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A PHYLOGENETIC HOT SPOT FOR EVOLUTIONARY NOVELTY IN MIDDLE AMERICAN TREEFROGS

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Among the various types of evolutionary changes in morphology, the origin of novel structures may be the most rare and intriguing. Here we show statistically that the origins of different novel structures may be correlated and phylogenetically clustered into "hot spots" of evolutionary novelty, in a case study involving skull elements in treefrogs. We reconstruct phylogenetic relationships within a clade of Middle American treefrogs based on data from 10 nuclear and four mitochondrial genes and then analyze morphological evolution across this tree. New cranial elements are rare among anurans and tetrapods in general, but three novel elements have evolved within this clade, with a 40% increase in the number of skull roof elements in some species. Two of these elements also evolved in a related clade of treefrogs, and these two novel elements may have each evolved repeatedly within one or both clades. The molecular phylogeny suggests striking homoplasy in cranial morphology and shows that parsimony and Bayesian analyses of the morphological data have produced misleading results with strong statistical support. The origins of the novel elements are associated with an overall increase in the ossification of dermal skull roof elements (suggesting peramorphosis) and with the evolution of a novel adaptive behavior. Our study may be the first to statistically document significant phylogenetic clustering and correlation in the origins of novel structures, and to demonstrate the strongly misleading effects of peramorphosis on phylogenetic analysis.

KEY WORDS: **Amphibians, heterochrony, homoplasy, novelty, phylogeny, skull morphology.**

Evolutionary changes in morphology typically fall into one of several classes (Carroll et al. 2005; Futuyma 2005). These classes include changes in size or shape of pre-existing structures, changes in the number of serially homologous features (e.g., limbs, vertebrae), and individualization and diversification of structures within a set of serially homologous features. Perhaps the most intriguing type of change is the origin of entirely novel structures, those that are not simply modifications of pre-existing structures.

In some ways, the evolution of such novelties (sensu Müller and Wagner 1991) is difficult to explain, in that there is no obvious variation in these traits upon which selection can act prior to their appearance (unlike variation in the shape or size of a pre-existing structure; Müller and Wagner 1991). Many well-known higher taxa are defined by the origin of novel structures (e.g., the shell of turtles), but it remains an open question whether such novelties are common or rare and whether they are randomly scattered across the Tree of Life or clustered in particular clades. Given that novel structures are invisible to natural selection before they arise (Müller and Wagner 1991), one might expect them to generally be rare and phylogenetically scattered and that different novel structures would arise independently of each other. Alternately, there may be "hot spots" or phylogenetic

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clusters of the origins of novel structures among taxa in some cases.

We define phylogenetic hot spots of novelty as clades within which: (1) one or more novel structures originate repeatedly among species that are more closely related to each other than expected from a random selection of species within the group (i.e., multiple origins of a single trait are clustered among species; see also Sanderson 1991), and/or (2) origins of different novel structures are correlated with each other among taxa (i.e., origins of different novel structures are clustered together in particular species or clades). These hot spots may be associated with environmental changes (Jablonski 2005), developmental changes (e.g., overcoming an ancestral constraint; Müller and Wagner 1991), key innovations, and/or particular points in the history of life (e.g., the Cambrian explosion). However, despite considerable interest in evolutionary novelty and innovation (e.g., Müller and Newman 2005) and many potential examples, such clusters have not been explicitly documented in a statistical phylogenetic context. In this paper, we document a phylogenetic hot spot for the origin of novel cranial elements in a clade of treefrogs (Hylidae).

Novel cranial elements are uncommon in tetrapods in general and anurans in particular. Most tetrapods share the same elements that were present in the earliest tetrapod lineages, and many tetrapod clades are diagnosed by losses or modifications of these elements (Carroll 1988; Hanken and Hall 1993). Relatively few clades are diagnosed by the origin of entirely new skull bones. In anurans, a basic set of 14 ossified elements is present in the skull of most species (exclusive of the mandible and hyoid apparatus) and almost all elements clearly are homologous with those in the skulls of early tetrapods (for a review of anuran cranial morphology see Duellman and Trueb 1986; Trueb 1993). Some elements are lost in various anuran lineages (i.e., quadratojugal, neopalatine, vomer, columella), especially those lineages in which there is an overall decrease in cranial ossification and body size (e.g., Yeh 2002). Yet, the origin of new elements beyond this basic set is rare (Duellman and Trueb 1986; Trueb 1993). Many of the origins of novel elements within anurans are confined to an informal group within the treefrog family (Hylidae) called the "casque-headed" hylids (Trueb 1970; Duellman and Trueb 1986; Trueb 1993).

Detailed anatomical work by Trueb (1970) revealed the presence of three novel cranial elements (dermal sphenethmoid, internasal, prenasal) among two genera of Middle American casque-headed hylids (*Pternohyla, Triprion*). The dermal sphenethmoid is a distinct dermal element that overlays the endochondral sphenethmoid (an ossification associated with the anterior braincase, which occurs in all anurans), just anterior to the frontoparietal elements. The prenasal is a well-developed, unpaired dermal element anterior to the premaxilla that articulates with the maxillae and nasals. The internasal is a small dermal element anterior and dorsal to the premaxillae. Trueb (1970) also found that two of these novel elements (dermal sphenethmoid and prenasal) also occur in South American casque-headed hylids, including the genera *Aparasphenodon*, *Corythomantis*, *Osteocephalus*, *Osteopilus*, and *Trachycephalus*. However, her study predated the development of modern methods for the analysis of phylogeny and character evolution.

