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VERTEBRAL EVOLUTION AND THE DIVERSIFICATION OF SQUAMATE REPTILES

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Taxonomic, morphological, and functional diversity are often discordant and independent components of diversity. A fundamental and largely unanswered question in evolutionary biology is why some clades diversify primarily in some of these components and not others. Dramatic variation in trunk vertebral numbers (14 to >300) among squamate reptiles coincides with different body shapes, and snake-like body shapes have evolved numerous times. However, whether increased evolutionary rates or numbers of vertebrae underlie body shape and taxonomic diversification is unknown. Using a supertree of squamates including 1375 species, and corresponding vertebral and body shape data, we show that increased rates of evolution in vertebral numbers have coincided with increased rates and disparity in body shape evolution, but not changes in rates of taxonomic diversification. We also show that the evolution of many vertebrae has not spurred or inhibited body shape or taxonomic diversification, suggesting that increased vertebral number is not a key innovation. Our findings demonstrate that lineage attributes such as the relaxation of constraints on vertebral number can facilitate the evolution of novel body shapes, but that different factors are responsible for body shape and taxonomic diversification.

KEY WORDS: **Body shape, disparity, morphological diversity, rate of evolution, supertree, taxonomic diversity.**

Explaining patterns of organismal diversity is a central aim of evolutionary biology (Darwin 1859; Schluter 2000; Ricklefs 2004; Pennisi 2005). Historically, species richness has been the primary proxy for diversity (Mitter et al. 1988; Davies et al. 2004; Fernandes et al. 2004; Lynch 2009), but other types of diversity may be equally relevant (Hunter 1998; Roy et al. 2004). For example, genetic (Bowen 1999; Ferguson 2002), morphological (Foote 1997; Harmon et al. 2003; Sidlauskas 2008), and functional or ecological diversity (Wainwright 2007; Young et al. 2007; Mouchet et al. 2010) are all components of organismal diversity. Although these components of diversity sometimes show similar patterns, they are often discordant, with complex relationships among them (Foote 1993; Roy et al. 2004; Collar and Wainwright 2006; Adams et al. 2009). The occurrence of cryptic species (Wiens and Penkrot 2002; Bergmann and Russell 2007), geographic mismatches between morphological and taxonomic diversity (Roy et al. 2001; Neige 2003), and many-to-one mapping of phenotypes on function (Alfaro et al. 2004) are compelling examples of these complex relationships. Thus, the questions of why some clades are diverse along some axes of diversity and not others, and why clades differ in these various types of diversity are important in evolutionary biology.

Many ecological and historical processes have been proposed as drivers of diversification within clades (Ricklefs 2004), including ecological release (Archibald and Deutschmann 2001; Bininda-Emonds et al. 2007), competition (Abrams 1986), key innovations (Mayr 1963; Hunter 1998), and constraints (Wagner 1988; Schwenk and Wagner 2003; Futuyma 2010). In this study, we focus on the role of key innovation and constraint in the diversification of squamate reptiles in the context of their vertebral evolution. A key innovation is a structure or property that allows

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a taxon to perform novel functions, thereby facilitating access to new niches, and ultimately resulting in increased rates of diversification (Mayr 1963; Schluter 2000; Gavrilets and Losos 2009). Testing for key innovation has typically involved establishing a positive relationship between the presence of a trait and species richness (e.g., Liem 1973; Hodges and Arnold 1995; Alfaro et al. 2009), but has largely ignored other aspects of diversity (Hunter 1998).

The influences of constraints on diversification are widely discussed yet poorly understood (Wagner 1988; Schwenk and Wagner 2003; Futuyma 2010). Constraints can either be structural, resulting from universal physical laws (Gillooly et al. 2001), or evolutionary, resulting from limits on the evolved organismal phenotype or body plan (Wake 1991; Cullinane 2000; Gould 2002). No matter how constraints are defined, they act to limit the variation in phenotypes, disallowing certain evolutionary options, and ultimately limiting diversification (Wagner 1988; Cullinane 2000; Brakefield 2006; Harmon et al. 2010). Conversely, the relaxation of constraints acting on a morphological trait may lead to increased morphological and functional diversification, the invasion of new niches, and ultimately allowing for increased speciation, as may be seen in adaptive radiation (Schluter 2000).

Vertebral number is a trait that is often perceived as being under a high degree of constraint. A case in point is mammals (Slijper 1946), in which almost all species have seven cervical vertebrae (Galis 1999) due to strong stabilizing selection (Galis and Metz 2007). However, in many other taxa, including some clades of fish (Ward and Brainerd 2007), salamanders (Parra-Olea and Wake 2001), and lizards (Greer 1991), this constraint is relaxed, and the number of vertebrae is highly variable. These clades often exhibit pleomerism, where species with increased vertebral number have more elongate bodies (Presch 1975; Greer 1990; Parra-Olea and Wake 2001), and consequently, the evolution of body shape in such groups is in large part mediated through the evolution of vertebral number (Ward and Brainerd 2007). However, vertebral length is also an important determinant of body proportions, and so evolutionary changes in body shape can be mediated primarily through changes in vertebral number, changes in vertebral length, or both. Indeed both aspects of vertebral morphology are involved in body shape evolution in fish (Ward and Brainerd 2007), and with only seven vertebrae, the long neck of the giraffe is exclusively the result of vertebral lengthening (van Sittert et al. 2010).

Vertebral morphology and number has far-reaching consequences for organismal function and ecology. Vertebral number influences body flexibility and locomotor performance in many clades, particularly fish and squamate reptiles (Brainerd and Patek 1998; Porter et al. 2009; Bergmann and Irschick 2010), and the same may be true for vertebral shape (Buchholtz and Schur 2004). In turn, body shape influences diet (Pough et al. 1997), and habitat use in squamates (Melville and Swain 2000; Van Damme and Vanhooydonck 2002). Furthermore, body shape and vertebral number are heritable and often the target of selection (Shine 2000; Walsh et al. 2006; Yamahira et al. 2006). These studies demonstrate a relationship between vertebral number, body shape, locomotor function, and niche use, but they do not address how these aspects relate to morphological and taxonomic diversification at a macroevolutionary level, and this is what we set out to accomplish.

