

Phylogenetic analysis of landmark data and the morphological evolution of cranial shape and diets in species of *Myotis* (Chiroptera: Vespertilionidae)

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Abstract Species in genus *Myotis* exhibit a pattern of cranial variation associated with insectivorous, facultative piscivorous, and truly piscivorous diets, which has not been studied in a phylogenetic context. Variation in landmark configurations of five cranial structures in 22 *Myotis* species was analyzed with phylogenetic methods to infer evolution of shape. Our goals were to detect changes in cranial morphology and to correlate these with concerted changes among diets. A reference phylogeny was estimated using a combined data matrix with previously available Cyt-b and RAG2 sequences and our five configurations of landmarks. We included the insectivorous *Kerivoula papillosa* Temminck, 1840, *Noctilio leporinus* Linnaeus, 1758 (piscivorous), and *N. albiventris* Desmarest, 1818 (insectivorous) as out-groups. The optimization of five landmark configurations on the combined phylogeny shows no evidence of convergent shape changes among species with similar piscivorous diets. Our findings document that facultative piscivory does not imply the same particular morphotype. In four cranial features, there is small shape change between estimated ancestral shapes and seven observed descendant shapes for the piscivorous species. Only the mandible

shows major changes from insectivorous ancestors to facultative piscivorous or piscivorous *Myotis*.

Keywords Piscivory · Geometric morphometrics · *Myotis* · Phylogenetic morphometrics

Introduction

Patterns of cranial morphological evolution in bats have recurrently been explained by diversification in feeding habits (Swartz et al. 2003; Monteiro and Nogueira 2009; Nogueira et al. 2009; Dumont et al. 2012). The specializations to certain diets have generated functional demands that apparently determine cranial shape evolution (Freeman 1981, 1984; Swartz et al. 2003; Nogueira et al. 2005, 2009). For example, the evolution of a skull phenotype in the most ecologically diverse Phyllostomidae has been explained in relation to the ecological opportunity for the species to incorporate new resources (fruits, nectar, etc.) in its diet and the move to new adaptive zones. This family includes insectivorous, frugivorous, nectarivorous, sanguinivorous, and carnivorous species (Dumont et al. 2012). Adaptive radiation as an explanation of ecological and species diversification has also been suggested in other families of insectivorous bats (Swartz et al. 2003; Lewis-Orrit et al. 2001) and in the origin of piscivory in species of genus *Myotis* (Ruedi and Mayer 2001). Piscivory in bats appeared in parallel also in *Noctilio leporinus* (Noctilionidae).

The genus *Myotis* is the most diverse (Ruedi and Mayer 2001; Stadelmann et al. 2004) with 105 species distributed worldwide covering varied habitats and foraging strategies, including several facultatively piscivorous species (*M. ricketti* Thomas, 1894, *M. daubentonii* Huhl, 1817, *M. capaccinii* Bonaparte, 1837, *M. adversus* Horsfield, 1824,

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M. macrotarsus Waterhouse, 1845, *M. albescens* Geoffroy, 1806, and *M. stalkerii* Thomas, 1910), and one species, *M. vivesi* Ménègaux, 1901 with a truly piscivorous diet (Blood and Clark 1998). Facultative species of *Myotis* are mainly insectivores but occasionally feed on fish (Whitaker and Findley 1980; Flannery 1995; Law and Urquhart 2000; Siemers et al. 2001; Aihartza et al. 2008). Facultative piscivorous species were initially grouped in the same subgenus due to similar external characteristics associated with their common diet, for example the laterally compressed and large claws used to catch prey on the water surfaces (Findley 1972; Norberg and Rayner 1987; Lewis-Oritt et al. 2001). However, a phylogenetic analysis showed that the ecomorph of facultative piscivorous species was not monophyletic (Ruedi and Mayer 2001; Stadelmann et al. 2004). Diet has been related to foraging strategy in *Myotis* species. For example, bats that glean over for prey are more selective than aerial-hawking species (Burles et al. 2008); likewise trawling species catch fish on water (Stadelmann et al. 2004). Members of the genus *Myotis* are thus an excellent model to examine correlated changes in the morphological characters associated with the evolution of piscivory from insectivorous ancestors (Lewis-Oritt et al. 2001). However, patterns of cranial and mandibular morphological evolution in species of *Myotis* remain unexplored. In this paper, we test an ecological question in an evolutionary context, using landmark data to map shape changes along a phylogeny. Inferences of morphological ancestral states and directionality of change are important in providing the phylogenetic pattern that can be further analyzed for the associated functional utility as required in hypotheses of adaptation (Grandcolas et al. 2004).

In the present paper, we document how cranial shape has changed in relation to the diversification of three diets within species of *Myotis* (Vespertilionidae). Our goals were to address the following two specific questions: (1) are there shared changes in cranial morphology among species with the same diet? and (2) how has cranial morphology changed from insectivorous ancestors to piscivorous and facultative piscivorous species? We test the hypothesis that changes in cranial morphology would be similar between species with the same diet. This study uses a phylogenetic approach to analyze shape changes in our newly generated landmark data from five structures of cranial morphologies. We investigated a sample of 22 bat species of the genus *Myotis* as exemplars of all subgeneric groups (Findley 1972; Ruedi et al. 2013) and three different ecomorphs and diets. In addition, we included *Kerivoula papillosa*, an insectivorous species, and two species of genus *Noctilio* as out-groups, one of them being piscivorous (*N. leporinus*) and the other insectivorous (*N. albiventris*). By optimizing diets and five geometric morphometric shape characters on a combined molecular and morphometric phylogeny, we

reconstructed ancestral landmark configurations, which were used to make inferences about the evolution of skull and jaw shapes in relation to diet.

Materials and methods

Molecular data

Twenty-five DNA sequences of two molecular markers were obtained from GenBank (Supplementary material 1). We used 1140 bp of complete Cyt-b with ATG as a start codon and AGA as stop codon (Ruedi and Mayer 2001; Stadelmann et al. 2004). We also included a fragment of 1054 bp from a recombination activating protein gene (RAG2) from a recent phylogenetic study for *Myotis* (Ruedi et al. 2013). We aligned Cyt-b and RAG2 sequences with the multiple sequence alignment progressive method (FFT-NS-2) with the usual parameters (scoring matrix for nucleotide sequences 200PAM/K=2 and gap opening penalty: 1.53) operating in the program MAFFT 7 (Katoh et al. 2002). These sequences in combination with landmark data were used to build a reference phylogeny for optimization of shape characters and diets.

Morphometric data

Five different landmark configurations (shape characters) were defined to describe the cranial morphology in a subsample of 25 species (Supplementary material 1) included in the molecular study by Ruedi et al. (2013) (Fig. 1). These 2D landmark data came from a total of 421 specimens (Table 1) deposited in five mammal collections (Supplementary material 2). Specimen identifications on labels were corroborated (LaVal 1973; López-González 2001; Dietz and Von Helversen 2004; Simmons 2005; Larsen et al. 2012). We selected specimens from the same biogeographic region and we checked distributions following Simmons (2005). Digital photographs of lateral and ventral views of skulls, and lateral views of mandibles were acquired using a reflex camera Nikon D3100 with a DX Nikkor 18–55 mm lens. Small specimens were photographed with a Leica Z16 APOA “Macroscope” modular zoom system at the bioinformatics unit (Instituto de Biología, UNAM).

