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Independent origins of a novel atympanic middle ear system within Chamaeleonidae

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Abstract

The evolution of the vertebrate ear is a complicated story of convergence, co-option, loss of function, and occasional regaining of said function. An incredible variety of structures has been adopted as sound receptors, but only chameleons are known to have a bony airborne sound receiver. In some chameleons, the pterygoid bone captures sound vibrations and relays them to the inner ear via a connection to the extracolumella. The distribution of this unique hearing system has not been examined across Chamaeleonidae. Here, I report on dissections on 12 species across four genera and describe their middle ear anatomy for the first time. Half of these species were found to have a link between their extracolumella and pterygoid, and ancestral state reconstruction supports four independent acquisitions of this novel sound-conduction pathway. Species with this pathway tend to have a gular pouch, which seems to produce biotremors and possibly airborne sound, suggesting that this hearing system plays some role in intraspecific communication. Three species were also μ-CT scanned using enhanced contrast to investigate differences in the musculature surrounding the middle ear cavity. In species with a middle ear connected to the pterygoid, the muscles directly lateral to the pterygoid insert farther anterior onto the mandible, which may serve to minimize dampening of vibrations on the pterygoid. Together, these data suggest that the ear plays a more significant role in the lives of some chameleons than has been recognized, and that parallelism is common in the evolution of the ear.

KEYWORDS
chameleon, co-option, diceCT, exaptation, hearing, middle ear

1 | INTRODUCTION

The ear is arguably the most evolutionarily labile of the vertebrate sensory systems. The sense of hearing can play an important role in navigation and orientation, localization of resources and habitat, detection of prey and predators, and intraspecific communication (Fay, 2009; Fay & Popper, 2000; Gans, 1992; Slabbekoorn, 2018), and this wide range of functions is reflected in its morphology. While the structure of the inner ear is relatively conserved (Popper & Fay, 1997), the pathway by which sound vibrations reach the inner ear from outside the body exhibits astounding variation among vertebrates. Specializations in this pathway typically serve to amplify sound vibrations as they pass to the inner ear (Wever & Lawrence, 1954). Though this amplification is less important for aquatic taxa, many of which lack a specialized pathway, the difference in acoustic impedance between air and water makes such amplification essential for a terrestrial tetrapod ear.

Vertebrates have evolved a myriad of ways to solve the problems of sound amplification and impedance matching. Several teleost lineages have independently evolved anterior extensions of their swim bladder, which amplify sound vibrations that can then spread to the nearby inner ear (Braun & Grande, 2008). In the Otophysi, vibrations in the swim bladder are transmitted to the inner ear via a set of highly specialized vertebrae termed the Weberian apparatus (Fay & Popper, 1974). Most tetrapod middle ears recruit the stapes or columella as a sound conductor, as this bone has a convenient articulation with the otic capsule (Westoll, 1943). Most tetrapods capture sound vibrations using a tympanum exposed to the air. This membrane transfers vibrations to the stapes/columella via the bony malleus and incus in extant mammals and the cartilaginous extracolumella in amphibians, reptiles, and birds. The ratio of the area of the tympanic membrane to that of the stapes/columella footplate is great enough to amplify vibrations and allow the detection of airborne sound (Wever & Lawrence, 1954). Evidence from embryonic development and the fossil record demonstrates that the tympanum and its connection with the middle ear ossicle(s) have evolved multiple times throughout Tetrapoda (Clack, 1993; Kitazawa et al., 2015; Lombard & Bolt, 1979; Martin & Luo, 2005). However, many tetrapods do not rely solely on the tympanum for sound reception, and several clades have lost the tympanum entirely, only to replace it with another means of sound reception. Frogs and salamanders have evolved the opercularis system, in which the opercularis muscle is hypothesized to communicate vibrations to the operculum and/or stapes to be relayed to the inner ear (Capshaw & Soares, 2016). This system is especially labile in taxa with relaxed selection for intraspecific communication. Some atympanic frogs use the resonant properties of their mouth to amplify mating call frequencies and divert those vibrations directly to the tissue surrounding the otic capsule (Boistel et al., 2013). Amphisbaenid lizards lack a tympanum, so the extracolumella instead extends into the skin of the lower jaw beneath a large, shield-like scale capable of transmitting airborne sound (Toerien, 1963; Wever, 1978). Odontocetes use fat masses around their mandible and throat to divert high-frequency echolocation signals to their inner ear (Cranford et al., 2008). The diverse structures that constitute the vertebrate ear provide a fascinating system for studying evolutionary processes like convergence, co-option, and novelty.

