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Combining Phylogenomics and Fossils in Higher-Level Squamate Reptile Phylogeny: Molecular Data Change the Placement of Fossil Taxa

JOHN J. WIENS[1,*], CAITLIN A. KUCZYNSKI[1], TED TOWNSEND[2], TOD W. REEDER[2], DANIEL G. MULCAHY[3], AND JACK W. SITES JR.[3]

[1]*Department of Ecology and Evolution, Stony Brook University, Stony Brook, NY 11794-5245, USA;*
[2]*Department of Biology, San Diego State University, San Diego, CA 92182-4614, USA; and*
[3]*Department of Biology, Brigham Young University, Provo, UT 84602, USA;*
[*]*Correspondence to be sent to: Department of Ecology and Evolution, Stony Brook University, Stony Brook, NY 11794-5245, USA; E-mail: wiensj@life.bio.sunysb.edu.*

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Abstract.—Molecular data offer great potential to resolve the phylogeny of living taxa but can molecular data improve our understanding of relationships of fossil taxa? Simulations suggest that this is possible, but few empirical examples have demonstrated the ability of molecular data to change the placement of fossil taxa. We offer such an example here. We analyze the placement of snakes among squamate reptiles, combining published morphological data (363 characters) and new DNA sequence data (15,794 characters, 22 nuclear loci) for 45 living and 19 fossil taxa. We find several intriguing results. First, some fossil taxa undergo major changes in their phylogenetic position when molecular data are added. Second, most fossil taxa are placed with strong support in the expected clades by the combined data Bayesian analyses, despite each having >98% missing cells and despite recent suggestions that extensive missing data are problematic for Bayesian phylogenetics. Third, morphological data can change the placement of living taxa in combined analyses, even when there is an overwhelming majority of molecular characters. Finally, we find strong but apparently misleading signal in the morphological data, seemingly associated with a burrowing lifestyle in snakes, amphisbaenians, and dibamids. Overall, our results suggest promise for an integrated and comprehensive Tree of Life by combining molecular and morphological data for living and fossil taxa. [Combined analysis; fossils; lizards; morphology; phylogeny; snakes; squamates.]

The Tree of Life includes both living and extinct taxa, and many major and minor branches are known only from fossils. But how can a complete phylogeny of living and fossil taxa be reconstructed? For living taxa, it is now possible to obtain molecular data sets that include dozens of genes and tens of thousands of characters (e.g., Rokas et al. 2003; Takezaki et al. 2004; Dunn et al. 2008; Hackett et al. 2008; Wiens et al. 2008), and new technologies are poised to increase these numbers even further. In contrast, most fossil taxa can be placed in phylogenies based only on morphological data sets, which are typically limited to a few hundred characters at most. This disparity raises some pivotal questions for the future of systematics. How can molecular data for living taxa and morphological data for fossil taxa be integrated to estimate a complete Tree of Life? Can advances in molecular phylogenetics lead to improvements in our understanding of the relationships of fossil taxa?

One approach for integrating molecular and fossil data is to include both types of data in a single combined data matrix. This approach generally involves obtaining morphological data from both living and fossil taxa and then combining these morphological data with molecular data for the living taxa only (e.g., Gatesy et al. 2003; Asher et al. 2005; Rothwell and Nixon 2006; Manos et al. 2007; O'Leary and Gatesy 2008). However, whether this practice actually improves phylogenetic accuracy for the fossil taxa is not yet clear.

Recent computer simulations (Wiens 2009) offer cause for cautious optimism. They suggest that adding molecular data might dramatically improve phylogenetic accuracy for fossil taxa, at least under certain conditions. These conditions include 1) when the molecular data are accurate (of course), 2) when fossil taxa do not greatly outnumber living taxa in the matrix, and 3) when there are many morphological characters but they exhibit high homoplasy. Perhaps most importantly, these simulations revealed very few conditions where including molecular data consistently decreased phylogenetic accuracy for fossil taxa.

A review of seven empirical case studies (Wiens 2009) showed somewhat mixed results, however. In four cases (Shaffer et al. 1997; Asher and Hofreiter 2006; Rothwell and Nixon 2006; Manos et al. 2007), the addition of molecular data improved the phylogenetic resolution of fossil taxa in the combined analysis (i.e., polytomies involving their placement were fully or partially resolved). Yet, the molecular data did not radically alter the placement of these fossil taxa. In the three other case studies (Gatesy et al. 2003; Asher et al. 2005; O'Leary and Gatesy 2008), there were strongly supported conflicts between the molecular and morphological data, and relationships among fossil taxa were generally more poorly resolved after molecular data were added (although some taxa also changed positions). Based on these examples, there is only limited evidence that molecular data can dramatically change the placement of fossil taxa, and none of these previous studies focused on such changes.

Here, we show that addition of molecular data can lead to major shifts in the phylogenetic placements of fossil taxa in a case study involving the higher level phylogeny of squamate reptiles (i.e., lizards and

snakes). Our study addresses the phylogenetic placement of snakes within squamates, combining data from molecules, morphology, and fossils.

The placement of snakes has been particularly problematic, given that morphological data tend to place snakes in a clade with other limb-reduced squamates, particularly those that burrow. Estes et al. (1988) presented the first modern analysis of higher level squamate relationships. Their analysis placed snakes with the burrowing amphisbaenians and dibamids (although they disbelieved this result), which also have an elongate, limb-reduced, snake-like body form. Subsequent morphological analyses produced similar trees (e.g., Hallermann 1998; Lee 1998; Kearney 2003; Evans et al. 2005). Some authors have suggested that snakes are nested inside of the lizard clade Anguimorpha (e.g., Lee 1998), which ostensibly includes a group of giant, extinct, marine reptiles called mosasaurs, as well as monitor lizards (Varanidae), and several other families (e.g., anguids, helodermatids, xenosaurids). Another recurring theme is the placement of iguanian lizards at the base of squamate phylogeny, which has been strongly supported by nearly every morphology-based analysis of squamate phylogeny.

Townsend et al. (2004) presented a radically different hypothesis for squamate relationships based on slow-evolving nuclear DNA sequences and some mitochondrial DNA data. They placed gekkonids and dibamids at the base of squamate phylogeny rather than iguanians. They reconstructed snakes as outside of anguimorphs, in a polytomy with iguanians and anguimorphs, far from the root. Amphisbaenians were the sister group of lacertids. Subsequent molecular studies with increased numbers of nuclear loci (e.g., Vidal and Hedges 2005; Fry et al. 2006) provided further confirmation of these novel results.

Conrad (2008) presented the most extensive phylogenetic analysis of squamate morphology to date, including 363 characters and 222 taxa (both living and fossil). Like previous morphological studies, this tree placed most elongate, limb-reduced burrowing squamates in a single clade (including dibamids, amphisbaenians, and snakes; but note that not all snakes are burrowers). This clade was nested inside Scincidae, which also contains many limb-reduced burrowers. Conrad (2008) then erected a new classification intended to formalize these results, including the clade of primarily burrowing taxa (i.e., Scincophidia).

Lee (2009) recently analyzed the placement of snakes using a combined analysis of molecular and morphological data. He examined 248 morphological characters among 28 taxa (19 living and 9 fossil), 9 nuclear loci (6,192 characters; from Vidal and Hedges 2005), and mitochondrial DNA data (from Townsend et al. 2004). Both his morphological and combined data analyses place snakes within Mosasauria (mosasaurids, aigialosaurids, dolichosaurids, *Adriosaurus*), with snakes and "mosasaurs" making up the sister to anguimorphs.

In this paper, we test the phylogenetic placement of snakes within squamates, combining morphological data from Conrad (2008) for 45 living taxa and 19 fossil taxa, and new molecular data (from 22 nuclear genes) from 45 living taxa. More importantly, we address the potential for molecular data to change the placement of fossil taxa and increase branch support. We also evaluate whether analyses of squamate morphology have been misled by morphological convergence and whether this influences both parsimony- and model-based methods.

