

ORIGINAL ARTICLE

Geometric morphometric study of the skull shape diversification in Sciuridae (Mammalia, Rodentia)

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Abstract

It is generally accepted that the high phenotypic diversity of mammals is a combined result of developmental constraint and ecological adaptation, although the influence of these endogenous and exogenous factors varies in different mammal groups. The rodent family Sciuridae represents an ideal candidate for examining phenotypic diversity in relation to phylogeny and ecological adaptations. In the present study, we investigate the effects of phylogeny and lifestyle on the skull shape in different species of Sciuridae by applying geometric morphometric methods. In addition, we investigate the importance of allometry on sciurid skull shape, because results from geometric morphometrics sometimes dispute those of traditional morphometry. Here, we identify significant associations between patristic distances obtained from molecular phylogeny and shape distances in all 3 views of the cranium and the lateral view of the mandible. Multivariate regression demonstrates that shape differences among lifestyle categories are substantial, especially in the dorsal and ventral structures after the influence of phylogeny is taken into account. Allometry plays an important role in the shape variation, although its importance on different skull structures varies. Our results indicate that complex structures of this highly diverse mammal group, which occupies different niches, are affected by ecological factors and developmental constraint.

Key words: adaptation, allometry, geometric morphometrics, phylogeny, Sciuridae

INTRODUCTION

The rodent family Sciuridae consists of 51 genera and 271 species and is found in a variety of habitats

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throughout the world (Nowak 1999; Thorington & Hoffmann 2005). As the third most diverse family in Rodentia, Sciuridae is a good example of complex adaptive radiation (Mercer & Roth 2003) and an ideal system for understanding the occurrence and maintenance of morphological diversity (Perez *et al.* 2009). Sciurids have a high diversity in phenotype, with body forms and modes of life quite differentiated (Nowak 1999). Nearly half of squirrels are solitary diurnal tree dwellers, living in the forest and leaping among trees. Flying squirrels

have adopted a peculiar gliding locomotion and become more active at night (Essner 2007), while the remaining species usually spend considerable time on the open ground, digging intricate burrow systems and living in complex social structures. After millions of years of evolutionary adaptation, these ecological and behavioral differentiations gradually result in ecomorphological differences (Perez *et al.* 2009).

The process of morphological diversification in these species has been widely investigated in an evolutionary context (Cardini & Tongiorgi 2003; Cardini & O'Higgins 2004; Cardini *et al.* 2005; Caumul & Polly 2005; Cardini & Thorington 2006; Michaux *et al.* 2008) but such studies tend to focus on a single genus (e.g. *Marmota*) or a single structure (mainly the mandible) (Cardini 2004; Cardini & O'Higgins 2004). For the Sciuridae family as a whole, the ecomorphological adaptations are seldom discussed (Michaux *et al.* 2008). Consequently, a comprehensive analysis of this group is needed.

Morphological diversification could be interpreted as the consequence of the contrasting impacts of developmental constraint and adaptation to ecological factors (Meloro *et al.* 2011). Numerous studies concerned with morphological variation have detected strong phylogenetic signal and have confirmed the importance of developmental forces in the postnatal morphogenesis, which is consistent with the view that biological structures of closely related species tend to resemble each other because of shared ancestry (Losos 2011). However, there is an opposing view suggesting that genetically-related species could diverge in shape to a considerable degree. Such shape divergence is perhaps a response to natural selection, particularly during adaptation to habitat shifts (Klingenberg & Ekau 1996). Sciurid morphology can be used as a model to test the congruence between morphological change and phylogenetic divergence because the characteristics of their skeleton are considered to be both conservative and inclined to convergence (Cardini 2003). Michaux *et al.* (2008) perform a multivariate analysis of variance (MANOVA) to assess the contribution of taxonomic grouping (tribes) to the sciurid mandible shape, and find a significant association between shape variation and the tribal level placement of these animals. However, 'tribe' is a discrete taxonomic category and the phylogenetic distances among species are not considered in their analysis. It is still unclear whether interspecific skull morphological changes synchronize with genetic divergence. Genetic distances calculated from DNA sequences or the topology of phylogenetic trees could provide more accurate quantitative

estimates for phylogenetic differentiation (Caumul & Polly 2005; Cardini & Elton 2008).

In the present study, we aim to explore how and to what extent phylogeny and lifestyle have influenced the cranial and mandible shape of Sciuridae. Because ecological adaptations can be nested within phylogenetic history (Losos 2011; Meloro & O'Higgins 2011), we will also test the relationships between shape and lifestyle by taking phylogenetic history into account. In addition, the traditional hypothesis that claimed conservatism and frequent convergence in the skeleton of squirrels was due to size similarity (Black 1963; Hafner 1984) is also tested. Recent studies on marmots (Cardini 2003; Cardini & O'Higgins 2004, 2005) suggest that allometric effects had been overestimated. We adopt landmark-based geometric morphometric methods as the main approaches here since these techniques are capable of uncovering and decomposing evolutionary and ecological patterns in complex structures (Bookstein 1986).

