

# Homeotic effects, somitogenesis and the evolution of vertebral numbers in recent and fossil amniotes

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**The development of distinct regions in the amniote vertebral column results from somite formation and *Hox* gene expression, with the adult morphology displaying remarkable variation among lineages. Mammalian regionalization is reportedly very conservative or even constrained, but there has been no study investigating vertebral count variation across Amniota as a whole, undermining attempts to understand the phylogenetic, ecological, and developmental factors affecting vertebral column variation. Here, we show that the mammalian (synapsid) and reptilian lineages show early in their evolutionary histories clear divergences in axial developmental plasticity, in terms of both regionalization and meristic change, with basal synapsids sharing the conserved axial configuration of crown mammals, and basal reptiles demonstrating the plasticity of extant taxa. We conducted a comprehensive survey of presacral vertebral counts across 436 recent and extinct amniote taxa. Vertebral counts were mapped onto a generalized amniote phylogeny as well as individual ingroup trees, and ancestral states were reconstructed by using squared-change parsimony. We also calculated the relationship between presacral and cervical numbers to infer the relative influence of homeotic effects and meristic changes and found no correlation between somitogenesis and *Hox*-mediated regionalization. Although conservatism in presacral numbers characterized early synapsid lineages, in some cases reptiles and synapsids exhibit the same developmental innovations in response to similar selective pressures. Conversely, increases in body mass are not coupled with meristic or homeotic changes, but mostly occur in concert with postembryonic somatic growth. Our study highlights the importance of fossils in large-scale investigations of evolutionary developmental processes.**

constraint | development | *Hox* genes | segmentation | paleontology

**S**omitogenesis, somatic growth, and *Hox* gene expression are primary factors driving the formation of the vertebrate body axis (1, 2). Somitogenesis occurs via the rhythmic budding of embryonic somites from the anterior part of the presomitic mesoderm, until a point when the latter has shrunk to such a degree that no further somites can be formed (3). Periodic somite formation is controlled by a molecular oscillator or “segmentation clock” (4), whereas the speed of the clock is highly variable across different vertebrate lineages. For example, the segmentation clock in snakes ticks much faster than in a mouse, resulting in a higher number of relatively smaller somites (3). Because the ossified vertebrae are derived from the embryonic somites through resegmentation (1), vertebral numbers can provide insights into the speed of the segmentation clock and the pattern of somitogenesis. In addition, the relative size of vertebrae relative to the number of vertebrae provides information about the rate of somatic growth (5, 6).

Besides the marked variation in the number and sizes of vertebrae, the vertebrate body axis is also highly regionalized. In tetrapods, this regionalization is expressed in the formation of presacral, sacral, and caudal vertebral series, and the presacral portion can be further subdivided into cervical and dorsal series (with the latter

subdivided still further into thoracic and lumbar vertebrae in mammals). These regional identities result from different *Hox* gene expressions along the vertebrate body axis (7, 8); consequently, the vertebral column can be viewed as a result of the transformation of repeated developmental modules into different evolutionary modules (9, 10). *Hox* genes are activated in the lateral somite precursors in the epiblast, so the timing of *Hox* activation during gastrulation largely determines the position of expression domains of *Hox* genes along the anteroposterior body axis, whereas the stem cell-derived medial somite precursors control segmentation (11). Because of the spatial dissociation between segmentation and axial regionalization, it has recently been suggested that the two processes are uncoupled (11, 12), despite some indication of crosstalk between the segmentation machinery and *Hox* patterning (11, 13).

The vertebral column has recently attracted the interest of many evolutionary and developmental biologists because the formative roles of somitogenesis, somatic growth, and *Hox* gene expression are coupled with easily observable adult phenotypes. For example, there have been investigations into vertebral count variation in numerous taxa, including fish, amphibians, reptiles, and mammals (5, 9, 14–21). Most of these studies ask whether, and how, vertebral counts are affected by developmental constraints and modular evolution, and how changing vertebral counts effect body size evolution. Mammals in particular have attracted much attention because, with few exceptions, their precaudal vertebral counts are highly conserved, or constrained (15, 17). By contrast, reptiles (including birds) are more variable in their vertebral numbers; the most dramatic example are snakes, with some taxa possessing >300 precaudal vertebrae (5). However, all of these comparisons have suffered from the fact that there has, so far, been no comprehensive treatment of vertebral count variation across Amniota as a whole, and none that have taken full account of extinct taxa that include morphologies that are unrepresented in the modern biota.

