). These groups are not supported as highly as they are in Wiens et al. (2012) (also see Appendix 1). Additionally, Iguania and Dibamidae have essentially switched places as basal and more derived clades with Squamata. This switch would most certainly require many reversals of morphological characters with Iguania as part of Toxicofera and with Dibamidae as the most basal group. Therefore, it is highly likely that the morphological characters have a significantly lower impact on the structure of the final tree than do the molecular characters. It is possible that the signal and number of nucleotides are overwhelming any signal that is coming from the much smaller number of morphological characters. With such a difference in size between the morphological and molecular datasets, combining them as is may be unproductive. The next section applies a potential solution to this problem.

IV. Genes as Characters

Introduction

Now with the ability to sequence the entire genomes of taxa, molecular phylogenetic datasets are bigger than ever, with tens to hundreds of thousands of nucleotide sequences for each taxon. In a combined framework of molecular and morphological data, it becomes

increasingly likely that the strong signal of the large molecular data may swamp any signal in the smaller morphological dataset (Doyle, 1992). This systematic error tends to result in a one-sided contribution of the data to the resulting phylogeny. This section discusses the application of a new method in which genes, rather than nucleotides, are treated as the characters of interest. This also attempts to solve the problems of data heterogeneity and long branch attraction, both of which may be due to the large size of phylogenomic datasets. Additionally, as genes are the units of inheritance and selection, not nucleotides, this approach is conceptually and theoretically capable of reducing systematic error (Doyle, 1992; Lu et al., 2013). Finally, a dataset consisting of the genes as characters data combined with the AToL Squamate morphology dataset is analyzed and compared to the results of Section III to assess whether there truly is a power in numbers problem when combining large molecular datasets with smaller morphological datasets.

The method, first put forth by Doyle (1992), codes gene trees as character state trees similar to the coding method of Brooks Parsimony Analysis, acting as "a completely polarized multistate transformation series" (Brooks, 1990; Doyle, 1992). Lu et al. (2013) further developed this method as an elaborated parsimony analysis. Each gene is treated as a single, equally-weighted character and the haplotypes of that gene represent the character states. Step-matrices, as determined for each gene, are used to describe the transformation series of the character states. The step-matrices are then incorporated into a parsimony analysis, resulting in a comprehensive approach that takes into account the differences between the taxa that are recorded by each gene. Lu et al. (2013) applied this method to turtle systematics and found low bootstrap support for relationships that had previously been strongly supported by analyses of large molecular (nucleotide and amino-acid) datasets. Unfortunately, the method is not without its limitations. Since the analysis is based on a parsimony framework, it can only be performed using PAUP* or TNT, both of which limit the number of character states to 32 (there hypothetically exists a 64-bit version of PAUP* that supports up to 64 character states; however, it has still yet to be released under the beta version) (Goloboff et al., 2003; Goloboff et al., 2008; Swofford, 2003). Additionally, the analysis is limited by the gene sampling, both for the dataset and for individual taxa. For example, Wiens et al. (2012) contains only 44

genes, many of which are only sequenced for a subset of the total 161 species. This limits the number of characters in the parsimony analysis to 44, which likely affects the importance of each gene in providing support for nodes in the analysis. More sampled genes will provide more characters for the analysis, providing more certainty that the true evolutionary history is being sampled.

Methods and Materials

32 extant taxa were selected from Wiens et al. (2010b) (22 loci, 15,794 nucleotides, 45 extant taxa) in order to cover all major Squamata taxonomic groups (see Figure 1). In order to analyze more loci, the data for 44 genes for the same taxa from Wiens et al. (2012) were used instead (33,717 nucleotides). For the combined analysis, the morphological data for the same 32 taxa were extracted from Gauthier et al. (2012) and added to the genes as characters dataset. For comparison, the unaltered DNA data from Wiens et al. (2012) for the 32 selected taxa were also analyzed.

RAxML v. 8 was used to separate the genes from Wiens et al. (2012) into separate files (using the -f s option) and to calculate the pairwise distances independently for each gene (using the -f x option combined with the best-known ML tree passed via -t)(Stamatakis, 2014). The output of these analyses was parsed using an in-house R script that produced step-matrices for all of the genes in which the maximum distance was set to 10 and all other values were scaled accordingly (R Core Team, 2014). These step-matrices were then included in a nexus file that was coded by hand for the gene characters and haplotype character states (0123456789 and ABCDEFGHJKMNPQRSTUVWXY) using Mesquite v. 2.75 (Maddison and Maddison, 2011). Taxa that were not sampled for a particular gene were coded as missing data ("?"). The data set was analyzed with PAUP* v. 4.0 Beta 10 (Swofford, 2003). PAUP* corrected any pairwise distances to satisfy triangle inequality. 1,000 bootstrap replicates were performed with stepwise addition, random addition sequence (10 replicates each), and TBR branch-swapping. The majority-rule consensus tree of these bootstrap results is presented in Figure 14 (with nodes collapsed for values less than 50% [left] and 95% [right]).

