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SHORT COMMUNICATION

Does nasal echolocation influence the modularity of the mammal skull?

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Abstract

In vertebrates, changes in cranial modularity can evolve rapidly in response to selection. However, mammals have apparently maintained their pattern of cranial integration throughout their evolutionary history and across tremendous morphological and ecological diversity. Here, we use phylogenetic, geometric morphometric and comparative analyses to test the hypothesis that the modularity of the mammalian skull has been remodelled in rhinolophid bats due to the novel and critical function of the nasal cavity in echolocation. We predicted that nasal echolocation has resulted in the evolution of a third cranial module, the 'nasal dome', in addition to the braincase and rostrum modules, which are conserved across mammals. We also test for similarities in the evolution of skull shape in relation to habitat across rhinolophids. We find that, despite broad variation in the shape of the nasal dome, the integration of the rhinolophid skull is highly consistent with conserved patterns of modularity found in other mammals. Across their broad geographical distribution, cranial shape in rhinolophids follows two major divisions that could reflect adaptations to dietary and environmental differences in African versus South Asian distributions. Our results highlight the potential of a relatively simple modular template to generate broad morphological and functional variation in mammals.

Introduction

Modularity is a widespread attribute of biological systems that explains both the integration within and the autonomy among organismal features (Goswami, 2007). Whereas integration maintains certain relationships that are necessary for proper function and high performance of structures (Cheverud, 1996), autonomy among parts allows for components to change independently. This can facilitate adaptive responses to conflicting selective pressures, the evolution of complex phenotypes, morphological, ecological and taxonomic diversity (e.g. Williams & Nagy, 2001; Yang, 2001; Tokita *et al.*, 2007; Esteve-Altava *et al.*, 2013). The vertebrate skull is a classic example in which the evolution of independent modules has allowed for tremendous diversity in form and function (e.g. de-coupling of oral and pharyngeal jaws in cichlid fishes, Hulsey *et al.*, 2006). Nevertheless, some groups of vertebrates, such as mammals, also appear to exhibit relatively few, highly integrated and evolutionarily stable cranial modules while still achieving a high diversity in morphology and ecology (Goswami, 2006; Porto *et al.*, 2009). Comparative studies suggest that the mammalian skull is comprised by two modules, the braincase and the rostrum, both of which could be the result of conserved developmental processes in skull morphogenesis and selection to maintain module functionality (Marroig *et al.*, 2009; Porto *et al.*, 2009). Across mammals, the primary roles of the skull are biting/feeding, housing sensory organs and encasing the brain, but a few lineages have evolved novel, fitness-relevant functions that could have created pressures for more partitions beyond the two-module scheme. We explore whether

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functional demands imposed by one of these functions has resulted in a different pattern of cranial modularity in a diverse lineage of bats.

Bats are one of the most morphologically diverse groups of mammals. In several clades of microchiropteran bats, the skull, and specifically the nasal cavity, has been co-opted to function in echolocation. Emission of sounds through the nasal cavity enables bats to call and chew at the same time, an adaptation that might be especially important when eating large prey items (Jones & Teeling, 2006). Nasal echolocation evolved multiple times from the more ancestral oral echolocation; it occurs in the Old World horseshoe bats (Rhinolophidae), Old World leaf-nosed bats (Hipposideridae), Old World slit-faced bats (Nycteridae) and the New World leaf-nosed bats (Phyllostomidae) (Wilson & Reeder, 2005). In rhinolophids, the nasal cavity forms large resonating chambers (Fig. 1), a striking morphology that is uncommon in mammals. The size and shape of the nasal chambers vary greatly across rhinolophid bats, and this variation might produce differences in frequency of echolocation calls across species (Odendaal & Jacobs, 2011). Although constituted by a radiation within one single genus, *Rhinolophus*, these bats are widely distributed across Africa, Asia and Oceania, where the occupy habitats ranging from savannahs to tropical forests (Nowak & Walker, 1994). Biogeographical analyses suggest that rhinolophids arose in Asia and subsequently dispersed into Europe and Africa, with the African group and Asian assemblage diverging approximately 35 million years ago (Stoffberg *et al.*, 2010). Occupation of such diverse habitats has likely selected for differences in cranial morphology within the genus.

