doi:10.1111/j.1558-5646.2008.00430.x

RATES AND PATTERNS IN THE EVOLUTION OF SNAKE-LIKE BODY FORM IN SQUAMATE REPTILES: EVIDENCE FOR REPEATED RE-EVOLUTION OF LOST DIGITS AND LONG-TERM PERSISTENCE OF INTERMEDIATE BODY FORMS

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Received December 12, 2007
Accepted May 10, 2008

An important challenge in evolutionary biology is to understand how major changes in body form arise. The dramatic transition from a lizard-like to snake-like body form in squamate reptiles offers an exciting system for such research because this change is replicated dozens of times. Here, we use morphometric data for 258 species and a time-calibrated phylogeny to explore rates and patterns of body-form evolution across squamates. We also demonstrate how time-calibrated phylogenies may be used to make inferences about the time frame over which major morphological transitions occur. Using the morphometric data, we find that the transition from lizard-like to snake-like body form involves concerted evolution of limb reduction, digit loss, and body elongation. These correlations are similar across squamate clades, despite very different ecologies and >180 million years (My) of divergence. Using the time-calibrated phylogeny and ancestral reconstructions, we find that the dramatic transition between these body forms can occur in 20 My or less, but that seemingly intermediate morphologies can also persist for tens of millions of years. Finally, although loss of digits is common, we find statistically significant support for at least six examples of the re-evolution of lost digits in the forelimb and hind limb.

KEY WORDS: Ancestral state reconstruction, divergence-time estimation, evolutionary rate, limb reduction, macroevolution, morphology, phylogeny, Squamata.

A major goal of evolutionary biology is to explain the remarkable diversity in morphology among multicellular organisms, and especially the dramatic transitions in body form or body plan that sometimes occur between and within clades (Futuyma 2005). Unquestionably, the most important trend in the study of body-form evolution in recent years has been the burgeoning of evolutionary developmental biology (evo–devo; Carroll et al. 2005). Nevertheless, the evo–devo approach is not necessarily the most efficient

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or appropriate way to answer every question in this area, nor is a strictly paleontological approach.

Analyses of extant taxa using phylogeny-based comparative methods also have much to offer. Although phylogeny-based comparative methods are widely used in evolutionary biology in general, their application to understanding major transitions in body form remains limited. Yet, reconstructions of ancestral character states and analyses of character correlation across a large clade can potentially reveal the number and direction of body-form changes, the evolutionary steps by which these changes have occurred, and whether they occurred the same way in every lineage (e.g., Hibbett 2004). Coupled with recent methods for estimating the divergence times of clades (e.g., Sanderson 2002, 2003; Thorne and Kishino 2002; Drummond et al. 2006), a phylogenetic approach can also help make inferences about the tempo or rate at which these changes occur in extant taxa. However, few previous studies (if any) have combined ancestral state reconstruction with time-calibrated phylogenies to estimate the general time frame over which one body form evolves into another, and how long taxa that are seemingly intermediate between these body forms persist (although there is obviously a large literature on inferring rates of evolution of quantitative characters from the fossil record [e.g., Gingerich 2001] and from comparisons of extant populations and species [e.g., Lynch 1990; Hendry and Kinnison 1999]). In this article, we apply a multifaceted phylogenetic approach to the evolution of major changes in body form in reptiles.

Squamate reptiles (lizards and snakes) offer an appealing model system with which to address questions about major changes in body form. Squamate reptiles consist of roughly 8200 species (Uetz 2007). Although most squamate species are characterized by a lizard-like body form with four pentadactyl limbs (Pough et al. 2004), an elongate, snake-like body form has independently evolved at least 26 times (Wiens et al. 2006). The most successful of these origins gave rise to modern snakes (∼3000 species) and amphisbaenians (∼165 species), but a snake-like morphology has also evolved independently in seven phylogenetically diverse squamate families (Anguidae, Cordylidae, Dibamidae, Gerrhosauridae, Gymnophthalmidae, Pygopodidae, and Scincidae; Wiens et al. 2006; Uetz 2007). Some families contain multiple origins of this body form (e.g., anguids, gymnophthalmids, scincids), and both the fully limbed, lizard-like body form and the limbless, snake-like body form may even occur within the same genus (e.g., the scincid genus *Lerista*; Cogger 1992). Thus, the evolution of snake-like body form offers a system in which there is extensive replication of body-form changes (making rigorous statistical analyses possible), and in which many changes seem to occur among closely related species (facilitating comparisons of their ecology, development, and function).

Here we address the patterns and rates of morphological change in body form across squamates using a phylogenetic approach. Most previous studies on the evolution of limb-reduced body form either focused only on small taxonomic groups (e.g., *Bachia*, Presch 1975; Kohlsdorf and Wagner 2006; *Chalcides*, Caputo et al. 1995; Greer et al. 1998; *Hemiergis*, Greer and Choquenot 1989; Shapiro 2002; *Lerista*, Greer 1987, 1990; *Scelotes*, Whiting et al. 2003), lacked phylogenetic information (e.g., Lande 1978; Choquenot and Greer 1987; Greer 1987, 1990, 1991; Caputo et al. 1995; Greer et al. 1998), or focused only on digit number and limb loss (e.g., Whiting et al. 2003; Kearney and Stuart 2004; Kohlsdorf and Wagner 2006). Wiens and Slingluff (2001) performed phylogeny-based analyses of body-form transitions within anguid lizards, but it is uncertain how applicable their results are across all squamates. Wiens et al. (2006) assembled a morphometric dataset and molecular phylogeny (with branch lengths in units of estimated time) for 258 squamate species to estimate how many times snake-like body form evolved. They documented repeated origins of two limb-reduced ecomorphs: a short-tailed burrowing morph, and a long-tailed, surface-dwelling (or "grass swimming") morph (Fig. 1). However, that study said little about how these transitions between lizard-like (pentadactyl) and snake-like (limbless) morphologies occurred. In other words, they focused on the morphological "end-products," but not the rates or pathways by which they evolved. Here, we use the morphometric data and phylogeny of Wiens et al. (2006) to address several questions about these dramatic transitions in body form in squamates. Specifically, we address whether the patterns of change in the evolution of snake-like body form are the same across clades and ecomorphs, the time frame over which these body-form transitions occur, and whether these changes are unidirectional.

Previous studies have suggested that the origins of snake-like body form involve the correlated evolution of three characters: elongation of body length, reduction in limb length, and loss of digits (reviewed in squamates and then tested in anguids by Wiens and Slingluff 2001). Here, we test whether these changes are correlated across all origins of snake-like squamates, or whether they are associated only with particular clades or ecomorphs. We address whether there are thresholds of limb size at which digits are lost (e.g., Lande 1978) and thresholds of body elongation at which limb reduction and digit loss occur. We also evaluate whether the origin of snake-like body form occurs through similar patterns of digit and limb loss in different clades or ecomorphs.

We take advantage of our time-calibrated phylogeny (Wiens et al. 2006) to ask questions about the rate at which these dramatic transitions in body form occur. Specifically, how much time is needed for the complete transition from a lizard-like to snake-like morphology? Can it occur in a few thousand years or does it require a hundred million? Furthermore, many taxa have only partial limb reduction (e.g., three fingers and three toes). Do these seemingly transitional morphologies merely represent fleeting intermediates on the way to complete limblessness or are

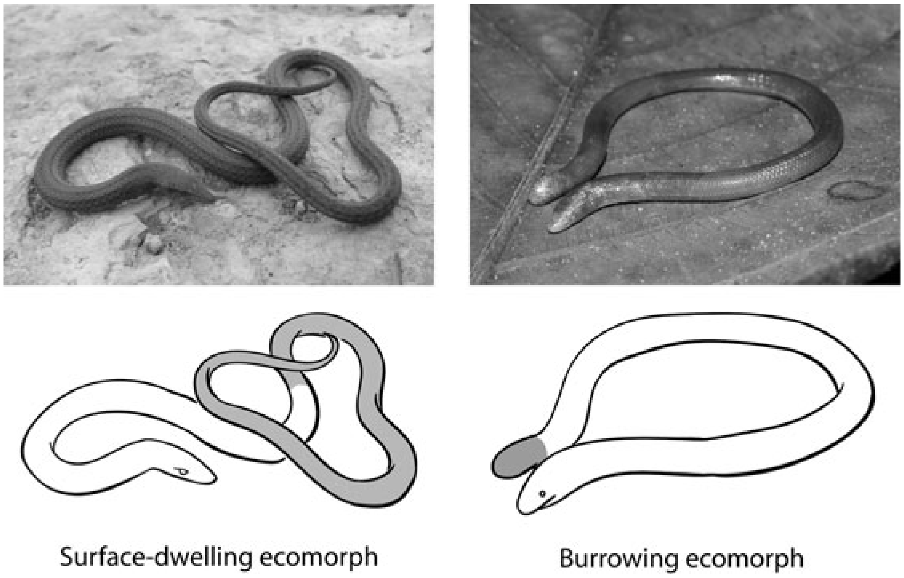

Figure 1. Exemplars of the two ecomorphs of snake-like squamate reptiles. The pygopodid, *Lialis burtonis* (left), is typical of the long-tailed surface-dwelling ecomorph. The dibamid, *Dibamus* sp. (right), is representative of the short-tailed burrowing ecomorph. Note the similar body shapes but differences in the relative lengths of the trunk (unshaded) and tail (shaded) between the two ecomorphs. Photos courtesy of Matthew Fujita (*Lialis*) and Rebecca Chong (*Dibamus*).

they stable end points that are maintained over long evolutionary time scales? How do these estimates compare to our estimates of the time for the complete transition between lizard-like and snake-like body forms? Many previous studies have constructed "morphoclines" of limb reduction from extant taxa (e.g., Stokely 1947; Gans 1975; Presch 1975; Lande 1978; Renous and Gasc 1979; Choquenot and Greer 1987; Greer 1987, 1990, 1991; Caputo et al. 1995; Greer et al. 1998), with the implicit assumption that taxa with partial limb reduction are in a transitory phase in the process of becoming limbless.

Finally, we test whether limb reduction is unidirectional, and if digits or limbs can re-evolve from ancestors that lacked these structures. Dollo's law (Gould 1970; Wagner 1982; Bull and Charnov 1985; Marshall et al. 1994), the notion that once a complex structure is lost in evolutionary time it cannot re-evolve in exactly the same way, continues to influence how researchers interpret analyses of morphological evolution. For example, Whiting et al. (2003) and Kearney and Stuart (2004) found evidence that indicated that there was re-evolution of lost limbs in scincids and amphisbaenians, respectively, but interpreted their results as showing repeated limb loss instead (even though this was not the pattern shown by their parsimony analyses of their datasets). More recently, Kohlsdorf and Wagner (2006) found very strong support for digit re-evolution in *Bachia* lizards based on statistical phylogenetic analyses. In light of these recent findings, we revisit the question of digit re-evolution and assess the evidence for this phenomenon across all major squamate lineages. To do this, we develop an approach that combines maximum-likelihood ancestral state reconstruction, a statistical model of ordered digit transformation, and a prior distribution of tree lengths and rates of digit change to estimate probabilities of ancestral digit number for each node on the phylogeny.

Materials and Methods

PHYLOGENY

The phylogenetic framework for this study (Figs. 4–7) is the "superchronogram" of 258 squamate species constructed by Wiens et al. (2006). It represents a synthesis of recent molecular phylogenetic analyses of squamates (both within and between families) and is particularly useful in that the branch lengths represent estimates of time from relaxed molecular clock analyses, and thus are comparable throughout the tree. Taxon sampling was intended to span squamate phylogeny and to capture as many independent origins of snake-like body form as possible, given the available molecular data (Wiens et al. 2006). Although the tree contains only 258 of the ~8200 species of lizards and snakes, many of the species excluded clearly are not relevant to the evolution of limb reduction (e.g., the ~1400 species of iguanian lizards which show no limb reduction and the ~2500 species of advanced snakes which lack limbs). The support for individual clades (e.g., from bootstrapping in the original studies) is shown in figures 3 and 4 of Wiens et al. (2006).