RAxML v. 8 and PAUP* v. 4.0 Beta 10 were both used to analyze the unaltered DNA data from Wiens et al. (2012) for the 32 selected taxa (Stamatakis, 2014; Swofford, 2003). The

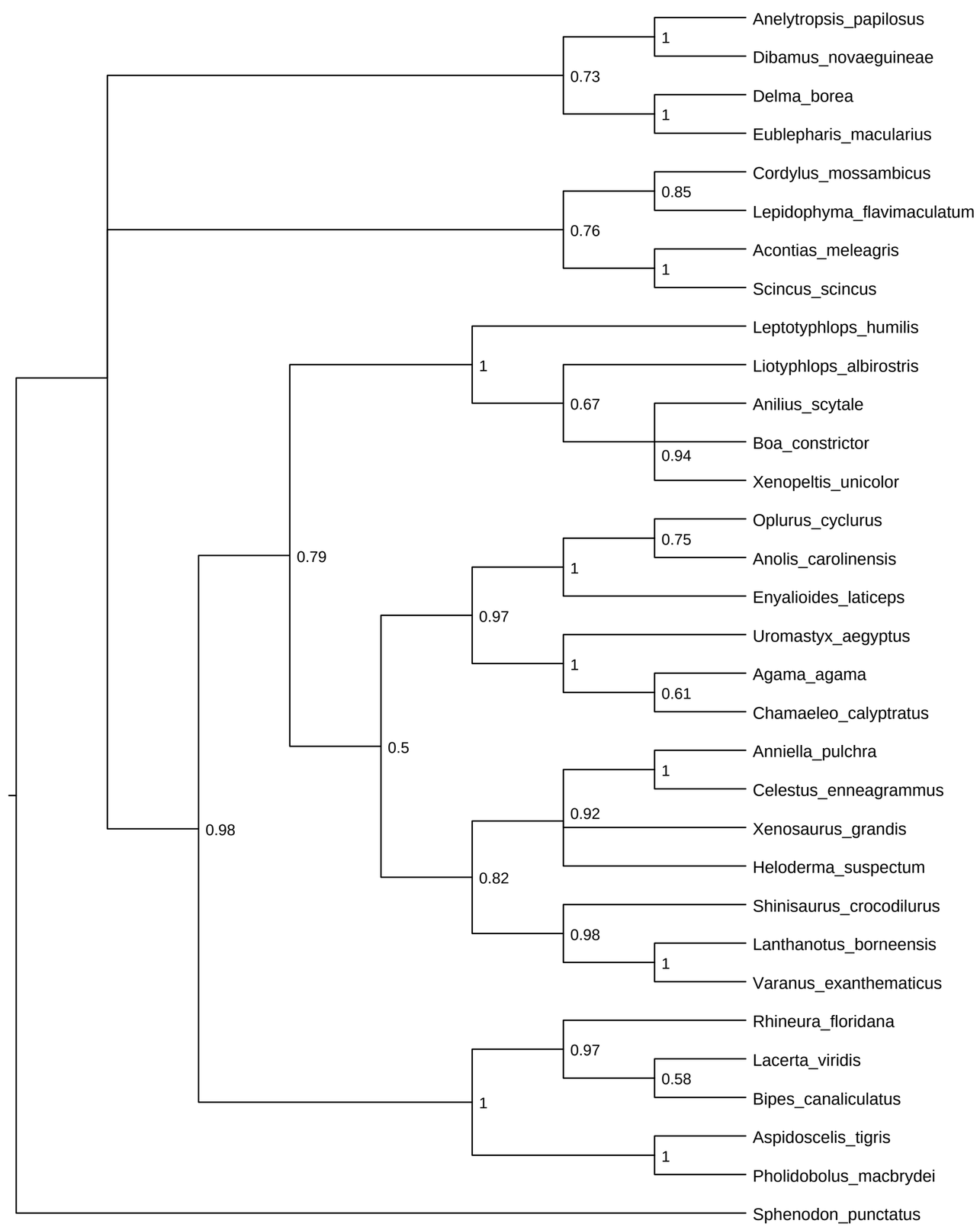

Figure 14: Consensus trees based on PAUP* parsimony analysis of the Squamate Genes as Characters data. Node values represent support values from the bootstrap trees. Nodes with less than .50 bootstrap support have been collapsed in the phylogeny on the left. Nodes with less than .95 bootstrap support have been collapsed in the phylogeny on the right.

