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Phylogenetic Signal in Bone Microstructure of Sauropsids

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Abstract.—In spite of the fact that the potential usefulness of bone histology in systematics has been discussed for over one and a half centuries, the presence of a phylogenetic signal in the variation of histological characters has rarely been assessed. A quantitative assessment of phylogenetic signal in bone histological characters could provide a justification for performing optimizations of these traits onto independently generated phylogenetic trees (as has been done in recent years). Here we present an investigation on the quantification of the phylogenetic signal in the following bone histological, microanatomical, and morphological traits in a sample of femora of 35 species of sauropsids: vascular density, vascular orientation, index of Haversian remodeling, cortical thickness, and cross-sectional area (bone size). For this purpose, we use two methods, regressions on distance matrices tested for significance using permutations (a Mantel test) and random tree length distribution. Within sauropsids, these bone microstructural traits have an optimal systematic value in archosaurs. In this taxon, a Mantel test shows that the phylogeny explains 81.8% of the variation of bone size and 86.2% of the variation of cortical thickness. In contrast, a Mantel test suggests that the phylogenetic signal in histological traits is weak: although the phylogeny explains 18.7% of the variation of vascular density in archosaurs, the phylogenetic signal is not significant either for vascular orientation or for the index of Haversian remodeling. However, Mantel tests seem to underestimate the proportion of variance of the dependent character explained by the phylogeny, as suggested by a PVR (phylogenetic eigenvector) analysis. We also deal with some complementary questions. First, we evaluate the functional dependence of bone vascular density on bone size by using phylogenetically independent contrasts. Second, we perform a variation partitioning analysis and show that the phylogenetic signal in bone vascular density is not a by-product of phylogentic signal in bone size. Finally, we analyze the evolution of cortical thickness in diapsids by using an optimization by squared change parsimony and discuss the functional significance of this character in terms of decreased buoyancy in crocodiles and mass saving in birds. These results are placed in the framework of the constructional morphology model, according to which the variation of a character in a clade has a historical (phylogenetic) component, a functional (adaptive) component, and a structural (architectural) component. [Archosauria; bone histology; Diapsida; periosteal ossification; phylogenetically independent contrasts; random tree-length distribution; regressions on distance matrices; variation partitioning.]

The potential usefulness of bone microstructure in systematics has been discussed for over one and a half centuries. A continuum of opinions about the factors that generate bone histodiversity has been expressed that emphasized either the phylogenetic or the functional aspects. At one extreme, some authors have assumed that bone microstructural characters include diagnostic phylogenetic information. For instance, Queckett (1849), Zavattari and Cellini (1956), and Dittmann (2003) dealt with the problem of the utilization of bone histological characters to identify vertebrate bone samples. Houde (1986, 1987) analyzed the variation of bone vascular organization in birds in a cladistic framework and concluded that some bone tissue types represent synapomorphies of Palaeognathae or Neognathae. At the other extreme, many authors have argued that bone microstructural characters may not include much phylogenetic information but reflect ontogenetic and functional factors. Amprino (1947) proposed that variation in the growth rate of bone tissue determines variation in its structure. Some quantitative works (e.g., Castanet et al., 1996, 2000; Margerie et al., 2002, 2004) have studied this functional relationship, now named ''Amprino's rule.'' Several studies (e.g., Lanyon, 1984; Thomason, 1985; Currey and Alexander, 1985; Carter et al., 1991; Margerie, 2002; Margerie et al., 2005) have provided evidence supporting Wolff's law (1892), which states that bone microstructure predominantly reflects adaptations to biomechanical constraints. If Amprino's rule or Wolff's law are correct, bone histodiversity may be only indirectly linked to, and poorly correlated with, the phylogeny.

From our point of view, this dichotomy between historicism and functionalism is misleading for two reasons. First, as noted by Darwin (1859: 217), adaptations inherited from ancestors (phylogenetic signal) originated in the past by natural selection and should, therefore, ultimately be considered as functional effects. Second, as has recently been stated by Ricqlès et al. (2004), "the old hope to find phylogenetic characters at the tissue level is now rooted in the premise that all intrinsic character-states of the semaphoront, at all levels of organization (from molecules to ethology), *should* have a taxonomic value, as they necessarily become synapomorphies at *some* level on the cladogram hierarchy" (unless they are autapomorphies at the level of terminal taxa with an homoplastic pattern of variation). According to these authors, all intrinsic character states (including those related to bone microstructure) should show a phylogenetic signal at some level of the phylogeny (Ricqlès et al., 2004). In this context, the classical question of whether the variation of a microstructural character is the outcome of functional factors or of phylogeny is not entirely appropriate because microstructural characters may have both a functional significance and a systematic value at some level of the phylogeny. As noted by Blomberg and Garland (2002), "casting phylogenetic inertia and adaptation by selection as alternative hypotheses may be inappropriate." Some recent papers show empirical analyses of microstructural traits

framed in this new integrative approach. These studies analyzed both the adaptive significance of microstructural traits and the phylogenetic variation of these characters by using parsimony. For instance, Rensberger and Watabe (2000) have shown that, whereas mammals, *Tupinambis* (Lepidosauria), and ornithischian dinosaurs share the presence of osteocyte canaliculi aligned parallel with the direction of bone growth (this is probably the primitive condition), coelurosaurs (Ornithomimidae and Aves) share the presence of extensively branching canaliculi in a random directional pattern (derived character state), which may be correlated with high bone growth rates. In the same context, Padian (2001), Padian et al. (2001), and Ricqlès et al. (2001) have suggested that the presence of an extensive fibrolamellar matrix may be a synapomorphy of dinosaurs, or a synapomorphy of ornithodirans if pterosaurs are included in the analysis (Padian and Horner, 2002), which may also reflect high bone growth rates. The presence of parallel-fibered matrix may be the primitive condition at this phylogenetic level (Padian, 2001). These conclusions were reached by considering these histological features as discrete (canaliculi aligned with the direction of growth versus canaliculi in a random directional pattern; fibrolamellar matrix versus parallel-fibered matrix) and by using parsimony.

In spite of the great development of methods to quantify phylogenetic signal for continuous characters (Legendre et al., 1994; Blomberg and Garland, 2002; Cubo et al., 2002; Freckleton et al., 2002; Blomberg et al., 2003; Laurin, 2004), so far, only two preliminary quantitative analyses have been carried out to assess the influence of the phylogeny on the variation of bone histological traits (Castanet et al., 2001; Cubo et al., 2001), and a single quantitative analysis has been carried out on bone microanatomical characters (Laurin et al., 2004). However, studying character evolution on a cladogram using parsimony is meaningful only if heritable character variation includes a phylogenetic signal (Cubo et al., 2002; Laurin, 2004).