We were unable to replicate the phylogenetic results of Kearney and Stuart (2004) for amphisbaenian phylogeny. Our analyses of their molecular dataset using parsimony and Bayesian analysis reveal that, contrary to Figure 3 of their paper, the placement of *Blanus* is ambiguous (results not shown). Because placement of this limbless taxon may affect the estimated ancestral state of the limbed *Bipes*, we initially performed all ancestral state reconstruction analyses with the same tree produced by Wiens et al. (2006), but with three alternate phylogenetic resolutions for *Blanus*. To estimate the chronogram used in the ancestral reconstruction analyses, we used an input tree that placed *Blanus* as the sister taxon to *Bipes* (as per Wiens et al. 2006), another that placed *Blanus* as the sister taxon to all other amphisbaenians but *Rhineura* (Kearney and Stuart 2004), and a third that placed *Blanus* as the sister taxon to all other nonrhineurid amphisbaenians exclusive of *Bipes*. For the latter two trees, we reestimated the branch lengths of the superchronogram with penalized likelihood in r8s, version 1.71 (Sanderson 2002, 2003) as done by Wiens et al. (2006). Both r8s reanalyses resulted in chronograms that inferred a polytomy for the interrelationships of *Bipes*, *Blanus*, and the other nonrhineurid amphisbaenians (i.e., regardless of the placement of *Blanus*, the branch between *Blanus* and its sister taxon is always extremely short). For the ancestral state reconstruction analyses, this polytomy was resolved by assigning a very small branch length (1 year) to the relevant branches. Regardless of the placement of *Blanus*, the results of the ancestral state reconstruction analyses were highly similar.

TESTING HYPOTHESES OF CORRELATED CHARACTER EVOLUTION

Regression analyses of independent contrasts were used to test hypotheses of correlated change among morphometric variables. Morphometric data from Wiens et al. (2006) were used (see their appendix 1), which consist of eight variables for the same 258 species included in the phylogeny. These variables are snout-vent length (SVL; tip of the snout to the posterior extent of the cloacal opening), head length (HL; tip of the snout to the posterior corner of the eye), tail length (TL; posteriormost extent of the cloaca to the top of the outstretched tail), total length (SVL + TL), fore- and hind limb length (FLL and HLL; from the posterior corner of the limb to the tip of the longest outstretched digit), and numbers of externally recognizable forelimb and hind limb digits.

We coded snake "pelvic spurs" and limbs consisting of only a small flap or stump as also having a single digit, but we acknowledge that, in many cases, these are not true anatomical digits and may instead be other externally visible, vestigial limb bones. For example, with one exception (*Delma tincta,* a species not sampled for this study), the externally visible pelvic limbs in pygopodids do not possess any phalangeal elements, and most species lack metatarsals as well (Kluge 1976). Within snakes, the pelvic spurs consist of femurs with a distal, keratinized spur (Bellairs 1950; Mlynarski and Madej 1961). However, for both flaps and spurs, coding these structures as single digits captures the idea that a highly reduced limb is present, with one or possibly no digits. Similarly, we acknowledge that our assessment of limb loss was based on the external morphology alone, but the presence or absence of external limbs may be the most relevant variation in terms of natural selection.

An average of six adult specimens per species were included, but many more individuals were examined but excluded because they were seemingly subadults or possessed damaged or regenerated tails. There may be minor differences in body proportions and body size between sexes and populations. When possible, we attempted to incorporate this by sampling from both sexes and throughout the species' range and pooling the data. Regardless, these morphological differences seem minor relative to the magnitude of differences between species, which are the primary focus of this article.

Independent contrasts (Felsenstein 1985) were calculated for each natural log-transformed variable using COMPARE 4.6b (Martins 2004). The value of 1 was added to all variables prior to log transformation, given that the natural log of zero is undefined and some limbless taxa have values of zero for some variables. The topology and branch lengths from the "superchronogram" of Wiens et al. (2006) were used to calculate contrasts.

Following previous studies (e.g., Lande 1978; Wiens and Slingluff 2001), we constructed indices of relative limb reduction and body elongation by scaling the raw variables of head and body length by head length (distance between the eye and the snout), as opposed to using absolute measures of limb and body lengths (analyses of which would be confounded by body size allometry). Independent contrasts of limb and body-length measurements were regressed against contrasts in head length (forcing the regression line through the origin; Garland et al. 1992) and the residuals were saved. Head-length-corrected morphometric variables (referred to as relative trunk length, limb length, and total length; rSVL, rFLL, rHLL, rTL) were then regressed (forced through the origin) to detect significant correlated change. Significant *P*-values were determined using a sequential Bonferroni-correction with $P \leq 0.05$ as a threshold (Rice 1989). All statistical analyses were conducted using SPSS 14.0.

To test for a relationship between body elongation and limb reduction, we regressed rSVL, rTL, and rTotal against rFLL and rHLL. We also tested for a relationship between digit number and both relative limb size (regressed against head length) and absolute limb size (raw contrasts) for each limb. To determine if these relationships are consistent between and within clades, we also performed three additional sets of regression analyses, each restricted to one of the three clades in which there have been multiple origins of limb-reduced body form: Anguidae, Gymnophthalmidae, and Scincidae. For each of these sets of analyses, independent contrasts were recalculated after pruning out the irrelevant taxa (i.e., those outside each clade). Otherwise, all statistical methods were the same as for the squamate-wide analysis.

Previous authors (Wiens and Slingluff 2001; Wiens et al. 2006) found evidence that body elongation results in two different limb-reduced ecomorphs that differ in the relative lengthening of the trunk (SVL) or tail (Fig. 1). We conducted phylogenetic generalized least squares (PGLS) analysis of variance (ANOVA) analyses (Ord and Martins 2006) to determine how variation in these variables is related to the ecomorph categories, after accounting for the phylogeny. To incorporate the effect of body size, we log-transformed morphometric variables (after adding the value of 1.0 to all variables), regressed all variables against head length, and used the residuals for subsequent analyses (note that in previous analyses we used residuals based on independent contrasts, whereas here we use nonphylogenetic residuals as input into the phylogenetic comparative analyses). We used the general linear model formulation of the PGLS ANOVA using COMPARE 4.6b by separately regressing relative SVL, tail length, forelimb length, hind limb length, and total lengths (y) against two different dummy variables (x) coded 0 or 1 (variable $1 =$ is/is not a burrowing ecomorph; variable $2 =$ is/is not a surface-dwelling ecomorph) for a total of 10 tests. The overall correlation coefficient (R) represents deviations from the mean of the comparison group and was tested for significance using a t-test (Ord and Martins 2006). Species were assigned to ecomorph categories following Wiens et al. (2006), which was based on a nonphylogenetic principal components analysis of all taxa. The present analyses differ in that we here test the morphological variation underlying these ecomorph categories in a phylogenetic context.

DETECTING THRESHOLD RELATIONSHIPS BETWEEN LIMB LENGTH AND DIGIT NUMBER

Based on studies of selected scincids and gymnophthalmids, Lande (1978) found that there are distinct thresholds of body proportions at which limb shortening and digit loss occur (e.g., no taxa with limb lengths less than 50% of the snout-eye length retain multiple digits). To determine if similar thresholds exist across all squamate reptiles, we constructed graphs of raw digit numbers versus relative limb lengths (number of fingers vs. FLL/HL and number of toes vs. HLL/HL), relative total body length ([SVL + TL]/HL), and relative SVL (SVL/HL). These graphs were visually inspected for general trends. For the few species in which the number of digits was variable (e.g., sexually dimorphic pelvic spurs in some male snakes and *Dibamus*), digit number was rounded up to the next whole number. We used the raw data (i.e., without log-transformation or phylogenetic correction) to make the results easier to visualize and interpret.

PATTERNS OF LIMB REDUCTION IN THE FORELIMBS AND HIND LIMBS

We tested if digit losses were correlated in the forelimbs and hind limbs, and if the rate of digit loss in one limb was greater than in the other. First, to test for correlated digit loss in forelimbs and hind limbs, we conducted regression analyses (forced through the origin) comparing independent contrasts of forelimb and hind limb digit number, using the methods described above. Note that no methods are presently available to test for the correlation between two discrete, multistate characters in a phylogenetic context.

To determine whether changes in digit numbers were more common in the fore or hind limbs, we used ancestral reconstruction (see below) to examine points in the phylogeny where digit change occurred in one limb and assessed whether the other limb had the same number of digits. In cases in which there was unequal loss of digits or limbs, we tested if digit loss was more common on the forelimb or hind limb using a binomial test (using SPSS 14.0) where the number of trials is the number of cases of asymmetric loss (n), the number of successes is the number of times the forelimb exceeded the hind limb in the extent of digit loss (k), and assuming an equal probability of loss in each limb ($P = 0.5$). Only unambiguously reconstructed states were used; a state was considered to be unambiguously reconstructed if it had a posterior probability ≥ 0.95 (see below).

Digits seem likely to both develop and be lost in an ordered sequence (e.g., Alberch and Gale 1985; Shapiro 2002). Therefore, we reconstructed ancestral states for digit numbers using an ordered model, in addition to an unordered model. Character state ordering is straightforward in a parsimony framework (a transition between five digits and four digits is given a weight of "1," from five to three digits a weight of "2," etc.). However, ordering in a maximum-likelihood framework is more complicated because transitions between character states should be modeled as rates of character change. Even though existing computer programs allow one to reconstruct character states in a likelihood framework using unordered or user-specified models (e.g., Mesquite; BayesMultiState ver. 1.0.2), the specific probabilities that one should use to create an ordered model have rarely (if ever) been discussed.

For example, Kohlsdorf and Wagner (2006) attempted to implement a statistical, ordered model of digit transformation with the program BayesMultiState version 1.0.2 (Pagel 1994; Pagel and Lutzoni 2002; now BayesTraits ver. 1.0), but did not provide an explicit rate matrix. According to T. Kohlsdorf (pers. comm.), they assumed a model in which the rate of transition between digit states is inversely proportional to the number of digits that are changing. Thus, a single-digit transformation is assumed to occur more frequently than a two-digit transformation. Although this model approaches a strictly ordered model by penalizing morphological changes involving more than one digit, it does not absolutely restrict changes of more than one digit to pass through an intermediate state, as one would expect under an ordered parsimony model. This subtle difference could potentially inflate the probability of reconstructing an ancestor with many more or less digits than the descendent lineage (although we also think it is unlikely that this would be a major source of error).

Here, we employed a model that assumes single-digit transitions evolve at equal rates (i.e., change between 0 and 1 digits has the same rate as 4 to 5 digits) and prohibits instantaneous transitions greater than one digit (e.g., no direct changes from 5 to 2 digits). The model is represented by the following six state (zero to five digits) matrix:

$$Q = \begin{pmatrix} -\alpha & \alpha & 0 & 0 & 0 & 0 \\ \beta & -(\beta+\alpha) & \alpha & 0 & 0 & 0 \\ 0 & \beta & -(\beta+\alpha) & \alpha & 0 & 0 \\ 0 & 0 & \beta & -(\beta+\alpha) & \alpha & 0 \\ 0 & 0 & 0 & \beta & -(\beta+\alpha) & \alpha \\ 0 & 0 & 0 & 0 & \beta & -\beta \end{pmatrix} \mu,$$

where α and β are the instantaneous rates of change for digit gains and losses, respectively, and μ is a factor that scales the mean rate of change to 1.0. Note that this is an instantaneous rate matrix and not a probability matrix. Changes of more than one digit are permitted at each node, but because they are forced to occur through an intermediate state, the probability of a large change is much lower than a small change.

We initially implemented this model in BayesTraits version 1.0 to separately reconstruct ancestral states for both fingers and toes using our "superchronogram." However, the reconstructions were largely ambiguous, even in clades with all extant taxa possessing the same character state. This problem may be a result of using branch lengths in millions of years, numbers that are too large to use for ancestral state reconstructions (because the exponentiations of these numbers needed to calculate ancestral states are too large to hold in computer memory).

We therefore developed a computer program that calculates Bayesian posterior probabilities of ancestral states by integrating over scaled tree lengths and rates of digit gain and loss using Markov Chain Monte Carlo (the details of this process are provided in the Appendix). Thus, we were able to both preserve the relative lengths of branches without arbitrarily choosing a single scaling factor, and implement the ordered model of character change (above) without assuming equal rates of gains (α) and losses (β). MCMC chains were run for 1.1 million generations, sampling every 1000th generation. The first 100,000 generations were discarded as "burnin" and the remaining 10,000 samples were used to calculate the posterior probabilities of the ancestral state of each digit on all nodes. To test the hypothesis that the rate of gains and losses are equal, we compared the posterior probability distribution of the rate parameter (i.e., the relative rate of gains to losses estimated from the data) to the prior probability of equal gains and losses (0.50) using the Bayes factor (see Appendix).