Here, we examined two hypotheses regarding vertebral evolution and diversification in squamates. First, we tested the hypothesis that a relaxation of constraint on vertebral number in certain clades of squamates coincides with increases in body shape and taxonomic diversification. Given the documented relationships among vertebral number, body shape, and niche use discussed above, we predicted that a relaxation of constraint on vertebral number evolution would coincide with increased rates of body shape evolution. This would facilitate the occupation of a wider range of niches, ultimately leading to increased speciation rates due to increased ecological opportunity associated with filling those niches (Simpson 1944; Mayr 1963; Schluter 2000). Second, we tested the hypothesis that the acquisition of many vertebrae represents a key innovation, and so has allowed for further body shape and taxonomic diversification. The evolution of many vertebrae is frequently associated with a snake-like body shape and is a common theme in vertebrate evolution (Parra-Olea and Wake 2001; Wiens et al. 2006; Ward and Brainerd 2007; Brandley et al. 2008). Under various circumstances, one might expect increased vertebral numbers to contribute to either increases or decreases in diversification rates. An increase in diversification would be expected if a snake-like body shape represents a key innovation that has allowed the invasion of new adaptive zones (Simpson 1944; Mayr 1963). We predict that the evolution of more vertebrae will coincide with increased taxonomic diversification rate, as could be argued for snakes themselves, which account for ∼38% of extant squamate species (Greene 2000; Uetz et al. 2007). However, the alternative outcome, a decrease in diversification rate, is possible if a snake-like body shape represented an evolutionary dead-end: a steep adaptive peak that constrained further evolution, as documented with piscivory in centrarchid fish (Collar et al. 2009).

We tested these hypotheses using phylogenetically informed measures of constraint, body shape diversification, and taxonomic diversification. We used the rate of evolution of vertebral number as our measure of constraint on this trait (O'Meara et al. 2006). We used two measures of body shape diversity: the rate of evolution in body shape variables and their lineage density or disparity (Sidlauskas 2008). The rate of evolution and disparity reveal different aspects of morphological diversity because the former can be interpreted as how easily a trait changes through time and the latter as how easily new areas of morphospace are explored (O'Meara et al. 2006; Sidlauskas 2008). Our measure of taxonomic diversification was species richness, adjusted for age of the most recent common ancestor (MRCA) of each clade (Magallón and Sanderson 2001; O'Meara et al. 2006). We chose squamate reptiles as a model system to test our hypotheses because a snake-like body has evolved >25 times in this taxon (Brandley et al. 2008) and because trunk vertebral numbers vary over an order of magnitude—from 14 in a dwarf chameleon to over 300 in some snakes (Polly et al. 2001; this study). With ∼8400 species, body shapes ranging from snake-like to very short bodied, and with some clades being more diverse in body shape than others (Wiens et al. 2006), squamates are both morphologically and taxonomically diverse. To test our hypotheses, we constructed a supertree including 1375 species of squamates, and compiled large vertebral and morphometric datasets.

Methods

SUPERTREE CONSTRUCTION

Data collection and coding

We constructed a supertree phylogeny of squamates that constitutes a maximally inclusive estimate of phylogeny, and a most parsimonious synthesis of all published phylogenies (Sanderson et al. 1998; Bininda-Emonds et al. 1999; Sanderson and Driskell 2003; Baum and Ragan 2004; Cardillo et al. 2004). In a supertree, conflict between source trees is interpreted as incongruence among data as opposed to homoplasy (Bininda-Emonds 2004). We produced a supertree of the Squamata, concentrating on lizards and sampling only a few snakes.

We used the Web of Science and Biological Abstracts online databases to identify source papers published between 1980 and 2006 (Cardillo et al. 2004). We conducted searches using the terms "phylog*" or "systemat*" or "clad*" and lizard taxon names (e.g., "Agami*") from Pianka and Vitt (2003). We also searched the Literature Cited sections of all of these publications. To be included, each publication had to: (1) present a formalized phylogenetic analysis of real data, (2) state the data type and analyses used, and (3) not be a pseudoreplicate of later work (Bininda-Emonds et al. 2004). When multiple phylogenies appeared within a study, we included multiple trees when they were based on independent datasets (different sequences, or morphology vs. DNA). We used multiple independent trees in preference to total evidence trees to allow inclusion of multiple phylogenetic signals. We also used phylogenies that presented clade support values over those that did not, and those phylogenies explicitly stated as preferred by authors of the original publications (Bininda-Emonds et al. 2004).

We converted phylogenies to matrix representation with parsimony (MRP) form by hand in Microsoft Excel (Bininda-Emonds et al. 2002; Bininda-Emonds et al. 2005). We coded taxa not included in a given study as missing, and used a hypothetical all-zero outgroup to root source trees (Sanderson et al. 1998; Baum and Ragan 2004). We also added a backbone taxonomy to the data matrix, including no missing data and consisting of 29 characters with no hierarchical structure that represented higher taxa of squamates (Bininda-Emonds and Sanderson 2001; taxa listed in Pianka and Vitt 2003). We chose MRP because it is computationally efficient, widely used, and performs well in obtaining an accurate phylogeny (Bininda-Emonds and Sanderson 2001; Eulenstein et al. 2004). We corrected all included species names for synonymy (Bininda-Emonds et al. 1999; Bininda-Emonds et al. 2004) using the Reptile Database (Uetz et al. 2007). Finally, we concatenated all MRP spreadsheets into a master matrix using the seqCat Perl script, written by and available from O. R. P. Bininda-Emonds.

We weighted each character by the clade support from the original published analysis, an approach that yields more accurate phylogenetic reconstructions because higher weighted characters are more robustly supported by the source data (Bininda-Emonds and Sanderson 2001; Salamin et al. 2002; Bininda-Emonds 2004; Davies et al. 2004; Moore et al. 2006). We included clades that had bootstrap or posterior probability support >50% in the original studies. In cases where two studies included large sets of common data but each included some new data or taxa, we down-weighted their characters by half (Bininda-Emonds et al. 2004; Gatesy and Springer 2004). Finally, we down-weighted characters from studies that did not present clade support values to 10% of the maximum possible weight, allowing the inclusion of their phylogenetic signal, while allowing more robust studies to overrule them (Gatesy and Springer 2004). It is important to note that this weighting scheme does not provide clade support values for the supertree, which is extremely difficult to do because of computational limitations and nonindependence of MRP characters (Bininda-Emonds et al. 1999; Moore et al. 2006).

Supertree phylogenetic analysis

We ran the MRP analysis using PAUP*4b10 (Swofford 2002). We used a parsimony ratchet (Nixon 1999), also used with other supertrees (Cardillo et al. 2004), to analyze the data because a standard heuristic search was prohibitively computationally intensive. We used PAUPrat version 1.0 software (Sikes and Lewis 2001) to produce scripts for the parsimony ratchet, which used 20 replicates of 200 iterations. For each replicate, we used a random starting seed for character weight perturbation, TBR branch swapping, one replicate of a stepwise random taxon addition sequence, and an additive weighting scheme. We retained one tree per iteration to maximize speed of analysis, and perturbed the weights of 15% of characters at each iteration. We retained only the shortest trees from each replicate and calculated a strict consensus of them using PAUP*.

