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Contrasting patterns of *RUNX2* repeat variations are associated with palate shape in phyllostomid bats and New World primates

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Establishing the genetic basis that underlies craniofacial variability in natural populations is one of the main topics of evolutionary and developmental studies. One of the genes associated with mammal craniofacial variability is *RUNX2*, and in the present study we investigated the association between craniofacial length and width and *RUNX2* across New World bats (Phyllostomidae) and primates (Catarrhini and Platyrrhini). Our results showed contrasting patterns of association between the glutamate/alanine ratios (Q/A ratio) and palate shape in these highly diverse groups. In phyllostomid bats, we found an association between shorter/broader faces and increase of the Q/A ratio. In New World monkeys (NWM) there was a positive correlation of increasing Q/A ratios to more elongated faces. Our findings reinforced the role of the Q/A ratio as a flexible genetic mechanism that would rapidly change the time of skull ossification throughout development. However, we propose a scenario in which the influence of this genetic adjustment system is indirect. The Q/A ratio would not lead to a specific phenotype, but throughout the history of a lineage, would act along with evolutionary constraints, as well as other genes, as a facilitator for adaptive morphological changes.

An understanding of the genetic basis associated with craniofacial variability in animal natural populations is a major focus of modern evolutionary and developmental studies^{1–5}. Until recently, broad phenotypic differences were thought to be the result of several gene mutations, each one with relatively small effects⁶. This proposal was based on extrapolations from studies with housekeeping genes in model species, as well as on the high capacity of population genetics to predict the results of allelic frequency changes in natural populations⁷. However, through the use of large-scale DNA sequencing, current studies of genes that either regulate the development process or code for receptors and signalling complex metabolic networks indicate that some mutations can have a large effect on morphology^{2,8,9}.

One of these genes, *RUNX2* (Runt-related transcription factor 2), was suggested to have a large effect on mammals' morphological variation, specifically on the craniofacial variability^{10–12}. *RUNX2* is one of the most important transcription factors associated with bone tissue formation, and is responsible for promoting cell transitions among cell lineages^{13,14} being essential for osteogenesis^{15,16}. The *RUNX* family is composed by three genes that share the same DNA binding motif (Runt Domain): *RUNX1*, *RUNX2*, and *RUNX3*. However, *RUNX2* is the only one that contains a tandem amino acid repeat region that precedes this domain¹⁷. The indicated repeat motif presents one segment rich in glutamate residues (polyQ), followed by a segment rich in alanine (polyA)¹⁰. This

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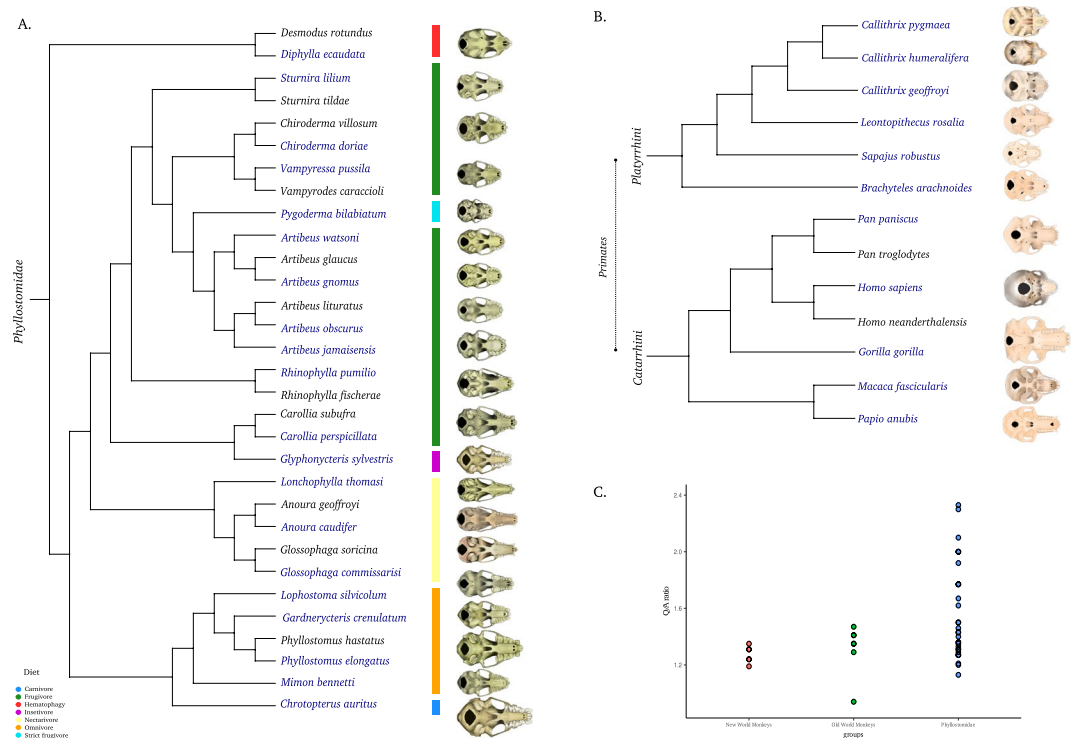


Figure 1. Phylogenies of sampled phyllostomid bats (A) and primates (B) (Old World and New World monkeys) showing a wide range of craniofacial diversity; (C) distribution of Q/A ratios for each group analysed. Species names in blue indicate the skull represented in the figure.

region appears to play an important role in the repression and activation of essential proteins for craniofacial development¹⁸. Clinical genetic studies have demonstrated that haplo-insufficiency of the *RUNX2* gene is associated with cleidocranial dysplasia (CCD) in humans, an autosomal dominant disease characterized by systemic skeletal anomalies, causing shortening of the face, abnormalities in the permanent dentition, as well as stature effects in affected individuals^{19–24}.

Fondon and Gardner²⁵ evaluated the correlation between different amino acid repeat motifs present in developmental genes and their effects across dog breeds with diverse skeletal and craniofacial phenotypes. They found a positive correlation between the ratio of polyglutamines (Q) to polyalanines (A) in the Q/A repeat domain of the *RUNX2* gene and craniofacial length. Similar patterns of significant positive correlation between the *RUNX2* repeat and facial length was also observed in other mammals belonging to the order Carnivora¹⁰. In the same study, based on functional tests, Sears *et al.*¹⁰ found significant correlations between the ratios of polyglutamines to polyalanines in the *RUNX2* tandem repeat region and *RUNX2* transcriptional activity. Other studies also provided evidence for the polyQ and polyA regions as being a mechanism of fine tuning in the activation and repression of the *RUNX2* protein^{10,16}. Conversely, Pointer *et al.*¹¹ found no correlation between *RUNX2* sequence and face length across a variety of non-carnivoran placental mammals. Also, recently, Newton *et al.*¹² demonstrated that *RUNX2* does not drive craniofacial diversity in marsupials. Taken together, these previous studies suggest that Q/A repeat domain of *RUNX2* might play a role in craniofacial evolution in some mammalian lineages but not others. Therefore, the role of the Q/A repeat domain of *RUNX2* in craniofacial evolution lineage must be investigated independently for each lineage.