The term "phylogenetic signal" refers to pure patterns that can be described, for continuously varying characters, as "the component of variation in a trait or of covariation between traits, attributable to the position of taxa within a phylogeny" (Starck and Ricklefs, 1998: 248) or as "the tendency for related species to resemble each other more than they resemble species drawn at random from the tree" (Blomberg and Garland, 2002). These patterns are the outcome of phylogenetic constraint (sensu Starck and Ricklefs, 1998: 248). As reviewed by Blomberg and Garland (2002), many modern researchers "equate phylogenetic inertia with nonadaptive (or maladaptive) phenotypic stasis." However, as noted by these authors, we cannot equate stasis to absence of selection because random mutation and genetic drift can produce phenotypic change (neutral character evolution). Therefore, stasis cannot be assumed as the null hypothesis for character evolution because it may be the outcome of stabilizing selection (Blomberg and Garland, 2002). Other processes generating a phylogenetic signal have been discussed by Harvey and Pagel (1991: 38–48) and Edwards and Naeem (1993). However, the data and methods of our study do not allow inferences on causal processes generating phylogenetic signal. Consequently, this paper deals only with patterns.

Various methods are available to quantify the phylogenetic signal for continuous characters (Blomberg and Garland, 2002; Freckleton et al., 2002). Regressions on distance matrices tested for significance by using permutations (Mantel, 1967) have been widely used to quantify phylogenetic signal in several kinds of characters, such as parasite species richness in Cyprinidae (Morand, 1997); morphological, life-history, behavioral, and ecological traits in extant birds (Böhning-Gaese and Oberrath, 1999; Böhning-Gaese et al., 2000); bone microstructural traits and bone shape in extant birds (Castanet et al., 2001; Cubo et al., 2001, 2002; Cubo, 2003) and in lissamphibians (Laurin et al., 2004); and body shape in Percidae (Guill et al., 2003).

Here we present a quantitative assessment of phylogenetic signal in the variation of bone microstructural traits in sauropsids by using two methods, regressions on distance matrices tested for significance by using permutations (Mantel test, Mantel, 1967) and the distribution of the squared length of continuous characters on random trees (Faith and Cranston, 1991). Below, this method is described by the more compact expression "random tree-length distribution." This analysis is important to justify the use of bone microstructure in systematics and in character optimizations on phylogenetic trees. We also deal with some complementary questions. First, we evaluate the functional dependence of bone vascular density on bone size by using phylogenetically independent contrasts (Felsenstein, 1985). Second, we test the hypothesis that the phylogenetic signal in bone vascular density is a by-product of phylogenetic signal in bone size due to character correlation. This hypothesis seems plausible because size is frequently linked to the phylogeny (Cheverud et al., 1985; Gittleman et al., 1996; Böhning-Gaese and Oberrath, 1999; Laurin, 2004). To test this, we perform a variation partitioning analysis (Desdevises et al., 2003). Finally, we analyze the evolution of cortical thickness in diapsids through an optimization by squared-change parsimony (Maddison, 1991).

Material and Methods

We have analyzed diaphyseal cross sections of a sample of femora of 35 species of adult sauropsids (Fig. 1). We have chosen femora because the other long bones are deeply modified by locomotor adaptations in Neognathae; there are adaptations to flight in forelimb bones and adaptations to bipedalism in hindlimb bones other than the femur (for instance, the fusion of the tibia to some tarsal elements to form the tibiotarsus, and the fusion of tarsal and metatarsal elements to form the tarsometatarsus). Bone sections corresponding to the 5 species of Chelonia, the 11 species of Lepidosauria, and the 3 species of Crocodylia used in this study belonged to a preexisting collection (J. Castanet, Pierre et Marie Curie University, Paris). Bone sections for the 16 Neognathae of the sample were especially prepared for this study using the same methods that were used to prepare the

