Acta Zoologica (Stockholm) **90**: 110–122 (April 2009) doi: 10.1111/j.1463-6395.2008.00328.x

Microanatomical diversity of the humerus and lifestyle in lissamphibians

Aurore Canoville and Michel Laurin

UMR 7179, Université Paris 6 – Pierre et Marie Curie, 4 place Jussieu, Case 19, 75251 Paris, France

Keywords:
bone compactness, ecology, habitat, amphibians, evolution

Accepted for publication:
4 April 2008

Abstract

Canoville, A and Laurin, M. 2009. Microanatomical diversity of the humerus and lifestyle in lissamphibians. — *Acta Zoologica* (Stockholm) **90**: 110–122.

A study of body size and the compactness profile parameters of the humerus of 37 species of lissamphibians demonstrates a relationship between lifestyle (aquatic, amphibious or terrestrial) and bone microstructure. Multiple linear regressions and variance partitioning with Phylogenetic eigenVector Regressions reveal an ecological and a phylogenetic signal in some body size and compactness profile parameters. Linear discriminant analyses segregate the various lifestyles (aquatic vs. amphibious or terrestrial) with a success rate of up to 89.2%. The models built from data on the humerus discriminate aquatic taxa relatively well from the other taxa. However, like previous models built from data on the radius of amniotes or on the femur of lissamphibians, the new models do not discriminate amphibious taxa from terrestrial taxa on the basis of body size or compactness profile data. To make our inference method accessible, spreadsheets (see supplementary material on the website), which allow anyone to infer a lissamphibian lifestyle solely from body size and bone compactness parameters, were produced. No such easy implementation of habitat inference models is found in earlier papers on this topic.

Aurore Canoville and Michel Laurin, UMR 7179, Université Paris 6 – Pierre et Marie Curie, 4 place Jussieu, Case 19, 75251 Paris, France.
E-mail: aurore.canoville@free.fr and laurin@ccr.jussieu.fr

Introduction

Extant tetrapods inhabit a great diversity of habitats (aquatic, amphibious or terrestrial). During their evolution, the shifts between these three lifestyles were sometimes accompanied by significant morphological modifications (Duellman and Trueb 1986). Lifestyle appears to be reflected in the skeleton at various levels ranging from gross morphology to histological structure (de Buffrénil *et al.* 1986; Bininda-Emonds *et al.* 2001). One of the most frequent and noticeable adaptations to an aquatic lifestyle is the modification of limbs into paddles (Carroll 1985). In the absence of such obvious adaptations, it is often difficult to assess the ecology of a taxon solely on the basis of morphology. It is therefore difficult to infer the habitat of most Palaeozoic tetrapods. However, at the histological level, the presence of large amounts of calcified cartilage is often associated with an aquatic lifestyle.

The relationship between bone microanatomy and lifestyle in extant tetrapods has been intensively studied (Fawcett 1942; Wall 1983; de Buffrénil *et al.* 1986; de Buffrénil and Schoevaert 1988; Stein 1989; Fish and Stein 1991; Leclair *et al.* 1993; Castanet *et al.* 2001; Steyer *et al.* 2004; Scheyer and Sander 2007). As a general rule, the long bones of terrestrial taxa have a large medullary cavity and a compact cortical region. In contrast, the long bones of aquatic vertebrates generally have a smaller medullary cavity. Furthermore, two divergent adaptations in bone global compactness in aquatic taxa were highlighted in previous studies: secondarily aquatic taxa that live in shallow water or have recently returned to an aquatic lifestyle show pachyostotic bones (de Ricqlès and de Buffrénil 2001), where the compact, heavy bones are usually interpreted as an adaptation to reduce buoyancy (Taylor 2000). In the extant fauna, sirenians (Fawcett 1942; de Buffrénil and Schoevaert 1989) and some lissamphibians (Laurin *et al.* 2004) show high pachyostosis. Conversely, active deep divers, such as extant cetaceans, have hydrodynamic, rather than hydrostatic, depth control favoured by an inertia reduction that results in low compactness of the cortex and in rather spongy bones (de Buffrénil *et al.* 1986).

Few studies have used quantitative methods to investigate the relationship between bone microstructure and lifestyle in extant vertebrates. Most of them were performed on mammals (Wall 1983; de Buffrénil *et al.* 1986; Stein 1989; Fish and Stein 1991). The relationship between skeletal microstructure and lifestyle in extant lissamphibians has rarely been studied. Nevertheless, this group is especially relevant to studies on the relationship between skeleton and habitat because of its great diversity of lifestyles (strictly aquatic, amphibious, strictly terrestrial). Leclair *et al.* (1993) and Castanet and Caetano (1995) worked on small taxonomic samples (of, respectively, seven and four species each represented by several individuals) of anurans. They concluded that the proportion of the body mass represented by the skeleton of anurans is lower in aquatic taxa than in terrestrial taxa. Recently, Laurin *et al.* (2004) studied the compactness profile of femoral cross-sections and body size of 46 species of lissamphibians (23 species of Anura and 23 species of Caudata) using several tests that incorporate phylogenetic information. They showed that the return to a fully aquatic lifestyle is associated with an increase in the compactness of the femur and an increase in body size.

The main purpose of our study is to determine how the compactness of the humerus of lissamphibians evolves in response to the return to an aquatic lifestyle. Our approach is complementary to the study of Laurin *et al.* (2004) because we have extracted new data for the humerus from a similar taxonomic sample of lissamphibians. We also use different statistical tests, some of which consider phylogenetic relationships.

Most previous studies on bone microstructure have not considered phylogenetic effects. Indeed, the phylogenetic value of bone histological characters has long been debated. Some authors assumed that these data were phylogenetically informative (Padian *et al.* 2001); in contrast, Castanet *et al.* (2001) concluded, after performing quantitative tests, that bone histological characters primarily reflected the ecology of a taxon rather than its phylogenetic affinities. Nowadays it is accepted that bone histological and microanatomical characters can supply both functional and phylogenetic information (Laurin *et al.* 2004). However, few studies have attempted to quantify these signals (Laurin *et al.* 2004; Cubo *et al.* 2005; Germain and Laurin 2005; Kriloff *et al.* 2008).

The present study compares results obtained from various bones, which might respond differently to a change in habitat because each is subjected to different mechanical stresses (de Margerie *et al.* 2005). Several reasons led us to study the humerus. Previous analyses (Laurin *et al.* 2004; Germain and Laurin 2005; Kriloff *et al.* 2008) show that inference models obtained from one bone (e.g. the femur) cannot be used to obtain inferences based on another bone (e.g. the radius). Fossils of lissamphibians are rare and precious, and producing histological sections is a destructive process so it would be advantageous to be able to base inferences on as many kinds of bones as possible. Furthermore, the proximodistal gradient of compactness in the bones of sea cows (de Buffrénil and Schoevaert 1989) suggests that proximal limb bones may yield a stronger ecological signal than more distal bones. Therefore, comparable studies need to be performed on other long limb bones to determine which yields the most reliable ecological signal.

Finally, to make our inference method accessible, we produced spreadsheets (see supplementary material on the website) that allow anyone to infer a lissamphibian lifestyle solely from body size and bone compactness parameters.

Materials and Methods

Our analysis involved the humeri of 47 individuals representing 37 species and 27 genera of extant lissamphibians (see Appendix A on the website). Most large clades of Anura and Caudata are represented in our sample, as well as the most highly aquatic lissamphibians such as *Cryptobranchus alleganiensis* (Daudin, 1803), *Andrias japonicus* (Temminck, 1836), *Amphiuma means* Garden in Smith, 1821, and Pipidae Gray, 1825, as well as their most terrestrial relatives such as Bufonidae Gray, 1825 and *Hypsiboas boans* (Linnaeus, 1758). Moreover, we sampled the various lifestyles fairly evenly (Appendix B on the website: 12 aquatic species, 11 amphibious species and 14 terrestrial species). Most body size classes are represented in our sample, from taxa of minute body size (Plethodontidae Gray, 1850; Hynobiidae Cope, 1859) to some of the largest extant lissamphibians such as *Andrias japonicus*, *Amphiuma means*, *Siren lacertina* Linnaeus and Österdam, 1766, and *Rhinella marina* (Linnaeus, 1758; best known as *Bufo marinus*). Finally, all the bones represent adults to avoid bones that were actively growing at the time of death and structural differences that reflect ontogenetic variations.