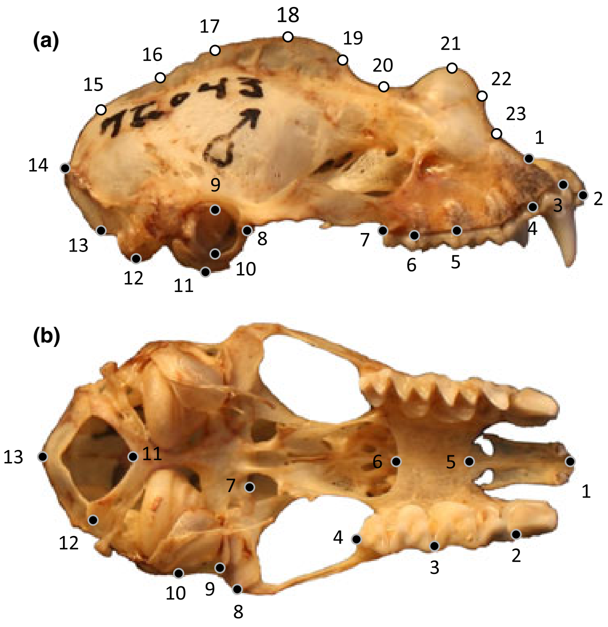


Fig. 1 Skull of *Rhinolophus hildebrandti* in lateral (a) and ventral (b) views showing the cranial landmarks (filled circles) and semilandmarks (open circles) used in geometric morphometric analyses. Description of landmarks is provided in Table S1.

Changes to patterns of modularity in the vertebrate skull can sometimes evolve rapidly (Sanger *et al.*, 2012), even when morphological changes are underlined by conserved molecular mechanisms (Abzhanov, 2010; Mallarino *et al.*, 2011). In New World leaf-nosed bats, Monteiro and Nogueira (2009) found that integration patterns of the mandible have evolved during relatively recent ecomorphological transitions related to diet. Rapid evolution of morphology and integration patterns has also been found in numerous cranial and post-cranial traits within the genus *Anolis* (Kolbe *et al.*, 2011; Sanger *et al.*, 2012). The rapid evolution of patterns of integration could be the result of direct selection on the patterns of covariation among traits or modification of developmental processes due to adaptive changes in life-history traits. Here, we investigate the evolution of skull morphology and modularity in the recent radiation of rhinolophid bats. We use phylogenetic, geometric morphometric and comparative analyses to test the hypothesis that skull modularity has been remodelled by natural selection in rhinolophids due to the novel and critical function of the nasal cavity in echolocation. We predict that nasal echolocation has resulted in the evolution of a third cranial module (the 'nasal dome'), in addition to the two cranial modules conserved across mammals. We also examine, within a phylogenetic framework, whether there are similarities in the evolution of the shape of skull modules among species that occupy the same geographical regions, which could indicate specialization to ecologically similar environments.

Materials and methods

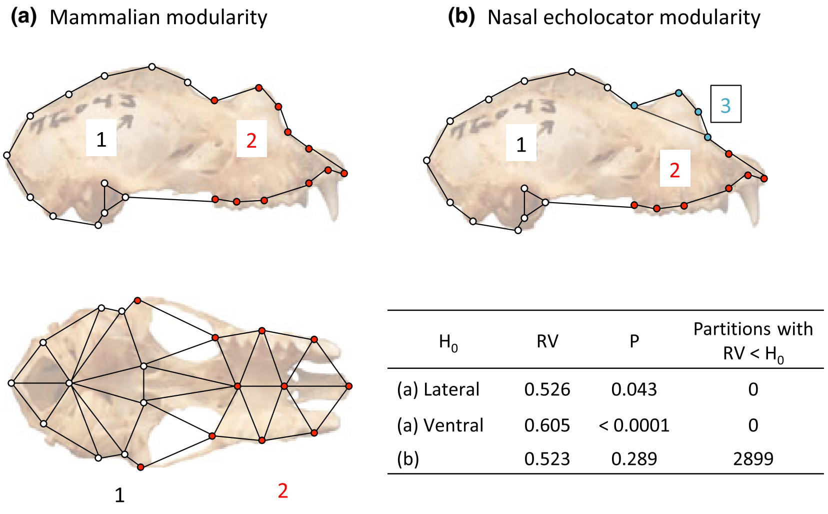
Our sample included skulls from 73 specimens representing 22 rhinolophid species ($N = 3–5$ specimens, Fig. S1). We used a Canon Rebel XSi mounted on a copy stand to take digital photographs of the skulls in ventral and lateral views. Dorsal views were not included because these did not provide additional information on skull shape variation; bats have many fused cranial bones and this hinders the placement of dorsal landmarks. We placed each skull on a custom-made platform that allowed us to consistently align the specimens with respect to a horizontal plane, with the palate (ventral view) or midline (lateral view) parallel to the camera lens to avoid any distortion in the photographs that could alter shape analyses (Zelditch *et al.*, 2004).

