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Molecular evidence and marine snake origins

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A molecular phylogeny was used to refute the marine scenario for snake origins. Nuclear gene sequences suggested that snakes are not closely related to living varanid lizards, thus also apparently contradicting proposed relationships between snakes and marine mosasaurs (usually considered to be varanoids). However, mosasaurs share derived similarities with *both* snakes and living varanids. A reanalysis of the morphological data suggests that, if the relationships between living taxa are constrained to the proposed molecular tree, with fossil forms allowed to insert in their optimal positions within this framework, mosasaurs cluster with snakes rather than with varanids. Combined morphological and molecular analyses also still unite marine lizards with snakes. Thus, the molecular data do not refute the phylogenetic evidence for a marine origin of snakes.

Keywords: phylogeny; molecular systematics; evolution; mosasaurs; lizards; snakes

1. INTRODUCTION

Two contrasting scenarios have been commonly proposed regarding the origin of snakes. The terrestrial, burrowing theory was based on the unusual structure of snake eyes (e.g. Walls 1942; Underwood 1970) and was further supported by primitive position of fossorial blindsnakes among living snakes. By contrast, the similarities between snakes and extinct marine lizards such as mosasaurs and dolichosaurs led to the suggestion that they had marine ancestors (see Lee & Caldwell (2000) for review). This has been supported by some recent phylogenetic analyses that have found marine lizards to be the closest relatives of snakes (e.g. Lee & Caldwell 2000; Lee 2005; but see Rieppel & Zaher 2000), and recent descriptions of fossil marine snakes with limbs (Caldwell & Lee 1997; Rage & Escuillié 2000; but see Tchernov *et al.* 2000).

Recently, Vidal & Hedges (2004; see also Townsend *et al.* 2004) used two nuclear genes to elucidate squamate phylogeny. Although few higher-level ('suprafamilial') groupings of squamates were robustly resolved (see figure 1*d*), the data strongly united living varanids with other anguimorph lizards, rather than with snakes. If the marine mosasaurs and dolichosaurs are most closely related to living varanids, they too must be excluded from close relationships with snakes, thus undermining much of the phylogenetic evidence for the marine hypothesis. While such an interpret-

ation is tempting, other possibilities need to be considered. First, mosasaurs and dolichosaurs share anatomical similarities both with living varanids (e.g. Russell 1967; Carroll & DeBraga 1992) and snakes (e.g. Caldwell & Lee 1997), causing all three to cluster together in some cladistic analyses (e.g. Lee & Caldwell 2000). If snakes and living varanids are widely separated in the new molecular phylogeny, mosasaurs and dolichosaurs might either group with living varanids (as suggested by Vidal & Hedges 2004) or could instead group with snakes. The latter possibility was discounted by Vidal and Hedges based on the published literature, which usually suggested close relationships between marine lizards and varanids. However, it has been argued that mosasaurs and dolichosaurs exhibit traits indicating they are not closely related to living varanids (e.g. Caldwell 1997). Furthermore, the phylogeny (varanids (marine lizards, snakes)) found in some recent studies (e.g. Lee & Caldwell 2000) indicates that derived traits shared between marine lizards and varanids should also be found in snakes. In addition to the traits found in all three taxa, marine lizards and snakes must share additional synapomorphies. Homoplasy might weaken this pattern: nevertheless, if the molecular phylogeny is accepted and varanids and snakes are assumed to be widely separated, marine lizards could be expected to cluster more strongly with snakes than with varanids. This possibility is investigated here.