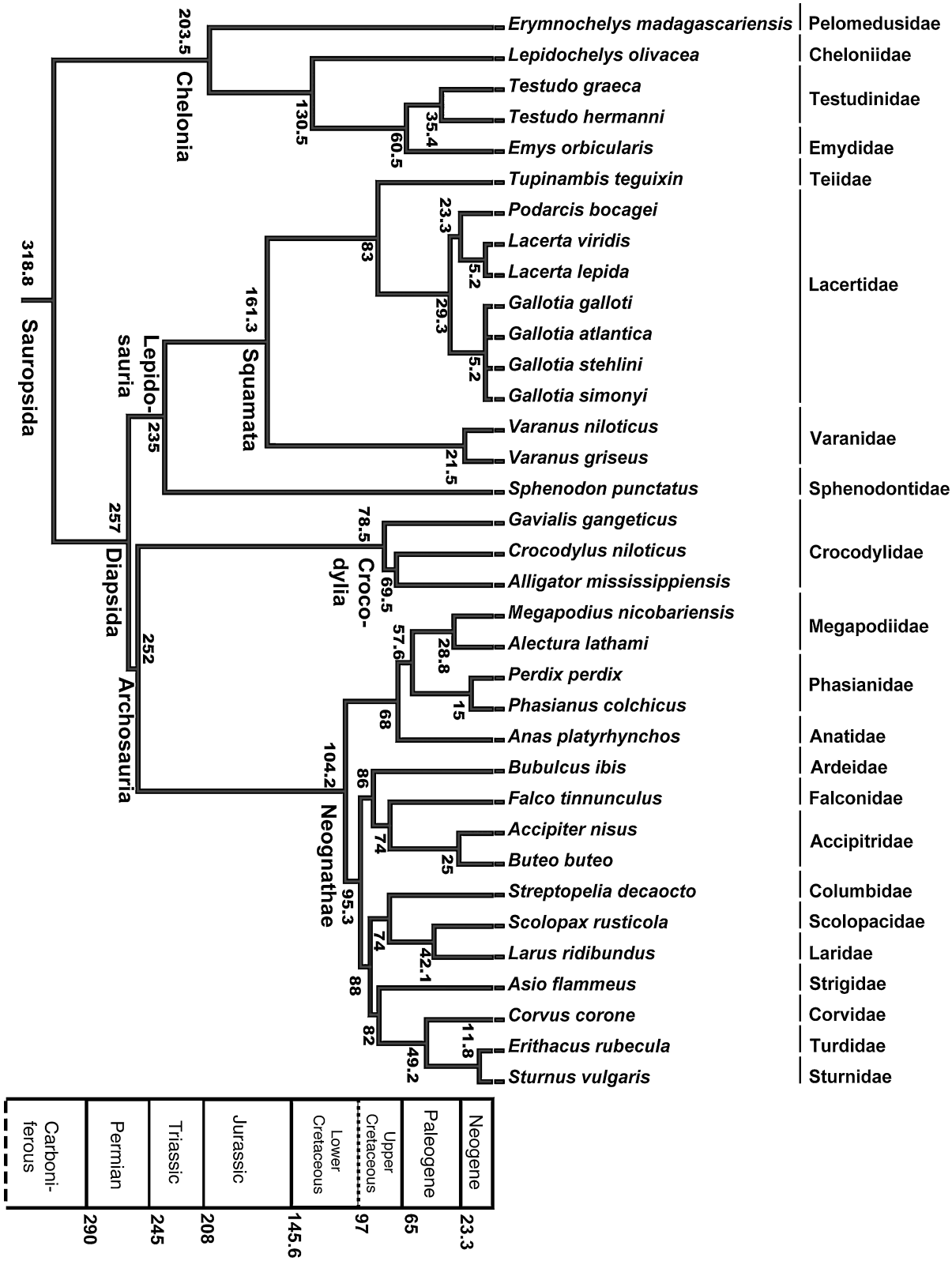

FIGURE 1. Phylogenetic relationships and divergence times in millions of years of the 35 species of adult Sauropsids used in this study. The phylogeny within Chelonia follows Gaffney (1990), Rougier et al. (1995), and Hirayama (1998); within Lepidosauria, Estes (1982), Estes et al. (1988), Rieppel (1988), and Caldwell (1999) were followed. The phylogeny of Crocodylia follows Brochu (1997). For birds, topology of basal Neognathae and within Galloanserae was taken from Cracraft (2001), Livezey and Zusi (2001), van Tuinen and Hedges (2001), Paton et al. (2002), and Mayr and Clarke (2003) and topology within Neoaves was modified from Livezey and Suzi (2001). Basal divergence times within Neognathae were taken from Waddell et al. (1999) and van Tuinen and Hedges (2001), whereas the more recent ones were determined by using the oldest known fossil of each "family" (Unwin, 1993).

preceding samples. This sample may seem biased towards birds, but this is not the case, because 57% of the extant species of sauropsids belong to this taxon, and only 46% of the sampled species are birds.

We used wild animals that died of natural causes and were conserved frozen in the collections of the Muséum National d'Histoire Naturelle (Paris) and the Pierre et Marie Curie University (Paris). Animals were dissected and femora removed. Bones were decalcified with nitric acid (3%). Thin sections (14 to 16 μm thick) were obtained at the diaphyseal level (i.e., minimal diameter of the shaft) using a freezing microtome. Sections were stained with Ehrlich's hematoxylin, like the preexisting bone sections used in this study. Examination was carried out by transmission light microscopy. Sections were digitalized with a C3030 digital camera (Olympus, Japan) mounted on a binocular microscope (Zeiss, Germany) and analyzed by using Adobe Photoshop 7.0. We have measured two kinds of continuous microstructural traits (histological and microanatomical characters), as well as a morphological character (bone cross-sectional area). See Figure 2 for a graphical description of these traits. Other microstructural traits like bone tissue types (Francillon-Vieillot et al., 1990) are discrete and, consequently, are not entirely appropriate for the statistical analyses used in the present study. The various tissue types are not always easy to delimit objectively and, more importantly, they are complex characters resulting from various combinations of values of the characters included in our study. Thus, we preferred working on the basic continuous characters.

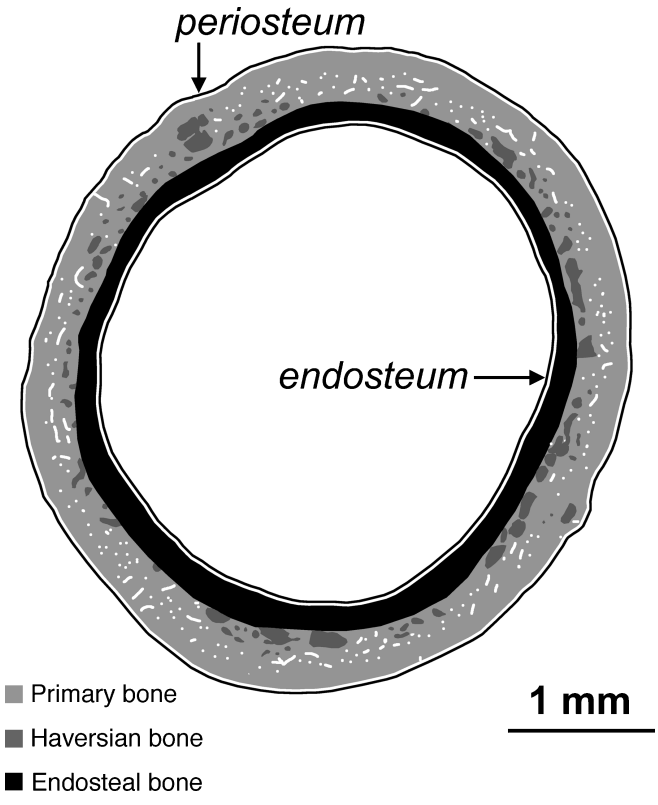

FIGURE 2. Schematic representation of the analyzed characters in a femoral diaphyseal cross section of *Corvus corone*. White dots are longitudinal canals containing blood vessels that run approximately parallel to the longitudinal axis of the long bone, appearing circular in cross sections; white lines are transverse canals containing blood vessels that run obliquely or orthogonally to the longitudinal axis of the long bone, appearing elongated in cross sections.

The histological characters include:

(1) *Vascular density*. This is the ratio of total vascular canal area (*Av*) to primary bone area (*Ap*). Total vascular canal area (*Av*) is the surface occupied by vascular canals in primary bone tissue. Primary bone area (*Ap*) refers to the total bone cross-sectional area (*A*), excluding the haversian bone area (*Ah*), the endosteal bone area (*Ae*), and the medullary cavity area (*Mc*). That is, $Ap = A - (Ah + Ae + Mc)$.
(2) *Vascular orientation*. This is the ratio of longitudinal vascular canal area (*Al*) to the total vascular canal area (*Av*). Total vascular canal area (*Av*) is the sum of longitudinal vascular canal area (*Al*) and transverse vascular canal area *(At)*. That is, *vascular orientation* $= Al/(Al + At)$. Longitudinal canals contain blood vessels that run approximately parallel to the longitudinal axis of the long bone, appearing circular in cross sections. Transverse canals contain blood vessels that run obliquely or orthogonally to the longitudinal axis of the long bone, appearing elongated in cross sections.
(3) *Index of Haversian remodeling* in cortical bone. This is the ratio of haversian bone area (*Ah*) to primary bone area (*Ap*). Haversian remodeling is a mechanism of destruction of primary compact bone and its reconstruction into secondary compact bone.

The single microanatomical character is relative thickness of cortical bone (*k*, "*cortical thickness*" throughout the paper), the ratio of inner diameter/outer diameter (Currey and Alexander, 1985). Character *k* varies between 0 and 1 (lower values are associated with thicker bone walls and higher values correspond to thinner bone walls).

The included morphological character is bone cross-sectional area, an estimator of bone size (*A*, "*bone size*" throughout the paper): $A = Ap + Ah + Ae + Mc$.