Geometric morphometric analyses

We used a geometric morphometric approach to identify the major axes of skull shape variation among rhinolophid bats. The tps packages (Rohlf, 2005) were used to place landmarks in ventral and lateral views of the skull (Fig. 1). A total of 14 landmarks and eight semi-landmarks were placed in lateral view, and six landmarks were placed in ventral view (Fig. 1, Table S1). We then used MorphoJ (Klingenberg, 2011) to conduct geometric morphometric analyses of shape variation, allometry and modularity. First, we conducted separate Procrustes superimpositions and calculated centroid size for each species. For the ventral view, which had symmetrical landmarks, the Procrustes superimposition accounted for 'object symmetry' of the skull by reflecting landmarks across the midline to find an average landmark position (Klingenberg *et al.*, 2002). From the specimen data, we calculated the average values of Procrustes coordinates and centroid size for each species and used these data in our comparative analyses. As the skulls of rhinolophid bats vary in size (head–body length: 35–110 mm, Nowak & Walker, 1994), and skull similarities may be explained by evolutionary relationships (e.g. Cardini & Elton, 2008), we tested the skull shape data for allometric correlations and phylogenetic effect. Klingenberg & Gidaszewski's (2010) phylogenetic permutation test for multivariate shape data (10 000 permutations) on a pruned version of Jones *et al.* (2002) phylogeny revealed that there was a significant phylogenetic signal for skull shape (ventral view, $P = 0.037$; lateral view, $P = 0.002$), but not for centroid size (ventral view, $P = 0.439$; lateral view, $P = 0.345$). Given that there was phylogenetic signal on skull shape, we tested for allometry in our skull shape data using a permutation test while accounting for phylogenetic relationships among species (10 000 iterations). This test yielded a significant effect ($P < 0.0001$ for both ventral and lateral views), and thus all subsequent analyses were performed on shape variables that were also size-corrected. To correct for size, we conducted a multivariate regression of the phylogenetically independent contrasts (PIC) of Procrustes coordinates on the PIC of centroid size and computed shape residuals (Klingenberg, 2009; Klingenberg & Marugán-Lobón, 2013). For ANOVAS testing the effect of geographical distribution (below), these residuals were summarized through a phylogenetic principal component (PC) analysis that computes the PC scores in the original species space (Revell, 2012). Of these PCs, the first three explained more than 70% of the variation and thus were used in subsequent analyses. Phylogenetic size correction and PCA were performed using functions in the phytools library (Revell, 2012) for R.

Tests of modularity hypotheses

We examined the hypothesis of mammalian cranial modularity (two modules: braincase and rostrum, Fig. 2) in both the lateral and ventral views. In the lateral view, we also contrasted the mammalian cranial modularity hypothesis to a 'nasal echolocator' hypothesis, in which the dome located above the nostrils constituted an additional module in the rostrum (three modules: braincase, rostrum and nasal dome). We tested all hypotheses in MorphoJ (Klingenberg, 2011) using covariance matrices generated from residuals of a multivariate regression of PICs of Procrustes coordinates of landmarks on PICs of centroid size. Given that all random partitions of the skull would not be biologically realistic, we edited the adjacency graphs based initially on Delaunay triangles to outline the potential partitions of the skull and only considered contiguous partitions during randomization procedures (Fig. 2, Klingenberg, 2009). Additionally, we ran the same tests of our hypotheses considering all sets of possible partitions. To test modularity hypotheses in the rhinolophid skull, we



H_0	RV	P	Partitions with RV < H_0
(a) Lateral	0.526	0.043	0
(a) Ventral	0.605	< 0.0001	0
(b)	0.523	0.289	2899

Fig. 2 Adjacency graphs of landmarks and results for each of the modularity hypotheses tested on rhinolophid skulls. In the mammalian modularity hypothesis (a), the skull is composed by two modules: (1) the braincase and (2) the rostrum. In the nasal echolocator hypothesis (b), the skull is composed by these two modules and an additional one, the nasal dome (3). RV coefficients and statistical significance for these hypotheses are shown for the mammalian modularity hypothesis in lateral and ventral view, and the nasal echolocator hypothesis in the lateral view.

used Klingenberg's application of the RV coefficient (Robert & Escoufier, 1976; Klingenberg, 2009). This metric reveals covariation among sets of landmarks, which in turns allows to test the prediction that shape covariation should be stronger within modules than between modules, if the modularity hypothesis is true. The RV coefficient ranges from 0 (no covariation) to 1 (perfect covariance), which represents the strength of association between two sets of variables (Robert & Escoufier, 1976). Therefore, high RV coefficients indicate covarying landmarks that can be interpreted as biological modules. We compared the RV coefficients generated for our two modularity hypotheses to RV coefficients estimated from 10 000 random partitions. RV coefficients below the cut-off of 95% of the random contiguous partitions of the skull indicate modularity with a similar significance to a *P*-value of 0.05 (Klingenberg, 2009).