To test the robustness of our results to a very different model, we also reconstructed ancestral states using a model in which transitions between all digit states may occur at equal rates (i.e., the widely used unordered model; Appendix).

TEMPO OF BODY-FORM EVOLUTION

We also combined our ancestral state reconstructions with our time-calibrated phylogeny to estimate the approximate timeframe for the transition from a fully limbed lizard-like body form with five digits to an elongate snake-like body form lacking one or both sets of limbs. We first identified a given extant species or clade that lacked one or both sets of limbs (i.e., the end point; note that missing limbs were always associated with an elongate, snake-like body form, see Results). We then determined the most recent ancestor of that clade which was reconstructed as having a pentadactyl, lizard-like morphology (the starting point) with significant posterior probability ($\geq$ 0.95). The age of this ancestor was determined from the chronogram. From this date, we then subtracted the age of the species or clade lacking limbs to obtain the time frame over which this transformation occurred.

We acknowledge that this analysis is potentially compromised by several factors. First, adding more species could reveal that this transformation happened more quickly (e.g., if a branch from a limbed ancestor to limbless descendant has a length of 20 My, a fully limbed species that is added with an ancestor that falls along the middle of the branch would suggest that the transition may have taken only 10 My or less). However, it seems unlikely that adding species could show that a given transformation happened more slowly. Furthermore, this transformation could occur at any point along a given branch, and so could be more rapid than estimated here, and thus our approach offers estimates of the maximum amount of time over which this transformation occurs.

Second, the inferred ages are statistical estimates and thus are subject to error. Nevertheless, the 95% credible intervals for age estimates for major clades are generally less than 10 million years (My) (Wiens et al. 2006), based on resampling characters in the RAG-1 gene. However, we acknowledge that there are additional sources of uncertainty in these age estimates (e.g., the ages and phylogenetic positions of taxa used as fossil calibration points), the most important of which may be the root age of Squamata (only a single date was considered by Wiens et al. (2006)). However, a recent study that also addressed the ages of squamate clades (Hugall et al. 2007) yielded roughly similar dates to those used here (i.e., most within 10–20 My). The taxon sampling of Hugall et al. (2007) was too limited to determine if the differences would strongly influence most of the dates relevant to our study. Another study of squamate divergence dates (Vidal and Hedges 2005) estimated dates that were substantially older than those estimated by Wiens et al. (2006) or Hugall et al. (2007), but Hugall et al. (2007) argued that these older dates may be an artifact of two problematic fossil calibration points and an inappropriate root age for Squamata.

We also combined ancestral reconstruction and divergence date estimation to determine how long intermediate stages of body-form evolution (i.e., some digits lost but one or both limbs still retained) have persisted. Our basic logic was that if a clade of two or more species is reconstructed as having an intermediate morphology and that morphology is also retained in the extant species, then the crown-group age of that clade provides a minimal estimate for how long the intermediate morphology has persisted. However, only a limited number of taxa could be included in this analysis. For example, an intermediate state had to be present in at least two descendent species and their common ancestor. With only a single species with intermediate morphology (i.e., 1 to 4 digits), it is impossible to estimate when this morphology evolved and how long it has persisted.

RE-EVOLUTION OF LOST LIMBS AND DIGITS

To determine whether extant taxa have re-evolved digits and/or limbs that were previously lost, we again compared extant morphologies to ancestral state estimates of ancestral digit number (0 digits = no external limbs). If extant taxa with a given number of digits are reconstructed as having ancestors lacking limbs or having fewer digits, and if those reconstructions had significant statistical support (posterior probability ≥ 0.95), then we considered there to be strong evidence for the re-evolution of those lost structures. In some cases, we combined the probabilities of reconstructed ancestral digit number if the number of digits was less than that in the extant species. For example, if an extant species with five digits had an ancestor with posterior probability of 0.50 for having four digits and 0.50 for having three digits, we considered this to be significant support for the re-evolution of at least one digit (i.e., an ancestor with three or four digits gave rise to a descendant with five). These analyses were based primarily on the reconstructions using the ordered likelihood model as described above, but results were also confirmed using the unordered model.

Results

ANALYSES OF CHARACTER CORRELATION

Regression analyses of phylogenetically independent contrasts (Table 1, Fig. 2) show highly significant correlations between increases in relative SVL and total length and decreases in relative forelimb and hind limb lengths. There is no significant correlation between relative tail length and relative limb lengths. The number of fingers and toes is closely related to both relative (rFLL and rHLL) and absolute (FLL and HLL) limb length.

Table 1. Regression analyses of pairs of variables associated with transitions from lizard-like to snake-like body form in squamates.

Independent variable	Dependent variable	df	R^2	*P*
rSVL	rFLL	257	0.303	**<0.001***
rSVL	rHLL	257	0.333	**<0.001***
rTL	rFLL	257	0.023	**0.015***
rTL	rHLL	257	0.004	0.288
rTotal	rFLL	257	0.205	**<0.001***
rTotal	rHLL	257	0.155	**<0.001***
rFLL	Fingers	257	0.799	**<0.001***
rHLL	Toes	257	0.723	**<0.001***
FLL	Fingers	257	0.802	**<0.001***
HLL	Toes	257	0.736	**<0.001***
Fingers	Toes	257	0.747	**<0.001***

All results are based on independent contrasts. Variables with the prefix "r" (e.g., rSVL), forelimb length (rFLL), hind limb length (rHLL), tail length (rTL), and total length (rTotal) represent regression residuals from regression of independent contrasts of SVL, FLL, HLL, TL, and Total with these variables against contrasts of head length. Significant *P*-values at $\alpha \leq 0.05$ are shown in bold; *P*-values that are significant after table-wide sequential Bonferroni-correction are identified with an asterisk.

Most of the same relationships are significant when restricted to anguids, gymnophthalmids, and scincids (Table 2). Thus, these relationships seem to apply both within and between major squamate clades. The exceptions involve the tail and total body lengths. Within anguids, increases in both relative tail and total length are correlated with decreases in relative limb length. In gymnophthalmids and scincids, limb shortening is significantly correlated with increases in relative SVL and total length (gymnophthalmids only), but not with tail length. These differences seem to be related to differences in the frequency of the ecomorphs among these clades (Wiens et al. 2006); most snake-like anguids belong to the long-tailed surface-dwelling ecomorph, whereas all snake-like gymnophthalmids and scincids belong to the short-tailed burrowing ecomorph (see below).

Results of the PGLS-ANOVA analyses of the ecomorph categories are provided in Table 3. Body-form changes in burrowers involve increasing overall relative total length ($P = 0.003$) and SVL ($P < 0.001$), whereas decreasing both relative limb lengths ($P < 0.001$) and tail length ($P = 0.002$). The surface-dwelling ecomorph is similar in that they tend to have a high relative total length ($P = 0.009$) and reduced forelimb ($P = 0.024$) and hind limb (not significant) lengths, but this elongation is achieved primarily through lengthening the tail ($P = 0.004$) rather than SVL ($P = 0.704$). These results are consistent with the results from nonphylogenetic analyses, which show that species of the burrowing ecomorph tend to have a tail length that is roughly half of the SVL, species of the surface-dwelling ecomorph have a

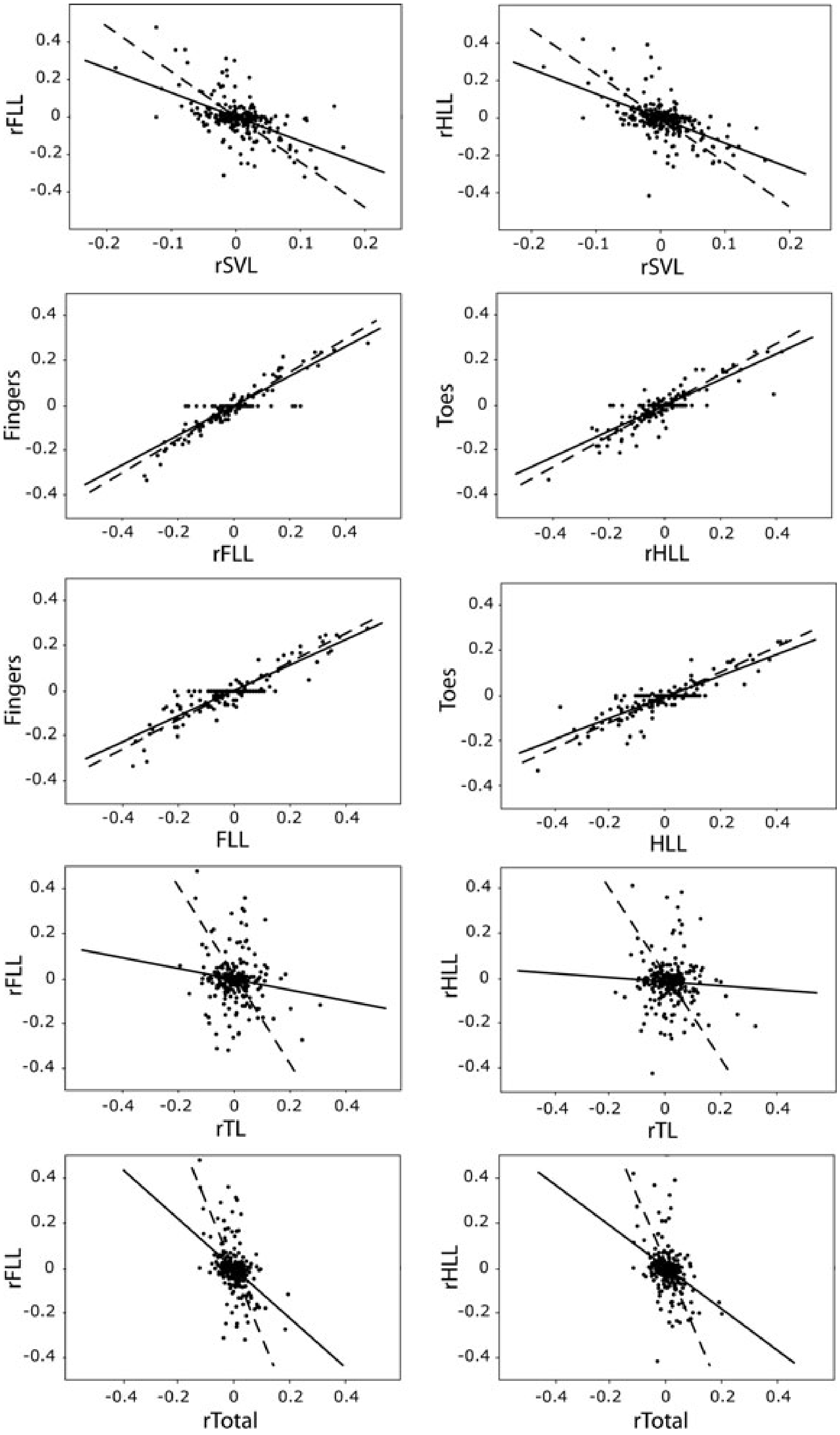

Figure 2. Linear regression analyses (forced through the origin) of pairs of morphological variables for squamate reptiles, based on phylogenetically independent contrasts, showing significant relationships between body elongation, limb reduction, and digit loss. Relative snout-vent length (rSVL), forelimb length (rFLL), hind limb length (rHLL), tail length (rTL), and total length (rTotal) are residuals from regression of independent contrasts of SVL, FLL, HLL, TL, and Total with contrasts of head length. The solid line represents the best-fit line of the ordinary least squares (OLS) regression, and the dotted line is the reduced major axis (RMA) regression. See Table 1 for further results.

Table 2. Regression analyses of pairs of variables associated with transitions from lizard-like to snake-like body form in squamates, conducted separately for three different clades each containing multiple origins of snake-like body form.