2. METHODS AND RESULTS

The first set of analyses evaluated the position of mosasaurs, dolichosaurs and other marine lizards in the context of the new molecular results. The morphological data (Lee 2005; see Electronic Appendix) is the osteological data of Lee & Caldwell (2000), modified as suggested by Rieppel & Zaher (2000; see §3), comprising 248 characters for 33 terminal taxa. All multistate characters were unordered to counter a previously suggested bias (Lee & Caldwell 2000; Rieppel & Zaher 2000) of ordered characters towards the marine hypothesis. Parsimony searches using PAUP* (Swofford 2000) employed 100 random additions and default heuristic search settings, bootstrap values are based on 1000 replicates, and branch support and partitioned branch support (PBS; see Gatesy *et al.* 1999) values were calculated using TREEROT v.2a (Sorenson 1999). The unconstrained tree for the morphological data is shown in figure 1*a*; there is strong support for the section of the tree that places marine lizards (mosasaurs, aigialosaurs, dolichosaurs and *Adriosaurus*) and limbed marine snakes (the pachyophiids *Pachyrhachis* and *Haasiophis*) as a paraphyletic assemblage on the snake stem.

A resolved molecular tree for 19 out of the 33 terminal taxa in the morphological dataset was presented in Vidal & Hedges (2004; see figure 1*d* here). The morphological dataset was thus analysed (using the 'backbone constraints' PAUP* command) with the 19 sequenced taxa constrained to the molecular tree and the other taxa left unconstrained and allowed to insert into their optimal positions. The two optimal trees (figure 1*b*) represent the best trees for the morphological data that are totally consistent with the molecular backbone: each tree was 753 steps long and placed the marine lizards and pachyophiids as a paraphyletic assemblage on the snake stem. The analysis was repeated with the same molecular backbone constraint plus the further constraints that mosasaurs