MATERIALS AND METHODS

Samples

We obtained 211 adult specimens (24 species within 16 genera) from several Chinese collections: the Institute of Zoology (IOZ), Chinese Academy of Sciences (CAS); the Kunming Institute of Zoology (KIZ), CAS; the Northwest Institute of Plateau Biology, CAS; and the Qinghai Institute for Endemic Disease Prevention and Control (Table 1). Specimens were all collected in the wild in China, although most are not endemic Chinese species. They were identified as adults by the presence of the upper third molar and the formation of occlusal surfaces (Cardini 2003; Perez *et al.* 2009). These species were chosen mainly for 2 reasons. First, they are good representatives of the morphological diversity, including all 4 lifestyles from different habitats. Second, most of them have been investigated in previous molecular systematic studies (Mercer & Roth 2003; Herron *et al.* 2004; Steppan *et al.* 2004), and, therefore, a pre-existing phylogenetic framework is available for reference. Previous studies demonstrate that sexual dimorphism is negligible in Sciuridae when compared to interspecific differences (Cardini & O'Higgins 2004; Michaux *et al.* 2008), then we combine males and females in the following analysis. Following the descriptions of Nowak (1999), Mercer and Roth (2003) and Steppan *et al.* (2004), 4 lifestyles were defined: arboreal–arboreal, no gliding and diurnal; gliding–arboreal, gliding and nocturnal; semi-terrestrial–spend considerable time on

ground, no burrowing and diurnal; and terrestrial–terrestrial, fossorial and diurnal (Table 1).

Photographing and digitization

For each skull, 4 standardized 2D images were collected using a Fujifilm Finepix S7000 (Fuji, Tokyo, Ja-

pan) digital camera: the cranial dorsal view (including the nasal, frontal and parietal bones), the cranial ventral view (including jugal, upper tooth row, auditory bulla, and palatine, basisphenoid and basioccipital bones), the cranial lateral view (including premaxilla, maxilla and temporal bones) and the hemimandible view (in-

Table 1 Sample size (*N*), lifestyles and gene sequence accession numbers in GenBank

Taxa	<i>N</i>	<i>Cytb</i>	Lifestyle
<i>Belomys pearsonii</i> (Gray, 1842)	3	AB126245.1	Gliding
<i>Petaurista yunanensis</i> (Anderson, 1875)	10	DQ072110.1	Gliding
<i>Petaurista xanthotis</i> (Milne-Edwards, 1872)	14	DQ072111.1	Gliding
<i>Pteromys volans</i> (Linnaeus, 1758)	10	EU919160.1	Gliding
<i>Hylopetes alboniger</i> (Hodgson, 1836)	6	DQ093187.1	Gliding
<i>Sciurus vulgaris</i> Linnaeus, 1758	10	8573078	Arboreal
<i>Callosciurus erythraeus</i> (Pallas, 1779)	11	AB043877.1	Arboreal
<i>Tamiops mccllellandii</i> (Horsfield, 1840)	10	EF539333.1	Arboreal
<i>Tamiops swinhoei</i> (Milne-Edwards, 1874)	10	EF539334.1	Arboreal
<i>Dremomys pernyi</i> (Milne-Edwards, 1867)	10	EF539336.1	Semi-terrestrial
<i>Dremomys pyrrhomerus</i> (Thomas, 1895)	7	EF539342.1	Semi-terrestrial
<i>Dremomys rufigenis</i> (Blanford, 1878)	10	EF539341.1	Semi-terrestrial
<i>Spermophilus dauricus</i> Brandt, 1843	10	AF157899.1	Terrestrial
<i>Spermophilus undulatus</i> (Pallas, 1778)	10	AF157912.1	Terrestrial
<i>Tamias sibiricus</i> (Laxmann, 1769)	11	FJ655262.1	Semi-terrestrial
<i>Marmota himalayana</i> (Hodgson, 1841)	10	GQ329721.1	Terrestrial
<i>Marmota baibacina</i> Kastschenko, 1899	3	AF100714.1	Terrestrial
<i>Marmota sibirica</i> (Radde, 1862)	6	AF143938.1	Terrestrial
<i>Trogopeterus xanthipes</i> (Milne-Edwards, 1867)	10	Unavailable	Gliding
<i>Ratufa bicolor</i> (Sparrman, 1778)	10	Unavailable	Arboreal
<i>Callosciurus pygerythrus</i> (I. Geoffroy Saint-Hilaire, 1833)	10	Unavailable	Arboreal
<i>Menetes berdmorei</i> (Blyth, 1849)	6	Unavailable	Semi-terrestrial
<i>Sciurotamias davidianus</i> (Milne-Edwards, 1867)	10	Unavailable	Terrestrial
<i>Aeretes melanopterus</i> (Milne-Edwards, 1867)	4	Unavailable	Gliding
Outgroups			
<i>Apodontia rufa</i> (Rafinesque, 1817)		AJ389528.1	
<i>Graphiurus murinus</i> (Desmarest, 1822)		AJ225115.1	
<i>Glis glis</i> (Linnaeus, 1766)		5835484	
<i>Glirulus japonicus</i> (Schinz, 1845)		AB218868.1	
<i>Eliomys quercinus</i> (Linnaeus, 1766)		AJ225030.1	
<i>Dryomys nitedula</i> (Pallas, 1778)		AJ225116.1	
<i>Muscardinus avellanarius</i> (Linnaeus, 1758)		AJ225117.1	

cluding condylar, coronoid, angular process and lower tooth row). Image standardization was important for the substitution of 3D structures by 2D images. This was achieved by setting skull position, camera lens plane and camera lens–skull distance (60 cm here) the same when photographing separate specimens (Perez *et al.* 2009). Scale was given for each photo by placing a ruler parallel to the longitudinal axis of the cranium or the mandible.

Landmark digitization was conducted with the software tpsDig2 2.16 (Rohlf 2010a). To digitize evenly distributed points along the contour of structures, Make-Fan6 (Sheets 2003) was used to set up guidelines before digitization. We selected 8 landmarks and 3 semilandmarks to describe the dorsal cranium, 8 landmarks and 7 semilandmarks for the lateral cranium, 18 landmarks and 10 semilandmarks for the ventral cranium, and 11 landmarks and 10 semilandmarks for the mandible (Monteiro *et al.* 2003; Cardini & O’Higgins 2004; Cardini *et al.* 2005) (Fig. 1). Detailed information referring to the locations of these landmarks and semilandmarks is given in Table 2.