Dating the supertree

Prior to dating, we pruned the tree to include only species for which we had vertebral count data using the Perl script TreePruner (available from O. R. P. Bininda-Emonds). Dating procedures followed those of Bininda-Emonds et al. (2007). We downloaded all nonmammalian vertebrate sequences from GenBank as flat files and used the Perl Script GenBankStrip (available from O. R. P. Bininda-Emonds) to identify genes with sequences for >30 lizard species and to compile files with longest gene sequences for each available species for each gene (Bininda-Emonds et al. 2007). We aligned gene sequence datasets using Clustal X (Thompson et al. 1997) and corrected them by eye using BioEdit version 7.0 (Hall 1999). The aligned sequence dataset included data for 26 genes (see Supporting information). For some genes, sequenced sections produced multiple largely independent blocks of data, and so we divided these genes into separate sections. ND2 and RNR2 were divided into two sections, and RAG-1 was divided into three sections. We then standardized the taxonomy between the supertree and the sequence datasets, retained a single sequence per species, and added outgroup sequences for *Mus musculus* and *Gallus gallus*.

Once we compiled sequence data in this way, we used ModelTest version 3.06 (Posada and Crandall 1998) to determine the best model of sequence evolution for each gene with AICc values (Burnham and Anderson 1998). We recorded model weights and used model-averaged parameters when the dominant model weight (w_i) was <0.95. For each ModelTest run, we constrained the analysis to use a version of the supertree that was pruned to include only species that were represented in each gene dataset using the Perl script autoMT (available from O. R. P. Bininda-Emonds). We then calculated the branch lengths for each pruned supertree using each gene's sequence data and model parameter estimates in PAUP*. We also ran a rapid bootstrap maximum likelihood analysis with 1000 bootstrap replicates and the GTRCAT model using RAxML (Stamatakis 2006), resulting in a gene-estimated sample of bootstrap trees for each gene. Finally, we compiled information on 26 dated fossils (see Supporting information) from Wiens et al. (2006) and the literature, with the help of J. A. Schulte.

We dated the supertree using the branch lengths calculated from each gene dataset fitted to the supertree topology, the sample of unconstrained bootstrap trees for each gene dataset, and a set of fossil calibration points using the Perl script relDate (Bininda-Emonds et al. 2007), which implements a local clock model that smoothes substitution rates across sister groups (Purvis et al. 1995; Bininda-Emonds et al. 2007). We used an updated version of relDate (Bininda-Emonds, unpubl. ms.) that fixes an earlier-reported bug (Bininda-Emonds et al. 2008), weights the relative date estimates derived from each gene according to the degree of congruence between it and the supertree topology, and finally interpolates branch lengths for which there are no sequence or fossil data using a pure birth model. Dates obtained using this approach are heavily reliant on the root age (Bininda-Emonds et al. 2007), which we took to be 227 Ma for the divergence between *Sphenodon* and the Squamata (Wiens et al. 2006; Ricklefs et al. 2007). We treated all other fossil dates as minimum age constraints. Together, fossil and molecular data provided date estimates for 550 of 830 (66.3%) nodes of the supertree. The remaining 280 nodes were interpolated by relDate, as described above. We used this approach because of its past success on large trees, computational efficiency, use of rate smoothing, interpolation of dates based only on gene-estimated dates (Bininda-Emonds et al. 2007), and its ability to account for the degree of gene conflict with the supertree topology (O. R. P. Bininda-Emonds, pers. comm.).

MORPHOLOGICAL DATA COLLECTION

We collected trunk vertebral numbers for 1375 species and morphometric data for 635 of these species (see Supporting information). PJB took the majority of radiographs at the Smithsonian Museum of Natural History and the Museum of Comparative Zoology at Harvard using the museums' Varian (Salt Lake City, UT) digital x-ray systems. We laid specimens flat on the x-ray surface and used scale bars to prevent image distortion and allow morphometric data collection. From the radiographs, we recorded the number of trunk vertebrae, defined as those anterior to the sacral vertebrae. We assumed that intraspecific variation in vertebral number is less than interspecific variation, and so we maintained species sample sizes low so as to maximize the taxonomic sampling, but attempted to include three specimens per species. In addition to these x-rays, we radiographed a series of *Phrynosoma taurus* from the University of Texas at Arlington, and borrowed film radiographs of some rare species from the Field Museum of Natural History, the University of Michigan Museum of Zoology, and M. C. Brandley. Finally, we added presacral vertebral counts from the literature (see Supporting information). We calculated species modal trunk vertebral number for use in subsequent analyses.

For our morphometric data, we measured one specimen per species and only specimens with undistorted radiographs. We measured head length (HL) and width (HW), body length (BL) and maximum width (BW), hindlimb length (HLL) and width (HLW), and hind foot length (HFL) to the nearest 0.01 mm using Image J (Rasband 2002). Body length was not homologous to standard snout-vent length measurements because the cloaca was not identified in the radiographs. Hence, we defined BL as the distance from the tip of the snout to the posterior-most extent of the pelvis at the midline. We defined HLL as the distance from the head of the femur to the tip of the longest toe and HLW as the distance perpendicular to the long axis of the femur at the origin of the hind limb from the body. We defined HFL as the distance from the proximal edge of the tarsals to the tip of the longest toe. We also noted the presence or absence of the front limb, because the orientation of the front limb often precluded taking accurate measurements.

DATA ANALYSIS

Measures of tempo and mode of evolution

We defined 50 mutually exclusive clades that together encompassed all of the Squamata. We defined clades as major recognized taxonomic units within the squamates, or as major resolved subclades within those taxa. We also defined some as taxa of particular interest in vertebral evolution. For example, the scincid genus *Lerista* was of particular interest because of high species richness and variation in vertebral number (Greer 1987, 1990). Although these criteria are arbitrary, this is unavoidable, as using only recognized taxa of a given rank (e.g., Family-level taxa) is also arbitrary.

To prepare our morphometric dataset for clade-level analyses, we first removed the effects of body size and analyzed it using principal component analysis (PCA) using a correlation matrix in SYSTAT version 11. To remove the effects of body size while taking phylogeny into account, we calculated phylogenetically independent contrasts (PICs) (Felsenstein 1985; Garland et al. 1992) of all log-transformed morphometric variables, and regressed each through the origin on HL, which we used as a proxy for size (Wiens and Singluff 2001; Brandley et al. 2008). We then used the PIC slope to calculate size-corrected trait values following Blomberg et al. (2003), and used these trait values as input for the PCA. We used PC-1 and PC-2 species factor scores in our calculations of rate of evolution and disparity in body shape for each of the 50 clades.

For each of our 50 defined clades, we calculated the rate of taxonomic diversification, rate of trunk vertebral number diversification, rate of PC-1 and PC-2 diversification, disparity of trunk vertebral number, and disparity of PC-1 and PC-2. To calculate rates of taxonomic diversification, we tallied the number of extant species in each clade using the online Reptile Database (Uetz et al. 2007) and obtained the age of the MRCA from our supertree. We then calculated rate of taxonomic diversification assuming a pure-birth model, for the crown group using equation (4) of Magallón and Sanderson (2001).

We calculated rates of vertebral and morphometric evolution and ancestral states for vertebral number and the first two PCs using Brownie version 2.0 (O'Meara et al. 2006). This is a maximum likelihood approach that fits a Brownian motion model of evolution to traits. The rate parameter, σ^2, is estimated using the MRCA as the distance from root to tip of each clade's phylogeny. Maximum likelihood ancestral state reconstructions match those of other software, and are consistent with those from squared-change parsimony (Schluter et al. 1997; O'Meara et al. 2006). This approach is more powerful than estimating rates from PICs (O'Meara et al. 2006).