Clade	Dependent variable	Independent variable	df	R^2	P
Anguidae	rSVL	rFLL	22	0.637	**<0.001***
	rSVL	rHLL	22	0.553	**<0.001***
	rTL	rFLL	22	0.455	**<0.001***
	rTL	rHLL	22	0.235	**0.019***
	rTotal	rFLL	22	0.664	**<0.001***
	rTotal	rHLL	22	0.435	**<0.001***
	rFLL	Fingers	22	0.827	**<0.001***
	rHLL	Toes	22	0.810	**<0.001***
	FLL	Fingers	22	0.776	**<0.001***
	HLL	Toes	22	0.798	**<0.001***
	Fingers	Toes	22	0.676	**<0.001***
Gymnophthalmidae	rSVL	rFLL	38	0.634	**<0.001***
	rSVL	rHLL	38	0.578	**<0.001***
	rTL	rFLL	38	0.000	0.999
	rTL	rHLL	38	0.007	0.604
	rTotal	rFLL	38	0.199	**0.004***
	rTotal	rHLL	38	0.231	**0.002***
	rFLL	Fingers	38	0.941	**<0.001***
	rHLL	Toes	38	0.821	**<0.001***
	FLL	Fingers	38	0.818	**<0.001***
	HLL	Toes	38	0.567	**<0.001***
	Fingers	Toes	38	0.893	**<0.001***
Scincidae	rSVL	rFLL	92	0.348	**<0.001***
	rSVL	rHLL	92	0.358	**<0.001***
	rTL	rFLL	92	0.051	**0.030**
	rTL	rHLL	92	0.013	0.270
	rTotal	rFLL	92	0.038	0.061
	rTotal	rHLL	92	0.073	**0.009***
	rFLL	Fingers	92	0.894	**<0.001***
	rHLL	Toes	92	0.895	**<0.001***
	FLL	Fingers	92	0.830	**<0.001***
	HLL	Toes	92	0.857	**<0.001***
	Fingers	Toes	92	0.815	**<0.001***

All results are based on independent contrasts. Variables with the prefix "r" (e.g., rSVL), forelimb length (rFLL), hind limb length (rHLL), tail length (rTL), and total length (rTotal) represent regression residuals from regression of independent contrasts of SVL, FLL, HLL, TL, and Total with these variables against contrasts of head length. Significant P-values at $\alpha \leq 0.05$ are shown in bold; P-values that are significant after table-wide sequential Bonferroni-correction are identified with an asterisk.

tail length that is 2.3 times the SVL (on average), and species of the lizard-like morph typically have a tail that is (on average) 1.5 times the SVL (Wiens et al. 2006).

THRESHOLDS

With few exceptions, both ecomorphs show smaller relative limb lengths and digit number associated with greater relative SVL (Fig. 3). All external vestiges of the forelimb are absent in species in which the SVL/HL exceeds ~22 (except in *Bipes*, which has an elongate body but well-developed forelimbs) and when the Total/HL exceeds ~62. However, small hind limb elements persist in species with much higher degrees of body elongation (SVL/HL $\leq$ ~60, Total/HL $\leq$ ~72) relative to the forelimb (Fig. 3).

There is no evidence for a general threshold of total length of SVL that would suggest an initiation point for the loss of digits, in that the range of relative body proportions of the five-digit and four-digit taxa broadly overlap. However, there do seem to be thresholds for all four relative body measurements (TL, SVL, FLL, and HLL) after which digits are always lost. With one exception (*Bipes biporus*), no species with a total length > 40 times its head length or SVL > 15 times its head length possesses five

Table 3. **Results of the phylogenetic generalized least squares (PGLS) ANOVA analyses assessing morphological variation related to the ecomorph categories.**

Variable	Burrower			Surface-dweller		
	N	*R*	*P*	*N*	*R*	*P*
rSVL	89	0.67	**<0.001***	25	0.08	0.704
rTL	89	−0.32	**0.002***	25	0.55	**0.004***
rFLL	89	−0.53	**<0.001***	25	−0.45	**0.024***
rHLL	89	−0.61	**<0.001***	25	−0.26	0.209
rTotal	89	0.31	**0.003***	25	0.51	**0.009***

Variables with the prefix "r" (e.g., rSVL), forelimb length (rFLL), hind limb length (rHLL), tail length (rTL), and total length (rTotal) represent regression residuals from regression of SVL, FLL, HLL, TL, and Total with these variables against head length. *P*-values that are significant at $\alpha \leq 0.05$ are shown in bold; *P*-values that are significant after table-wide sequential Bonferroni-correction are identified with an asterisk.

fingers. Similarly, no species with a TL/HL > 35 or SVL/HL > 15 possesses five toes. Also, no species with less than five digits has TL/HL < 20, although several five-digit species possess TL/HL less than this value. Five-digit and four-digit species have very similar relative SVLs (SVL/HL < ∼18) whereas species with less than three digits tend to be more elongate and never possess a relative SVL < ∼10.

The relationship between relative limb lengths and digit numbers is similar to that found in the analyses of body elongation. There is no apparent threshold of relative limb length for the initiation of digit loss, although digit loss is always present when FLL is approximately equal to or less than HL, and HLL is ∼1.5 times HL or less. Additionally, there is a considerable range of relative limb length values that is only occupied by five-digit species (FLL/HL < ∼2, HLL/HL < ∼3.8). The relationship between digit loss and absolute limb size is very similar to the relative limb loss in that there is morphospace only occupied by pentadactyl and limbless species. In the forelimb, no species with a forelimb longer than ∼11 mm possesses less than five digits, whereas none less than 2 mm possesses more than one digit. With one exception (*Teius teyou,* HLL = 72.4 mm), no species with a hind limb longer than 15.8 mm possess less than five digits, whereas all species with a hind limb less than 3 mm have one or no digits.

Although there is a general relationship between decreasing relative limb length and digit loss, this relationship is highly variable. Unlike the total and SVL body lengths, species with four, three, or two digits possess a broad range of relative limb lengths (Fig. 3). However, there is a distinct difference between those species with two or more digits, and those with one or none. In the forelimb, all species with an FLL less than half their HL possess one digit or are limbless. Similarly, all species with an HLL less than half their HL possess one digit or are limbless, with the exception of *Scelotes bipes.*

PATTERNS OF DIGIT REDUCTION IN THE FORE AND HIND LIMB

Regression analyses of forelimb and hind limb digit number (Table 1) indicate that digit loss in the forelimb and hind limb is correlated. Many cases of digit loss appear as direct transitions from pentadactyl to limbless morphology, with intermediate taxa (if there were any) either unsampled or extinct. However, there are several species with 1–4 digits on both the fore and hind limb, which show differences in the extent of digit loss between fore and hind limbs (Table 4; Figs. 4–7). Overall, there are 13 cases in which more digits were lost in the forelimb than in the hind limb. In contrast, there are only four examples of digit loss in the hind limb exceeding that in the forelimb, a statistically significant disparity (two-tailed $P = 0.049$).

The pattern of limb loss is similar to that for digits. There are at least 10 cases in which complete loss of the forelimb is coupled with the loss of the hind limb, and this is almost certainly an underestimate due to ambiguous ancestral state reconstructions (Figs. 4–7). But among cases of asymmetry between loss of fore and hind limbs, loss of forelimbs is more common (Table 5). There are at least seven transitions to a body plan lacking forelimbs but retaining hind limb elements, but only two examples of the opposite pattern (although this result is not significant, two-tailed $P = 0.180$). Among the species examined, only *Bipes* both possess forelimbs and lack hind limbs. However, some species of *Bachia* also possess a similar morphology (Kohlsdorf and Wagner 2006), although these were not among the *Bachia* species included in our analyses.

We also note that the posterior distributions of the character-change rate parameter from the ancestral reconstruction analyses (Fig. 8) are very similar for digits on both the fore and hind limbs. This result indicates that rates of digit loss in both limbs are almost identical (but see above).

TIME FRAME FOR MAJOR MORPHOLOGICAL TRANSITIONS AND PERSISTENCE OF INTERMEDIATES

The estimated amount of time for the transition from a pentadactyl, tetrapod body form to a snake-like body form lacking one or both sets of limbs (Table 6; Figs. 4–7) ranges from ∼16 to ∼176 My. However, with the outliers *Dibamus* (176 My) and two clades of snakes (∼101 and ∼129 My) removed, the range is from 16 to 69 My, with a median of ∼50 My. Fifteen lineages fit the criterion of possessing seemingly intermediate morphologies (one to four digits) in both extant species and their reconstructed

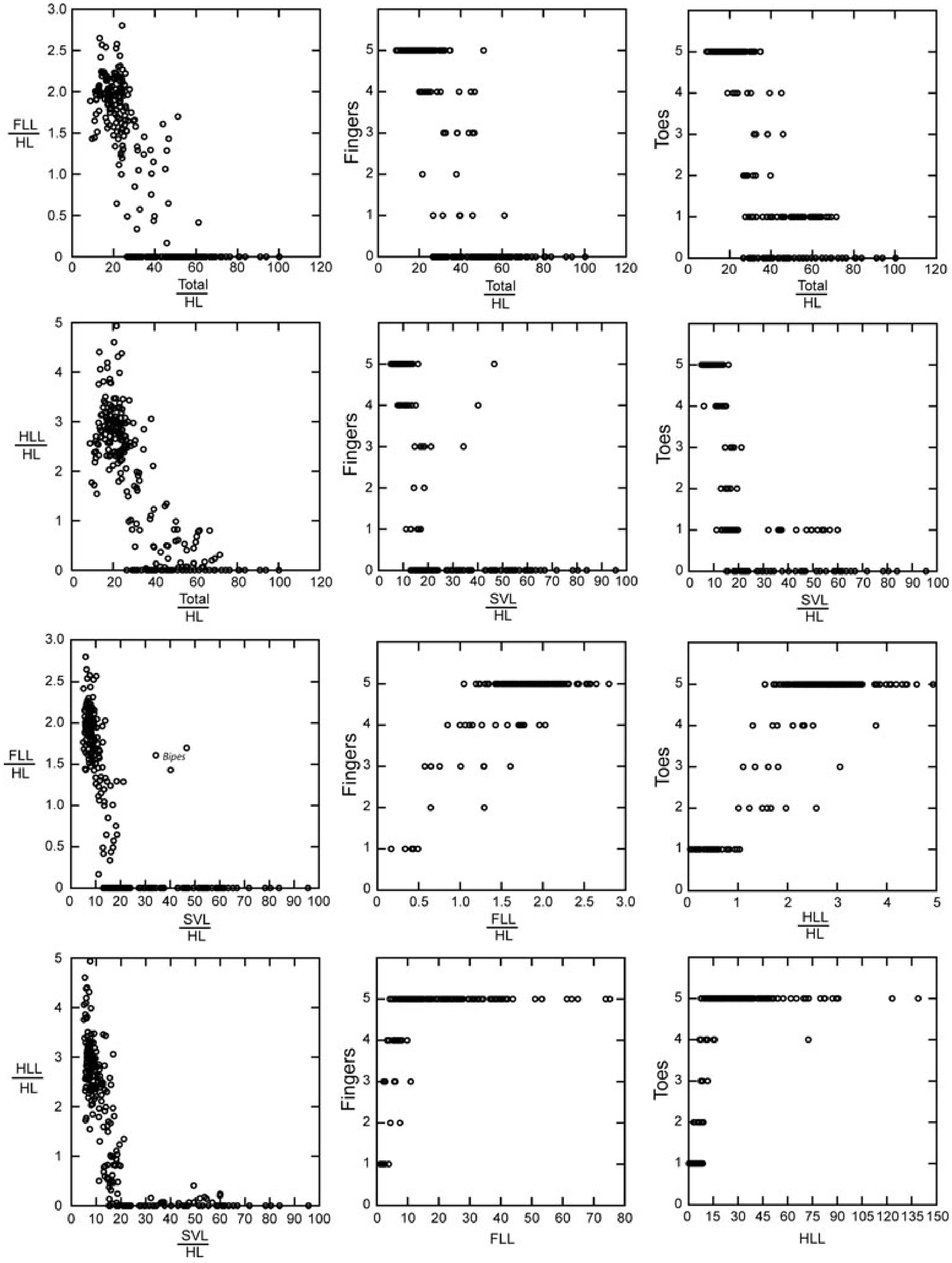

Figure 3. Plots of various body proportions involved in the transition from lizard-like to snake-like body form, showing thresholds of relative limb size and body elongation at which changes in digit number occur. Snout-vent length (SVL), forelimb length (FLL), and hind limb length (HLL) were each divided by head length (HL) to obtain measures of body elongation and limb reduction.

Table 4. Species or clades in which the extent of digit loss differs in the fore and hind limb, based on unambiguous ancestral reconstructions.