PTHREADS version of RAxML conducted 200 bootstraps, taking into account separate partitions for each codon position for each gene (132 partitions total). The majority-rule consensus tree of these bootstrap results is presented in Figure 15 (with nodes collapsed for values less than 50% [left] and 95% [right]). PAUP* v. 4.0 Beta 10 conducted 10,000 bootstrap replicates with stepwise addition, simple addition sequence (reference taxon = *Sphenodon punctatus*), and TBR branch-swapping. The majority-rule consensus tree of these bootstrap results is presented in Figure 16 (with nodes collapsed for values less than 50% [left] and 95% [right]).

Finally, PAUP* v. 4.0 Beta 10 was used to analyze the 32 taxa subset of the unaltered AToL Squamate morphology dataset and the combined dataset consisting of the genes as characters and morphology data for the same 32 taxa. 10,000 bootstrap replicates were performed on the 32 taxa morphology dataset with stepwise addition, simple addition sequence (reference taxon = *Sphenodon punctatus*), TBR branch-swapping, and a time limit of 3600 seconds per replicate. The majority-rule consensus tree of these bootstrap results is presented in Figure 17 (right). 1,000 bootstrap replicates were performed on the combined genes and morphology dataset with stepwise addition, simple addition sequence (reference taxon = *Sphenodon punctatus*), TBR branch-swapping, and a time limit of 3600 seconds per replicate. The majority-rule consensus tree of these bootstrap results is presented in Figure 17 (left). A partition homogeneity test, or incongruence length difference test, performed by PAUP* with the morphology and genes as characters data treated as separate partitions produced a p-value of .001 (Farris et al., 1994). These incongruence test results should be treated as preliminary, but it does imply that these two partitions may reflect different evolutionary histories (Barker and Lutzoni, 2002; Darlu and Lecointre, 2002; Planet, 2006).

Majority rule consensus trees and bootstrap values for the parsimony and RAxML analyses were computed using SumTrees v. 3.3.1, part of the DendroPy v. 3.12.1 Python package (Sukumaran and Holder, 2010). Bootstrap values have been added to the branches using FigTree v. 1.4 and Adobe Acrobat (Rambaut, 2012).

Results

The genes as characters analysis (Figure 14) did not result in a different topology from that of Wiens et al. (2012) (see Figure 15 and Appendix 1). However, it did result in a significant