We calculated disparity as lineage density (D_2) for each clade for vertebral number and a morphospace defined by PC-1 and PC-2 (Sidlauskas 2008) as the quotient of the sum of morphometric branch lengths and the area of morphospace occupied by each clade. We calculated morphometric branch lengths for each clade by reconstructing ancestral states for each node on the supertree and for each trait using Mesquite version 2.6 (Maddison and Maddison 2010), with all branch lengths set equal to unity (Sidlauskas 2008). We then calculated Euclidean distances along each branch, between ancestor and each descendent and summed all of these distances for each clade. Vertebral number distances were one-dimensional, and distances based on PC-1 and PC-2 were two-dimensional. We calculated the area of morphospace occupied by each clade as the sum of spans of the major axes of the bounding hyperellipsoid (Sidlauskas 2008) in R Core Development Team (2008).

Statistical analysis

We corrected for multiple comparisons by using the Benjamini-Hochberg (B-H) method (Benjamini and Hochberg 1995), which controls both the type I error and false discovery rates, while maintaining a high and stable power (Williams et al. 1999; Moran 2003). The more commonly used sequential Bonferroni correction (Rice 1989) suffers from decreased power as the number of comparisons increases (Garcia 2004). With the B-H method, we calculated the critical *P*-value as: $P_{crit} = i\alpha/m$, where i is the rank of the comparison, when all comparisons are ordered from lowest to greatest *P*-value, α is the desired type I error rate (0.05), and m is the total number of comparisons (Williams et al. 1999). We also evaluated effect sizes with R^2 values because many of our sample sizes were large, making it possible for very small effects to be statistically significant. We interpreted only analyses that were significant following the B-H correction and that had $R^2 > 0.15$.

To test our first hypothesis, that a relaxation of constraint on vertebral number coincides with body shape and taxonomic diversification, we analyzed relationships among clade-level values of taxonomic diversification rate, and rates of evolution and disparities of body shape (PC-1 and PC-2) and trunk vertebral number with PICs (Felsenstein 1985; Garland et al. 1992), using Mesquite version 2.6 (Maddison and Maddison 2010). We did multiple regressions among PICs, including log-transformed vertebral number as a covariate to ensure that the rate of evolution in vertebral number, and not vertebral number, was driving observed relationships. We tested the assumption that PICs follow a Brownian motion model by testing for significant correlations between PICs and their standard deviations (Garland et al. 1992) and their estimated node heights (Freckleton and Harvey 2006). Because many of the clade-level PICs were not well standardized when calculated with temporal branch lengths, we set all branch lengths equal to 1, which yielded well-standardized PICs. Our approach strengthens previous approaches that have considered single transitions in rate or disparity (e.g., O'Meara et al. 2006; Sidlauskas 2008; Collar et al. 2009), by treating these variables as continuous and using regression analysis to consider multiple instances of trait evolution without having to identify each one. Thus, we combined the power of using rates and disparity of evolution, (O'Meara et al. 2006; Sidlauskas 2008), with the power of historical testing using multiple evolutionary events (Jensen 1990).

We tested our second hypothesis that increases in the number of vertebrae, and an associated elongate body shape, coincides with body shape and taxonomic diversification in several ways. We tested for clade-level relationships between ancestral reconstructions of vertebral number with rates and disparity of body shape evolution, and between taxonomic diversification rate and ancestral reconstructions in body shape and vertebral number using PICs, as described above.

We also conducted species-level analyses to test whether taxonomic diversification rate was related to the morphological traits trunk vertebral number, PC-1, and PC-2, by using macroCAIC (Agapow and Isaac 2002), as implemented in the CAIC package in R Core Development Team (2008). This approach is a modification of PICs to test whether a continuous trait influences species richness that uses species as the units of analysis (Agapow and Isaac 2002). The rate difference and proportion dominance ratio formulae for calculating the contrasts for species richness gave identical results, so we present only results from the former because it considers diversification rate by taking phylogenetic branch lengths into account (Agapow and Isaac 2002).

Finally, we used the Binary State Speciation and Extinction (BiSSE) model to evaluate whether elevated numbers of trunk vertebrae relative to the ancestral state influenced rates for speciation and extinction (Maddison et al. 2007). We fitted the BiSSE model, a character-independent model of speciation and extinction (two parameters estimated), and several intermediate models, while taking incomplete taxonomic sampling into account, using Diversitree (FitzJohn et al. 2009), an R package (R Core Development Team 2008). We coded species with trunk vertebral numbers >27 with a 1 and those with fewer vertebrae with a 0. This cut-off was chosen because it is close to the ancestral number of trunk vertebrae for squamates (25, exhibited by *Sphenodon*). To ensure that our results were robust to our selection of cut-off, we also fit an unconstrained BiSSE model to our data, using a cut-off where species with >29 vertebrae were coded with a 1, as this corresponds to coding all well-represented vertebral numbers with a 0 (Fig. 2). Finally, we fit an unconstrained Multiple State Speciation and Extinction (MuSSE) model (FitzJohn et al. 2009), where vertebral number was represented using three character states: <27, 28–49, and >49 trunk vertebrae, to further evaluate our results for robustness. We fitted all models at least five times with independent random parameter starting points to evaluate whether some analyses detected local instead of global maxima. We fitted 10 replicate three-state MuSSE models due to problems with convergence with many replicates. These problems, associated with significantly increased numbers of parameters that needed to be estimated prevented us from fitting more complex multistate models or from treating vertebral number as a continuous character (FitzJohn 2010). We compared the fit of these models using AICc (Burnham and Anderson 1998). The results of these models are robustly supported by the results from our PIC-based analyses.

Results

SUPERTREE OF THE SQUAMATA

The weighted supertree analysis included 2538 taxa prior to pruning, was based on 3923 parsimony-informative pseudocharacters, and yielded 165 equally most parsimonious trees of length 312,230. The supertree was derived from 318 source trees published in 295 source publications (Supporting information). Most source trees were derived from morphological and mtDNA characters, although nuclear DNA sequences and other sources of data were also represented (Supporting information). The pruned and dated supertree includes the 1375 species for which we had vertebral count data (Supporting information). The supertree is consistent in many respects with recent molecular phylogenies (Townsend et al. 2004; Vidal and Hedges 2004) in allying the Iguania with the Varanidae and Anguidae; suggesting a close relationship among the Lacertidae, Gymnophthalmidae, and Teiidae; and placing the Cordyloidea as sister to the Scincidae and Xantusiidae (Fig. 1A). The supertree also resolved the Gekkota as sister to other squamates (Townsend et al. 2004). However, the placement of the Serpentes as sister to the Amphisbaenia may be the result of conflicting phylogenetic signal from source studies (Fig. 1A). The placement of these groups has always been equivocal and varied due to their highly derived nature (Estes et al. 1988; Lee 1998; Caldwell 1999; Harris et al. 2001; Townsend et al. 2004).