Materials and Methods

Taxon Sampling

The 45 living taxa were selected with four goals in mind: 1) including all major squamate clades, 2) addressing the possible placement of snakes in anguimorphs (e.g., many snakes and anguimorphs were included), 3) addressing the hypothesis that burrowing limb-reduced squamates form a clade (e.g., various scincids and amphisbaenians were included), and 4) matching taxon sampling for morphological and molecular data sets as closely as possible (see below). We included only a small fraction of all ~8400 extant squamate species (Uetz and Hallermann 2009), but sampling more extant taxa would only add more species to major clades already represented. We included a rhynchocephalian (*Sphenodon punctatus*) as an outgroup to represent the well-established sister group to squamates; other living outgroups are only distantly related to squamates. Fossil taxa were selected to optimize 1) inclusion of major clades and 2) relevance to the origin of snakes. We used only a fraction of the taxa sampled by Conrad (2008) to prevent the combined matrix from being dominated by taxa lacking comparable phylogenomic data (see Wiens 2009).

Conrad (2008) used a mixture of species and higher clades as terminal taxa. For the combined analysis, we used a single species from the molecular data set to represent each higher taxon that was coded as a single terminal by Conrad (2008). Specifically, we used *Sphenodon* for Rhynchocephalia, *Amphisbaena fuliginosa* for Amphisbaenidae, *Rhineura floridana* for Rhineuridae, *Trogonophis wiegmanni* for Trogonophidae, *Anilius scytale* for Aniloidea, *Boa constrictor* for "other Macrostomata," *Strophurus ciliaris* for Diplodactylinae (Gekkonidae), *Cordylus mossambicus* (Cordylidae) for Cordyloidea (Cordylidae + Gerrhosauridae), *Pholidobolus macbrydei* for Gymnophthalmidae, *Aspidoscelis tigris* for Teiinae (Teiidae), *Lacerta viridis* for Lacertidae, *Acontias meleagris* for Acontinae (Scincidae), *Feylinia polylepis* for Feylininae (Scincidae), and *Scincus scincus* for Scincinae (Scincidae). Cases in which Conrad (2008) coded a genus but did not mention a specific species are not listed.

In some cases, Conrad (2008) represented a higher taxon with a genus or species that differed from one we sampled for molecular data. In these cases, we used the following species in the molecular data set to represent the taxon in the morphological data set (with the latter in parentheses): *Chamaeleo calyptratus* (*Rhampholeon*:

Chamaeleonidae), *Anolis carolinensis (A. vermiculatus)*, *Varanus acanthurus* (*V. eremius*), *Varanus salvator (V. varius)*, *Typhlops jamaicensis* (*T. lineolatus*), *Leptotyphlops humilis* (*L. goudottii*), *Eublepharis macularius* (*Hemitheconyx*; Eublepharinae, Gekkonidae), and *Lialis burtonis* (*Pygopus*). In most cases, the taxa involved are the only representatives of the higher taxon in each data set, and so these replacements should have little impact on the results and little choice was involved. For Varanidae, we used an estimated phylogeny (Ast 2001) to select matching species as much as possible. Finally, some taxa sampled for molecular data were not included as separate taxa in the morphological data set (e.g., many snake families) and were excluded.

Morphological Data and Analysis

Following Conrad (2008), we did not order any characters nor make other modifications to his data. However, we removed one character because it was based solely on the biogeographic ranges of species. Parsimony analyses used PAUP* 4.0b10 (Swofford 2002), with a heuristic search with 1000 random addition sequence replicates, each with tree-bisection-reconnection (TBR) branch swapping, and retaining all shortest trees. To assess support, we used nonparametric bootstrapping (Felsenstein 1985), with 500 pseudoreplicate data matrices, each analyzed with a heuristic search with 10 replicates and TBR branch swapping. Bootstrap (bs) values $\geq$70% were considered strongly supported (Hillis and Bull 1993; but see their caveats).

Bayesian analyses were performed using the Mk model (Lewis 2001) with a parameter for rate variation among characters (Γ). Analyses were implemented in MrBayes, version 3.1.2 (Huelsenbeck and Ronquist 2001), with two replicate searches with 20 million generations each (sampling every 1000 generations), using default priors. Trees sampled before achieving stationarity were discarded as burn-in, and stationarity was identified based on plots of log likelihoods over time, average standard deviation of split frequencies between runs $\leq$0.01, and using Tracer version 1.4 (Rambaut and Drummond 2004). The phylogeny was estimated from the majority rule consensus of the pooled post burn-in trees from the two replicates. Clades with posterior probabilities (Pp) $\geq$0.95 were considered strongly supported (e.g., Alfaro et al. 2003; Erixon et al. 2003; Huelsenbeck and Rannala 2004).

Molecular Data and Analysis

DNA sequence data were obtained from 22 nuclear protein-coding loci. (Primer sequences in Appendix S1, and GenBank and voucher numbers in Appendix S2, both available from http://www.sysbio.oxfordjournals.org; TreeBASE accession TB2:S10908.) Some DNA data were used in a previous study of snake phylogeny (Wiens et al. 2008), including data for all 6 extant snakes and 5 other taxa used in this study (of 45 total), for 11 genes. Genes were selected based on an extensive comparison among nuclear genomes of *Fugu*, *Gallus*, and *Homo* (Townsend et al. 2008). Gene regions that were selected were 1) apparently single copy, 2) contained within a single exon, 3) short enough to amplify and sequence with one pair of reactions ($\sim$500–1000 base pairs, bp), and 4) evolving at an appropriate rate (i.e., variable among squamate families). Standard methods of DNA extraction, amplification, and sequencing were used. Sequence data were generated in the labs of T.W.R., J.W.S., and J.J.W. All sequences for a given gene were generated in the same lab, and the same individual specimen was used for a given species in all three labs. Alignment was done by eye after translating nucleotide sequences to amino acid sequences (using MacClade, version 4.0; Maddison D.R. and Maddison W.P. 2000).

For each gene, preliminary analyses were conducted using parsimony to detect possible contamination and other errors. When a representative of one species had an identical or nearly identical sequence to another, the gene was resequenced for one or both taxa. However, we did not exclude sequences merely because they conflicted with previous taxonomy or with phylogenies from other genes.

Some species proved difficult to amplify and/or sequence for a given gene even after designing new primers. Taxa that lacked data for a given gene were coded as missing ("?") for that gene. The 22 genes ranged in their completeness from 80% to 100% of the 45 species (mean = 94%; Table 1). Individual species had from 2.8% to 45.5% missing data cells overall (mean = 10.2% and median = 7.9%). Simulation and empirical studies suggest that missing data need not preclude taxa from being accurately placed in an analysis, particularly when many characters are sampled (e.g., Wiens 2003; Driskell et al. 2004; Philippe et al. 2004; Wiens et al. 2005b; Wiens and Moen 2008).

The molecular phylogeny was estimated primarily based on combined parsimony and Bayesian analyses of the 22 loci, with a total of 15,794 characters. Parsimony and Bayesian analyses were conducted with PAUP* and MrBayes as described above.

For Bayesian analyses, genes were partitioned by codon positions. We tested whether partitioning improved model fit by analyzing each gene separately and comparing likelihood values from partitioned and unpartitioned analyses using Bayes factors (e.g., Nylander et al. 2004; Brandley et al. 2005). Partitioning was strongly supported for all 22 genes (Bayes factor $>$70). For each gene region, we selected the best-fitting model using MrModeltest version 2.0 (Nylander 2004), using the Akaike information criterion. We assumed that partitions within genes had the same overall model as the entire gene. We prefer this approach (relative to testing for separate models within genes) because simulations (Posada and Crandall 2001) show that correct choice of a complex model may be difficult, given only a few hundred characters (i.e., even if the simulated model is complex, a simple model may erroneously be chosen).

