Zoological Journal of the Linnean Society, 2024, XX, 1–20
https://doi.org/10.1093/zoolinnean/zlad180
Advance access publication 19 January 2024
Original Article

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Comparative anatomy of the vocal apparatus in bats and implications for the diversity of laryngeal echolocation

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ABSTRACT

Most of over 1400 extant bat species produce high-frequency pulses with their larynx for echolocation. However, the debate about the evolutionary origin of laryngeal echolocation in bats remains unresolved. The morphology of the larynx is known to reflect vocal adaptation and thus can potentially help in resolving this controversy. However, the morphological variations of the larynx are poorly known in bats, and a complete anatomical study remains to be conducted. Here, we compare the 3D laryngeal morphology of 23 extant bat species of 11 different families reconstructed by using iodine contrast-enhanced X-ray microtomography techniques. We find that, contrary to previously thought, laryngeal muscle hypertrophy is not a characteristic of all bats and presents differential development. The larynges of Pteropodidae are morphologically similar to those of non-bat mammals. Two morphotypes are described among laryngeal echolocating bats, illustrating morphological differences between Rhinolophoidea and Yangochiroptera, with the main variations being the cricothyroid muscle volume and the shape of the cricoid and thyroid cartilages. For the first time we detail functional specialization for constant frequency echolocation among Rhinolophoidea. Lastly, the nasal-emitting taxa representing a polyphyletic group do not share the same laryngeal form, which raises questions about the potential modular nature of the bat larynx.

Keywords: Chiroptera; cricothyroid muscle; functional adaptation; larynx; mammalian nasopharyngeal morphology; vocal tract; X-ray microtomography

INTRODUCTION

Bats (order Chiroptera) constitute the second largest mammalian order after Rodentia in term of species diversity with over 1400 described species (Simmons and Cirranello 2020). They are unique mammals that are capable of self-powered flight and can use echolocation to navigate in dark environments, enabling them to colonize all continents except Antarctica and to exploit, and radiate into, unoccupied ecological niches (Griffin 1944, Rayner 1988, Teeling 2009).

Extant bats are classified into two suborders: Yinpterochiroptera (yinpterochiropterans) uniting the family Pteropodidae (pteropodids) and the super family Rhinolophoidea (rhinolophoids), which include Rhinolophidae, Rhinonycteridae, Hipposideridae, Rhinopomatidae, Craseonycteridae, and Megadermatidae; and Yangochiroptera (yangochiropterans) comprising all other recognized families (Simmons and Cirranello 2020). Members of yangochiropterans and rhinolophoids conduct echolocation by generating

Received 25 June 2023; revised 9 October 2023; accepted 6 November 2023

high-frequency sound produced by the larynx, whereas pteropodids do not echolocate or employ a primitive form of echolocation using tongue and/or wing clicks (Yovel *et al.* 2011, Boonman *et al.* 2014, Chaverri *et al.* 2018). Among laryngeal echolocating bat families, there are considerable differences in echolocation strategies, such as constant frequency (CF), frequency modulated (FM), high-duty cycles (HDC), low-duty cycles (LDC), nasal or oral emitters (e.g. Fenton *et al.* 2012). These differences between bat families have raised questions regarding the origin and evolution of laryngeal echolocation. Nowadays, two competing hypotheses are debated. The first explains a single and common origin of laryngeal echolocation for all bats with a common ancestor able to laryngeally echolocate, followed by a loss of the ability in the pteropodids (e.g. Veselka *et al.* 2010, Wang *et al.* 2017). The second hypothesis supports the idea that laryngeal echolocation appeared independently multiple times in the different bat clades (e.g. Davies *et al.* 2013, Nojiri *et al.* 2021). This second hypothesis implies that pteropodids conserved the same characteristics as the common ancestor of all bats and that, independently, both rhinolophoids and yangochiropterans acquired the ability of laryngeal echolocation by convergence.

Many researchers have examined variation in the ear (e.g. Davies *et al.* 2013, Wang *et al.* 2017, Nojiri *et al.* 2021), but very few have undertaken comparative studies of the sound-producing organ, the larynx (Brualla *et al.* 2023), probably due to the different preservation of the two organs through time. The inner ear is located in the petrosal part of the temporal bone, which is one of the stiffest, and therefore best-preserved bones of the cranium when studying museum specimens of extant species and fossils. In contrast, the larynx mainly comprises delicate soft tissues that are not preserved in fossils or dried museum specimens. Laryngeal soft tissues in bats are small but structurally very complex because most bats are small, making macroscopic dissections difficult. Today, researchers may study small organisms in a non-destructive way by using a combination of staining protocols for soft tissues and X-ray microtomography scanning (e.g. Metscher 2009, Gignac *et al.* 2016, Santana *et al.* 2019, Nojiri *et al.* 2021). These technologies enable laryngeal anatomy to be studied in greater detail, potentially yielding critical information to further resolve the origin of laryngeal echolocation in bats.

The larynx is an essential mammalian organ due to its contributions to several vital functions, including vocalization (e.g. Harrison 1995, Shiba 2010). The morphology of the mammalian larynx is described as relatively conserved (Harrison 1995, Saigusa 2011), with only minor morphological changes having been reported (Brualla *et al.* 2023). The mammalian larynx is comprised of four cartilages (one thyroid, one cricoid, and a pair of arytenoids) (Fig. 1) that support seven pairs of intrinsic muscles (cricoarytenoid dorsalis, cricoarytenoid lateralis, cricothyroid, oblique arytenoid, transverse arytenoid, thyroarytenoid, and vocalis muscles) (Negus 1949, Harrison 1995, Hoh 2005, 2010, Saigusa 2011, König and Liebich 2020, Brualla *et al.* 2023). The muscle contraction on the larynx acts to incline the thyroid caudally and tilt the arytenoid cartilages laterally, adducting or abducting the vocal folds and putting them in position to vibrate and produce sound (e.g. Harrison 1995, Finck and Lejeune 2010, Metzner and Müller 2016, Brown and Riede 2017). The MyoElastic-AeroDynamic (MEAD) theory adds that the elasticity of the vocal folds plays a role in the different frequencies emitted by mammals (van den Berg 1958, Brudzynski 2009, Brown and Riede 2017, Švec *et al.* 2021). Laryngeal specializations to different ecologies and behaviours, such as larger laryngeal structures, presence of air sacs, or of a dorsal tracheal membrane, have been found in several mammals (Harrison 1995, Thomas *et al.* 2004, Reidenberg and Laitman 2010). Because bats are volant mammals that produce high-frequency vocalizations, in addition to common morphological patterns shared with other mammals, their laryngeal structures are expected to have some unique traits associated with the adaptative evolution of vocalization (Suthers 2004, Metzner and Schuller 2010; Metzner and Müller, 2016; Brualla *et al* 2023). For instance, bats have been described as having a similar laryngeal morphology to other mammals, presenting only minor variations for aspects of the cartilages (shape and mineralization patterns) and some modifications in the muscle size and activity (e.g. Denny 1976, Harrison 1995, Elemans *et al.* 2011, Carter 2020). Hypertrophied intrinsic muscles have been linked to vocalization needs, especially for laryngeal echolocation. High-frequency vocalizations observed in bats require high subglottal pressure, which is possible because of the greatly developed laryngeal muscles regulating the tension on the vocal folds (Elemans *et al.* 2011, Ratcliffe *et al.* 2013, Grinnell *et al.* 2016). Indeed, laryngeal echolocation is produced by the larynx from the contraction of the different intrinsic laryngeal muscles that twist the arytenoid cartilages and tilt the thyroid cartilage. This muscular action closes and tenses the vocal folds that possess thin vocal membranes that vibrate extremely fast, enabling bats to produce high frequency sounds (e.g. Metzner and Müller, 2016).

Our literature review of pioneer studies of bat laryngeal morphology (e.g. Elias 1907, Denny 1976), indicated that (i) the larynges of bats can be classified into two main morphotypes, one specific to rhinolophoids and the other found only in their relatives, belonging to the superfamily Vespertilionoidea of yangochiropterans (vespertilionoids), and that (ii) the different forms of the laryngeal components found in different bat taxa could be correlated with their variations of frequencies, rate of calls, and amplitudes during sound production (Brualla *et al.* 2023). As such, the precise descriptions, comparisons and interpretations of the potential different laryngeal forms of bats may be used for elucidating the evolutionary history of laryngeal echolocation. However, the anatomy of the larynx and its relation to ecological diversity remains to be investigated for most bat species (Brualla *et al.* 2023). Illustrating the amount of anatomical variation among bat larynges may challenge the commonly held idea that mammalian larynges are morphologically uniform and evolutionarily constrained. Formulizing the patterns of laryngeal forms could also result in better understanding of the evolutionary success of bats, by demonstrating great modifications of the larynx in relation to different niches, ecologies, but also vocal productions.

Here, for the first time, we describe the patterns and magnitude of variation in the bat larynx (cartilages and muscles), discussing the diversity of forms and their potential collation into morphotypes, using iodine contrast-enhanced X-ray microtomography, and virtual dissection (Fig. 1). In this study,

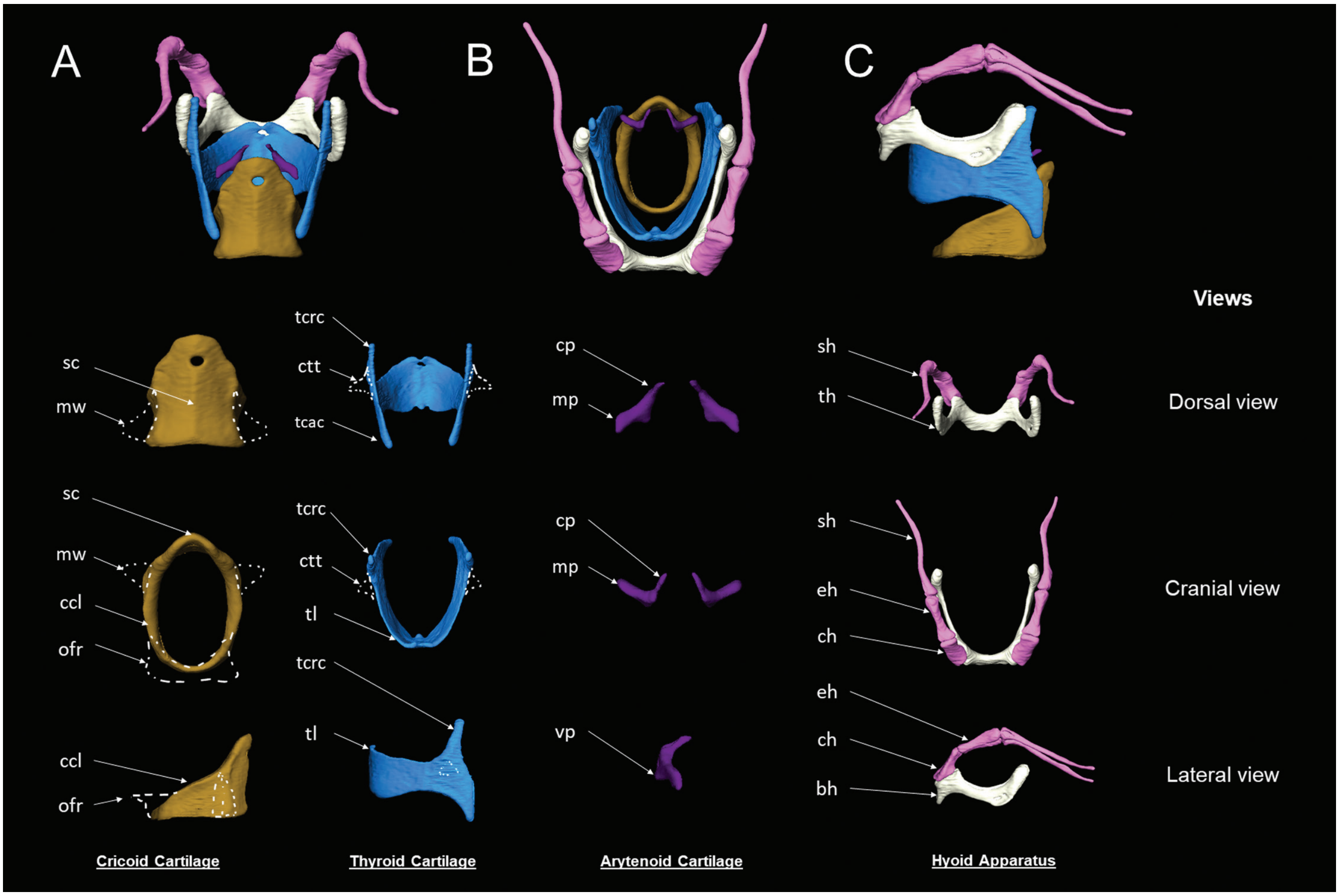

Figure 1. Detailed visualization of the laryngeal cartilages of *Eonycteris spelaea* and the specific components found in several bat species (dashed lines). A, dorsal view; B, cranial view; C,: lateral view. See text for abbreviations.

we investigated whether different laryngeal forms are present in bats, or whether bat larynges are similar to the general mammalian body plan, e.g. those of other laurasiatherians (Harrison 1995, Evans and De Lahunta 2012). As a working hypothesis based on previous research (reviewed in: Brualla *et al.* 2023), we predict that bat larynges display several distinct features compared to the general mammalian scheme and that discrete morphotypes are distributed across the phylogeny. We show that this morphological diversity is unique among mammals, which underscores the remarkable diversity and ecological success of Chiroptera.