Transition	Clade	Ecomorph
Fewer digits on the forelimb than hind limb		
	Anguidae	
	Ophisaurus+Anguis	Surface-dweller
	Ophiodes striatus	Surface-dweller
	Gymnophthalmidae	
	Calyptommatus	Burrower
	Colobodactylus dalcyanus	Primitive
	Gymnophthalmini	Primitive
	Nothobachia ablephara	Burrower
	Pygopodidae	Surface-dweller
	Scincidae	
	Lerista bipes	Burrower
	Plestiodon reynoldsi	Burrower
	Pygomeles braconnieri	Burrower
	Some *Scelotes*	Burrower
	Sphenops sphenopsiformis	Burrower
	Voeltzkowia	Burrower
Fewer digits on the hind limb than forelimb		
	Amphisbaenia	
	Bipes	Burrower
	Gymnophthalmidae	
	Bachia dorbignyi	Burrower
	Scincidae	
	Anomolopus macayi	Burrower
	Teiidae	
	Teius teyou	Primitive

"Primitive" refers to the fully-limbed, lizard-like ecomorph, which gave rise to the long-tailed surface-dwelling and short-tailed burrowing ecomorphs of snake-like squamates.

common ancestors (Table 7; Figs. 4–7). These lineages have persisted from ~9 to ~63 My (median ~27 My).

DIGIT RE-EVOLUTION

The posterior distributions of the character change rate parameters for the Bayesian ancestral state reconstruction analyses are very similar (Fig. 8) and indicate an overwhelming bias toward loss of limb elements rather than gains. Additionally, log-transformed Bayes factors strongly reject an equal rate of gains and losses (fingers: Bayes factor = −154.1; toes: Bayes factor = −134.8). Yet, despite implementing a model biased heavily against digit gains, our ancestral state reconstructions of digit number nonetheless reveal statistically significant support for six instances of the re-evolution of lost digits (Table 8; Figs. 4–7). However, we note that this number could be substantially higher depending on the resolution of the many ambiguous ancestral state reconstructions (Figs. 4–7). The results of the unordered analyses (not shown) are similar to those from the ordered model and there are no differences that have strong support statistically (i.e., there are no cases in which, for a given node, one state is significantly supported by one method and a different state is significantly supported by the other method). The major difference is that many of the nodes in anguids and scincids that are ambiguously reconstructed using the ordered model are strongly supported as either pentadactyl or limbless (the latter offering more support for the hypothesis of digit re-evolution). Except when explicitly noted, the results and discussion will be limited to the ordered analyses as we think this model is more biologically realistic and, given the greater ambiguity of the results, more conservative.

Digit re-evolution is more common in the forelimb (five examples) than in the hind limb (one example) and most cases involve the re-evolution of a single digit (Table 8). However, there are two cases involving more extensive limb re-evolution. In the scincid *Scelotes mirus* the re-acquisition of at least two digits in the forelimb seems to have occurred (Fig. 5). In the amphisbaenian genus *Bipes* there is significant support for the re-evolution of a pentadactyl forelimb from ancestors with potentially far fewer digits (Fig. 6); indeed, it is possible that the ancestor of *Bipes* was completely limbless, although our results are ambiguous. This result is consistent regardless of the placement of *Blanus* (results not shown).

In contrast to the ordered model results, the reconstructions using the unordered model (not shown) support the absence of forelimbs in the common ancestor of *S. mirus* and *S. arenicolus* (0.94; marginally nonsignificant), and the ancestor of amphisbaenians (1.0), and thus suggest the independent re-evolution of entire, pentadactyl forelimbs in both of these lineages.

Discussion

SHARED AND DIVERGENT PATTERNS OF CHANGE

Snake-like body form has evolved repeatedly in squamate reptiles, a group with ~8200 species (Uetz 2007) that is at least 180-million-year old (Vidal and Hedges 2005; Wiens et al. 2006; Hugall et al. 2007). This pattern of repeated evolution raises the obvious question: does this macroevolutionary transition occur in similar ways or dissimilar ways across the tree? Given the age and phylogenetic diversity of the taxa involved, one might expect that there would be intrinsic differences in the morphological evolution of this character complex in different clades. Furthermore, snake-like body form is associated with two distinct ecomorphs in squamates (Fig. 1): relatively long-tailed surface dwellers and short-tailed burrowers (Wiens et al. 2006; this study).

Nevertheless, several lines of evidence show the similarity in these patterns. Across squamates, there are strong relationships between limb reduction, body elongation, and digit loss (Fig. 2). When the three groups in which snake-like body form

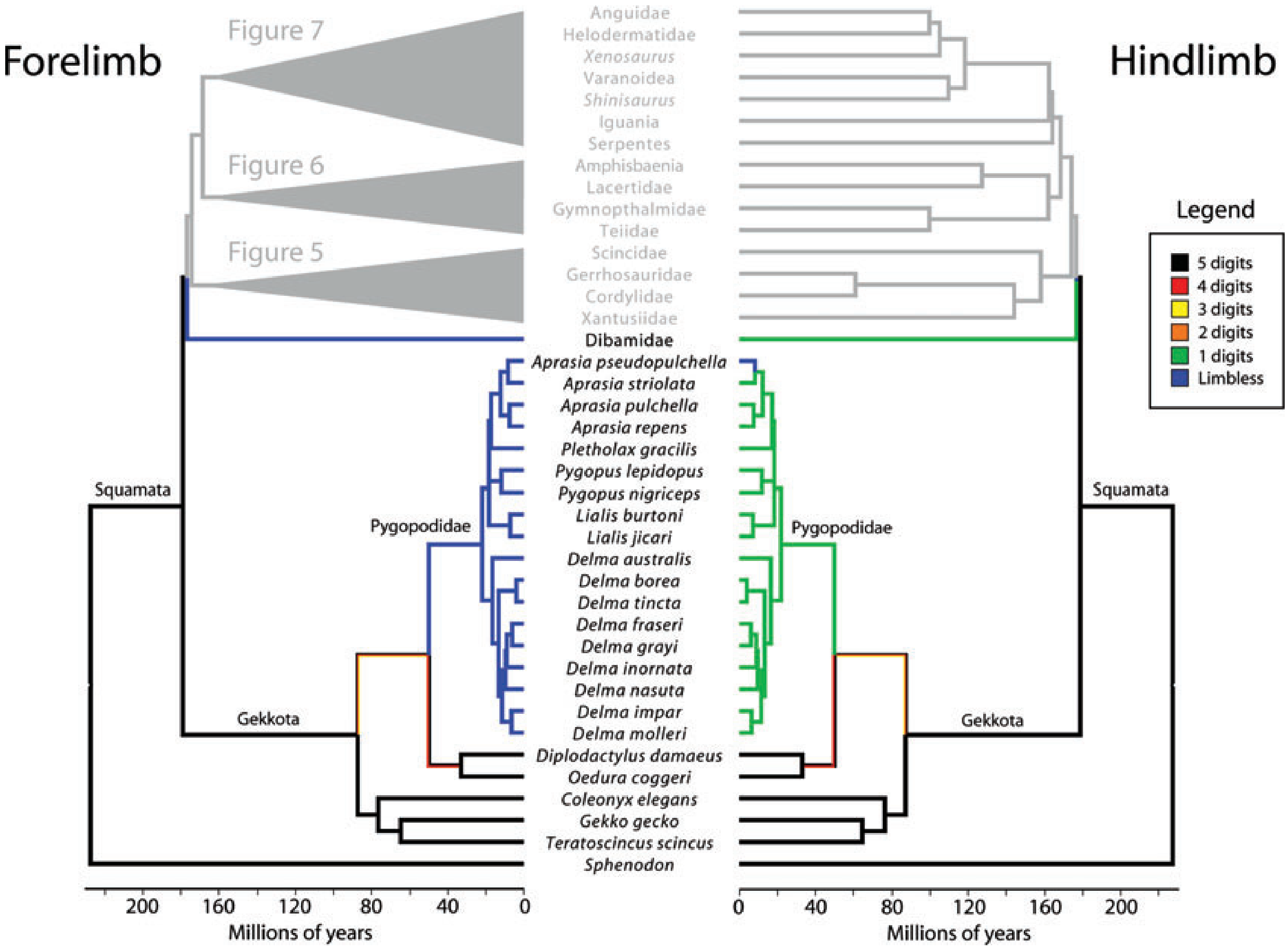

Figure 4. Chronograms and estimated ancestral states of digit numbers in the forelimb and hind limb of squamate reptiles, with emphasis on gekkotans. Solid color branches represent character states with a cumulative posterior probability ≥ 0.95. Branches with ambiguous ancestral reconstructions are indicated in multiple colors; these colors represent the multiple states whose posterior probabilities sum to ≥ 0.95.

evolves most frequently (anguids, gymnophthalmids, scincids) are considered separately and compared to each other, with few exceptions (see below) these same relationships are also seen and are generally similar between groups (Table 2). Examination of thresholds of digit loss and limb reduction further confirms that these relationships are similar across clades and ecomorphs (Fig. 3). Although pentadactyl species may possess a broad range of relative and absolute limb lengths, there are values at which digit loss always occurs. For example, all sampled digit-reduced species possess an absolute forelimb length less than 11 mm and hind limb length less than ~15 mm. Finally, our results suggest that digit loss in the forelimb is usually accompanied by loss in the hind limb (Table 1). When asymmetry exists, there is a general bias toward loss of forelimbs and forelimb digits (Tables 4, 5). The similar patterns of change among taxa and similar correlations among traits are intriguing given the diverse ecologies of the species and immense phylogenetic time scale of the lineages (>180 My).

There is also some evidence for divergent patterns of change. Although the PGLS-ANOVA analyses suggest that both burrowing and surface-dwelling ecomorphs are elongate (Table 3), there is strong evidence that overall body elongation occurs in two different ways. In the burrowing ecomorph, elongation occurs via elongation of the trunk (i.e., SVL). In contrast, in the surface-dwelling ecomorph, this elongation seems to be due mostly to increases in tail length. Consequently, some of the differences in patterns of character correlation among clades (Table 2) seem to be due to differences in the relative frequencies of ecomorphs among clades (i.e., anguids contain both ecomorphs, but gymnophthalmids and scincids contain only burrowers).

The other striking example of divergent patterns of change is the difference in which set of limbs are lost among clades (Table 5). In many limb-reduced taxa, the forelimbs are lost and the hind limbs are retained as small vestiges with a single digit (e.g., *Ophisaurus*, Pygopodidae, many snakes). However, in *Bipes* and some *Bachia*, relatively well-developed forelimbs with 3–5

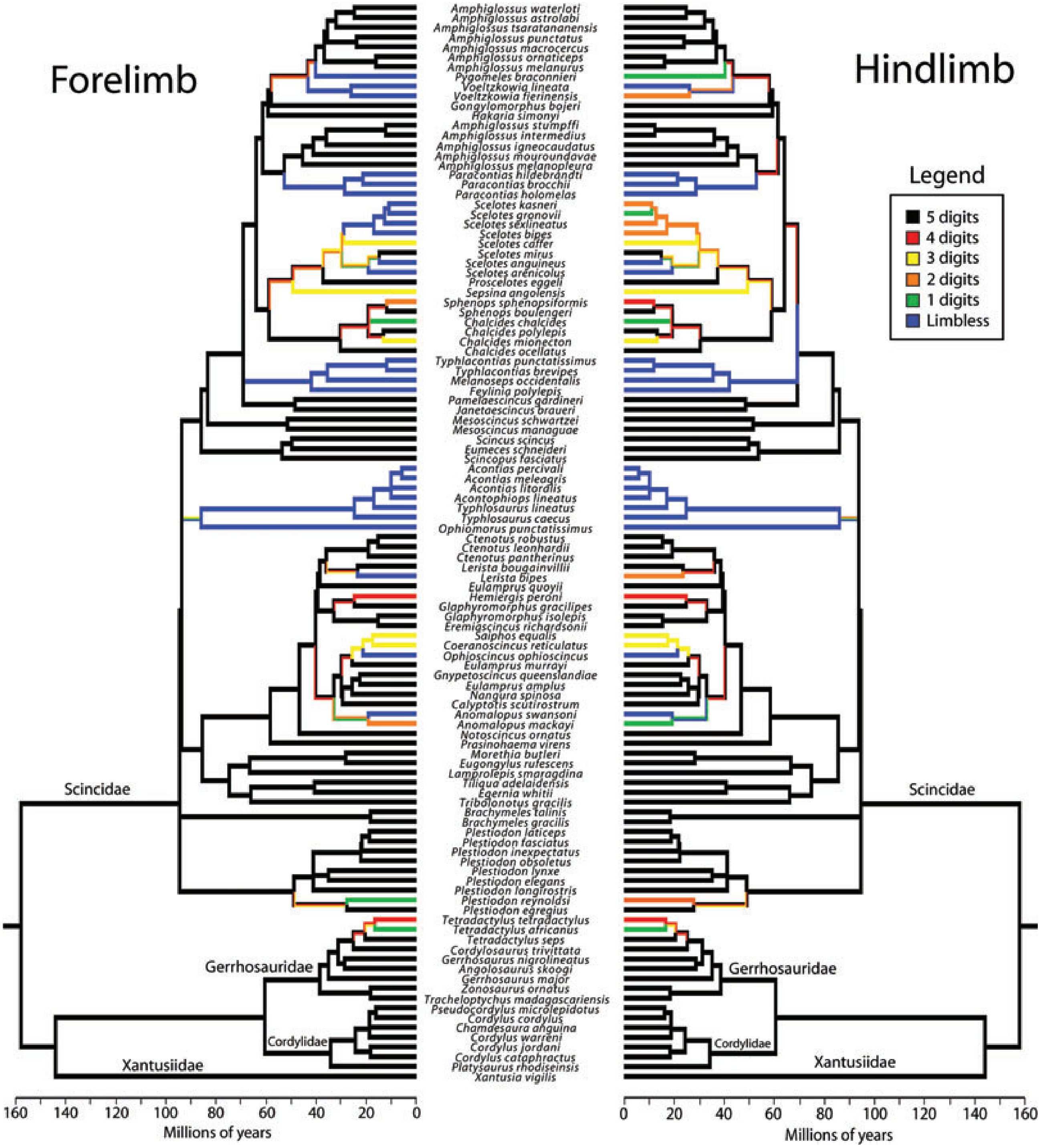

Figure 5. Chronograms and estimated ancestral states of digit number in the forelimb and hind limb of scincomorph squamate reptiles. Solid color branches represent character states with a cumulative posterior probability ≥ 0.95. Branches with ambiguous ancestral reconstructions are indicated in multiple colors; these colors represent the multiple states whose posterior probabilities sum to ≥ 0.95.