VERTEBRAL NUMBER AND BODY SHAPE VARIATION

Trunk vertebral numbers varied over an order of magnitude—from 14 in a dwarf chameleon (*Brookesia superciliaris*) to 120 in a pygopodid (*Aparisia pseudopulchella*), to 136–293 in the various snakes that we sampled (Fig. 2). The modal trunk vertebral number was 26, possessed by 350/1375 species sampled. The ancestral state, as determined by comparison to the extant squamate outgroup, *Sphenodon punctatus*, was 25 vertebrae and this was shared with 93 other species sampled.

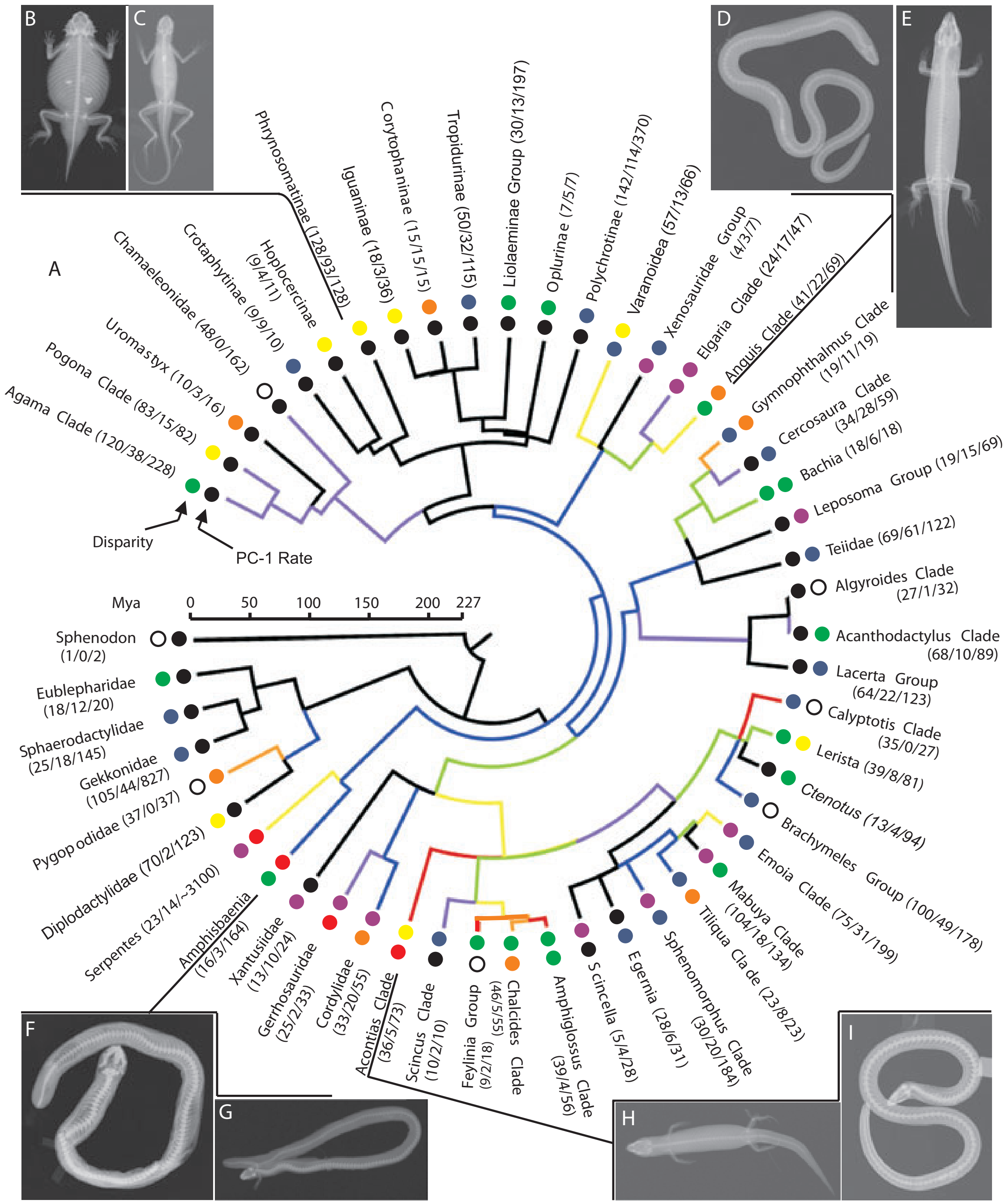

Figure 1. The supertree, simplified to clades described in the text (A). Branch lengths are proportional to time. Colors of branches and of inner and outer rings of dots (in sequence: black, purple, blue, green, yellow, orange, red) indicate rates of vertebral number evolution, rate of PC-1 evolution, and disparity in body shape (all low to high), respectively. Open circles indicate an unknown state. Numbers in parentheses indicate vertebral sample size, morphometric sample size, and total extant species richness for each clade. Radiographs (B–I) show body shapes of representatives from the underlined clades.

The first component from the PCA on phylogenetically size-corrected morphometric data explained 61.9% of the variance in the data. It represented a trade-off between body length, and all limb and digit characteristics (Table 1). Species that loaded highly positively on PC-1 had relatively short bodies and well-developed limbs with five digits. We, therefore, interpret PC-1 as an index of elongation, separating lizard-like from snake-like species (Fig. 3). PC-2 explained 21.5% of variance, and represented variation associated with head and body width (Table 1). We interpret PC-2 as an index of robustness, where heavy-bodied species with broad heads, such as *Phrynosoma*, and snakes belonging to the Scolecophidia (e.g., Typhlopidae) have high PC-2 factor scores,

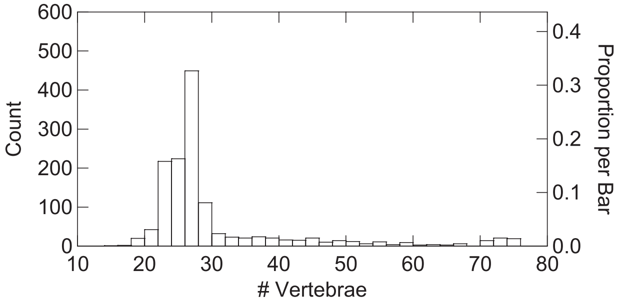

Figure 2. A histogram of trunk vertebral numbers for 1375 squamate species sampled in this study. The three right-most bars represent bins of unequal size to accommodate the long tail of the distribution.

and gracile species such as *Lialis*, and *Anolis* have low PC-2 factor scores (Fig. 3). PC-3 explained 7.4% of variance and is not considered further.