MATERIALS AND METHODS

X-ray microtomography (XMT) imaging data were collected from adult specimens, comprising 23 species of bats. One species of Eulipotyphla (*Suncus murinus* Linnaeus 1766) was also studied as an outgroup. The bat sampling comprises two species of non-laryngeal echolocating bats (two Pteropodidae) and eight species of laryngeal echolocating bats (three Hipposideridae, one Rhinopomatidae, two Rhinolophidae, one Megadermatidae, and one Craseonycteridae) among the yinpterochiropteran suborder and 13 species of laryngeal echolocating bats (three Emballonuridae, three Phyllostomidae, two Mormoopidae, one Molossidae, and four Vespertilionidae) among the yangochiropterans (Table 1). The sample composition was chosen to illustrate the spectrum of diversity present in bats, by detailing the morphology of the different bat families. We sampled six out of seven families inside yinpterochiropterans (86% of the families), and five of 14 families in the yangochiropterans (36%). Thus, the majority of yinpterochiropterans and the families with the most species diversity among yangochiropterans are represented in this study. Phylogenetic relationships between species were produced from the Timetree (Kumar *et al.* 2022). Adult body mass within the sample ranged from 2 g (*Craseonycteris thonglongyai* Hill 1974) to 60 g (*Eonycteris spelaea* Dobson 1871). The different laryngeal echolocation strategies were also represented in the sample, with inclusion of constant frequency (CF), frequency modulated (FM), high-duty cycle (HDC), and low-duty cycle (LDC) laryngeal echolocators. Reference to the bats at a generic level will be made throughout the paper for brevity, except for multiple species comparisons within a genus. Specimens originate from multiple museum collections (Australia, Japan, Thailand, the United States of America, and Vietnam) (Table 1). We gathered specimens stored in 70% ethanol from the collections of the University Museum of the University of Tokyo, the Vietnam Academy of Science and Technology, Chulalongkorn University Museum of Natural History, and the East Tennessee State University. We also used public datasets from Morphosource.org provided by the University of Michigan Museum of Zoology and Stony Brook University.

Table 1. Species scanned, their source, collection ID and laryngeal echolocation strategy.

Family	Species	Source	ID (+ DOI/ARK)	Laryngeal echolocation strategy
Eulipotyphla	*Suncus murinus*	University Museum of The University of Tokyo	KATS_835A	Nil
Pteropodidae	*Eonycteris spelaea*	Vietnam Academy of Science and Technology	VN18-026	Nil
Pteropodidae	*Macroglossus sobrinus*	Vietnam Academy of Science and Technology	VN15-017	Nil
Hipposideridae	*Aselliscus dongbacanus*	Vietnam Academy of Science and Technology	VTTu15-013	CF-HDC
Hipposideridae	*Coelops frithii*	Vietnam Academy of Science and Technology	VN19-196	CF-LDC
Hipposideridae	*Hipposideros larvatus*	Vietnam Academy of Science and Technology	VN18-209	CF-HDC
Rhinolophidae	*Rhinolophus cornutus*	University Museum of The University of Tokyo	JP21-025	CF-HDC
Rhinolophidae	*Rhinolophus macrotis*	Vietnam Academy of Science and Technology	VN11-089	CF-HDC
Megadermatidae	*Lyroderma lyra*	Vietnam Academy of Science and Technology	VN17-535	FM-LDC
Craseonycteridae	*Craseonycteris thonglongyai*	Chulalongkorn University Museum of Natural History	CUMZ(M)220213-002	FM/CF-LDC
Rhinopomatidae	*Rhinopoma hardwickii*	University of Michigan Museum of Zoology	UMMZ:Mammals:159357 (https://doi.org/10.17602/M2/M96954)	FM/CF-LDC
Emballonuridae	*Saccolaimus mixtus*	Australian Museum	A3257	FM-LDC
Emballonuridae	*Saccopteryx bilineata*	Stony Brook University	L-LD:PE160 (http://n2t.net/ark:/87602/m4/393533)	FM-LDC
Emballonuridae	*Taphozous melanopogon*	Vietnam Academy of Science and Technology	VN17-0252	FM-LDC
Phyllostomidae	*Artibeus jamaicensis*	East Tennessee State University	AJ001	FM-LDC
Phyllostomidae	*Desmodus rotundus*	University of Michigan Museum of Zoology	UMMZ:Mammals:112960 (https://doi.org/10.17602/M2/M170159)	FM-LDC
Phyllostomidae	*Rhinophylla fischerae*	Stony Brook University	L-LD:PE101 (http://n2t.net/ark:/87602/m4/394474)	FM-LDC
Mormoopidae	*Pteronotus quadridens*	Stony Brook University	L-LD:DR098 (http://n2t.net/ark:/87602/m4/393837)	CF-LDC
Mormoopidae	*Pteronotus* cf. *rubiginous*	University of Michigan Museum of Zoology	UMMZ:Mammals:74643 (https://doi.org/10.17602/M2/M97786)	CF-HDC
Molossidae	*Molossus molossus*	Stony Brook University	L-LD:PE156 (http://n2t.net/ark:/87602/m4/393783)	FM-LDC
Vespertilionidae	*Kerivoula hardwickii*	Vietnam Academy of Science and Technology	VN11-0043	FM-LDC
Vespertilionidae	*Myotis albescens*	Stony Brook University	L-LD:PE008 (http://n2t.net/ark:/87602/m4/393605)	FM-LDC
Vespertilionidae	*Myotis ater*	Vietnam Academy of Science and Technology	VN19-016	FM-LDC
Vespertilionidae	*Myotis siligorensis*	Vietnam Academy of Science and Technology	VTTu14-018	FM-LDC

CF, constant frequency; FM, frequency modulated; HDC, high-duty cycles; LDC, low-duty cycles.

Image acquisition

As data collection was undertaken from different museum collections, other fixatives and iodine-staining protocols than the one cited above are present in the sample, making the data preparation non-uniform. Iodine staining was undertaken to enhance the contrast and visualize the soft tissues during scanning (Metscher 2009, Gignac *et al* 2016). The staining protocol used ethanol with 1% iodine for 14 days (Gignac *et al.* 2016).

All specimens were then XMT scanned with different scanners and protocols (parameters and voxel sizes) depending on the team acquiring the data. A voxel size between 10 and 30 μm was required to be able to reconstruct the different components with sufficient resolution: the average thickness of the laryngeal cartilage was between 50 and 350 μm for most species. Manual reconstruction of the three intrinsic cartilages of the larynx (cricoid, thyroid, and arytenoids) and of the entire hyoid apparatus was undertaken using the brush tool and interpolation function in AMIRA 5.3.3 software (ThermoFisher). All the intrinsic muscles were reconstructed with the same method (cricothyroid, cricoarytenoid dorsalis, cricoarytenoid lateralis, oblique arytenoid, transverse arytenoid, thyroarytenoid, and vocalis muscles), to which we added the thyrohyoid muscle for its connection with the hyoid apparatus and its potential role in laryngeal echolocation (e.g. Novick and Griffin 1961, Griffiths 1983). The reconstructed 3D surfaces were saved as STL files and analysed during the current study for anatomical comparisons. All 3D surface models are available on the repository website MorphoMuseum (https://doi.org/10.18563/journal.m3.219) or Morphomuseum (Table 1).

We focused on morphological descriptions and comparison of the size and shape of laryngeal structures. We did not quantify ossification or degree of mineralization of the cartilage, because the specimens were acquired by different researchers using different protocols, especially regarding staining and parameters of the XMT scanners. Hence, the images contain different contrast for potentially the same degree of mineralization from one specimen to another. For the muscles, we compared the overall morphology, presence/absence, attachment points/areas, and size range. Precise volume measurements were not taken as ethanol preservative and iodine are known to cause soft-tissue shrinkage (Vickerton *et al.* 2013). We chose to maximize sampling by incorporating specimens that were stained under different protocols, rather than applying strict exclusion criteria. Lastly, we provide a complete description of all the cartilages and muscles, followed by detailed comparisons between clades and laryngeal echolocation strategies.

During reconstruction, certain groups of muscles were classed as single entities to facilitate identification and reconstruction. Thus, the oblique and transverse arytenoid muscles have been merged as arytenoid muscles. Similarly, the thyroarytenoid and vocalis muscles have been merged as thyroarytenoid muscles. The complete list of species with the state of reconstruction of each component are provided (Supporting Information, Table S1), illustrating specimens with missing data due to the different acquisition protocols and staining processes. Overall, the sampling and qualitative descriptions remain robust and the information extracted from the 3D reconstruction, and detailed in the result part of this study, are of great value for further investigations in bat biology. The following abbreviations have been used for the figures: a, arytenoid muscle; ac, arytenoid cartilage; bh, basihyal; cad, cricoarytenoid dorsalis muscle; cal, cricoarytenoid lateralis muscle; cc, cricoid cartilage; ccl, cricoid cartilage laminae; ch, ceratohyal; cp, corniculate process; ct, cricothyroid muscle; ctt, cranial thyroid tubercle; eh, epihyal; ha, hyoid apparatus; mp, muscular process; mw, muscular wings; ofr, outwardly flared rim; mc, median crest; sh, stylohyal, ta, thyroarytenoid muscle; tc, thyroid cartilage; tcac, thyroid caudal cornua; tch, tracheal chambers; tcrc, thyroid cranial cornua; th, thyrohyal; thm, thyrohyoid muscle; tl, thyroid laminae; vp, vocal process.

RESULTS

Cartilages

Cricoid cartilage

The cricoid cartilage displays the greatest variation among the laryngeal components investigated in this study (Fig. 2). Within yinpterochiropterans, the cricoid cartilage of the two species of pteropodids is similar to that observed in the shrew species, *Suncus murinus*. The main difference observed in bats is the development of a thin median crest on the dorsal part of the cricoid cartilage. Hipposiderids (Hipposideridae) and rhinolophids (Rhinolophidae) species have similar cricoid forms, being narrower than in pteropodids, and with an extreme development of the median crest and of muscular wings (Fig. 2A). The cricoid cartilage form observed in craseonycterids (Craseonycteridae) and rhinopomatids (Rhinopomatidae) species are highly similar. They have a median crest and muscular wings with less development than the hipposiderids, and a cranial development of the ventral part of the cartilage (Fig. 2A). The megadermatids (Megadermatidae) species possess a cricoid cartilage similar to that of craseonycterids, with less ventral development (Fig. 2A). Yangochiropteran families have variable cricoid cartilage forms (Fig. 2B). The emballonurids (Emballonuridae) possess the most caudally elongated, tube-shaped cartilage. A prominent median crest is present on the cranial part of the dorsal region of the cricoid, with oval concavities on each side of the crest (Fig. 2B). The phyllostomids species present interspecific variations and the thinnest cartilages. Their cricoid has a triangular shape instead of being more oval or rounded as in yinpterochiropterans. *Artibeus jamaicensis* (Leach 1821) exhibits an expansion of the cranioventral part of the cricoid, not seen in the other phyllostomids (Fig. 2B). All phyllostomids have a reduced median crest and muscular wings. The cricoid cartilage of mormoorpids is more flattened and elongated ventrally than in phyllostomids and possesses an outwardly flared rim on the ventral part (Fig. 2B). The muscular wings and median crest in both *Pteronotus* (Gray 1838) species are more developed than most yangochiropterans (except for the emballonurids). The molossids (Molossidae) have a similar cricoid cartilage to the rhinopomatids, but with more prominent muscular wings and a large outwardly flared rim. The cricoid cartilage of the vespertilionids is similar, morphologically, to that of the molossids, with thin cartilage as in phyllostomids and muscular wings such as in rhinolophids. The median crest of both vespertilionids and molossids is prominent cranially, similarly to other yangochiropteran species (Fig. 2B).