TABLE 1. Basic properties of the 22 nuclear protein-coding genes used in phylogenetic analyses

Gene	Length (bp)	Variable characters	Parsimony-informative characters	Taxa (% of 45 total)	Best-fitting model
ADNP	801	378	271	40 (89)	HKY + I + Γ
BDNF	676	251	180	45 (100)	GTR + I + Γ
BMP2	645	311	253	39 (87)	GTR + I + Γ
CAND1	759	279	218	44 (98)	GTR + I + Γ
DLL1	570	269	205	36 (80)	GTR + I + Γ
DNAH	722	357	302	43 (96)	GTR + I + Γ
ECEL	570	341	253	43 (96)	GTR + I + Γ
ENC1	888	351	277	42 (93)	GTR + I + Γ
FSHR	753	374	292	44 (98)	GTR + I + Γ
FSTL	622	299	207	39 (87)	HKY + I + Γ
GPR	509	290	183	36 (80)	GTR + I + Γ
LRRN1	681	267	207	40 (89)	GTR + Γ
NGFB	582	362	272	44 (98)	GTR + I + Γ
NTF3	510	342	276	44 (98)	GTR + I + Γ
PNN	1050	685	506	44 (98)	GTR + I + Γ
RAG1	1091	563	449	44 (98)	GTR + I + Γ
SINCAIP	486	293	229	40 (89)	GTR + I + Γ
SLC30A1	555	309	256	42 (93)	HKY + I + Γ
SLC8A1	996	395	344	45 (100)	GTR + I + Γ
TRAF6	642	405	324	45 (100)	HKY + I + Γ
VCPIP1	801	338	275	42 (93)	SYM + I + Γ
ZEB2	885	360	244	45 (100)	HKY + I + Γ

Notes: Models were selected using MrModeltest version 2, using the AIC. Full names of the genes are provided in Townsend et al. (2008).

To ensure that results were not an artifact of over-partitioning in the Bayesian analyses, we repeated the molecular analyses using likelihood analyses with different partitioning strategies (66 partitions, 3, and none). We used RAxML (version 7.0.5; Stamatakis 2006), with 200 bootstrap replicates coupled with 40 heuristic searches, with the GTR + I + Γ model (based on Table 1). Analyses with 66 partitions yielded an identical topology to that from Bayesian analyses, with similar branch lengths and support values (not shown). Although internal branches in the Bayesian analysis are slightly longer overall (mean = 0.0332 vs. mean = 0.0242), the correlation between lengths of matched internal branches is nearly perfect ($r^2 = 1.000$). Likelihood results with fewer partitions are also very similar in terms of topology, support, and branch lengths (not shown).

We did not explore the use of coalescent-based species-tree methods (e.g., Edwards et al. 2007) for these data, despite their potential advantages, because it would be difficult to incorporate the fossil taxa in such analyses.

Combined Analysis

In general, we consider the combined analysis to offer the best estimate of phylogeny rather than separate analyses of the morphological or molecular data. We performed parsimony and Bayesian analyses of the combined molecular and morphological data as described above. However, for the Bayesian analysis we used 50 million generations, given that stationarity was achieved more slowly. Although we performed combined analyses both including and excluding the 19 fossil taxa, the relationships among living taxa were very similar between analyses, and so (for brevity) results excluding the fossil taxa are not presented.

Do Molecular Data Increase Branch Support for Fossil Taxa?

We tested whether the addition of molecular data increases the support for the localized placement of fossil taxa within the combined data tree, a question that has not been addressed previously. Support in Bayesian analyses was assessed based on Pp, whereas bootstrap values were used for parsimony. For species with a single sister species (in our analysis), we used the support value for the branch uniting them. For a species placed as sister to more than one species, we used the average of the support values for the branch immediately below and above that species (following Wiens et al. 2005b). We acknowledge that there is some nonindependence of these values (e.g., two fossil sister species may share the same support values). For parsimony analyses, it was difficult to determine bootstrap support for clades with values $< 50\%$ (and differences between very low values were of limited interest). We assigned these clades a value of 49%. Similarly, taxa involved in polytomies were given values of 49%, even if some branches in the polytomy had higher values. We used a nonparametric Wilcoxon signed rank test (in Statview) to compare support values for all 19 fossil taxa before and after addition of the molecular data for each method.

RESULTS

Parsimony analysis of the morphological data for the 45 extant taxa and 19 fossil taxa yields 442 trees of length 1704 (Fig. 1). The strict consensus tree shows the fossil taxon *Huehuecuetzpalli* as the sister group to all other squamates. Iguanians are supported as sister to Scleroglossa (all living squamates exclusive of Iguania), and monophyly of Scleroglossa has moderate support (bs = 61%). However, many relationships within Scleroglossa are unresolved (Fig. 1). There is strong support for a

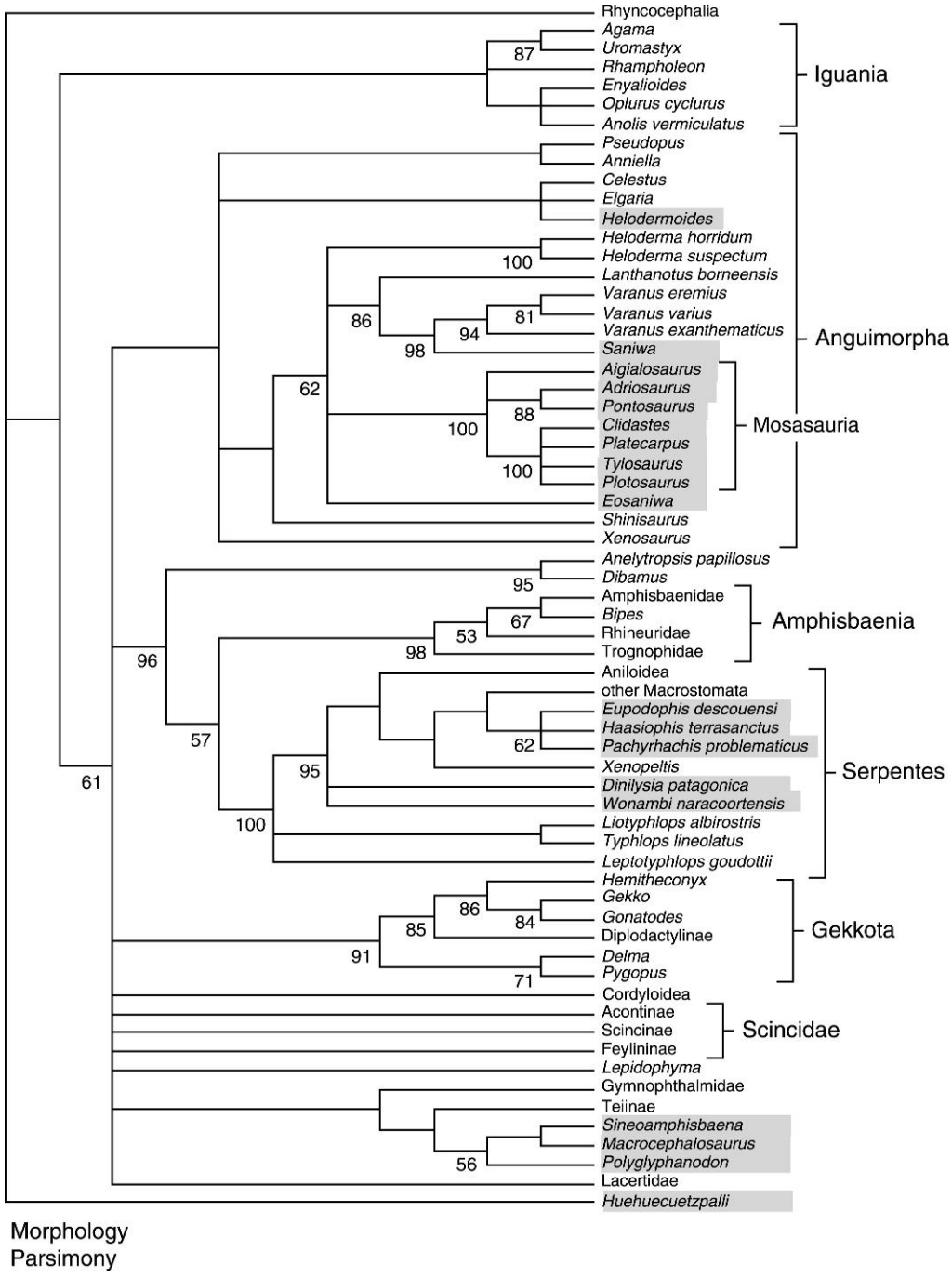

FIGURE 1. Phylogeny of squamate reptiles based on parsimony analysis of the morphological data alone (data from Conrad 2008). The tree shown is a strict consensus of 442 trees of length 1704 steps. Fossil taxa are indicated with gray shading. Numbers adjacent to nodes are bootstrap values ≥50%.

clade uniting Amphisbaenia, Dibamidae, and Serpentes. Mosasauria is strongly supported as monophyletic and is placed in Anguimorpha, as are the fossil anguimorphs *Eosaniwa*, *Helodermoides*, and *Saniwa*. The fossil snakes sampled (*Dinilysia*, *Eupodophis*, *Haasiophis*, *Pachyrachis*, *Wonambi*) are placed in snakes above scolecophidians (*Leptotyphlops, Liotyphlops, Typhlops*).