This study is based on detailed anatomical drawings of mid-diaphyseal cross-sections (Figs 1–3; Appendix B on the website), both because compactness profiles can vary within a single bone (the metaphysis is generally spongier than the diaphysis) and because the mid-diaphyseal level should yield the strongest ecological signal. In fact, while spongy bone is usually present in the metaphysis of vertebrates of all lifestyles, at the mid-diaphyseal level it is common only in aquatic tetrapods. More precisely, by 'mid-diaphyseal' we mean the region of the diaphysis that is narrowest and where the cortical compacta is thickest. It is not necessarily the midpoint between the proximal and the distal extremities of the bone.

All cross-sections were drawn with a camera lucida, digitized and transformed into binary images using PHOTOSHOP 7.0. Bone was marked as black and all other surfaces (medullary cavity, vascular and resorption spaces) as white. Finally, all cross-sections were analysed using BONE PROFILER (Girondot and Laurin 2003).

BONE PROFILER, already used by Laurin *et al.* (2004, 2006), Steyer *et al.* (2004), Germain and Laurin (2005) and Kriloff

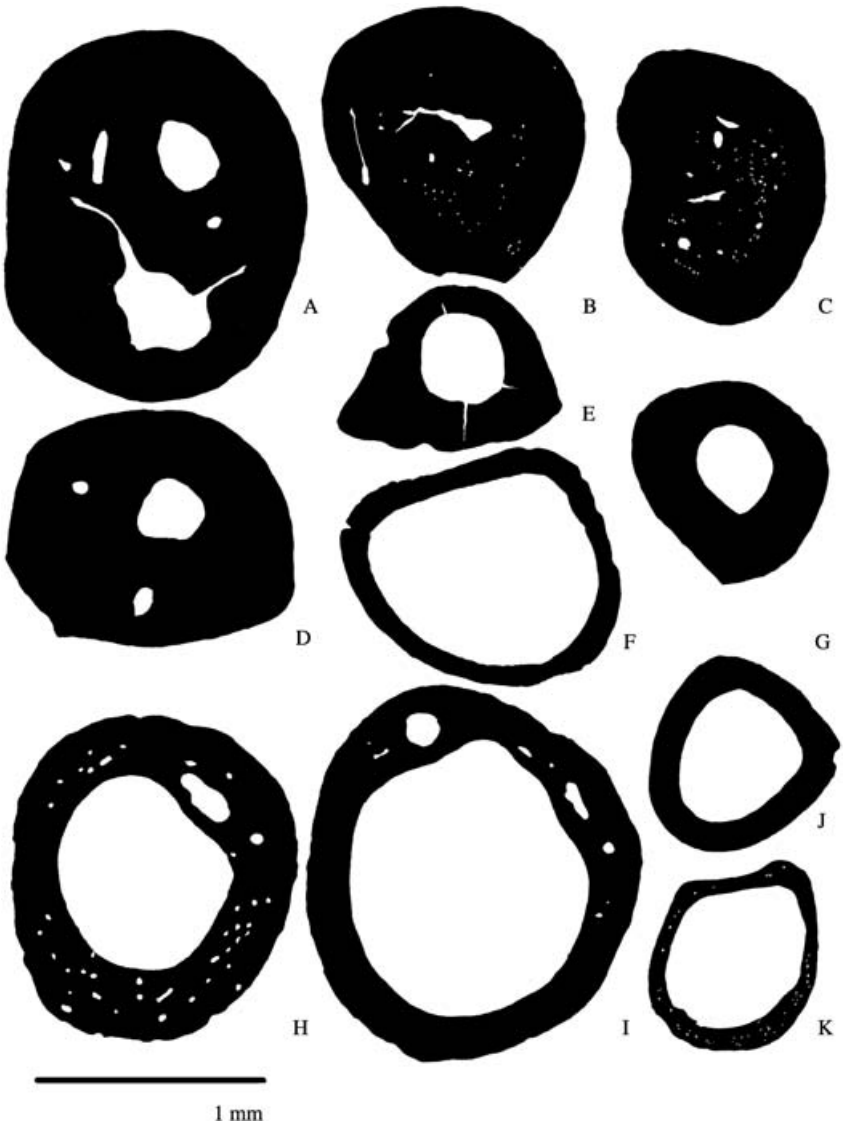

Fig. 1—Drawings of mid-diaphyseal cross-sections of humeri of small aquatic (**A**–**E**), amphibious (**F**–**G**) and terrestrial (**H–K**) anurans. Taxa are arranged according to their lifestyle and phylogenetic affinities. Scale: 1 mm. —**A**. *Pipa carvalhoi*, —**B**–**D**. *Xenopus leavis* (three individuals), —**E**. *Bombina orientalis*, —**F**. *Discoglossus* sp., —**G**. *Ascaphus truei*, —**H**–**I**. *'Bufo' pentoni* (two individuals), —**J**. *Chiromantis rufescens*, —**K**. *Pelobates fuscus*.

et al. (2008), allows the extraction of several compactness profile parameters that describe a mathematical model. This model shows the distribution of the bony tissue on the cross-section along the radius of the bone. This method is advantageous because it is able to reveal differences in the distribution of the bony tissue between aquatic and terrestrial taxa, even if the global compactness is equal in both groups.

Bone Profiler divides each drawing of a bone section into 51 concentric zones and 60 radial sectors (each of which measures 6° in width). Then, the compactness (i.e. the ratio of the area occupied by bone tissue to the total area of the cross-section) is measured in each of the 3060 cells and in the whole section (see Laurin *et al.* 2004; figure 3). These data are used to establish 60 compactness profiles on a complete section. Bone compactness C as a function of the distance to the centre d can be described by a sigmoidal function. The graph (Laurin *et al.* 2004; figure 2), which is associated with the function, is called the compactness profile.

$$C(d) = \frac{1}{1 + e^{\frac{1}{S}(P-d)}}(Max - Min) + Min$$

Four main parameters are extracted from this model (Appendix B on the website). P is the relative distance from the centre to the point of inflection, where the most abrupt change in compactness is observed. P is then proportional to the size of the medullary cavity. This parameter has a relationship with the corticodiaphyseal index (CDI), which has often been used in traditional comparative osteology (Castanet and Caetano 1995): it corresponds to 1 – CDI.