Geographical variation of skull shape

We used the IUCN database (http://www.iucnredlist.org/) to gather data on the geographical range of all rhinolophid species included in this study. According to the distribution of species investigated, the geographical ranges were grouped into four major areas: Africa (characterized by small ranges in Central and Southern Africa), Mediterranean (comprising Southern Europe, Northern Africa and Middle East), South-East Asia and Oceania (New Guinea and Australia). These were assigned when more than 80% of the species' distribution was located in a given area. We used these data as explanatory variables of the variation in skull shape across species (PC scores of skull shape) in phylogenetic ANOVAs using the Jones *et al.* (2002) phylogeny. Tukey's honest significance difference test was performed on ANOVA results to identify which and how species from different geographical regions differed in skull shape.

Results and discussion

Modularity of rhinolophid skulls

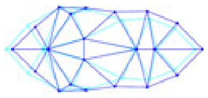
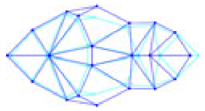
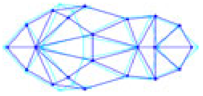
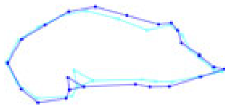
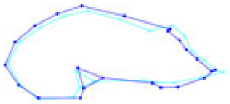
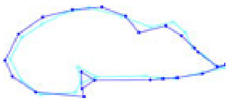
Due to the novel function of the nasal cavity in echolocation in *Rhinolophus*, we hypothesized that macroevolutionary changes in this region would be decoupled from changes in other parts of the rostrum. However, the evolutionary trends in the skulls of these nasal-echolocating bats are highly consistent with the patterns of integration and modularity described in other mammals (Porto *et al.*, 2009). The mammalian modularity hypothesis, where the braincase and rostrum constitute separate modules, was supported in rhinolophids by both the data on skull shape from the ventral view (Fig. 2, RV coefficient: 0.605, $P < 0.0001$, no partition schemes had lower RV than hypothesis, 13 contiguous partitions considered) and the lateral view (RV coefficient: 0.526, $P = 0.043$, no partition schemes had lower RV than hypothesis, 25 contiguous partitions considered). This hypothesis was also supported even when all possible partitions of the skull were considered. The nasal echolocator (3-module) hypothesis was not supported in any analysis (RV coefficient: 0.523, $P = 0.289$; 2899 partitions with lower RV than hypothesis).

Unlike other animals, in which patterns of integration seem to have been more easily shaped by environmental selection (Beldade *et al.*, 2002; Sanger *et al.*, 2012), mammals have maintained the same two major cranial modules during their 100 + million-year history and over an extraordinary diversity in ecologies and cranial shapes (Goswami, 2006; Porto *et al.*, 2009; Drake & Klingenberg, 2010). Our results might be explained by the proposition that the two cranial modules reflect constraints imposed by their different cellular origins during embryonic development; the rostrum derives from neural crest and the braincase derives from the neurocranium (Cheverud, 1996; Marroig & Cheverud, 2007), although these two phenotypic modules do not seem to be integrated in developmental timing across placentals (Goswami, 2007). In comparisons using a mouse genetic variance/covariance matrix, Porto *et al.* (2009) found that the broad modular partitioning of mammal skulls seems to be linked to underlying genetic factors. Internal stabilizing selection on the form and function of the skull is likely a mechanism maintaining these potentially conserved genetic patterns of integration of the mammalian skull (Cheverud, 1996) and might explain their persistence during the diversification of the lineage. The diversity of mammalian dietary ecologies might be further facilitated by variation in the musculature and the shape and number of teeth, which is a system that is modular itself (Stock, 2001). Further comparative studies across mammalian taxa are required to investigate whether and how cranial integration imposes limitations to intra- and interspecific variation, and how it may be shaped by natural selection.