Geometric morphometrics analysis

After digitization, the configuration of 2D landmark coordinates for each specimen was prepared. Generalized Procrustes Analysis (Adams *et al.* 2004) was applied to align and superimpose these configurations, and effects of location, rotation and scale differences were removed. Semilandmarks were allowed to slide to make sure the bending energy among specimens was minimized. Thus, the 24 mean shapes (consensuses) for all 24 species were calculated for the following analyses (Rohlf & Slice 1990; Bookstein 1991; Meloro *et al.* 2008).

Relative warp (RW) analysis, which is mathematically equivalent to a principal components analysis on the residual covariation matrix of the aligned configuration coordinates after superimposition when $\alpha = 0$ (Rohlf 1993; Caumul & Polly 2005), was carried out to survey the patterns of variation among the 24 species. This analysis reduces shape variables with RWs of vectors and allows shape changes to be visualized with deformation grids. The first 5 RWs (which explained approximately 89%–96% of the total variances of each structure in this analysis) were used for the following multivariate analyses. Deformation grids computed using thin plate splines (Bookstein 1991) were used to visualize the extreme shape variability on the first 2 axes of RW scores, along with scatter plots showing the distribution pattern of the 24 data points. All the above analyses were conducted using the software tpsRelw 1.49 (Rohlf 2010b).

Allometry analysis

Size was computed for each specimen as the natural log transformed centroid size (CS, the square root of the summed squared distances of the landmarks from their centroid) (Slice *et al.* 1996). For the purpose of the following analysis, which is concerned with interspecific variations, size is represented by the species mean lnCS. Allometric shape variability among species was explored using the multivariate regression, with lnCS as the independent variable and the shape variables as dependents (Meloro *et al.* 2008). Multivariate regression was performed using the software tpsRegr version 1.28 (Rohlf 2003b), which can predict the proportions of size-explained shape variation and allow the visualization of allometric shape changes. Goodall’s *F*-tests, (Goodall 1991) were performed here.

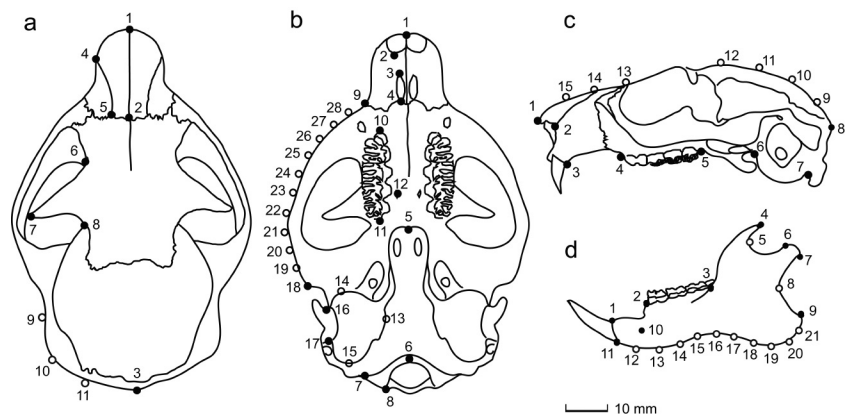


Figure 1 Landmarks and semilandmarks on the dorsal (a), ventral (b), lateral (c) and mandible (d) views of the skull in squirrels. Landmarks are given as black dots while semilandmarks are white circles.

Table 2 Definition of landmarks and semilandmarks

Landmarks	Landmark definition
Dorsal	
1	Anterior (midsagittal) tip of the nasal
2	Meeting point between nasal and frontal along the midsagittal plane
3	Most posterior point of the parietal along the midsagittal plane
4	Anterior tip of suture between nasal and premaxilla
5	Suture between nasal, frontal, and premaxillary
6	Supraorbital notch
7	Tip of the postorbital process of the frontal
8	Posterior base of the postorbital process
9–11	Semilandmarks: describing the occiput outline
Ventral	
1	Anterior (midsagittal) tip of the premaxilla
2	Posterior extremity of the incisor alveolus
3–4	Extremities of incisive foramen
5	Antermost point in the mesopterygoid fossa
6	Antermost point of foramen magnum
7–8	Lateral tips of the occipital condyle
9	Most lateral point of the rostrum along the suture between premaxilla and maxilla
10–11	Posterior and anterior end of the tooth row
12	Posterior palatine foramen
13	Most lingual surface point of the tympanic bulla
14	Antermost point of the tympanic bulla
15	Most posterior point of the tympanic bulla
16–17	Anterior and posterior tip of the external auditory meatus
18	Posterior tip of the zygomatic arch
19–28	Semilandmarks: describing the outline of zygomatic bone
Lateral	
1	Anterior extremity of the suture between nasals
2	Antermost point of the suture between nasals and premaxillary
3	Point between the premaxillary and the posterior end of incisor
4–5	Posterior and anterior end of the toothrow
6	Most ventral meeting point between tympanic bulla and alisphenoid
7	Most ventral meeting point between mastoid process and tympanic bulla
8	Most posterior point of the parietal along the midsagittal plane
9–15	Semilandmarks: describing the vault and nasal outline
Mandible	
1	Upper extreme anterior point of the incisor alveolus
2	Anterior extremity of the tooth row (premolar alveolus)
3	Intersection point of the dental ridge and the coronoid process
4	Tip of the coronoid process
5	Antermost point on the curve of coronoid process
6–7	Anterior and posterior tip of the condyle process
8	Antermost point on the curve of the posterior boundary of the mandible
9	Posterior extremity of the angular process
10	Mental foramen
11	Antero-ventral border of incisive alveolus
12–21	Semilandmarks: describing the bottom outline of mandible