Amniota is the tetrapod clade that is composed of Synapsida (crown mammals and their fossil relatives) and Reptilia (including birds), and is diagnosed by a suite of adaptations for terrestrial habits, including extraembryonic membranes (22). The oldest representatives of modern amniote crown clades have been recovered from Triassic deposits (23).

Here, we examine variation of presacral vertebral counts across all major amniote clades (*SI Methods* and *SI Appendix*), fossil and

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extant. We focus on the presacral portion of the body axis because it is regionalized in most amniotes, and because the caudal skeleton is a single, anatomically undifferentiated region whose segmentation is temporally disjunct from the presacral region and is derived by terminal addition at the tailbud, a different patterning than in the presacral region (9, 24). As a result, changes in caudal somite counts are uninformative with respect to the relative roles of both homeotic and meristic changes within specific regions of the axial skeleton. Additionally, the posterior parts of the vertebral column such as the sacral and caudal regions are often not preserved in fossils and therefore are difficult to study. By including fossils, we trace the evolutionary developmental history of extant taxa through the inclusion of their extinct relatives and examine developmental patterns in different ecologies within a comparative phylogenetic framework. In this study, we ask whether homeotic and somitogenetic patterns in the presacral region are correlated with each other as well as within and across major clades, whether similar ecologies and morphological specializations convergently release or constrain presacral patterning in different lineages, and whether changes in presacral patterning reflect changes in body size.

## Results

Our analysis presents ancestral state reconstructions for presacral and cervical series counts across all major amniote clades (Figs. 1 and 2 and *SI Appendix*). We define cervical vertebrae as a rostrally situated subset of presacral elements that can be distinguished from more caudally situated vertebrae by either topological position (rostral to the shoulder girdle) or discrete morphology (25). We used the ratio between the number of cervical vertebrae to all presacral vertebrae as a reference for the relative proportions of different axial regions along the body axis (referred to as CP ratio hereafter). Because both the phylogenetic and developmental relationships between homeotic and somitogenetic variation are poorly constrained, we used phylogenetic independent contrasts (PIC) and phylogenetic generalized least-squares regression (PGLS) to test whether presacral vertebral numbers among amniotes are correlated with the CP ratio while minimizing the potential effects of phylogenetic autocorrelation (26, 27; see *Methods*). No significant correlation exists for any of the included amniote taxa, and neither PIC nor PGLS analyses resulted in a significant correlation between the two variables ( $r^2 = 0.06$ , correlation coefficient =  $-0.25$ ) when autocorrelation was removed, arguing against the hypothesis that somitogenesis and regionalization are linked processes (see *SI Appendix* for details).

In Synapsida, only moderate deviations from the ancestral amniote condition (CP = 0.23) were recorded, remaining roughly in the range of  $\pm 0.04$ . The lowest ratio was found in the basal synapsid *Hapiodus* (CP = 0.2), whereas the highest ratios occur in dinocephalian therapsids (CP = 0.275), crown mammals (CP = 0.27), and the closely related traversodontid cynodonts (CP = 0.27).

We recorded a remarkable amount of variation in the CP ratio among reptile lineages. In parareptiles, there is both a decrease in ratio relative to the ancestral amniote condition, as well as an increase. The strongest decrease can be seen in millerettids (CP = 0.17) and *Macroleter* (CP = 0.195). The strongest increase in ratio was recorded for mesosaurs (CP = 0.36), as well as *Eunotosaurus* and *Nyctiphruretus* (CP = 0.33 in both cases).

In eureptiles, a remarkable amount of plasticity is evident at the base of the clade. In the nondiapsid taxa, low CP ratios, reaching as low as 0.125 (*Paleothyris*), are frequently observed. Much higher ratios relative to the ancestral amniote condition are also found in basal diapsids, with the highest values in *Claudiosaurus* (CP = 0.33), *Coelurosauravus* (CP = 0.38), and drepanosaurids (CP = 0.33). The highly derived marine ichthyopterygians/*Hupehsuchus* and thalattosaurs show at their base only marginally higher ratios relative to the ancestral condition (CP = 0.24 and 0.27, respectively). Eosauropterygians, which are marine too, possess a CP ratio of 0.47,

whereas placodonts, the sister taxon to plesiosaurs and other eosauropterygians, exhibit a CP ratio of 0.32.