Results from Bayesian analysis of the morphological data (including fossil taxa) are similar (Fig. 2). However, many polytomies from the parsimony tree (Fig. 1) are well resolved and strongly supported in the Bayesian analysis. *Huehuecuetzpalli* is again placed as the sister to all other squamates. Iguania and Scleroglossa are each strongly supported as monophyletic. Anguimorpha is weakly placed as sister to all other scleroglossans. Mosasauria is strongly supported as monophyletic and is placed deep within Anguimorpha. The clade consisting of amphisbaenians, dibamids, and snakes is

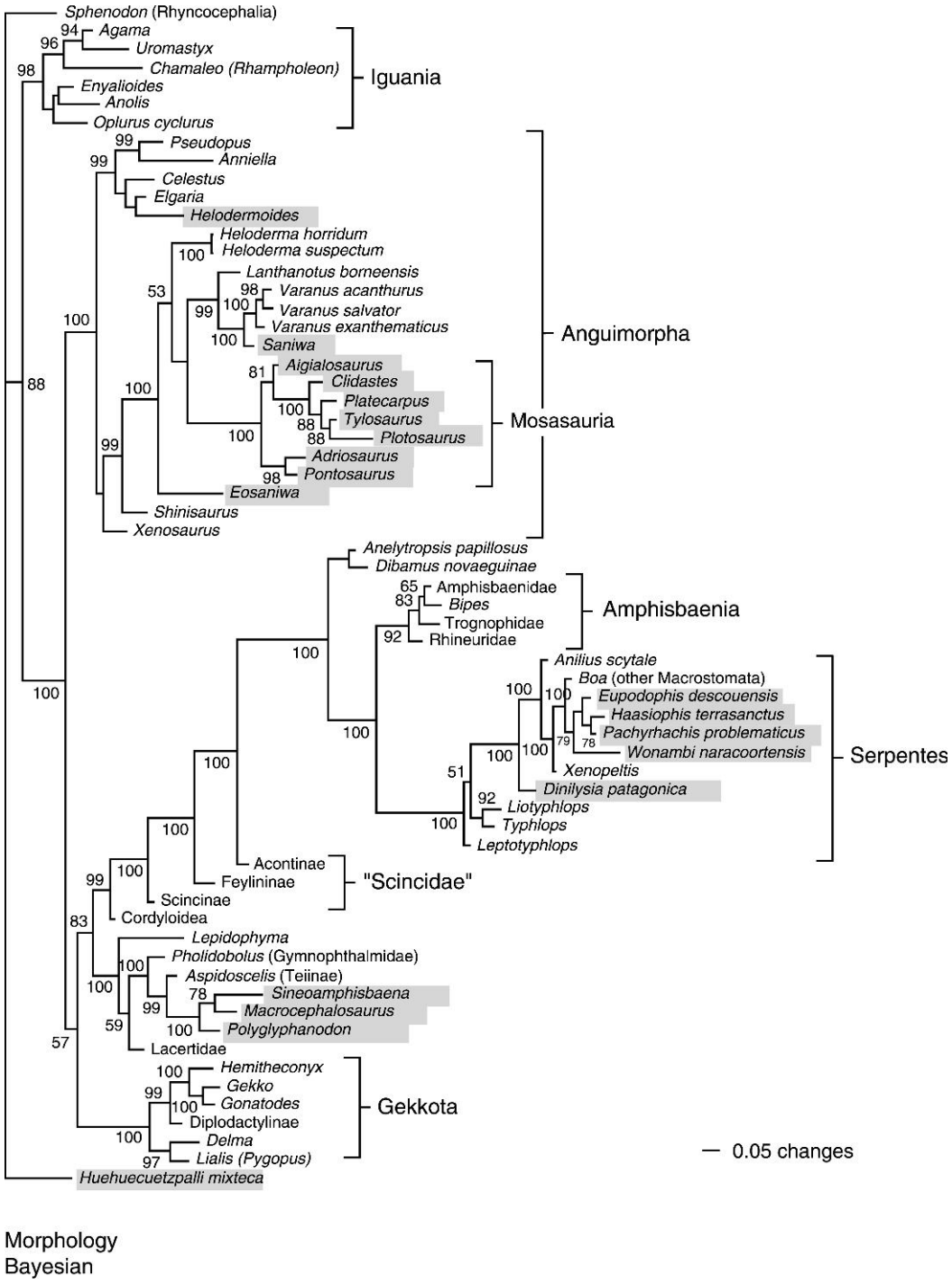

FIGURE 2. Phylogeny of squamate reptiles based on Bayesian analysis of the morphological data alone (data from Conrad 2008). Fossil taxa are indicated with gray shading. Numbers adjacent to nodes are Pp values $\geq$0.50 (Pp are multiplied by 100 in figure).

again strongly supported and is nested inside Scincidae (Scincophidia; Conrad 2008).

Parsimony analysis of the molecular data (22 nuclear loci) yields a single strongly supported tree (29,616 steps; Fig. 3) similar to that from Townsend et al. (2004) and subsequent authors. Snakes are placed as sister to iguanians, but with only moderate support (bs = 62%). The molecular data do not support the hypothesis that snakes, amphisbaenians, and dibamids are nested inside of skinks (Scincidae). Strangely, the molecular data show Amphisbaenia as nonmonophyletic in that there is strong support for placing Rhineuridae outside and Lacertidae within in both parsimony and Bayesian analyses (Figs. 3 and 4).