Tracheal chambers

Tracheal chambers are cartilaginous bullae on the first tracheal rings, caudal to the cricoid cartilage (Fig. 3). They are unique features found only in the hipposiderids and rhinolophids. The rostral pair of chambers is large and laterally distributed and the lower pair is small and dorsally positioned. The rhinolophids have greater size variation between the upper and lower pairs

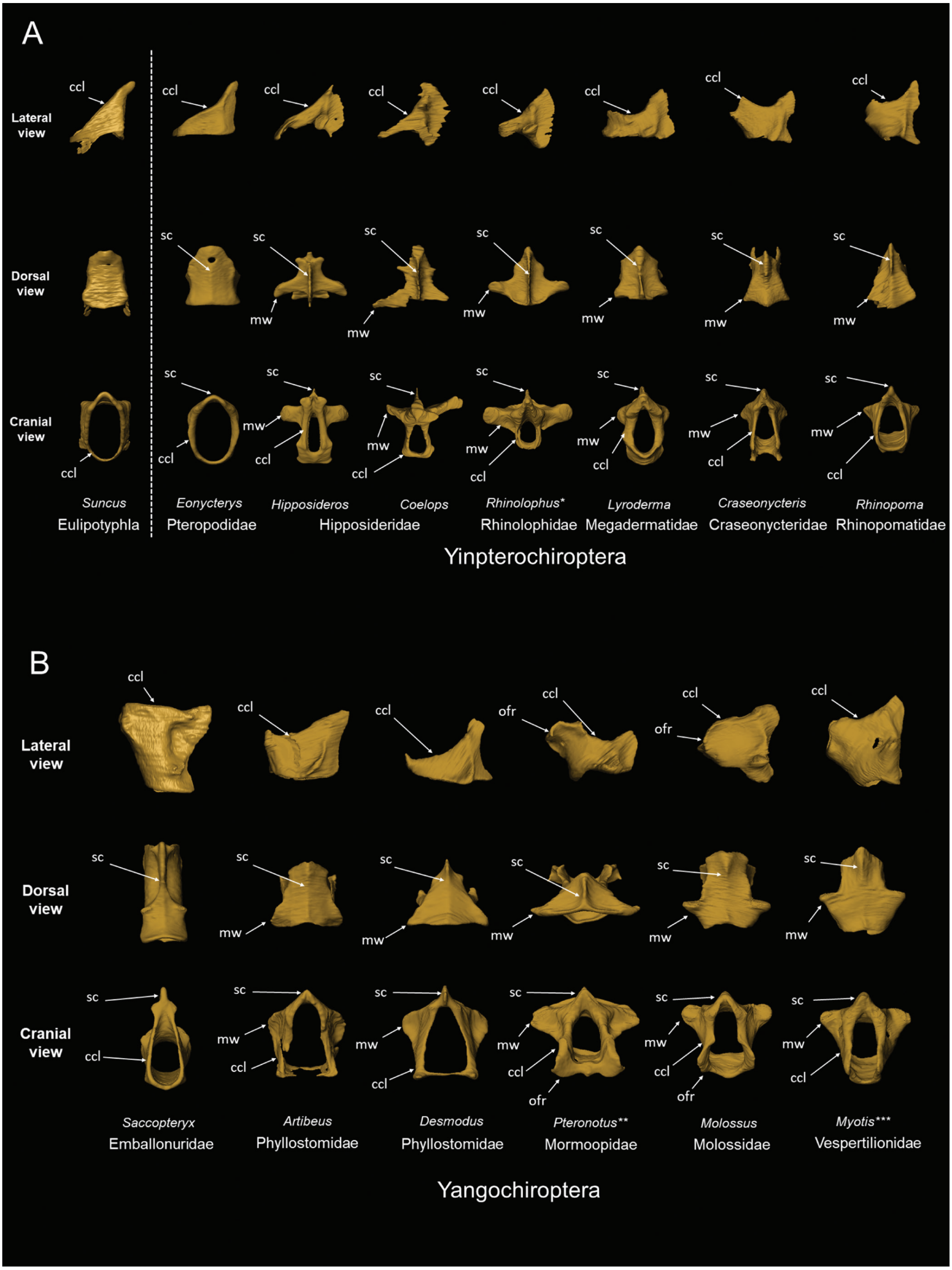

Figure 2. Comparison of the different cricoid forms. A, outgroup and Yinpterochiroptera species; B, Yangochiroptera. * *Rhinolophus cornutus*; ** *Pteronotus quadridens*; *** *Myotis albescens*. See text for abbreviations.

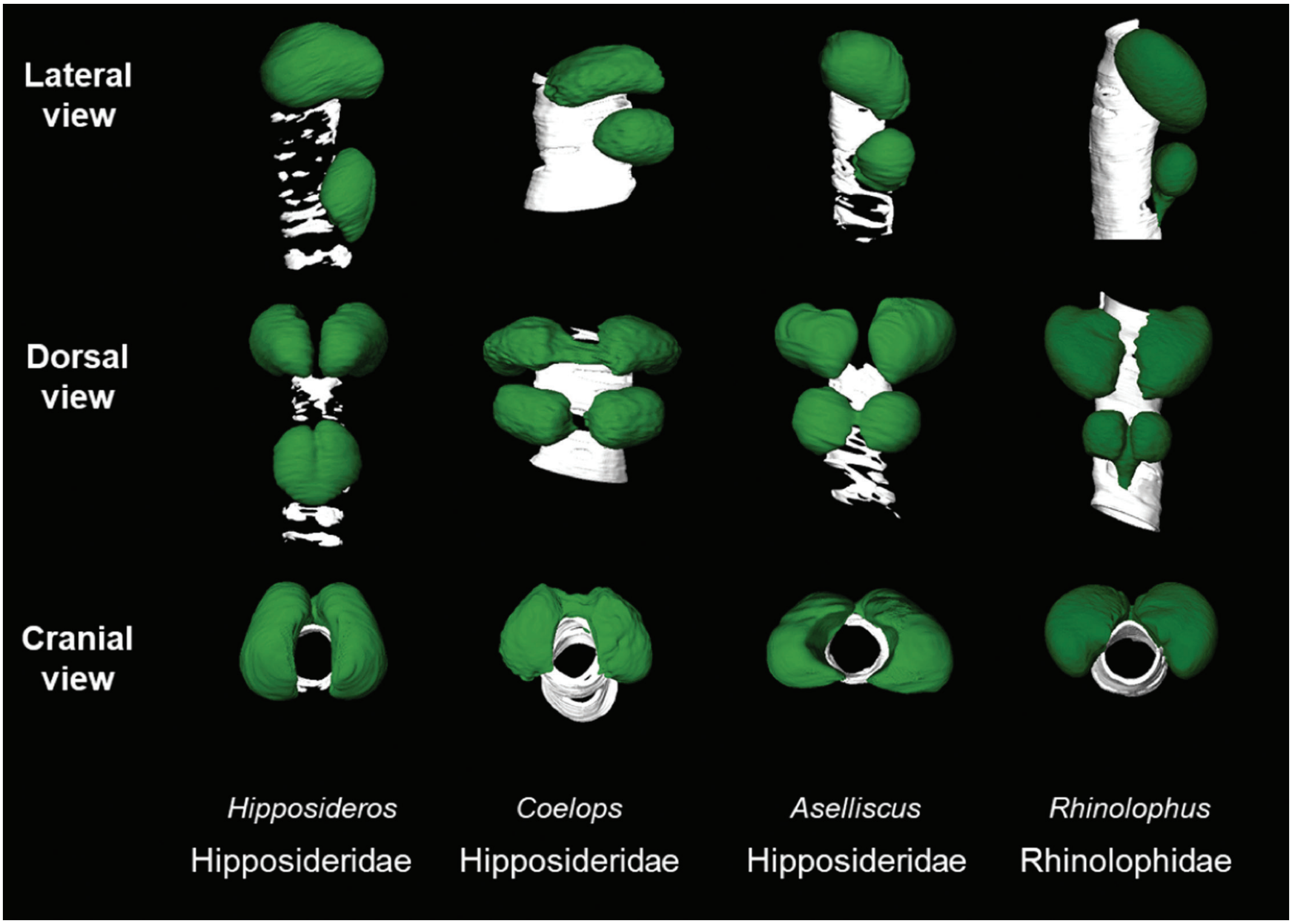

Figure 3. Comparison of the different tracheal chambers in *Hipposideros larvatus*, *Coelops frithii*, *Aselliscus dongbacanus*, and *Rhinolophus cornutus*.

than the hipposiderids (Fig. 3). Other families of rhinolophoids, such as the craseonycterids, megadermatids, and rhinopomatids, do not possess these tracheal chambers.

Thyroid cartilage

The pteropodids possess a thyroid cartilage similar in shape to that observed in the outgroup, with a U shape and the two cornua oriented craniocaudally (Fig. 4A). Hipposiderids and rhinolophids species have a more acute angle formed by the junction of the two laminae making a rounded V shape. Also, their cranial cornua are thinner than in pteropodids and oriented more ventrally. Their caudal cornu is extremely reduced and the cricothyroid joint is larger and more robust than that in other bats (Fig. 4A). The thyroid cartilage of megadermatids species is similar to the rhinolophids. The craseonycterids and rhinopomatids share a similar morphology, with thinner laminae than the other yinpterochiropterans. Their cornua are also different with the cranial cornu being ventrally oriented, parallel to the thyroid laminae, and the caudal cornu more dorsally oriented than in pteropodids (Fig. 4A). Yangochiropteran species possess a distinct thyroid cartilage form with a straighter V shape and extremely elongated caudal cornua (Fig. 4B). They also possess a small cartilaginous development laterally, a cranial thyroid tubercle. The emballonurids have the most narrowed V shape and the longest caudal cornua. The phyllostomids have a wider V-shaped thyroid and their cranial cornua are well developed, oriented ventrally along the laminae. *Rhinophylla fischerae* (Carter 1966) possess large thyroid laminae compared to other species (Fig. 4B). The mormoorpids species have different thyroid form than the phyllostomids, with a shorter and more robust caudal cornu, and a straighter V shape. The molossids thyroids are highly similar to those of the mormoorpids. Lastly, the vespertilionids have an extremely developed cranial cornu and cranial thyroid tubercle in *Myotis albescens* (Geoffroy 1806). Otherwise, their thyroid is similar to that observed in phyllostomids (Fig. 4B).