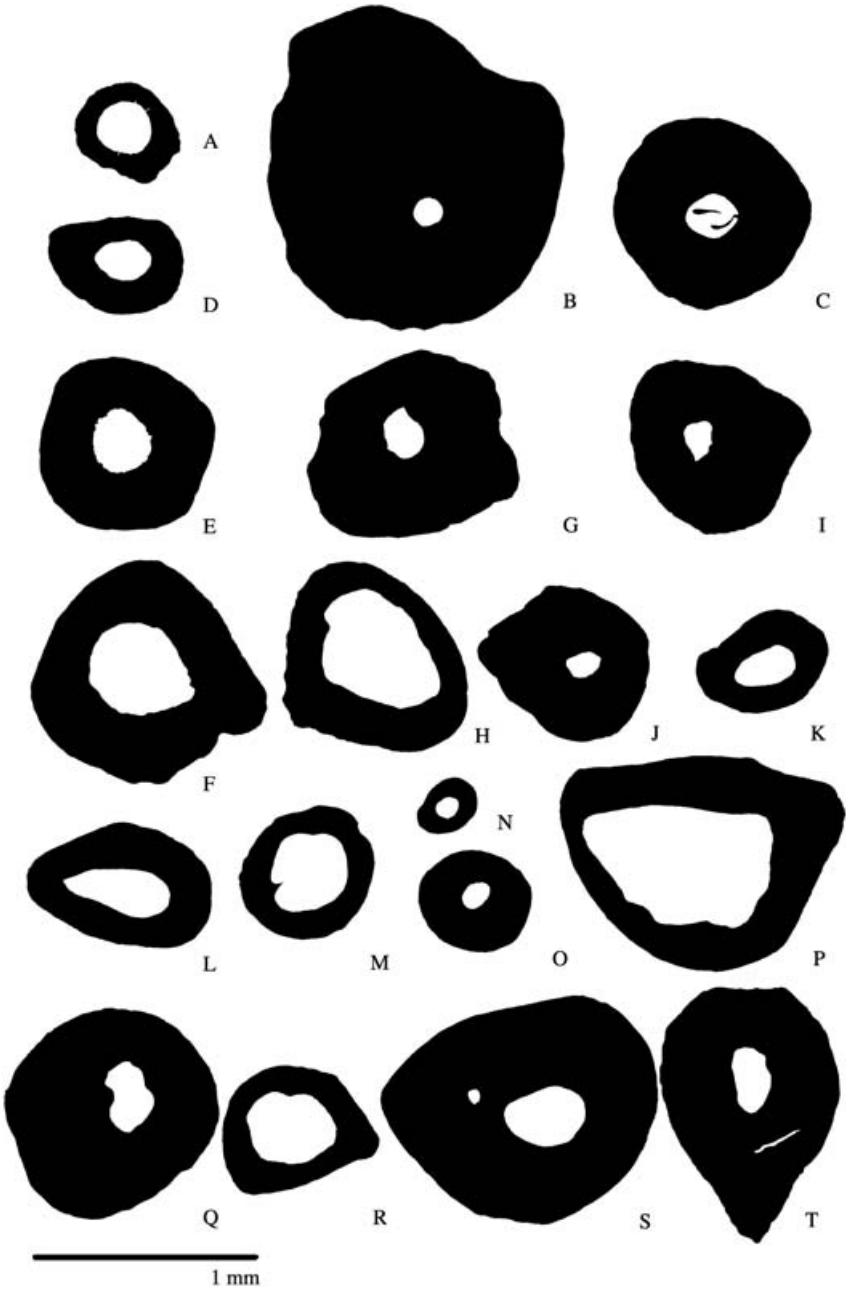

Fig. 2—Drawings of mid-diaphyseal cross-sections of humeri of small aquatic (**A**–**C**), amphibious (**D**–**K**) and terrestrial (**L–T**) caudates. Taxa are arranged according to their lifestyle and phylogenetic affinities. Scale: 1 mm. —**A**. *Proteus anguinus*, —**B**. *Siren lacertina*, —**C**. *Amphiuma means*, —**D**. *Onychodactylus fischeri*, —**E**. *Desmognathus quadramaculatus*, —**F**, **H**. *Pleurodeles waltl* (two individuals), —**G**, **I**, **J**. *Triturus marmoratus* (three individuals), —**K**. *Triturus alpestris*, —**L**. *Salamandrella keyserlingii*, —**M**. *Ambystoma opacum*, —**N**. *Desmognathus ocrophaeus*, —**O**. *Desmognathus monticola*, —**P**–**S**. *Salamandra salamandra* (four individuals), —**T**. *Salamandra lanzai.*

S is the reciprocal of the slope at the inflection point and generally reflects the width of the transition zone between the cortical compacta and the medulla. Finally, *Max* and *Min* are, respectively, the upper and the lower asymptotes and often correspond to the compactness in the outermost cortex and in the centre of the medullary region. These four indices are calculated using data on the 51 zones, disregarding the sectors (which are merged in this case).

Other parameters calculated by the software are included in our analyses (Appendix B on the website): the angular parameters *Srad*, *Prad*, *Minrad* and *Maxrad* calculated using 6° sectors (their values are the average of 60 estimates, one for each sector), the compactness in the centre of the bone section (*Cc*), the compactness in the periphery of the cross-section (*Cp*) and the global compactness of the bone cross-section (*Cg*). We also incorporated body size parameters (Presacral length: *PLg*, and bone maximal diameter: *MD*, log-transformed or not) into the analyses (Appendix B on the website) because an initial examination of many sections revealed that the bones of small animals usually have a

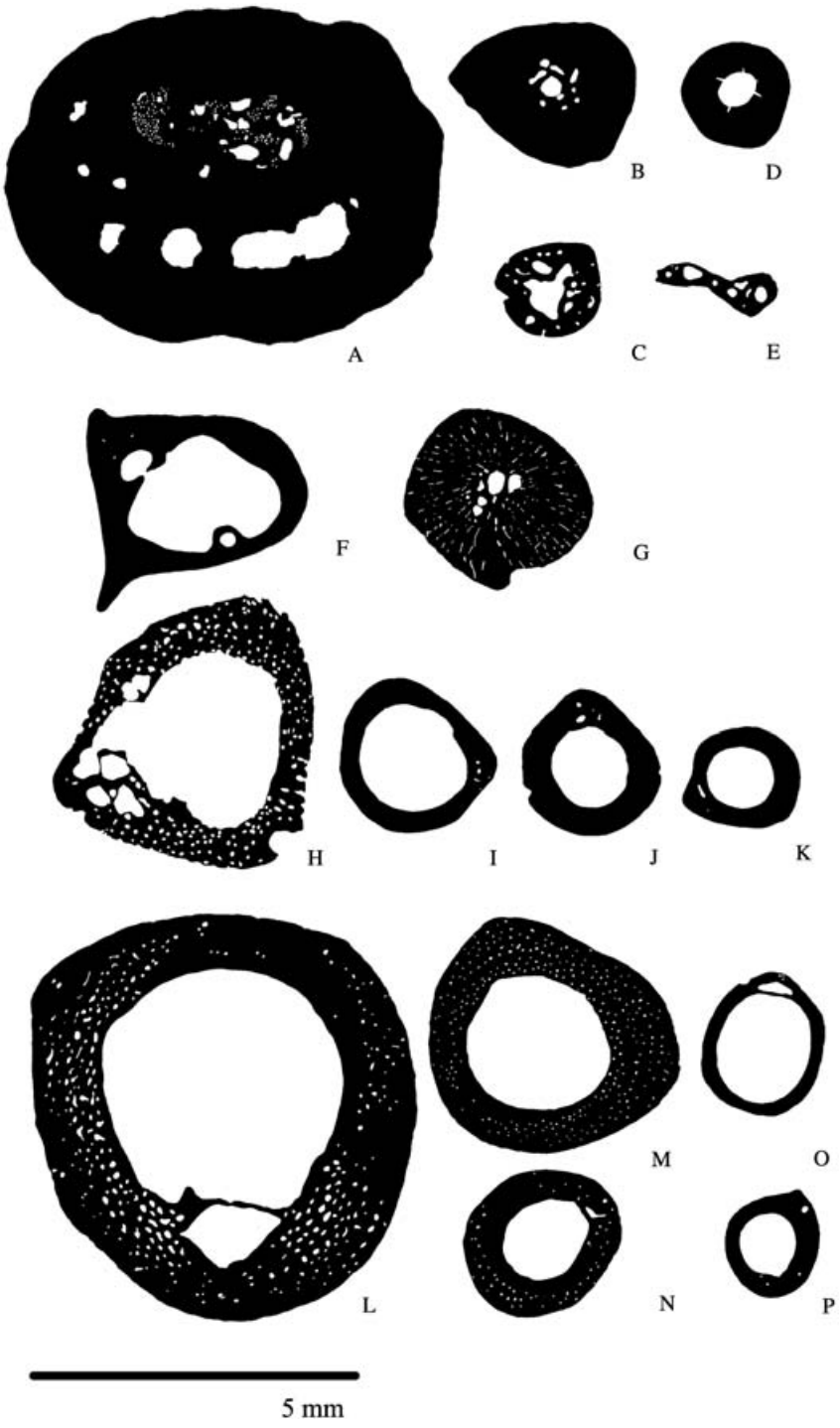

Fig. 3—Drawings of mid-diaphyseal cross-sections of humeri of medium-sized aquatic (**A-D**, **F**–**G**), amphibious (**H**–**K**) and terrestrial (**E**, **L**–**P**) caudates (**A**–**E**) and anurans (**F**–**P**). Taxa are arranged according to their lifestyle and phylogenetic affinities. Scale: 5 mm. —**A**. *Andrias japonicus*, —**B**. *Cryptobranchus alleganiensis*, —**C**. *Ambystoma andersoni*, —**D**. *Necturus maculosus*, —**E**. *Salamandra atra*, —**F**. *Telmatobius culeus*, —**G**. *Pipa pipa*, —**H**. *Lithobates catesbeianus*, —**I**. *Lithobates vaillanti*, —**J**. *Lithobates forreri*, —**K**. *Pelophylax ridibundus*, —**L**, **M**. *Rhinella marina* (two individuals), —**N**. *Bufo bufo*, —**O**. *Hypsiboas boans*, —**P**. *Pachymedusa dacnicolor*.

simpler structure than those of large animals. Furthermore, body size might contain ecological information, because it has already been suggested to differ between aquatic and terrestrial taxa (Bininda-Emonds *et al*. 2001; Laurin *et al*. 2004). Presacral length corresponds to the length from the atlas to the sacrum (in cm), except for anurans, in which it was measured to the posterior end of the urostyle. Bone diameter refers to the maximal diameter of the cross-section (in mm). Finally, lifestyle is coded as follows: 0 = aquatic, 1 = amphibious and 2 = terrestrial. These states are defined by the relative amount of time spent in water: > 90% for aquatic taxa, between 20% and 90% for amphibious taxa and

< 20% for terrestrial taxa. We have coded the habitat of all species into these three categories using both primary literature and compilations, such as Duellman and Trueb (1986) and Goin *et al.* (1978).