Variation of skull shape across the geographical range of rhinolophids

We found a significant relationship between skull shape and geographical distribution across the rhinolophids studied. In the ventral view, the first three principal components (PC) of phylogenetically adjusted data explained 79.04% of the variation in skull shape, and PC1 and PC3 were related to geographical distribution (Table 1, Table S2). In the lateral view, the first three PC explained 71.23% of the variation in skull shape, and PC2 was significantly related to geographical distribution (Table 1, Table S3). Altogether, the trends in both views indicate that species living in Africa and Mediterranean regions tend to have a proportionally taller skull, larger braincase module and a narrower

Table 1 Major trends of cranial shape variation across phylogenetic principal components (PC) of shape coordinates in rhinolophid skulls. Wireframe graphs (dark blue outlines) show a change in 0.1 units of Procrustes distance in the positive direction of the PC with respect to the mean shape (light blue outline). Mean PC scores for the species' geographical ranges (Africa, Mediterranean, Oceania and South-East Asia) are shown, along with results for a phylogenetic ANOVA (*P*-values in parentheses) bold values are in significant.

	%Variance	Africa	Med	Oceania	SE Asia	F (*P*)
Ventral view						
PC1 	36.36	–0.017	–0.031	–0.004	0.019	**10.310** **(0.0004)**
PC2 	32.15	0.024	0.029	–0.004	0.011	1.407 (0.275)
PC3 	10.53	–0.016	–0.003	0.005	0.003	**5.870** **(0.006)**
Lateral view						
PC1 	43.81	–0.019	0.002	0.033	-0.010	2.154 (0.129)
PC2 	17.37	0.037	0.042	–0.004	0.003	**8.670** **(0.001)**
PC3 	10.05	0.006	0.000	–0.009	0.001	0.786 (0.517)

rostrum with a smaller nasal dome. In turn, species living in SE Asia and Oceania have a broader skull and a more robust rostrum with a larger nasal dome (Figs S2 and S3).

Upon phylogenetic corrections, the geographical trends in cranial shape could reflect adaptations to the dietary and echolocation demands encountered by species in each region. A broader and shorter rostrum in SE Asian and Oceanic species would increase the mechanical advantage of the skull at a given gape angle (Greaves, 2000; Clausen *et al.*, 2008; Santana *et al.*, 2010) and reduce torsional loads applied to the rostrum as bat chew on hard prey (Santana *et al.*, 2012). These traits would allow species to achieve high levels of bite performance and thus consume a broader spectrum of prey by accessing harder prey items (Dumont *et al.*, 2012). Differences in the size of the nasal chambers are related to differences in echolocation call frequency in nasal-emitting bats (Armstrong & Coles, 2007; Odendaal & Jacobs, 2011), and these are in turn closely associated with the habitat types bats occupy and their diet (Aldridge & Rautenbach, 1987; Bogdanowicz *et al.*, 1999; Schnitzler & Kalko, 2001). To fully interpret the adaptive value of the morphological trends found across rhinolophid skulls, future studies should quantify the bite force and physical properties of prey items consumed by these bats, as well as the openness of their microhabitats. Comparative analyses on these data would also allow us to determine whether the integration of the rostrum is a response to linked functional traits. For example, larger and harder insects, which require stronger bites to process, might be easier to detect with echolocation frequencies produced by larger nasal chambers, and *vice versa*.

Over evolutionary time, the emergence or loss of modularity patterns are expected to follow similar trends as other phenotypic traits (Schwenk & Wagner, 2004). Nevertheless, this study contributes to the body of research that has demonstrated a persistent pattern of cranial integration along the evolutionary history of mammals, while testing this pattern at a smaller taxonomic scale. As predicted by the mammalian modularity hypothesis, the diversity in cranial shape in a recent radiation of nasal-echolocating bats is underlined by variation in two modules: the braincase and the rostrum, which in turn seem to be modelled by broad differences in habitat. Further studies that combine developmental, anatomical and ecological information will be important in understanding the origins of the two-module model and how it allows mammals to achieve strikingly high levels of morphological and functional diversity.

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Supporting information

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

Table S1 Description of the cranial landmarks and semilandmarks used.

Table S2 Results of multiple pair-wise comparisons from Tukey's honest significance test on skull ventral shape variables across geographical regions.

Table S3 Results of multiple pair-wise comparisons from Tukey's honest significance test on skull lateral shape variables across geographical regions.

Figure S1 Phylogeny of the Rhinolophus species included in this study, coded for their geographical distribution.

Figure S2 Scatterplot of ventral skull shape across rhinolophid species.

Figure S3 Scatterplot of lateral skull shape across rhinolophid species.

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