One of the most distinctive sound conduction pathways is found in several species of chameleon. The tympanum has been lost in all chameleons, possibly as a result of specializations in the feeding apparatus (Tucker, 2017), so the ears of most chameleons can only detect substrate vibrations through bone conduction (Wever, 1978). As a result, the middle ear is vestigial in many chameleons, its structures reduced or sometimes completely obliterated (Brock, 1940; Engelbrecht, 1951; Frank, 1951; Siebenrock, 1893; Toerien, 1963). However, early anatomical descriptions of *Chamaeleo chamaeleon* document a well-developed middle ear cavity and columella (Parker, 1881; Versluys, 1898), which prompted a series of dissections and physiological experiments by Wever (1968, 1969, 1978) that demonstrated that several chameleons have adopted alternate routes for sound transmission. Members of the genus *Chamaeleo* were found to have an anterior process of the extracolumella that attaches to the quadrate ramus of the pterygoid, which in all chameleons is dorsoventrally expanded into a thin plate with an unossified membrane at the posterior end contacting the quadrate. His experiments showed that vibrations passing through the tissue of the side of the head are collected by the wide pterygoid plate and associated membrane and directed to the inner ear via the extracolumella and columella, which allows these animals to hear low frequency sounds (~200–600 Hz) almost as well as lizards with tympanic ears (Wever, 1968, 1978). Some members of the genera *Trioceros* and *Kinyongia* instead have an extracolumella that is partially embedded in the depressor mandibulae, which acts as a sound receiver in these species, albeit a less effective one than the pterygoid plate (Wever, 1969, 1978). This second pathway is partially present in *Chamaeleo*, as well, though the connection between the extracolumella and the depressor mandibulae is weaker and less useful for sound conduction (Wever, 1978).

Recent work has demonstrated that several species within the genera *Bradypodion*, *Chamaeleo*, *Furcifer*, and *Trioceros* use their gular pouch to produce vibrations to communicate with conspecifics (Huskey et al., 2020). Species in which Wever found a connection between the extracolumella and pterygoid plate were also shown to have a well-developed gular pouch. Species lacking this connection also lacked a gular pouch. The presence of a gular pouch in several genera other than *Chamaeleo* raises the possibility that the pterygoid ear is more widespread within Chamaeleonidae than previously thought. An expanded survey of chameleon middle ear anatomy is therefore timely, as it may reveal a complex evolutionary history of the chameleon ear and its role in social behavior.

I performed dissections on museum specimens of a wide array of chameleons to assess how widespread the pterygoid ear is within Chamaeleonidae. I also μ-CT scanned specimens of three species using the diffusible iodine-based contrast-enhanced computed tomography (diceCT) technique of Gignac and Kley (2014) and Gignac et al. (2016) to further investigate the morphology of the middle ear cavity. These scans were used to compare the arrangements of jaw adductor muscles surrounding the pterygoid plate between species that are hypothesized to use this bone for hearing and those that are not.

2 | MATERIALS AND METHODS

2.1 | Institutional abbreviations

CM, Carnegie Museum of Natural History, Pittsburgh, PA, USA; FMNH, Field Museum of Natural History, Chicago, IL; UCMVZ, University of California Berkeley Museum of Vertebrate Zoology, Berkeley, CA; UF, Florida Museum of Natural History, Gainesville, FL; UWBM, University of Washington Burke Museum of Natural and Cultural History, Seattle, WA; VTPE, Virginia Polytechnic Institute and State University, Blacksburg, VA; YPM, Yale Peabody Museum, New Haven, CT.