Arytenoid cartilage

The arytenoid cartilages are the most challenging part to reconstruct due to their low degree of mineralization and their small size (Fig. 5). In pteropodids, the structures appear different to the outgroup, and all other yinpterochiropteran families illustrate similar shape but with variation in size and the degree of development. The rhinolophids have well-developed arytenoid cartilages, with a caudally extended muscular process, a prominent corniculate process, and a short, vocal process (Fig. 5A). The emballonurids have bulky cartilages with extremely developed corniculate and vocal processes. The phyllostomid species

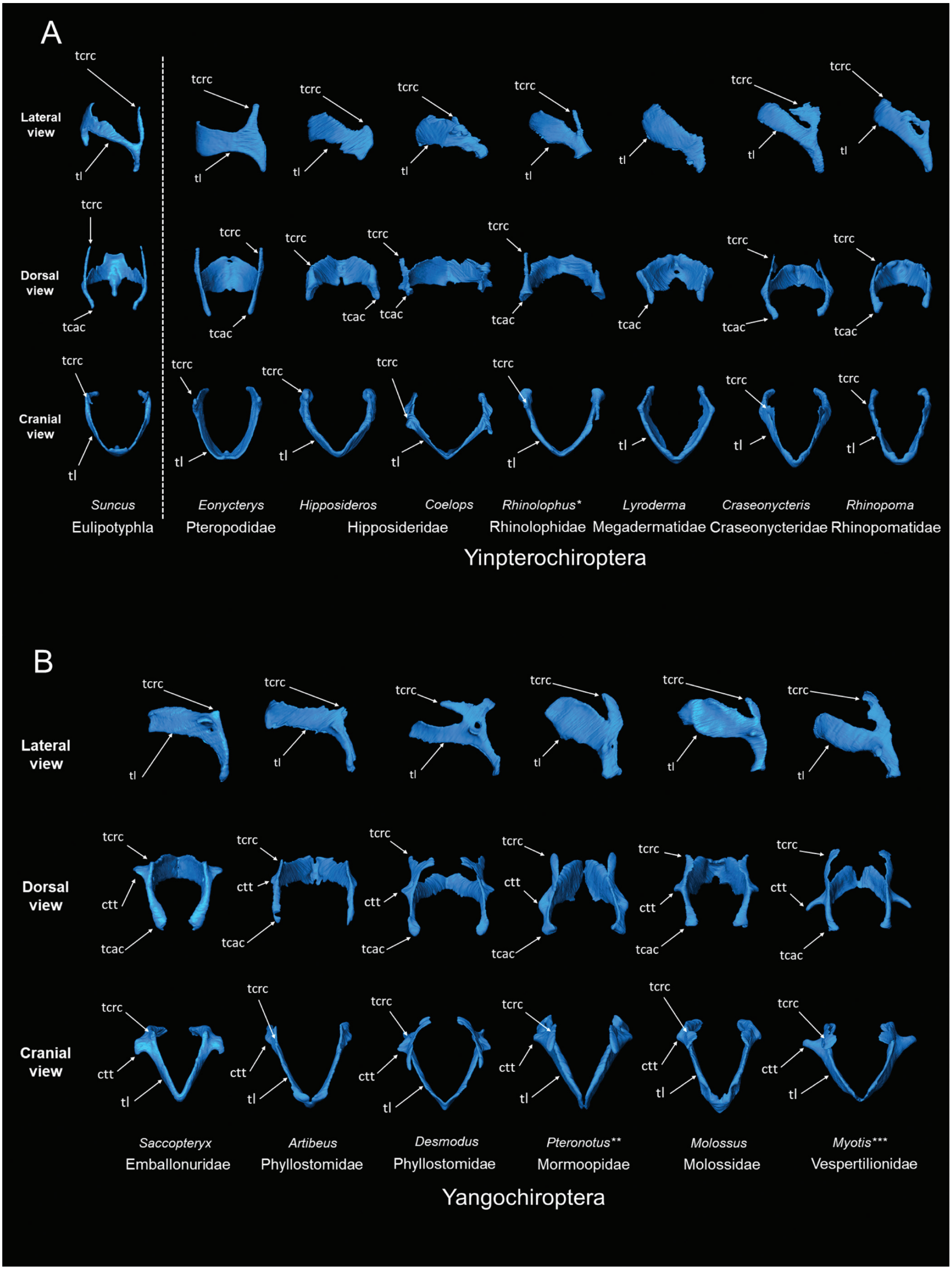

Figure 4. Comparison of the different thyroid forms. A, outgroup and Yinpterochiroptera species; B, Yangochiroptera. * *Rhinolophus cornutus*; ** *Pteronotus quadridens*; *** *Myotis albescens*. See text for abbreviations.

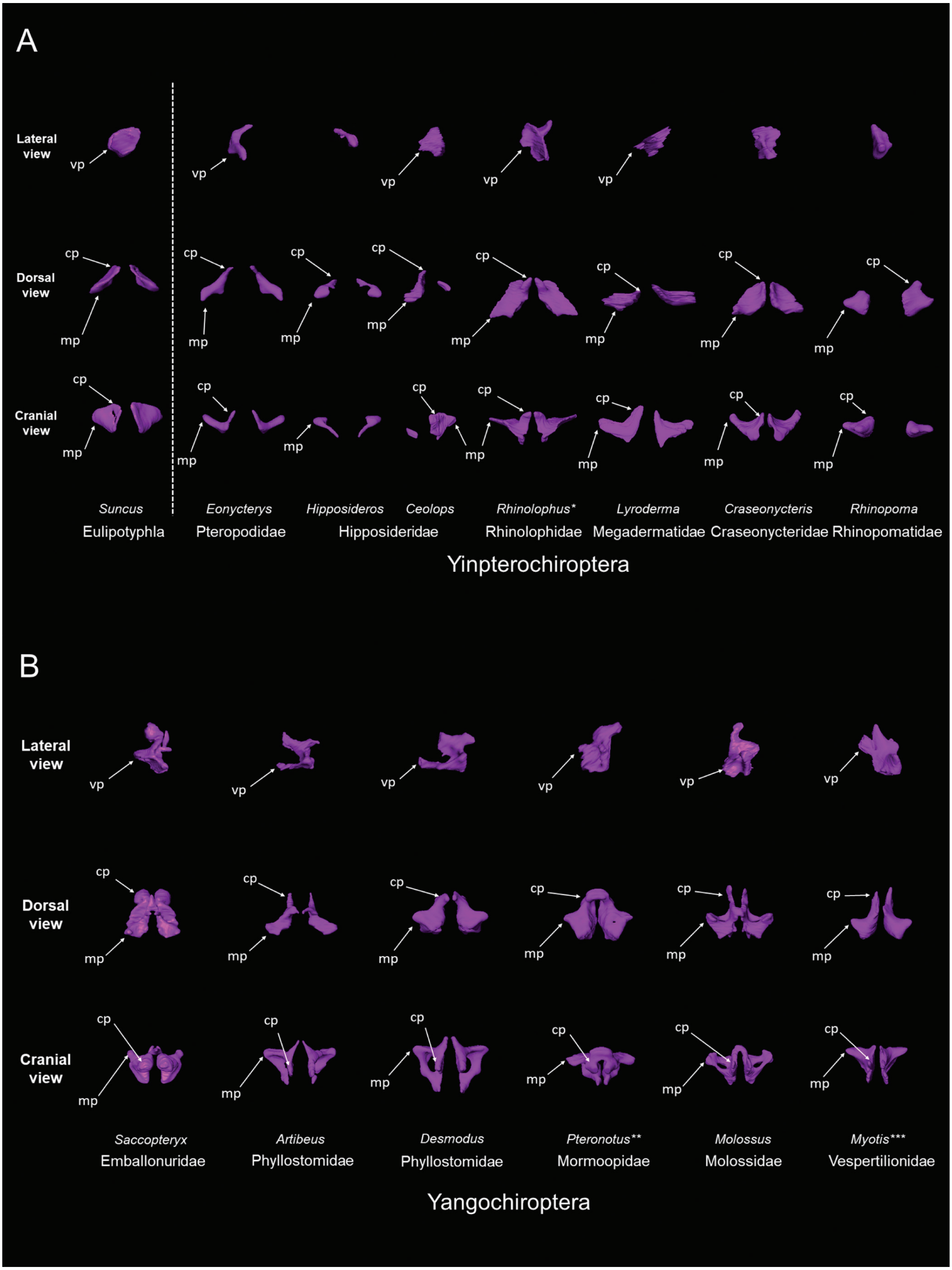

Figure 5. Comparison of the different arytenoid cartilage forms. A, outgroup and Yinpterochiroptera species; B, Yangochiroptera. * *Rhinolophus cornutus*; ** *Pteronotus quadridens*; *** *Myotis albescens*. See text for abbreviations.

have arytenoid cartilages developed more ventrally and laterally, with a shorter muscular process, large vocal process, and an elongated and curved corniculate process (Fig. 5B). In the mormoorpids, the cartilages are bulky but with short vocal and corniculate processes. The molossids possess similar arytenoid cartilages to the phyllostomids and mormoorpids but with a shorter vocal process. Lastly, the vespertilionids have a more robust and simple shape than the molossids (Fig. 5B).

Hyoid apparatus

The hyoid apparatus is variable in size and shape among bats (Fig. 6). The hyoid apparatus in pteropodids is similar to the outgroup, with only a more curved basal portion. The stylohyal cartilage is not in direct contact with the tympanic bone in pteropodids and *Suncus* (Ehrenberg 1832) (Fig. 6A). In all other bat species, the paddle-shaped tip of the stylohyal is in contact or fused with the tympanic bone. The hyoid apparatus in rhinolophids and hipposiderids are similar to one another, with a reinforced basihyal ventrally. The craseonycterids possess a short basal portion that presents a separation between basihyal and thyrohyals (Fig. 6A). The hyoid apparatus of rhinopomatids was difficult to reconstruct due to resolution issues and little can be inferred from our reconstruction, which may be artefactual. *Saccopteryx bilineata* (Temminck 1838) appears to have a longer greater cornu than all other species, with the development of the thyrohyals dorsally (Fig. 6B). The form of the hyoid apparatus in the phyllostomids and mormoorpids is similar to the other bats, with some internal development of the thyrohyals. The molossid species have similar shape and size but with the development of lateral tubercles on the thyrohyals, similar to the cranial thyroid tubercles (Fig. 6B). The vespertilionids have the most developed of these tubercles on their thyrohyals, which have a wing shape, flattened laterally. Also, the thyrohyals in vespertilionids species are separated from the basihyal, as in craseonycterids. Overall, the basihyal of yangochiropterans is comparatively smaller than in rhinolophoids.

Muscles

Cricothyroid muscle

Muscle volume is lower in pteropodids than in other bats, similar to the outgroup (Fig. 7). The hypertrophied muscle in rhinolophids is similar to that in hipposiderids but does not cover the ventral part of the cricoid cartilage (Fig. 7C). The cricothyroid muscle of the megadermatids is well developed and bulky. In *Rhinopoma hardwickii* (Gray 1831), the muscle is thinner than in *Lyroderma lyra* (Geoffroy Saint-Hilaire 1810), and the craseonycterid has a more cranially developed muscle (Supporting Information, Fig. S1). In all yangochiropterans, the cricothyroid muscle is extremely well developed and covers the thyroid cartilage externally but presents some variations (Fig. 7D; Supporting Information, Fig. S1). In emballonurids, the ventral part of the larynx is not covered by muscle. The phyllostomids and mormoorpids have a common shape with a developed cricothyroid muscle that almost covers the lateral and ventral portions of the larynx. Finally, the molossids and vespertilionids possess an extremely well-developed and bulky cricothyroid muscle, which does not cover the ventral part of the larynx (Fig. 7D).

Cricoarytenoid dorsalis muscle

The cricoarytenoid dorsalis muscle is relatively voluminous in bats. In pteropodids and *Suncus*, the muscle consists of one, thin, merged layer of muscle (Fig. 7A, B). In the hipposiderids, rhinolophids, and megadermatids, the muscle is hypertrophied and separated in two by the median crest (Fig. 7C; Supporting Information, Fig. S1). In *Rhinopoma* (Geoffroy Saint-Hilaire 1818) and *Craseonycteris* (Hill 1974), the size of the muscle is reduced. The emballonurids have a thin and extremely elongated muscle due to the tube shape of the cricoid cartilage. Their cricoarytenoid dorsalis muscle is also not in contact with the dorsal part of the cricoid but mainly with the elongated caudal cornua of the thyroid cartilage. The muscle observed in the phyllostomids and molossids is similar in shape to the one in the rhinopomatids, but thinner. The cricoarytenoid dorsalis muscle of *Pteronotus* is short but bulky and separated by the median crest (Supporting Information, Fig. S1). Finally, in the vespertilionid species, the muscle is elongated and relatively thin (Fig. 7D).

Cricoarytenoid lateralis muscle

The cricoarytenoid lateralis muscle varies in size and shape among the different bat families. The muscle is hypertrophied in yinpterochiropterans, except for the pteropodids, and relatively small in yangochiropterans and non-bat mammals (Fig. 7; Supporting Information, Fig. S1). In pteropodids, the cricoarytenoid lateralis muscle is shorter than the muscle observed in *Suncus*. The rhinolophids have an extremely bulky muscle compared to the other yinpterochiropterans (Fig. 7C). The megadermatids, rhinopomatids, and craseonycterids possess hypertrophied muscles but of shorter length than in rhinolophids. The muscle in emballonurids, mormoorpids, and molossids is highly similar to the small one of pteropodids, and it is elongated in the phyllostomids species as in *Suncus* (Supporting Information, Fig. S1). Lastly, the cricoarytenoid lateralis muscle of the vespertilionids is similar to that found in the phyllostomids but slightly curved (Fig. 7D).