In the present study we combine phylogenetic comparative methods and morphological integration analysis to investigate the association between craniofacial length and width with *RUNX2* Q/A ratios across two mammalian lineages displaying large variation in skeletal terms, the New World leaf-nosed bats (Family Phyllostomidae) and Primates (Catarrhini and Platyrrhini). The investigation of these taxa is of interest to understand the developmental mechanism involved in the expression of *RUNX2*.

The New World leaf-nosed bats, Family Phyllostomidae, is a Neotropical lineage that evolved during the last 30 million years²⁶ and represents one of the most ecologically diverse families of mammals²⁷. They present a marked diversity in feeding specialisations, including species that consume insects, blood, small vertebrates, fruits, nectar and pollen. Feeding specialisation is accompanied by morphological adaptations, such as conspicuous craniofacial variability (Fig. 1A)^{27–29}. Few studies have explored the genetic basis underlying diversity in craniofacial phenotype in bats. One of the rare studies to consider this subject was conducted by Phillips *et al.*³⁰, who examined the evolutionary dynamics of the *Pax9* transcription factor and craniofacial diversification in bats. Subsequently, Ball *et al.*¹⁶ found an association between the *RUNX2* variation and cranial bone density in bats and other mammals. On the other hand, the craniofacial morphological variation across phyllostomid lineages has been widely documented through biomechanical, functional, and developmental studies^{27,29,31–42}.

From a comparative perspective, primates show remarkable morphological variation (Fig. 1B). The Catarrhini evolved during the last 35 million years⁴³ and represents a monophyletic lineage encompassing all Old World monkeys (OWM) and apes, including humans. Catarrhines exhibit a diverse range of life histories, habitat use, and dietary strategies, and their geographic distribution includes tropical ecosystems in Africa and Asia⁴⁴. The evolutionary diversification of the Platyrrhini (NWM) occurred over a period of more than 30 million years, driven by diet and size-related selection^{45,46}. Species from both Catarrhini and Platyrrhini display diverse cranial morphologies and remarkable variation in body size. Among the studies with hominids, Green *et al.*⁴⁷ hypothesized that an evolutionary change in *RUNX2* affected the upper body and cranial morphology of modern humans. Additionally, as demonstrated by Lindskog *et al.*⁴⁸, humans and macaques showed differences in protein and RNA expression levels in the *RUNX2* functional network genes (e.g. *RUNX1*, *SMAD3*, *SOX6*, and *SATB*). Other genomic studies showed a correlation between *RUNX2* and nose bridge breadth in current human populations⁴⁹. However, in several other primate lineages, such as the NWM, little is known about the genetic basis that controls the fine-tuning of craniofacial phenotypic variation.

Results

Morphological variation and *RUNX2* repeat variability. Craniofacial variation is a conspicuous trait of phyllostomid bats. We observed marked differences in palate shape across species representing all feeding strategies (electronic supplementary material, Table S1 and Fig. 1A,B). The nectarivorous species (*Anoura caudifer* and *A. geoffroyi*; *Glossophaga commissarisi* and *G. soricina*; *Lonchophylla thomasi*) presented the longest (0.83–0.90 length/GM) and narrower face (0.51–0.54 cm width/GM), what is expected as a morphological adaptation to a diet based on nectar. Alternatively, the hematophagous (*Desmodus rotundus* and *Diphylla ecaudata*) showed the shortest (0.44–0.51 length/GM), but broader (0.60–0.62 width/GM) face, revealing a more robust cranium. *Glyphonycteris sylvestris*, an insectivorous species, also presented a long face (0.81 length/GM, 0.64 width/GM). Interestingly, frugivorous species presented the greatest variation of palate shape (0.60 length/GM, 0.78 width/GM, *Artibeus gnomus*, and 0.74 length/GM, 0.80 width/GM, *Chiroderma villosum*). The strictly frugivorous *Pygoderma bilabiatum* presented the shortest (0.54 length/GM) and a moderately broad (0.69 width/GM) palate among the frugivorous lineage. We observed all major forms of palate shape (short, long, and intermediate⁵⁰); in phyllostomids. Primates also presented variation in craniofacial morphology (0.60–0.94 length/GM; 0.42–0.52 width/GM). Overall, among primates, *Papio anubis* presented the more elongated face (0.94 palate length/GM, 0.47 width/GM).

Similarly, great variation in *RUNX2* Q/A repeats and flanking control regions was evident both in phyllostomid bats and primates (Table 1). Overall, phyllostomid bats presented greater variation in number of glutamate and alanine residues than did primate species (Fig. 1C). Frugivorous phyllostomids presented the widest range, with a Q/A ratio varying from 1.21 to 2.33 (Table 1 and Fig. 1C).

Correlation between Q/A ratios and palate length and width. Spearman's correlation test for the phyllostomid bats data showed a significant negative correlation between Q/A ratio and palate length $r = -0.51$ ($p = 0.01$) and significant positive correlation between palate width and Q/A ratio, $r = 0.55$ ($p < 0.01$) (Fig. 2A,B). Spearman's correlation test for the primates data (NWM and OWM analysed together), and for OWM (analysed separately) showed no significant association between the Q/A ratio and craniofacial morphological traits (NWM + OWM: palate length \times Q/A ratio: $r = 0.44$, $p = 0.12$; palate width \times Q/A ratio: $r = 0.07$, $p = 0.80$; OWM: palate length \times Q/A ratio: $r = 0.58$, $p = 0.17$; palate width \times Q/A ratio: $r = 0.29$, $p = 0.52$). For NMW (analysed separately), we found a positive marginally significant association between palate length and Q/A ratio ($r = 0.79$, $p = 0.05$) (Fig. 2C).

Bayesian PCM was also used to estimate the correlation coefficient between palate and Q/A ratios in both groups, corrected for phylogenetic relatedness, evolutionary rates and divergence times. In accordance with the previous findings, these analyses showed a positive correlation between Q/A ratio and palate width ($r = 0.2910$; $pp = 0.94$), and a negative correlation between Q/A ratio and palate length ($r = -0.2260$; $pp = 0.13$) for phyllostomid bats (Table 2). The craniofacial variation of primates in general did not correlate with Q/A ratios. However, when the dataset is split in two subgroups (NWM and OWM), we found a significant correlation between palate length and Q/A ratios for NWM ($r = 0.583$, $pp = 0.93$).

For phyllostomids, the PGLS results showed a phylogenetic signal (Pagel's lambda = 1) associated with palate length and width, and with zygomatic width (Table 3). The regression between phenotypic traits and Q/A ratios showed that palate width is positively associated with *RUNX2* variation (adjusted $R^2 = 0.19$, $p = 0.01$). For primates (NWM and OWM analysed together) the PGLS results showed a phylogenetic signal associated with palate length and width. In that case, we found no significant association between phenotypic traits and Q/A ratios (Table 3). For NMW (analysed separately), the PGLS results presented phylogenetic signal associated with palate length and width, and a positive marginally significant association between palate length and Q/A ratio (adjusted $R^2 = 0.55$, $p = 0.06$) (Table 3). Finally, in the OWM, we found phylogenetic signal associated with palate length and no association between phenotypic traits and Q/A ratios in the PGLS analyses (Table 3).