Some statistical tests performed below incorporate phylogenetic information. A phylogeny that incorporates branch lengths (proportional to divergence time in million years before present and established using the oldest fossil attributable to each clade or, when fossils were unavailable, using molecular estimates) had to be produced (Fig. 4). This supertree was compiled from three recently published phylogenies (Laurin *et al.* 2004; Wiens *et al.* 2005; Marjanovii and Laurin 2007) and was constructed using MESQUITE (Maddison and Maddison 2006).

To assess the phylogenetic and ecological signal in the compactness profile and body size parameters, a variation-partitioning method with Phylogenetic eigenVector Regressions was used (Desdevises *et al.* 2003). This comparative analysis consists of comparing two or more characters across species or a character and an environmental variable, while accounting for phylogenetic inertia. First, the phylogenetic-distance matrix was produced using the STRATIGRAPHIC TOOLS FOR MESQUITE (Josse *et al.* 2006) and then converted into a matrix of principal coordinates (PCs) using the R PACKAGE (Casgrain *et al.* 2004). The PC analysis (in the R PACKAGE) generated $n - 1$ PCs for n species, that is, 36 PCs in this analysis. However, only a subset of the resulting $n - 1$ axes can be used in the analyses because otherwise no degree of freedom would be left. These axes were selected using a broken-stick model or a backward-elimination procedure, depending on the analysis. Then a succession of multiple linear regressions (with 999 permutations) was performed using the software PERMUTE (Casgrain 2005).

To detect the phylogenetic signal, each compactness profile parameter and body size parameter was considered a dependent variable and the lifestyle and some PCs were considered the independent variables. We used backward-elimination procedures to determine which PCs contributed significantly to the explanation of each parameter (so 15 backward-elimination procedures were performed).

To detect the ecological signal, the dependent variable was the lifestyle and the independent variables were the compactness profile parameters, the body size parameters and the PCs representing the phylogeny. In that case, the PCs were selected using a broken-stick model (Diniz-Filho *et al.* 1998). Actually, the backward-elimination procedure kept too many PCs, which distorted the results because few degree of freedom had been left. We used a backward-elimination procedure (in PERMUTE) to determine which variables show the most important ecological signal.

Furthermore, a linear discriminant analysis was carried out using STATISTICA 6 (StatSoft France 2003). This method gives the probabilities of the modelled lifestyle and does not require linearity between the categories of discrete variables (here, the lifestyle). This model does not consider phylogenetic relationships. The dependent variable is once again the lifestyle, and the independent variables are the compactness profile parameters and body size parameters. Backward-elimination or forward-selection procedures can be used to identify which variables distinguish the various lifestyles.

Results

The partitioning method performed using PERMUTE (Casgrain 2005) demonstrated that most bone compactness parameters and body size parameters (except *Prad* and *Cg*) contain phylogenetic information (Table 1).

The broken-stick model retained only the first PC (PC1), which represents 62.38% of the phylogenetic variance of the 37 species. Only this PC was used in the analyses of ecological signal. The backward-elimination procedure retained the parameters *P*, *Max*, *Prad* and *Cc* as the most significant variables (Table 2). A variance partitioning analysis shows that these parameters exhibit an ecological signal ($P = 0.002$; Table 3), and that they explain 41.98% of the lifestyle variance of the 37 species of lissamphibians. The part of lifestyle variance explained by the phylogeny alone (1.57%) is not statistically significant ($P = 0.450$). An important fraction (56.88%) remains unexplained by the variables studied (Table 3).

Table 1 Proportion of the observed variance in each character explained by the phylogeny and probability that this variance is unrelated to this phylogeny

Dependent variable	Fraction related to phylogeny only	*P*-value
PLg	0.6711	0.004**
Ln(PLg)	0.2396	0.044*
MD	0.5171	0.008**
*Ln(10*MD)*	0.2647	0.006**
S	0.3379	0.032*
P	0.3125	0.004**
Min	0.9922	0.001***
Max	0.7481	0.001***
Srad	0.8091	0.022*
Prad	0.1687	0.052
Minrad	0.9991	0.001***
Maxrad	0.6349	0.003**
Cg	0.1579	0.052
Cc	0.9374	0.002**
Cp	0.6983	0.001***

To detect the phylogenetic signal, each compactness profile parameter and body size parameter was considered as a dependent variable and the lifestyle and some principal coordinate axes (PCs) were considered as the independent variables. We used backward-elimination procedures to determine which PCs contributed significantly to the explanation of each parameter.

*Significant at a 0.05 threshold, **significant at a 0.01 threshold, ***significant at a 0.001 threshold.

See Materials and Methods for abbreviations.