We followed the hierarchy of levels of integration of bone by Francillon-Viellot et al. (1990), according to which bone cortical thickness belongs to the (micro)anatomical level of integration and vascular density, vascular orientation, and the index of Haversian remodeling belong to the histological level of integration. See Appendix 1 for quantitative microstructural data.

The Reference Phylogeny

The phylogeny (topology and divergence times) was compiled from Gaffney (1990), Rougier et al. (1995), and Hirayama (1998) for Chelonia; Estes (1982), Estes et al. (1988), Rieppel (1988), Caldwell (1999) for Squamata; and Brochu (1997) for Crocodylia (Fig. 1). The position of turtles is still controversial (Rieppel and Reisz, 1999; Zardoya and Meyer, 2001); we have considered

that they are outside Diapsida (Fig. 1), as many paleontological studies have argued (Laurin and Reisz, 1995; Lee, 1995, 2001). For most sauropsid taxa, the age of the divergences was assessed using (whenever possible) the oldest known fossil that appeared to be part of each dichotomy between any pair of terminal taxa. When nested clades seem to appear simultaneously in the fossil record (thus suggesting a zero-length internal branch), we interpolated plausible divergence dates between calibrated dates. For recent divergences between terminal taxa, in the absence of relevant information from the fossil record, we enforced a 5.2 million year minimal branch length (this corresponds to the beginning of the Pliocene).

Recent molecular (van Tuinen and Hedges, 2001; Paton et al. 2002) and morphological (Cracraft, 2001; Livezey and Zusi, 2001; Mayr and Clarke, 2003) studies have reached similar conclusions regarding the relationships among major groups of extant birds: within Neognathae, Galliformes and Anseriformes are each other's closest relatives, and they are grouped in Galloanserae; Galloanserae and Neoaves (all other Neognathae) are sister-groups (Fig. 1). Molecular (Waddell et al., 1999; van Tuinen and Hedges, 2001; Paton et al., 2002), paleontological (Cracraft, 2001), and combined molecular and paleontological studies (Dyke and van Tuinen, 2004) have concluded that the divergences Galloanserae-Neoaves, Galliformes-Anseriformes, and the basal divergences within Neoaves occurred prior to the Cretaceous-Tertiary boundary. Divergence times used for the split between major groups of neognaths were Galloanserae-Neoaves: 104.2 Mya (van Tuinen and Hedges, 2001); Galliformes-Anseriformes: 68 Mya (Waddell et al., 1999). Other divergence dates were more difficult to estimate because, contrary to other sauropsid taxa, no detailed bird phylogeny include extinct taxa. Thus, the known fossils are difficult to place in a phylogeny. Therefore, within Neoaves, the first occurrence in the fossil record of each taxon traditionally considered a "family" (Unwin, 1993) was used to determine the minimal age of divergence between this "family" and its sister-group (we recognize that taxonomic levels are subjective and therefore we put the words "family" and "genus" within quotation marks). We have used half the age of the "family" as an estimate of the divergence between the two "genera" of a given "family" (each "family" included one or two "genera").

The divergence dates were required because all the comparative statistical methods that we use require branch lengths, that can correspond to evolutionary time separating included terminal taxa (Felsenstein, 1985). Other methods can be used to determine branch lengths, including number of changes in the phylogeny, but in the absence of a complete data matrix of the included taxa, the only other way of obtaining an estimate of evolutionary divergence between the taxa would be to assume unitary branch lengths, which corresponds to a speciational model of character evolution. Using a speciational model would be unwarranted because this requires knowing the total number of species (extant and extinct) in the clade studied (Cubo, 2003: 100); nobody has access to this information because the fossil record is incomplete.

Regressions on Distance Matrices

This method was described by Mantel (1967). Firstly we computed pairwise phylogenetic distances using the consensus phylogeny (Fig. 1). For each pair of species, the microstructural dissimilarity was quantitatively assessed using the absolute value of the difference between the character states. Two distance matrices were constructed: the phylogenetic distance matrix (the sum of branch lengths connecting two taxa, in My) and the microstructural dissimilarity matrix. Afterwards, the microstructural dissimilarity (the dependent variable) was regressed on the phylogenetic distance (the independent variable). The significance of the regression coefficient (R^2) could not be tested using a parametric test because the values of the phylogenetic distance matrices (the independent variables) are not normally distributed, and a normal distribution is a fundamental condition of parametric testing. In these cases, significance of statistics must be tested through randomization tests (Harvey and Pagel, 1991: 152–155). Therefore, the significance of the (R^2) parameter was tested by a permutation test (Mantel, 1967) using Permute 3.4a9 (distributed by P. Casgrain), a software that can perform regressions on distance matrices as described by Legendre et al. (1994). Each regression and its statistics were recomputed 9999 times by repeatedly randomizing the values of the microstructural dissimilarity matrix to obtain a null distribution against which to test the significance of the statistics of the regression on the original dataset. Contrary to the independent contrast analysis, no transformation of data or branch lengths were performed.

Random Tree-Length Distribution

A phylogenetic signal can also be detected in a character by determining if the character requires fewer steps on the reference phylogeny than on most randomly generated trees, provided that the phylogeny has been produced using other characters. In the case of continuous characters, squared length (rather than number of steps) of the character over the tree can be used (Maddison, 1991). The squared length is the statistic for a continuous character equivalent to the number of steps for discrete characters. It is the sum of the square of changes between each node or between nodes and terminal taxa. Squared change parsimony minimizes this statistic, and in the version that we used (weighted square-change parsimony, implemented in Mesquite), what is minimized is the sum over all branches of the squared change divided by branch length (Maddison, 1991). If the number of steps of the character of interest is less on the chosen phylogeny than in at least 95% of the randomly generated trees, we can conclude (using a 5% threshold) that the evolution of this character is associated with this tree (i.e., there is a phylogenetic signal in this character). These simulations were performed by the TreeFarm package of modules of Mesquite (Maddison and Maddison, 2002; Maddison et al., 2002). We used

a recently implemented simulation algorithm of the TreeFarm package of Mesquite that randomly permutes the taxa (along with their character values) on the tree, while holding the topology as well as the branch lengths constant. This procedure has the advantage of yielding random trees that have a branch length distribution identical to that of the reference tree. This is desirable because the squared length of a character over a tree depends on tree depth (Maddison, 1991). No transformation of data or branch lengths were performed in this analysis.