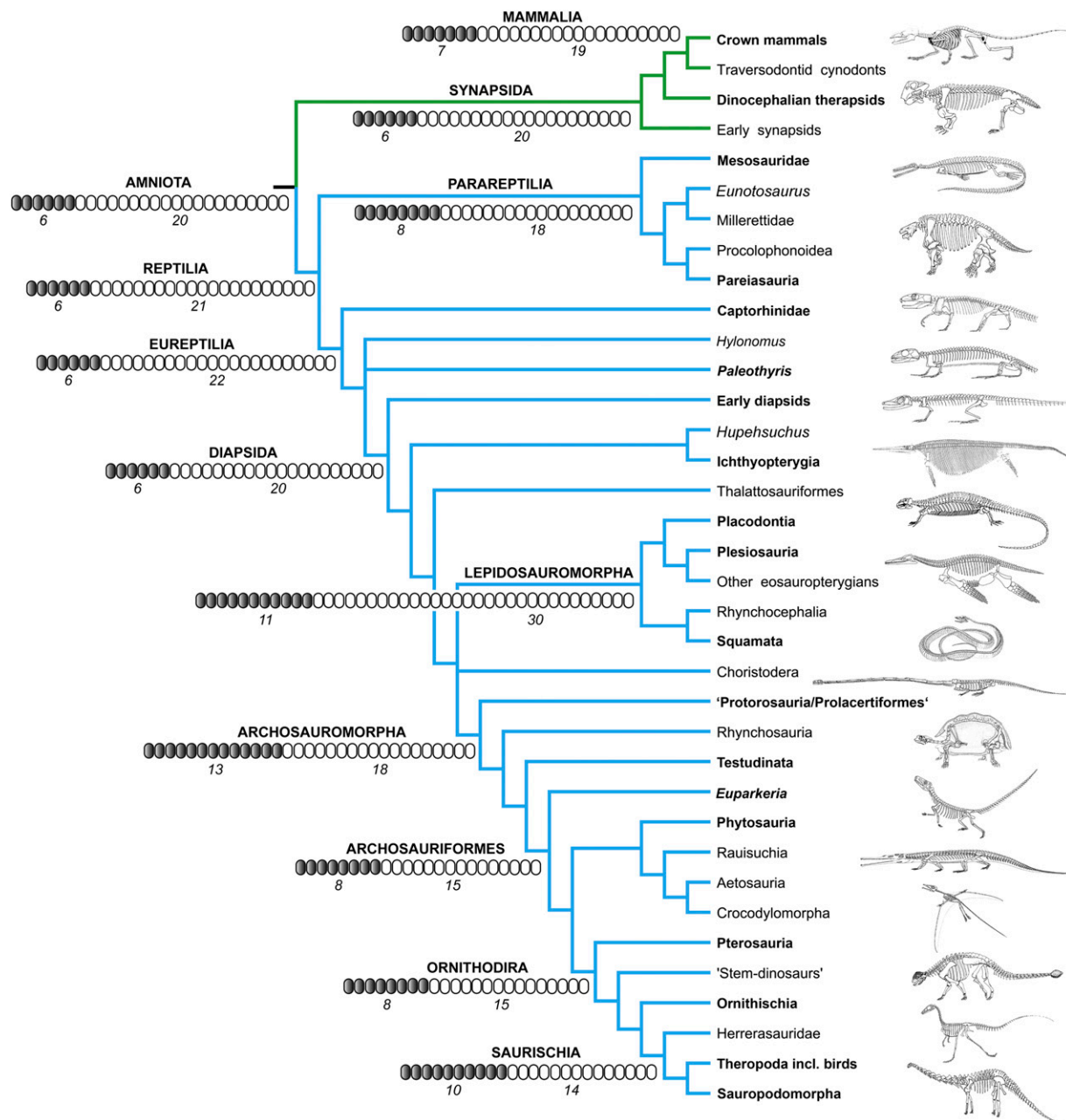
Lepidosauromorpha includes the taxa with the strongest deviations from the ancestral amniote condition; the CP ratios found include the lowest among all amniotes. Squamata as a whole has an ancestral CP value of 0.08. In rhynchocephalians, the aquatic pleurosaurids have a CP ratio of 0.18, whereas the terrestrial species such as the tuatara remain comparatively close to the ancestral amniote condition (CP = 0.28).