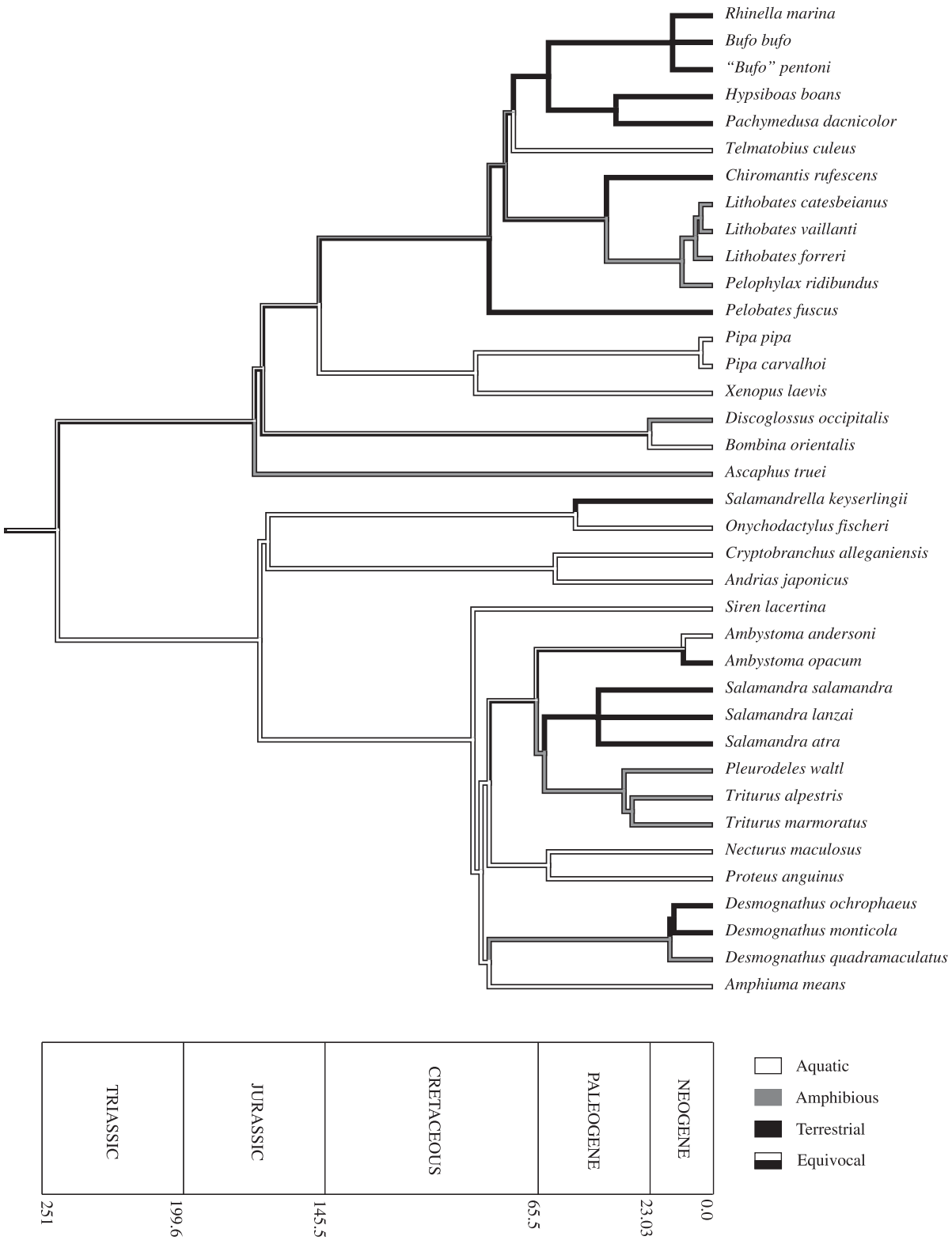

Fig. 4—Phylogeny used in this study. This supertree is a compilation of three recently published phylogenies (Laurin *et al*. 2004; Wiens *et al*. 2005; Marjanovii and Laurin 2007). The lifestyle is optimized as an ordered character using parsimony. The geological time is expressed in million years before present.

Table 2 Parameters that reflect the lifestyle

Parameter	Backward elimination
PLg	**3**
Ln(PLg)	**9**
MD	**11**
*Ln(10*MD)*	**12**
S	**2**
P	0.002**
Min	**7**
Max	0.003**
Srad	**6**
Prad	0.015*
Minrad	**5**
Maxrad	**10**
Cg	**8**
Cc	0.002**
Cp	**1**
PC 1	**4**

Numbers in bold indicate the step at which the parameter has been removed from the backward-elimination analysis. Probability that the variation of characters is random with respect to lifestyle is obtained by multiple linear regressions for lifestyle as the dependent variable with a backward-elimination procedure.
*Significant at a 0.05 threshold, **significant at a 0.01 threshold.
See Materials and Methods for abbreviations.

Table 3 Results of the partitioning method performed with the habitat as the dependent variable and *P*, *Max*, *Prad*, *Cc* and the principal coordinate axis 1 as independent variables

	R^2	*P*-value
Fraction related to ecology	0.4198	0.002**
Fraction related to ecology and phylogeny	–0.0044	
Fraction related to phylogeny	0.0157	0.450
Residual variation (unexplained fraction)	0.5688	

**Significant at a 0.01 threshold.

The first results suggest that bone compactness evolves with lifestyle. There is an ecological signal in the parameters *P* and *Prad*. Aquatic taxa tend to have a lower value of these parameters (Table 4). The medullary cavity is smaller in aquatic taxa than in their terrestrial and amphibious relatives. Furthermore, *Cc*, which reflects the compactness in the centre of the cross-section, has a higher value in aquatic taxa than in other taxa (Table 4). The values of these parameters suggest that there is a difference between aquatic taxa on the one hand and amphibious and terrestrial taxa on the other hand. However, there is no clear separation between amphibious and terrestrial taxa. Aquatic taxa tend to be larger than terrestrial and amphibious taxa and have higher values of the parameter *S (*the transition zone between the cortical compacta and the medulla is broadest in aquatic taxa) (Table 4).

A simple examination of the drawings (Figs 1–3) confirms these conclusions. In anurans (Figs 1 and 3) and caudates (Figs 2 and 3), aquatic species generally have a thicker cortical compacta and a smaller medullary cavity than their terrestrial relatives. The transition zone between the cortical compacta and the medullary cavity seems to be wider in aquatic taxa than in amphibious and terrestrial taxa. This microanatomical difference of the humerus according to lifestyle is well illustrated by the comparison between the fully aquatic *Xenopus laevis* (Daudin, 1802; Fig. 1B–D) and the terrestrial *Pelobates fuscus* (Laurenti, 1768; Fig. 1K). Furthermore, the humeri of small lissamphibian species (Figs 1 and 2) seem to have a much simpler structure than the bones of mid-sized animals (Fig. 3). Humeral mid-diaphyseal cross-sections of *Pipa pipa* (Linnaeus, 1758; Fig. 3G), *Lithobates catesbeianus* (Shaw, 1802; Fig. 3H) or *Rhinella marina* (Fig. 3L,M) show several vascular and resorption spaces. Nevertheless, examination of the drawings reveals no microanatomical variations that might reflect phylogenetic affinities.

Three discriminant analyses were performed using STATISTICA.

First, only the parameters selected in the variance partitioning method (*P*, *Max*, *Prad* and *Cc*; Table 2) were included as independent variables. This discriminant function correctly attributed the lifestyle of 21 species (56.7%) and the correlation index (R^2) between observed lifestyles and inferred lifestyles was 0.3137. Nevertheless, errors are not randomly distributed and affect only adjacent states, with the exception of *Pipa carvalhoi* (Miranda-Ribeiro, 1937) and *Desmognathus monticola* Dunn, 1916 (Table 5; Appendix C, column I, on the website).

Table 4 Mean (left) and standard deviation (right) of parameters of the humerus cross-sections showing an ecological signal in lissamphibians of each lifestyle

	Ln(PLg)	*S*	*P*	*Max*	*Prad*	*Maxrad*	*Cc*
0	2.561/0.935	0.0472/0.035	0.3403/0.1871	1.0002/0.0147	0.3441/0.1803	0.9955/0.0103	0.141/0.294
1	1.542/0.443	0.0243/0.0188	0.5114/0.1817	0.9987/0.0035	0.5142/0.1798	0.9938/0.0207	–0.003/0.003
2	1.619/0.563	0.0243/0.0098	0.5542/0.1921	0.9955/0.007	0.5342/0.1692	0.9961/0.0078	0.053/0.21

For the lifestyle, 0 = aquatic, 1 = amphibious, 2 = terrestrial.
See Materials and Methods for abbreviations.

Table 5 Values of the constants of the discriminant functions used to infer the lifestyle of lissamphibians (Appendices C and D, on the website)

	Discriminant function I (Appendix C, column I)			**Discriminant function II (Appendix C, column II)**			**Discriminant function III (Appendix C, column III)**	
	0	1	2	0	1	2	0	1
Origin ordinate	–6657.05	–6560.75	–6510.46	–5365.66	–5199.46	–5227.55	–5446.81	v5294.18
Ln(PLg)	–	–	–	55.40	52.26	52.56	55.43	52.40
S	–	–	–	4163.19	4051.86	4063.18	4225.57	4118.67
P	–999.83	–971.40	–958.91	–	–	–	–	–
Max	13319.35	13220.71	13169.80	–	–	–	–	–
Prad	826.25	804.77	793.51	–	–	–	–	–
Maxrad	–	–	–	10 437.96	10 281.26	10 308.97	10 597.97	10 455.50
Cc	322.28	312.30	310.58	–	–	–	–	–

See Materials and Methods for abbreviations.

Second, both backward-elimination and forward-selection procedures were used to identify the variables that most contributes to the model. *Ln(PLg)*, *S* and *Maxrad* were included in the discriminant analysis. This function correctly attributes the lifestyle of only 59.5% of taxa; the correlation index (R2) between observed lifestyles and inferred lifestyles is 0.2850. However, the lifestyles of 66.7% of the aquatic taxa and 85.7% of the terrestrial taxa are correctly inferred (Table 5; Appendix C, column II, on the website). The aquatic lissamphibians *Pipa carvalhoi*, *Bombina orientalis* (Boulenger, 1890), *Proteus anguinus* Laurenti, 1768 and *Xenopus laevis* are inferred to be terrestrial. Most amphibious lissamphibians are actually modelled as terrestrial (81.82%). Finally, graphic representations of these discriminant analyses show that the aquatic lifestyle is well separated from the amphibious and terrestrial relatives (Fig. 5A,B). However, the amphibious and terrestrial lifestyles overlap broadly. Data for *Lithobates catesbeianus* (Shaw, 1802) were removed from the graphic representation of the second discriminant model (Fig. 5B) because of extreme values (with the inclusion of the *Lithobates catesbeianus* data the graphic was oblate and difficult to understand). The individual of *Lithobates catesbeianus* included in this analysis has a high value of parameter *S* for an amphibious lissamphibian and by far the lowest value of *Maxrad* (Appendix B, on the website). The transition between the cortical compacta and the medulla seems to be broad and the compactness in the outermost cortex is low, because of many vascular cavities (Fig. 3H).