2.2 | Materials

All specimens were from museums or university teaching collections. They were acquired by wild capture or donations from zoos or pet trade breeders. The following species were dissected: *Chamaeleo calyptratus*, *Chamaeleo gracilis*, *Chamaeleo gracilis* or *Chamaeleo senegalensis*, *Chamaeleo zeylanicus*, *Calumma brevicorne*, *Furcifer lateralis*, *Furcifer oustaleti*, *Furcifer pardalis*, *Furcifer verrucosus*, *Kinyongia multituberculata*, *Trioceros cristatus*, *Trioceros ellioti*, *Trioceros jacksonii*, *Trioceros johnstoni*, *Trioceros melleri*, and *Trioceros montium* (see Appendix 1 for details of each specimen). Most dissected specimens were formalin fixed and stored in alcohol, but the specimens at VTPE and UWBM were frozen and thawed.

The breeder from which VTPE acquired the *Kinyongia* specimen used in this study listed the species as *Kinyongia fischeri*. However, DNA evidence has revealed a multitude of species that were once lumped together under this species name, and it is unlikely that this specimen represents a true member of *K. fischeri* (Mariaux et al., 2008). Using the identification key provided by that study, the VTPE specimen is identified as *K. multituberculata* based on the posterior extent of the dorsal crest and the presence of horns in a female.

2.3 | Methods

This work will follow terminology used by Wever (1978) for chameleon ear structures and Anderson and Higham (2013) for muscles.

Specimens from the FMNH, UF, and UWBM were dissected at the University of Washington under a Wild M5 dissecting microscope and imaged using Leica Acquire V1.0 (© Leica Microsystems, Buffalo Grove, IL) on a Leica M125 microscope. Photographs of these specimens were taken with a Canon macro lens EF 100 mm. I dissected the lateral side of the head on the left side of each specimen (photos were mirrored to conform to