Correlation between Q/A ratios and morphological integration index. The morphological integration index (ICV), obtained for phyllostomid bats, exhibited strong negative correlation with Q/A ratios ($r = -0.6$, $p = 0.02$; Fig. 3A). Additionally, the evolutionary flexibility index was strongly correlated with Q/A ratios ($r = 0.7$, $p < 0.01$; Fig. 3B), and the constraints index exhibited negative correlation with Q/A ratios ($r = -0.6$, $p = 0.03$; Fig. 3C). Spearman's correlation test for the primates data analysed together and separately showed no significant association between the Q/A ratio and ICV, flexibility and constraints indexes. The lack of correlation observed for primates data might be due to the relatively small sample sizes for both NWM and OWM clades.

Group	Feeding habit	Species	Palate (mm)/GM			RUNX2		
			Length/GM	Width/GM	Zygomatic/GM	Q	A	Q/A Ratio
New World leaf-nosed bats	Carnivory	<i>Chrotopterus auritus</i>	0.71	0.62	1.10	23	17	1.35
		<i>Artibeus glaucus</i>	0.64	0.75	1.10	20	10	2.00
	Frugivory	<i>Artibeus gnomus</i>	0.60	0.78	1.11	21	10	2.1
		<i>Artibeus jamaicensis</i>	0.70	0.76	1.11	23	13	1.77
		<i>Artibeus lituratus</i>	0.67	0.80	1.10	23	10	2.3
		<i>Artibeus obscurus</i>	0.68	0.77	1.12	23	13	1.77
		<i>Artibeus watsoni</i>	0.63	0.75	1.06	21	9	2.33
		<i>Carollia perspicillata</i>	0.73	0.63	1.07	19	15	1.27
		<i>Carollia subrupha</i>	0.74	0.63	1.04	18	14	1.29
		<i>Chiroderma doriae</i>	0.72	0.79	1.12	26	13	2.00
		<i>Chiroderma villosum</i>	0.74	0.80	1.11	23	12	1.92
		<i>Rhinophylla fisherae</i>	0.69	0.67	1.09	20	14	1.43
		<i>Rhinophylla pumilio</i>	0.68	0.67	1.07	17	13	1.31
		<i>Sturnira lilium</i>	0.63	0.67	1.18	20	12	1.67
		<i>Sturnira tildae</i>	0.66	0.68	1.21	17	14	1.21
		<i>Vampyressa pusilla</i>	0.67	0.78	1.11	23	13	1.77
		<i>Vampyrodes caraccioli</i>	0.71	0.82	1.14	21	13	1.62
	Strict frugivory	<i>Pygoderma bilabiatum</i>	0.54	0.69	1.27	22	11	2.00
	Hematofagy	<i>Desmodus rotundus</i>	0.44	0.60	1.24	18	12	1.5
		<i>Diphylla ecaudata</i>	0.51	0.62	1.23	19	13	1.46
	Insectivory	<i>Glyphonicteris sylvestris</i>	0.81	0.64	1.03	20	15	1.33
	Nectarivory	<i>Anoura caudifer</i>	0.90	0.51	0.99	21	14	1.5
		<i>Anoura geoffroyi</i>	0.91	0.52	0.99	19	14	1.36
<i>Glossophaga commissarisi</i>		0.85	0.54	0.99	21	15	1.4	
<i>Glossophaga soricina</i>		0.86	0.54	0.99	20	14	1.43	
<i>Lonchophylla thomasi</i>		0.83	0.54	1.00	18	15	1.2	
Omnivory	<i>Lophostoma silviculum</i>	0.77	0.60	1.04	19	15	1.27	
	<i>Mimon benettii</i>	0.75	0.66	1.07	17	13	1.31	
	<i>Gardnerycteris crenulatum</i>	0.72	0.69	1.01	17	15	1.13	
	<i>Phyllostomus elongatus</i>	0.75	0.70	1.09	19	15	1.27	
Primates	NWM	<i>Leontopithecus rosalia</i>	0.77	0.52	1.26	23	17	1.35
		<i>Sapajus robustus</i>	0.80	0.42	1.40	21	16	1.31
		<i>Brachytheles arachnoides</i>	0.80	0.42	1.34	21	16	1.31
		<i>Callithrix hummelifera</i>	0.72	0.51	1.32	21	17	1.24
		<i>Callithrix pygmaea</i>	0.72	0.47	1.26	19	16	1.19
		<i>Callithrix geoffroyi</i>	0.74	0.49	1.29	21	17	1.24
	OWM	<i>Pan paniscus</i> ^a	0.73	0.51	1.33	16	17	0.94
		<i>Pan troglodites</i> ^b	0.75	0.51	1.29	25	17	1.47
		<i>Macaca fasciculari</i> ^a	0.88	0.49	1.32	24	17	1.41
		<i>Gorilla gorilla</i> ^b	0.75	0.46	1.25	22	17	1.29
		<i>Homo sapiens</i> ^b	0.60	0.47	1.26	23	17	1.35
		<i>Homo neanderthalensis</i> ^{c*}	0.63	0.49	1.38	23	17	1.35
		<i>Papio anubis</i> ^b	0.94	0.47	1.23	24	17	1.41

Table 1. Species examined in this study with craniofacial measurements and RUNX2 Q/A ratios. GM: geometric mean. ^aNCBI, ^bENSEMBL, ^cUCSC, *http://neandertal.ensemblgenomes.org/Homo_sapiens/Transcript/Summary?db=core;t=ENST00000359524.

Discussion

Frugivorous phyllostomid bats: a hotspot of variation. The Phyllostomidae family includes subfamilies that are recognised mainly by their feeding habits and associated morphological adaptations^{27,28,51}. Phytophagous phyllostomids (frugivorous and nectar feeding species) evolved in nested clades among ancestral animalivorous species, and previous studies have proposed that the insectivorous ancestor of all phyllostomids also fed on plants^{38,31,51,52}. In the present study, the largest variation in the Q/A ratios was found in frugivorous phyllostomids (Q/A ratio varying from 1.21 to 2.33; see Table 1). The feeding specialisation in the subfamily Stenodermatinae varies from primary frugivory (feed on fruits and complement diet with other items, e.g. *Artibeus*, *Carollia*, *Sturnira*,