In this paper, we develop a multilocus molecular phylogeny for the Middle American casque-headed hylids and use this phylogeny to analyze the origins of novel structures and their potential morphological and functional correlates (including hyperossification, adaptive behavior, and body size). We contrast our phylogenetic results with those of a recent morphological analysis of this clade (Duellman 2001). We also extend our analyses of morphological evolution across hylids to include the South American casque-headed species. Our study may be the first to statistically demonstrate phylogenetically clustered hot spots for the origins of novel structures among extant taxa. Our results also show the potential for phylogenetic analyses of morphology to be strongly misled by heterochronic processes that may underlie these hot spots.

Materials and Methods

PHYLOGENETIC ANALYSIS OF MOLECULAR DATA

Recent phylogenetic analyses of hylid frogs suggest that the genera *Anotheca* (one species), *Pternohyla* (two species; but synonymized with *Smilisca* in many recent taxonomies), *Smilisca* (six species), and *Triprion* (two species) form a clade (*Smilisca* clade hereafter), that this clade is closely related to the genera *Isthmohyla* and *Tlalocohyla*, and that *Hyla* is a more distant outgroup (Faivovich et al. 2005; Smith et al. 2005, 2007; Wiens et al. 2005a, 2006). However, these previous studies have not included all the relevant species (e.g., Faivovich et al. 2005; Smith et al. 2005; Wiens et al. 2005a, 2006) or else found only weak support for relationships among the casque-headed species (Smith et al. 2007). None have examined morphological character evolution in a phylogenetic context.

We included 10 of 11 species in the *Smilisca* clade (neither tissues nor DNA of the rare *Pternohyla dentata* were available) along with outgroup taxa belonging to *Hyla* (one species), *Isthmohyla* (four species), and *Tlalocohyla* (three species). In general, we sequenced a single individual per species. However, we sequenced additional individuals for both species of *Triprion* and for *Anotheca spinosa* for several genes, given the surprising relationships found among these species (see Results). Furthermore, we also combined our data with sequences from the literature (e.g., Faivovich et al. 2005) for several species. These analyses showed that conspecific individuals do cluster together, and supported the use of a single individual to represent each species (results not shown).

For each of the 18 sampled species, we sequenced up to 10 nuclear genes (beta-crystalin [Cry-b], exons 2 and 3 of cellular myelocytomatosis [c-myc], exon 2 of sodium-calcium exchanger 1 [NACX], proopiomelanocortin A [POMC], prostaglandin E receptor 4 [PTGER], protein tyrosine phosphatase, nonreceptor type 12 [PTPN], recombinase activating gene 1 [RAG-1], rhodopsin [Rho], sevenin absentia [SIA], tensin 3 [TNS3]) and four mitochondrial genes, including some adjacent tRNAs (mitochondrial ribosomal small subunit [12S], large subunit [16S], cytochrome *b*, NADH dehydrogenase subunit I [ND1]). Primers are given in Appendix S1 (see online Supplementary Material), and basic properties of these genes are described in Table 1. In a few cases, we were unable to amplify a given gene for a given species despite repeated attempts, and these taxa were treated as having missing data in the combined analysis. Some sequences were previously published and all GenBank numbers are listed in Appendix S2 (see online Supplementary Material). Specimen localities and voucher numbers are given in Appendix S3 (see online Supplementary Material). We used standard methods of DNA extraction and PCR amplification, and purified PCR products were sequenced using an ABI 3100 automated sequencer. Sequences were aligned following Wiens et al. (2005a) using Clustal X 1.8.1 (Thompson et al. 1994).

Our primary estimate of phylogeny was a partitioned Bayesian analysis of the combined data. However, we performed separate parsimony and Bayesian analyses of each gene to identify potential contamination (and other problems). For each gene, we used MrModeltest version 2.0 (Nylander 2004) to identify the best-fitting model of sequence evolution (using hierarchical likelihood ratio tests and the Akaike information criterion). We then used comparison of Bayes factors to determine whether additional partitions (stems and loops, different codon positions) were supported within each gene (Nylander et al. 2004; Brandley et al. 2005; Wiens et al. 2005a). We also performed parsimony and Bayesian analyses of the combined nuclear genes and the combined mitochondrial genes to identify possible cytonuclear genealogical discordance. Bayes factors were also used to evaluate whether partitions were supported between genes in combined analyses. Each Bayesian analysis used two replicate searches of 2.0×10^6 generations each, with default priors. Bayesian analyses were implemented using MrBayes version 3.1.2 (Huelsenbeck and Ronquist 2001). Clades with posterior probabilities ≥ 0.95 were considered strongly supported (e.g., Wilcox et al. 2002; Alfaro et al. 2003; Huelsenbeck and Rannala 2004). Parsimony analyses were performed using PAUP* version 4.0b10 (Swofford 2002), using heuristic searches with 100 random taxon addition sequence replicates. Nonparametric bootstrapping was used to evaluate clade support (Felsenstein 1985) and clades with bootstrap values $\geq$70% were considered strongly supported (Hillis and Bull 1993).