digits are present and external hind limbs are absent (Figs. 6 and 7).

Intriguingly, it is no longer clear that *Bipes* represents an intermediate stage in the process of limb loss (given that it is not the sister taxon to all amphisbaenians according to recent phylogenetic evidence; Kearney and Stuart 2004). Thus, the surprising deviation from the typical pattern may be associated with the re-evolution of forelimbs (see below). There has also been re-evolution of limbs and digits in *Bachia* (Kohlsdorf and Wagner 2006), although this does not seem to explain the bias

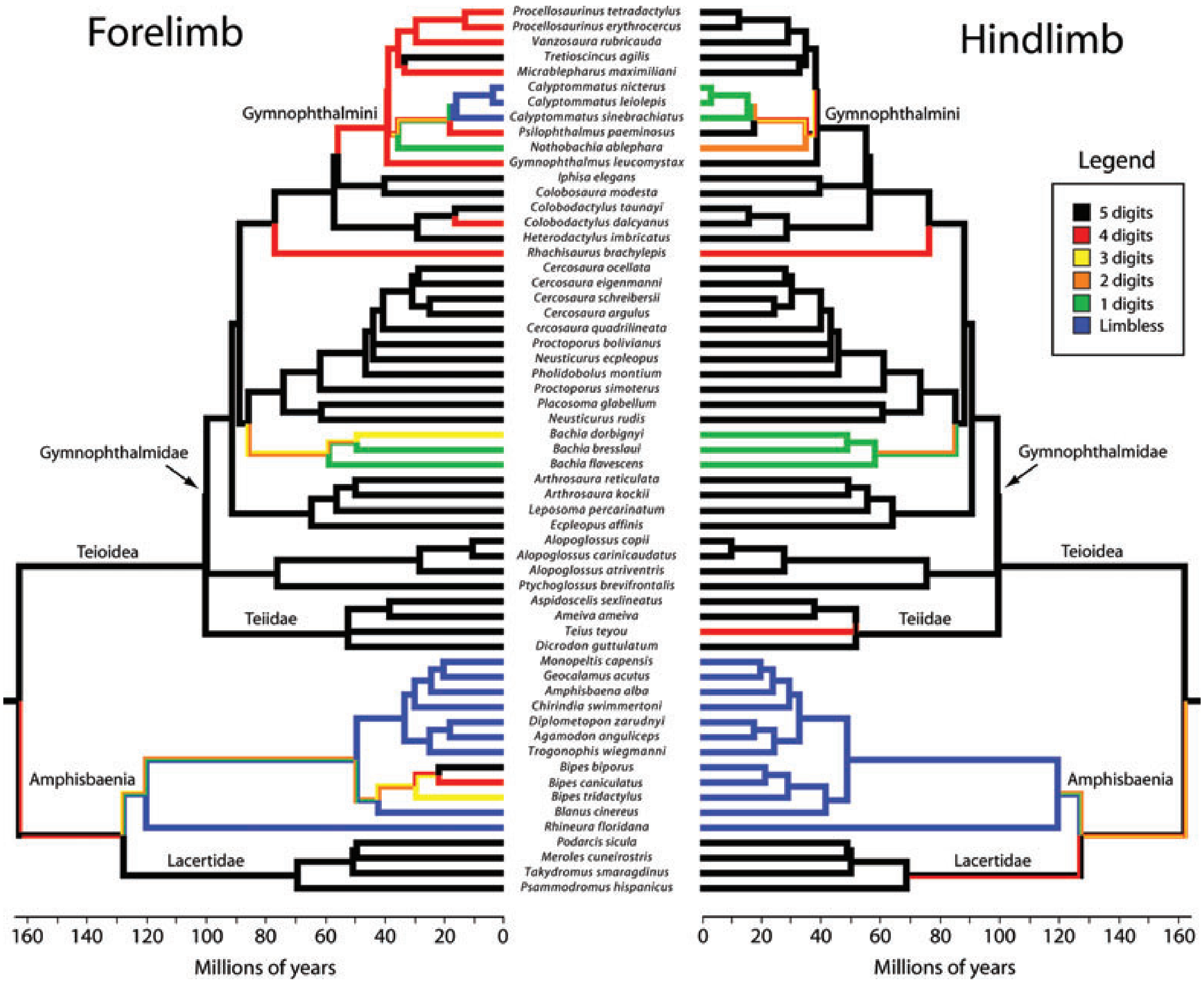

Figure 6. Chronograms and estimated ancestral states of digit number in the forelimb and hind limb of amphisbaenian, lacertid, gymnophthalmid, and teiid squamate reptiles. Solid color branches represent character states with a cumulative posterior probability ≥ 0.95. Branches with ambiguous ancestral reconstructions are indicated in multiple colors; these colors represent the multiple states whose posterior probabilities sum to ≥ 0.95.

toward hind limb loss in this group (given that the basal, limb-reduced *Bachia* possess forelimbs and lack hind limbs). There is no obvious phylogenetic or ecological explanation for these differences in the order of limb loss (Table 5). For example, *Bachia* belong to the Gymnophthalmidae, in which other limb-reduced taxa have lost only the forelimbs. Similarly, forelimbs are lost before hind limbs in both burrowing and surface-dwelling forms.

What might explain the generally similar patterns of body-form evolution across squamates? The two most obvious candidates are natural selection for similar function and intrinsic biases in development. These hypotheses are not mutually exclusive, and cannot be readily distinguished with our data. The association between morphologies and ecologies strongly suggests that, in general, natural selection drives the repeated evolution of these ecomorphs (Wiens et al. 2006). However, it may be that the similarity in the morphological changes by which these ecomorphs evolve (and the similarity between them) is also related to shared aspects of development. Some important exceptions (e.g., *Bachia*, *Bipes*) would seem to argue against a universally shared developmental program across squamates, however.

TEMPO OF CHANGE

In this article, we combine estimates of the ages of clades with ancestral trait reconstructions to make inferences about the rate at which major transformations in body form occur. To our knowledge, relatively few studies (if any) have combined divergence time estimation and ancestral reconstruction in this manner before. This combined approach should be applied cautiously, however, because there is the potential for errors in estimating both ancestral states (e.g., Schultz et al. 1996; Oakley and Cunningham 2000; Wiens et al. 2007) and divergence dates (e.g.,

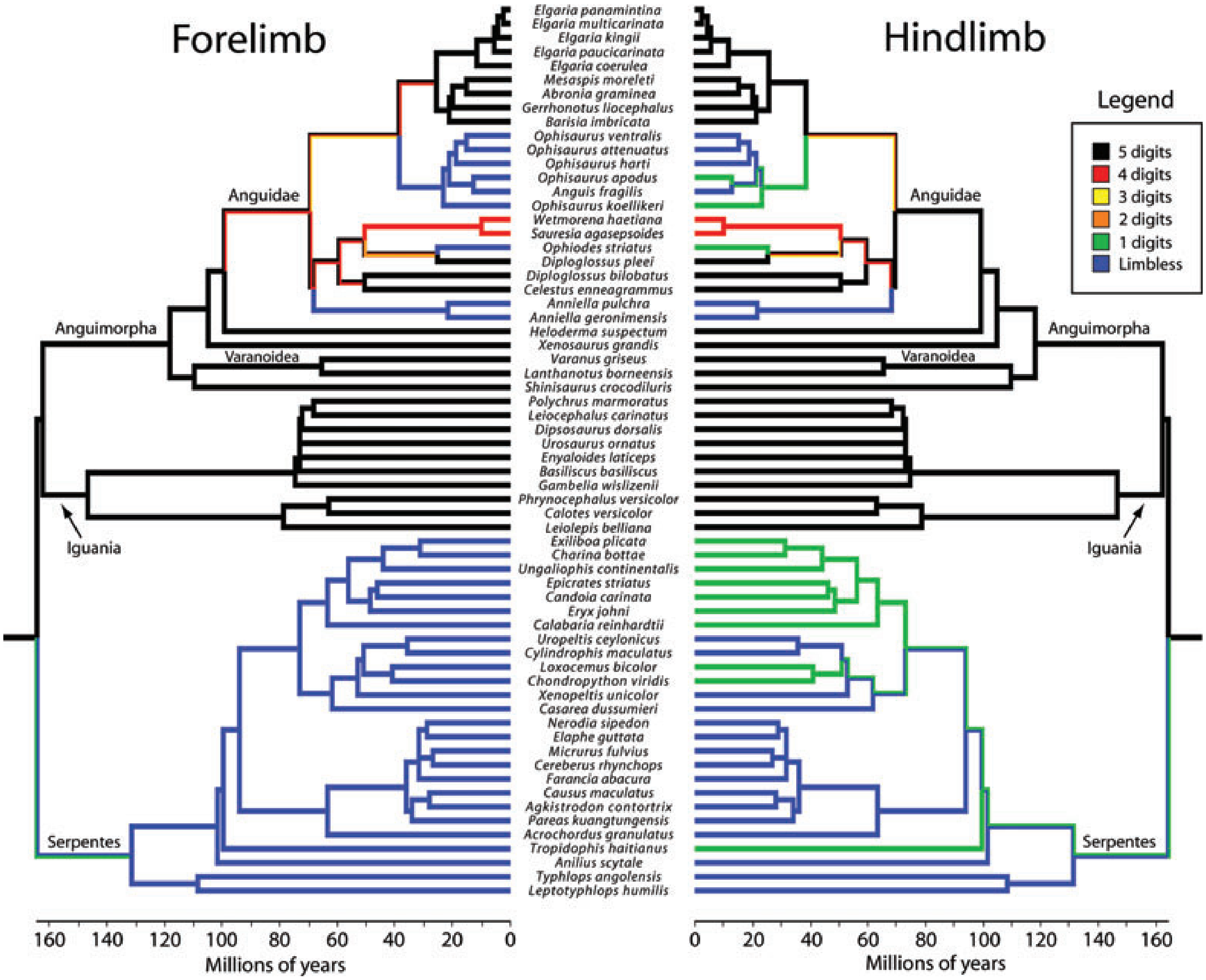

Figure 7. Chronograms and estimated ancestral states of digit number in the forelimb and hind limb of anguimorph and iguanian squamate reptiles. Solid color branches represent character states with a cumulative posterior probability ≥ 0.95. Branches with ambiguous ancestral reconstructions are indicated in multiple colors; these colors represent the multiple states whose posterior probabilities sum to ≥ 0.95.

Graur and Martin 2004; Pulquério and Nichols 2007). Despite these caveats, this combined approach reveals two major results in squamates.