Third, if we consider only two lifestyles (0 = aquatic vs. 1 = amphibious or terrestrial) and the parameters *Ln(PLg)*, *S* and *Maxrad*, this new model attributes a correct lifestyle to 33 species (89.2%). Errors still affect *Pipa carvalhoi*, *Bombina orientalis*, *Proteus anguinus* and *Xenopus laevis* (Table 5; Appendix C, column III, on the website).

For the two most efficient discriminant models, a file is available on-line (Appendix D on the website). Anyone can infer the lifestyle of an extinct lissamphibian solely from the parameters *P*, *Max*, *Prad*, *Cc* or *Ln(PLg)*, *S* and *Maxrad*, using these spreadsheets.

Discussion

Our microanatomical characters correspond to a level that is intermediate between a truly histological level, at which many authors argue there is no phylogenetic signal (Castanet *et al.* 2001), and a gross morphological level, which is known to contain phylogenetic signal (Laurin 2004). We expected that at least some bone compactness parameters would contain phylogenetic information. Our results suggest that most of these parameters and body size exhibit a phylogenetic signal, and this corroborates the conclusions of Cubo *et al.* (2005).

We did not use the method of Phylogenetically Independent Contrasts (Felsenstein 1985) or the random tree generation algorithm in Mesquite (Maddison and Maddison 2006) to detect a phylogenetic signal because of statistical artefacts. Multiple attempts to eliminate these artefacts by branch length or data transformation (or both) failed to solve this problem. We therefore only used the variance partitioning method with Phylogenetic eigenVector Regressions (Desdevises *et al.* 2003) to detect a phylogenetic signal in body size and bone compactness profile parameters. Our results suggest that these parameters could be optimized onto the phylogeny to infer the lifestyle of hypothetical ancestors.

Our findings are consistent with those of Laurin *et al.* (2004) on the femora of lissamphibians. Aquatic lissamphibians seem to be larger than their terrestrial and amphibious relatives. Moreover, the humeri of aquatic lissamphibians have smaller medullary cavities than the humeri of amphibious and terrestrial taxa. This observed increase in compactness in the most aquatic lissamphibians is not surprising. After all, aquatic lissamphibians are neither very active nor deep divers, and our results illustrate the widespread trend toward increasingly compact bones in aquatic taxa that have recently returned to an aquatic lifestyle or live in shallow water (Fish and Stein 1991; de Ricqlès and de Buffrénil 2001). Our findings

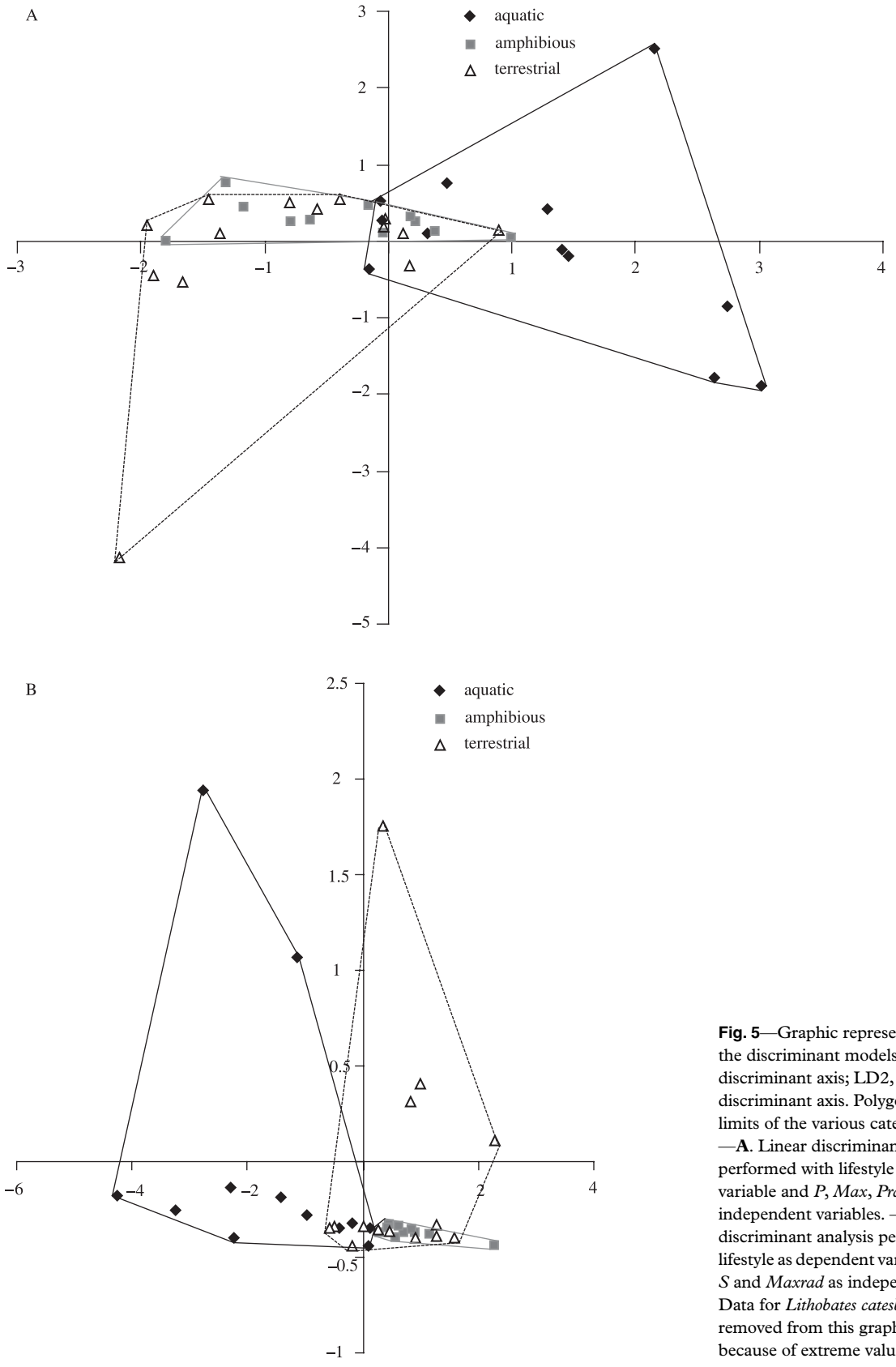

Fig. 5—Graphic representations of the discriminant models. LD1, first discriminant axis; LD2, second discriminant axis. Polygons represent the limits of the various categories of lifestyle. —**A**. Linear discriminant analysis performed with lifestyle as dependent variable and *P*, *Max*, *Prad* and *Cc* as independent variables. —**B**. Linear discriminant analysis performed with lifestyle as dependent variable and *Ln(PLg)*, *S* and *Maxrad* as independent variables. Data for *Lithobates catesbeianus* were removed from this graphic representation because of extreme values.

might at first glance seem to conflict with the results of two other studies on the relationship between skeletal mass and compactness and lifestyle in anurans (Leclair *et al.* 1993; Castanet and Caetano 1995). Leclair *et al.* (1993) found that in ranids the relative mass of the skeleton in terrestrial species can be twice that of aquatic species. Castanet and Caetano (1995) found that amphibious anuran species have a lower relative skeletal mass and a smaller corticodiaphyseal index (i.e. a greater parameter *P* and a lower compactness) than more terrestrial relatives. However, the contradiction is more apparent than real because we have not studied the relative mass of the skeleton, which is not necessarily closely correlated with bone compactness. Moreover, the study of Castanet *et al.* (1993) included no aquatic species, and the differences which we documented are mostly between aquatic and more terrestrial (including amphibious) species.