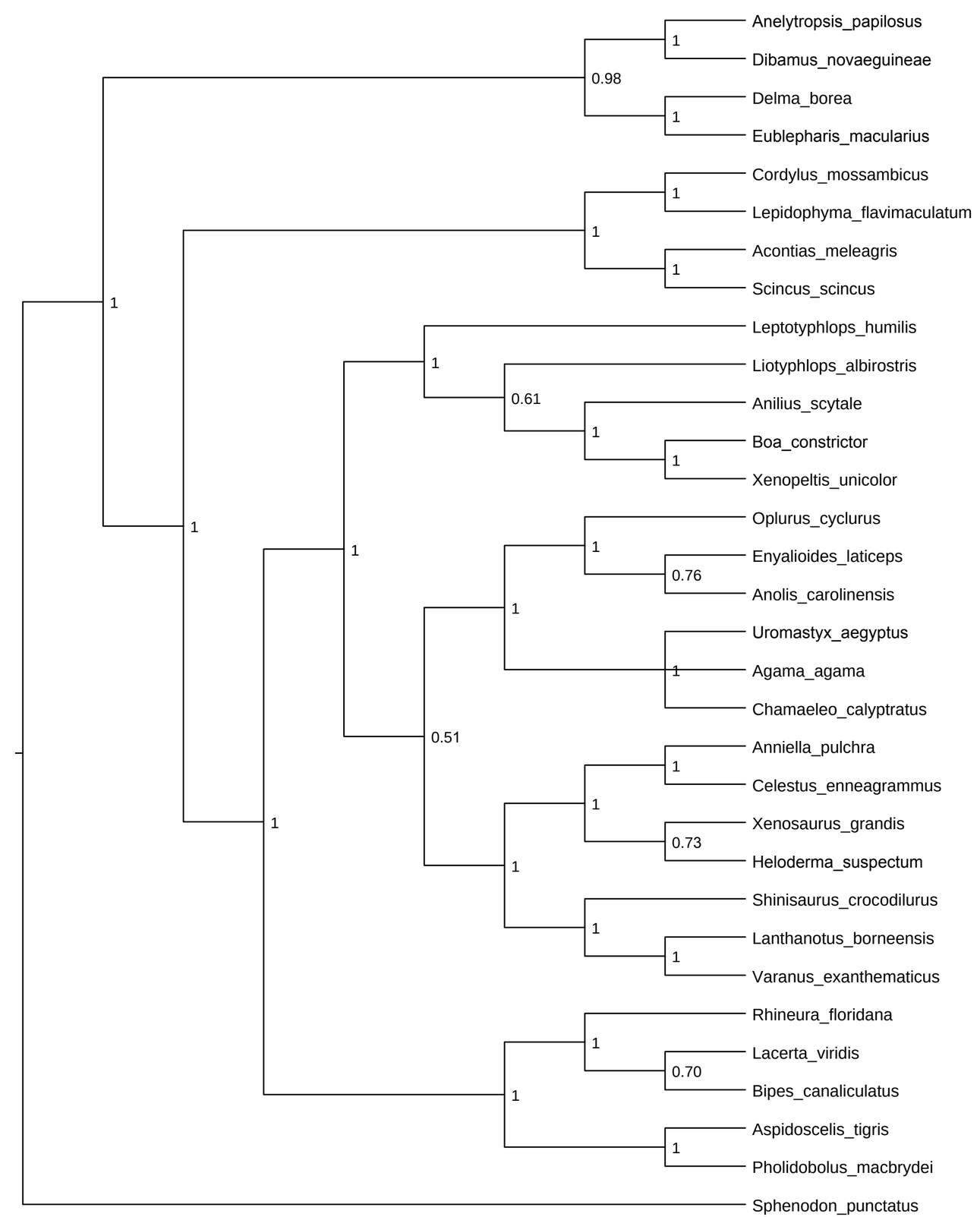

Figure 15: Consensus trees based on RAxML maximum likelihood analysis of 32 selected taxa from the Wiens et al. 2012 Squamate DNA data. Node values represent support values from the bootstrap trees. Nodes with less than .50 bootstrap support have been collapsed in the phylogeny on the left. Nodes with less than .95 bootstrap support have been collapsed in the phylogeny on the right.