Phylogenetic signal

To estimate evolutionary inertia (defined as the percentage of shape variation explained by neutral evolution along the phylogenetic tree) (Diniz-Filho *et al.* 1998), we compared morphometric distance matrices and the phylogenetic distance matrix using the Mantel test (Mantel 1967) and the software NTSYSpc 2.10e (Rohlf 2002). This procedure has been widely used in the past decade in studies of various organisms (Monteiro & Abe 1999; Harvati & Weaver 2006; Michaux *et al.* 2007; Cardini & Elton 2008; Perez *et al.* 2009) and it has advantages over multivariate regression analysis in which phylogenetic information might be lost because only the first few eigenvectors of the phylogenetic distance matrix are retained (Diniz-Filho *et al.* 1998). For each Mantel analysis, a permutation test was calculated with 9999 permutations for a 1-tailed significance *t*-test, with the proportion of correlations larger than or equal to the observed correlation giving the *P*-value. A conservative significance threshold of 0.01 was chosen to decrease the probability of type I errors in these tests. For the preparation of the matrices compared in the Mantel tests, the morphometric pairwise Procrustes distance matrices describing the total pattern of shape variation among species were calculated using the aligned landmark coordinates of configurations in tpsSmall (Rohlf 2003a). The phylogenetic distance (patristic distance) derived from the topological structure of a Bayesian tree constructed using the cytochrome *b* (*Cytb*) gene sequence matrix was calculated using the software Mesquite version 2.74 (Maddison & Maddison 2010). Outgroup taxa were excluded in this analysis.

For the above 24 species, 18 *Cytb* sequences of 18 species were available in GenBank (<http://www.ncbi.nlm.nih.gov>) and were subject to phylogenetic tree construction (Table 1). These sequences were aligned using ClustalW in MEGA 5.05 (Tamura *et al.* 2011) and the best-fit gene substitution model (TIM3 + I + G) was chosen using jModelTest 0.1.1 (Posada 2008). Phylogenetic relationships were estimated by applying Bayesian Metropolis method coupled with Markov Chain Monte Carlo phylogenetic method (MCMC) using the software MrBayes 3.1.2 (Huelsenbeck *et al.* 2001; Ronquist & Huelsenbeck 2003). We ran 4 MCMC chains (3 heated chains and 1 cold chain) simultaneously, with 1.0×10^6 generations, retaining 1 sample out of every 1000 (for a total of 1000 trees). Runs were monitored for convergence, and the first 500 samples were discarded; the resulting tree and clade-credibility values were a summary of 500 samples. *A. rufa* (sister group of Sciuridae)

and 6 species from Gliridae (sister group of Sciridae + Aplodontidae, including *M. avellanarius*, *D. nitedula*, *E. quercinus*, *G. japonicus*, *Gl. glis* and *Gra. Murinus*) were selected as outgroups (Table 1).

Lifestyle influence estimation

To estimate the influence of lifestyle differences on skull shape variation, we applied multivariate regression analyses using a combination of 3 dummy variables to represent the 4 lifestyle categories defined above. This is equivalent to a MANOVA. To overcome bias due to the small sample size, we repeated the analyses using the first 5 (which explained approximately 90% shape variations or more) as well as the first 2 RW axes (Meloro & O'Higgins 2011) as dependent variables.

Phylogenetic generalized least squares

Biological traits are not independent variables. Phylogeny can introduce morphological covariation indirectly through factors like lifestyle and size, which themselves may nest in the phylogenetic structure (Caulmul & Polly 2005; Meloro *et al.* 2011). Therefore, to test whether there is significant correlation between shape variation and lifestyle (or size), the phylogenetic signal needs to be partialled out. This can be achieved using methods such as phylogenetic generalized least squares (PGLS) analysis (Rohlf 2001). The regression formula of PGLS is expressed as $Y = XB + \epsilon$, where *Y* represents the dependent variable (the matrix of the first 5 RW scores in this study), *X* the dependent variables (here the vector of lnCS or lifestyle dummy variables), *B* is the partial regression coefficients, and ϵ is the error term (Rohlf 2001; Adams 2008). The error term here is the phylogenetic covariance matrix obtained from the constructed phylogenetic tree, using the software Mesquite 2.74. All the regression processes of shape variations on size or lifestyle were performed in NTSYSpc 2.10e (Rohlf 2002).

RESULTS

Shape variation inferred from geometric morphometrics

In the dorsal view, 18 RWs were extracted, with the first 5 accounting for 96.14% of the total variance: RW1 accounts for 51.34%, RW2 for 25.50% and RW3 10.55%. 2D scatter plots based on the first 2 RWs (with the percentages of the explained variation) are shown in Fig. 2a. Four shape deformation grids (at the highest