PHYLOGENETIC ANALYSIS OF MORPHOLOGY

For comparison to the molecular results, we re-analyzed the morphological data matrix of Duellman (2001) for the *Smilisca* clade and outgroups using parsimony and Bayesian methods. We also used these data to evaluate character evolution within the group.

Table 1. Summary of genes used in phylogenetic analyses.

Gene	Total characters	Variable characters	Parsimony-informative characters	Likelihood model
Mitochondrial				
12S	892	285	184	GTR+I+Γ
16S	1280	421	254	GTR+I+Γ
cytochrome *b*	385	161	139	HKY+I+Γ
ND1	1082	540	422	HKY+I+Γ
Nuclear				
c-myc	787	93	30	HKY+I+Γ
Cry-b	314	99	29	HKY+Γ
NACX	1326	169	61	GTR+I+Γ
POMC	508	123	71	GTR+I+Γ
PTGER	466	44	27	GTR+I
PTPN	823	142	41	HKY+Γ
RAG-1	993	220	147	GTR+I+Γ
Rho	316	51	24	HKY+Γ
SIA	388	53	24	K80+Γ
TNS3	579	111	33	HKY+Γ

Duellman's (2001) matrix did not include *Anotheca spinosa*, but data were obtained from Duellman (2001) and Wiens et al. (2005a) and added to the data matrix (Appendix S4; see online Supplementary Material). Parsimony and Bayesian analyses followed the methods described above, but Bayesian analyses used the model of Lewis (2001) with a parameter for rate variation among characters (Mk + Γ). We also performed parsimony and Bayesian analyses of the combined molecular and morphological data, using either equal weighting of all molecular and morphological characters (parsimony) or a separate partition for the morphological characters (Bayesian analysis).

RECONSTRUCTING MORPHOLOGICAL CHARACTER EVOLUTION

The evolution of individual morphological characters was reconstructed using parsimony (with MacClade version 4.0; Maddison and Maddison 2000), and maximum likelihood (using Mesquite, version 1.05; Maddison and Maddison 2004) using the topology and branch lengths from the Bayesian analysis of the combined molecular data. Maximum likelihood reconstructions used a single estimated rate for gains and losses of each trait. Note that for brevity and simplicity we only illustrate a single parsimony reconstruction and merely comment on alternate reconstructions and the likelihood results. Importantly, our statistical analyses of clustering and trait correlation are not dependent on any particular parsimony or likelihood reconstruction.

ANALYSES OF TRAIT CORRELATION AND CLUSTERING

To test for the phylogenetic clustering and correlation of the origin of novel cranial elements across hylids, we expanded our taxon sampling to include additional hylids for which both phylogenetic and morphological data were available, including many representatives of the South American casque-headed hylids (all of which belong to the tribe Lophiohylini; Faivovich et al. 2005). Wiens et al. (2006) estimated a phylogeny for almost all hylid genera using combined nuclear and mitochondrial data, with branch lengths based on estimated divergence dates (using penalized likelihood; Sanderson 2002). However, taxon sampling within the *Smilisca* clade was not extensive in that study. We therefore combined the phylogeny and branch lengths from that study with those of the present study by using time as a common currency. Thus, we estimated a chronogram for the *Smilisca* clade based on our new molecular phylogeny for the group and then added this chronogram to that of Wiens et al. (2006; using 100 million years ago as the root age for Neobatrachia). We first used the age of the root of the *Smilisca* clade estimated by Wiens et al. (2006) as the root age for a new penalized likelihood analysis of the *Smilisca* clade, using methods for penalized likelihood analysis described by Smith et al. (2005). The resulting chronogram for the *Smilisca* clade was then manually added to the chronogram of Wiens et al. (2006) to create a phylogeny with comparable branch lengths for all 70 hylid taxa (Appendix S5; see online Supplementary Material).

Note that the casque-headed hylids are the only hylids reported to have the novel cranial elements (e.g., Trueb 1970; Duellman and Trueb 1986; Duellman 2001); other hylid taxa might have been included in theory (although we lacked morphological data for many), but most would be of limited relevance to our analyses of the evolution of novel elements. Adding many taxa that lacked these novel cranial elements would presumably have very little impact on our analyses of the clustering or correlation of these structures, especially given that all major clades of hylids are already represented. However, we do note that there are scattered reports of novel cranial elements in some nonhylid anurans, such as paired prenasal bones in bufonids (Pramuk 2000).

Morphological data (Appendix S6, see online Supplementary Material) were obtained primarily from Wiens et al. (2005a), supplemented with data from Trueb (1970), Trueb and Tyler (1974), Duellman (1974, 2001). Given the similar anatomical position and morphology of the prenasal and internasal (particularly when the prenasal first develops; Trueb 1970), most analyses were run treating the prenasal and internasal as the same trait. However, results were generally similar when treating these elements as distinct (results not shown).

We tested whether the origins of novel cranial elements are correlated with each other and whether they are phylogenetically clustered across the tree using the chronogram for 70 taxa. We tested for correlated evolution of different novel elements using the likelihood method of Pagel (1994) implemented in Discrete, version 4.0 (Pagel 1998). For each pair of characters tested, we estimated the log-likelihood for the model of evolution in which the characters evolve independently and that in which they evolve dependently and compared the likelihoods using the likelihood-ratio test statistic. Based on simulations (Pagel 1998), the test statistic generally follows a chi-squared distribution with four degrees of freedom.