Two-dimensional landmark coordinates from images were obtained with the program TpsDig 2.16 (Rohlf 2008). Five configurations were registered separately, four of them correspond to cranial developmental regions in mammals, and are also of biomechanical relevance in diet differences (Porto et al. 2009). The ventral view of skull contains portions from other skull modules; it was analyzed as a separate unit. Lateral views of skulls (411 photographs) were

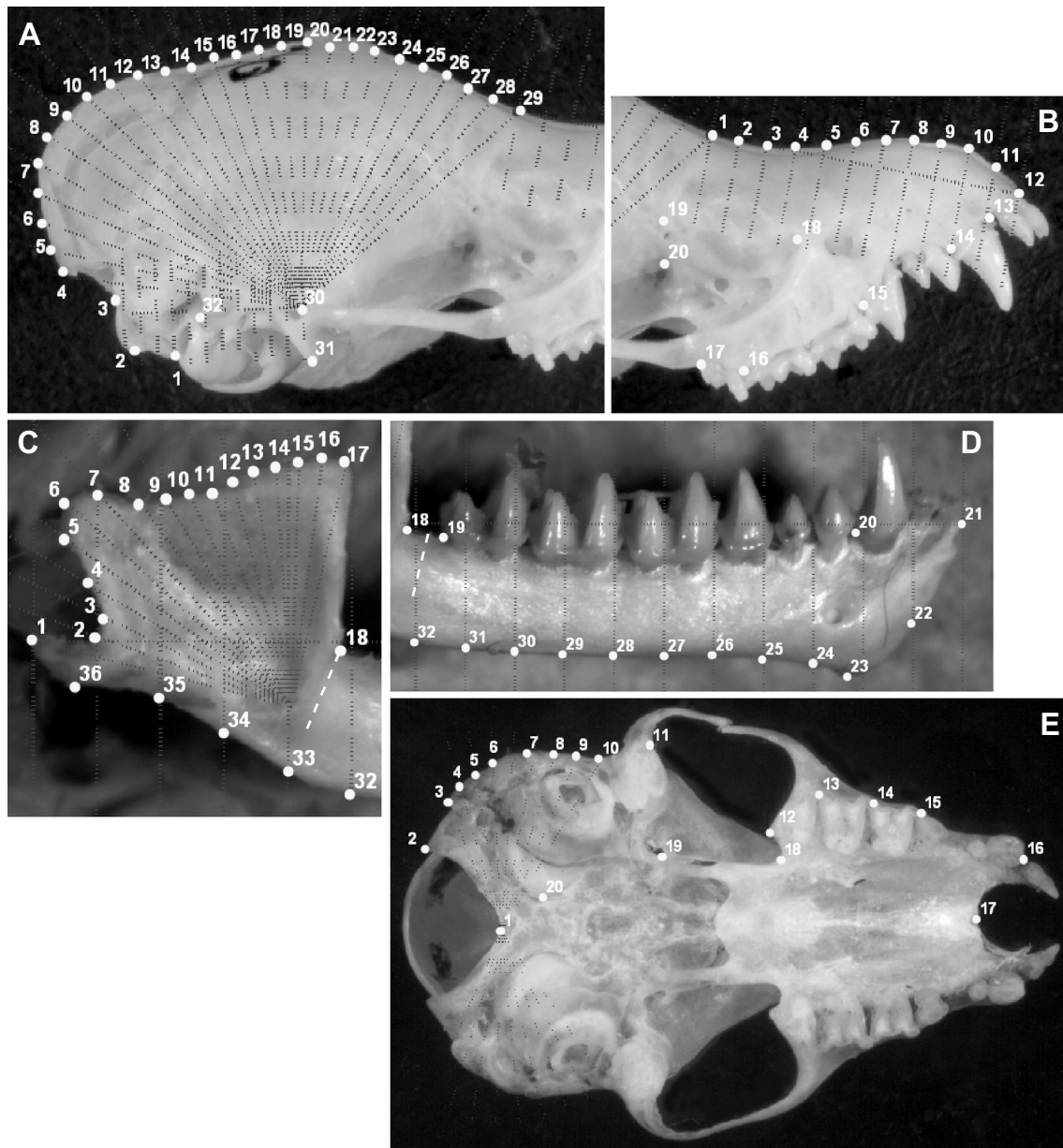


Fig. 1 Five configurations of points devised to register the shape of five cranial characters in *Myotis*. **a** Character 1, parieto-occipital region with 32 points, 1–32 lateral view; **b** character 2, frontal-maxillary region with 20 points, 1–20 lateral view; **c** character 3, mandible

process area with 22 points, 1–18, 33–36 lateral view; **d** character 4, dental row with 14 points, 19–32, white dashed line connects the initial and last points, lateral view; **e** character 5, skull with 20 points, 1–20 ventral view

divided into two structures (Fig. 1a, b). The first data set comprises the parieto-occipital region and it was registered with a set of 8 landmarks and 24 semi-landmarks (Fig. 1a). The second configuration is the frontal-maxillary region; it was registered with a collection of ten landmarks and ten semi-landmarks (Fig. 1b). The mandibles were divided into two regions in 421 photographs of lateral views. The third configuration is the processes region, which comprises three masticatory processes where muscles are inserted (angular, condylar, and coronoid), and it was registered

with 4 landmarks and 18 semi-landmarks (Fig. 1c). The fourth structure is the alveolar region; it was registered with a configuration of 4 landmarks and 10 semi-landmarks (Fig. 1d). The fifth configuration corresponds to the ventral view of the skulls; we registered a set of 13 landmarks and 7 semi-landmarks on 407 photographs (Fig. 1e).