Thyroarytenoid muscle

The pteropodids and phyllostomids have thin thyroaryenoid muscles. In hipposiderids and rhinolophids, the thyroarytenoid muscles are elongated and hypertrophied (Fig. 7B, C). The megadermatids, craseonycterids, and rhinopomatids species have more developed thyroarytenoid muscles than the pteropodids but less hypertrophied than in the hipposiderids. Similarly, the emballonurids have a well-developed muscle. The mormoorpids have a ventrally elongated muscle, relatively similar to the one in pteropodids. The molossid thyroarytenoid muscles have a similar elongated shape but are bulkier. Finally, the vespertilionids have thyroarytenoid muscles similar to those in hipposiderids (Fig. 7D).

Arytenoid muscles

The smallest muscles relative to larynx size are those in the rhinolophids and non-bats, and the most voluminous arytenoid muscles are observed in the phyllostomids (Fig. 7). The muscles are bulky in *Lyroderma* (Peters 1872), *Craseonycteris*, and *Rhinopoma*. The arytenoid muscles are similar in the emballonurids and *Craseonycteris*. The phyllostomids possess the

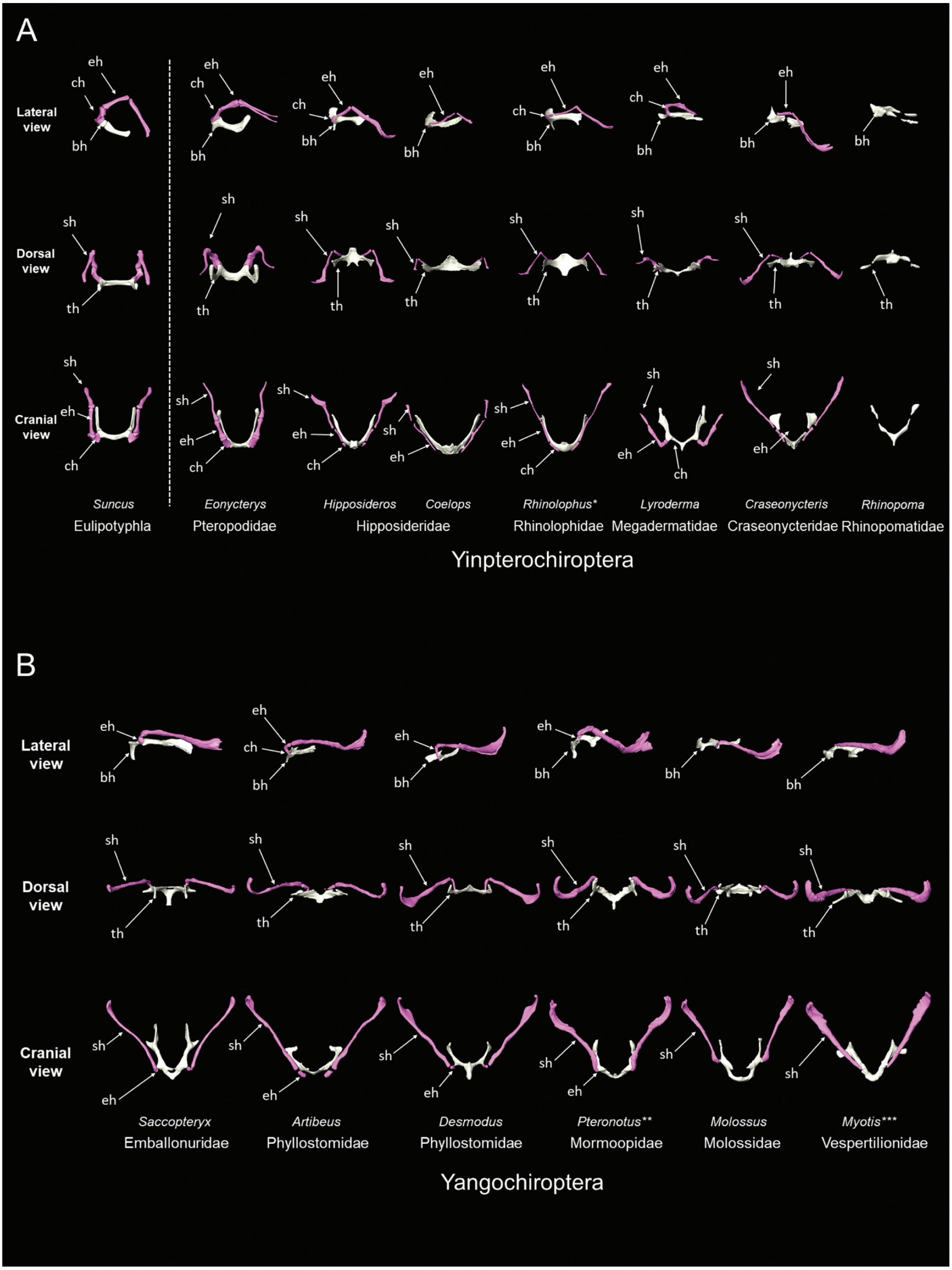

Figure 6. Comparison of the different hyoid forms. A, outgroup and Yinpterochiroptera species; B, Yangochiroptera. * *Rhinolophus cornutus*; ** *Pteronotus quadridens*; *** *Myotis albescens*. See text for abbreviations.

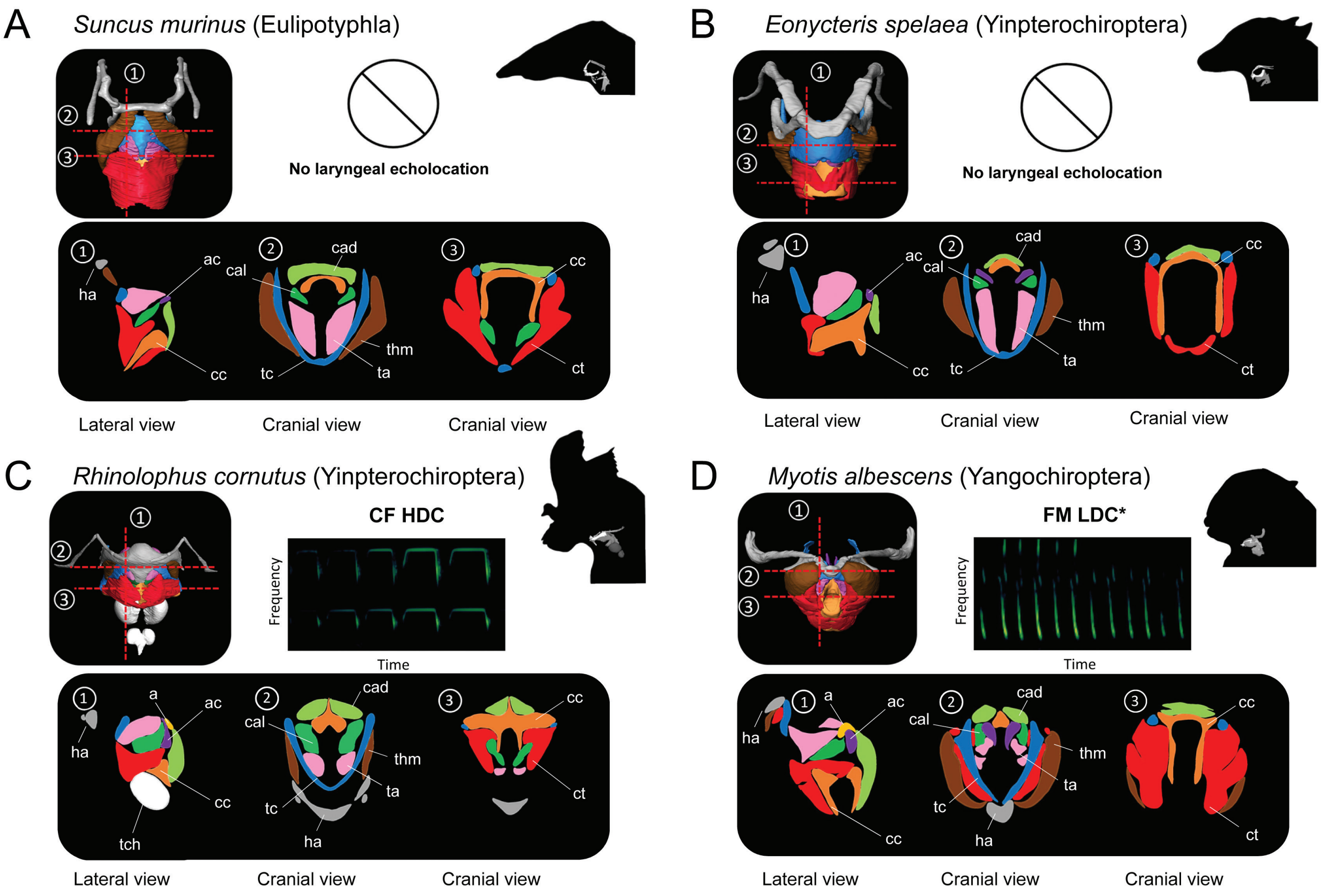

Figure 7. Muscle comparison of the different bat clades with the outgroup *Suncus murinus*, considering the main laryngeal echolocation strategies. A, *Suncus murinus*; B, *Eonycteris spelaea*; C, *Rhinolophus cornutus*; D, *Myotis albescens*. 1, sagittal section of the larynx, at mid-distance between the median crest and the cricothyroid joint. 2, horizontal section of the larynx, along the main axis of the thyroid cartilage. 3, horizontal section of the larynx, parallel to section 2, through the cricothyroid joint. * The spectrogram for the FM LDC echolocation strategy is from *Scotophilus kuhlii* (Leach 1821), another vespertilionid that has similar echolocation calls to *Myotis* sp. See text for abbreviations.

most ventrally and laterally well-developed muscles. *Pteronotus*, *Molossus molossus* (Pallas 1766), and *Myotis* (Kaup 1829) have arytenoid muscles of reduced size compared to those of phyllostomids (Fig. 7D).

Thyrohyoid muscle

This muscle also illustrates size variations between taxa. For some, the insertion is on the thyrohyal (Fig. 7); for *Suncus* it is on the basihyal. Rhinolophids have small thyrohyoid muscles, different in size from the hypertrophied thyrohyoid muscles observed in the vespertilionids and mormoopids (Fig. 7C, D). The hipposiderids and pteropodids have similar muscle size and shape. In rhinolophids the muscle is relatively thin. In contrast, the thyrohyoid muscle is bulky in *Craseonycteris*, and also elongated in *Rhinopoma* and *Lyroderma*. The thyrohyoid muscle is also elongated in the emballonurids species, and the one in phyllostomid species is similar to the one in megadermatids. *Pteronotus* has an extremely craniocaudally elongated muscle and *Molossus* (Geoffroy Saint-Hilaire 1805) has a similar shape but of shorter length (Supporting Information, Fig. S1). Lastly, the vespertilionids have an extremely hypertrophied muscle in contact with the cranial thyroid tubercle and the tubercle on the thyrohyals. The insertion in *Taphozous melanopogon* (Temminck 1841), *Kerivoula hardwickii* (Horsfield 1824), and *Myotis* is on the basihyal (Fig. 7D).

DISCUSSION

Our observations of the larynx yielded new insights into the understanding of anatomical diversity among bats and revised the previous propositions made by different studies (and reviewed in: Brualla *et al.* 2023). Different potential factors may impact observed variation in the laryngeal anatomy of bats, such as phylogenetic relationships, diet, and the capability of laryngeal echolocation, which for the latter is a highly demanding skill in terms of vocalization capacities. This study links morphological changes in bat larynges to different laryngeal echolocation strategies through the hypertrophied musculature and the different laryngeal cartilage forms. We noticed that the laryngeal cartilages in vespertilionids and phyllostomids have previously been described as thinner than in all other bat families (e.g. Elias 1907, Denny 1976, Carter 2020). Our results confirm this assertion, and we suggest that the thickness of the cartilage might be correlated with the use of laryngeal echolocation, as the phyllostomids rely on other senses, rather than only echolocation, to find their food, thus, involving less pressure on the laryngeal structures.