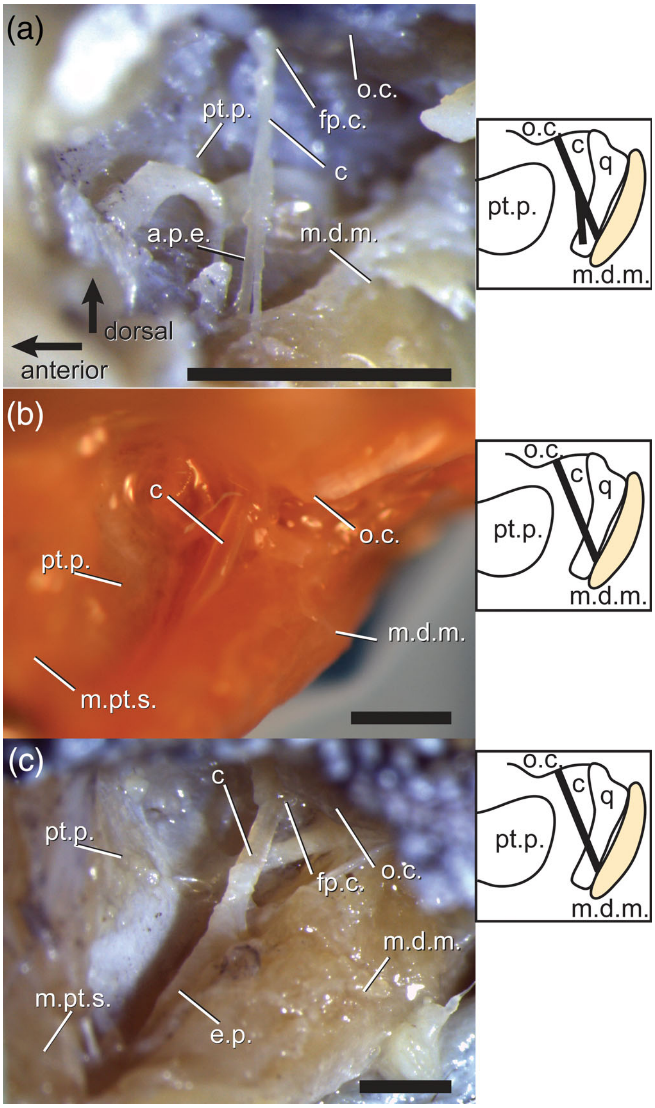

FIGURE 1 Middle ear dissections of chameleons with no pterygoid ear in the genus *Trioceros* in medial view, showing the extracolumella morphology. (a) *Trioceros ellioti*, (b) *Trioceros jacksonii*, and (c) *Trioceros johnstoni*. Schematics summarize the relevant morphology in each species. The columella and extracolumella are represented by a thick, black bar in the schematics. Scale bars 1 mm. Abbreviations: a.p.e., anterior process of the extracolumella; c, columella; e.p., extracolumellar plate; fp.c., footplate of the columella; m.d.m., depressor mandibulae muscle; m.pt.s., pterygoideus muscle superficial head; o.c., otic capsule; pt. p., pterygoid plate; q, quadrate

intuitive anatomical directions). The skin was cut along with the medial edge of the mandible and the posterolateral edge of the quadrate (Appendix 2). Connective tissue was cut between the depressor mandibulae and the hyoid apparatus to access the middle ear cavity. Gently prying the side of the head away from the rest of the body allowed observation of the columella. This bone, along with the associated extracolumella, was contained within a membrane, which was carefully removed for the clarity of the photographs.

Ancestral state reconstruction was implemented to explore the evolutionary history of the pterygoid ear in chameleons. Presence or absence of a pterygoid ear was revealed by dissections in this study or noted in the preexisting literature (Appendix 3). Species known to have a heavily reduced columella were assumed to lack a pterygoid ear, as vibration transfer to the inner ear would be nearly impossible without a functioning columella. This analysis used the phylogeny of Tolley et al. (2013). I used the stochastic character mapping method of Huelsenbeck et al. (2003), which uses a Markov chain Monte Carlo approach to generate posterior probabilities for trait distribution. This procedure was replicated 100 times, and the combined posterior probability was used to assess the likelihood of evolutionary scenarios. The analysis was completed using the programming language R (v. 3.5.3, R Core Team, 2019) and the package phytools (Revell, 2012).

Three species were μ-CT scanned to visualize the middle ear cavity and surrounding tissues: *Ch. chamaeleon*, *Ch. senegalensis*, and *T. jacksonii* (see Appendix 1 for details of each specimen). These specimens were initially fixed in formalin and then stored in a 70% ethanol solution. The *T. jacksonii* specimen (UWBM 8333) has been stored since 1976, so it exhibits more tissue degradation than the other specimens. It should be noted that the *Ch. chamaeleon* specimen (UCMVZ 2364801) was somewhat smaller than the typical adult size for this species and may not have reached full maturity. Scans were performed using the diceCT method of Gignac and Kley (2014) and Gignac et al. (2016). Each specimen was scanned once without staining to get an isolated model of the skull and mandible. These initial scans were conducted at Friday Harbor Laboratories using a Skyscan 1173 scanner and the following parameters: voltage 65 kV, current 123 μA, voxel size 44 (*Ch. chamaeleon* and