Trunk vertebral numbers and body shape coevolved in squamates. There was a strong significant relationship between PICs of log-transformed vertebral number and the degree of elongation (PC-1), where species with more vertebrae had lower PC-1 values, so were more serpentine ($R^2 = 0.478$, $t_{436} = -24.11$, $P < 0.0001$; Fig. 4). There was no relationship between trunk vertebral number and the degree of robustness (PC-2) because of low effect size ($R^2 = 0.121$, $t_{436} = 3.06$, $P = 0.0023$). These species-level analysis results are further supported by clade-level results, where clade ancestral vertebral number included as a covariate and was significantly related to clade ancestral PC-1 and PC-2 values, again with low effect size for PC-2 (Table 2A).

VERTEBRAL NUMBER EVOLUTION, AND BODY SHAPE AND TAXONOMIC DIVERSIFICATION

Our hypothesis that relaxed constraint on vertebral number facilitates body shape and, ultimately, taxonomic diversification was partially supported. The rate of trunk vertebral evolution was significantly related to aspects of body shape evolution. Specifically, we found that clades that evolved vertebral numbers quickly evolved along the index of elongation (PC-1) more quickly and had greater disparity in body shape than clades with lower rates of vertebral number evolution (Table 2A). Despite a strong pattern of pleomerism in squamates (Fig. 4), these relationships were not confounded by vertebral number itself, which we included as covariate (Table 2A). The rate of evolution in vertebral number was not related to the rate of evolution in the degree of robustness (PC-2), or to clade disparity in vertebral number (Table 2A). Furthermore, there was no relationship with clade ancestral body shape (PC-1 and PC-2), which was significantly related to the covariate, vertebral number (Table 2A). Despite this clear relationship between the degree of constraint on vertebral number and body shape diversification, rates of vertebral number evolution, body shape evolution, or their disparities were not significantly related to the rate of taxonomic diversification (Table 2B).

Table 1. PCA loadings, eigenvalues, and percent variance explained for the first two PCs for body shape variables analyzed. $n = 635$. Factor loadings with 95% confidence intervals excluding zero are in bold.

Variable	PC-1	PC-2
Head width	0.1927	**0.5993**
Body length	**−0.3521**	**0.3557**
Body width	0.1946	**0.6282**
Thigh width	**0.4003**	0.1445
Hind limb length	**0.4173**	0.0140
Foot length	**0.3843**	0.0030
Front digit no.	**0.3964**	−0.2265
Hind digit no.	**0.4023**	−0.2176
Eigenvalue	4.95	1.72
% Variance	61.89	21.52

Our second hypothesis, that the evolution of high numbers of vertebrae, which tend to be associated with snake-like body shapes (Fig. 4), allows both morphological and taxonomic diversification, was rejected. We found no significant relationships between ancestral vertebral numbers, taxonomic diversification rate, and body shape evolution either at the clade level, using standard PICs, or the species level, using macroCAIC (Table 3). These findings were strongly supported using the BiSSE and MuSSE models, taking incomplete taxonomic sampling into account. Here, the simplest, or character-independent, model fit our data far better than the next best model, which was the unconstrained BiSSE model with a vertebral number cut-off of 30, as opposed to 28. The MuSSE model with vertebral number characterized with three character states fit our data the poorest (Table 4). All models that estimated rates of speciation and/or extinction as dependent on vertebral number fit the data much more poorly than the character-independent model (Table 4). A lack of a significant relationship between vertebral number and body shape evolution further supports the assertion that the tests of our first hypothesis (Table 2) were not confounded by the observed pattern of pleomerism (Fig. 4).

Discussion

DOES THE DEGREE OF CONSTRAINT ON VERTEBRAL NUMBER EXPLAIN PATTERNS OF DIVERSITY?

We have demonstrated that a relaxation of constraint on the evolution of vertebral number has coincided with increased body shape diversification in the Squamata, partially supporting our hypothesis. Clades with increased rates of evolution in vertebral number have evolved a greater disparity of body shapes more quickly than clades with lower rates of vertebral number evolution (Table 2).

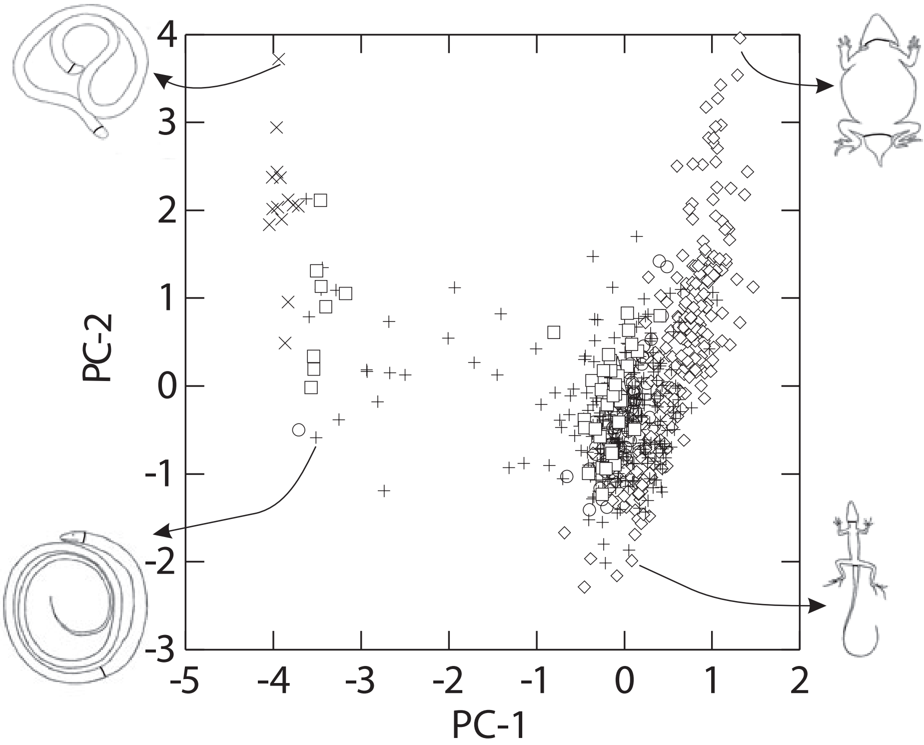

Figure 3. A plot of squamate morphospace defined by PC-1 (an index of elongation) and PC-2 (an index of robustness). Symbols identify species to major clade: ◊ Iguania, ☐ Varanoidea, + Scincoidea, ∘ Gekkota, × Serpentiformes. Representative body shapes are shown in each corner, with the extent of the head and tail demarcated. $n = 635$.

Such a relationship between vertebral and body shape evolution is important to understanding how body shape can evolve through changes in its vertebral components (Ward and Brainerd 2007). One might expect that a high rate of vertebral number and body shape evolution would contribute to taxonomic diversification because they would enable occupation of a wider variety of niches (Melville and Swain 2000; Van Damme and Vanhooydonck 2002; Bergmann and Irschick 2010), but this was not the case (Table 2). Therefore, our work generally supports the idea that relaxation of constraints on vertebral number can facilitate the evolution of novel phenotypes, but there appears to be a decoupling between the evolution of form and the evolution of more species. This suggests that the effects of constraints may be differential for different components of diversity, something also found in cetaceans and triggerfish (Slater et al. 2010; Dornburg et al. 2011).