Statistical morphometric analyses were conducted with the IMP program series (Sheets 2014). Procrustes coordinates were extracted from five alignments of configurations for the 25 species pooled together (Supplementary

Table 1 Number of specimens for the 25 species included in morphometric and phylogenetic analyses

Species	Skull lateral view (modules 1 and 2)	Mandible (modules 3 and 4)	Skull ventral view (module 5)
<i>Myotis adversus</i>	17	18	18
<i>M. albescens</i>	16	17	17
<i>M. bechsteinii</i>	8	8	7
<i>M. bocagii</i>	10	16	14
<i>M. capaccinii</i>	20	24	24
<i>M. dasycneme</i>	5	5	5
<i>M. daubentonii</i>	17	14	18
<i>M. dominicensis</i>	6	6	5
<i>M. emarginatus</i>	7	7	5
<i>M. evotis</i>	25	24	24
<i>M. brandtii gracilis</i>	3	3	3
<i>M. horsfieldii</i>	25	26	26
<i>M. keaysi</i>	25	23	23
<i>M. leibii</i>	20	21	19
<i>M. macrotarsus</i>	13	13	14
<i>M. myotis</i>	20	20	18
<i>M. nattereri</i>	13	13	13
<i>M. nigricans</i>	28	28	28
<i>M. riparius</i>	28	25	26
<i>M. thysanodes</i>	12	12	11
<i>M. velifer</i>	30	30	33
<i>M. vivesi</i>	27	31	22
<i>Noctilio leporinus</i>	31	32	29
<i>N. albiventris</i>	3	3	3
<i>Kerivoula papillosa</i>	2	2	2
Total	411	421	407

material 1). Separately for each configuration, we used a Procrustes superimposition to align landmark coordinates with CoordGen 8h. In addition, semi-landmarks were aligned with SemiLand 8 by sliding points along curves using the minimum Procrustes distance criterion to remove the difference along the curve in semi-landmark positions between the reference form and each specimen (Gunz and Mitteroecker 2013). Instead of calculating partial warps as shape variables, we used the average Procrustes coordinates of each species in our simultaneous parsimony analyses to reconstruct a combined molecular-morphometric phylogeny as the reference tree for the inference of shape change.

Phylogenetic analyses of molecular and geometric morphometric data

First, molecular phylogenetic analyses of 25 sequences of Cyt-b and RAG2 were executed in Winclada (Nixon 2002) using Ratchet with 10% of characters sampled for reweighting, performing 200 iterations (Nixon 1999). Support was calculated with Jackknife resampling (1000 iterations, with 36% of character removal). The molecular tree from our subset of 25 terminals was saved and compared with the previously published phylogeny by Ruedi et al. (2013), with 95 terminals (Supplementary material 3a).

Next, morphometric phylogenetic analyses of five geometric morphometric characters, comprising two mandibular and three cranial structures (Fig. 1), were conducted to test if the piscivorous and the five facultative piscivorous species in a landmark-only phylogenetic analysis group together. The aligned “x, y” Procrustes coordinates of the species mean shape for each structure were analyzed under parsimony as proposed Catalano et al. (2010). Five landmark data blocks in 25 terminals were used for tree search in the program TNT 1.5 (Goloboff et al. 2015). Each landmark configuration, regardless of the number of “x, y” points, was weighted in such a way that the contribution to the total score of each configuration is similar to that of a single character. In this procedure for tree search, TNT simultaneously optimizes the total tree score and the landmark configurations at internal nodes by iteratively calculating Fermat/Geometric medians (Catalano and Goloboff 2012). This analysis was executed initially approximating landmark positions with a 10×10 grid, nesting Sankoff 2 times, using iterative-pass for landmark optimizations and realigning during TBR (Goloboff and Catalano 2016); we performed 1000 replicates, each replicate starting from a random addition sequence followed by Tree fusing and Ratchet algorithms with default settings. The topology of the best tree from morphometric data alone was saved and compared with the molecular phylogenetic tree (Supplementary material 3b).

Third, the combined data matrix with aligned Cyt-b and RAG2 sequences and the five average configurations of aligned landmarks for 25 species were also analyzed in TNT to produce a reference phylogeny. We used the same parameters argument values as with morphometric data alone, running 1000 replicates. Support for the combined tree was calculated also using a Jackknife resampling (same settings as with molecular data alone) in the program TNT. Our combined molecular and morphometric phylogeny was used to optimize how cranial morphology has changed from insectivorous ancestors to piscivorous and facultative piscivorous *Myotis* species (Figs. 2, 3).

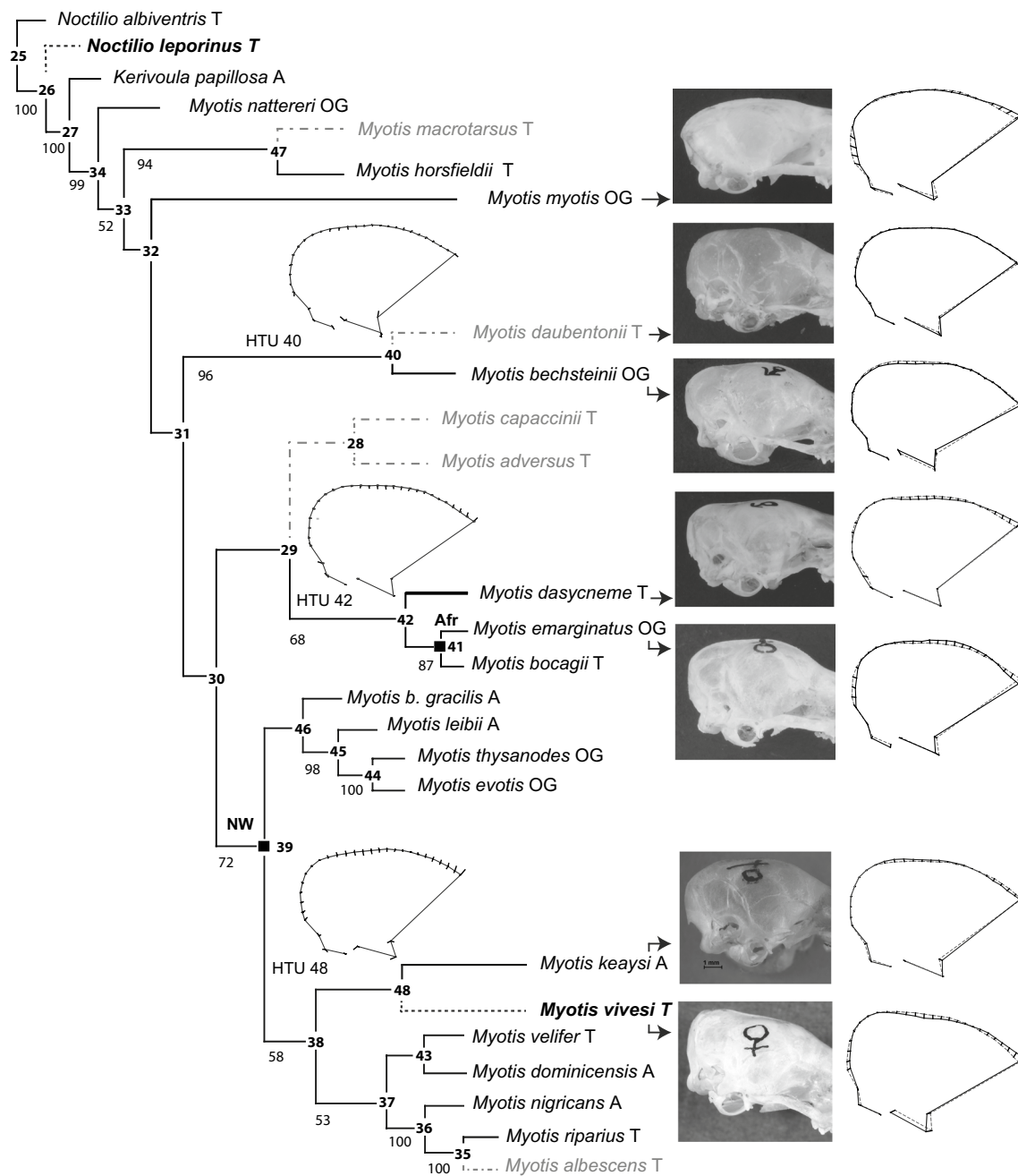
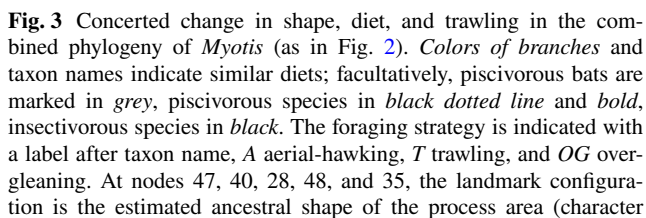