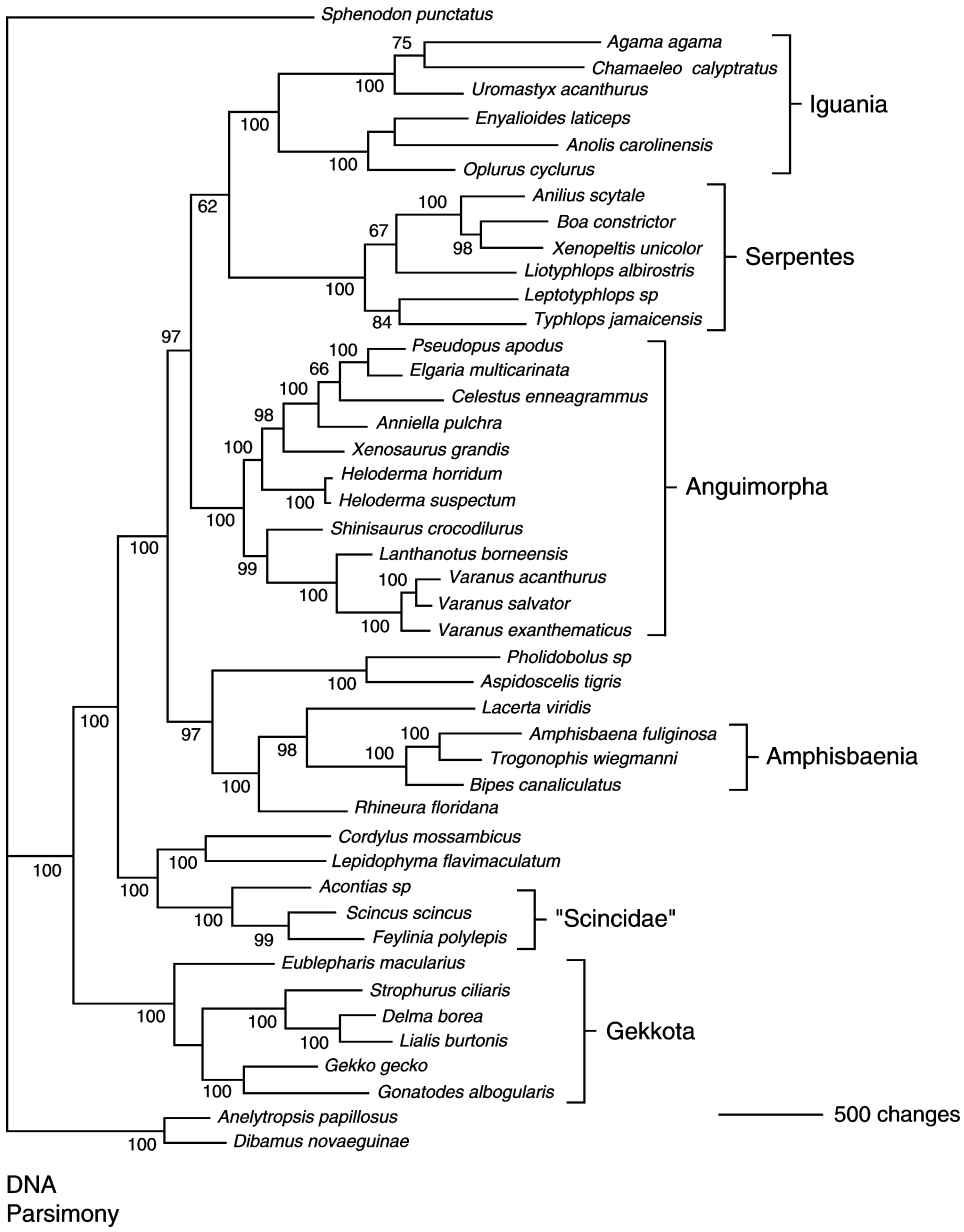

FIGURE 3. Phylogeny of squamate reptiles based on parsimony analysis of molecular data only, showing the single shortest tree (length = 29,616 steps). Numbers adjacent to nodes are bootstrap values ≥50%.

The Bayesian molecular tree is very similar to the parsimony tree (Fig. 4). These trees differ primarily in that in the Bayesian tree, dibamids and gekkotans are placed as sister taxa at the base of the tree (rather than dibamids being sister to all other extant squamates). Furthermore, in the Bayesian tree, iguanians and anguimorphs are placed together as the sister group to snakes, with very weak support.

Parsimony analysis of the combined data for living and fossil taxa yields 6 trees (31,522 steps; Fig. 5). For extant taxa, these trees are very similar to those from molecular data alone. However, some fossil taxa show very different placements when molecular data are added, relative to the tree from morphology alone. Most notably, the morphological data alone place *Huehuecuetzpalli mixteca* at the base of squamates, whereas addition of molecular data places this taxon deeply nested in the tree as sister to Iguania. Although the branch linking Huehuecuetzpalli with Iguania is not strongly supported, many of the nodes that separate Huehuecuetzpalli from its original position near the root of the tree are strongly supported (i.e., bs > 70%).

Similarly, the placement of *Sineoamphisbaena* also changes, from a clade with *Polyglyphanodon* and *Macrocephalosaurus* (morphology only) to the sister to amphisbaenians (combined data), although the relevant branches are not strongly supported. Furthermore, *Polyglyphanodon* and *Macrocephalosaurus* are no longer

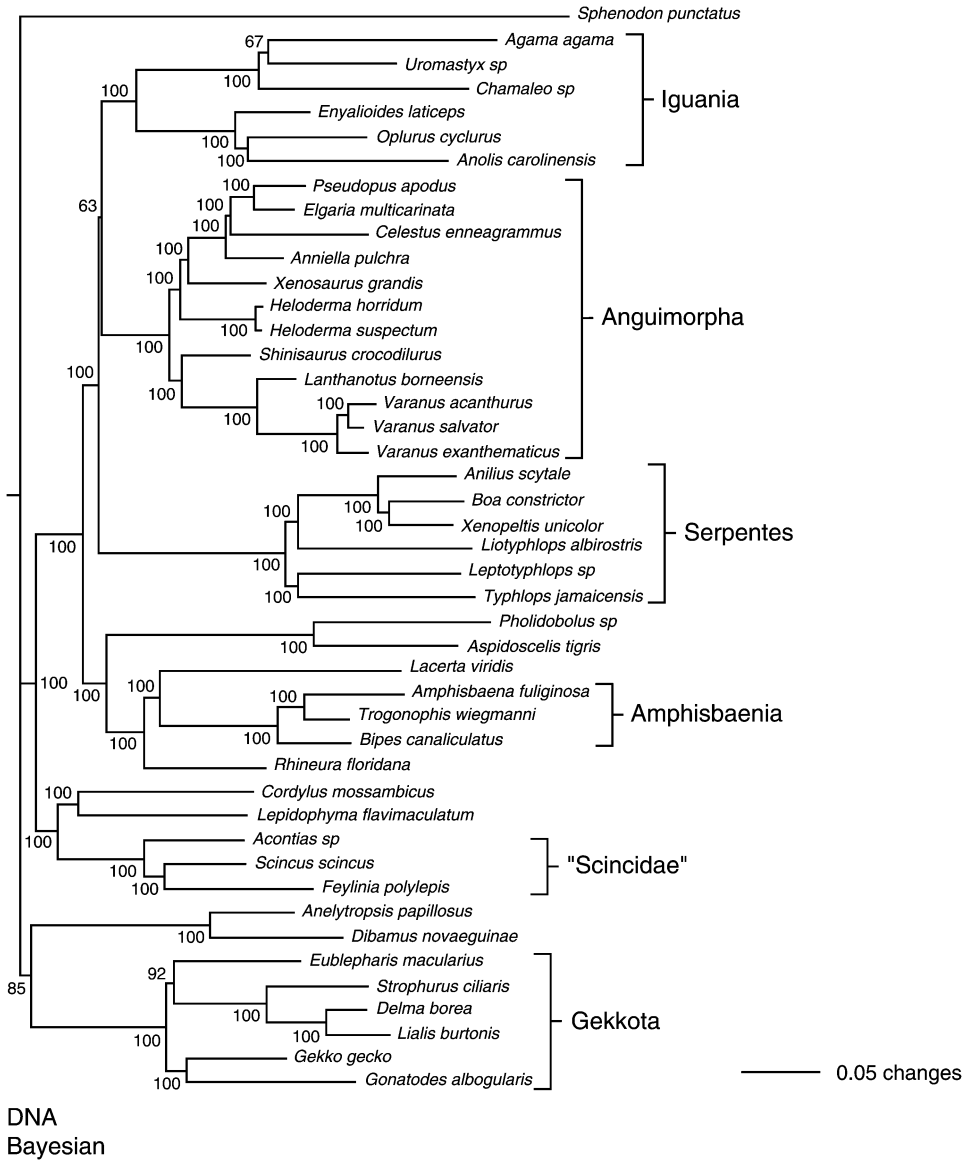

FIGURE 4. Phylogeny of squamate reptiles based on Bayesian analysis of molecular data only. Numbers adjacent to nodes are Pp values ≥0.50 (Pp are multiplied by 100 in figure).

placed as the sister group to Teiidae, but instead as the sister group to Teiidae + Gymnophthalmidae. Within Anguimorpha, molecular data help resolve the placement of fossil taxa unresolved by morphological data alone (e.g., *Eosaniwa*, Mosasauria). Furthermore, the molecular data lead to a shift in the position of the fossil anguid *Helodermoides*, from a clade with *Celestus* and *Elgaria* to the sister taxon of *Pseudopus*. Within snakes, the molecular data help resolve the placement of the fossil taxa *Dinilysia* and *Wonambi*. Intriguingly, addition of the morphological data also leads to a change in the tree for extant taxa, with monophyly of Amphisbaenia being strongly supported.

In the Bayesian analysis of the combined data for all taxa (Fig. 6), *Huehuecuetzpalli* undergoes a similar dramatic shift in placement after addition of the molecular data, from the base of the squamate tree (morphology only) to the sister group of Iguania. However, most branches separating this species from its basal position are not strongly supported (e.g., Pp = 0.74), and these same branches become strongly supported when the analysis is rerun with this taxon removed (not shown). The position of the fossil anguimorph *Eosaniwa* also changes when molecular data are added, from being weakly placed as the sister group of helodermatids, varanids, and mosasaurs (morphology only) to being strongly supported as the sister taxon of helodermatids alone (combined).