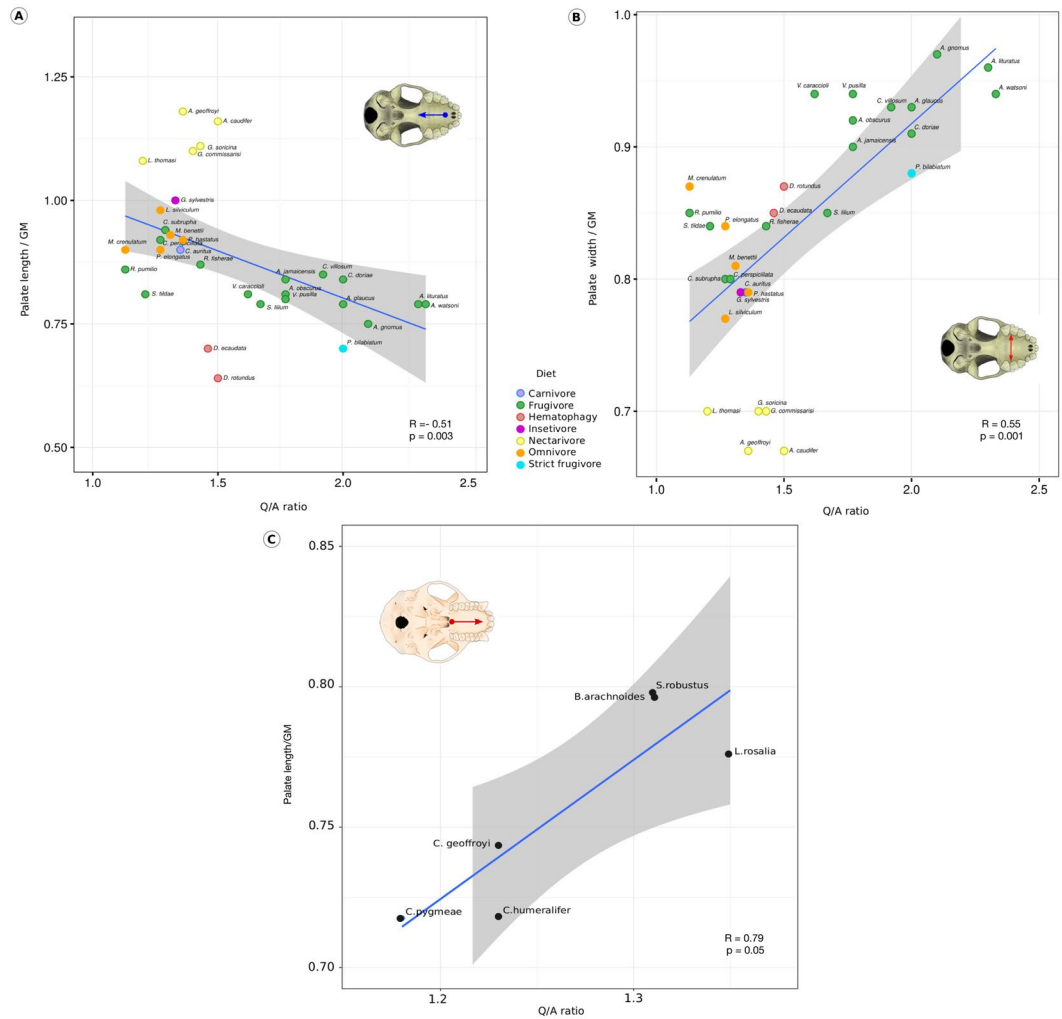


Figure 2. Correlations between Q/A ratios and palate lengths (A), phyllostomids; (C) New World monkeys and width (B), phyllostomids).

	PL	pp	PW	pp	ZW	pp
Phyllostomids vs. Q/A ratio	-0.226	0.13*	0.291	0.94*	0.004	0.51 ^{ns}
Primates vs. Q/A ratio	0.095	0.63 ^{ns}	-0.017	0.47 ^{ns}	-0.116	0.34 ^{ns}
NWM vs. Q/A ratio	0.583	0.93*	0.237	0.71 ^{ns}	0.275	0.73 ^{ns}
OWM vs. Q/A ratio	0.051	0.56 ^{ns}	-0.087	0.40 ^{ns}	-0.176	0.30 ^{ns}

Table 2. Bayesian coefficient of correlation (estimated with CoEvol program, see material and methods section for details) between craniofacial measures (PL, palate length; PW, palate width; ZW, zygomatic width) and Q/A ratio. Statistical significance is given by posterior probability (pp). *Statistically significant; ^{ns} Not significant.

Vampyroides, *Vampyressa*) to obligate frugivory (feed on hard fruits, e.g. *Pygoderma bilabiatum*). The origin of the Stenodermatinae clade is associated with a significant increase in the species diversification rate and a high bite performance skull^{27,53}. The anatomical form of the palate in frugivorous phyllostomids (broad structure⁵⁰) likely favoured a mechanical advantage for this group. When frugivorous vertebrates emerged ca. 25–38 Myr ago, among all other lineages of bats, only phyllostomids fed on fruits³¹. Thus, they explored a new adaptive zone that likely required remarkable cranial and dental adaptations. Sorensen *et al.*⁵⁰ suggested that the diverse palate shapes of phyllostomids are the result of subtle evolutionary changes in later developmental events.

Magnitude of skull integration in bats and primates: flexibility to evolve. In contrast to the remarkable stability observed in the patterns of covariation in the mammalian skull^{54–57} the magnitudes of inter-trait association, estimated by the average squared phenotypic correlation between traits (r^2), are highly variable⁵⁶. For the same cranial measurements, primates (chimpanzees, humans, and gorillas) and Chiroptera

Group	Model	r^2	Estimate	p	Pagel's lambda
Phyllostomid bats	Q/A ratio x PL	0.06	-0.09	0.09	1000
	Q/A ratio x PW	0.19	0.08	0.01	1000
	Q/A ratio x ZW	-0.03	0.02	0.72	1000
Primates	Q/A ratio x PL	-0.07	0.04	0.63	994
	Q/A ratio x PW	-0.09	-0.01	0.90	933
	Q/A ratio x ZW	-0.06	-0.06	0.59	0
Only NWM	Q/A ratio x PL	0.55	0.27	0.06	1000
	Q/A ratio x PW	-0.18	0.32	0.66	1000
	Q/A ratio x ZW	-0.06	0.31	0.44	0
Only OWM	Q/A ratio x PL	-0.18	0.04	0.77	1000
	Q/A ratio x PW	-0.11	-0.03	0.55	0
	Q/A ratio x ZW	-0.09	-0.09	0.51	0

Table 3. Results of phylogenetic generalized least squares (PGLS) models exploring the putative association between *RUNX2* Q/A ratios and relative palate-length and width in phyllostomid bats and primates, depicting the magnitude of phylogenetic signal (Pagel's lambda).

(*Molossus molossus*, *Myotis nigricans*, and *Artibeus lituratus* - Family Molossidae, Vespertilionidae, and Phyllostomidae, respectively) have r^2 values under 0.10, whereas Carnivora (*Cerdocyon thous*, *Leopardus*, *Eira*, *Nasua* - Family Canidae, Felidae, Mustelidae, and Procyonidae, respectively) display values on the order of 0.12–0.20, and marsupials have r^2 values of 0.30–0.50^{56,58}.

The high variability in the magnitude of inter-trait correlations has potential important consequences for the evolution of these clades⁵⁹. Higher magnitudes of skull integration are associated with constrained evolutionary responses to selection, which would reduce adaptive flexibility to evolve in directions other than those driven by the tight inter-trait associations, at least on short-term^{46,59,60}. Alternatively, lower magnitudes indicate that most traits are weakly connected and that there is more potential to evolve across several axes of variation. Species with lower magnitudes of trait association could evolve in more directions, therefore presenting less potential evolutionary constraint and greater evolutionary flexibility⁵⁹. Thus, the different integration magnitudes in phyllostomid bats in relation to the *RUNX2* gene (Fig. 3A–C) together with the probable differences in the reaction norms of this gene within this clade, could explain the differences in the genotype–phenotype relationships observed, as well as the contrasting patterns described for other lineages of mammals.