Fig. 2 Phylogeny of *Myotis* obtained from the parsimony analysis of combined molecular (Cyt-b, 1140 pb, RAG2, 1054 pb) and landmark data of five cranial configurations in TNT. Single optimal tree, with internal nodes **numbered in bold** for reference in the optimizations of diets, foraging strategy, and landmark shape data. Numbers below branches are Jackknife values. Squares on the nodes represent African and New world clades. Branch lengths are not proportional to changes. Colors of branches and taxon names indicate similar diets; facultatively, piscivorous bats are marked in grey, piscivorous species in black dotted line and bold, insectivorous species in black.

The foraging strategy is indicated with a label after taxon name, A aerial-hawking, T trawling, and OG over-gleaning. Synapomorphies for geometric morphometric data are shown only at three branches. At nodes 40, 42, and 48, there are three ancestral configurations to illustrate changes in the cranial parieto-occipital region (character 1). In all landmark configurations, deformation vectors at each point indicate displacements from the ancestral configurations to each of the descendant configurations, as optimized with spatial parsimony with TNT

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Evolution of cranial shapes

We investigated if changes in cranial morphology are shared among species with the same diet, and if these changes were homologous or convergences using parsimony phylogenetic procedures to analyze geometric morphometric data (Catalano et al. 2010). Variations in five configurations of cranial morphologies were tested for homology and homoplasy by directly mapping shape characters over our reference tree, as with any other type of data (Nixon and Carpenter 2012). We computed the retention index and the consistency index to estimate shape changes that can be interpreted as synapomorphy, or as homoplasy. Furthermore, for a simple measure of phylogenetic variability of landmark data along the tree, we used the difference of the minimum parsimony score and the maximum parsimony score for each cranial configuration. Details of these parsimony score estimations are found in Supplementary material 4.

Our combined molecular and morphometric phylogeny was used to optimize how cranial morphology has changed from insectivorous ancestors to piscivorous and facultative piscivorous species. We tested the hypothesis that changes in cranial morphology would be similar between species with the same diet by also quantifying the relative amount of shape change from ancestors to 22 descendants. We calculated procrustes distances (PD) along the 22 terminal branches to quantify the amount of shape change from the most recent hypothetical shape of the insectivorous ancestors (es) to each of the insectivorous, piscivorous, or facultative descendant species. We estimated a *t* test to ask if the average procrustes distance in 16 insectivorous species was different from the average change in six facultative and truly piscivorous species using the program STATISTICA 10 (Statsoft 2010). Since branch lengths in the 22 terminals are different, we also compared the relationship between amount of shape change (PD) and branch lengths.

Concerted changes in shape and diet

To evaluate if changes in shape are related to changes in diet (piscivorous, facultative piscivorous, and insectivorous), both characters were optimized on the reference tree, and concerted changes in both characters were inspected. When characters are single variables (discrete or continuous), searching for a concerted pattern of change is pursued with implementations under a probabilistic framework (comparative methods, Pagel 1994; Maddison 2000). However, when a shape character consists of a multivariate character, only a more “descriptive” approach is presented here for estimation of a concerted pattern of change between the optimization of the five shape characters (configurations 1–5, as five landmark coordinate sets) and

diet type (three discrete states) using parsimony in TNT. Moreover, since diet is related to foraging strategy, we also estimated if changes in foraging (aerial-hawking, overgleaning, and trawling) are related to changes in diet using pairwise comparisons module in Mesquite (Maddison 2015). We inspected optimizations to consider changes in diet or foraging in the branches leading to the piscivorous or five facultatively piscivorous species, and evaluating if the change in shape also occurs in those branches. Results are presented in Figs. 2 and 3.

Results

Phylogenetic analyses

Parsimony analysis of Cyt-b and RAG2 sequences in 25 terminals resulted in a single optimal tree, 2357 steps (Supplementary material 3a). In this molecular tree, there is a clade with all the species distributed in the New World. The Asian species *M. brandtii gracilis* Eversmann, 1845 (Benda and Tsulina 2000) appears inside the nearctic clade. Our molecular tree also recovers the African clade with the two species we sampled. Relevant to our question of the evolution of shape, the truly piscivorous species *M. vivesi* is sister to the insectivorous species *M. keaysi* Allen, 1914 (Supplementary material 3a).

The topology of the single best tree from geometric morphometric data describing variation in three cranial and two mandibular characters has a total score of 14.6501. Relationships among species of *Myotis* in the morphometric tree recover clades not found in the analyses of molecular data alone (Supplementary material 3a) or in the combined analysis (Fig. 2). In the phylogenetic morphometric tree (Supplementary material 3b), the truly piscivorous *M. vivesi* is sister to a group of two insectivorous species *M. evotis* Allen, 1864 and *M. leibii*, Audubon and Bachman, 1842. The five facultative piscivorous species are not grouped in phylogenetic analyses of five morphometric shape characters.

The parsimony analysis of the combined geometric morphometric and molecular data found one most parsimonious tree (score = 2373.25; Fig. 2). Relationships among species of *Myotis* in this combined tree recover the Neotropical and African clades, also found in the analyses of molecular data alone (Supplementary material 3a). Three of five facultative piscivorous species (*M. albescens*, *M. daubentonii*, and *M. macrotarsus*) are dispersed in the combined tree, each one sister to a different insectivorous species, and the other two (*M. capaccini* and *M. adversus*) were grouped together. The truly piscivorous *M. vivesi* is sister to a clade of insectivorous species. This combined tree is used as reference to optimize shape and diet changes (Figs. 2, 3).