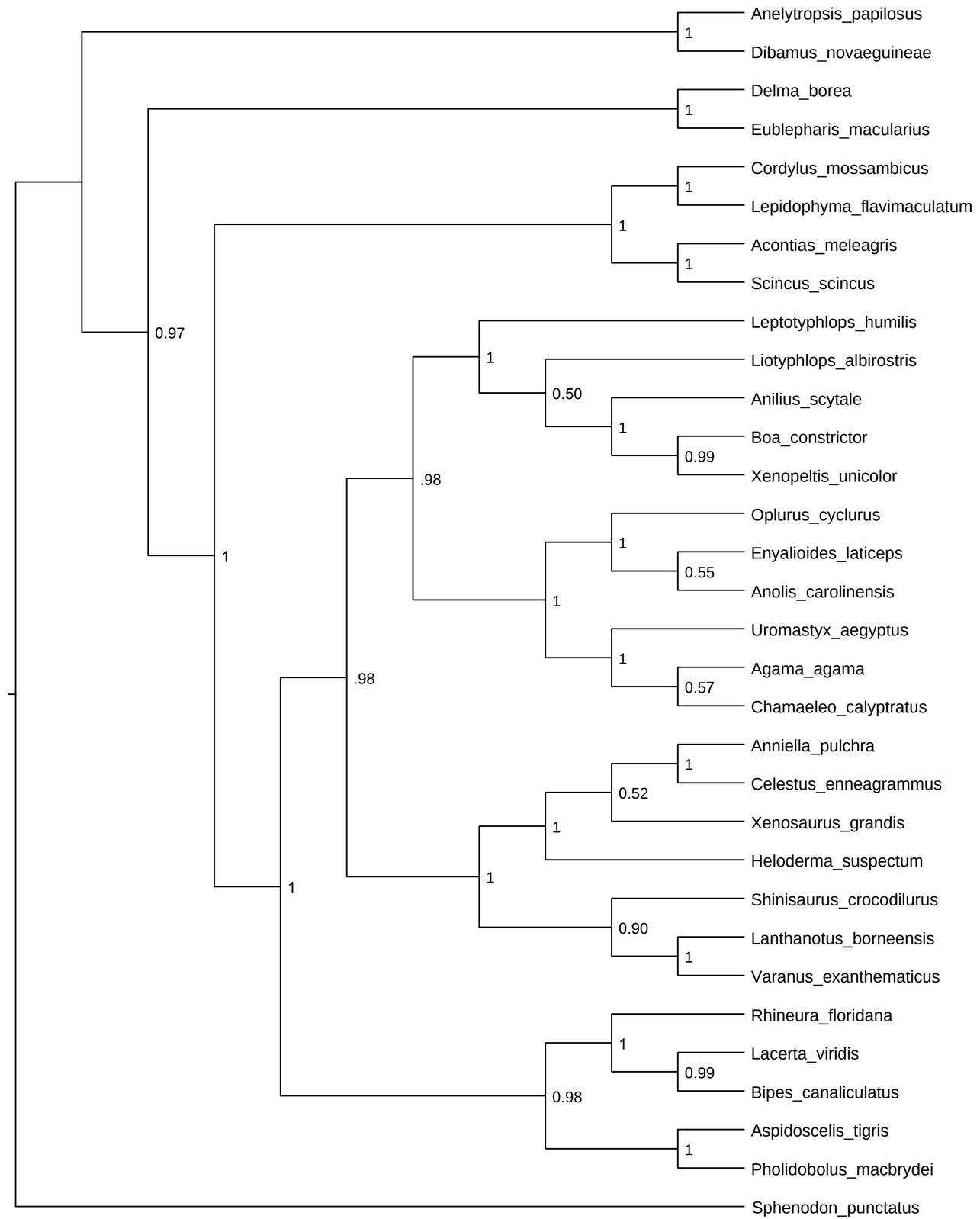

Figure 16: Consensus trees based on PAUP* parsimony analysis of 32 selected taxa from the Wiens et al. 2012 Squamate DNA data.
Node values represent support values from the bootstrap trees. No nodes have been collapsed in the phylogeny on the left.
Nodes with less than .95 bootstrap support have been collapsed in the phylogeny on the right.