Phylogenetically Independent Contrasts

We tested the functional dependence of bone vascular density on bone size in sauropsids by using phylogenetically independent contrasts (Felsenstein, 1985; Harvey and Pagel, 1991; Garland et al., 1992). Values for both variables at all nodes in the phylogeny were estimated and standardized sister-taxon contrasts (differences weighted by branch lengths) for both variables were computed by using CAIC (Purvis and Rambaut, 1995). This method should be a priori adequate because it partitions the evolutionary change into constrasts (corresponding to the changes that occurred between the taxa), in such a way that the phyletic change included in a contrast is not included in the other contrasts (Starck and Ricklefs, 1998: 257). We used a phylogenetic tree with the same topology as in the preceding analyses. Both branch lengths and morphological data were transformed in order to avoid violations of statistical and evolutionary assumptions of the phylogenetically independent constrasts method. A minimum value of 20 My between two successive nodes or between a node and terminal taxon was imposed and, afterwards, a cubic root transformation was carried out. For the morphological variable, a log10 transformation was performed on bone size and a square root transformation was carried out on bone vascular density. We tested the normality of contrasts by using three different tests : Kolmogorov-Smirnov, Lillefors, and Shapiro-Wilk. For all tests, the distribution of contrasts was not significantly different from a normal distribution. We also tested evolutionary assumptions (the evolution of continuous characters was modeled as a random-walk process) and statistical assumptions (homogeneity of variance of the residuals in regression analysis) of the independent contrasts analysis. Finally, a regression through the origin for the contrasts in bone vascular density against the contrasts in bone size was carried out.

The Variation Partitioning Method

We tested whether the phylogenetic signal in bone vascular density is a by-product of phylogenetic signal in bone size by using a variation partitioning analysis. This method was developed by Desdevises et al. (2003) and can quantify the portion of the variation of bone vascular density exclusively explained by the phylogeny, the portion exclusively explained by bone size and, finally, the portion explained by the covariation between phylogeny and bone size. It has been argued (Desdevises et al., 2003) that regression on distance matrices underestimates the percentage of the variation of the dependent variable (e.g., bone vascular density) explained by the independent variables (e.g., phylogeny and bone size) because the coefficient of correlation between distance matrices derived from variables is always smaller than the coefficient of correlation on the original variables (Legendre, 2000). Furthermore, the Mantel test is known to be less powerful than the test of Pearson correlation. Consequently, we have performed our analysis of variation partitioning using the raw histological and size data (instead of distance matrices), but for consistency, we performed the same transformations as for the independent contrast analysis (including those for branch lengths). The phylogeny was expressed in the form of principal coordinates, which were computed from the phylogenetic distance matrix via principal coordinate analysis (Diniz-Filho et al., 1998) in *R* (Casgrain et al., 2004). Two methods were used to select the principal coordinates to be used in the variation partitioning method. The first three coordinates (explaining 70.6% of the phylogenetic variance) were selected by using a broken-stick model (Diniz-Filho et al., 1998). In a second analysis, all the principal coordinates that were significantly correlated to the dependent variable (eight) were selected (Desdevises et al., 2003) that explain 66.7% of the phylogenetic variance.

The portion of the variation of bone vascular density exclusively explained by the phylogeny, by bone size, and by the covariation existing between phylogeny and bone size were quantified using multiple linear regressions and tested for significance by using a permutation test (see Desdevises et al., 2003). The significance of the portion exclusively explained by the phylogeny was tested by performing a partial regression between bone vascular density and the phylogenetic principal coordinates, using bone size as a covariable. Similarly, the significance of the portion exclusively explained by bone size was tested by performing a partial regression between bone vascular density and bone size, using the phylogenetic principal coordinates as covariables (Desdevises et al., 2003).

Optimization Using Squared-Change Parsimony

We were interested in the evolution of cortical thickness in diapsids because of the biomechanical significance of this character. Cortical thickness was optimized using squared-change parsimony in Mesquite (Maddison and Maddison, 2002). The branch lengths were derived from those shown in Figure 1, but a minimal length of 20 My was imposed on each branch (internal or terminal), and the resulting lengths were transformed by raising them to the power 0.33. As mentioned above, this transformation removed violations in statistical and evolutionary assumptions in the phylogenetically independent contrast analysis. For consistency, we have used the same branch length transformations here. The results obtained by using

squared-change parsimony are equivalent to those obtained by using the generalized linear model approach (Cunningham et al., 1998) and one-parameter maximum likelihood model (Webster and Purvis, 2002), two methods that also use branch length data.

RESULTS

Phylogenetic Signal

Within sauropsids, results obtained by using regressions on distance matrices and random tree-length distribution show that bone microstructural traits have an optimal systematic value among archosaurs (Table 1, Fig. 3). In this taxon, the phylogeny explains 81.8% of the variation of bone size and 86.2% of the variation of cortical thickness. In contrast, phylogenetic signal in histological traits are weak: although the phylogeny explains 18.7% of the variation of vascular density, the phylogenetic signal is not significant for vascular orientation or for the index of Haversian remodeling in archosaurs. For all the characters studied, the phylogenetic signal is not significant in Chelonia (five species), probably because of the small sample size. Crocodylia (three species) could not be tested separately for the same reason. Cortical thickness could not be measured in Chelonia because of the absence of a well-defined medullary cavity. Instead, in this group, the center of the bone is occupied by cancellous bone. The orientation of vascular canals could not be analysed in Lepidosauria because this variable is a ratio and the absence of vascularization in most of the studied species results in a mathematical indetermination. Finally, the index of Haversian remodeling in cortical bone could not be measured in Chelonia or in Crocodylia because of the absence of a well-defined medullary cavity in most of these taxa.

TABLE 1. Quantification of phylogenetic signal by using regressions on distance matrices tested for significance by using permutations (Mantel test) and tree-length distribution.

Variable	Clade	Species	Tree-length distribution P	Regressions on distance matrices R^2	Regressions on distance matrices P
Bone size	Sauropsids	33	0.0039**	0.044	0.0335*
	Diapsids	28	0.0093**	0.049	0.0173*
	Archosaurs	17	0.0360*	0.818	0.0248*
	Chelonia	5	0.282	0.025	0.8731
	Lepidosauria	11	0.1331	0.024	0.4036
	Neognathae	16	0.2044	0.0001	0.8566
Bone cortical thickness	Diapsids	28	0.0000***	0.536	0.0001***
	Archosaurs	17	0.0021**	0.862	0.0163*
	Lepidosauria	11	0.8363	0.069	0.2076
	Neognathae	16	0.1085	0.003	0.4864
Bone vascular density	Sauropsids	35	0.0000***	0.042	0.0104*
	Diapsids	30	0.0000***	0.140	0.0002***
	Archosaurs	19	0.0075**	0.187	0.0101*
	Chelonia	5	0.8820	0.185	0.2727
	Lepidosauria	11	0.0108*	0.136	0.0837
	Neognathae	16	0.0002***	0.077	0.0042**
Bone vascular orientation	Sauropsids	27	0.8460	0.002	0.8187
	Diapsids	22	0.8173	0.002	0.8338
	Archosaurs	19	0.6383	0.008	0.5206
	Chelonia	5	0.744	0.182	0.4687
	Neognathae	16	0.5986	0.0001	0.8508
Index of Haversian remodeling	Diapsids	27	0.3687	0.0026	0.5424
	Lepidosauria	11	0.1812	0.0009	1.0000
	Neognathae	16	0.4615	0.0000	0.9259