and living varanids had to form a clade, and pachyophiids had to be the sister group of snakes, as suggested by Vidal & Hedges (2004; see their fig. 2 and figure 1*d* here). The single tree from this analysis (figure 1*c*) was 780 steps long and significantly worse than the two previous trees (one-tailed Templeton test $p < 0.01$). Thus, if the molecular tree for certain living forms is assumed to be fully correct, and the remaining taxa are unconstrained, mosasaurs and other marine lizards still emerge near snakes. This arrangement is significantly supported over their suggested alternative position near varanids.
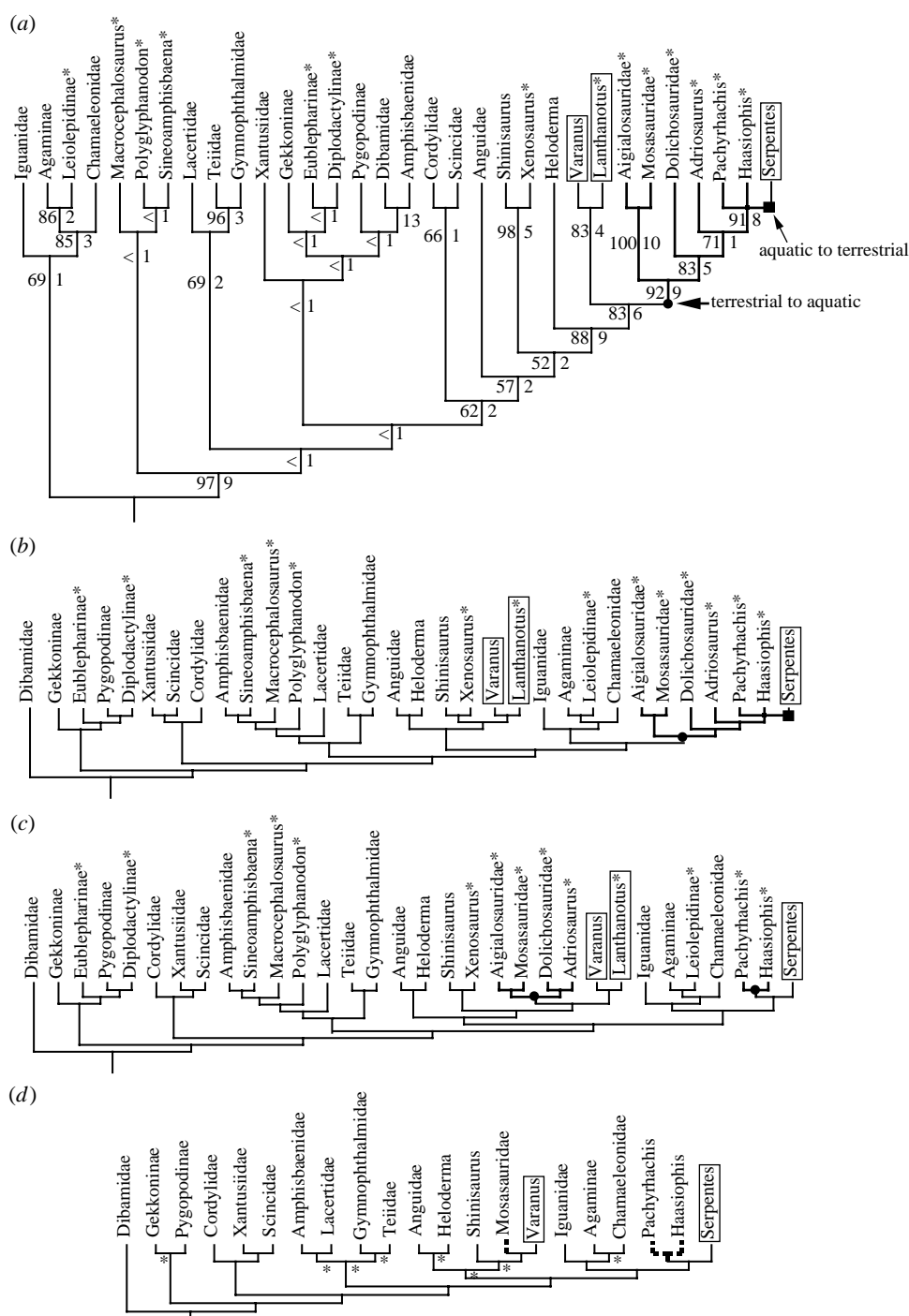
Figure 1. (a) The strict consensus of the two most parsimonous trees (634 steps) from the morphological data (Electronic Appendix, item 1), with bootstrap frequencies ('<' denotes less than 50%) to the left of each branch, and branch (Bremer) support to the right. Marine forms in bold, varanids and snakes in boxes. (b) The consensus of two trees (each 753 steps) that results if the morphological data are analysed with relationships between certain living taxa constrained to the topology suggested by molecular data (d) and other taxa (asterisks) are unconstrained; note that marine lizards and pachyophiids both group with snakes. (c) The tree (780 steps) that results if the morphological data are analysed with the molecular backbone constraints in (b); mosasaurs are additionally constrained to group with varanids, and pachyophiids with snakes (d). (d) The relationships between major lineages of living squamates, as suggested by molecular data (Vidal & Hedges 2004). Asterisks denote strongly corroborated clades (greater than 70% bootstrap), and the hypothesized position of mosasaurs and pachyophiids (Vidal & Hedges 2004) is also indicated. The fully resolved tree, and the tree retaining only strongly corroborated nodes (asterisks), were used as backbone constraints as discussed in the main text.