The mice genome informatics database (http://www.informatics.jax.org/vocab/mp_ontology/MP:0020039) lists several known skeleton phenotypes compiled so far and associated with *RUNX2* mutants. In this context, we could assume that delayed or increased bone ossification (both endochondral and intramembranous) might explain the opposed association of *RUNX2* variation and/or integration and facial forms we observe in different groups of mammals. By affecting bone ossification the locus potentially can impact allometric size variation within population, which in turn would usually impact mostly facial traits due to its late development (see^{61,62} for craniofacial development in mammals). Consequently it would lead to an increase or decrease in correlation among facial traits and thus to a larger or smaller morphological integration. Thus, the strong positive correlation found between evolutionary flexibility index and the *RUNX2* gene (Fig. 3B) suggests a potential association between the high potential ability for craniofacial traits evolution in phyllostomid bats and *RUNX2* variation. The increased ability to promote bone tissues formation might have played a fundamental role during transitions to feeding specializations, thus facilitating evolutionary change. Finally, since the ratio of glutamine to alanine residues in the *RUNX2* protein might influence the regulation of bone development, our results indicate that Q/A ratio can act as a key feature for natural selection to operate in these species.

Long, short, or broad faces: what is the impact of *RUNX2*? Our results clearly showed contrasting patterns of association between tandem repeats of *RUNX2* Q/A and palate shape in the highly diverse groups of phyllostomid bats and primates (Tables 1, 2 and Fig. 2). Until recently, studies on marsupials and placental mammals, including domestic dog breeds, evidenced two contrasting patterns: i) a correlation of Q/A ratios and face lengths¹⁰; or ii) a non-association of these traits^{11,12}. Sears *et al.*¹⁰ suggested a pattern of increase in face length of carnivores due to a higher Q/A ratio. In contrast, the results obtained in our study point to a reduction in length and a widening of the maxilla, which would lead to shorter and broader faces in phyllostomid bats. However, in NWM, the result is consistent with that described for the order Carnivora. Overall, our results support the main idea proposed by Sears *et al.*¹⁰, reinforcing the role of the Q/A ratio as a flexible genetic mechanism that would rapidly change the time of ossification of the skull throughout development.

In this context, the findings of the present study propose a complex scenario, where the permissiveness of this genetic adjustment system created by the variability in the Q/A ratios of the *RUNX2* gene is indirect, and does not lead to a specific phenotype. However, throughout the history of a lineage (at the species or family level), *RUNX2* variability acts with external factors and evolutionary constraints, together with other genes, as a facilitator for the adaptive morphological changes currently found. In the case of phyllostomids, these changes seem to have been selected by dietary constraints, corroborating the phenotype–environment correlation^{27,32,33,41,63}. Overall, it should be noted that many genetic, epigenetic, and environmental attributes modulates the expression of genetic variance and interact with developmental networks to produce the available phenotypic variation. Therefore, the

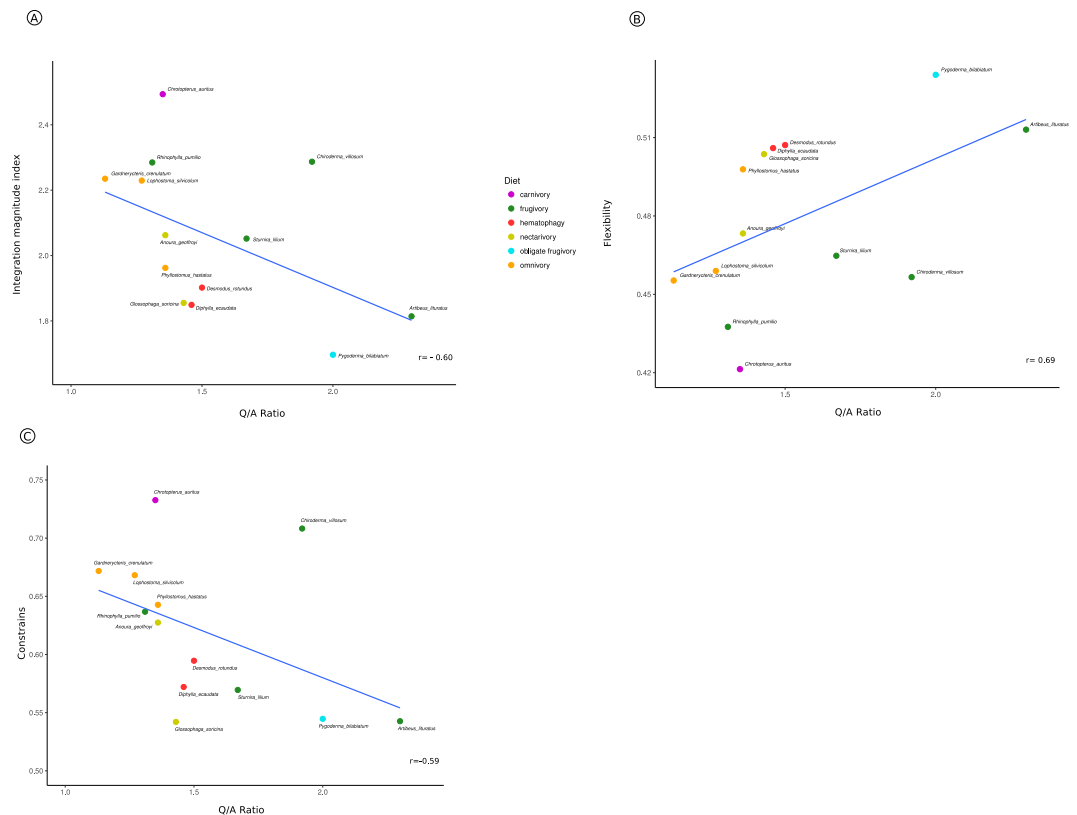


Figure 3. (A) Plot of the overall integration magnitude (ICV) and Q/A ratio for phyllostomid bats. (B) Plot of the evolutionary flexibility index and Q/A ratio for phyllostomid bats. (C) Plot of the evolutionary constraints index and Q/A ratio for phyllostomid bats.

results of the present study seem to agree with Alberch's⁶⁴ view on the conceptualisation of genotype-phenotype association, i.e. genes do not directly specify the development or the shape of an organism; they are only one of the several causal factors that jointly determine the phenotype. Thus, developmental events are able to ambiguously affecting, or be affected, by gene expression.

Material and Methods

RUNX2 sequence data. Samples from 31 phyllostomid bat species from a wide range of feeding habits and craniofacial morphologies were surveyed in this study (electronic supplementary material, Table S1). Tissues and skulls individually paired are deposited in the Department of Zoology collection at the Fundação Universidade Regional de Blumenau (FURB). Additionally, six New World primate species (*Leontopithecus rosalia*, *Spajus robustus*, *Brachytheles arachnoides*, *Callithrix hummelifera*, *Callithrix pygmaea*, *Callithrix geoffroyi*), from the Laboratório de Evolução Molecular of the Departamento de Genética, Universidade Federal do Rio Grande do Sul, were used. Five species of Catarrhini (*Pan paniscus*, *Papio anubis*, *Gorilla gorilla*, *Pan troglodytes*, and *Macaca fascicularis*) were considered using the ENSEMBL (www.ensembl.org, release 86, [A. Yates]) and NCBI (<http://www.ncbi.nlm.nih.gov/genbank/> [Benson]) genomic databases.