Finally, our data, like those of Germain and Laurin (2005) and Laurin *et al.* (2004), discriminate the aquatic lifestyle from the others. Our third binary discriminant model (Appendix C, column III, on the website) established with humerus compactness profile parameters seems to be more powerful than the model of Laurin *et al.* (2004) on the femur. However, contrary to what we expected based on the decreasing proximodistal gradient of compactness in sea cows, the humerus does not appear to provide a more reliable ecological signal than the radius, since the ternary inference model of Germain and Laurin (2005) had a 71% success rate (compared with 57–60% in our ternary models). Nevertheless, these studies are based on different taxonomic samples (lissamphibians and amniotes, respectively). An additional survey on the humeral compactness profile of amniotes has to be produced.

Erroneous modelling of *Proteus anguinus* may be explained by its relatively small body size, which is atypical of aquatic lissamphibians, or by its neotenic features. Nevertheless, its less paedomorphic relative *Necturus maculosus* (Rafinesque, 1818), and more distantly related paedomorphic urodeles, such as *Siren lacertina* or the partially metamorphosed *Amphiuma means*, are correctly inferred and show common aquatic features. The presacral length of our specimen of *Bombina orientalis*, which is also small for an aquatic anuran, and its bone microanatomy may not be optimal in its environment but may reflect other factors (historical, developmental, etc.). Errors of inferences are more difficult to explain for the pipid species *Pipa carvalhoi* and *Xenopus laevis*, which exhibit typical aquatic adaptations (Fig. 2A–D: a broad cortical compacta, a small medullary cavity and a wide transition zone between the cortical compacta and the medullary cavity). Microanatomical and body size data do not perfectly segregate terrestrial from amphibious lissamphibians.

Even if a relationship with the habitat is demonstrated in this study, many other parameters might influence bone architecture; the determinism of bone microanatomy is known to be very complex. A partial phylogenetic determinism was highlighted in many previous studies (Laurin *et al.* 2004; Cubo *et al.* 2005; Germain and Laurin 2005; and the present study). Moreover, some taxon-dependent microanatomical features have been noticed. For example, compactness profile characters of turtles evolve in response to changes of the habitat in a different way from other amniotes (Germain and Laurin 2005), and various histological characters that are correlated with habitat in other amniotes show no such correlation in turtles (Scheyer and Sander 2007). This phenomenon could be linked to a phylogenetic effect or biomechanical constraints, such as the presence of a carapace (Scheyer and Sander 2007; Kriloff *et al.* 2008).

Biomechanical constraints largely influence bone architecture (Castanet *et al.* 2001). Locomotion has a strong effect on wall thickness and cross-sectional geometric properties of long bone (Meers 2002; de Margerie *et al.* 2005). Graviportal adaptations also condition bone structure. The long bones of the elephant limbs are massive and lack a marrow cavity, according to Ramsay and Henry (2001). Furthermore, specific and individual body sizes have been identified as factors affecting long bone organization. Examination of many sections reveals that the bones of small animals have notably a much simpler structure than bones of large organisms (de Ricqlès 1976; Laurin *et al.* 2004; Cubo *et al.* 2005).

Bone microstructure exhibits also many characteristics that reflect ontogeny, growth trajectory, pathology or ageing (Castanet *et al.* 1993, 2001) and metabolism (de Ricqlès 1983). Our specimen of *Lithobates catesbeianus* appears to be an actively growing individual (explaining the considerable number of osteons).

Bone microanatomy also depends on environmental constraints, such as temperature or food and water availability (de Buffrénil and Francillon-Vieillot 2001; Meers 2002). Thus, Meers (2002) noted that captive crocodilians exhibit unusual cross-sectional geometric properties (such as a larger cortical area relative to wild conspecifics) that may result from different nutrition or different locomotor activity pattern.

Physiological factors also influence bone microanatomy. De Buffrénil and Francillon-Vieillot (2001) demonstrated that bone compactness in the rear limbs of the Nile monitor (*Varanus niloticus*) is largely influenced by the sex of the specimen and the reproductive status. In males, femoral compactness increases during ontogeny, whereas in females, it tends to decrease. In addition to this long-term trend, gravid females have lower femoral compactness than non-gravid females because of cortical resorption linked with ovogenesis.

Finally, several studies showed that bone structure variation depends on the identity of the skeletal element considered (de Buffrénil and Francillon-Vieillot 2001; Laurin *et al.* 2004; de Margerie *et al.* 2005; Germain and Laurin 2005). The errors in modelling can be explained by the combined effect of most of the factors mentioned above, and possibly other, still unidentified but relevant factors.

Despite the few problematic cases noted above, we have provided a reliable (up to 89% accuracy), simple way to infer

the habitat of early lissamphibians, such as *Vieraella* (an early anuran, from the Lower Jurassic of South America; Carroll 1988) or *Karaurus* (the oldest known urodele from the Upper Jurassic of Russia; Carroll 1988). Lack of material and space constraint precluded such palaeobiological inferences in this study, but such analyses might lead to a better understanding of habitat use by lissamphibians in the Mesozoic and Cenozoic.

Acknowledgements

All persons who have lent or given specimens or histological sections for this study are thanked. This includes J. Castanet (Université Paris 6), V. de Buffrénil (Université Paris 6), H. Francillon-Vieillot (Université Paris 7), F. Renoult and D. Robineau (both in the Laboratoire d'Anatomie Comparée, Musée National d'Histoire Naturelle, Paris), and A. S. Severtsov (Moscow State University). Furthermore, the authors are very grateful to M.-M. Loth and H. Lamrous for the histological preparations and are indebted to D. Germain for his advice and aid. D. Marjanovii is also thanked for his proofreading and his comments on this manuscript. Last but not least, we thank the *Acta Zoologica* editorial staff for its efficient handling of this manuscript, as well as an anonymous referee and T. Scheyer for their constructive review of an earlier draft of this manuscript.

References

Bininda-Emonds, O. R. P., Gittleman, J. L. and Kelly, C. K. 2001. Flippers versus feet: comparative trends in aquatic and non-aquatic carnivores. – *Journal of Animal Ecology* **70**: 386–400.

de Buffrénil, V. and Francillon-Vieillot, H. 2001. Ontogenic changes in bone compactness in male and female Nile monitors (*Varanus niloticus*). – *Journal of Zoology* **253**: 539–546.

de Buffrénil, V. and Schoevaert, D. 1988. On how the periosteal bone of the delphinid humerus becomes cancellous: ontogeny of a histological specialization. – *Journal of Morphology* **198**: 149–164.

de Buffrénil, V. and Schoevaert, D. 1989. Données quantitatives et observations histologiques sur la pachyostose du squelette du dugong, *Dugong dugon* (Müller) (Sirenia, Dugongidae). – *Canadian Journal of Zoology* **67**: 2107–2119.

de Buffrénil, V., Sire, J.-Y. and Schoevaert, D. 1986. Comparaison de la structure et du Volume squelettiques entre un delphinidé (*Delphinus delphis* L. –) et un mammifère terrestre (*Panthera leo* L.). – *Canadian Journal of Zoology* **64**: 1750–1756.

Carroll, R. L. 1985. Evolutionary constraints in aquatic diapsid reptiles. – *Palaeontology* **33**: 145–155.

Carroll, R. L. 1988. *Vertebrate Paleontology and Evolution*. W. H. Freeman, New York.

Casgrain, P. 2005. Permute! Montreal. Available from http://www.bio.umontreal.ca/Casgrain/en/labo/permute/index.html.

Casgrain, P., Legendre, P. and Vaudor, A. 2004. The R Package for multidimensional and spatial analysis. Montreal. Available from http://www.bio.umontreal.ca/Casgrain/fr/labo/R/index.html.

Castanet, J. and Caetano, M. H. 1995. Influence du mode de vie sur les caractéristiques pondérales et structurales du squelette chez les amphibiens anoures. – *Canadian Journal of Zoology* **73**: 234–242.

Castanet, J., Cubo, J. and de Margerie, E. 2001. Signification de l'histodiversité osseuse: le message de l'os. – *Biosystema* **19**: 133–147.

Castanet, J., Francillon-Vieillot, H., Meunier, F. J. and de Ricqlès, A. 1993. Bone and individual aging. In Hall, B. K. (Ed.): *Bone*, Vol. 7, pp. 245–283. CRC Press, London.

Cubo, J., Ponton, F., Laurin, M., de Margerie, E. and Castanet, J. 2005. Phylogenetic signal in bone microstructure of sauropsids. – *Systematic Biology* **54**: 562–574.