The aquatic choristoderes have a high CP ratio of 0.43, and so do archosauromorphs, with a CP ratio of 0.41. The values remain high throughout the various archosauromorph lineages, which also applies to turtles (CP = 0.44) despite their very low count of only 18 presacrals. The paraphyletic prolacertiforms differ strongly from each other, with some showing comparatively low CP ratios of  $\approx 0.3$ ; however, this is still much higher than the ancestral amniote count. The highest ratios among prolacertiforms are found in *Tanystropheus* (CP = 0.48) and *Dinocephalosaurus* (CP = 0.51), although it should be noted that these values are only marginally lower than those in many other archosauromorph clades. Also in archosauriforms, the ratios remain rather constant and approximate the ancestral archosauromorph condition. However, in Ornithodira a relatively higher presacral count is recorded, in both pterosaurs and dinosaurs. In the large-bodied and long-necked sauropodomorphs the CP ratio is initially 0.4 and thus almost identical to the ancestral archosauromorph pattern, whereas in the more derived sauropod clades Macronaria and Diplodocoidea the CP ratios increase to 0.52 and 0.54, respectively. Birds have among the highest ratios of all archosaurs (CP = 0.64).

## Discussion

**Somitogenesis vs. Regionalization.** Our PIC and PGLS analyses did not provide any statistical support for a correlation of homeotic regionalization with somitogenesis. Developmental connections between regionalization and segmentation are controversial. Experimental grafts of Fgf8 beads in developing embryos reveal that *Hox* expression domains track segment boundaries when the size and number of somites change, suggesting crosstalk between the processes (11), and the effects of notch signaling on both *Hoxd* expression and segmentation suggest a common coordinating mechanism (13). Conversely, *Hox* gene mutations do not impact somite number, and changes in somite numbers do not demonstrate corresponding changes in the size or extent of axial expression domains, suggesting independence of the processes (3, 11, 12, 28). Our results indicate that segmentation and homeotic regionalization act separately at higher-order phylogenetic levels, and that if transcription mechanisms exist that link the processes in model taxa, then they are decoupled in the evolution of new body plans.

Independence of the two processes allows us to use the CP ratio in relation to the presacral number to evaluate if the observed variation in vertebral counts is due to either homeotic change or alteration of somitogenesis. For example, an increased rate of somitogenesis in the absence of any modified homeotic effect should result in a higher number of cervical and dorsal vertebrae, but the relative proportions of axial regions formed from the different *Hox* gene expressions along the body axis should remain the same. If the CP ratio equals the ancestral condition but the overall presacral count is increased, then changes in somitogenesis, with no change in *Hox* gene expressions, were most likely responsible. By contrast, a decrease in ratio with no change in total presacral count would indicate that homeotic changes must have been involved in the trunk region; however, if the presacral count is increased, then both homeotic and somitogenetic changes must have been in effect. Lastly, an increase in this ratio would occur either if the relative number of cervical vertebrae increased because of homeotic changes (with the total presacral count remaining constant) or if the presacral count decreased because of altered somitogenesis but with no corresponding (homeotic) effects on the cervical region.



**Fig. 1.** Phylogeny of the major amniote groups treated in this article, based on a compilation from several sources (23, 53, 54). The position of turtles follows current hypotheses (50, 51). See *SI Appendix* for a detailed phylogeny of all taxa treated in this article. Circles at major nodes illustrate ancestral states for cervical (gray) and dorsal (white) vertebrae, based on rounded numbers (in italics) derived from the ancestral reconstructions. The skeletal reconstructions of taxa in bold are modified from several sources (*SI Appendix*) and serve to illustrate the diversity of skeletal structures and the associated vertebral numbers. The figure is not to scale.

In synapsid evolution leading to living mammals, we found a remarkable conservatism relative to the ancestral amniote condition. In fact, the only observable changes in the developmental program are a slight decrease in cervical number in the basal synapsid *Haptodus* and the evolution of a slightly longer neck in dinocephalian therapsids, traversodontid cynodonts, and crown mammals. All of these modifications were possibly caused by moderate homeotic effects rather than by meristic change.

By contrast, reptiles display a much higher degree of plasticity (Fig. 2). Among parareptiles, indications for changes in either somitogenesis or *Hox* expression domains can be observed throughout the clade. For example, the marine mesosaurs have a

high cervical number but overall show only a slight increase in the presacral count, indicating largely homeotic modifications with only minor meristic changes. At the same time, the enigmatic *Eunosaurus* has the lowest presacral count among all amniote taxa considered in this study, which obviously must be largely due to changes in somitogenesis, whereas, interestingly, the cervical count is practically unaffected. Eureptiles behave even more plastic: at the origin of the clade in the Carboniferous, eureptiles already show concerted changes in somitogenesis and regionalization, resulting in higher presacral counts but a relatively lower number of cervicals relative to the ancestral condition. Among early diapsid reptiles, several taxa possess relatively longer necks



trasting cases of neck elongation is the unsolved discussion on the functional factors related to this phenomenon (38).