Nevertheless, the fossil taxa (as a group) are far less sensitive to the addition of molecular data in the Bayesian analysis. For example, relationships of the fossil taxa *Macrocephalosaurus, Polyglyphanodon*, and

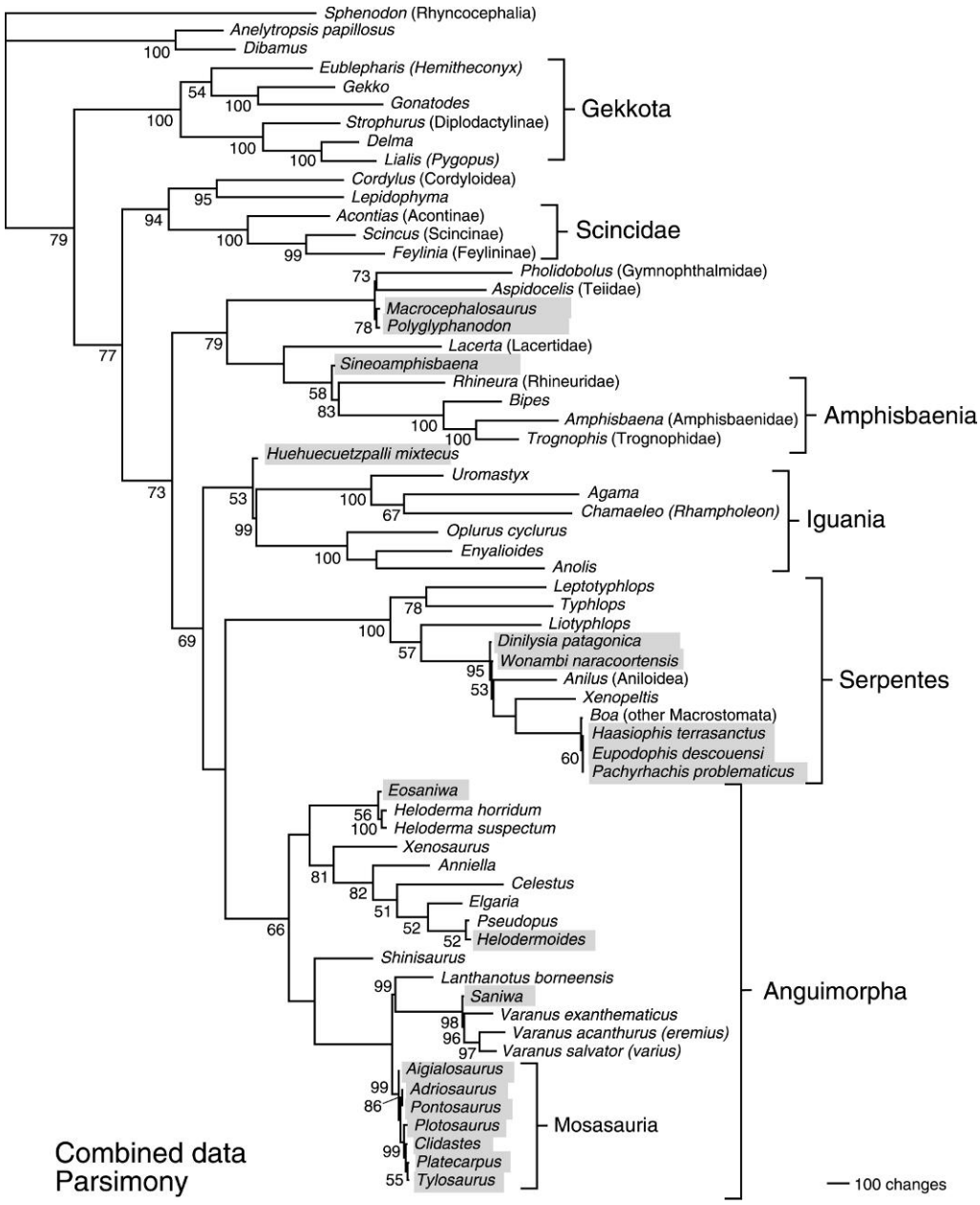

FIGURE 5. Phylogeny of squamate reptiles based on a parsimony analysis of the combined molecular and morphological data. The tree shown is 1 of 6 trees of length 31,522 steps. The tree was selected arbitrarily to illustrate the branch lengths. The only clades that are collapsed in a strict consensus of the 6 trees are those among some fossil snakes (*Eupodophis, Haasiophis, Pachyrhachis*) and some mosasaurs (i.e., the relationships between *Aigialosaurus*, *Adriosaurus* + *Pontosaurus*, and *Clidastes* + *Platecarpus* + *Plotosaurus* + *Tylosaurus*, and relationships within the latter clade); these collapsed clades also have very short branch lengths. Fossil taxa are indicated with gray shading. Numbers adjacent to nodes are bootstrap values ≥50%. Names in parentheses after some terminal taxa indicate the name for the corresponding terminal taxon used in the morphological data set of Conrad (2008) and Figs. 1 and 2.

Sineoamphisbaena change considerably after addition of molecular data in the parsimony analyses. In the Bayesian analyses, they are strongly supported as monophyletic and the sister group of teiids, both with and without molecular data. In summary (Fig. 7), only 7 clades are identical before and after the addition of the molecular data in the parsimony analyses, whereas 14 are identical in the Bayesian analyses.

For both parsimony and Bayesian analyses, we find that support values for the localized placement of the 19 fossil taxa do not significantly differ after addition of molecular data ($P > 0.10$ for both methods). For parsimony, the mean bs support for the fossil taxa is 60% with morphological data alone and 66% with molecular data added. In contrast, the mean Bayesian Pp is 0.84 with morphology alone and 0.80 with molecular data added.