Table 2 Estimates of variability in five shape characters among species of *Myotis*

Module	<i>s</i>	<i>m</i>	<i>g</i>	<i>g-m</i>	<i>h</i>	<i>ci</i>	<i>ri</i>
1. Cranial parieto-occipital	2.77	1.96	4.52	2.49	0.81	0.71	0.68
2. Frontal-maxillary region	2.47	1.85	5.31	3.47	0.62	0.75	0.82
3. Mandibular process area	2.99	2.08	4.45	2.37	0.91	0.70	0.62
4. Mandible dental row	1.15	0.82	5.08	4.26	0.33	0.71	0.92
5. Ventral view of skull	1.82	1.31	4.95	3.64	0.51	0.72	0.86

Parsimony scores were estimated from Manhattan distances in TNT with the following metrics: *s* actual steps of a character on the combined tree, *m* minimum possible steps for the character alone, *g* minimum steps on a bush, *g-m* phylogenetic variability, *h* = *s-m* homoplasy index for a character, *ci* = *m/s* character consistency index, *ri* = *g-s/g-m* retention index

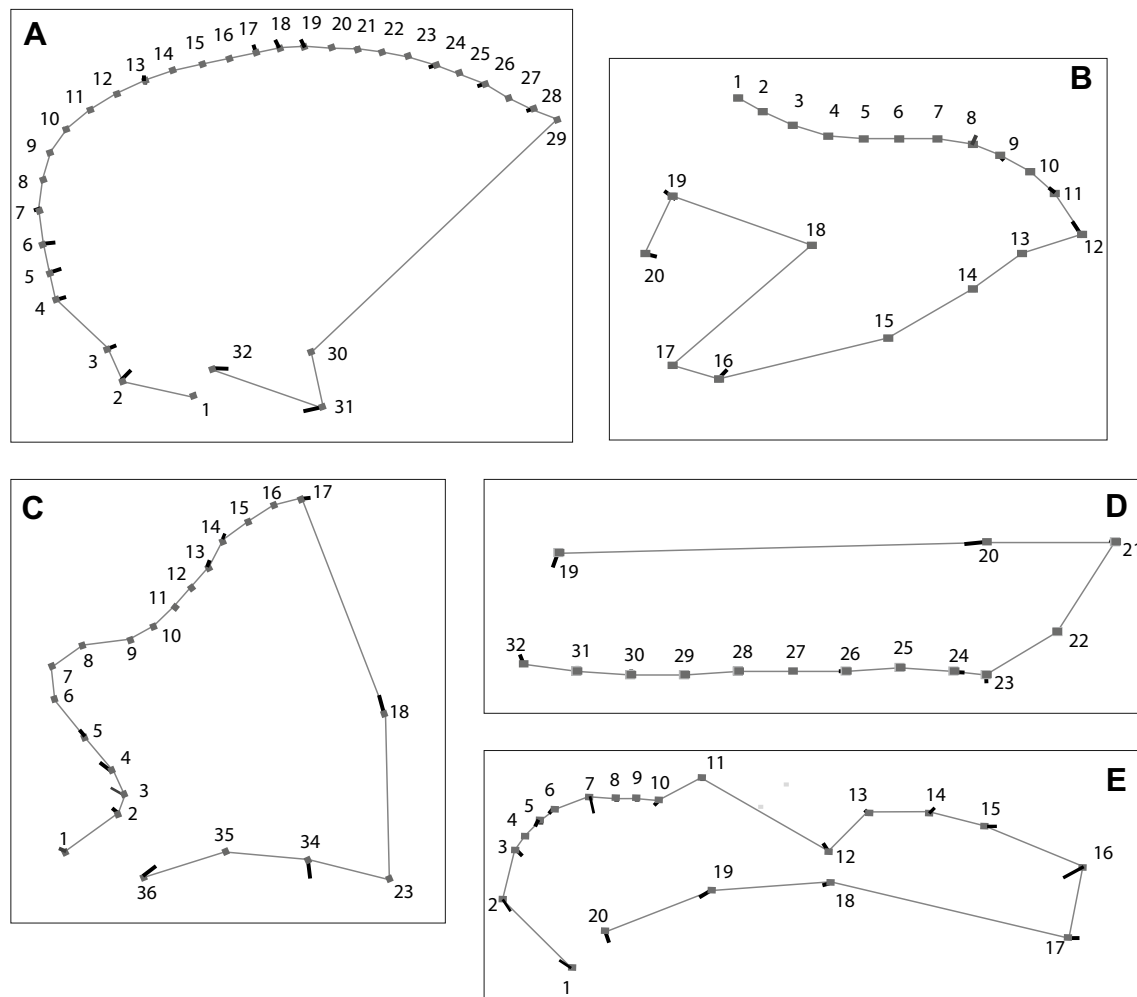


Fig. 4 Five geometric morphometric synapomorphies for *Myotis*. Landmark points in each shape character correspond to those in Fig. 1. The lines depict multivariate vectors of landmark displacements from the reference ancestral shapes at tree node 34 in Fig. 2, as optimized with spatial parsimony in TNT. These vectors are not comparable between drawings of configurations a–e. **a** Shape change in

the landmark configuration for the parieto-occipital region (character 1); **b** shape deformation in the frontal-maxillary region (character 2); **c** change in the process area (character 3); **d** landmark displacements in the configuration of the dental row (character 4); **e** displacements in the ventral view of skull (character 5)

Shape synapomorphy and homoplasy

Our analyses of the evolution of five cranial shapes reveal changes in landmark configurations consisting of synapomorphy and homoplasy (Table 2). Parsimony scores in four cranial features (characters 1, 2, 4, 5) suggest that most shape change can be interpreted as synapomorphy. Only the mandible shows homoplasious changes from insectivorous ancestors to facultative piscivorous or piscivorous *Myotis*. The parieto-occipital region (character 1) changes as a synapomorphy for *Myotis* (Fig. 4a), although later major changes are optimized as autoapomorphies in *M. myotis* Borkhausen, 1797, *M. bechsteinii* Kuhl, 1817, *M. dasycneme* Boie, 1825, *M. emarginatus* Geoffroy, 1806, and also in *M. vivesi* (Fig. 2). This is the shape character with an average consistency index ($ci=0.71$, Table 2). The frontal-maxillary region (character 2) shows most of the change at node 32 (Fig. 4b), and there are only small changes in the branches leading to *M. bechsteinii*, *M. myotis*, and *M. vivesi*. This is the shape character with the highest consistency index ($ci=0.75$). Similarly, the shape of the mandible alveolar region (character 4) changes as a synapomorphy for *Myotis* (Fig. 4d), and then there is practically no change in the rest of the tree. This is the shape character with the lowest homoplasy, highest phylogenetic variability, and highest retention index ($h=0.33$, $g-m=4.26$, $ri=0.92$, Table 2). Shape changes in the ventral region of skull (character 5) are also a synapomorphy for *Myotis* (Fig. 4e); later, there is some shape change in the piscivorous *M. vivesi*. This shape character has low homoplasy ($h=0.51$). In contrast, the shape of mandible processes region (character 3) changes as a synapomorphy for *Myotis* (Fig. 4c); however, later multiple independent changes are in the facultative piscivorous species *M. capaccinii*, *M. daubentonii*, *M. macrotarsus*, *M. albescens*, and in the piscivorous *M. vivesi*. This is the shape character with the highest homoplasy, the lowest consistency index, and the lowest phylogenetic variability ($h=0.91$, $ci=0.70$, $g-m=2.37$, Table 2).