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

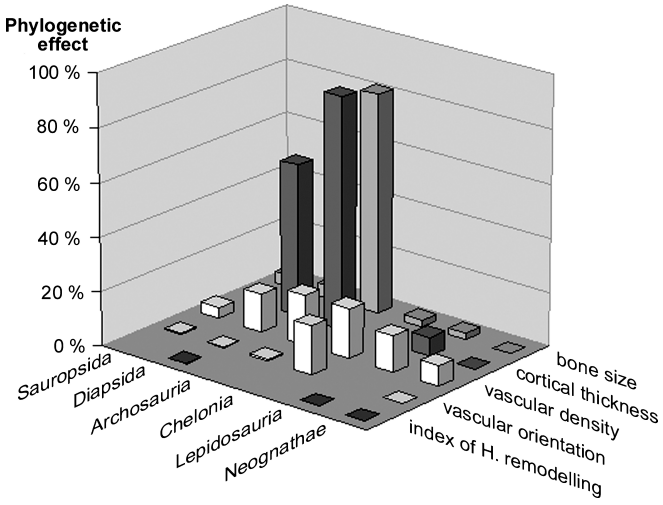

FIGURE 3. Histogram showing the proportion of the variance of the various bone microstructural traits explained by the phylogeny as shown by Mantel tests (Table 1). Bone microstructural traits have an optimal systematic value in archosaurs (the phylogeny explains 81.8% of the variation of bone size and 86.2% of the variation of cortical thickness). In contrast, the phylogenetic signal in histological traits is variable and weaker, but these values are likely to be underestimated (see text for detail).

Results obtained by using regressions on distance matrices tested for significance using permutations (Mantel test) are very similar to those obtained by using random tree-length distribution. In all cases but one, when a method detected a significant phylogenetic signal, the other method also detected a significant effect. The only exception is vascular density in Lepidosauria: although we have found a significant phylogenetic signal by using random tree-length distribution ($P = 0.0108$), the effect was not significant by using regressions on distance matrices ($P = 0.0837$). Probabilities are often slightly lower using random tree-length distribution than regressions on distance matrices. This systematic difference may be linked to the fact that regressions on distance matrices requires a linear relationship between phylogenetic distance and morphological differences, and this requirement may not be met (Laurin et al., 2004). Moreover, it should be kept in mind that the regressions on distance matrices underestimate the percentages of variation of morphological characters explained by phylogeny and that this test is conservative (Legendre, 2000; Desdevises et al., 2003).

Size Effect

The phylogenetically independent contrasts analysis (Felsenstein, 1985) suggests that bone size explains 18.3% of the variance of bone vascular density (slope = 0.031, $P = 0.016$, $n = 30$ contrasts; Fig. 4). Because the phylogenetic signal in bone size is strong (81.8% of the variance explained) compared with that found in bone vascular density (18.7% of the variance explained) in archosaurs

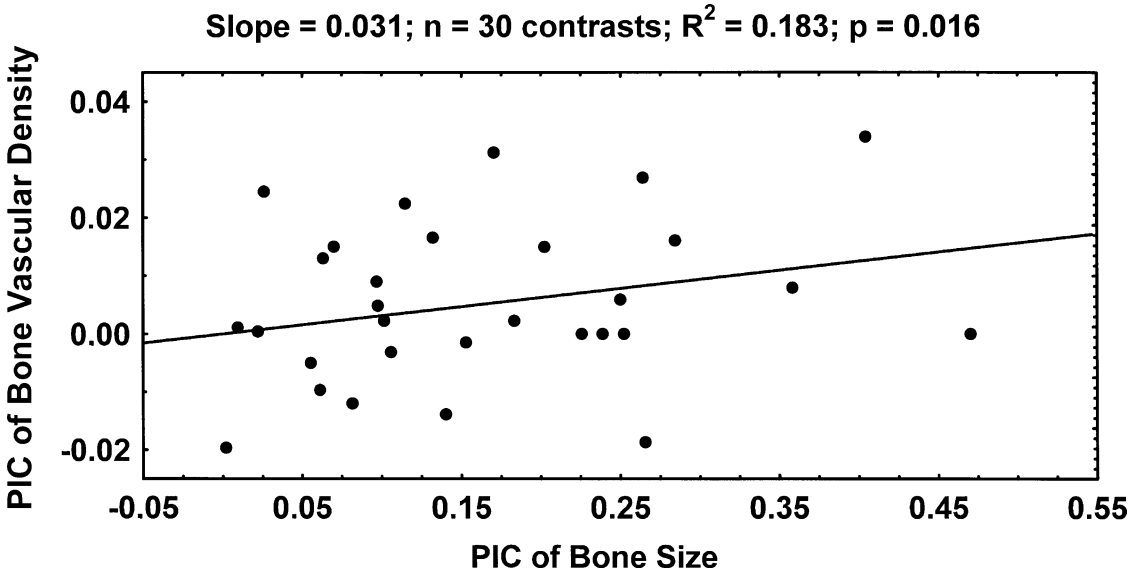

FIGURE 4. Linear regression of phylogenetically independent contrasts (PIC) of bone vascular density on PIC of bone size in sauropsids showing a significant relationship between these variables.

(Fig. 3), we wondered whether the phylogenetic signal in bone vascular density was a by-product of phylogenetic signal in bone size due to character correlation. To answer this question, we performed a variation partitioning analysis. When we used the phylogenetic principal coordinates selected by reference to a broken-stick model, 2.8% ($P = 0.341$) of the variation of bone vascular density was exclusively explained by bone size, 51.5% of the variation was exclusively explained by the phylogeny ($P = 0.0003$), and 23.4% of the variation was explained by the covariation existing between the phylogeny and bone size (Table 2). Unfortunately, the variation partitioning method cannot test for the significance of this last portion (Desdevises et al., 2003). When all the phylogenetic principal coordinates that were significantly related to the dependent variable (bone vascular density) were selected for the analysis, the percentages exlusively explained by bone size and phylogeny were, respectively, 2.0% ($P = 0.445$) and 67.8% ($P = 0.0002$), and 24.2% (untested) of the percentage was explained by the covariation between the phylogeny and bone size (Table 2). The variation partitioning analyses show that a significant part of the variation of bone vascular density is exclusively explained by the phylogeny (51.5% and 67.8%, depending on the method of selection of phylogenetic principal coordinates). Therefore, we can conclude that most of the phylogenetic signal in bone vascular density is not a by-product of phylogenetic signal in bone size due to character correlation.