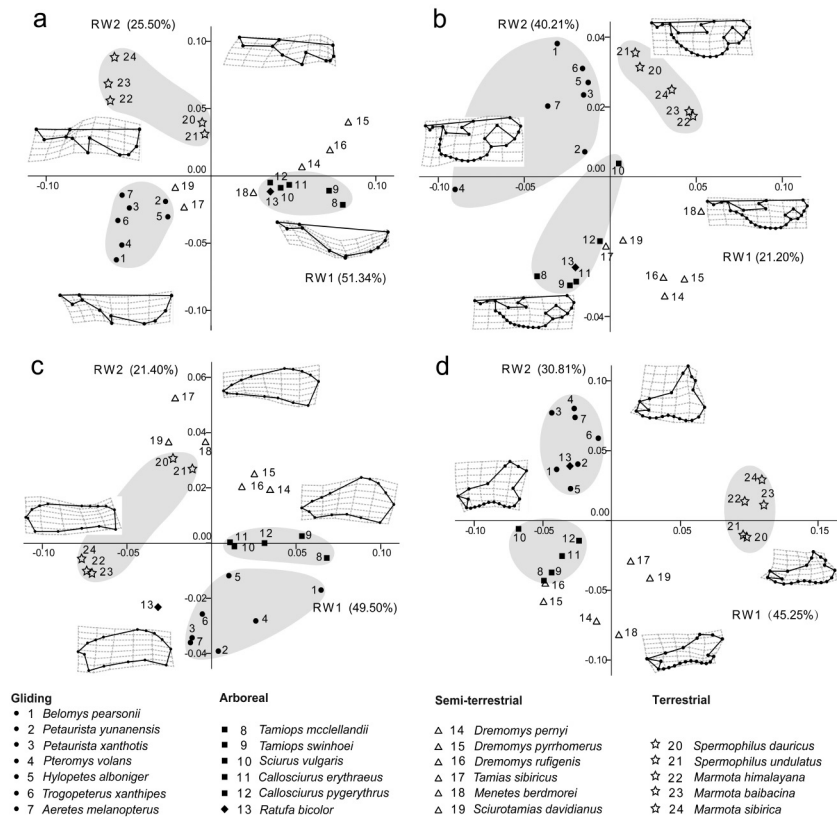


Figure 2 Scatter plots of the first 2 relative warp (RW) axes that describe shape change in dorsal (a), ventral (b) and lateral (c) cranium and mandible (d) of 24 squirrels. Species are labeled according to life-style and transformation grids visualize the deformation depicted by thin plate splines of extreme RW scores relative to the consensus (regular grid not shown).

and lowest value of the first and second axis) are presented to show shape variation among species. Four life-style groups are significantly clustered, with the arboreal, gliding and terrestrial groups (each shadowed in Fig. 2a) having no overlap, while the semi-terrestrial group is somewhat scattered. The deformation grids show that the dorsal shape variation mainly occurs in the relative size of nasal and postorbital processes, which are structures important in olfaction and vision. The roundness of the vault is also significantly different. The largest nasal bone and postorbital process and the flattest vault are possessed by terrestrial species, followed by gliding and then arboreal individuals. *Dremomys* shows greater similarity to arboreal groups, while the other 3 semi-terrestrial species show intermediate shapes and are scattered in the central region of the plot.

In the ventral view, 23 RWs were extracted, with the first 5 together accounting for 89.07% of the total variance: RW1 accounts for 40.21%, RW2 for 22.20% and RW3 11.82%. Four groups are significantly different-

ated on the first 2 RWs in the scatter plot (Fig. 2b). The shape deformations mainly occur in the relative length of the palatine and the upper tooth row, and the relative size of the zygomatic arch and the auditory bulla. Zygomatic arch and auditory bulla are important structures for vision and audition, and their expansion and enlargement are significant in both gliding and terrestrial species, indicating the functional importance of the structures in these organisms. Semi-terrestrial species share some variations with the arboreal group on the second RW. Both exhibit a relatively long and narrow profile of the cranium compared to those of the terrestrial and gliding species.

Similar results were obtained for the lateral view of the cranium. A total of 23 RWs were extracted, with the first 5 accounting for 92.36% of the total variance: RW1 accounts for 49.50%, RW2 for 21.40% and RW3 10.84%. In the scatter plot of the first 2 RWs, the 4 life-style groups are significantly distinct (Fig. 2c). *Dremomys* shows more similarity to the arboreal group in the first RW, while *Tamias*, *Menetes* and *Sciurotamias* occur

closer to the terrestrial group. *Ratufa* is a clear outlier of the arboreal group and resembles terrestrial and gliding groups on the first and second RW, respectively. The deformation grids indicate that shape deformations mainly occur in the relative heights of the snout, the roundness of the cranial vault and occiput, and the relative lengths of the diastema, upper tooth row and auditory bulla. Terrestrial species have a relatively higher snout, even higher than their occiput, while the arboreal and semi-terrestrial groups have a more inflated vault and a relatively higher occiput. Gliding species have a relatively longer diastema and larger bulla, and the outline of the vault is neither too flattened nor too inflated.

Finally, 23 RWs were extracted for shape variation of the mandible, with the first 5 accounting for 92.27% of the total variance: RW1 for 45.25%, RW2 for 30.81% and RW3 8.61%. Four groups are significantly separated on the first 2 RWs in the scatter plot (Fig. 2d). The terrestrial species are prominently differentiated from other groups, clustering at the positive extreme of the first RW. The gliding group clusters at the positive end of the second RW, as does *Ratufa*. The semi-terrestrial group shows greater similarity to arboreal species than to the terrestrial group on both RWs. Significant deformations appear both in the corpus and ramus mandible. The relative depth of the mandible decreases prominently toward the positive orientation of the first RW and the negative end of the second, while the relative size of the ramus and the relative height of corpus mandible increase in the opposite direction. Further differences in the ramus relate to the relative size of coronoid, condyloid and angular processes. The shape variations related to ramus reflect differences in the temporalis attachment and feeding habitat.