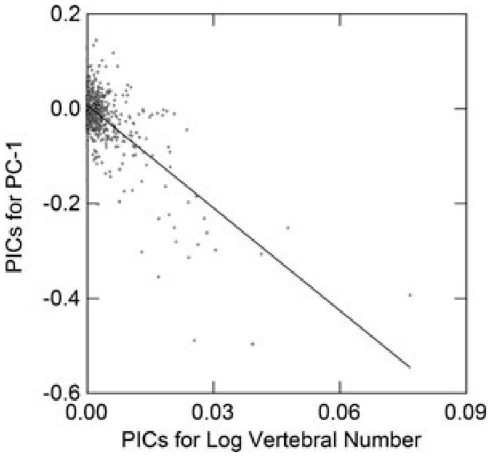

Figure 4. Phylogenetically independent contrasts of log trunk vertebral number and PC-1. A regression line through the origin is drawn for reference. $n = 635$.

Two major aspects of body shape variation in many vertebrates are the degree of elongation and the degree of robustness or body width (this study; Ward and Brainerd 2007; Bergmann et al. 2009; Goodman 2009). In squamates, relaxed constraints on vertebral number have allowed for the diversification of clades in the degree of body elongation, but not the degree of body robustness (Table 2). This result makes sense because numbers of vertebrae contribute to body length (Ward and Brainerd 2007; Brandley et al. 2008), but not body width, despite the fact that the two features sometimes coevolve to produce long, attenuated body shapes (Parra-Olea and Wake 2001; Greer and Wadsworth 2003). Although vertebral evolution is one way of evolving different body shapes, other skeletal elements are also involved, which allow for changes in robustness as well. For example, there is little variation in vertebral number in the Phrynosomatinae

Table 2. Analyses related to testing the effects of a relaxation of constraints on diversification. Presented are relationships of phylogenetically independent contrasts for squamate clades among listed variables. "Vert #" represents log-transformed trunk vertebral number; when in parentheses, it is the covariate. *P*-values significant after correction for multiple comparisons are in bold.

Variable 1	Variable 2	df	R^2	t	P
(A) Rate of Vert # evolution	Rate: PC-1	47	0.219	2.56	**0.0139**
	(Vert #)			1.15	0.2549
	Ancestor: PC-1	47	0.639	−0.38	0.7091
	(Vert #)			−7.79	**<0.0001**
	Disparity: PC-1, 2	43	0.284	−3.97	**0.0003**
	(Vert #)			0.92	0.3634
	Disparity: Vert #	49	0.098	−0.86	0.3938
	(Vert #)			−1.52	0.1346
	Rate: PC-2	47	<0.001	0.30	0.7684
	(Vert #)			−0.25	0.8022
	Ancestor: PC-2	47	0.275	−0.42	0.6777
	(Vert #)			3.95	**0.0003**
(B) Taxon div. rate	Rate: Vert #	49	<0.001	−0.04	0.9677
	Rate: PC-1	47	<0.001	0.04	0.9662
	Rate: PC-2	47	0.105	−2.33	0.0244
	Disparity: Vert #	49	<0.001	0.01	0.9927
	Disparity: PC-1, 2	45	0.010	−0.65	0.5168

Table 3. Analyses related to testing the effects of high vertebral number on diversification. Presented are relationships of phylogenetically independent contrasts for squamates. Clade-level analyses relate ancestral reconstructions of vertebral number and taxonomic diversification rate with listed variables. Species-level analyses relate vertebral number, PC-1, and PC-2 values to taxonomic diversification rate using macroCAIC. "Vert #" represents log-transformed trunk vertebral number.

	Variable 1	Variable 2	df	R^2	t	P
Clade level	Ancestor: Vert #	Rate: PC-1	47	0.123	2.33	0.0245
		Rate: PC-2	47	0.001	−0.12	0.8777
		Disparity: PC-1, 2	43	0.035	−1.23	0.2275
	Taxon div. rate	Ancestor: Vert #	49	0.001	0.22	0.8256
		Ancestor: PC-1	47	0.002	−0.26	0.7936
		Ancestor: PC-2	47	0.005	−0.47	0.6393
Spp. level	Taxon div. rate	Vert #	437	0.002	−0.57	0.5689
		PC-1	241	0.009	1.78	0.0763
		PC-2	241	0.004	−0.20	0.8416

(Fig. 1; range of 4, which is comparable to mammals—Slijper 1946), yet this clade includes species ranging from very gracile to very robust (Bergmann et al. 2009). In contrast, the *Acontias* clade exhibits far more variation in the degree of elongation of body shape and trunk vertebral numbers range from 28 to 101 (this study).

DOES THE EVOLUTION OF MANY VERTEBRAE EXPLAIN PATTERNS OF DIVERSITY?

Whether the evolution of many vertebrae represents a key innovation in squamates is of particular interest in light of the significant relationship between the degree of constraint on vertebral number and morphological diversification, and the strong pattern of pleomerism. We found that although a snake-like body shape has evolved many times (Brandley et al. 2008), it has neither spurred on nor inhibited diversification, a pattern also seen in salamanders (Parra-Olea and Wake 2001). Importantly, we observed this lack of relationship with diversification both taxonomically and in terms of body shape diversification, and so, while many vertebrae do coincide with a snake-like body shape, they do not coincide with the ability to evolve new body shapes or to speciate. This has important implications for the diversification of snakes, suggesting that although the origin of snakes may be associated with the evolution of increased numbers of vertebrae, their subsequent radiation is associated with other factors not studied by us. Snakes were purposely under-represented in our analyses because they have no intermediate forms between snake-like and lizard-like, are highly derived, and have diversified into many niches subsequent to their evolution (Greene 2000). The selective pressures that were important during their evolution are likely no longer present (Shine 1986), and so including a large sample of Serpentes would have produced an outlier in our analyses. The diversification of snakes is a separate question from the evolution of a snake-like body shape, and its explanation does not lie with the number of vertebrae they possess.