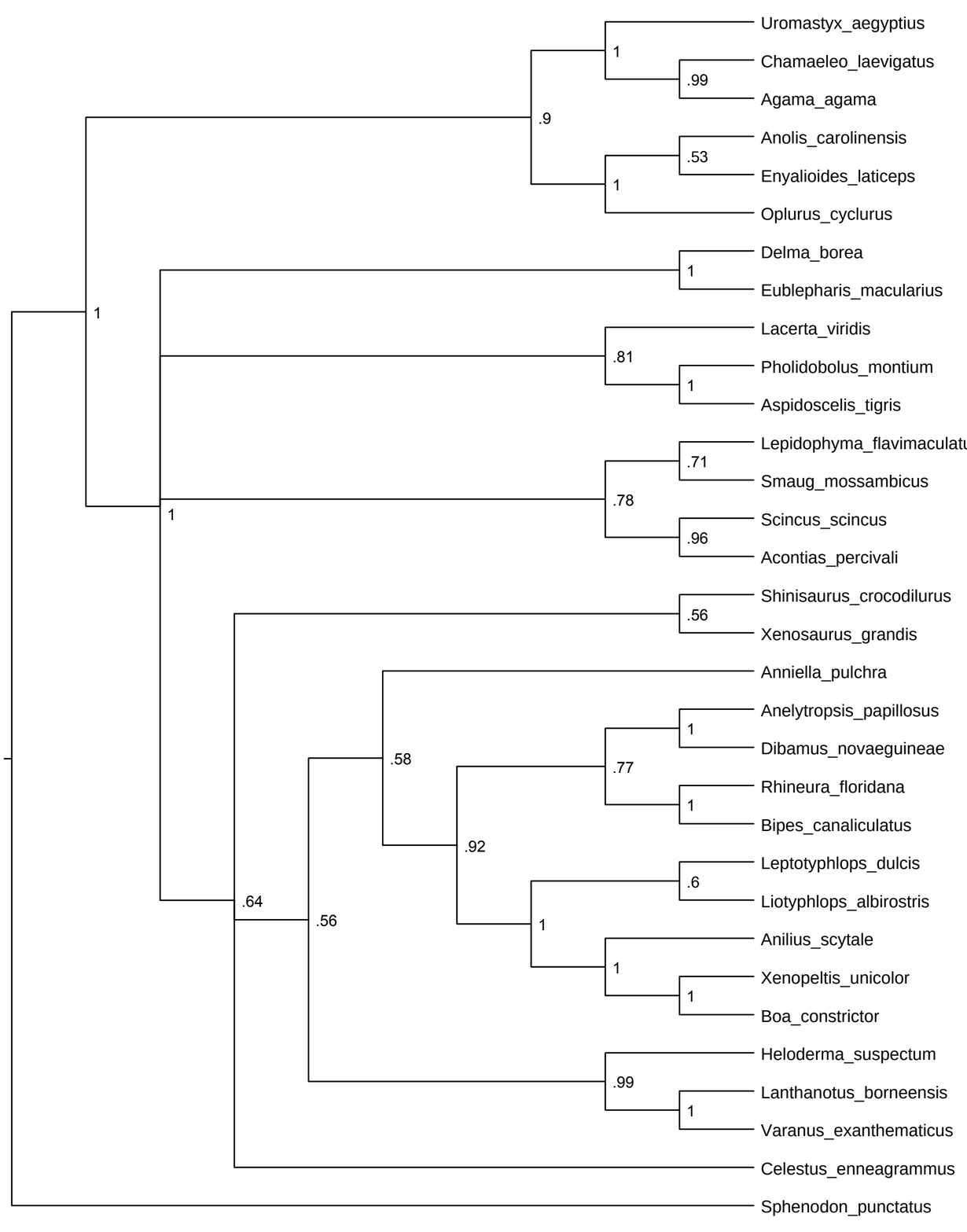

Figure 17: (left) Consensus tree based on PAUP* parsimony analysis of 32 selected taxa from the AToL Squamate morphology data set combined with the Squamate Genes as Characters data set. (right) Consensus tree based on PAUP* parsimony analysis of 32 selected taxa from the AToL Squamate morphology data set. Node values represent support values from the bootstrap trees. Nodes with less than .50 bootstrap support have been collapsed.

drop in support for nearly all of the nodes in the tree, ranging from drops by as little as .01 to drops by as much as .25 or even more (bootstrap values). This results in much less support for the entire topology, which can be seen by comparing Figure 14 (right) and Figure 15 (right). Even when compared to the results of the parsimony analysis of the DNA data for the 32 selected taxa (Figure 16), the genes as characters resolution remains significantly lower for almost all nodes. The support values in all of these results (and those of all of the other analyses in this report) are summarized in Appendix 2. Surprisingly, there are a small number of nodes with increased support under the genes as characters method: *Agama* + *Chamaeleo* and *Liotyphlops* + *Anilius* + *Boa* + *Xenopeltis*.

The analysis of the genes as characters dataset combined with the morphology dataset (only the 32 selected taxa) (Figure 17 [left]) resulted in a topology very similar to that of the parsimony analysis of only the morphology dataset (Figure 17 [right]). However, interestingly, many node support values increased when the genes as characters data was added to the morphology data. The only notable decreases in support are for the *Anniella* + *Dibamidae* + *Amphisbaenia* + *Serpentes* clade (from .77 to .58) and Autarchoglossa (from .75 to <.5).

Discussion

The drop in support in the genes as characters analysis results likely has two origins. First, the genes as characters methods takes into the amount of variation among the different genes. Therefore, if the genes do not agree with each other, under this method, the amount of support for the nodes will decrease. If the nucleotides were treated as the characters, the disagreement would be overwhelmed by the sheer number of characters in the analysis. This leads to the second reason: there are only 44 characters in this analysis. If a gene isn't sampled for one of the taxa, that reduces the amount of possible data for that taxa by more than 2%. The more disagreement at a node, the more that disagreement will become apparent with fewer characters to use as data for that node. This is likely the cause of the drastic shift of support for some of the nodes in the results as compared to the parsimony and maximum likelihood analyses of the DNA data for the 32 selected taxa.

Adding this dataset to the entire morphology dataset causes a similar problem to that of combining the morphology dataset with the entire DNA dataset. The signal of the morphology

swamps the signal of the genes merely due to the number of characters that are conveying that signal. Additionally, the genes as characters signal is much weaker than the pure DNA signal, so it is further weaker against the morphology dataset. However, it is apparent that is still has an effect on the results of the combined analysis, as does adding the morphology data to the molecular data. In fact, the addition of the genes as characters data was able to cause the support for Autarchoglossa to decrease by more than 25%, which is impressive based on the fact that it is already at a disadvantage due to sheer numbers. With broader gene sampling, this method could be extremely valuable to teasing out variability among genes within a molecular dataset. Furthermore, treating genes as character rather than nucleotides brings morphology and DNA closer to an equal match in combined analyses.

V. Conclusion

This report has investigated numerous new and old methods for inferring squamate systematics. Unfortunately, it appears the state of a complete Tree of Life for Squamata remains unfinished. The analysis of squamate morphological data with the Mkv model under both a Bayesian and maximum likelihood framework produces a much more resolved topology for Squamata; however, relationships above the family level remain in disagreement with those in the results of analyses of squamate molecular datasets. Even through the combination of morphological and molecular data, these discordances remain unresolved, and these differences cause a high degree of irresolution and low node support. Results of the analyses of the combined dataset tend to agree more strongly with the analyses of only the molecular data, lending truth to the hypothesis that the signal from the molecular data may be swamping any signal from the morphological data. Although one would expect the molecular data to contain more noise than the morphological data, merely due to the nature of both types of data and the ways in which the data are collected, it seems that the inclusion of more than 30,000 nucleotides overpowers the 779 morphological characters.