To test for the phylogenetic clustering of the repeated origins of novel structures, we first scored each species in the combined-taxon chronogram for the presence of any novel cranial element. We then tested whether the mean phylogenetic distance (Webb et al. 2002) among taxa with novel cranial elements was less than expected by chance (indicating significant clustering) by randomly permuting the observed states among taxa 999 times and calculating the phylogenetic distance for each replicate, using Phylocom, version 3.40 (Webb et al. 2006). We tested whether origins of novel elements are clustered in hylids overall by including all 70 taxa. We then tested whether the origins in the *Smilisca* clade formed a significant cluster by deleting all Lophiohylini and repeating the analyses, and then tested Lophiohylini by deleting the *Smilisca* clade. Analyses using the mean-nearest-phylogenetic-neighbor distance gave generally similar results, as did analyses

using equal branch lengths, and these results are not reported. The analyses were then repeated for each of the novel elements separately. Although some taxa may share the same novel element because they share a common ancestor, the issue of nonindependence among taxa should be obviated by the randomization, and analyses deleting potentially redundant taxa (i.e., those not representing separate origins) gave similar results.

We hypothesize that novel cranial elements might arise as a by-product of an overall increase in skull roof ossification and might become fixed if they have some adaptive value. Previous studies (Trueb 1970, 1993; Duellman 2001; Jared et al. 2005) suggest that evolution of novel skull elements may be associated with an overall increase in dermal skull roof ossification (for which we use the shorthand expression "hyperossification" hereafter) and an unusual behavior called "phragmosis." Phragmosis involves use of the head to "plug" a burrow in which the animal is hiding, for defense and/or to reduce water loss (Trueb 1970; Duellman 2001; Jared et al. 2005). We tested whether the origin of novel cranial elements is associated with evolution of dermal skull roof hyperossification and with phragmosis, using likelihood analyses as implemented in Discrete (see above).

To address the relationship between the origin of novelty and hyperossification, the 70 hylid taxa were also scored for four traditional qualitative systematic characters that appear to be indicative of extensive ossification of dermal cranial elements in anurans: (1) bony contact between nasal and frontoparietal elements, (2) exostosis (dermal sculpturing) on one or more cranial elements, (3) bony contact between the squamosal and frontoparietal, and (4) bony contact between the zygomatic ramus of the squamosal and maxilla (data in online Appendix S6). Different combinations of states are present in different taxa. We therefore used two indices of hyperossification to summarize this variation as a binary character for Discrete; taxa were scored as being hyperossified if they had any of these four states (minimum index) or if they had all four (maximum index). We tested for a significant relationship between hyperossification (using both indices) and the presence of each novel element. Describing hyperossification with qualitative states facilitated the testing of hypotheses of correlated evolution with the novel elements.

We scored phragmosis as present in those taxa in which the behavior has been reported in the literature (online Appendix S6). Phragmotic taxa seem to show distinctive behavior, cranial morphology, and microhabitat usage (e.g., Jared et al. 2005). Nevertheless, scoring taxa as absent is somewhat problematic, given that few studies specifically report the absence of this behavior. We tested for a significant relationship between the presence of each of the three novel elements and phragmosis using Discrete, but with the caveat that the taxonomic extent of phragmosis may be underestimated.

Previous studies (e.g., Hanken 1985) also suggest that evolutionary novelty may be associated with major changes in body size. We obtained data from the literature on maximum male body sizes for the 70 hylid taxa included in the previous analyses (online Appendix S6; sources available from S.A.S.) and tested for an association between independent contrasts of body size and each of the novel elements. Tests were implemented using Analysis of Traits, version 3.1 (Ackerly 2006), a module of Phylocom version 3.40 (Webb et al. 2006). In short, this method involves a *t*-test between independent contrasts on branches on which a given discrete character changes and those branches on which there is no change.

Results

Phylogenetic analyses of 14 genes (Table 1) confirm recent molecular studies (Faivovich et al. 2005; Smith et al. 2005, 2007; Wiens et al. 2005a, 2006) that show that *Anotheca, Pternohyla, Smilisca,* and *Triprion* form a clade (the *Smilisca* clade), that an *Anotheca–Triprion* clade is sister taxon to a *Smilisca–Pternohyla* clade, and

Table 2. Support of different genes for different topologies within the *Anotheca–Triprion* clade based on Bayesian analysis. In some cases, the entire *Anotheca–Triprion* clade was not supported as monophyletic, but two of the three species were supported as sister taxa: * = *Anotheca+T. petasatus*; ** = *Anotheca+T. spatulatus*. Note that discordance among trees from different genes may reflect both incongruence between the gene and species trees (which may be especially likely for nuclear genes due to retained polymorphism) or failure of the estimated tree to reflect the phylogeny of the gene.