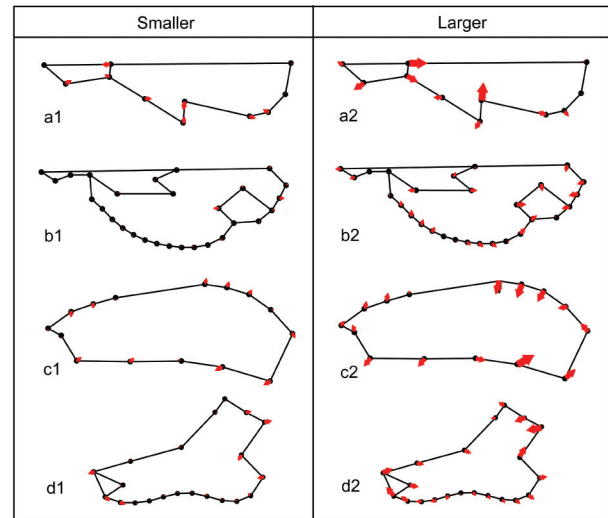


Figure 3 Allometric shape deformations from the consensus shapes (not shown) to smaller specimens (a1, b1, c1 and d1) and larger specimens (a2, b2, c2 and d2), with the direction and length of the arrows representing the orientation and extent of shape changes; a–d are dorsal, ventral, lateral cranium and mandible, respectively.

Allometric shape changes

The shape variation explained by size and Goodall's *F*-test result are presented in Table 3. The regressions are all statistically significant, with the highest percentage of size explained variation of 38.88% for the lateral cranium, and the lowest for the ventral view (11.72%). Allometric shape deformations are illustrated in Fig. 3. Large species are characterized by deformation in the

Table 3 Percentages of size-explained shape changes (with the results of the significance tests) and the results of Mantel tests for the association between shape and phylogenetic distance matrices

	Size explained (%)	Goodall's <i>F</i> -test			Phylogeny
		<i>F</i>	df	Significance	r^2 (%)
Dorsal	21.76	6.1400	18, 396	$P < 0.001$	0.389*
Ventral	11.72	2.9282	52, 1144	$P < 0.001$	0.181*
Lateral	38.88	14.0166	26, 572	$P < 0.001$	0.224*
Mandible	20.35	5.6342	38, 836	$P < 0.001$	0.477*

r^2 , Phylogenetic inertia estimated as a percentage of morphometric distances explained by phylogenetic distances. *Means significant and P -value is 0.0002 (P -value is the probability that random permutations of matrices yield values of r higher or equal to the observed ones. This probability was estimated by 9999 random permutations).

Figure 4 Bayesian tree based on aligned *Cytb* sequences. Posterior probabilities at all nodes are 1.00 unless indicated otherwise.

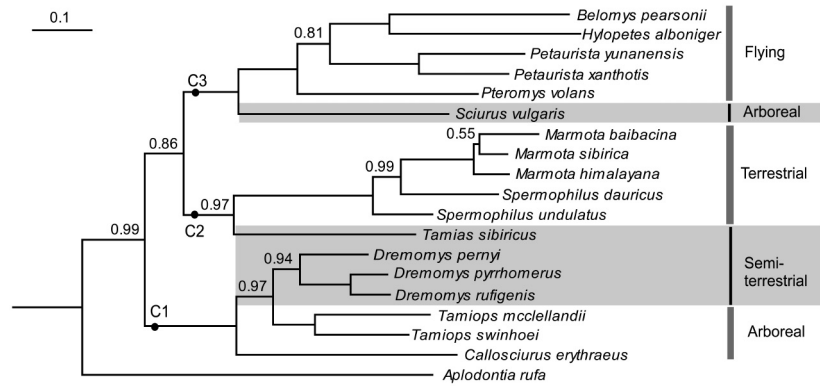


Table 4 Results of the Wilks' λ significance tests of multivariate regression of skull shape on lifestyle category using different numbers of variables (5 RWs and 2 RWs) representing skull shape

	5 RWs			2 RWs		
	Wilks' λ	$F_{(20, 54)}$	P	Wilks' λ	$F_{(4, 38)}$	P
Dorsal	0.0005	23.624	<0.0001	0.0557	30.755	< 0.0001
Ventral	0.0064	9.685	<0.0001	0.0524	31.985	< 0.0001
Lateral	0.0201	6.068	<0.0001	0.1005	20.461	< 0.0001
Mandible	0.0080	8.881	<0.0001	0.0193	58.875	< 0.0001

RW, relative warp.

Table 5 Results of the Wilks' λ significant test of phylogenetic generalized least squares analyses of shape (5 relative warps) on size and lifestyle

	Shape on size			Shape on lifestyle		
	Wilks' λ	$F_{(10, 24)}$	P_1	Wilks' λ	$F_{(20, 34.1)}$	P_2
Dorsal	0.3113	1.901	0.0957	0.0337	3.034	0.0021**
Ventral	0.2551	2.351	0.0419**	0.0662	2.162	0.0230**
Lateral	0.1521	3.755	0.0038**	0.1049	1.661	0.0938
Mandible	0.6408	0.598	0.7998	0.0872	1.853	0.0549

**Significant regressions ($P_1, P_2 < 0.05$).

postorbital process, auditory bulla, vault outline, cheek tooth rows and condyloid and angular processes. As size increases, the lateral profile of the cranium and the mandible both become more compressed, while in the dorsal view, the nasal bone broadens and the interorbital region contracts. In the ventral view, cheek tooth rows tend to be larger as size increases, while the bulla and condyle become compressed.

Phylogenetic signal in shape variance

In the phylogenetic tree based on *Cytb* of 18 species, 3 lineages are formed (c1–c3) (Fig. 4) corresponding to clades III, IV and V in the study of Mercer and Roth (2003) and clades D, E and C in Stepan *et al.* (2004). In the context of this phylogeny, the correlations between the phylogenetic distance matrix (i.e. patristic

distance) and each of the 4 skull Procrustes distance matrices were tested by Mantel analyses. The results show that all matrix associations are statistically significant (Table 4). The estimated evolutionary inertia is highest for the mandible and lowest for the ventral.