Patterns of cranial and mandibular morphological evolution

The detailed analyses of the anatomical locations of landmark displacements and the quantification of shape changes with PD in the 22 terminal branches document that the evolution of cranial shape in five facultative species of *Myotis* is not convergent in relation to their common diet. There are no shared changes of cranial shape from insectivorous ancestors to facultative or truly piscivorous species (Figs. 2, 3). Four shapes (characters 1, 2, 4, 5) show minor changes only in three of the five branches from insectivorous ancestors to piscivorous and facultative piscivorous species. The parieto-occipital region (character 1) with

the second highest homoplasy (0.81) changes mostly at the sagittal crest, only in the piscivorous species *M. vivesi*. There are small changes in the position of the glenoid fossa (landmarks 30–31, Fig. 2) only in *M. daubentonii*. This character has no change in the other four facultative piscivorous species *M. albescens*, *M. capaccinii*, *M. macrotarsus*, and *M. adversus*. The frontal-maxillary region (character 2, homoplasy index 0.62) presents autoapomorphic changes at the nasal region (landmarks 1–12) in the piscivorous species *M. vivesi* and one facultative piscivorous *M. daubentonii* (not illustrated). This character does not change in the other four facultative piscivorous species. The alveolar region (character 4) shows practically no change from insectivorous ancestors to the piscivorous species *M. vivesi* and the five facultative piscivorous species. The ventral region of the skull (character 5) has only small changes at the maxillary region (landmarks 12–18). In the piscivorous *M. vivesi*, the skull is extended, but in one facultative piscivorous, *M. daubentonii*, the skull is shortened (not illustrated). This shape character does not change in the other four facultative piscivorous species.

Among the five structures, only the mandible (character 3, homoplasy index 0.91) shows major changes in the five transitions from insectivorous ancestors. Landmark displacements are located in the area of the two mandibular processes region; however, these changes are not shared by all facultative piscivorous and piscivorous species (arrows, Fig. 3). There are three different patterns of shape change associated with changes in diet. First, in three species, shape change consists in a reduction of the height between the condylar process and the coronoid process (double headed arrows, Fig. 3). The outline between the condylar process and coronoid process goes from a soft sigmoid curve, as in the insectivorous *M. bechsteinii*, to an almost straight line in the facultative piscivorous *M. capaccinii*, *M. daubentonii*, and *M. albescens*. This deformation is interpreted as an extension of the condylar process (character 3, landmarks 4–8) together with a contraction of the coronoid process (character 3, landmarks 13–17, arrows, Fig. 3). The second pattern is observed in the other two facultative piscivorous species (nodes 28, 47, Fig. 3), where shape change in the mandible only involves the condylar process, with no change in the position of the coronoid process. The condylar process expands the mandible in *M. macrotarsus*, whereas in *M. adversus*, changes are more subtle in this direction. A third different pattern of shape change is observed in the mandible of the truly piscivorous *M. vivesi*. The condylar process and the coronoid process move toward each other, which results in a unique contraction of the area between these two processes. Thus, our detailed analyses of the locations of landmark displacements in each particular feature reveal that shape changes are not shared in species with common diets. Moreover, the quantification

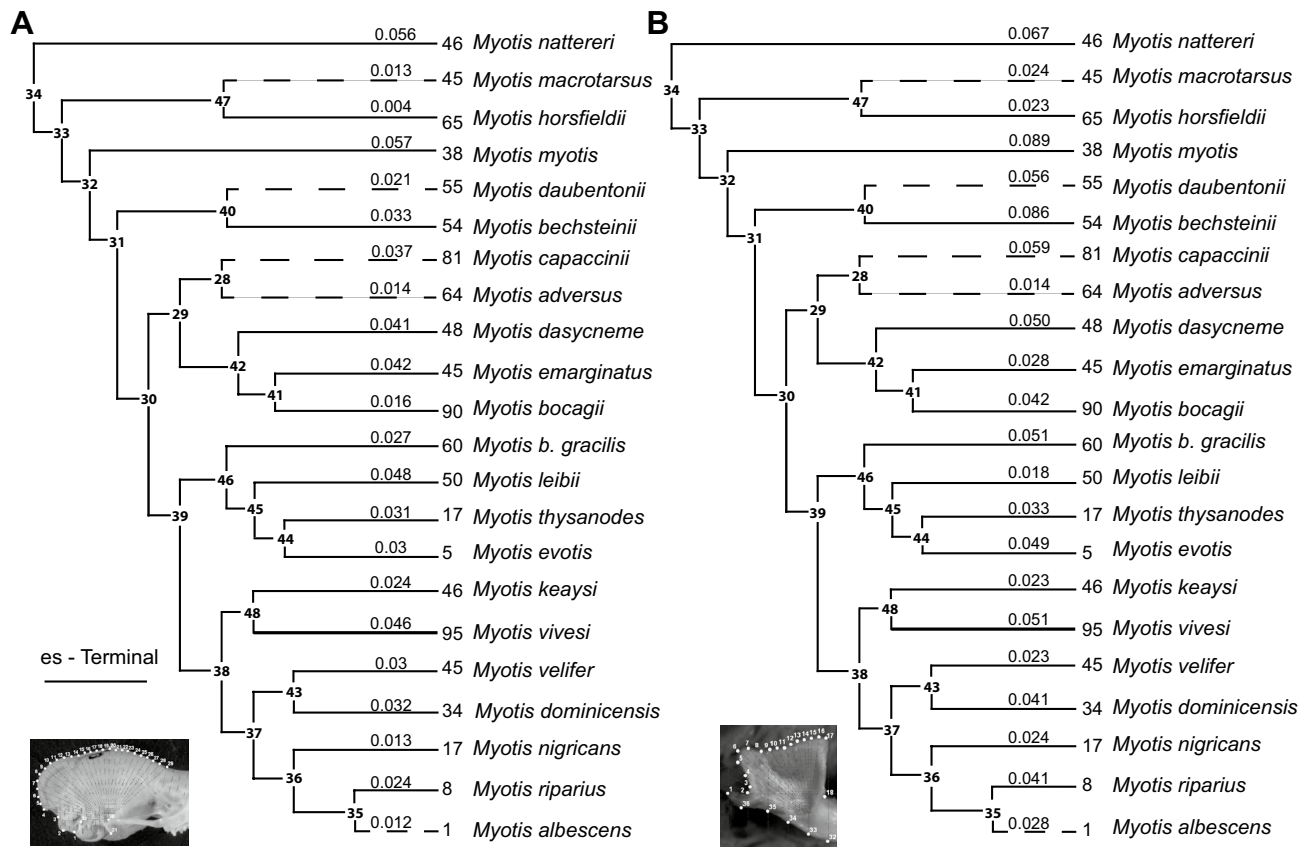


Fig. 5 Comparisons of differences in shape changes between reconstructed ancestors and observed shapes, as inferred by phylogenetic morphometric methods. These comparisons illustrate the effect of branch lengths (numbers at the tip of branches) in the estimation of shape change. Branch lengths were estimated by the reconstruction of changes of the combined data set using TNT. Numbers above branches are the amount of change (procrustes distances) between most recent common ancestors (es) and species in *Myotis*, as esti-

mated by linear parsimony. The reference tree and node numbers are the same as in Fig. 3, but with out-groups pruned. Dashed lines indicate facultatively piscivorous species and bold lines indicate piscivorous species. **a** Procrustes distances from ancestral estimated shapes to terminals for the cranial parieto-occipital region (character 1); **b** Procrustes distances from ancestral estimated shapes to terminals for the mandibular process area (character 3)

of most recent changes reveals that the amount of shape change is also independent of the change in diet.