The above analyses were repeated with the molecular 'backbone constraint' tree modified by collapsing all nodes, except the eight with greater than 70% bootstrap (see asterisks in figure 1d). Thus, only strongly supported molecular nodes were enforced, and weaker nodes (less than 70% bootstrap) were allowed to be overturned by morphological data. The best morphological trees consistent with this conservative molecular backbone again united marine lizards and pachyophiids with snakes (2 trees, each 711 steps). The optimal trees that satisfied this conservative molecular backbone and also placed marine lizards with varanids, and pachyophiids with snakes (*fide* Vidal & Hedges 2004) were 728 steps long (8 trees). Six of these were significantly worse than
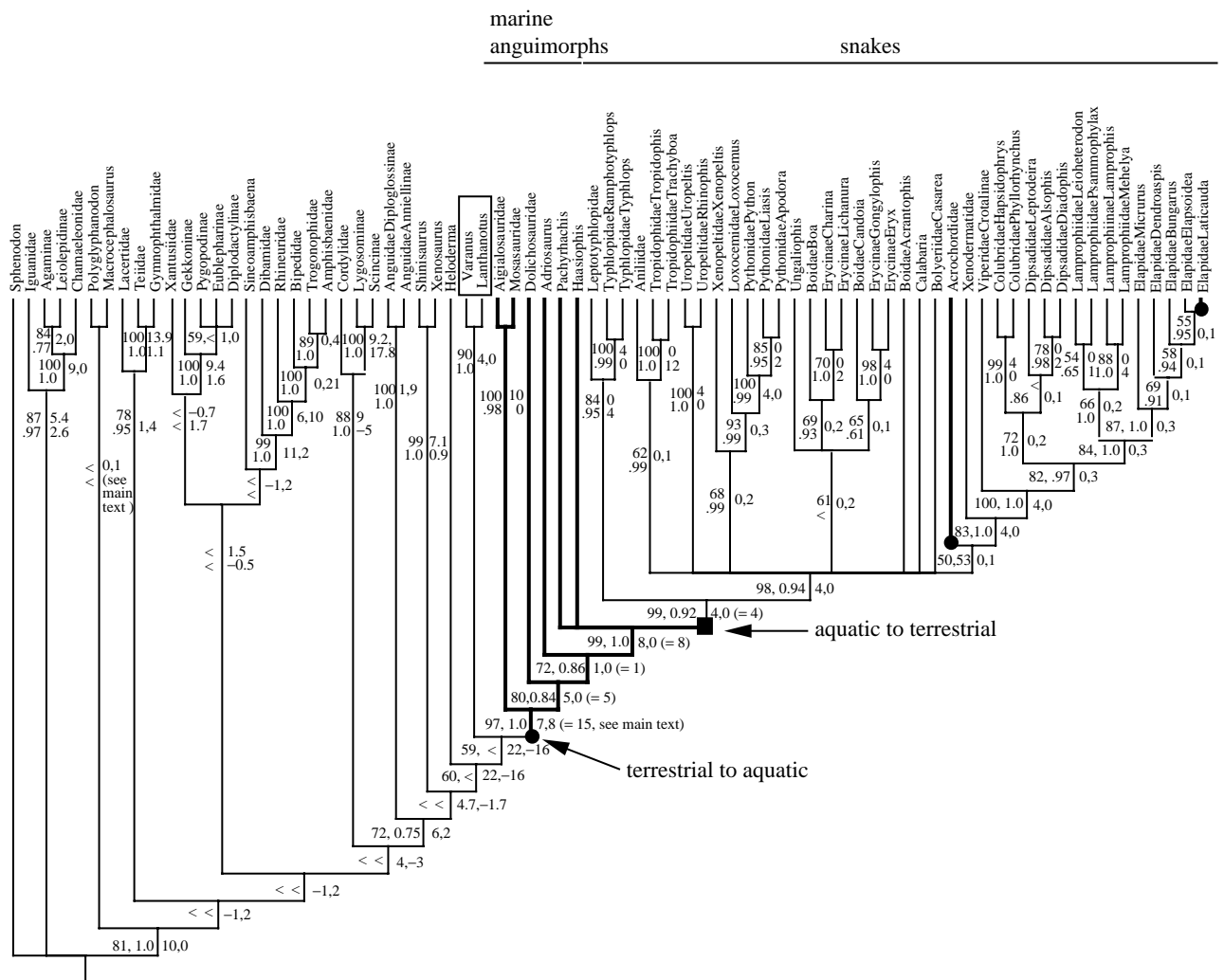
Figure 2. The strict consensus of the 80 most parsimonious trees (2871 steps) that result from the combined morphological and molecular data (see Electronic Appendix, item 2). On the left of each branch are parsimony bootstrap frequencies and Bayesian posterior probabilities. '<' denotes less than 50% bootstrap or 0.5 posterior probability. Partitioned branch support for morphology and molecules is shown on the right of each branch; the sum of these figures is the overall branch or Bremer support. Marine lizards and pachyophiids form a paraphyletic assemblage basal to snakes, implying a marine phase in snake origins. Marine forms in bold, varanids in boxes.