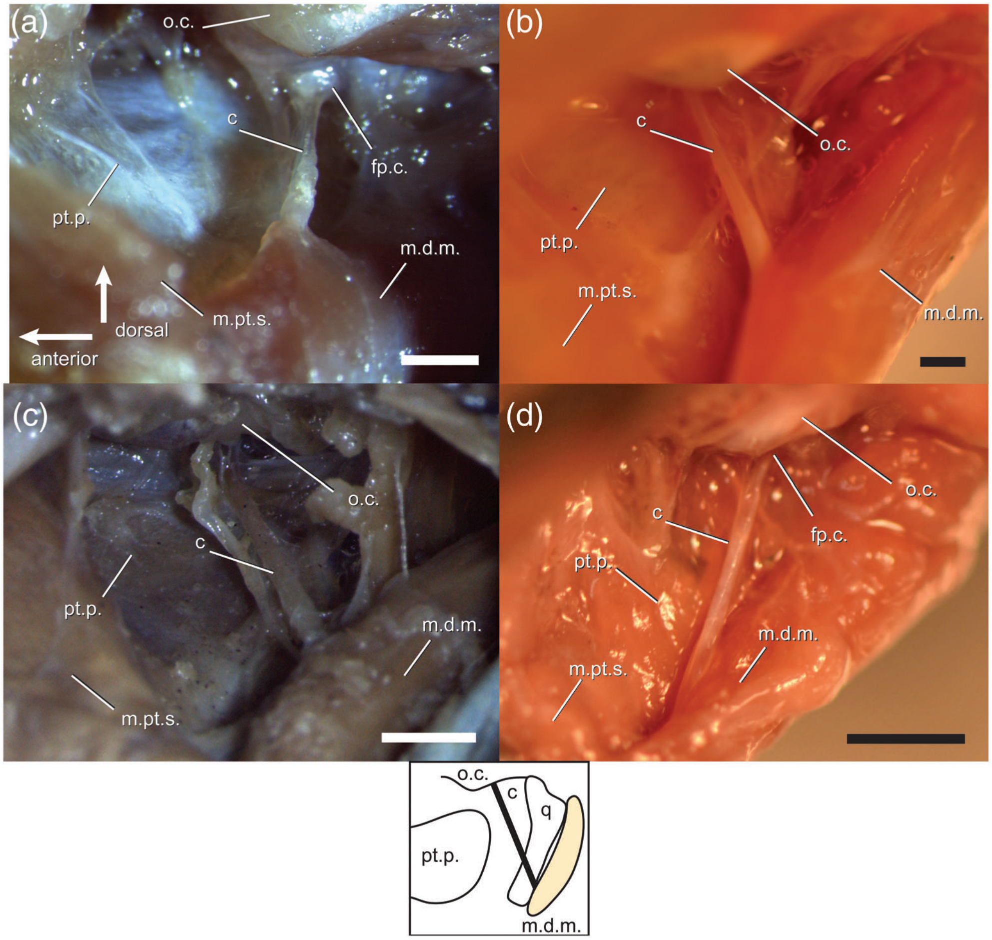

FIGURE 2 Middle ear dissections of chameleons with no pterygoid ear in several genera in medial view, showing the extracolumella morphology. (a) *Calumma brevicorne* (mirrored), (b) *Furcifer oustaleti* (mirrored), (c) *F. verrucosus*, and (d) *Kinyongia multituberculata* (mirrored). Schematic summarizes the relevant morphology in all species. The columella and extracolumella are represented by a thick, black bar in the schematic. Scale bars 1 mm. Abbreviations: c, columella; fp.c., footplate of the columella; m.d.m., depressor mandibulae muscle; m.pt.s., pterygoideus muscle superficial head; o.c., otic capsule; pt.p., pterygoid plate; q, quadrate

Ch. jacksonii) and 47 (*Ch. senegalensis*) μm. I used Avizo V9.2.0 (© FEI VSG, Hillsboro, OR) to isolate the skulls and mandibles in the scan via grayscale thresholding and create surface renderings. The specimens were then soaked in a 3% w/v Lugol's iodine (I_2KI) solution for 6 weeks, and this solution was replaced every 2 weeks. Scans were taken at 2 and 4 weeks into the soaking process at the University of Washington Computed Tomography Facility using an NSI X5000 scanner. When the muscles had reached a sufficient level of contrast, the specimens were scanned a final time with the Skyscan 1173 scanner with the following parameters: voltage 65 kV, current 122 μA, and voxel size 35 μm. Avizo was again used to manually segment all muscles surrounding the middle ear cavity and generate surface renderings. The mandible, quadrate, and pterygoid were also segmented in the final scan to aid in aligning the muscular and skeletal models, which was executed manually in Meshmixer V3.5.474 (©Autodesk, San Rafael, CA, USA).