Our finding that it is a high rate of vertebral evolution, and not high vertebral numbers, that has led to body shape diversification is well illustrated by several examples. The Amphisbaenia, a clade where all species have elongate bodies and many vertebrae, has low rates of vertebral number evolution and low body shape disparity (Fig. 1). In contrast, in clades with high rates of vertebral evolution (e.g., the *Anguis* and *Acontias* clades), disparities in vertebral number and body shapes are both high (Fig. 1). However, we note that changes in the degree of body elongation

Table 4. **Comparison of models of speciation and extinction, accounting for incomplete taxonomic sampling. For each model, $n = 1375$, and the −ln Likelihood, AICc, and Di are presented. The parameter estimates are also presented for each model: q_{01} and q_{10} are the rates of forward and backward evolution between character states (vertebral number), λ_x is the speciation rate under each character state, and μ_x is the extinction rate under each character state. BiSSE 28 and BiSSE 30 indicate unconstrained BiSSE models with 28 and 30 vertebrae as the cut-off between the two possible states. Not all the parameters for the MuSSE model are shown because the model fit so poorly.**

Model	q_{01}	q_{10}	λ_0	λ_1	μ_0	μ_1	−lnL	AICc	Di
Char. indep.			0.0268		4.31×10^{-8}		−2218	−4444	Best
BiSSE 28	0.0007	0.0036	0.0768	0.0708	0.0250	0.0199	6516	13,008	17,452
BiSSE 30	0.0010	0.0013	0.0841	0.0517	0.0329	5.56×10^{-6}	6479	12,933	17,377
Equal λ	0.0007	0.0033	0.0749		0.0225	0.0253	6516	13,012	17,456
Equal μ	0.0007	0.0034	0.0756	0.0733	0.0234		6516	13,012	17,456
MuSSE	0.0007	0.0050	0.0761	0.0819	0.0243	0.0277	6620	13,191	17,635

can be mediated either through changes in vertebral number or length (Ward and Brainerd 2007; van Sittert et al. 2010), and we only examined the former in this study.

MECHANISMS FOR THE EVOLUTION OF VERTEBRAL NUMBER

One of our findings is that the degree of constraint on vertebral numbers among squamate clades has evolved: some are highly constrained whereas others are extremely variable (Fig. 1). In mammals, an association between aberrant vertebral numbers and a high incidence of cancer, mediated through a high metabolic rate has been used as evidence of strong stabilizing selection for invariable vertebral number (Galis 1999; Galis and Metz 2007). Low metabolic rates and low incidence of cancer in nonavian reptiles (Galis 1999) can explain some of our findings: squamate clades with low constraint on vertebral number have relaxed stabilizing selection on this trait due to the low incidence of cancer. However, this hypothesis does not explain why some squamate clades exhibit little variation in vertebral number (e.g., Gekkonidae, Phrynosomatinae—Fig. 1). In these clades, body shape evolution still occurs, but along axes unrelated to vertebral number, such as in the degree of robustness. This lack of variation in some clades may be explained by constraints imposed by their natural history (de Querioz 2002). For example, the possession of adhesive pads in the Gekkonidae might constrain their body shape disparity and rate of vertebral number evolution, both of which are low (Fig. 1A).

Ultimately, it is the genetic mechanism controlling vertebral number that has likely changed in response to selection on body shape. In snakes, the anterior and posterior expansion of *HoxC6* and *HoxC8* expression domains is associated with loss of the front limbs and an expansion of thoracic vertebrae (Cohn and Tickle 1999). There is also decreased stabilizing selection on the *HoxD* cluster in snakes relative to other amniotes (Di-Poï et al. 2010). However, these genes are involved in changes in vertebral identity, not in changes in vertebral numbers. Vertebral number is controlled by a clock-and-wavefront mechanism (Pourquie 2003), where the boundaries of somites, which are vertebral precursors, are determined by a posteriorly moving wavefront of antagonistic retinoic acid and Wnt/FGF gradients (Gomez and Pourquié 2009) interacting with the cyclic expression of *lunatic fringe*, which forms the clock component (Pourquie 2003). Increased vertebral number in snake-like squamates is a result of an increase in the clock rate, which produces more somites (Gomez et al. 2008). When the constraint on vertebral number is relaxed, selection may be influencing the regulatory elements of *lunatic fringe*, mediating body shape diversification. The evolution of gene regulatory elements may be important for allowing phenotypes to evolve without changing major pleiotropic genes (Jablonski 2000), which is also hypothesized for *Hox* genes (Di-Poï et al. 2010).

DIVERSIFICATION ALONG MULTIPLE AXES

Our findings of discordance between morphological and taxonomic diversification add to the studies showing that these components of diversity often do not coincide (Foote 1993; Alfaro et al. 2004; Roy et al. 2004; Alfaro et al. 2005; Adams et al. 2009). There is growing consensus that taxonomic, morphological, and functional/ecological aspects of diversity are all important to understanding clade diversification (Foote 1997; Roy et al. 2004; Bursatte et al. 2008; McPeek 2008; Mouchet et al. 2010). An important outcome of this work is that it broadens the persistent question of why some clades diversify and others do not, to why some clades diversify along different axes from other clades, and why they diversify along one axis and not another.

Our research clearly shows that some clades are morphologically diverse but taxonomically poor, whereas others are the opposite. Well-studied examples of adaptive radiation exhibit this pattern as well, with Darwin's finches (Grant 1986) and the Hawaiian silversword alliance (Baldwin and Sanderson 1998) being taxonomically depauperate but morphologically rich. In contrast, the

Polychortinae, which includes *Anolis* lizards (Losos 2009), is taxonomically rich, but exhibits relatively low morphological disparity when compared to other squamate clades (Fig. 1A). These examples suggest that such discordance between these types of diversity may be a common feature in the tree of life. In addition to discordance between morphological and taxonomic diversification, our study illustrates how a trait (vertebral number), may not be related to any metric of diversification, but its rate of evolution may be.

Having evolved dozens of times, a snake-like body shape is a major theme in vertebrate evolution (Parra-Olea and Wake 2001; Pough et al. 2004; Ward and Brainerd 2007; Brandley et al. 2008), and so it is tempting to hypothesize that elevated numbers of vertebrae that often underlies this body shape constitute some sort of key innovation. Our findings in squamates directly reject this notion, suggesting instead that the rate of evolution of vertebral number has allowed for body shape diversification. What remains to be seen is whether there are common selective pressures resulting in this relaxed constraint on vertebral numbers, similarities in underlying standing genetic variation (Schluter 1996), or whether historical contingency (Jensen 1990; de Querioz 2002) has played a role in the repeated evolution of a snake-like body shape. Although we clearly show a relationship between vertebral diversity and body shape diversity, the question of explaining taxonomic diversity remains elusive. One possible bridge between morphological and taxonomic diversity is functional and ecological diversity (Wainwright 2007; Mouchet et al. 2010). The outstanding question here is whether clades of squamates (or other taxa) with increased morphological diversity actually fill a wider variety of niches. This is an important prediction made by a hypothesis of adaptive radiation (Schluter 2000), and deserves further study.

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Supporting Information

The following supporting information is available for this article:

Table S1. The number of taxa for which the gene was represented, the number of base pairs (bp) used, the type of gene (mt = mitochondrial; nc = nuclear), the best fitting model of sequence evolution, the model's relative weight (w_i), and the ln Likelihood of each model are presented for each gene.

Table S2. Fossil names, ages, and the node on the supertree that it dates and references are presented.

Table S3. Number of source studies and trees differ when multiple trees were used from a given study. Data type counts exceed source tree counts because some trees included multiple data sources.

Supporting Information may be found in the online version of this article.