The adoption of the genes as characters method results in an understanding of the degree of variability among the genes sampled by Wiens et al. (2012). The drop in support for nearly all nodes of the tree supports the proposal that the variation within the molecular dataset may be masked by the number of characters. Analyzing fewer characters that are more

sensitive to this variability will produce more reliable topologies and support values. Combining this genes as characters data with the morphological dataset reversed the power in numbers problem. Sampling more genes, both for individual taxa and for the entire dataset, may result in a more reliable combined dataset that reconciles the signals of both the genes as characters data and the morphological data. However, it is possible that analyzing combined datasets, especially those that consist of two or more datasets with drastically different signals, may not be solved by finding an equal weighting between the two datasets. Even with relatively similar numbers of characters, heterogeneity within and between the datasets may ultimately lead to low resolution.

The ultimate goal of this report is to open a new avenue for research, particularly in squamate systematics, but also in systematics as a field. While the results presented here are by no means groundbreaking, they reveal that there may be potential for solving the relationships of Squamata. There are a number of potential next steps to discover the proximal and ultimate causes of the discordance between these two datasets. First, gene trees should be produced to analyze whether any of the 44 genes have significantly different signals than the others. Congruence tests could also be used to assess the similarities and differences between the genes. Next, as stated above, the best case scenario for combining the morphological and molecular data is for the two combined datasets to consist of a similar number of characters. Therefore, combining the morphological dataset with each gene separately may yield productive results. This would also assess the variability between the genes and how the different number of characters and/or strength of the evolutionary signal of each gene dataset impact the analyses of the combined dataset. Greater sampling of both genes and morphological characters will make both datasets stronger, especially for the genes as characters method, as discussed above. The inclusion of more fossil taxa in the morphological dataset will possibly increase resolution of the relationships between higher order groups, narrowing the current morphological gaps between these groups. These taxa will not affect the molecular data, and may potentially only benefit the combined analyses. Finally, sensitivity analyses should be run on both the morphological and the molecular analyses as in Gauthier et al. (2012). These and other assessments should be conducted to determine whether there are

any rogue taxa that may be significantly affecting the results of the analyses. Most of these next steps have already been started and will hopefully resolve the problems presented here.

Data

All data are available upon request from the authors in both PHYLIP and Nexus format. The R script for step-matrix production is also available upon request. The morphological data descriptions will be available in a future publication and will be made available online.

Acknowledgments

I am truly indebted to the advisory of Jacques Gauthier over the course of this intensive research project. He and his research group have contributed to the development and completion of this project by answering my many questions, discussing many unintuitive aspects of systematics and squamate phylogenetics, and helping me feel welcome in the lab. Additionally, many thanks must be given to Thomas Near, without who I would be running all of these analyses and making all of these conclusions totally blindly. Lastly, I must also credit Elisabeth Vrba for her absolutely wonderful teaching and advisement, David Evans for his superior academic advising over the past four years, and the entire Geology and Geophysics Department of Yale University for providing such a wonderful atmosphere for learning and performing research.

This work was supported in part by the facilities and staff of the Yale University Faculty of Arts and Sciences High Performance Computing Center.