the optimal trees (one-tailed Templeton test $p < 0.05$; p between 0.05 and 0.1 for the remaining two).

In addition to forcing the morphological data to conform to a rigid molecular backbone (see above), the two datasets were also analysed together. The morphological data matrix described above was combined with the molecular alignment of Vidal & Hedges (2004). The combined data matrix can be found in the Electronic Appendix. Parsimony analyses employed searches with at least 50 random additions, and 200 bootstrap replicates. Bayesian analyses (using MRBAYES 3; Ronquist & Huelsenbeck 2003) employed the default 'standard' model for morphology and the likelihood models for each gene identified by Vidal & Hedges (2004). Three cold and one heated Markov Chain Monte Carlo chains for each of 5 million generations were run, sampling every 50 generations after stationarity was reached (beyond the first 10 000 sampled trees).

The combined parsimony analysis resulted in a topology largely consistent with the morphological data alone (figure 2), and PBS values revealed that the morphological signal (although weak) is still stronger than the molecular signal. The PBS values further reveal that the molecular data contain hidden support (Gatesy *et al.* 1999) for a snake–marine lizard clade. The molecular PBS for this clade is +8, indicating that enforcing a constraint that splits marine lizards and snakes results in a new set of relationships between extant taxa that is molecularly less parsimonious. A similar phenomenon results in positive molecular PBS for the (*Polyglyphanodon* + *Macrocephalosaurus*) clade. The Bayesian analysis resulted in a similar topology; most nodes with high parsimony bootstraps also

appeared on the Bayesian tree with high posterior probabilities (figure 2). In both combined analyses, marine lizards again formed a paraphyletic assemblage between anguimorphs and snakes.

3. CONCLUSIONS

Mosasaurs have been hypothesized to be related to living varanid lizards and/or snakes. A recent molecular phylogeny (Vidal & Hedges 2004) demonstrated that living varanids and snakes are only distantly related, and assumed that because mosasaurs and other marine lizards must be related to varanids, they cannot be related to snakes. However, evaluation of the morphological data suggests instead that even if living varanids and snakes are assumed to be widely separated (as per the molecular data), mosasaurs cluster with snakes. Thus, while the molecular data indicate distant relationships between varanids and snakes, this result does not yet refute affinities between mosasaurs and snakes. Furthermore, in a combined analysis, the morphological signal prevails over the molecular. These results do not demonstrate

that the morphological signal is correct. However, they demonstrate that the molecular signal, then based on less than 1 kb of data (but see Townsend *et al.* 2004), was relatively weak for large sections of the tree (figure 1*d*). Furthermore, if a combined approach is adopted (contentious given the extensive morphological–molecular conflict revealed in the PBS values), significantly more molecular data will be needed to robustly overturn (i.e. refute) the morphological results. All these results highlight the need to explicitly incorporate fossils in phylogenetic analyses (Gauthier *et al.* 1988), instead of making assumptions about their position based on published literature (especially when this literature contains disagreements; e.g. Carroll & DeBraga 1992; Caldwell 1997).

Finally, it should be acknowledged that certain characters used in this morphological dataset are contentious. While an attempt has been made to incorporate recent critiques (e.g. Rieppel & Zaher 2000), there will be ongoing debate about certain characters and the resultant trees, in particular the putative characters uniting varanids, mosasaurs and snakes (see Kluge 2003; Lee 2005). However, this issue is outside the scope of the present study. The results of any morphological analysis could be refuted if the primary character codings are reinterpreted. The aim here is to evaluate a very different claim (Vidal & Hedges 2004): that new molecular data *alone* refute hypothesized relationships between snakes and marine lizards. For the comprehensive morphological dataset considered here, this is not the case. Marine lizards still group robustly with snakes in the face of the new molecular data, an arrangement consistent with an aquatic origin of snakes.

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