Marine mammals, such as cetaceans and sirenians, have similar patterning of the axial skeleton relative to that seen in short-necked marine reptiles, in which the overall number of presacrals may increase but the ancestral cervical count is largely retained (17, 39). This suggests that although the cervical region of mammals may be interpreted as developmentally constrained (40), the dorsal region does appear to be able to respond to certain selective pressures in a similar way to those of reptiles with equivalent ecologies.

Pinnipeds, comprising the walruses, sea lions, and seals, are largely aquatic and do not possess any modifications of the ancestral carnivoran pattern, instead they retain the highly conserved number of 20 thoracolumbar vertebrae typical of other carnivores (41). The same conservatism is also observed in basal placodont reptiles, whose vertebral numbers are similar to the ancestral condition. Consequently, both of these clades indicate that either marine habits need not to be coupled with changes in vertebral number or that the basal taxa examined retained largely terrestrial habits. It is noteworthy, however, that derived placodonts possess a turtle-like carapace over the trunk, which correlates with a remarkable decrease in vertebral number. Other taxa with extensive dermal armor, a shell, or strongly modified thoracic ribs (bearing extensive plates or accessory ossifications) also possess low presacral counts, including turtles, the parareptile *Eunotosaurus*, and ankylosaurian dinosaurs. Although we did not find any statistical support for these observations, it is interesting that among placental mammals the armored armadillos also have a reduced presacral count (40, 42). In addition, it appears that altered somitogenesis always occurs in the trunk regions of these taxa, whereas their cervical series are unaffected. This example may be another indication that both mammals and reptiles rely on the same developmental responses under similar selective pressures.

A vertebral formula of 8 cervical, 10 trunk, and 2 sacral vertebrae (with a varying number of caudal vertebrae) is highly conserved among both fossil and recent turtles. The recently described basalmost turtle *Odontochelys semitestacea* (43), from the Late Triassic of China, departs from this general pattern in possessing only 9 trunk vertebrae. This low number probably represents a reduction from that present in the ancestor of turtles, assuming either archosauromorph affinities (Fig. 1) or an alternative, more basal position of turtles relative to living diapsids (44).

The aforementioned examples demonstrate that although taxon-specific variation is present in all amniote lineages, specific ecological/morphological adaptations are consistently coupled with similar underlying developmental mechanisms.

**Size and Vertebral Formulae.** Increasing body size by increasing vertebral numbers within the axial skeleton (pleomerism) is a primary mechanism of body size increase in fish, some lissamphibians, and snakes (5, 21). The role of pleomerism in other amniote groups has not been investigated. Our observations clearly demonstrate that the dramatic increases in body size seen in many dinosaur taxa are not accompanied by an increase in vertebral number. All dinosaur taxa show a generally conservative presacral vertebral count that is relatively close to the ancestral amniote condition. Derived sauropods, the macronarians and diplodocoids, also fit this pattern despite the evolution of impressively long necks in these clades, which leads us to infer that within sauropods, neck elongation was not only due to elongation of individual vertebrae but also the result of homeotic effects, causing somewhat higher cervical and lower dorsal numbers than in many other dinosaurs (45). Additional meristic changes may occur in some individual sauropod species, as in *Mamenchisaurus* and *Euhelopus*, which both possess 30 presacral vertebrae, the highest number present among the sauropod taxa sampled in this study. This pattern is similar to that described for marine reptiles (see above), which indicates that whatever the respective ecology,

high cervical counts in an elongated neck appear to be under the primary influence of homeotic changes. By contrast, body size per se seems to play only a marginal role (if any) in the evolution of sauropod vertebral counts. Similarly, giant snakes do not reach final body size through significant increases in vertebral number but through postembryonic somatic growth (5), as must also have been the case in dinosaurs.

Conversely, small amniotes often possess high vertebral counts. This is best exemplified in squamates, where numerous fossorial lineages including amphisbaenians, dibamids, and snakes attain adult body lengths as small as 100 mm (46, 47) but possess the highest vertebral counts among amniotes. This observation provides additional support for the observation that segmentation and body size are not positively correlated within amniote lineages. We therefore hypothesize that factors other than size, for example habits and locomotion, may be more important in driving the evolution of high or low vertebral counts in many amniote clades (21).