Additionally, UCSC (<https://genome.ucsc.edu/index.html>) and Neandertal Ensembl (http://neandertal.ensemblgenomes.org/data_info.htm) genomes were accessed to obtain modern human and Neandertal data. The DNeasy Blood & Tissue Kit (Qiagen, Germantown, MD, USA) was used to extract and isolate genomic DNA from muscle samples for the 37 species. The polymerase chain reaction (PCR) was carried out to amplify the target *RUNX2* exon, using primers and conditions described by Pointer *et al.*¹¹, standardising the PCR conditions for the different species surveyed. Shrimp alkaline phosphatase and exonuclease I enzymes (GE Healthcare, Chicago, IL, USA) were used to purify the PCR products. Amplicons were sequenced in an ABI3730xl (Applied Biosystems Inc., Foster City, CA, USA) sequencer, using the same primers employed in the PCR for both DNA strands. Sequences were analysed using the ClustalW algorithm, implemented in the Codon Code Aligner software (CodonCode Corporation, Dedham, MA, USA)⁶⁵. A prior screening test was performed, which considered only the sequences presenting conserved regions flanking the Q/A motif. These regions were then used as reference markers for the Q/A motif. The Q/A ratio for each specimen was calculated by dividing the number of repeated glutamine residues by the number of repeated alanine residues in tandem^{10,11} (Table 1).

Samples and linear measurements. We measured 31 phyllostomid skulls (the same specimens sequenced for *RUNX2*) using a digital calliper, and obtained the primate data set employing a combination of craniometric data recorded by Marroig and Cheverud⁶⁶ and Oliveira *et al.*⁵⁴ using a Microscribe 3DMX. From

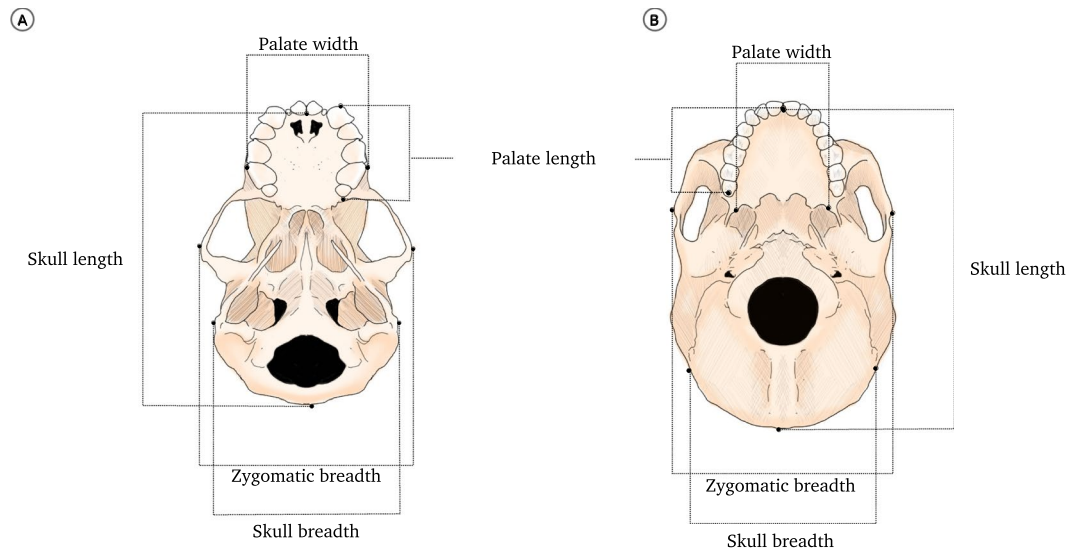


Figure 4. Craniofacial measurements recorded in this study. (A) Ventral view of the phyllostomid bat skull. (B) Ventral view of a primate skull. See also the electronic supplementary material, Table S2, for detailed descriptions of the measurements used in this study.

these data, we obtained five linear measurements from the ventral crania of all specimens: palate length, palate width, skull length, skull width, and zygomatic breadth (Fig. 4, see also the electronic supplementary material, Table S2, for detailed measurement descriptions). These linear measurements were chosen to reflect variations in skull form among species and to represent the dimensions of palate width and length both in phyllostomid bats and primates^{54,63,66,67}.

Additionally, evolutionary alterations in palate morphology are thought to underlie much of the feeding specialisations in bats⁵⁰. We calculated the geometric mean for all measurements and standardised the palate length and width for each specimen by dividing each measurement by the geometric mean (GM) (Table 1). Only skulls from adult specimens were measured. For both primates and bats, specimens were considered adults when the basisphenoid and basioccipital joint was completely fused.

Statistical analysis. In an initial set of analysis, we performed Spearman's correlation test to investigate the association between *RUNX2* tandem repeat ratios and palate length and width. Additionally to this traditional statistical method, a Bayesian phylogenetic comparative method (PCM) approach was implemented in CoEvol 1.4 software⁶⁸ to investigate the correlation between *RUNX2* Q/A ratio and palate length and width, taking into account phylogenetic relationships, evolutionary rates, and divergence time under a Brownian motion model of evolution. In the CoEvol results, a posterior probability (pp) close to 1 represents a strong statistical support for a positive correlation, and a pp close to 0 indicates a supported negative correlation. Two data sub-sets for each taxonomic group (bats and primates) were analysed: I, morphometric measures for each species, corrected by geometric mean of all measures and II, phylogenetic relationships estimated from neutral markers (i.e., mitochondrial genes obtained from public databases). We ran CoEvol for 30,000 MCMC (total number of cycles) and considered the analysis finished when all effective sample size values reached at least 300⁶⁸.

Finally, we performed a phylogenetic generalised linear model (PGLS) using the R function (pgls) of the “caper” R package⁶⁹ to investigate the magnitude of phylogenetic signal associated with phenotypic (palate length and width - dependent variables) and genotypic (Q/A region - independent variable) changes. This function fits a linear model controlling for phylogenetic non-independence between data. We also used this parameter for comparative analyses with the results of previous studies on carnivores¹⁰ and other placental mammals¹¹. We assume that all the three analytical methods employed in our study are integrative and represents different levels of complexity, ranging from a more general exploratory analysis (Spearman's correlation) to a more complex phylogenetic comparative methods (PGLS and Bayesian).

Q/A ratios and morphological integration index. The magnitudes of integration among traits were obtained by using the coefficient of variation of the eigenvalues (ICV⁷⁰). This index was calculated for primates^{56,59} and phyllostomids (Rossoni et al. *manuscript in preparation*) as the standard deviation of the eigenvalues ($\sigma_{(\lambda)}$) divided by the average of the eigenvalues (λ). The flexibility and constraints index were also provided from the author's cited above. The evolutionary flexibility index captures the ability of a species to respond in direction of selection, while the evolutionary constraints index captures the relative influence of the line of least resistance (first principal component) on the direction of the evolutionary responses⁵⁹. These measurements are presented in the electronic supplementary material, Table S3 (see also^{58,59,70} for a detailed description of these index estimates). We performed Spearman's correlation test in order to investigate the association between *RUNX2* tandem repeat ratios and ICV, flexibility and constraints index.