An Application: The Evolution of Cortical Thickness

The discovery of a significant phylogenetic signal in cortical thickness in diapsids ($R^2 = 0.536$, $P = 0.0001$; Table 1) validates the optimization of this character onto the independently generated phylogenetic tree and the study of the pattern of character evolution. This optimization was carried out by using squared-change parsimony and shows that the last common ancestor of this group probably had moderately thick bone walls (estimated $k = 0.469$; Fig. 5). Both the last common ancestor of lepidosaurs and the last common ancestor of archosaurs appear to have retained the primitive condition

TABLE 2. Partitioning analysis on the variance of bone vascular density using phylogenetic eigenvector analysis (PVR) and two different methods to select phylogenetic principal coordinates (PCAs). Significance of components a and c were tested as explained in Desdevises et al. (2003); portions b and d cannot be tested (Desdevises et al., 2003).

	Explained proportion of the variance of bone vascular density (%)			
Type of variance explained	Only size-related (a)	Covariance between size and phylogeny (b)	Only phylogenetic (c)	Unexplained (d)
Partitioning using a broken stick model to select phylogenetic PCAs (three axes retained, representing 70.6% of the phylogenetic variance)	2.8 ($P = 0.341$)	23.4	51.5 ($P = 0.0003$)	22.3
Partitioning using multiple regressions with permutations to select phylogenetic PCAs (eight axes retained, representing 66.7% of the phylogenetic variance)	2.0 ($P = 0.445$)	24.2	67.8 ($P = 0.0002$)	6.0

FIGURE 5. Optimization of bone cortical thickness onto the independently generated phylogenetic tree of diapsids by using squared change parsimony. The branch lengths were derived from those shown in Figure 1 but a minimal length of 20 My was imposed on each branch (internal or terminal), and the resulting lengths were transformed by raising them to the power 0.33 to remove violations in statistical and evolutionary assumptions.

for diapsids (moderately thick bone walls: estimated $k = 0.461$ and 0.476, respectively; Fig. 5). Two major events presumably took place during the evolution of cortical thickness in Archosauria. On the one hand, cortical thickness increased drastically in Crocodylia ($k = 0.220$ in *Alligator mississippiensis*, the only species of this group for which k could be measured). On the other hand, cortical thickness decreased dramatically in birds (estimated k for the last common ancestor of birds = 0.681; Fig. 5).

Discussion

According to the constructional morphology model (Seilacher, 1970; Gould, 2002; Cubo, 2004), variation of a character in a clade has three components (aspects): historical (phylogenetic), functional (adaptative), and structural (architectural). However, the proportion of the character variation explained by each of these three components (aspects) can overlap. In fact, Darwin (1859: 217) already noted that "natural selection acts by either now adapting the varying parts of each being to its organic and inorganic conditions of life; or by having adapted them during past periods of time." Gould (2002: 1055) viewed the genesis of presently adaptive features "either by immediate construction for a current role, or by adaptive origin in an ancestor, with subsequent maintenance by homology in descent," and therefore agrees with Darwin's point of view. According to these hypotheses, we can consider that while immediate adaptations to current conditions (the first of Darwin's categories, autapomorphies with functional significance for discrete characters) are "pure" functional effects, past adaptations inherited by homology (the second of Darwin's categories; synapomorphies with functional significance for discrete characters) are simultaneously "deep" functional effects and phylogenetic signal (Cubo, 2004). Neutral character evolution would explain a part of the remaining variation.

In this paper, we have quantified separately the historical (phylogenetic signal) and the functional (size effect) components of bone vascular density variation by using, for the first purpose, regressions on distance matrices and tree length distribution and, for the second one, phylogenetically independent contrasts. Although these methods are appropriate to quantify separately the historical and the functional components of character variation, they fail to quantify the proportion of the variation explained by the covariation between these factors. We have used the variation partitioning method (Desdevises et al., 2003) to quantify the portion of the variation of bone vascular density exclusively explained by the phylogeny (synapomorphies without functional significance) that exclusively explained by functional factors (autapomorphies with functional significance) and that explained by the covariation between both factors. This last portion can be called "phylogenetically structured functional variation" by analogy with the term "phylogenetically structured environmental variation" proposed by Desdevises et al. (2003) for ecological variables, and corresponds with the "deep" functional effects, or synapomorphies with functional significance quoted above.

Size Effect

Femora of small sauropsids of our sample are avascular (*Gallotia, Lacerta, Podarcis, Sphenodon*) or almost avascular (*Erithacus, Sturnus*). In these specimens, the *periosteum* (a connective tissue located at the external bone surface; Fig. 2) and the *endosteum* (located at the internal bone surface, surrounding the medullary cavity; Fig. 2) bring sufficient nutriments and oxygen to bone cells. As body size and bone size (measured in linear dimensions) increase in different lineages, the volume of cortical bone increases proportionally to bone length raised to the third power, but the surfaces of *periosteum* and *endosteum* that bring nutriments and oxygen to bone cells increase roughly proportionally to bone length raised to the second power. The presence of bone vascularization in larger sauropsids may compensate for this geometric constraint. Therefore, we expected a positive relationship between bone vascular density and bone size.

The independent contrasts analysis shows evidence for this functional relationship ($R^2 = 0.183$, $P = 0.016$, $n = 30$; Fig. 4). The variation partitioning analysis shows that the sum of the variance of bone vascular density explained by bone size and by the covariance between bone size and the phylogeny is 26.2% of the total variance (Fig. 6). This value is higher than that obtained by using independent contrasts (18.3%, see above), suggesting that this last method removes some of the phylogenetically structured functional variation of bone vascular density (the portion of the variance explained by the covariance between phylogeny and bone size). However, the proportion of the variance of bone vascular density attributable to size in the independent contrast analysis includes at least part of the variance explained by the covariance between size and phylogeny, as shown by a comparison with the results of the partitioning analysis.

Phylogenetic Signal

Our results show that one histological character displays a strong phylogenetic signal (bone vascular

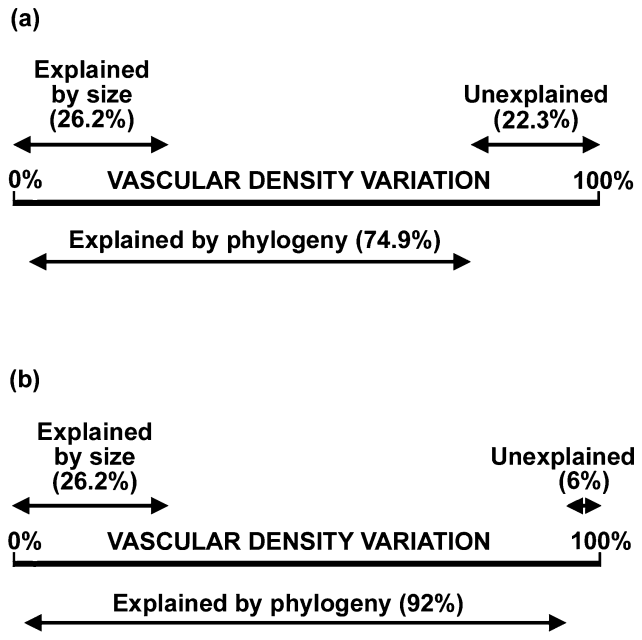

FIGURE 6. Partitioning analysis on the variance of bone vascular density according to (a) phylogenetic eigenvector analysis (PVR) using a broken stick model to select phylogenetic principal coordinates (PCAs) and (b) PVR using multiple regressions with permutations to select phylogenetic PCAs.