3 | RESULTS

3.1 | Dissections

The middle ear cavity is well-developed in all the species studied. As has been previously described in chameleons, the middle ear cavity is mostly separate from the pharynx, which is atypical in lizards (Christensen-Dalsgaard, 2011; Wever, 1978). The cavity is bounded laterally by the superficial head of the pterygoideus muscle, the pterygoid plate, and its associated membrane (Figures 1–4). Spanning this cavity is the columella, a simple rod with a footplate situated in the fenestra ovalis of the otic capsule. None of the specimens dissected here displayed reduction of the

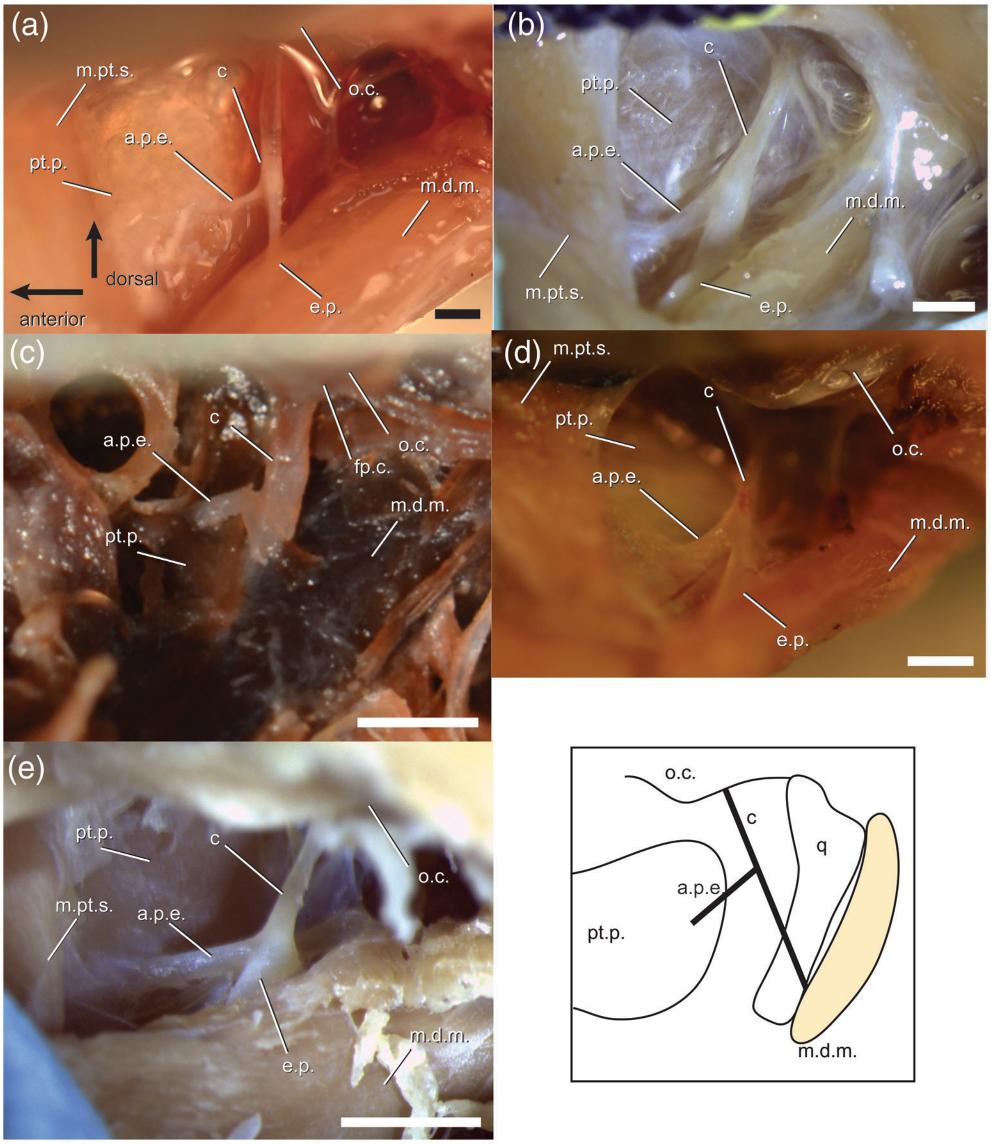