The shape of the cricoid arch in all bats studied herein has been previously described as a ring shape (e.g. Harrison 1995). We demonstrate that this is not the case for most of the families within rhinolophoids, which present an oval and narrowed cricoid arch (Fig. 2; Table 2). Also, all yangochiropterans, except for the emballonurids (that share a similar oval shape with the rhinolophoids), possess a more triangular shape of their cricoid arch (Fig. 2; Table 2). This reduction in size of the cartilages, such as the diameter of the cricoid arch, first, characterizes rhinolophoids and yangochiropterans from pteropodids and, second, opens more space between the cricoid and the thyroid laminae. It has been suggested that this surface reduction allowed for the muscles to develop, especially for the cricothyroid muscle involved in laryngeal echolocation (Fig. 7; Håkansson *et al.* 2022). To that, we add that the differential shape modification of the cricoid arch might be related to air pressure for high-frequency calls. Rhinolophids and hipposiderids (RH group) present the most narrowed cricoid arch, which might be correlated with the need for higher air pressure to produce their unique CF-HDC calls (Table 3; Fenton *et al.* 2012). Our observation agrees with the previous statement that the rhinopomatids and vespertilionids have similar laryngeal shapes (Robin 1881, Denny 1976). We have shown that similar ventral development of the cricoid cartilage and the similar shape of the thyroid laminae might have led authors to describe the laryngeal shape of rhinopomatids as close to that of vespertilionids (Fig. 2A, B). This ventral development of the cricoid cartilage has previously been described as exclusive to the megadermatids (Denny 1976), but we find the same pattern in other families, such as rhinopomatids and craseonycterids for yinpterochiropterans, and in the phyllostomids, molossids, and vespertilionids for the yangochiropterans (Figs 8, 9; Table 2). Further work is warranted to explain this development in these specific bat families. The cricoid cartilage of mormoorpids has also been noted as highly different from the one of phyllostomids (Griffiths 1978, 1983). Our results suggest a more complex situation. Phyllostomids present some intrafamilial variations, such as differential ventral development of the cricoid cartilage, and we suggest that morphological variation in the group may be correlated with its ecological diversity. The mormoopids have a cricoid cartilage craniocaudally flattened and the development of larger muscular wings, but still share an overall similar shape to the phyllostomids, especially regarding the triangular shape of the cricoid arch. Also, the mormoopids and molossids share a common development of an outwardly flared rim, with as yet unknown function. This rim has a different height in the *Pteronotus* species, as the cricoid cartilage of *Pteronotus quadridens* (Gray 1838) is more ventrally and cranially developed. In emballonurids, the tube shape of the cricoid cartilage is found in all species studied and it contradicts the previous idea that this shape is exclusive to *Taphozous* (Geoffroy Saint-Hilaire 1818) and *Saccolaimus* (Temminck 1938) (Fig. 2B; Robin 1881, Elias 1907, Brualla *et al.* 2023).

The development of a median crest on the dorsal part of the cricoid cartilage has previously been described only in pteropodids (Giannini *et al.* 2006), hipposiderids (Denny 1976), rhinolophids (e.g. Robin 1881), mormoorpids [*Pteronotus parnellii* (Gray 1843); Griffiths 1983], and vespertilionids (Robin 1881, Elias 1907). Our results show that the median crest is also developed to some degree on the cricoid

Table 2. Distribution of anatomical features in the different bat clades.

Clade		Cricoid Arch Shape	Muscular Wings	Median Crest	Cricoid Ventral Development	Tracheal Chambers	Thyroid Laminae Shape	Arytenoid Cartilage	Hyoid Apparatus	Styohyoid Shape	Muscle Hypertrophy
Non-bats and Pteropodidae		Rounded	-	-/ +	-	-	U	Average	Average	Drumstick	-
Rhinolophoidea	RH	Narrowed—Oval	+++	+++	-	++	Rounded V	Developed muscular process	Reinforced basihyal	Paddle	++ (CAD/CAL)
	MCR	Narrowed—Oval	++	++	++	-	Rounded V	Developed muscular process	Reinforced basihyal	Paddle	++ (CAD/CAL)
Yangochiroptera	Emballonuridae	Narrowed—Oval	+	++	++	-	Strict V + cranial tubercle	Developed vocal process	Shorten thyrohyals	Elongated and curved paddle	++ (CT)
	Phyllostomidae	Narrowed—Triangle	++	+	-/++	-	Strict V + cranial tubercle	Developed vocal process	Shorten thyrohyals	Elongated and curved paddle	++ (CT)
	Pteronotus sp.	Narrowed—Triangle	+++	+++	++ (flared rim)	-	Strict V + cranial tubercle	Developed vocal process	Reinforced basihyal	Elongated and curved paddle	++ (CT)
	Others	Narrowed—Triangle	++	++	-/++	-	Strict V + cranial tubercle	Developed vocal process	Shorten thyrohyals	Elongated and curved paddle	++ (CT)

CAD, cricoarytenoid dorsalis muscle; CAL, cricoarytenoid lateralis muscle; CT, cricothyroid muscle; MCR, Megadermatidae, Craseonycteridae and Rhinopomatidae group; RH, Rhinolophidae and Hipposideridae group; -, absent; +, reduced; ++, present; +++, extremely developed; -/++, absent in some species and present in others.

cartilage of all other bat families studied. In contrast to previous understanding, we postulate that it is a morphological feature shared among all bats despite differential development (Fig. 8; Table 2). The development of the median crest is still a source of debate concerning its role in laryngeal echolocation, as it can be visible on the cricoid cartilage of some pteropodids species, even being reduced (Fig. 2A; Giannini *et al.* 2006). Also, a similar median crest is observed in other mammals of large body size (e.g. in camel and donkey; Fig. 2; Eshra *et al.* 2016) to support the power of the muscle architecture (Harrison 1995),

Table 3. Distribution of anatomical features in the different laryngeal echolocation strategies.

Echolocation Type	Median Crest	Muscular Wings	Cricoid Ventral Development	Tracheal Chambers	Cricoid Arch Shape	Stylohyoid Shape	Muscle Hypertrophy
None or Non-LE	-/ +	-	-	-	Rounded	Drumstick	-
CF-HDC	+++	+++	-/++	-/++	Narrowed—Variable	Paddle	++ (CAD/CAL or CT)
CF-LDC	++	++	++	-	Narrowed—Oval	Paddle	++ (CAD/CAL)
FM-LDC	++	+/ ++	-/ ++	-/ ++*	Narrowed—Triangle	Elongated and curved paddle	++ (CT)

CAD, cricoarytenoid dorsalis muscle; CAL, cricoarytenoid lateralis muscle; CF, constant frequency; CT, cricothyroid muscle; FM, frequency modulated; HDC, high duty cycles; LDC, low duty cycles; LE, laryngeal echolocator; -, absent; +, reduced; ++, present; +++, extremely developed; -/++, absent in some species and present in others.

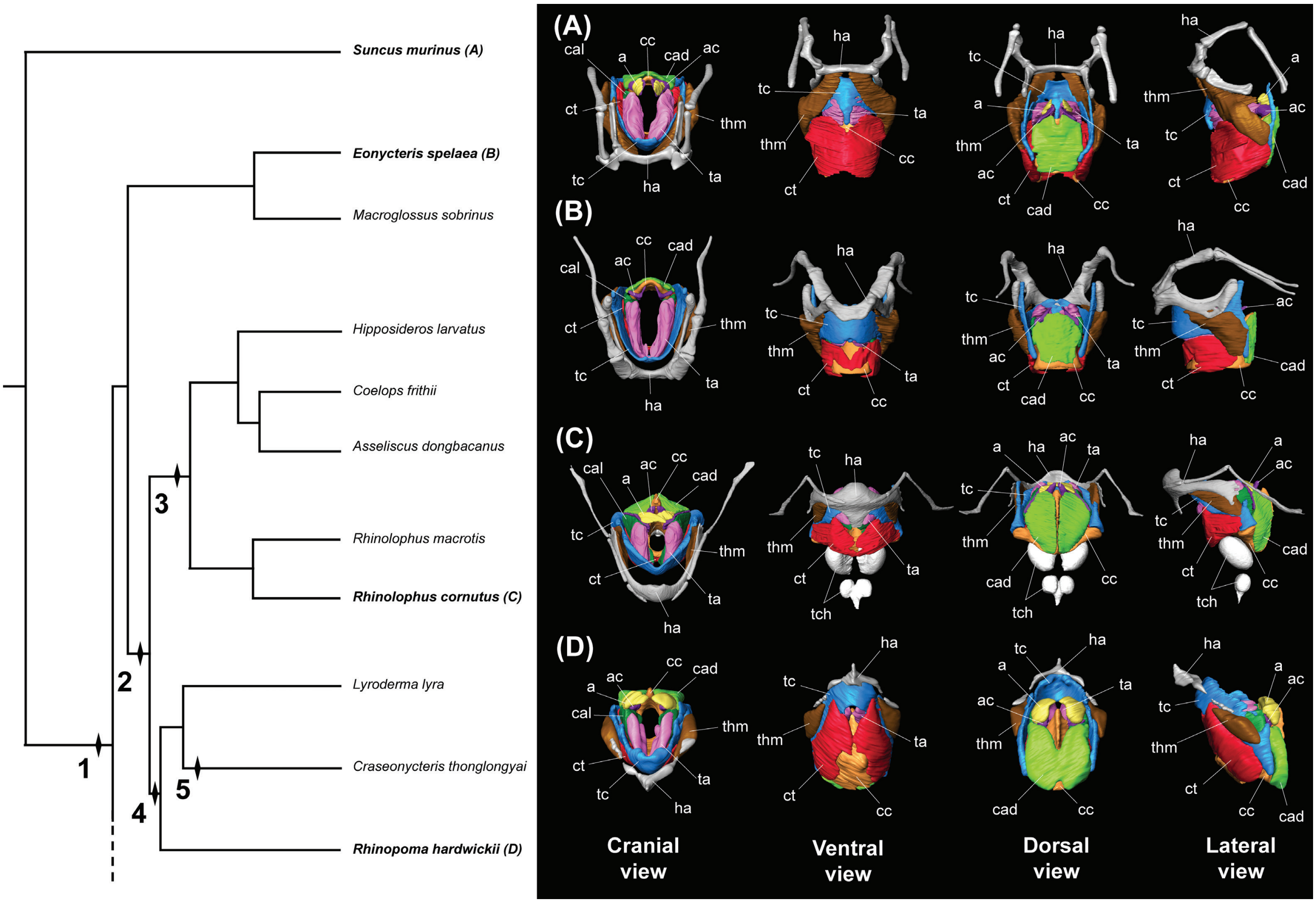

Figure 8. Diversity of laryngeal morphology in bats, with major traits mapped onto the phylogeny (Outgroup and Yinpterochiroptera). Laryngeal views from left to right: cranial view, ventral view, dorsal view, lateral view. Black lozenges represent the development of specific morphological traits: 1, development of the median crest; 2, hypertrophied muscles, stylohyoid in paddle shape, development of muscular wings, rounded V shape of the thyroid cartilage, oval narrowed laryngeal lumen; 3, extremely narrowed laryngeal lumen, extremely developed median crest and muscular wings, tracheal chambers; 4, ventral development of the cricoid; 5, thyrohyals and basihyal separated. See text for abbreviations.

and in smaller mammals such as dogs (Evans and De Lahunta 2012). We suggest that its presence in small mammals, such as bats and dogs, could be linked to their vocalization and/or to their requirements for breathing during intense activity such as running (dog) or flying (bat). Then, the extreme development of the median crest observed in the rhinolophids, hipposiderids, and *Pteronotus* species studied here could be a morphological feature linked to their CF-HDC laryngeal echolocation strategy (Fig. 7; Tables 2, 3; Supporting Information, Fig. S1). *Pteronopus* cf. *rubiginous* (Wagner 1843) in our dataset is one member of the *Pteronotus* cf. *parnellii* species group, which is the only group of the yangochiropteran suborder considered to be CF-HDC emitters (Dávalos 2006, Jones and Teeling 2006, Fenton *et al.* 2012, De Thoisy *et al.* 2014, Pavan and Marroig 2016; López-Baucells *et al.* 2017). *Pteronotus quadridens* has been described to be a CF-LDC emitter (Macías and Mora 2003, Mora and Macías 2011). We found that the cricoid cartilage of *P.* cf *rubiginous* is greatly reduced in height compared to *P. quadridens*, especially the cricoid arch, and this could express the differences between an HDC and LDC type of CF sound emissions. However, it is also essential to note that, despite a phylogenetic inertia that potentially constraints the overall laryngeal morphology among yangochiropterans, *P. quadridens* has a prominent median crest.