The comparison between PD values of 16 insectivorous and 6 piscivorous species reveals very similar amounts of shape change for the five characters considered (Supplementary material 5). All *T* tests of the average PD from ancestors to insectivorous ($n = 16$) and the average PD from ancestors to piscivorous species ($n = 6$) reveal no difference in relation to change in diet. This amount of shape change estimated from ancestors to descendants at a single branch (Fig. 5) seems to be independent from branch lengths. This pattern is present in the five cranial characters, but only illustrated for two shapes (Fig. 5), the parieto-occipital region (character 1), and the shape of mandible process area (character 3). The separate analysis of the Procrustes distances of shape change along the 22 exterior branches reveals that the largest shape changes are estimated on the branches with average lengths, as in the case of *M.*

nattereri Kuhl, 1817. Conversely, smallest shape changes are quantified on the largest branches, as in the case of *M. bocagii* Peters, 1870 (Fig. 5a). In general, the average shape changes are estimated on the shortest branches, as in the case of *M. evotis* (Fig. 5b).

Concerted change in shape, diet, and trawling

Optimizations with TNT uncover five concerted changes of five shapes and diets (half filled circles, Fig. 3). Four pairs consist of a shape change in the mandible (character 3) associated with a change in diet from an insectivorous ancestor to a facultative piscivorous descendant (nodes 40, 35 and 47, Fig. 3) or piscivorous descendant (node 48, Fig. 3), and one pair consists of a shape change associated with a facultative piscivorous ancestor (node 28, Fig. 3). Optimizations of landmark displacements at these internodes reveal changes in the mandibular processes

region, and in the height of the condylar and coronoid processes (double headed arrows in Fig. 3). Changes in diet are not related to changes in foraging, since results of pairwise comparisons in PDAP Mesquite show no pairs of concerted changes between foraging strategy and diet (Supplementary material 6).

Discussion

Phylogenetic analyses of molecular and geometric morphometric data in *Myotis*

Our parsimony analysis of 25 DNA sequences of *Myotis* basically recovered the same relationships as in the larger likelihood analysis of 95 terminals by Ruedi et al. (2013). The addition of five geometric morphometric characters with molecular data supports similar species relationships recovered with the molecular data alone, in our analyses or in the previous studies. The similarity of the topology of the combined analysis (Fig. 2) with the topology of the molecular characters (Supplementary material 3a) only reveals the minimal effect of different phylogenetic inference methods and of adding five morphometric characters. Although our study only sampled a subset of 22 out of 100 species of *Myotis* worldwide, the same broad inferences survive from the previous studies. For example, the ten morphologically diverse species we included were grouped within the New World clade (Node 39, Fig. 2), this clade includes all species from New World plus the Palearctic species *M. brandtii gracilis*, as in previously published phylogeny New World clade that includes Palearctic species (*M. brandtii*). In agreement with Ruedi and Mayer (2001), a general pattern in our combined phylogenetic hypothesis of *Myotis* (Fig. 4) is that the biogeographic distribution of the species appears to be a much better predictor of their phylogenetic position than their cranial and mandibular morphology. In contrast, the morphometric phylogeny based on the five shape characters (Supplementary material 3b) did not recover relationships congruent with the molecular tree (Supplementary material 3a). The Neotropical and the African clades were not present in the morphometric tree, or in the combined analyses of shape characters (Supplementary material 3b). Geometric morphometric data in five characters produced hierarchically structured relationships. Still, none of the combined and separate parsimony analyses of the five shapes (Supplementary material 3) grouped the facultative piscivorous species with presumably similar shapes.

The combined phylogeny of molecular and morphometric data shows no evidence that shape changes are convergent among species with similar diets. The optimization

of configurations on our reference tree revealed localized changes in the mandibular processes region (character 3, arrows, Fig. 3); however, there are no indications of similarity in cranial morphology (characters 1, 2, 4, and 5) among species with the same diet in the five facultative piscivorous species. These results along with concerted optimizations of shape and diet on our combined phylogeny (Fig. 3) imply that facultative piscivorous and piscivorous diets originated independently from trawling insectivorous ancestors in the diversification of *Myotis*.

Synapomorphy and homoplasy in the evolution of cranial shape characters

A generalization from our results of phylogenetic analyses of landmark variation is that some changes in each of the five cranial shapes can be interpreted as hypothesis of homology, and some are best interpreted as homoplasy (Table 2). The most evident synapomorphies in our geometric morphometric data are in the frontal-maxillary region (configuration 2, Fig. 4b), the mandibular alveolar region (character 4, Fig. 4d), and the ventral region of skull (character 5, Fig. 4e) with lowest homoplasy and highest retention index (0.82–0.96). The levels of homoplasy were highest for the cranial parieto-occipital region and the mandibular processes region (0.81 and 0.91, respectively). Our underlying assumption is that character parsimony scores (homoplasy index, consistency index, and the retention index) for each trait provide evidence to interpret phylogenetically informative changes in landmark configurations (Table 2). These parsimony scores are indicators of the levels of character synapomorphy and homoplasy (Wenzel 2002), and we suggest to routinely using these scores as measures of shape transformation and agreement of character changes in geometric morphometric data on a particular cladogram, just as it is standard for other character systems (Goloboff 1991; Schuh 2000). Our results suggest that landmark data should not be disqualified for supposedly lacking historical information, as generalized by Klingenberg and Gidaszewski (2010). Recently, it had been demonstrated that the inclusion of three or more landmark configurations improves the results of the phylogenetic analysis (Catalano and Torres 2017). As in the case of any other evidence, geometric morphometric data may contain historical information and the phylogenetic analyses of landmark coordinates will reveal which shape changes are homologous and which are homoplasious. Homoplasious shape changes detected in *Myotis* could be interpreted in an ecological sense separately for the cranial features (characters 1, 2, 4, 5) in relation to foraging strategies and the mandibular shape (character 3) in relation to diets. For example, in the case of mandibular processes region (character 3), the two patterns of changes in the facultative piscivorous species