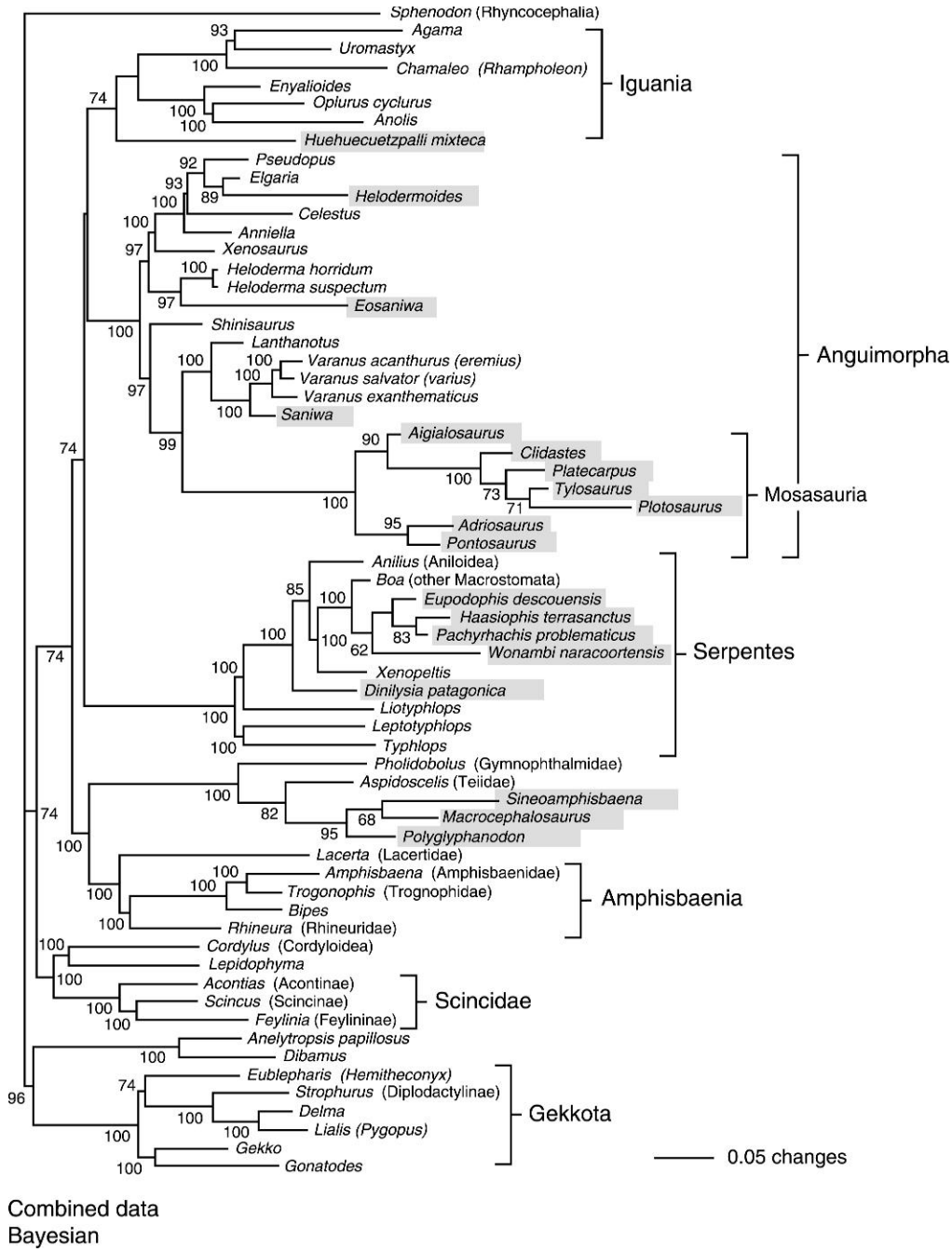

FIGURE 6. Phylogeny of squamate reptiles based on a Bayesian analysis of the combined molecular and morphological data. Fossil taxa are indicated with gray shading. Numbers adjacent to nodes are Pp values $\geq$0.50 (Pp are multiplied by 100 in figure). Names in parentheses after some terminal taxa indicate the name for the corresponding terminal taxon used in the morphological data set of Conrad (2008) and Figs. 1 and 2.

DISCUSSION

Integrating Molecules and Fossils

Our results from squamate reptiles have several intriguing implications for the integration of data from phylogenomics and fossils. First, we show instances in which addition of molecular data dramatically changes the placement of fossil taxa, especially in parsimony analyses (Fig. 7). For example, in both parsimony and Bayesian analyses, *Huehuecuetzpalli* shifts from the base of the tree (morphology alone) to a highly nested position as sister group of Iguania. This result demonstrates that it may be problematic to assume that fossil taxa can be placed within a molecular phylogeny based on their placement in analyses of morphological data alone. Yet, this assumption is standard practice in many studies, especially for estimation of divergence times using molecular clock–based methods with fossil calibration

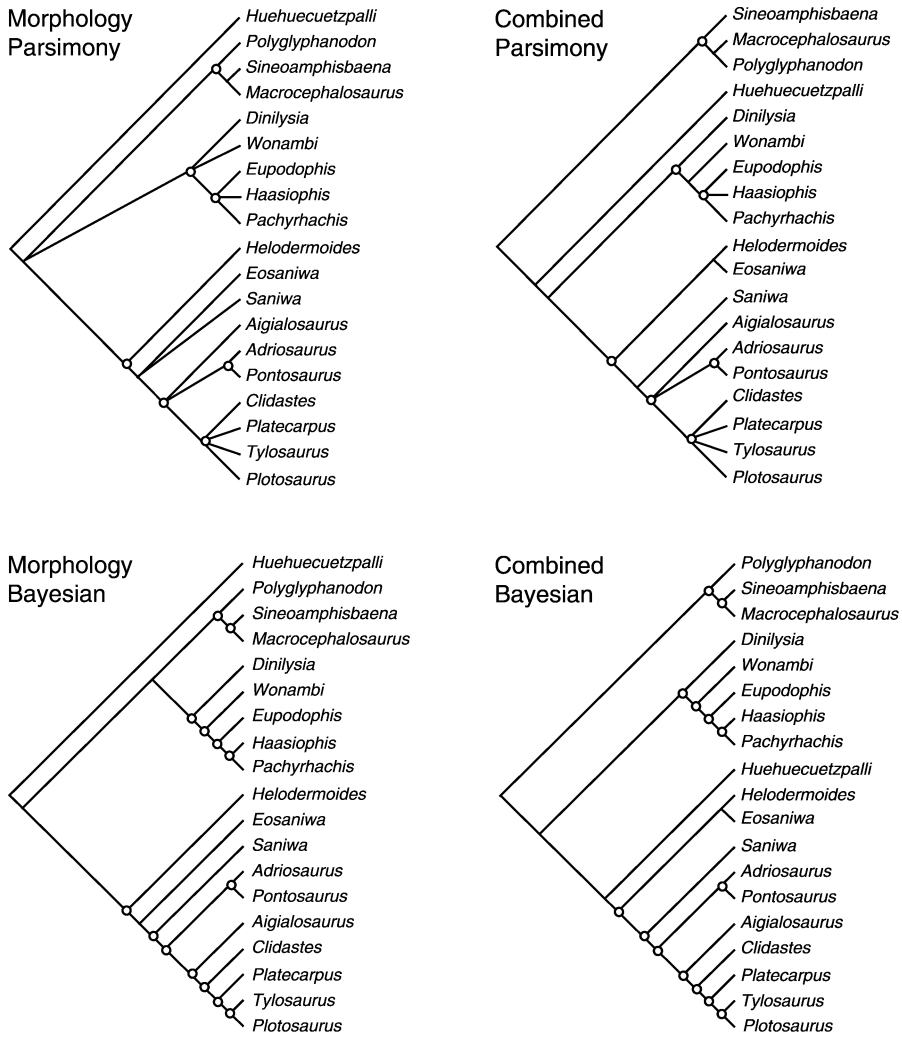

FIGURE 7. Phylogenies for the 19 fossil taxa alone, illustrating the effects of adding the molecular data on their estimated relationships. Open circles indicate clades that remain unchanged after the addition of the molecular data (for a given method).

points. This assumption may be especially problematic for groups like squamates and mammals in which molecular data suggest extensive phylogenetic rearrangements for living taxa.

The changes in the placement of fossil taxa are not simply caused by changes in the placement of the clades in which these taxa are nested. For example, some changes involve fossil taxa within Anguimorpha and are not solely caused by changing the position of Anguimorpha within squamates. Similarly, *Huehuecuetzpalli* is placed as sister to all other squamates by morphological data alone (see also Reynoso 1998) and so the change in its placement with the addition of molecular data is not an inevitable consequence of changing the position of Iguania within squamates.

Admittedly, for any empirical study, we cannot know if these changes in the position of fossil taxa caused by addition of molecular data actually make their placement more or less accurate (i.e., similar to the true phylogeny). However, simulations (Wiens 2009) suggest that molecular data can improve accuracy for fossil taxa under many conditions and almost never led to consistently worse estimates for the fossil taxa. Furthermore, given that the tree based on morphology alone contains aspects that may be incorrect (i.e., the burrowing clade of snakes, amphisbaenians, and dibamids; see below), we speculate that accuracy for the fossil taxa may be increased as well.

Second, we show that the addition of molecular data greatly improves resolution for fossil taxa in the parsimony analysis (Fig. 7). Many relationships are unresolved by morphology alone (Fig. 1), and the molecular data improve resolution for both living and fossil taxa (Figs. 5 and 7). This result suggests that molecular data can improve phylogeny estimation for fossil taxa and runs counter to the common practice of many studies focusing on the placement of fossil taxa, in which available molecular data for living taxa are excluded (e.g., Wible et al. 2007; Friedman 2008). Some previous studies have also found increased resolution when adding molecular data to analyses with fossil taxa (e.g., Shaffer et al. 1997; Asher and Hofreiter 2006; Rothwell and

Nixon 2006; Manos et al. 2007), although others have not (e.g., Gatesy et al. 2003; Asher et al. 2005; O'Leary and Gatesy 2008).

Third, we find that extensive missing data in the fossil taxa in the combined Bayesian analyses does not prevent these taxa from being placed in the expected clades with strong support (Fig. 6), despite claims that extensive missing data are generally problematic for Bayesian analyses (Lemmon et al. 2009; contra Wiens et al. 2005b; Wiens and Moen 2008). Instead, we find that fossil taxa with >98% missing data and >15,000 missing data cells each are generally placed with strong support in the groups expected by previous taxonomy in the combined Bayesian analyses (i.e., fossil snakes within snakes, fossil anguimorphs in Anguimorpha, with Pp = 1.00 for monophyly of both snakes and anguimorphs). Results for Bayesian analysis of the molecular data alone are similar; all species were placed with strong support in the clades suggested by previous taxonomy (Fig. 4), including those with the most missing data (e.g., *Anelytropsis* has 45% missing molecular data but is placed with the other dibamid, *Dibamus*, with Pp of 1.00; the scincid *Scincus* has 32% missing data and is placed with another scincid, *Feylinia*, with Pp of 1.00). Based on previous simulation studies (e.g., Wiens 2003; Wiens and Moen 2008), we expect that a high proportion of missing data in incomplete taxa will be problematic primarily when the overall number of characters in the analysis is small, and there are too few characters known for these taxa to accurately place them on the tree. In other words, the critical parameters are the quantity and quality of data that are present, not the amount or proportion of data that are absent.