**Conclusions.** Shortly after the origin of amniotes, reptiles and synapsids evolved very different degrees of plasticity in their vertebral numbers, with reptiles displaying much more variation. This discrepancy could be due to a developmental constraint in the synapsid lineage lacking in reptiles. Unfortunately, the current dearth of large-scale comparative ecological studies over extended temporal intervals prevents examination of the effects of these potentially different selective regimes on the evolutionary trajectories of these two lineages.

We demonstrate that “extreme ecologies” such as marine life habits seem to elicit the same developmental responses, independent of the phylogenetic position of the taxa involved. In addition, size does not appear to be a major factor in the evolution of vertebral counts.

Finally, our study emphasizes the importance of fossils when interpreting developmental evolutionary morphologies. The fossil record helps to recognize “the pattern of evolutionary change we want to explain” (48, page 530), and as such is indispensable for large-scale interpretations of evolutionary processes. As we have illustrated in our study, the fossil record can provide insights into ancient developmental mechanisms even if the developmental transformation itself is no longer present in a living taxon. By examining also Paleozoic amniotes, we have explored developmental programs that are 20–100 million years older than the oldest fossils of the living amniote groups.

Although our sampling of fossil clades is very comprehensive, the taxa considered in the analysis represent only a small subsample of the amniote evolutionary tree, because of various preservational biases of other known species and extinction so far not recorded in the fossil record. For example, our study considered 96 nonavian dinosaur genera, whereas Wang and Dodson (49) estimated that  $\approx 1,850$  genera once existed. As such, as more information from the fossil record becomes available, this could be used to further explore the evolutionary and developmental patterns discussed in this article.

## Methods

A total of 436 amniote taxa were considered in this study, including many recent taxa, dinosaurs, basal synapsids, and numerous stem members of recent and extinct clades. Data were obtained by critically reviewing the literature, supplemented with data from personal observations on original specimens (*SI Appendix*). In cases where a taxon possessed variable vertebral formulae, the mean value was used in the analysis. Identification of the cervicodorsal boundary was problematic in some taxa because of the transitional morphology that sometimes occurs in this region. Where personal observations of material were not possible, or where specimens were inadequately figured, information obtained from the primary literature was taken at face value; however, in most cases we were able to use several cues to establish the position of this boundary, including the position of the parapophysis relative to the neurocentral boundary, the shape of the ribs, or the presence/absence of ribs.

Data for each of the clades surveyed were supplied as follows: Synapsida (M.R.S.-V.); Ornithischia and nonavian Theropoda (P.M.B.); Aves (P.G.P.E.); Sauropodomorpha and Crurrotarsi (D.P.); Squamata (J.J.H.); Parareptilia, basal Eureptilia, Thalattosauriformes (J.M.); Trutudinata, Ichthyopterygia, Sauropterygia excluding Plesiosauria, basal Crurrotarsi, basal Diapsida (together with J.M.), Choristodera, Pterosauria (T.M.S.); and Plesiosauria (I.W.).

Individual composite phylogenies were constructed for the major taxonomic groups on which the vertebral counts were mapped. These composite phylogenies are informal supertrees, representing an evaluation of the current evidence based on each person's individual taxonomic expertise (SI Appendix). We also assembled a general tree of amniote relationships incorporating all of the major clades as well as basal, monospecific taxa representing independent lineages (Fig. 1). Perhaps the most controversial issue in the general amniote tree was the phylogenetic position of turtles (44). For the present study, we followed the latest molecular results (50, 51) and grouped turtles as the sister taxon to Archosauriformes.

Ancestral states for vertebral numbers were reconstructed by using squared-change parsimony, which minimizes the sum of squared changes along a rooted tree, in the software package Mesquite 2.6 (52). The ancestral states for the presacral and cervical counts of each taxonomic group were first reconstructed by using the individual phylogenies and then mapped onto the general amniote phylogeny, whereas the values for monospecific taxa were entered directly. Similar to the individual phylogenies, the ancestral states for

the major clades were then reconstructed by using the squared-change parsimony algorithm.

Because we were interested primarily in vertebral numbers and different regionalization patterns, we did not take measurements of individual vertebrae. Furthermore, metric data for individual vertebrae in complete presacral series of fossils are rare. As such, our study cannot be used to evaluate different patterns of somatic growth.

PIC and PGLS were calculated by using the software package COMPARE 4.6b (27), with resolved branch lengths set to 1 and polytomies artificially resolved with branch lengths of 0.001, which is effectively zero but satisfies the algorithmic requirements of the program.

The comparison of the means of the CP ratios and the deviations from the ancestral presacral amniote count was performed by a two-tailed *t* test in Microsoft Excel.

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