and the third morphotype in the truly piscivorous (Fig. 3) could be, indeed, related mostly to diet. Although there is not a common mandibular shape among all facultative piscivorous species, small changes in the masseter insertion area affect the mechanical advantage generated by the masseter muscle and, therefore, its performance in diet (Santana et al. 2010). In the case of the parieto-occipital region (character 1), shape changes seem not be related to diet but with foraging (Fig. 2). The largest changes were estimated in the transitions to insectivorous species, rather than in the origin of facultative piscivorous species (Fig. 5a). This shape change could be better explained by how insectivorous species find and capture preys using different echolocation calls and at least two possible foraging strategies (Norberg and Rayner 1987; Siemers et al. 2001; Fenton and Bogdanowicz 2002). The ability to detect prey in bats is related to the temporal muscle position, which is inserted on the sagittal crest (landmarks 4–29, character 1) and the position of the auditory apparatus on the skull (landmarks 30, 32, 1), parieto-occipital region (Freeman 1984; Veselka et al. 2010). The same detection ability determines if insectivorous species capture flying prey in open areas by aerial-hawking or amongst vegetation by over-gleaning (Findley 1972; Norberg and Rayner 1987; Fenton and Bogdanowicz 2002). In other cases, cranial shape changes in insectivorous species such as *M. emarginatus* or *M. dasycneme* (Fig. 3) could be related to the hardness of prey, an aspect of diet not considered here. The hardness of prey in combination with body size seems to be two determinant factors in cranial shape variation among insectivorous *Myotis* species (Ghazali and Dzeverin 2013). In a hypothetical sequence, the most recent ecomorph is ‘*Myotis*’ with a usually larger size and harder diets, whereas ‘*Leuconoe*’ and ‘*Selysius*’ were medium or small in size and usually preferring softer insects (Ghazali et al. 2016).

Concerted change in shape, diet but not trawling

Some localized changes in mandibular morphology (character 3) seem concerted with changes in diet from insectivorous to piscivorous and facultative piscivorous species (half filled circles, Fig. 3). The phylogenetic analysis of landmark data has succeeded in identifying three very localized patterns of changes in mandibular shape related to a transition in diet (Fig. 3). The three patterns of concerted change inferred in the facultative piscivorous species and in the truly piscivorous *M. vivesi* (Fig. 3) reveal that small differences in mandible shape could have common functional changes in the masticatory apparatus related to transitions to the same diet.

Our quantification of the amount of mandibular shape change indicates that minor changes in specific regions could improve the masticatory performance to switch diet

from insects to fish. The morphological change as estimated with Procrustes distance from insectivorous ancestors to piscivorous species in the mandibular shape of *Myotis* is rather modest compared with similar dietary specializations in other groups of bats. Carnivorous and sanguivorous have larger mastoid breadth and height of the skull at bullae bats in comparison with insectivorous and frugivorous bats (Van Cakenberghe et al. 2002). Size and shape differences have an effect in the masticatory performance of carnivorous bats. Freeman (1979) found that carnivorous bats use their canines and temporal muscles to help in capture of prey items, whether soft or hard, suggesting that wide faced bats have more powerful jaws than the insectivorous and a major relative importance of the pterygoid and zygomaticomandibularis muscles, increasing their ability to move the jaw transversely. The small changes from most recent insectivorous ancestors to piscivorous descendants in *Myotis* were not related even to the different amounts of molecular change, indicated by branch lengths (Baker 2002). Therefore, despite about 21 MYA of diversification in *Myotis*, the presumed adaptative radiation (Ruedi and Mayer 2001) is accompanied by a slightly modified generalist cranial morphology (Ruedi et al. 2013). Our results add another case in support for such generalization.

Shape deformations in the cranial and mandibular features of facultative piscivorous *Myotis* may be interpretable in view of the common foraging strategy. The external morphology of *Myotis* species has been separated in three ecomorphs, characterized by aerial-hawking foraging strategy (“*Selysius*”), by over-gleaning (“*Myotis*”), and by trawling (“*Leuconoe*”, Findley 1972; Norberg and Rayner 1987; Ruedi and Mayer 2001). These ecomorphs are not associated with a specific diet (Ghazali and Dzeverin 2013; Ghazali et al. 2016). The five facultative piscivorous species and the truly piscivorous species capture prey by trawling, using their claws and the uropatagium to capture prey and move it towards a wide opened mouth. The positions of condylar and coronoid process in the six piscivorous species indicate a vertically oriented temporal muscle, different from the insectivorous species. Three patterns of mandibular shape changes in these six species seem associated with changes in the position of muscles and gape of skulls (increase of insertion area), the position of processes and crest in skulls, all of which are related to the capacity to chew hard items, whether insects or fish (Freeman 1981; Dumont and Herrel 2003; Herrel et al. 2008). These morphological changes may be related to how wide a bat could open its jaws and the mechanical advantage generated by the muscle position, following a model of muscle stretching proposed by Herring and Herring (1974). If position of coronoid process and temporal muscle is involved in gape capacity in the piscivorous species of *Myotis*, our results suggest performance experiments to test the importance of

the mandibular processes region in how bats capture preys to include fish in their diet.

Adaptations have already been explored in linking cranial morphologies in bats to a sequence of dietary transitions from insectivores through omnivores to feeding on nectar, and fruit feeding (Wetterer et al. 2000; Monteiro and Nogueira 2009) or carnivores (Freeman 1984). In particular, wide faces and the height of the condylar process above the tooth row have been associated with greater attachment area for the masticatory muscles (Freeman 1984). Our results on the inferred phylogenetic pattern of change in the mandible process area (arrows, Fig. 3) suggest that this is one of the main structures with biomechanical relevance in relation to the type of food consumed and could be more informative in the case of more contrasting diets in other bat species, as Monteiro and Nogueira (2009) already found for Phyllostomidae. Of course, biomechanical variation should be further analyzed in *Myotis* to evaluate if there is an associated functional utility and performance advantage as hypotheses of adaptation in the transitions of diet. Considering the importance of hardness of preys, the small changes in the mandible process area of piscivorous species in *Myotis* could be an adaptation to catching prey with similar hardness (fish, insect larvae) near water. Our findings of subtle shape changes suggest a biomechanical solution in the mandible of piscivorous species of *Myotis*, which remains to be studied in detail. Biomechanical variation should be further analyzed in *Myotis* to evaluate if there is an associated functional utility and performance advantage as hypotheses of adaptation in the transitions of diet.

Conclusions

A phylogenetic approach to analyze morphometric variation allowed the hierarchical partitioning of total shape change in landmark data. Some shape deformation in each of the five structures of skull morphologies is best interpreted as synapomorphies for *Myotis*. Most of the shape changes in the cranial parieto-occipital region and mandibular processes region are interpreted as homoplasy; however, these changes are modest and are not shared among the five species of *Myotis* with the same facultative piscivorous diet. There are three different independent shifts in the mandibular processes region (shape 3) associated with the change in diet. These changes in mandibular morphology seem to be related to a common functional utility in facultative piscivorous and piscivorous species sharing a trawling foraging strategy.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Informed consent Informed consent was obtained from all individual participants included in the study.

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