Topology	Supporting genes
T. spatulatus (*Anotheca*, *T. petasatus*)	Mitochondrial: 16S, ND1, combined
	Nuclear: PTGER, PTPN, SIA, combined
Anotheca (*T. petasatus, T. spatulatus*)	Mitochondrial: 12S
	Nuclear: POMC
T. petasatus (*Anotheca, T. spatulatus*)	Nuclear: TNS3
Nonmonophyly of *Anotheca–Triprion* clade	Mitochondrial: cytochrome b*
	Nuclear: c-myc, Cry-b, NACX, RAG-1*, Rho**

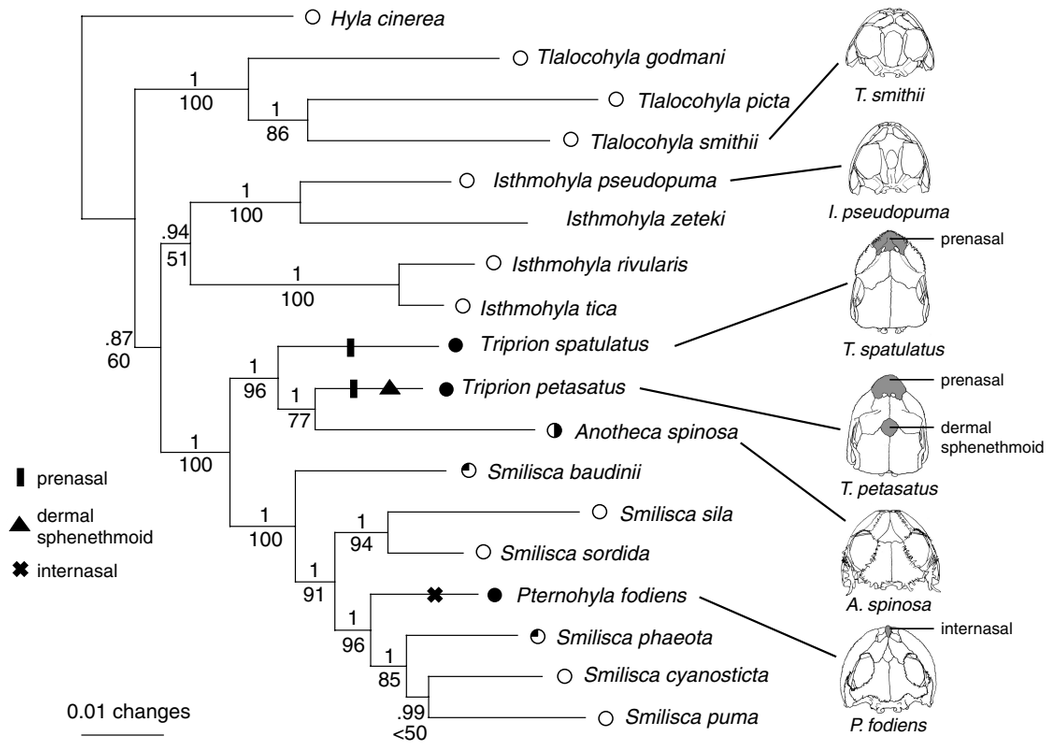

Figure 1. **Phylogeny of selected Middle American treefrogs (*Smilisca* clade and outgroups), based on a combined, partitioned Bayesian analysis of 10 nuclear genes and four mitochondrial genes. Numbers above each branch are Bayesian posterior probabilities, and numbers below are parsimony bootstrap values (Bayesian and parsimony analyses give similar trees). The reconstructed origins of novel cranial elements are also shown. For simplicity, only one of two most-parsimonious reconstructions for the prenasal is shown. The alternate reconstruction shows origin of the element in the ancestor of *Triprion* + *Anotheca* and loss in *Anotheca*. Maximum-likelihood reconstruction is ambiguous with regard to these two possibilities (note that our statistical analyses of clustering and correlation are not dependent on any particular parsimony or likelihood reconstruction for these nodes). Circles preceding each species name show the proportion of hyperossified traits present (open circle indicates none, closed circle indicates four of four present), with filled quadrants indicating specific character states: upper right quadrant = bony contact between nasal and frontoparietal; lower right = cranial exostosis; upper left = bony contact between squamosal and maxilla; lower left = bony contact between squamosal and frontoparietal. Skulls are redrawn from Duellman (2001). Note that the prenasal (when present) covers and obscures the premaxilla from dorsal view, and articulates with the maxillae and nasals. Similarly, the dermal sphenethmoid (when present) covers the endochondral sphenethmoid, and completely obscures the sphenethmoid from dorsal view, and articulates with the nasals and frontoparietals.**

that *Pternohyla* is nested inside of *Smilisca* (Fig. 1). Remarkably, however, we find strong support for the hypothesis that *Triprion petasatus* is more closely related to *Anotheca* than to *T. spatulatus* (Fig. 1).

This phylogeny implies striking homoplasy in the unusual morphological traits shared by the two species of *Triprion* (e.g., novel prenasal bone, modified maxillary flanges). One potential explanation for this surprising result is that the molecular phylogeny is wrong, possibly because of discordance between gene and species trees (e.g., through introgression, incomplete lineage sorting, or paralogy; Maddison 1997). However, paraphyly of *Triprion* is confirmed by multiple nuclear and mitochondrial genes (Table 2). Furthermore, analyses including additional individuals of *Anotheca* and *Triprion* for both nuclear and mitochondrial genes (12S, Cry-b, POMC, PTPN, SIA, TNS3) show that sampled individuals of each species form an exclusive (monophyletic) group (results not shown). These lines of evidence confirm that the apparent nonmonophyly of *Triprion* is not caused by discordance between gene and species trees, misidentification of specimens, or laboratory error.