Multivariate regression of shape on lifestyle

Accounting for most of the variance (the first 5 RWs), there are significant differences in skull shape among different lifestyles. Results of multivariate regression of the shape variation on dummy lifestyle variables for the 4 skull structures are shown in Table 4. All the regression analyses are significant (in all cases $P < 0.0001$), and when the same tests are performed using only the first 2 RW axes, the same pattern is found.

Phylogenetic generalized least squares analysis

When accounting for shared ancestry with PGLS, the allometric constraint on shape variations of the ventral and lateral cranium remains substantial, and the correlation of lifestyle with dorsal and ventral cranium shape variations also remains significant (Table 5). Conversely, PGLS analyses of the correlation of mandible shape with both size and lifestyle are non-significant. Hence, the PGLS analysis confirms the influence of size and/or lifestyle on the cranium shape changes after taking phylogeny into account, and demonstrates that mandible shape is not significantly associated with these factors.

DISCUSSION

Developmental constraint on skull shape variance in Sciuridae

It has been argued that the rates of molecular and morphological changes should be decoupled (Bromham *et al.* 2002) because phenotypic evolution is expected to be driven mostly by directional selection, which can lead to convergent evolution and the blurring of phylogenetic signal (Losos *et al.* 1998). However, there are also studies detecting good congruence between morphological and molecular patterns of differentiation (Omland 1997; Polly 2001; Renaud *et al.* 2007), like our results here. Variations in skull shape among the 18 representative species of the family Sciuridae are considerable, and all 4 structures retain phylogenetic signals in their shape. While based on a broader sample of taxa, our study has confirmed most results of previous analyses on the skull or mandible morphology of *Marmota* (Cardini 2003; Cardini & O'Higgins 2004, 2005; Cardini *et al.* 2005; Caumul & Polly 2005).

Different regions or subunits of a complex biological structure might have quite different conservative properties. As for the mammalian skull, the cranial base is expected to be the most constrained region because it usually attains adult size and shape at an early stage during ontogeny in various organisms, and bears many passages for vital nerves and vessels (Lieberman *et al.* 2000; Sperber 2001). This assumption is well demonstrated here, because variations in the ventral cranium exhibited the weakest signals in both phylogeny and allometry. Although shape variations are still statistically significant among different lifestyle groups, the extent of variation in the ventral view is obviously smaller when compared with the other 3 structures, as shown by the deformation grids (Fig. 2). Structures of vital importance tend to remain relatively unchanged within sciurid subclades.

Involved in feeding and certain other activities (e.g. digging), the mandible is supposed to show a greater association with mastication, diet and possibly habitat (Perez *et al.* 2009). Other studies on carnivores also show that mandible shape is strongly influenced by dentition complexity (Meloro *et al.* 2008, 2011). Phylogenetic signal has been reported in most morphological studies on the mandible, although to differing extents (Caumul & Polly 2005; Renaud *et al.* 2007). Strong phylogenetic signal is also observed in our data. Being involved in feeding, for a group of mostly omnivorous squirrels, it might be assumed that this structure is much more conservative at the macroevolutionary scale, and less influenced by lifestyle adaptations. This is confirmed in the PGLS analyses. When the phylogenetic covariance matrix is taken into the regression as an error term, the regressions of mandible shape both on size and lifestyle become insignificant (Table 5).

The interplay of dorsal and lateral cranium shape and phylogeny are also very apparent, which confirms the strong developmental constraint on skull shape evolution in Sciuridae. The proportion of shape variation explained by molecular evolutionary patterns in the dorsal and lateral cranium are higher than in the ventral cranium, but lower than in the mandible (Table 3). The dorsal structure retains a larger degree of phylogenetic signal in shape data than that of the lateral cranium. The phylogenetic information in the latter structure has probably been diluted because the subunits in this structure are more related to the trophic and sensory functions and might have experienced more directional selection.

Generally, a phylogenetic correlation with shape variance is hard to establish for a diverse rodent group like sciurids; they occupy heterogeneous habitats and adopt various lifestyles. From the results here we infer that natural selection might dilute phylogenetic correlation with shape evolution (Renaud *et al.* 2007), but only to a degree, with developmental influence still observed in this group.

Allometric shape change in the skull of squirrels

The variation in squirrel skull shape due to change in size is statistically significant in all 4 allometric analyses (Table 3). The result using the mandible is consistent with the previous study of Swiderski (2003), in which the researcher found allometry accounted for approximately 20.4%–24.7% of the adult mandible shape variation in the fox squirrel (*Sciurus niger* Linnaeus, 1758); here it is 20.35%. However, his speculation that allometry would have little power to direct the evolutionary changes in squirrels is not confirmed here, and, therefore, probably could not extend to traits other than the lower jaw as was proposed in his study. The proportion of shape changes explained by allometry is nearly 40% in the lateral structure of the skull. This allometric variation is independent from the evolutionary history, because in the PGLS analysis, the regression of lateral shape on size is still significant in the context of phylogenetic covariance. The large proportion of shape change in the lateral structure could probably be ascribed to the prominent dorso–ventral compression of the skull in the larger species, mainly the marmots. The relatively lower proportion for the ventral structure (11%) is possibly evidence for its conservative nature, as mentioned above. Allometric influence on the dorsal and mandible structures is great, but it is not independent from phylogeny, as indicated from the result of PGLS (Table 5).