Desdevises, Y., Legendre, P., Azouzi, L. and Morand, S. 2003. Quantifying phylogenetically structured environmental variation. – *Evolution* **57**: 2467–2652.

Diniz-Filho, J. A. F., de Sant'Ana, C. E. R. and Bini, L. M. 1998. An eigenvector method for estimating phylogenetic inertia. – *Evolution* **52**: 1247–1262.

Duellman, W. E. and Trueb, L. 1986. *Biology of Amphibians*. McGraw-Hill, New York.

Fawcett, D. W. 1942. The amedullary bones of the Florida manatee (*Trichechus latirostris*). – *American Journal of Anatomy* **71**: 271–309.

Felsenstein, J. 1985. Phylogenies and the comparative method. – *The American Naturalist* **125**: 1–15.

Fish, F. E. and Stein, B. R. 1991. Functional correlates of differences in bone density among terrestrial and aquatic genera in the family Mustelidae (Mammalia). – *Zoomorphology* **110**: 339–345.

Germain, D. and Laurin, M. 2005. Microanatomy of the radius and lifestyle in amniotes (Vertebrata, Tetrapoda). – *Zoologica Scripta* **34**: 335–350.

Girondot, M. and Laurin, M. 2003. Bone Profiler: a tool to quantify, model and statistically compare bone section compactness profiles. – *Journal of Vertebrate Paleontology* **23**: 458–461.

Goin, C. J., Goin, O. B. and Zug, G. R. 1978. *Introduction to Herpetology*. W. H. Freeman, New York.

Josse, S., Moreau, T. and Laurin, M. 2006. Stratigraphic Tools for Mesquite. Available from http://mesquiteproject.org/packages/stratigraphicTools/.

Kriloff, A., Germain, D., Canoville, A., Vincent, P., Sache, M. and Laurin, M. 2008. Evolution of bone microanatomy of the tetrapod tibia and its use in palaeobiological inference. – *Journal of Evolutionary Biology* **21**: 801–826.

Laurin, M. 2004. The evolution of body size, Cope's rule and the origin of amniotes. – *Systematic Biology* **53**: 594–622.

Laurin, M., Girondot, M. and Loth, M.-M. 2004. The evolution of long bone microanatomy and lifestyle in lissamphibians. – *Paleobiology* **30**: 589–613.

Laurin, M., Germain, D., Steyer, J.-S. and Girondot, M. 2006. Données microanatomiques sur la conquête de l'environnement terrestre par les vertébrés. – *Comptes Rendus Palev* **5**: 603–618.

Leclair, R. Jr, Lamontagne, C. and Aubin, A. 1993. Allométrie de la masse du squelette chez des amphibiens anoures. – *Canadian Journal of Zoology* **71**: 352–357.

Maddison, W. P. and Maddison, D. R. 2006. *Mesquite, a Modular System for Evolutionary Analysis*, Version 1.1. Available from http://mesquiteproject.org.

de Margerie, E., Sanchez, S., Cubo, J. and Castanet, J. 2005. Torsional resistance as a principal component of the structural design of long bones: comparative multivariate evidence in birds. – *The Anatomical Record Part A* **282A**: 49–66.

Marjanovii, D. and Laurin, M. 2007. Fossils, molecules, divergence times, and the origin of lissamphibians. – *Systematic Biology* **56**: 369–388.

Meers, M. B. 2002. Cross-sectional geometric properties of the crocodilian humerus: an exception to Wolff's law? *Zoological Journal (London)* **258**: 405–418.

Padian, K., de Ricqlès, A. and Horner, J. R. 2001. Dinosaurian growth rates and bird origins. – *Nature* **412**: 405–408.

Ramsay, E. C. and Henry, R. W. 2001. Anatomy of the elephant foot. In Csuti, B., Sargent, E. L. and Bechert, U. S. (Eds): *The Elephant's Foot: Prevention and Care of Foot Conditions in Captive Asian and Africans Elephants*, pp. 9–12. Iowa State University Press, Ames, Iowa.

de Ricqlès, A. 1976. Recherches paléohistologiques sur les os longs des tétrapodes. VII. Sur la classification, la signification fonctionnelle et l'histoire des tissus osseux des tétrapodes. – Deuxième partie. – *Annales de Paléontologie* **62**: 71–126.

de Ricqlès, A. 1983. Cyclical growth in the long limb bones of a sauropod dinosaur. – *Acta Palaeontologica Polonica* **28**: 225–232.

de Ricqlès, A. and de Buffrénil, V. 2001. Bone histology, heterochronies and the return of tetrapods to life in water: where are we? In Mazin, J. M. and de Buffrénil, V. (Eds): *Secondary Adaptation of Tetrapods to Life in Water*, Vol. 1, pp. 289–310. Dr Friedrich Pfeil, Munich.

Scheyer, T. M. and Sander, P. M. 2007. Terrestrial palaeoecology for basal turtles indicated by shell bone histology. – *Proceedings of the Royal Society of London Series, B* **274**: 1885–1893.

StatSoft France. 2003. STATISTICA (logiciel d'analyse de données), version 6. www.statsoft.com.

Stein, B. R. 1989. Bone density and adaptation in semiaquatic mammals. – *Journal of Mammalogy* **70**: 467–476.

Steyer, J.-S., Laurin, M., Castanet, J. and de Ricqlès, A. 2004. First histological and skeletochronological data on temnospondyl growth; palaeoecological and palaeoclimatological implications. – *Palaeogeography, Palaeoclimatology, Palaeoecology* **206**: 193–201.

Taylor, M. A. 2000. Functional significance of bone ballast in the evolution of buoyancy control strategies by aquatic tetrapods. – *Historical Biology* **14**: 15–31.

Wall, W. P. 1983. The correlation between high limb-bone density and aquatic habits in Recent mammals. – *Journal of Paleontology* **57**: 197–207.

Wiens, J. J., Bonett, R. M. and Chippindale, P. T. 2005. Ontogeny discombobulates phylogeny: paedomorphosis and higher-level salamander relationships. – *Systematic Biology* **54**: 91–110.

Supplementary Material

The following supplementary material is available for this article:

Appendix A Lissamphibian species sampled in our analysis and sources of biological material. Taxa are listed in the same order as in Fig. 4 and Appendix B. Abbreviations: MNHN, Collection of the Museum National d'Histoire Naturelle; P.c., Private collection without specimen number.

Appendix B Cross-section of humeri of lissamphibians studied for this analysis. When more than one specimen of a given species was used, these are numbered from 1 to X (up to 4). For the habitat : 0 = aquatic, 1 = amphibious, 2 = terrestrial. These states are defined by the relative amount of time spent in water: > 90% for aquatic taxa, between 20% and 90% for amphibious taxa and < 20% for terrestrial taxa. Abbreviation: LS, lifestyle; PLg, Presacral length; MD, maximal diameter of the cross-section; Cg, global compactness; Cc, compactness in the centre of the cross-section; Cp, compactness in the periphery of the cross-section.

Appendix C Probabilities of inferred lifestyle for each taxon obtained by the discriminant function calculated with **Column I**: parameters *P*, *Max*, *Prad* and *Cc* selected in the variance partitioning method (**Table 2**) and included as independent variables; **Column II**: *Ln(PLg)*, *S*, *Maxrad* selected both by a backward-elimination and a forward-selection procedure in STATISTICA and included as independent variables; **Column III**: *Ln(PLg)*, *S*, *Maxrad* and the two-states lifestyle. For Columns I and II, the lifestyle is coded as follow, 0 = aquatic, 1 = amphibious, 2 = terrestrial. For Column III, the lifestyle is coded as follow: 0 = aquatic, 1 = amphibious or terrestrial. All errors are marked in bold and errors between two non-adjacent states are marked by asterisks. R^2 corresponds to the coefficient of determination between observed lifestyles and inferred lifestyles for each model. SR is the success rate (proportion of correct lifestyle inferences).

Appendix D Inference models (spreadsheets) I.

Appendix D-a Discriminant function I.

Appendix D-b Detailed formula I.

Appendix D-c Discriminant function III.

Appendix D-d Detailed formula III.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1463-6395.2008.00328.x
(This link will take you to the article abstract).

Please note: Blackwell Publishing are not responsible for the content or functionality of any supplementary materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.