Mapping morphological traits onto this phylogeny using parsimony (Fig. 1) and likelihood (not shown) shows that three novel cranial elements have evolved within the group. The two species of *Triprion* share a prenasal element anterior to the premaxilla. Reconstructions suggest that the prenasal evolved in parallel in each species of *Triprion* or else evolved once in the ancestor of the *Anotheca–Triprion* clade and then was lost in *Anotheca* (distinguishing these hypotheses unambiguously is not possible with

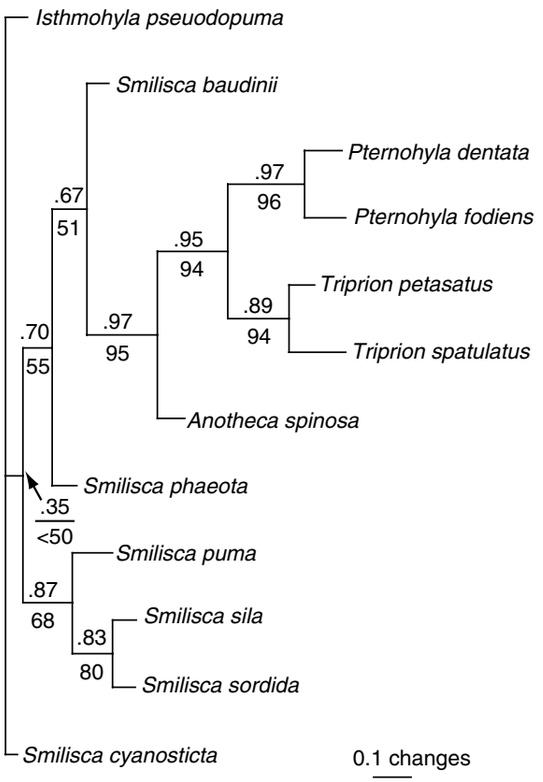

Figure 2. Phylogeny of *Smilisca* clade based on Bayesian analysis of 22 morphological characters. Numbers above each branch are Bayesian posterior probabilities, and numbers below are parsimony bootstrap values (parsimony and Bayesian analyses give similar results). Branch lengths represent the average lengths of each branch from the pooled post-burnin trees.

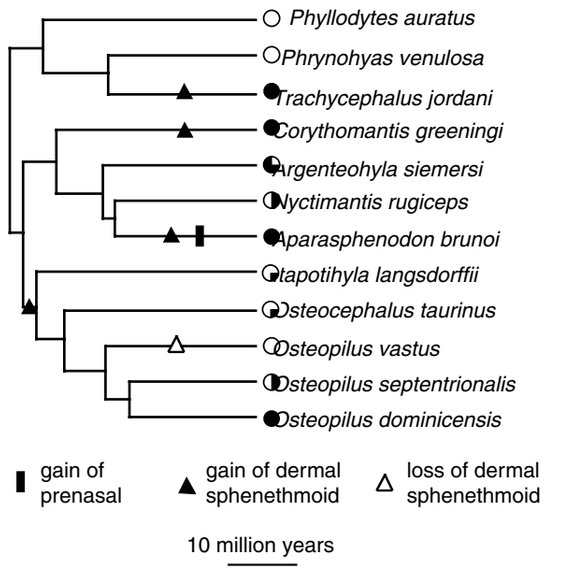

Figure 3. Phylogeny and evolution of novel cranial elements (dermal sphenethmoid, prenasal) within the hylid frog tribe Lophiohylini (topology and branch lengths from Wiens et al. 2006). One shortest parsimony reconstruction is shown for the dermal sphenethmoid (alternate reconstructions show three gains and two losses or two gains and three losses). Maximum likelihood reconstruction is ambiguous for many nodes for this character, but note that our statistical analyses of clustering and correlation are not dependent on any particular parsimony or likelihood reconstruction for these nodes. Branch lengths indicate estimated ages (in millions of years). Circles preceding each species indicate the proportion of character states indicative of hyperossification, as in Figure 1.

parsimony or likelihood given the available data). Another novel element, the dermal sphenethmoid, evolved in *T. petasatus*. A third novel element, the internasal, is unique to *Pternohyla fodiens*. However, this element is similar in position to the prenasal and may represent another origin of the same structure.

Bayesian and parsimony analyses of a morphological dataset for the *Smilisca* clade yield topologies that are very different from the topology supported by the molecular data (Fig. 2). Many of the conflicting branches are strongly supported by the morphological data (e.g., monophyly of *Anotheca–Pternohyla–Triprion* clade, *Pternohyla–Triprion* clade, and *Triprion*), and many character changes supporting these branches are associated with increased ossification of cranial elements. For example, the *Anotheca–Pternohyla–Triprion* clade is supported by a median contact of the nasals and by integumentary cranial co-ossification, the *Pternohyla–Triprion* clade is supported by lateral expansion of the maxillae, and *Triprion* is supported by evolution of the prenasal and further expansion of the maxillae. Other characters in this dataset include variation in adult external morphology and tadpole morphology (online Appendix S4). Parsimony and Bayesian analyses of the combined molecular and morphological data yield the same topology as that derived from the molecular data alone (except that the positions of *Smilisca cyanosticta* and *Smilisca phaeota* are reversed in the Bayesian analysis of the combined data relative to their positions in the tree from the molecular data alone).