Fourth, we find (disappointingly) that the molecular data do not significantly improve branch support for the localized placement of fossil taxa. Although there are specific instances where support improves considerably (e.g., *Eosaniwa*; Fig. 2 vs. Fig. 6), there is no significant trend. In our study, many fossil taxa have other fossil taxa as their closest relatives (e.g., mosasaurs), and there may be limited potential for molecular data to improve support in these cases. Even though addition of molecular data can change the placement of fossil taxa, the support for that placement may still depend on the morphological data.

Finally, although our main focus is the impact of molecular data on fossil taxa, we also show that addition of morphological data can change the placement of living taxa relative to analyses of molecular data alone. In our study, the molecular data alone (15,794 characters) show strong support for nonmonophyly of amphisbaenians (i.e., *Rhineura* outside amphisbaenians) in both parsimony and Bayesian analyses (bs = 98%; Pp = 1.00), possibly due to long-branch attraction. Yet, monophyly of amphisbaenians is strongly supported when the 363 morphological characters are added in both analyses (bs = 83%; Pp = 1.00). This result suggests the importance of combined analyses of molecular and morphological data for a comprehensive Tree of Life. An obvious alternative approach to integrating molecular and fossil data sets is to analyze the morphological data alone (with both living and fossil taxa), but to use the tree from molecular data to constrain relationships among living taxa (e.g., Manos et al. 2007). A potential disadvantage of this approach is that the molecular tree is assumed to be true and unchangeable, and there is no opportunity for the morphological data to contribute to estimating relationships among living taxa. Although the idea that the morphological data could influence relationships when they are so greatly outnumbered by the molecular data may seem far fetched, that is exactly what we find with amphisbaenians (see also Wiens 2005). Of course, we do not truly know if amphisbaenians are monophyletic, but this group has been recognized as such by morphologists for decades (e.g., Estes et al. 1988 and references therein) and has been supported in other molecular studies (e.g., Vidal and Hedges 2005). In a similar vein, simulations suggest that adding fossil taxa with morphological data alone can improve accuracy for relationships among living taxa (each having vastly greater numbers of molecular characters), apparently by subdividing long branches (Wiens 2005).

Misleading Phylogenetic Signal in the Morphological Data

Our results from squamate reptiles seem to provide a dramatic example of the problematic impacts of morphological convergence on phylogenetic analysis of morphology. Parsimony and Bayesian analyses of morphological data alone place snakes in a strongly supported clade with amphisbaenians and dibamids (Figs. 1 and 2), and in the Bayesian analyses, this clade is nested inside Scincidae with strong support (Scincophidia of Conrad 2008). This placement of snakes is strongly rejected by the molecular data and combined data analyses (and by traditional taxonomy), which leads us to suspect that this aspect of the morphological results may be incorrect.

The causes of this seemingly misleading signal are not clear but may be related to the burrowing habits of these taxa. Based on a molecular phylogeny and divergence time estimates, amphisbaenians, dibamids, and basal snakes represent the oldest lineages of burrowing squamates (Wiens et al. 2006). Our parsimony results show that the characters supporting this clade include loss of dermal skull bones (squamosal, supratemporal; characters 87 and 98), various modifications of the braincase (characters 133, 137, 140, 143, 144, 151, 154, and 155), and reduction of the eyes and related structures (characters 313–315). Many of these characters may be related to a subterranean lifestyle and use of the skull for burrowing. Furthermore, the subfamilies of skinks (Acontinae, Feyliniinae) that are placed closest to this snake–amphisbaenian–dibamid clade in the Bayesian morphological analysis are also burrowers (summary in Wiens et al. 2006).

Intriguingly, although amphisbaenians, dibamids, snakes, and acontine and feyliniine skinks all share an elongate snake-like body form with reduced limbs,

other taxa that have undergone body elongation and limb reduction are not placed in this clade by the morphological data alone. These other taxa include the pygopods (placed in Gekkota by molecular and morphological data) and the genus *Pseudopus* (placed in Anguidae by morphological and molecular data). These pygopods (*Delma, Pygopus*) and *Pseudopus* are notable in that they are surface dwellers rather than burrowers (reviewed in Wiens et al. 2006). Clearly, the dramatic morphological changes associated with body elongation and limb reduction are not sufficient to place species in this burrowing clade. But if one believes the results from the molecular and combined data, then homoplasy associated with the burrowing lifestyle seems to overwrite the true historical signal from nearly 200 myr of divergence across squamate phylogeny (e.g., Wiens et al. 2006) by placing the most basal lineages (i.e., dibamids) in a clade with one far from the root (i.e., snakes). However, one burrowing squamate (the anguid, *Anniella*) does not fall into this clade of burrowing taxa and instead is placed with a snake-like but surface-dwelling genus of the same family (*Pseudopus*). We also note that many snake species are not burrowers, although they retain many of the same morphological traits as basal snakes and other burrowing squamate clades (Pough et al. 2004; Vitt and Caldwell 2009).

Methodologically, these results are interesting in showing yet another example in which both parsimony and Bayesian analyses of morphological data seem to be misled with strong statistical support, at least for certain clades (see also Wiens et al. 2005a; Smith et al. 2007). Clearly, the application of Bayesian methods is no panacea for problems in morphological data analysis, particularly when suites of characters seem to change in tandem due to developmental or functional coupling. In fact, the Bayesian analysis seems to provide even stronger support for the unorthodox placement of snakes than does the parsimony analysis (e.g., the problematic placement of snakes within skinks is unresolved in the parsimony analysis but strongly supported by Bayesian analysis).

The Marine Origin of Snakes Reconsidered

A recent analysis (Lee 2009) addressed the possible marine origin of snakes and concluded that snakes are nested inside of a paraphyletic (and marine) Mosasauria, that basal snakes are marine, and that the snake–mosasaur clade is sister to Anguimorpha. Our results are based on a larger sampling of molecular and morphological characters and living and fossil taxa and clearly show that snakes are not nested inside of Anguimorpha or Mosasauria. Furthermore, Lee (2009) assumed that the marine fossil snakes *Haasiophis* and *Pachyrachis* are outside of a clade formed by other snakes (i.e., snakes exclusive of these two genera were treated as a single terminal taxon, such that no analysis can contradict this assumption). Our results show strong support for placing these genera within snakes above the burrowing scolecophidians and aniliids. Lee (2009) claimed that the "hypothesis for a marine origin of snakes is therefore consistent with the increasing molecular data bearing on this issue." However, our current results using more extensive molecular and morphological data do not support this hypothesis.

Conclusions

Our results show that molecular data can dramatically alter the placement of fossil taxa in the context of a combined analysis of morphological and molecular data. Along with recent simulations, these results suggest hope for reconstructing a complete Tree of Life that includes molecular and morphological data and fossil and living taxa, and in which the molecular data actively contribute to the accurate placement of fossil taxa for which molecular data are lacking. Our results also suggest the dangers of adaptive parallelism for phylogenetic analysis of morphology. In our study, parallel adaptations to a burrowing habitat in multiple lineages seem to erase the historical signal that has evolved over hundreds of millions of years and leads both parsimony and Bayesian methods to reconstruct an apparently incorrect clade with strong statistical support. This should be cause for some concern to paleontologists when reconstructing relationships among fossil taxa based on morphology alone. Conversely, we also show that morphological data can contribute to phylogeny estimation for living taxa in combined analyses, despite a staggering disparity in the number of characters in each data set. Finally, our results provide a well-resolved tree for most of higher level squamate phylogeny based on a greater sampling of molecular and morphological characters than in previous studies. This hypothesis will continue to be refined in the future with the addition of more living and fossil taxa and more genes.

Supplementary Material

Supplementary material can be found at http://www.sysbio.oxfordjournals.org/.

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