FIGURE 3 Middle ear dissections of chameleons with a pterygoid ear in the genus *Chamaeleo* in medial view, showing the extracolumella morphology. (a) *Ch. calyptratus* (mirrored), (b) *Ch. chamaeleon*, (c) *Ch. gracilis*, (d) *Ch. gracilis or senegalensis* (mirrored), and (e) *Ch. zeylanicus*. Schematic summarizes the relevant morphology in all species. The columella and extracolumella are represented by a thick, black bar in the schematic. Scale bars 1 mm. Abbreviations: a.p.e., anterior process of the extracolumella; c, columella; e.p., extracolumellar plate; fp.c., footplate of the columella; m.d.m., depressor mandibulae muscle; m.pt.s., pterygoideus muscle superficial head; o.c., otic capsule; pt.p., pterygoid plate; (q), quadrate

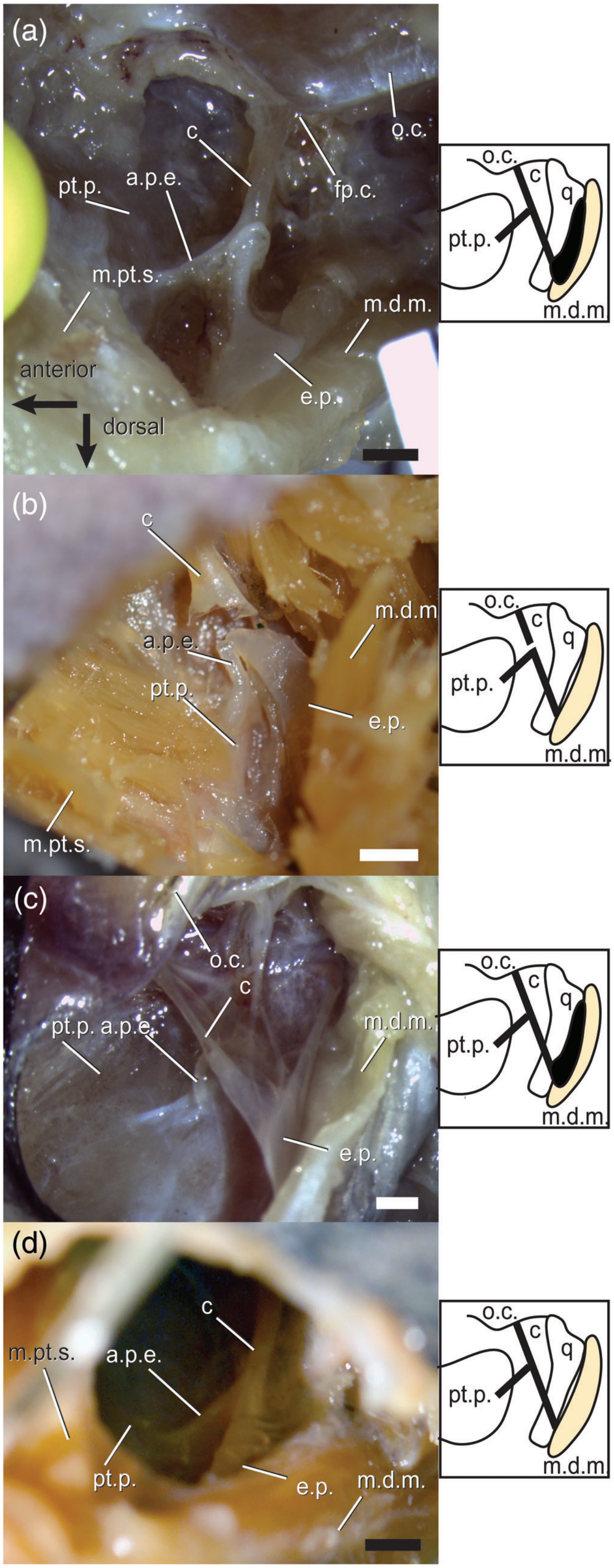

FIGURE 4 Legend on next page.