Recent morphological allometric analyses on marmots cast doubt on the prominence of allometry as a constraint on evolutionary change in sciurids (Cardini 2003; Cardini & O'Higgins 2004; Cardini *et al.* 2005). They ascribe the overestimation of the role of allometry (Hafner 1984; Heaney 1985; Velhagen & Roth 1997) to limitations of morphometrics based on the linear measurements. Although studies on marmots have taken advantage of the ability of geometric morphometrics to avoid confounding size and shape, these studies document divergence among populations of 1 species or among several species of a single genus, while none explore the allometric effect by taking all arboreal, gliding and terrestrial representatives into consideration. Hence,

their inferences are probably not generally applicable on a family-level scale.

The 24 species in our study represent a rather small part of the family, but include quite different forms with a large range of size differences. The results here indicate that allometric changes have probably diverged greatly at different phylogenetic levels, and that the sciurid skull might have evolved as a mosaic of characters composed of parts showing convergence mostly determined by size similarities, with other parts show less. Therefore, determining the importance of allometry requires increased species sampling in further analyses.

Lifestyle impacts on skull shape variation of squirrels

According to the multivariate regression (Table 4), skull shape shows a strong correlation with lifestyle. Variations in the dorsal and ventral cranium are still statistically significant when phylogeny has been partialled out in the PGLS process (Table 5). Variations in these structures mainly occur in the vault, postorbital process, zygomatic arch, auditory bulla and nasal bone. The rounded and inflated vault seems to be a conservative character in the arboreal group because both the early diverged arboreal *Ratufa* and the recent arboreal squirrels have this character state. These taxa are overlapping in the shape space for this character, even though some of them are genetically divergent (Fig. 2). However, the flying squirrels have developed a relatively aspheric vault and a more laterally expanded zygomatic arch, with constrictions in the interorbital and antorbital regions (Roth 1996). These variations make the orbits bigger and eyes wider apart, characteristics thought to be adaptations for triangulation of distance in preparation for gliding (Jackson 2000), and also found in the gliding marsupial *Petaurus*. Quite similar to the gliders, the terrestrial squirrels also developed a robust and posteriorly widened zygomatic arch, which helps them build up a typical triangular spade-like head important in fossorial movement (Shimer 1903; Stein 2000). The relative position and size of the postorbital process account for a large part of shape deformation in different groups. Robust postorbital processes and larger orbits indicate good eyesight for both gliding and terrestrial squirrels. The nocturnal gliders have to swiftly avoid tree branches and avian predators during a glide (Nowak 1999; Jackson 2000), while for the terrestrial species, which mainly reside in open grassland and mostly have a highly socialized system, good eyesight might be of great importance in detecting predators and in social com-

munication (Armitage 1962). The relatively larger auditory bulla in flying squirrels implies that during gliding auditory sense plays a role equal to vision. For the terrestrial squirrels, a larger auditory bulla reflects good low-frequency audition (Francescoli 2000) and could be interpreted as an adaptation for vocal communication and enemy detection underground (Schleich & Vassallo 2003), while the larger nasal bone is consistent with the importance of olfaction in squirrels inhabiting subterranean environments.

The lateral cranium profile confirms the overall shape differences mentioned above: the tree dwellers have groined vaults and slender nasals while their fossorial relatives have a rather flattened cranium and an anterodorsally-inclined occiput, which increases the available attachment area for the muscles from jaw and neck (Stein 2000). The gliding squirrels have an intermediate lateral shape, not too inflated or obviously flattened, with the occiput nearly vertical to the median sagittal plane of the skull. These changes might be advantageous in gliding when they stretch the whole body and keep all parts in a plane to minimize air resistance. The mandible is thought to be a conservative structure throughout the history of the sciurids (Emry & Thorington 1982), and in the present study this view is supported in the PGLS analysis. Lifestyle clearly impacts the skull shape but not the mandible, possibly because the latter structure retains a much stronger phylogenetic signal and its shape is probably more related to differences in diet rather than lifestyle.

Among all the species studied, the characteristics of *R. bicolor* are particularly interesting and should be emphasized. As an early established and strictly arboreal species, *R. bicolor* shows some different characters from the more recently evolved tree squirrels. Its dorsal and ventral cranium structures are quite similar to recent tree squirrels but the lateral cranium and mandible are more similar to the gliders (Figs 2 and 3). Thus, if the Sciuridae really have an arboreal ancestor (Thorington & Heaney 1981; Corbet & Hill 1992) and *Ratufa* has maintained the primitive traits on its skull, it could be inferred that the recently evolved tree dwellers and flying squirrels have both retained some ancestral characters and obtained new traits during their evolution.

Many studies on morphological adaptation in various organisms support the hypothesis that as a result of the great pressure from the external environment on morphological diversification, species coming to occupy a similar ecological niche share a similar cranial shape independent of phylogenetic relationships (Lo-

so *et al.* 1998; Harmon *et al.* 2005; Perez *et al.* 2009). The results for the dorsal and ventral cranium here also support this conclusion, while the phylogenetic signal dominates in the lateral and mandible views. However, structures discussed here are actually an integrated unit, so a further analysis on the shape as a whole would be more appropriate to avoid information redundancy or deficiency.

CONCLUSIONS

Skull and mandible shape exhibit strong phylogenetic signal in the Sciuridae. However, shape changes related to size and lifestyle play a significant role in Sciuridae evolution, especially in the cranium. The mandible exhibits the strongest phylogenetic signal and its associations with size and lifestyle are not significant after taking phylogeny into account. Our study suggests that it is important to combine molecular and morphological analyses to understand the morphological evolution of Sciuridae. For the first time, we demonstrate that this group is quite diverse and skulls of Sciuridae retain important phylogenetic and ecomorphological information.

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