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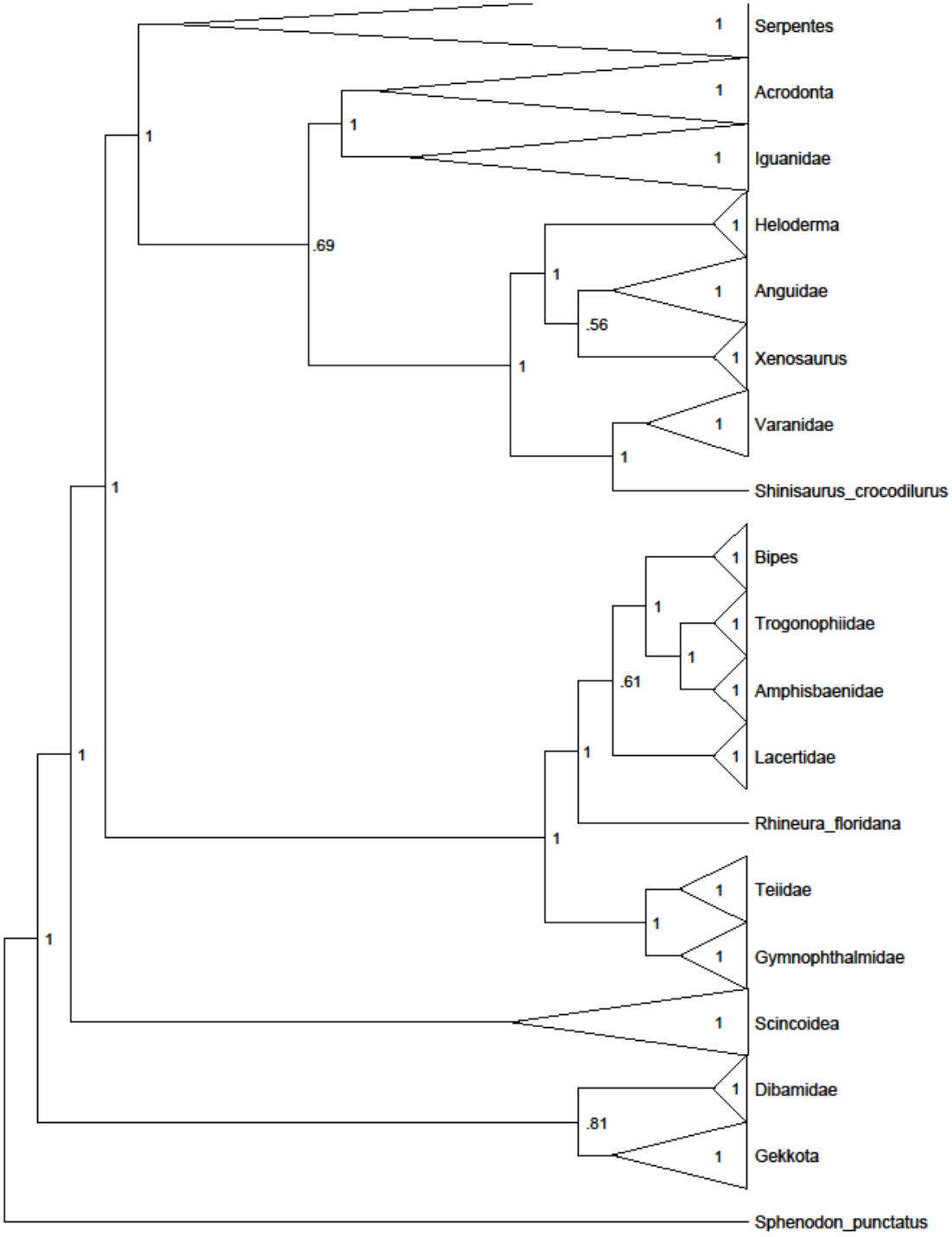
Appendix

Appendix 1

Results from a maximum likelihood analysis of the Wiens et al. (2012) dataset



Consensus tree based on RAxML rapid maximum likelihood analysis of the Wiens et al. 2012 Squamate DNA data. Node values represent support values from the bootstrap trees. All nodes have more than .50 support, so no nodes have been collapsed.

Appendix 2

Clade support under all analyses presented in main text (support for *monophyly*)

(P = Parsimony, BI = Bayesian inference, ML = maximum likelihood)

All clades are as defined in Figure 1 (left) unless noted.

	AToL (unpartitioned)			**AToL (partitioned)**		**Combined**			**DNA (32 Taxa)**		**Genes as Characters (P)**		
Clade	P	BI	ML	BI	ML	P	BI	ML	P	ML	Genes	Morph	Both
Iguania*	.91	Analysis incomplete	.98	1	.94	.8	Analysis incomplete	.95	1	1	.97	.73	.9
Acrodonta*									1	1	1	.78	1
Iguanidae	<.5		.5	1	<.5	<.5		.6	1	1	1	.91	1
Scleroglossa	<.5		.78	1	.86	<.5		<.5	<.5	<.5	<.5	1	1
Autarchoglossa	<.5		.7	1	.72	<.5		<.5	<.5	<.5	<.5	.75	<.5
Amphisbaenia	.99		.97	1	.95	1		1	<.5	<.5	<.5	.95	1
Anguimorpha	<.5		<.5	<.5	<.5	.94		.89	1	1	.82	<.5	<.5
Varanoidea*	.88		1	.8	.89	<.5		<.5	<.5	<.5	<.5	.98	.99
Dibamidae	1		1	1	1	1		1	1	1	1	1	1
Scincomorpha	<.5		<.5	<.5	<.5	<.5		<.5	<.5	<.5	<.5	<.5	<.5
Lacertoidea**	.93		.95	1	.88	.75		.83	.98	1	1	.73	.81
Scincoidea	<.5		<.5	<.5	<.5	<.5		<.5	1	1	.76	<.5	.78
Scincidae	<.5		<.5	<.5	<.5	.57		.53	1	1	1	.57	.96
Serpentes*	.98		1	1	.99	.89		.84	1	1	1	1	1
Gekkota*	.71		.81	1	.78	.66		.98	1	1	1	1	1
Toxicofera***	<.5		<.5	<.5	<.5	<.5		.53	.98	1	.79	<.5	<.5

*Includes stem members

**Includes Amphisbaenia in DNA and combined analyses

***As defined in Figure 1 (right)