A similar issue is found for muscular wings, previously described in the rhinolophids (Harrison 1995). We demonstrate that muscular wings exist in all the families studied, except for pteropodids, with different degrees of development (Figs 8, 9; Giannini *et al.* 2006). This trait illustrates, once again, the distinction between pteropodids and the other bat clades (Table 2). The function of these muscular wings was theoretically stated to support the insertion of more voluminous muscles linked to laryngeal echolocation [e.g. Elias (1907)] and our data confirmed that enlarged muscular wings support the insertion of the bulky cricothyroid muscle (e.g. Denny 1976, Harrison 1995). Additionally, it changes the size of the contact surfaces of the cricothyroid joint (especially for the rhinolophids and hipposiderids) (Figs 4A, 8, 9). This reinforced connection of the cricothyroid joint could have an important biomechanical role, especially in high-frequency sound production (Table 3). Like the median crest, the extreme development of the muscular wings in rhinolophids, hipposiderids and *Pteronotus* species studied, could be correlated to their unique CF-HDC echolocation strategy (Figs 8, 9).

Concerning the thyroid cartilage, the narrowed V shape is shared between all laryngeal echolocators, compared to the rounded U shape of the cartilage in pteropodids and non-bats (Giannini *et al.* 2006). This feature's variation could be anatomically compared to the narrowing of the cricoid arch, which differs among families. A distinction is present between rhinolophoids and yangochiropterans, with a stricter V-shaped thyroid cartilage in the yangochiropterans. The rhinolophoids seem to possess an intermediate morphology of the cartilage, being between that of pteropodids and of yangochiropterans. Functionally, the shape modification might be correlated to laryngeal echolocation as pteropodids are the only bats with the rounded U-shaped cartilage commonly seen in other mammals. However, no inference regarding specific echolocation strategy can be made from our results. We noticed that the thyroid cartilage of vespertilionids was described as unique and divided into two parts, the lateral one being more flattened (Robin 1881, Elias 1907). However, our results are not in accordance with this description as we find in *Myotis* a complete lamina of the thyroid (Fig. 4A). It is possible that extremely low degrees of mineralization on the thyroid, except on the cricothyroid joint, the cranial thyroid tubercle parts, as well as the ventral tip of the laminae, may have led previous researchers to consider the thyroid cartilage of vespertilionids as two separate mineralized entities. The thyroid cornu in bats have been studied only by Elias (1907), Denny (1976), and Giannini *et al.* (2006), mainly in vespertilionids, rhinopomatids, and pteropodids. Our results show that the size and orientation of the thyroid cornu appear different in the laryngeal echolocators compared to the pteropodids (and non-bats) (Giannini *et al.* 2006). The former group possesses dorsoventrally tilted cornua and the latter possesses more craniocaudally orientated cornua. We also describe a short and large caudal cornu in rhinolophids and hipposiderids, and an elongated caudal cornu in all yangochiropterans. This distinguishes rhinolophoids from yangochiropterans and changes the contact on the cricothyroid joint, potentially enabling specific laryngeal echolocation strategies (Figs 4A, 8, 9). The arytenoid cartilages have been poorly reconstructed due to their non-mineralization and their small size, but from our results we suggest that rhinolophoids and yangochiropterans have different arytenoid development. In rhinolophoids, the muscular process is greatly developed, potentially to support the bulky cricoarytenoids involved in CF-HDC laryngeal echolocation (Fig. 5A). For the yangochiropterans, the vocal process is elongated, and this might change the length of the vocal folds, then changing the frequencies emitted (Fig. 5B).

Our study confirms that the shape of the stylohyal is a main difference in the anatomy of the hyoid apparatus between laryngeal echolocating bats and non-laryngeal echolocators (Figs 6, 8, 9; Tables 2, 3; Simmons *et al.* 2008, Veselka *et al.* 2010). In laryngeal echolocators, the paddle-shaped tip of the stylohyal is in contact or fused with the tympanic bone (Nojiri *et al.* 2021, Snipes and Carter 2023). Conversely, in non-bats and pteropodids, the stylohyal is similar to a thin drumstick and disconnected from the tympanic bone (Simmons *et al.* 2008, Veselka *et al.* 2010). In addition, our results show a suspensory apparatus of the hyoid positioned more laterally with the basal portion in laryngeal echolocating bats compared to a more cranially positioned one in non-laryngeal echolocators. Also, the ceratohyal of all laryngeal echolocating bats is diminished in size or absent in some taxa compared to the pteropodids and non-bat mammals. We suggest that these differences could be associated with different needs in sound conduction between laryngeal echolocating bats and non-laryngeal echolocators (Table 3). Lastly, the basihyal is reinforced in all rhinolophoids, especially rhinolophids and hipposiderids, and it contrasts with the reduced basihyal of the yangochiropterans. We suggest that this reinforcement allows more strength during vocal production of CF-HDC calls in these taxa. Overall, most anatomical descriptions of the hyoid made by Sprague (1943) are verified in this study. However, the separation of the basihyal and thyrohyal bones in most of the vespertilionids and in the craseonycterids found in this study were not described previously (Fig. 6).

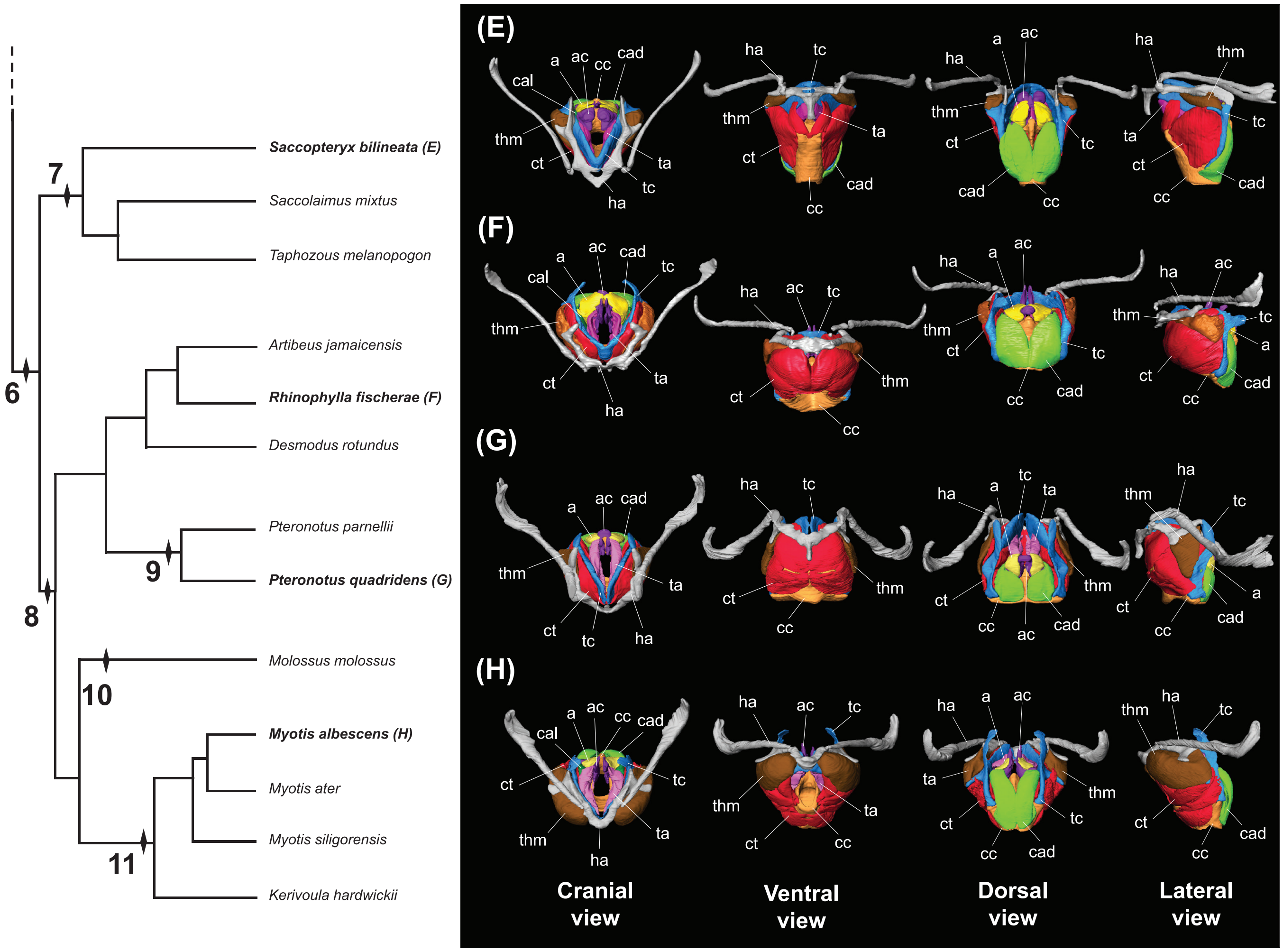

Figure 9. Diversity of laryngeal morphology in bats, with major traits mapped onto the phylogeny (Yangochiroptera). Laryngeal views from left to right: cranial view, ventral view, dorsal view, lateral view. Black lozenges represent the development of specific morphological traits: 6, hypertrophied muscles, stylohyoid in paddle shape, development of muscular wings, narrowed laryngeal lumen, elongated caudal cornu of the thyroid cartilage, narrow strict V shape of the thyroid cartilage, development of a cranial thyroid tubercle, insertion of the cricothyroid muscle on the cranial edge of the thyroid cartilage; 7, tube-shaped cricoid cartilage, extreme development of the median crest and the caudal cornu of the thyroid cartilage; 8, ventral development of the cricoid cartilage, triangular shape of the laryngeal lumen; 9, outwardly flared rim, extreme development of the muscular wings; 10, outwardly flared rim; 11, extreme development of the cranial thyroid tubercle, thyrohyals laterally elongated and separated from basihyal. See text for abbreviations.

The tracheal chambers have been described as potential Helmholtz resonators that could regulate sound amplitude, or potentially prevent the reverberation of the sound by tissue conduction from the lungs to the ears during vocal production (Denny 1976, Suthers *et al.* 1988, Harrison 1995). They are reported to be present in hipposiderids, rhinolophids, and nycterids (Nycteridae) (Robin 1881, Denny 1976, Harrison 1995, Brualla *et al.* 2023). Our results confirm the presence of these chambers in hipposiderids and rhinolophids, but we were not able to include nycterids in this study. No enlargement has been observed in the first tracheal rings of rhinopomatids, emballonurids, mormoorpids, and phyllostomids as compared to previous studies that note this feature (Sprague 1943, Denny 1976, Griffiths 1978, 1983, Griffiths and Smith 1991). The morphofunction of these features remains unclear and warrants further investigation with an expanded taxonomic sample. More precisely, as no tracheal chambers or enlargement has been identified in the larynges of *Pteropotus* species studied, these features may not be related to CF-HDC echolocation (Tables 2, 3).

The muscle hypertrophy observed in all laryngeal echolocators of this study possibly results from the necessity of high-speed call production. Recently, Håkansson *et al.* (2022) introduced a new understanding of bat hypertrophied musculature. In bats, superfast muscles are somewhat weaker than normal skeletal muscles, due to the balance between speed and force, the one diminishing if the other increases. To compensate for the force lost by adapting for speed, the muscles overgrow to keep their strength, because force is a function of a muscle's effective cross-sectional area. It has been proposed that the cricothyroid muscle is the main muscle involved in laryngeal echolocation and is hypertrophied in laryngeal echolocators (Figs 7, 8, 9; Griffiths 1983, Elemans *et al.* 2011). Here, we suggest a more complex scenario of muscle involvement. From our results, rhinolophoids are distinct from other bats by presenting hypertrophied cricoarytenoid (dorsalis and lateralis) and cricothyroid muscles, and the yangochiropterans possess relatively thinner cricoarytenoid muscles but present an extremely developed cricothyroid muscle that inserts more cranially on the thyroid laminae, overlapping the thyroid cartilage almost entirely (Fig. 7; Table 2; Supporting Information, Fig. S1). This distinction could be linked to the different echolocation strategies, CF calls being potentially produced with a reinforced action of bulky cricoarytenoid muscles (Table 3). The cricothyroid muscle of megadermatids is seen as uniquely complex (Griffiths and Smith 1991), but it appears to be only the two branches of the cricothyroid muscle that are well differentiated from one another compared to the other rhinolophoids. This specificity is also observed on the distantly related blood-feeding phyllostomid *Desmodus* (Wied-Neuwied 1826). We postulate that the cricothyroid muscle of the vespertilionids illustrates the greatest complexity, having several large and distinct fascicles (Fig. 7). The importance of a differential complexity of development in the cricothyroid muscle, present in all laryngeal echolocators, requires further clarification.