density), but two others do not (Tables 1, 2). Two reasons may explain the absence of phylogenetic signal in the index of Haversian remodeling and in bone vascular orientation. Haversian remodeling is an epigenetic mechanism of destruction/reconstruction of bone that has been shown to be linked to individual age (Kerley, 1965; Ericksen, 1991), biomechanical constraints (Rubin et al., 1996; Lee et al., 2002), and phospo-calcic metabolism (Ott, 2002). Therefore, considering that the index of Haversian remodeling depends on the individual experience (the "individual history") of each organism (it is not an intrinsic or inherited character-state), it is not surprising to find an absence of phylogenetic signal on this trait (although see Sander, 2000, who has found evidence for a strong phylogenetic signal on Haversian remodeling in sauropod dinosaurs). On the other hand, although bone vascular orientation can be influenced to some extent by epigenetic factors during ontogeny, the global influence of these factors seems to be small because this trait does not change after bone formation during ontogeny. In this case, the absence of phylogenetic signal may be explained by a fast evolutionary rate that may have produced a homoplastic pattern of variation. If so, a denser taxonomic sampling may be necessary to detect a phylogenetic signal in this character.

Our morphological and microanatomical characters (femoral cross-sectional area and cortical thickness) show a strong phylogenetic signal, which validates the frequent use of morphological characters in phylogenetic inference (e.g., Hennig, 1966; Laurin and Reisz, 1995; Lee, 2001; Mayr and Clarke, 2003) and corroborates recent results on bone microanatomy (Laurin et al., 2004).

An Application: The Evolution of Cortical Thickness

As discussed by Cubo et al. (2002) and Laurin (2004), the existence of a significant phylogenetic signal in a character (with a significant genetic determinism) should be verified prior to performing an optimization of this character onto an independently generated phylogenetic tree. This is one of the principal justifications for quantifying phylogenetic signal. In fact, the estimation of ancestral character states in a character for which there is no significant phylogenetic signal is probably meaningless. The finding of a significant phylogenetic signal in cortical thickness in diapsids (53.6% of the variance explained by the phylogeny) warranted the optimization of this character onto the phylogenetic tree. The pattern of evolution of cortical thickness deserves a careful analysis because of its biomechanical significance. For a given external diameter, bones with thicker walls have a higher density than bones with thin walls. The drastic increase in cortical thickness in Crocodylia (Fig. 5) and the resulting high skeletal density may be advantageous for aquatic life as it may counteract lung buoyancy and, therefore, facilitates diving and underwater locomotion (Ricqlès and Buffrénil, 2001; Laurin et al., 2004). The drastic decrease in bone wall thickness in birds (Fig. 5) and the resulting savings in skeletal mass is probably advantageous to flying animals (Currey and Alexander, 1985; Cubo and Casinos, 2000) because for a given external diameter, bones with thin walls are lighter than bones with thick walls, especially when air sacs are present. This conclusion is supported by an optimization, a procedure that is routinely used in comparative or evolutionary studies (e.g., Cunningham et al., 1998; Padian et al., 2001; Cubo et al., 2002; Laurin, 2004), and by the presence of a strong phylogenetic signal in this character. We propose that a test be routinely used to detect such a signal before optimizing a character, just like tests of normality or of homogeneity of variance are normally performed before applying parametric statistical tests.

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APPENDIX 1. Quantitative microstructural data. All characters but the last are dimensionless numbers (see text for more information).

Species	Bone vascular density	Bone vascular orientation	Index of Haversian remodeling	Bone cortical thickness	Bone size (mm^2)
Chelonia					
Erymnochelys madagascariensis	0.0018	0.1918	—	—	26.1615
Lepidochelys olivacea	0.0051	0.1080	—	—	200.0189
Testudo graeca	0.0016	0.1609	—	—	18.9839
Testudo hermanni	0.0104	0.0588	—	—	22.1177
Emys orbicularis	0.0005	0.2822	—	—	8.4834
Lepidosauria					
Tupinambis teguixin	0.0107	0.0250	0.0036	0.4983	26.6864
Podarcis bocagei	0.0000	—	0.0000	0.3870	0.3278
Lacerta viridis	0.0000	—	0.0000	0.2641	0.8714
Lacerta lepida	0.0000	—	0.0000	0.5442	2.9115
Gallotia galloti	0.0000	—	0.0000	0.3602	1.6138
Gallotia atlantica	0.0000	—	0.0000	0.5998	0.9237
Gallotia stehlini	0.0000	—	0.0000	0.4811	4.7595
Gallotia simonyi	0.0000	—	0.0000	0.6121	20.0549
Varanus niloticus	0.0082	0.6452	0.0000	0.4894	26.4178
Varanus griseus	0.0073	0.0940	0.0000	0.5267	9.8103
Sphenodon punctatus	0.0000	—	0.0000	0.4186	10.969
Crocodylia					
Gavialis gangeticus	0.0789	0.1887	—	—	—
Crocodylus niloticus	0.0035	0.1900	—	—	—
Alligator mississippiensis	0.0096	0.0763	—	0.2200	250.6296
Neognathae					
Megapodius nicobariensis	0.0212	0.1914	0.0069	0.8423	27.0349
Alectura lathami	0.0334	0.1350	0.0018	0.8988	85.1621
Perdix perdix	0.0398	0.1629	0.0000	0.7596	9.2377
Phasianus colchicus	0.0454	0.1268	0.0000	0.8071	35.0945
Anas platyrhynchos	0.0490	0.0879	0.0206	0.7599	16.699
Bubulcus ibis	0.0345	0.3401	0.0000	0.8484	18.5342
Falco tinnunculus	0.0329	0.1266	0.0197	0.8524	11.5163
Accipiter nisus	0.0362	0.1408	0.0073	0.8608	15.3269
Buteo buteo	0.0348	0.1223	0.0232	0.8471	35.7518
Streptopelia decaocto	0.0203	0.3021	0.0000	0.7980	7.5159
Scolopax rusticola	0.0195	0.2441	0.0046	0.8130	13.2076
Larus ridibundus	0.0160	0.2633	0.4403	0.7559	18.4636
Asio flammeus	0.0275	0.1822	0.0269	0.7752	14.1953
Corvus corone	0.0173	0.2113	0.1007	0.7578	13.716
Erithacus rubecula	0.0014	1.0000	0.0000	0.8253	1.1476
Sturnus vulgaris	0.0055	0.2180	0.0000	0.8259	5.2382