First, the dramatic transition from a fully limbed (i.e., all digits present) to a fully limbless body form has occurred in several clades in less than 30 My (Table 6). At the same time, we presently have no evidence that this process occurs in less than 16 My (Table 6). However, it is possible that future studies with more dense taxon sampling (e.g., within certain scincid genera such as *Brachymeles* and *Lerista*) may show that this change occurs more rapidly. Furthermore, our approach can generally only estimate the maximum amount of time over which this transformation occurred, given that we cannot tell when a change occurred along a single branch. Nevertheless, our analysis offers a rough estimate for the time frame over which this major change in body form occurs.

The second major result from our dated ancestral reconstructions is that morphologies that seemingly are intermediate between fully limbed and fully limbless (i.e., those with a reduced but nonzero number of digits) can persist for relatively long periods of time (Table 7), between 9 and 63 My. Importantly, in many cases, these intermediate morphologies seem to be retained for periods of time that are as long or longer than the time estimated for limbs to go from being well-developed to completely absent in other taxa (i.e., one cannot argue that the limbs are retained simply because there has not been enough time for them to disappear). Thus, we suggest that these intermediate morphologies may not be simply transitory points on the pathway between fully limbed and limbless morphologies. Instead, there may be selection specifically for the origin and long-term maintenance of these seemingly intermediate morphologies.

Table 5. Species or clades in which limb loss differs between the pectoral and pelvic girdles.

Transition	Clade	Ecomorph
Forelimbs absent, hind limbs present		
	Anguidae	
	Ophisaurus + Anguis	Surface-dweller
	Gymnophthalmidae	
	Calyptommatus	Burrower
	Pygopodidae	Mostly Surface-dweller, Burrower[2]
	Scincidae	
	Lerista bipes	Burrower
	Pygomeles braconnieri	Burrower
	Some *Scelotes*	Burrower
	Voeltzkowia fieriensis	Burrower
Hind limbs absent, forelimbs present		
	Amphisbaenia	
	Bipes	Burrower
	Gymnophthalmidae	
	Bachia[1]	Burrower

We define "limb" as any externally recognizable remnant of the limb bone.

[1] Species unsampled in this study. See Kohlsdorf and Wagner (2006).

[2] The burrower ecomorph of *Aprasia* is derived from a surface-dweller ancestor (Wiens et al. 2006).

One important implication of this hypothesis is that so-called intermediate morphologies may evolve under different selection pressures than those favoring fully limbless forms, and their patterns of morphology, development, and function may not necessarily be representative of an intermediate stage in the transition from fully limbed to fully limbless forms (although we, and most previous authors, have assumed that they are). Partially reduced limbs may be maintained by natural selection because they are somehow important in locomotion. Unfortunately, there are very few published studies that examine locomotion in fully (or nearly) limbless squamates (Gans and Gasc 1990; Gasc and Gans 1990; Walton et al. 1990), although one study focusing on two partially limb-reduced gymnophthalmids found that these species primarily use "limbless" forms of locomotion, such as lateral undulation and concertina, but may also simultaneously move their limbs (Renous et al. 1995). Observational evidence suggests that reduced limbs are used to balance the body, push through vegetation, and to assist burrowing in sandy soils (Bruno and Maugeri 1976; Orsini and Cheylan 1981; Wagner 2005). There is also evidence that the extremely small, sexually dimorphic hind limbs in male boiid snakes are used during copulation (Murphy et al. 1978), but it remains unknown if they are used in a similar fashion in other lineages with similar morphologies (e.g., dibamids and pygopodids).

DIRECTION OF CHANGE: RE-EVOLUTION OF LOST LIMBS AND DIGITS

The notion that digit and limb loss are unidirectional has long influenced the study of squamate limb evolution (e.g., Fürbringer 1870; Sewertzoff 1931; Stokely 1947; Gans 1975; Presch 1975; Lande 1978; Renous and Gasc 1979; Choquenot and Greer 1987; Greer 1987, 1990, 1991; Caputo et al. 1995; Greer et al. 1998;

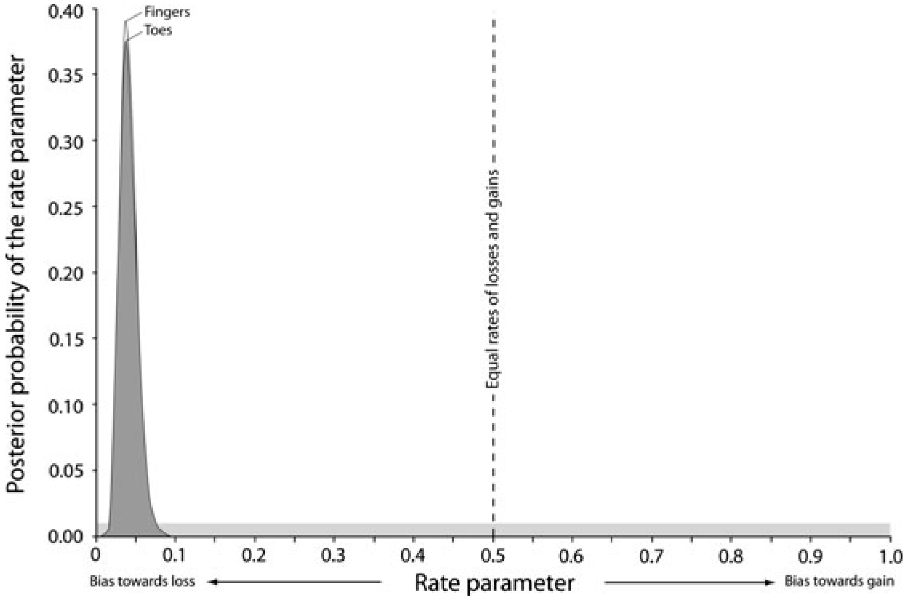

Figure 8. Posterior distributions of the rates of digit change estimated by MCMC ancestral reconstruction analyses for both the fingers and toes. We assumed a uniform (0,1) prior (gray box), and the mean of this distribution, 0.50, represents equal rates of losses and gains. Any deviation from the prior probability (0.50) indicates a bias toward digit losses (< 0.50) or gains (> 0.50).

Table 6. **Estimated time for the transition from a pentadactyl, tetrapod lizard-like ancestor to a snake-like morphology lacking forelimbs and/or hind limbs.**

Clade	Limbs lost	Time (My)	Ecomorph
Anguidae			
Anniella	Both	45.7	Burrower
Ophisaurus+*Anguis*	Forelimb	15.5	Surface-dweller
Ophiodes striatus	Forelimb	24.9	
Amphisbaenia	Both	42.5[1]	Burrower
Dibamidae			
Dibamus novaeguineae	Forelimb	176.4	Burrower
Gymnophthalmidae			
Calyptommatus	Forelimb	39.5	Burrower
Scincidae			
Acontine skinks	Both	68.7	Burrower
Anomalopus swansonii	Both	39.8[1]	Burrower
Feylinia+*Melanoseps*+*Typhlacontias*	Both	41.4[1]	Burrower
Lerista bipes	Forelimb	35.5[1]	Burrower
Ophioscincus ophioscincus	Both	29.4[1]	Burrower
Paracontias	Both	54.9[1]	Burrower
Pygomeles braconnieri	Forelimb	57.5[1]	Burrower
Scelotes bipes, S. gronovii, S. kasneri+*S. sexlineatus*	Forelimb	58.6[1]	Burrower
Some *Scelotes*	Both	58.6[1]	Burrower
Voeltzkowia	Forelimb	57.5[1]	Burrower
Voeltzkowia lineata	Both	57.5[1]	Burrower
Pygopodidae	Forelimb	28.2	Mostly Surface-dweller, Burrower[2]
Serpentes	Forelimb only	32.9[1]	Burrower
Typhlops+*Leptotyphlops*	Both	55.7[1]	Burrower
Acrochordus+other snakes	Both	100.8[1]	Burrower
Cylindrophis+*Uropeltis*	Both	128.8[1]	Burrower
		Median=50.3	

[1] **May be severely overestimated due to ambiguous ancestral state reconstructions.**

[2] **The genus *Aprasia* belongs to the burrowing ecomorph, but is derived from a surface-dwelling ancestor (Wiens et al. 2006).**

Whiting et al. 2003; Kearney and Stuart 2004). Although our analyses confirm that digit losses are indeed common (at least 70 examples), we found statistically significant support for at least six instances of re-evolution of lost digits. The majority of these gains are of one or two digits (but note that each digit consists of several phalangeal elements).

However, two cases involved relatively well-developed limbs (with many digits) that seemed to have re-evolved from highly limb-reduced ancestors. These cases involved the amphisbaenian genus *Bipes* and the scincid *Scelotes mirus*. These cases were also discussed by Whiting et al. (2003; *Scelotes*) and Kearney and Stuart (2004; *Bipes*), but these authors argued against re-evolution of lost digits, given that losses are so common in other clades. Within *Bipes*, there is statistically significant support for the re-evolution of pentadactyly through "intermediate" stages over the span of approximately 40 My (Fig. 6). Although the ordered analyses cannot resolve whether these limbs evolved from a completely limbless ancestor or not (due to ambiguity in the reconstructed state of the ancestor to living Amphisbaenia), the unordered analyses strongly support this hypothesis.

We note that there is some circumstantial evidence that suggests that re-evolved digits might develop through different pathways than digits that are ancestrally present (see also Kohlsdorf and Wagner 2006). Specifically, data on phalangeal formulae in *Bipes* and *Scelotes* show interesting parallels with those from *Bachia*, in which re-evolution of digits is reconstructed unambiguously (Kohlsdorf and Wagner 2006). In *Bachia,* the phalangeal formula of the seemingly re-evolved limbs differs from the primitive pattern among squamates (forelimb: 2-3-4-5-3; hind limb: 2-3-4-5-4) in being unusually uniform among digits (i.e., 0-2-2-2-2 in the forelimb and 2-2-2-2-0 in the forelimb). Based on phalangeal data from *Bachia* and developmental genetic data from mice, Kohlsdorf and Wagner (2006) suggested that the uniformity among digits might be explained by the identity of one digit being shared by multiple digits, a pattern similar to a known mutation (in mice) in the *Gli3* gene. In *B. biporus* the forelimb

Table 7. Evidence for long-term persistence of seemingly "intermediate" body forms in the transition from lizard-like to snake-like body form in squamates.

Clade	Finger number	Toe number	Age (My)
Amphisbaenia			
Bipes caniculatus+B. tridactylum	3, 4, or 5	0	28.4
Anguidae			
Ophisaurus koellikeri	0	1	38.2
Sauresia+Wetmorena	4	4	9.3
Gerrhosauridae			
Tetradactylus africanus+T. tetradactylus	3 or 4	3 or 4	16.3
Gymnophthalmidae			
Bachia	3 to 1	1	57.6
Calyptommatus	0	1	15.1
Gymnophthalmini	4 to 1	5	34.5
Pygopodidae	0	1	20.9
Scincidae			
Anomalopus mackayi	0	1	18.7
Coeranoscincus reticulatus+Saiphos equalis	3	3	20.8
Scelotes	3 to 0	3 to 0	29.7
Voeltzkowia fieriensis	0	2	25.9
Serpentes			
Calabaria reinhardtii+Boids	0	1	63.1
Chondropython viridis+Loxocemus bicolor	0	1	40.5
		Median =	27.2

Taxa represent extant clades with one to four digits on the fore and hind limb in both extant and reconstructed ancestors.

Table 8. Re-evolution of lost digits in squamate reptiles.

Lineage	Ancestral number of fingers	Extant number of fingers	Ancestral number of toes	Extant number of toes	Posterior probability of ancestral state
Amphisbaenia					
Bipes tridactylus	0,1, or 2	3			**0.981**[1]
Bipes biporus	2 or 3	4			**0.983**[1]
Bipes caniculatus	3 or 4	5			**0.984**[1]
Gymnophthalmidae					
Tretioscincus agilis	4	5			**0.978**
Scincidae					
Scelotes mirus	1, 2, or 3	5			**0.980**[1]
			1,2, or 3	5	**0.980**[1]

The taxa listed below have immediate ancestors that were reconstructed as possessing fewer digits in either the forelimb or hind limb than the extant species or clade (strongly implying the re-evolution of lost digits in these extant taxa). Posterior probabilities represent the probability of the estimated number of digits of the forelimb or hind limb, or fewer in the ancestor (i.e., posterior probabilities of multiple states are added if they are less than the extant taxon). Probabilities in bold are statistically significant ($\geq$ 0.95). Data are only presented for limbs in which a digit gain occurred.