The thyroarytenoid muscle of the rhinolophids has been described as extremely voluminous and both cricoarytenoid (dorsalis and lateralis) muscles of vespertilionid species have been considered bulky and hypertrophied (Robin 1881, Elias 1907). Our observations appear to contradict these descriptions. The thyroarytenoid muscle in rhinolophids is of normal volume compared to other bat families of this study, and vespertilionids species possess some of the less voluminous cricoarytenoid muscles, especially the cricoarytenoid dorsalis muscle (Figs 7, 8; Supporting Information, Fig. S1). On the other hand, our results agree with the previous research describing the cricoarytenoid muscles of emballonurids as underdeveloped (Elias 1907, Griffiths and Smith 1991, Griffiths *et al.* 1991). Indeed, we describe the cricoarytenoid dorsalis muscle as expanded caudally but relatively thin. The cricoarytenoid lateralis muscle is also relatively small, potentially due to the massive cricothyroid muscle of this family developed along the tube-shaped cricoid cartilage. Finally, the cricothyroid muscle of phyllostomids is described as thin and underdeveloped, which does not correspond to our results (Fig. 9; Griffiths 1978, 1982). Nonetheless, our results demonstrate some volume variations of the cricothyroid muscle in phyllostomids. *Desmodus* and *Rhinophylla* (Peters 1865) possess hypertrophied and developed muscles. Only *Artibeus* (Leach 1821) present developed but thin cricothyroid muscles. The cricothyroid muscle of phyllostomids probably illustrates a more complex interconnection between muscle mass, diet, and echolocation strategy. We find that the thyrohyoid muscle originates from the caudal tip of the caudal thyroid cornu in non-bat mammals and pteropodids but that it originates more cranially, from the laminae, in most of the laryngeal echolocators. This difference correlates with the idea that the thyrohyoid muscle, not being an intrinsic laryngeal muscle, could play a role in laryngeal echolocation by pulling the basihyal into the thyroid for sound conduction through vibration (Novick and Griffin 1961, Griffiths 1983, Snipes and Carter 2023). However, the thyrohyoid varies in its insertion areas (basihyal or thyrohyal bones) in different families, so it is difficult to fully understand its implication in sound production. A similar situation is observed for variation in the size of the thyroarytenoid among bats (Fig. 7A, D; Supporting Information, Fig. S1).

Overall, we show that the pteropodids (as non-laryngeal echolocating bats) share similar laryngeal morphology with other non-laryngeal echolocators, reinforcing the idea that laryngeal echolocation could be responsible for some of the morphological variation in other bat taxa (Figs 7, 8; Table 3). We observe almost no intrafamilial morphological variation (except for the phyllostomids). In the rhinolophoids, there are several main differences. The RH group possesses tracheal chambers, extreme development of the muscular wings and median crest, and extremely narrowed oval shape of the cricoid arch. The ventral development of the cricoid arch is specific to the MCR group (megadermatids, craseonycterids, and rhinopomatids) despite an overall laryngeal form relatively intermediate between the pteropodids and the RH group (Fig. 8; Table 2). In addition, among the MCR group, the megadermatids are relatively different from the two other families (lesser ventral development and muscular wings; Figs 2A, 4A; Table 2), and they also have a slightly different laryngeal echolocation strategy than the others (FM-LDC laryngeal echolocators with some short CF calls; Schmidt *et al.* 2011, Smarsh and Smotherman 2015). The craseonycterids and rhinopomatids have been described as CF-LDC (Surlykke *et al.* 1993, Fenton *et al.* 1995, Hiryu *et al.* 2016), with rhinopomatids species able to produce FM-LDC calls (Shah and Srinivasulu 2020). The CF calls are generally considered more behaviourally derived than the FM calls (Fenton *et al.* 2012), which could explain why the laryngeal morphology of the megadermatids appears less derived than the rhinopomatids and craseonycterids and still modified compared to the morphology of non-laryngeal echolocators (pteropodids and non-bats) (Fig. 8). In the same way, the species of the RH group possess a more derived morphology, which could be a sign of specialization to the CF-HDC laryngeal echolocation strategy (e.g. extreme development of median crest and muscular wings). Among hipposiderids, some differences are found in the laryngeal form of *Coelops frithii* (Blyth 1848) compared to other species, despite an overall similar morphology. *Coelops'* (Blyth 1848) laryngeal lumen and thyroid laminae are relatively wider than in other hipposiderids and are features that distinguish the overall laryngeal form of this species from *Hipposideros* (Gray 1831) and *Aselliscus dongbacanus* (Tu *et al.* 2016). This species is also distinctive among hipposiderids as it does not use long CF calls. Instead, it primarily employs FM calls with a short CF component at the end (Hughes *et al.* 2012), which may account for its morphological divergence from other hipposiderids observed here. All these differences express that laryngeal morphology appears constrained by phylogeny in rhinolophoids and that the extreme morphological variation found within the RH group is correlated with the unique laryngeal echolocation strategy used by these bats. Because yangochiropterans share unique morphological features among themselves, such as the triangular shape of the cricoid arch, the V-shaped thyroid cartilage, the cranial thyroid and hyoid tubercles, and the extreme development of the cricothyroid muscle, similar conclusions about a phylogenetic constraint may be drawn. However, yangochiropterans illustrate some morphological variation with the tube-shaped cricoid of the emballonurids, the thin diamond-shaped cricoid cartilage supporting thin muscles in the phyllostomids, and the outwardly flared rim on the cricoid cartilage of the molossids and mormoopids species. This diversity can be explained by the great diversity of species and ecologies among yangochiropterans. However, our sample size does not allow us to draw precise correlations. Despite similar shapes, the development of the cricoid and thyroid cartilages of *P. quadridens*, being greatly enlarged cranially, contrasts to the relatively thin cartilages of *P.* cf *rubiginous*. How these differences relate to echolocation strategy appears unclear; indeed, the 3D reconstruction of *P.* cf *rubiginous* has been difficult due to image quality issues, especially the non-mineralized part of some cartilages (Supporting Information, Table S1; MorphoMuseum repository 3D models 'https://doi.org/10.18563/journal.m3.219'). Still, we can infer that the strategy of constant frequency echolocation strategy has more constraints on the overall shape of the cartilages than the duty-cycle strategies, the first one being associated with the large development of the median crest and muscular wings.

Laryngeal echolocation is also characterized by two strategies of sound emission, through the nasal cavities or through the mouth (e.g. Pedersen 2000, Pedersen and Müller 2013). The nasal emission is a polyphyletic trait shared convergently among all rhinolophoids and with the nycterids, phyllostomids and several species of vespertilionids (Jakobsen *et al.* 2018). While bioacoustic research has been conducted to understand the differences in sound emissions, no specific laryngeal morphology stands out to differentiate nasal from oral emitters (Pedersen and Müller 2013). Similarly, we find no evidence of morphological patterns that differentiate the rhinolophoid and phyllostomid nasal emitters from the other oral emitters among yangochiropteran species. Such investigations require further research, and the qualitative aspect of this article prevents comment on these hypotheses. Furthermore, other parameters such as diet or flying habits could be influencing laryngeal anatomy, as they vary from one family to another. However, the larynx may be viewed in relation to the concept of modularity (Zelditch and Goswami 2021), whereby two levels of modularity could be implied to explain nasal sound emission in phylogenetically distant bat clades, despite the absence of a unifying morphology. The first level would consider the laryngeal components (cartilages and muscles) as modules that function (and potentially evolve) in interaction with the cartilage developing and functioning to support the muscle development and function. The second level of modularity would place the larynx as a module inside the throat, with the pharynx and the rostrum as other modules. In this case, the nasal sound emission could still be produced despite an independent evolution of the larynx, by adaptability of the other modules (pharynx and rostrum).

Perspectives for future studies

Further studies should focus on the collection of quantitative data to investigate the morphofunction of the laryngeal features described here. Although this study has a small number of species compared to the high number of bat representatives, and we cannot correlate the morphology for all bat species diversity to a specific functional specialization (e.g. the tube shape of *Taphozous*), this study can still illustrate an overall aspect of bats' laryngeal morphology. Also, our findings are consistent with the degrees of mineralization that have already been discussed in previous studies (e.g. Denny 1976, Carter 2020). Further work on the larynx should cover the different families not yet described in yangochiropterans, as well as species known to be morphologically unique, such as the hammer-headed bat (*Hypsignatus montrosus* Allen 1861), known for its mating vocalizations and possessing a larynx one-half the length of the spine, and for being sexually dimorphic (Langevin and Barclay 1990).

CONCLUSIONS

In the present study, we investigated the variation in the laryngeal anatomy of bats. We found that several forms were distributed across bat phylogeny, not specifically linked to muscle hypertrophy. We observed distinctive laryngeal features and forms between the pteropodids and the rhinolophoids, and between yangochiropterans and yinpterochiropterans (Figs 8, 9; Table 2). Rhinolophoids and yangochiropterans possess distinct laryngeal forms that we consider as two distinct morphotypes (Figs 8, 9; Table 2). The larynges of pteropodids share with non-bat mammals several features that are modified in rhinolophoids and yangochiropterans, such as the modifications of the cricoid cartilage shape and development of muscular wings, the V shape of the thyroid cartilage, the hypertrophy of most of the laryngeal muscles, and the paddle shape of the stylohyoid chain. The most distinctive aspect of the yangochiropteran laryngeal morphology is the extreme development of the cricothyroid muscle, overlapping the entire ventral and lateral surface of the larynx. Narrowed cricoid cartilage arch, extreme development of the median crest and muscular wings, enlargement of the cricothyroid joint, muscular process of the arytenoid cartilage, and of the basihyal bone may be associated with the CF-HDC echolocation strategy. No specific form or feature was found to unite the polyphyletic the nasal emitters. Nasal and oral emitters inside yangochiropterans share similar forms, distinct from the rhinolophoids (all nasal emitters), as the most morphological variations are distributed along the phylogeny.

These findings validate several previous statements regarding the laryngeal anatomy of bats and adjust or improve other observations thanks to the use of modern protocols (virtual dissection of contrast-enhanced X-ray microtomography images). This study offers new perspectives of research on the laryngeal morphofunction, its implication in different laryngeal echolocation strategies, and, to some extent, research on the evolutionary history of bats and their laryngeal echolocation.

SUPPLEMENTARY DATA

Supplementary data are available at *Zoological Journal of the Linnean Society* online.

ACKNOWLEDGEMENTS

We thank Cody W. Thompson for approving access to the scan data of the University of Michigan. We thank Laurel Yohe for providing staining protocol information and sharing her materials on Morphosource. We thank Kai Ito for his help with scanning.

This study was supported by City University of Hong Kong Start-up Grant (9610466), JSPS (21H02546, 21K19291, and 22KK0101), JST (JPMJFR2148) to D.K., and a grant from the Australian Research Council to L.A.B.W. (FT200100822).

CREDIT STATEMENT

D.K. and L.A.B.W. conceptualized the project and obtained funding.
D.K., L.A.B.W., V.T.T., T.N., R.T.C., T.N., T.W., and D.F. gathered samples and performed CT scanning.
D.K., L.A.B.W., and M.D. supervised the study.
N.L.M.B. curated the scan data, processed the 3D reconstructions, studied the data, and summarized the results.
N.L.M.B., L.A.B.W., and D.K. drafted the manuscript; all authors provided insights, critically revised the text and the figures.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

DATA AVAILABILITY

Surface data that support the findings in the present study are available from the Morphomuseum repository (https://doi.org/10.18563/journal.m3.219) or Morphosource.

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