The prenasal and dermal sphenethmoid also evolved in the hylid tribe Lophiohylini. Within Lophiohylini, there have been repeated origins of the dermal sphenethmoid and a single origin of the prenasal (Fig. 3). Maximum-likelihood analyses show a significant association between the evolution of the prenasal and dermal sphenethmoid across hylids, suggesting that the origins of these novel structures are correlated with each other (test statistic = 16.2500; $P < 0.05$). The origins of novel elements are significantly clustered within the *Smilisca* clade, within Lophiohylini, and within hylids in general (Table 3). Considering each novel element separately shows significant clustering of the internasal/prenasal in the *Smilisca* clade and of the dermal sphenethmoid in

Table 3. Results of tests for the phylogenetic clustering of species with novel skull elements within hylids in general, within Lophiohylini (i.e., when the *Smilisca* clade is excluded) and within the *Smilisca* clade (i.e., when Lophiohylini are excluded). The prenasal is present in a single species within Lophiohylini and the dermal sphenethmoid is present in a single species within the *Smilisca* clade, and so clustering was not assessed in these cases. * = $P < 0.05$, ** = $P < 0.01$.

	Mean phylogenetic distance		
	Any novel element	Pre- or internasal	Dermal sphenethmoid
Hylids	16.2500*	77.1176*	76.6836**
Lophiohylini	62.8128**	—	65.3817**
Smilisca clade	43.6459**	36.2793*	—

Lophiohylini, and that each element is significantly clustered in its occurrence across hylids in general. Although our taxon sampling within Lophiohylini is not extensive (constrained by the availability of both morphological and molecular data for the same species), the basic results should be robust to the addition of other species in that clade. Specifically, our major result in Lophiohylini is that there is an origin of the prenasal and many changes in the dermal sphenethmoid. Adding taxa might reveal more origins or changes in these traits, but not fewer.

Across all sampled hylids, there is a significant association between the origin of these novel elements and increased ossification of dermal skull roof elements, regardless of whether the minimum or maximum index for hyperossification is used (pre- or internasal and minimum hyperossification index, test statistic = 9.8032, $P < 0.05$; maximum index, test statistic = 20.5128, $P < 0.05$; dermal sphenethmoid and minimum index, test statistic = 26.9646, $P < 0.05$; maximum index, test statistic = 11.8684, $P < 0.05$). There is also a significant association between phragmotic behavior (i.e., using the head to plug burrow entrances) and origin of the prenasal (test statistic = 11.3886, $P < 0.05$), and the dermal sphenethmoid (test statistic = 13.0638, $P < 0.05$). There is no relationship between origin of the novel elements and body size (prenasal/internasal, $t = 0.7872$, df = 3, $P > 0.05$; dermal sphenethmoid, $t = 0.0353$, df = 5, $P > 0.05$).

Discussion

The evolution of novel structures may generally be uncommon, and is known to be rare in the tetrapod skull (Hanken and Hall 1993). In this paper, we use new phylogenies and phylogeny-based methods to elucidate two hot spots for the evolution of novel cranial elements in treefrogs, building on the anatomical study of Trueb (1970). Two or possibly three new cranial elements originate in these clades, with two novel elements present in some species (a 40% increase in dermal skull roof elements). Despite their relative novelty across anurans and tetrapods, we find that each element has likely evolved at least twice, either within or between these clades (given that the internasal is likely synonymous with the prenasal). The origins of these novel elements are associated with each other, with increased ossification of other dermal cranial elements, and with an unusual and seemingly adaptive behavior (phragmosis). Although changes in the overall rate of morphological evolution may be common between clades and through time (Futuyma 2005), this is possibly the first report to show hot spots (significant phylogenetic correlation and clustering) for the evolution of different novel structures.

In a similar study, Hanken (1985) reported extensive "novelty" in carpal morphology in a clade of salamanders (genus *Thorius)*. Comparison with our results reveals several intriguing differences. In *Thorius*, the putative novelties involve new configurations of pre-existing elements (e.g., fusions of carpals) rather than origins of novel structures. These unusual carpal morphologies consist mostly of relatively uncommon intraspecific variants that often are present only on one side of a given individual. Hanken (1985) emphasized that these unusual features were associated with miniaturized body size, but did not explicitly relate miniaturization to heterochrony or paedomorphosis (although some authors do, e.g., Alberch and Alberch 1981). Finally, he postulated that these limb skeletal variants had no functional or ecological significance.

In contrast, the dermal sphenethmoid, prenasal, and internasal in hylids are novel elements rather than apomorphic modifications of pre-existing structures. There are no reports of intraspecific variation in the presence of these structures, and we find no evidence of association between the origins of these traits and changes in body size. However, the origins of these elements are significantly associated with increased ossification of other dermal skull roof elements, and the latter may be related to a type of heterochrony called peramorphosis. Peramorphosis involves extension or acceleration of ancestral ontogenetic trajectories rather than truncation or deceleration, as in paedomorphosis (Alberch et al. 1979). Finally, association of these novel elements with phragmotic behavior suggests that they may have adaptive functional and ecological significance.