References

- Abzhanov, A. Bmp4 and Morphological Variation of Beaks in Darwin's Finches. *Science* **305**, 1462–1465 (2004).
- Abzhanov, A. *et al.* The calmodulin pathway and evolution of elongated beak morphology in Darwin's finches. *Nature* **442**, 563–567 (2006).
- Martínez-Abadías, N. *et al.* FGF/FGFR Signaling Coordinates Skull Development by Modulating Magnitude of Morphological Integration: Evidence from Apert Syndrome Mouse Models. *PLoS ONE* **6**, e26425 (2011).
- Sears, K. E. Quantifying the impact of development on phenotypic variation and evolution. *J. Exp. Zool. B Mol. Dev. Evol.* **322**, 643–653 (2014).
- Orr, D. J. A., Teeling, E. C., Puechmaille, S. J. & Finarelli, J. A. Patterns of orofacial clefting in the facial morphology of bats: a possible naturally occurring model of cleft palate. *J. Anat.* **229**, 657–672 (2016).
- Futuyma, D. J. *Evolutionary biology*. (Sinauer Associates, 1998).
- Popadic, A., Abzhanov, A., Rusch, D. & Kaufman, T. C. Understanding the genetic basis of morphological evolution: the role of homeotic genes in the diversification of the arthropod bauplan. *Int. J. Dev. Biol.* **42**, 453–461 (1998).
- Hoekstra, H. E. Genetics, development and evolution of adaptive pigmentation in vertebrates. *Heredity* **97**, 222–234 (2006).
- Manceau, M., Domingues, V. S., Linnen, C. R., Rosenblum, E. B. & Hoekstra, H. E. Convergence in pigmentation at multiple levels: mutations, genes and function. *Philos. Trans. R. Soc. B Biol. Sci.* **365**, 2439–2450 (2010).
- Sears, K. E., Goswami, A., Flynn, J. J. & Niswander, L. A. The correlated evolution of Runx2 tandem repeats, transcriptional activity, and facial length in carnivora. *Evol. Dev.* **9**, 555–565 (2007).
- Pointer, M. A. *et al.* RUNX2 tandem repeats and the evolution of facial length in placental mammals. *BMC Evol. Biol.* **12**, 103 (2012).
- Newton, A. H., Feigin, C. Y. & Pask, A. J. RUNX2 repeat variation does not drive craniofacial diversity in marsupials. *BMC Evol. Biol.* **17**, (2017).
- Komori, T. *et al.* Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *cell* **89**, 755–764 (1997).
- Javed, A. *et al.* runt Homology Domain Transcription Factors (Runx, Cbfa, and AML) Mediate Repression of the Bone Sialoprotein Promoter: Evidence for Promoter Context-Dependent Activity of Cbfa Proteins. *Mol. Cell. Biol.* **21**, 2891–2905 (2001).
- Levanon, D. & Groner, Y. Structure and regulated expression of mammalian RUNX genes. *Oncogene* **23**, 4211–4219 (2004).
- Ball, H. C. *et al.* Methods and insights from the characterization of osteoprogenitor cells of bats (Mammalia: Chiroptera). *Stem Cell Res.* **17**, 54–61 (2016).
- Ziros, P. G., Basdra, E. K. & Papavassiliou, A. G. Runx2: of bone and stretch. *Int. J. Biochem. Cell Biol.* **40**, 1659–1663 (2008).
- Schroeder, T. M., Jensen, E. D. & Westendorf, J. J. Runx2: A master organizer of gene transcription in developing and maturing osteoblasts. *Birth Defects Res. Part C Embryo Today Rev.* **75**, 213–225 (2005).
- Mundlos, S. *et al.* Mutations involving the transcription factor CBFA1 cause cleidocranial dysplasia. *Cell* **89**, 773–779 (1997).
- Otto, F., Kanegane, H. & Mundlos, S. Mutations in the RUNX2 gene in patients with cleidocranial dysplasia. *Hum. Mutat.* **19**, 209–216 (2002).
- Napierala, D. *et al.* Mutations and promoter SNPs in RUNX2, a transcriptional regulator of bone formation. *Mol. Genet. Metab.* **86**, 257–268 (2005).
- Hansen, L. *et al.* RUNX2 analysis of Danish cleidocranial dysplasia families. *Clin. Genet.* **79**, 254–263 (2011).
- Jaruga, A., Hordyjewska, E., Kandzierski, G. & Tylzanowski, P. Cleidocranial dysplasia and RUNX2-clinical phenotype-genotype correlation: Cleidocranial dysplasia and RUNX2. *Clin. Genet.* **90**, 393–402 (2016).
- Shibata, S., Suda, N., Suzuki, S., Fukuoka, H. & Yamashita, Y. An *in situ* hybridization study of Runx2, Osterix, and Sox9 at the onset of condylar cartilage formation in fetal mouse mandible. *J. Anat.* **208**, 169–177 (2006).
- Fondon, J. W. & Garner, H. R. Molecular origins of rapid and continuous morphological evolution. *Proc. Natl. Acad. Sci.* **101**, 18058–18063 (2004).
- Rojas, D., Warsi, O. M. & Dávalos, L. M. Bats (Chiroptera: Noctilionoidea) Challenge a Recent Origin of Extant Neotropical Diversity. *Syst. Biol.* **65**, 432–448 (2016).
- Dumont, E. R. *et al.* Morphological innovation, diversification and invasion of a new adaptive zone. *Proc. R. Soc. B Biol. Sci.* **279**, 1797–1805 (2012).
- Baker, R. J., Bininda-Emonds, O. R. P., Mantilla-Meluk, H., Porter, C. A. & Van Den Bussche, R. A. Molecular time scale of diversification of feeding strategy and morphology in New World Leaf-Nosed Bats (Phyllostomidae): a phylogenetic perspective. In *Evolutionary History of Bats* (eds Gunnell, G. F. & Simmons, N. B.) 385–409 (Cambridge University Press, 2012).
- Santana, S. E., Geipel, I., Dumont, E. R., Kalka, M. B. & Kalko, E. K. V. All You Can Eat: High Performance Capacity and Plasticity in the Common Big-Eared Bat, *Micronycteris microtis* (Chiroptera: Phyllostomidae). *PLoS ONE* **6**, e28584 (2011).
- Phillips, C. D., Butler, B., Fondon, J. W., Mantilla-Meluk, H. & Baker, R. J. Contrasting Evolutionary Dynamics of the Developmental Regulator PAX9, among Bats, with Evidence for a Novel Post-Transcriptional Regulatory Mechanism. *PLoS ONE* **8**, e57649 (2013).
- Freeman, P. W. Macroevolution in Microchiroptera: recoupling morphology and ecology with phylogeny. *Evol. Ecol. Res.* **2**, 317–335 (2000).
- Freeman, P. W. Form, function, and evolution in skulls and teeth of bats. (1998).
- Santana, S. E., Grosse, I. R. & Dumont, E. R. Dietary hardness, loading behavior, and the evolution of skull form in bats. *Evolution* **66**, 2587–2598 (2012).
- Santana, S. E., Dumont, E. R. & Davis, J. L. Mechanics of bite force production and its relationship to diet in bats: Bite force and diet in bats. *Funct. Ecol.* **24**, 776–784 (2010).
- Santana, S. E. & Cheung, E. Go big or go fish: morphological specializations in carnivorous bats. *Proc. R. Soc. B Biol. Sci.* **283**, 20160615 (2016).
- Santana, S. E., Strait, S. & Dumont, E. R. The better to eat you with: functional correlates of tooth structure in bats: Functional correlates of tooth structure in bats. *Funct. Ecol.* **25**, 839–847 (2011).
- Dumont, E. R. The effect of food hardness on feeding behaviour in frugivorous bats (Phyllostomidae): an experimental study. *J. Zool.* **248**, 219–229 (1999).
- Dumont, E. R. The effects of gape angle and bite point on bite force in bats. *J. Exp. Biol.* **206**, 2117–2123 (2003).
- Monteiro, L. R. & Nogueira, M. R. Adaptive radiations, ecological specialization, and the evolutionary integration of complex morphological structures. *Evolution* **64**, 724–744 (2010).
- Monteiro, L. R. & Nogueira, M. R. Evolutionary patterns and processes in the radiation of phyllostomid bats. *BMC Evol. Biol.* **11**, 1 (2011).
- Nogueira, M. R., Peracchi, A. L. & Monteiro, L. R. Morphological correlates of bite force and diet in the skull and mandible of phyllostomid bats. *Funct. Ecol.* **23**, 715–723 (2009).
- Nogueira, M. R., Monteiro, L. R., Peracchi, A. L. & de Araújo, A. F. B. Ecomorphological analysis of the masticatory apparatus in the seed-eating bats, genus *Chiroderma* (Chiroptera: Phyllostomidae). *J. Zool.* **266**, 355–364 (2005).
- Schrage, C. G. Timing the Origin of New World Monkeys. *Mol. Biol. Evol.* **20**, 1620–1625 (2003).
- Fleagle, J. G. *Primate adaptation and evolution*. (Elsevier - AP, 2013).
- Marroig, G. & Cheverud, J. M. Did natural selection or genetic drift produce the cranial diversification of neotropical monkeys? *Am. Nat.* **163**, 417–428 (2004).