[1] Combined posterior probabilities of ancestral states.

phalangeal formula is 3-3-3-3-3 (Zangerl 1945). Thus, the phalangeal formula is also modified in this species toward greater uniformity of digits, both in terms of gain (on digit 1) and loss (on digits 3 and 4) of phalangeal elements. In *S. mirus*, another species in which the limbs may have re-evolved, the formulae are 2-3-3-3-2 for the forelimb and 2-3-4-4-2 for the hind limb (M. C. Brandley, pers. obs.), patterns that also suggest greater uniformity among digits than the primitive formulae, but only through loss.

Another interesting difference between the normal and potentially re-evolved limbs involves the location of the pectoral girdle.

In *Bipes* the pectoral girdle is unusually close to the head, at the third cervical vertebrae rather than at the sixth cervical (or more posteriorly) as in other squamates (Kearney 2002). This difference suggests the possibility that the position of *Hoxc6* expression has shifted in *Bipes* (see Carroll et al. (2005) for a discussion of the effects of this gene).

These differences in the morphology of re-evolved limb elements (relative to the ancestral morphology) could be interesting subjects for future study. More research is also needed to assess why digits and limb re-evolve. Based on the relationship between decreasing limb size and digit number evidenced by this study, we speculate that digit re-evolution might be a pleiotropic effect of increasing limb size. The causes of limb re-evolution are even less clear.

Conclusions

A major challenge in evolutionary biology is to understand the transitions between dramatically different body forms. Here, we have analyzed the transitions from lizard-like to snake-like body forms in squamate reptiles. Based on morphometric analyses, we find that the evolution of these ecomorphs involves similar concerted changes in terms of body elongation, limb reduction, and digit loss, despite the immense time scale involved ($>$180 My) and different ecologies of the snake-like taxa (i.e., burrowers and surface-dwellers). We do find significant differences in how body elongation is achieved between the ecomorphs, and some differences in patterns of limb and digit loss between clades. We also find that the re-evolution of previously lost digits is surprisingly common, with at least six strongly supported cases, two of which may involve the re-evolution of entire, pentadactyl forelimbs from a limbless ancestor.

In this study, we have also used a relatively novel phylogenetic approach to study the evolution of body form. Specifically, we use a time-calibrated phylogeny to make inferences about the tempo at which these body-form transitions occur. From these analyses, we find that these transitions occur over roughly 20–70 million of years. But we also find that intermediate morphologies persist on time scales of tens of millions of years, suggesting that these intermediate morphologies are actively maintained by selection and are not simply fleeting transitory stages in the evolution from lizard-like to snake-like body forms.

Our research leaves open many areas for future exploration. The similar changes in body form that we document are presumably the result of similar developmental processes and selection pressures, but we are unable to parse out the relative importance of these factors at this point. The developmental basis for this transition remains poorly understood (but see Cohn and Tickle 1999; Shapiro 2002; Shapiro et al. 2003; Carroll et al. 2005), as are the functional consequences of limb reduction (e.g., Gans 1975; Gans and Gasc 1990; Gasc and Gans 1990). Even the basic anatomical changes that underlie the evolution of these ecomorphs would benefit from further study (e.g., vertebral number, phalangeal formulae). Based on our results, future studies should bear in mind that the seemingly intermediate limb-reduced morphologies may represent targets of selection that are independent of the extreme lizard-like and snake-like body forms, and that many taxa have regained limb elements as well as lost them.

ACKNOWLEDGMENTS

We thank T. Kohlsdorf for helpful clarification of methods, R. Chong and M. Fujita for the photographs, and A. Seago for the illustrations in Figure 1. We thank D. Moen, A. Seago, G. Wagner, and one anonymous reviewer for useful comments on the manuscript. This study was funded by the Koford grant from the Museum of Vertebrate Zoology, and NSF Doctoral Dissertation Improvement Grant (DEB 0709885) awarded to MCB; NIH grant GM 069801 and NSF grant DEB 0445453 awarded to JPH; and NSF grant EF 0334923 awarded to JJW. We thank T. Reeder for helping to compile the original morphometric dataset, and the many museum curators and collection managers who facilitated our access to specimens (listed in Wiens et al. 2006).

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Associate Editor: K. Crandall

APPENDIX

INFERRING PATTERNS OF CHARACTER CHANGE

We performed likelihood analyses of the finger and toe character data. The data consisted of character-state information for $s = 258$ squamate reptiles. The character states were coded 0, 1, 2, 3, 4, 5 and indicate the number of toes or fingers observed for each species, with "0" indicating the absence of toes/fingers. The data, in part, look like

Abronia graminea	5
Acontias litoralis	0
Acontias meleagris	0
⋮	⋮
Xenopeltis unicolor	0
Xenosaurus grandis	5
Zonosaurus ornatus	5

for the finger data and are coded in a vector $\mathbf{x} = (5, 0, 0, \ldots, 0, 5, 5)'$.

We assume that the species are related through a rooted phylogenetic tree (τ) with branch lengths $[\mathbf{v} = (v_1, v_2, \ldots, v_{2s-2})]$ estimated from other data, such as an alignment of DNA sequences. There is not enough information in a single character to estimate the tree and branch lengths, so we consider both to be fixed in this analysis. However, there is some information about the rate of change in the observed character, $\mathbf{x}$. For example, if the character has only one species with a different state, we would intuitively expect the rate of change for the character to be low. Similarly, if we observe many different states for the species, we would expect the rate of character change to be high. In this study, we consider the branch-length proportions, parameters we denote $\mathbf{p} = (p_1, p_2, \ldots, p_{2s-2})$ with the ith proportion calculated as $p_i = v_i / \sum_{j=1}^{2s-2} v_j$, to be fixed, but allow the tree length (T, the sum of the branch lengths) to be estimated from the data.

The character states evolve on the tree according to a continuous-time Markov model, with rate matrix $\mathbf{Q}$. The rate matrix describes the instantaneous rate of change from character state i to character state j. In this study, we consider two alternative rate matrices. The first model we consider allows a change from state i to any other state to occur in an instant of time

$$\mathbf{Q} = \begin{pmatrix} -5\alpha & \alpha & \alpha & \alpha & \alpha & \alpha \\ \beta & -(\beta+4\alpha) & \alpha & \alpha & \alpha & \alpha \\ \beta & \beta & -(2\beta+3\alpha) & \alpha & \alpha & \alpha \\ \beta & \beta & \beta & -(3\beta+2\alpha) & \alpha & \alpha \\ \beta & \beta & \beta & \beta & -(4\beta+\alpha) & \alpha \\ \beta & \beta & \beta & \beta & \beta & -5\beta \end{pmatrix} \mu,$$

where μ is a scaling factor that is chosen such that the mean rate of substitution is one. The second model we consider orders the character states $0 \longleftrightarrow 1 \longleftrightarrow 2 \longleftrightarrow 3 \longleftrightarrow 4 \longleftrightarrow 5$ and forces the evolution of the character to occur through intermediate states. This model has rate matrix

$$\mathbf{Q} = \begin{pmatrix} -\alpha & \alpha & 0 & 0 & 0 & 0 \\ \beta & -(\beta+\alpha) & \alpha & 0 & 0 & 0 \\ 0 & \beta & -(\beta+\alpha) & \alpha & 0 & 0 \\ 0 & 0 & \beta & -(\beta+\alpha) & \alpha & 0 \\ 0 & 0 & 0 & \beta & -(\beta+\alpha) & \alpha \\ 0 & 0 & 0 & 0 & \beta & -\beta \end{pmatrix} \mu.$$

For both models, we impose the constraint that $\alpha + \beta = 1$. The stationary probabilities of the models are denoted $\boldsymbol{\pi}$ and are calculated by solving the equation $\boldsymbol{\pi}\,\mathbf{Q} = \mathbf{0}$.

We estimate parameters of the model in a Bayesian framework, basing inferences on the posterior probability distribution of the parameters, which can be calculated using Bayes' theorem as

$$\mathbb{P}(T, \alpha, \beta | \mathbf{x}, \tau, \mathbf{p}, a, b) = \frac{\mathbb{P}(\mathbf{x} | T, \alpha, \beta, \tau, \mathbf{p})\, \mathbb{P}(T | a, b)\, \mathbb{P}(\alpha, \beta)}{\mathbb{P}(\mathbf{x} | \tau, \mathbf{p}, a, b),}$$

where $\mathbb{P}(\mathbf{x} | T, \alpha, \beta, \tau, \mathbf{p})$ is the likelihood, $\mathbb{P}(T | a, b)$ is the prior probability distribution of the tree length, $\mathbb{P}(\alpha, \beta)$ is the prior probability distribution for the rate parameters α and β, and $\mathbb{P}(\mathbf{x}|\tau, \mathbf{p})$ is the marginal likelihood. The likelihood is the probability of the data conditional on the parameters of the model taking specific values. We calculate the likelihood using the pruning algorithm first described by Felsenstein (1981), but condition on the character being variable (Lewis 2001). We assume that the tree length follows a gamma prior probability distribution with parameters a and b (Huelsenbeck et al. 2003). We further assume that the rate parameters α and β, which are constrained to sum to one, follow a flat Beta probability distribution.

The marginal likelihood involves a multidimensional integral over all possible combinations of tree lengths and rate parameters. We use Markov chain Monte Carlo (MCMC) to approximate the posterior probability distribution of T, α, and β (see Metropolis et al. 1953; Hastings 1970). The general idea is to construct a Markov chain that has as its state space the parameters of the statistical model and a stationary distribution that, in this case, is the posterior probability distribution of the parameters. Parameter values sampled from the chain are valid samples from the posterior probability distribution (Tierney 1996). We use two proposal mechanisms to update the tree length and the rate parameters. We propose a new tree length, T', by multiplying the old tree length, T, by a random factor

$$T' = T \times e^{\lambda(u-1/2)},$$

where λ is a tuning parameter specified at the beginning of the MCMC analysis and u is a uniform(0,1) random variable (Larget and Simon 1999). We propose a new value for α using a sliding window mechanism. A new value for α, denoted α' is proposed by adding a random factor to the original value of the parameter

$$\alpha' = \alpha + w(u - 1/2),$$

where w is the window size and u is a uniform(0,1) random variable. If the proposed value for α is negative or greater than one, we reflect the value into the acceptable region. The proposed value for β is then $\beta' = 1 - \alpha'$. The proposed state is accepted with probability

$$R = \min\left(1, \frac{\mathbb{P}(\mathbf{x}|T', \alpha, \beta, \tau, \mathbf{p})}{\mathbb{P}(\mathbf{x}|T, \alpha, \beta, \tau, \mathbf{p})} \times \frac{\mathbb{P}(T'|a, b)}{\mathbb{P}(T|a, b)} \times \frac{T'}{T}\right)$$

for updates of the tree length and with probability

$$R = \min\left(1, \frac{\mathbb{P}(\mathbf{x}|T, \alpha', \beta', \tau, \mathbf{p})}{\mathbb{P}(\mathbf{x}|T, \alpha, \beta, \tau, \mathbf{p})} \times \frac{\mathbb{P}(\alpha', \beta')}{\mathbb{P}(\alpha, \beta)}\right)$$

for updates of the rate parameters.

We evaluated the hypothesis that $\alpha = \beta$ using Bayes factors. The Bayes factor of a comparison of models M_1 and M_2 is calculated as the ratio of the marginal likelihoods

$$BF = \frac{\mathbb{P}(\mathbf{x}|M_1)}{\mathbb{P}(\mathbf{x}|M_2)}$$

or the ratio of the posterior odds to the prior odds of the two models

$$BF = \frac{\mathbb{P}(\mathbf{x}|M_1)}{\mathbb{P}(\mathbf{x}|M_2)} = \frac{\mathbb{P}(M_1|\mathbf{x})}{\mathbb{P}(M_2|\mathbf{x})} \Big/ \frac{\mathbb{P}(M_1)}{\mathbb{P}(M_2)}.$$

When one of the models is nested within the other, as is the case for our comparison of M_1: $\alpha = \beta$ and M_2: $\alpha \neq \beta$, the Bayes factor can be calculated using the Savage–Dickey Ratio (Verdinelli and Wasserman 1995). In short, the MCMC analysis is performed under the general model, and the ratio of the posterior density to the prior density at the restriction that $\alpha = \beta$ is evaluated. We fit the samples of α and β to a beta probability distribution to compute the ratio at the restriction.

The Bayes factor test of the hypothesis that $\alpha = \beta$ allows us to test for a bias in the pattern of character change.