What explains the origin of these novel elements in hylids? Despite the potential functional significance of these elements, natural selection can only act on existing phenotypic variation, and so it is unlikely to directly explain why genetic and developmental systems have created novel elements. Many anurans have heavily ossified skull roofs (e.g., Duellman and Trueb 1986; Trueb 1993), but phragmotic behavior appears to be rare. Increased skull roof ossification may facilitate both phragmosis and the origin of novel

elements. The prenasal and dermal sphenethmoid seem to further reinforce the heavily ossified skull roofs of phragmotic species. It is tempting to suggest a possible causal developmental association behind the observed correlation between increased growth of dermal skull roof elements, the deposition of dermal bone onto existing elements (i.e., exostosis), and the origin of novel dermal elements in hylids with heavily ossified skull roofs. Developmental studies of *T. petasatus* (Trueb 1970) show that these features develop during a similar time period in ontogeny (late postmetamorphic). Comparative and experimental developmental studies to elucidate the specific developmental mechanisms underlying the origins of these novel structures could be an exciting area for future research. Overall, we speculate that these novel elements may have originated as developmental by-products associated with increased skull roof ossification, and that both novel elements and increased ossification may have been favored by natural selection for structurally reinforcing the skull (possibly in association with phragmotic behavior). Hot spots of novelty seem especially likely if selection favors a developmental process that helps generate novelties and simultaneously favors fixation of those novelties.

The idea that the same feature may repeatedly evolve among closely related species has a long history in evolutionary biology (reviewed by Sanderson 1991). Some of this history is related to the idea of "orthogenesis," which suggests that species are predisposed to evolve certain traits, although orthogenesis itself has been largely discredited (Sanderson 1991; Futuyma 2005). Sanderson (1991) developed statistical phylogenetic methods for testing for such "homoplastic tendencies," but did not focus on novelties per se as we have done. His tests address whether changes in a given character: (1) occur near other changes in the same character on the tree, and (2) occur in only one localized subclade of the tree. The observations are then compared to expected patterns of random change based on simulations. Similarly, we have addressed whether the repeated origins of any novel elements are clustered (or "localized") in particular clades on the tree, and (not addressed by Sanderson) whether the origins of different novel structures are correlated in their origins on the tree.

Our results show remarkable evolutionary lability and homoplasy in cranial morphology among closely related species in the *Smilisca* clade. Although we cannot distinguish whether the *Triprion* morphotype evolved twice or evolved once and was lost in *Anotheca*, the overall homoplasy is striking (Fig. 1). Parsimony and Bayesian analyses of the morphological data alone show strong statistical support for the monophyly of *Triprion*, a *Pternohyla–Triprion* clade, and an *Anotheca–Pternohyla–Triprion* clade (Fig. 2). Many characters supporting these three clades are associated with increased ossification of skull roof elements. Our molecular and combined-data results strongly suggest that these clades are incorrect, and that the morphological analyses may have been misled by a correlated suite of traits associated with the repeated evolution of increased ossification in two different lineages (*Pternohyla* and *Anotheca* + *Triprion*). Previous studies have shown that paedomorphosis can strongly mislead parsimony and Bayesian analyses of morphology (Wiens et al. 2005b), and our study may be the first to demonstrate a parallel effect for peramorphosis. Bayesian analysis of morphology (using Lewis' [2001] likelihood model) may be more robust to stochastic homoplasy than parsimony, but it also seems to produce statistically well-supported, but incorrect, results when developmental coupling of traits (through paedomorphosis or peramorphosis) violates the fundamental assumption of character independence.

Given that *Triprion* seemingly is not monophyletic, the taxonomy of these frogs should be modified. Rather than lumping the distinctive genus *Anotheca* Smith 1939 into *Triprion* Cope 1866, we recommend resurrecting *Diaglena* Cope 1887 for *T. spatulatus* (a long-used generic name for this species). Thus, *Anotheca* is retained, and *Triprion* is restricted to *T. petasatus*. Our results also support the placement of *Pternohyla* in *Smilisca* (e.g., Faivovich et al. 2005; Wiens et al. 2006).

In summary, we have shown that origins of different novel morphological structures may be correlated with each other and clustered phylogenetically. Similar hot spots likely occur in many other clades of animals and plants, and the statistical methods used here can (in theory) be applied to any group. Our results also suggest that phylogenetic analyses of morphological data may be strongly misled by peramorphosis and the related nonindependence of characters. Thus, clades that are supported primarily by characters associated with peramorphosis should be viewed with appropriate caution, even if they are statistically well supported and defined by the seemingly rare origins of novel structures.

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Supplementary Material

The following supplementary material is available for this article:

Appendix S1. Primers used for DNA amplification and sequencing. F = forward primer; R = reverse primer.

Appendix S2. GenBank accession numbers for DNA sequences analyzed in this study.

Appendix S3. Locality and voucher information for specimens from which molecular data were obtained.

Appendix S4. Morphological data used in Bayesian and parsimony analyses.

Appendix S5. Tree and branch lengths (in millions of years before present) for 70 hylid taxa used in analyses of character evolution.

Appendix S6. Data matrix for 70 hylid taxa used in analyses of character evolution.

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