46. Marroig, G., Cheverud, J. M. & Wainwright, P. Size as a line of least evolutionary resistance: diet and adaptive morphological radiation in New World monkeys. *Evolution* **59**, 1128–1142 (2005).
47. Green, R. E. *et al.* A draft sequence of the Neandertal genome. *science* **328**, 710–722 (2010).
48. Lindskog, C. *et al.* Analysis of Candidate Genes for Lineage-Specific Expression Changes in Humans and Primates. *J. Proteome Res.* **13**, 3596–3606 (2014).
49. Adhikari, K. *et al.* A genome-wide association scan implicates DCHS2, RUNX2, GLI3, PAX1 and EDAR in human facial variation. *Nat. Commun.* **7**, 11616 (2016).
50. Sorensen, D. W. *et al.* Palate Variation and Evolution in New World Leaf-Nosed and Old World Fruit Bats (Order Chiroptera). *Evol. Biol.* **41**, 595–605 (2014).
51. Rojas, D. Vale, Ángel, Ferrero, V. & Navarro, L. When did plants become important to leaf-nosed bats? Diversification of feeding habits in the family Phyllostomidae: Evolution of feeding habits in phyllostomid bats. *Mol. Ecol.* **20**, 2217–2228 (2011).
52. Ferrarezi, H. & Gimenez, E. Systematic patterns and the evolution of feeding habits in Chiroptera (Archonta: Mammalia). *J. Comp. Biol.* **1**, 75–94 (1996).
53. Shi, J. J. & Rabosky, D. L. Speciation dynamics during the global radiation of extant bats: Bat speciation dynamics. *Evolution* **69**, 1528–1545 (2015).
54. De Oliveira, F. B., Porto, A. & Marroig, G. Covariance structure in the skull of Catarrhini: a case of pattern stasis and magnitude evolution. *J. Hum. Evol.* **56**, 417–430 (2009).
55. Goswami, A. Cranial Modularity Shifts during Mammalian Evolution. *Am. Nat.* **168**, 270–280 (2006).
56. Porto, A., Oliveira, F. B., Shirai, L. T., de Conto, V. & Marroig, G. The evolution of modularity in the mammalian skull I: morphological integration patterns and magnitudes. *Evol. Biol.* **36**, 118–135 (2009).
57. Goswami, A., Smaers, J. B., Soligo, C. & Polly, P. D. The macroevolutionary consequences of phenotypic integration: from development to deep time. *Philos. Trans. R. Soc. B Biol. Sci.* **369**, 20130254–20130254 (2014).
58. Porto, A., Shirai, L. T., de Oliveira, F. B. & Marroig, G. Size Variation, Growth Strategies, and the Evolution of Modularity in the Mammalian Skull. *Evolution* **67**, 3305–3322 (2013).
59. Marroig, G., Shirai, L. T., Porto, A., de Oliveira, F. B. & De Conto, V. The Evolution of Modularity in the Mammalian Skull II: Evolutionary Consequences. *Evol. Biol.* **36**, 136–148 (2009).
60. Steppan, S. J., Phillips, P. C. & Houle, D. Comparative quantitative genetics: evolution of the G matrix. *Trends Ecol. Evol.* **17**, 320–327 (2002).
61. Smith, K. K. Comparative Patterns of Craniofacial Development in Eutherian and Metatherian Mammals. *Evolution* **51**, 1663 (1997).
62. Koyabu, D. *et al.* Mammalian skull heterochrony reveals modular evolution and a link between cranial development and brain size. *Nat. Commun.* **5**, (2014).
63. Dumont, E. R. *et al.* Selection for Mechanical Advantage Underlies Multiple Cranial Optima in New World Leaf-Nosed Bats. *Evolution* **68-5**, 1436–1449 (2014).
64. Alberch, P. From genes to phenotype: dynamical systems and evolvability. *Genetica* **84**, 5–11 (1991).
65. Thompson, J. D., Higgins, D. G. & Gibson, T. J. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**, 4673–4680 (1994).
66. Marroig, G. & Cheverud, J. M. A comparison of phenotypic variation and covariation patterns and the role of phylogeny, ecology, and ontogeny during cranial evolution of New World monkeys. *Evolution* **55**, 2576–2600 (2001).
67. Dumont, E. R. Patterns of Diversity in Cranial Shape Among Plant-Visiting Bats. *Acta Chiropterologica* **6**, 59–74 (2004).
68. Lartillot, N. & Poujol, R. A Phylogenetic Model for Investigating Correlated Evolution of Substitution Rates and Continuous Phenotypic Characters. *Mol. Biol. Evol.* **28**, 729–744 (2011).
69. Freckleton, R. P., Harvey, P. H. & Pagel, M. Phylogenetic Analysis and Comparative Data: A Test and Review of Evidence. *Am. Nat.* **160**, 712–726 (2002).
70. Shirai, L. T. & Marroig, G. Skull modularity in neotropical marsupials and monkeys: size variation and evolutionary constraint and flexibility. *J. Exp. Zool. B Mol. Dev. Evol.* **314B**, 663–683 (2010).

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Author Contributions

G.L.S. and T.H. designed the study; T.F., D.M.R., R.G.J. and G.M. collected the morphological data; T.F., D.M.R., G.L.S. and T.H. performed the analysis; S.L.A., A.P., V.R.P.C. and M.C.B. contributed reagents/materials; T.F., D.M.R., F.M.S. G.L.S. and T.H. wrote the main manuscript.

Additional Information

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