columella. The distal end of the columella is flush with the cartilaginous extracolumella, which extends to the quadrate. Both the columella and extracolumella reside within a thin membrane, as in other lizards (Peterson, 1966). The extracolumella nestles between the quadrate and depressor mandibulae, and here it has a ligamentous connection to the quadrate. The extracolumella is expanded into a plate at its distal end.

As noted by previous work, *T. jacksonii* has no connection between the extracolumella and the pterygoid plate or membrane, and there is no distinct separation between the anterior and posterior processes of the extracolumella (Figure 1b; Wever, 1969). This morphology is also seen in *Ca. brevicorne*, *T. johnstoni*, *F. lateralis*, *F. oustaleti*, *F. verrucosus*, *K. multituberculata* (Figures 1c and 2). *T. ellioti* does have an extracolumella divided into an anterior process and a posterior process, as in *Chamaeleo*, but its anterior process does not contact the pterygoid plate or membrane (Figure 1a). Instead, the anterior process connects to the quadrate, while the posterior process connects to the depressor mandibulae. These two processes are more defined than they appear to be in Wever's (1978) drawings. The extracolumellar plate of *Ca. brevicorne* is embedded into the depressor mandibulae (Figure 2a). In specimens lacking the pterygoid hearing mechanism, physically pressing against the auditory area at the side of the head did not result in the displacement of the columella.

Previous work has documented a connection between the anterior process of the extracolumella and the pterygoid plate in *Ch. calyptratus* (Wever, 1978), which is confirmed here (Figure 3a). An anterior process contacting the membrane extending from the pterygoid plate was also seen in *Ch. chamaeleon* (Figure 3b), confirming the findings of Wever (1978) and contradicting those of Versluys (1898). This specialized ear arrangement was found in two additional members of the genus *Chamaeleo*: *Ch. gracilis* and *Ch. zeylanicus* (Figure 3c,d). A connection between the extracolumella and the pterygoid plate or membrane was also seen in several species

FIGURE 4 Middle ear dissections of chameleons with a pterygoid ear in several genera in medial view, showing the extracolumella morphology. (a) *Furcifer pardalis*, (b) *Trioceros cristatus*, (c) *T. melleri*, and (d) *T. montium*. Schematics summarize the relevant morphology in each species. The columella and extracolumella are represented by a thick, black bar in the schematics. Scale bars 1 mm. Abbreviations: a.p.e., anterior process of the extracolumella; c, columella; e.p., extracolumellar plate; fp.c., footplate of the columella; m.d.m., depressor mandibulae muscle; m.pt.s., pterygoideus muscle superficial head; o.c., otic capsule; pt.p., pterygoid plate; q, quadrate outside this genus: *F. pardalis*, *T. cristatus*, *T. melleri*, and *T. montium* (Figure 4). In species with an anterior process, the extracolumella still forms a plate attached to the quadrate. The extracolumella also forks anteriorly to contact the pterygoid plate or membrane just posterior to the edge of the superficial head of the pterygoideus. This part of the pterygoid is free from muscle attachments. In addition to this anterior process, some of these species also have elaborations of their extracolumellar plate that provides greater contact with the quadrate and/or depressor mandibulae. In *F. pardalis*, the extracolumellar plate is widened into a cup that fits snugly around the depressor mandibulae, reaching the dorsal end of the quadrate (Figure 4a). In *T. melleri*, the extracolumellar plate is greatly expanded dorsoventrally along the full height of the quadrate and depressor mandibulae (Figure 4c). The morphology in *T. cristatus* is especially unique, as the columella and extracolumella articulate at a distinct joint rather than grading into one another as seen in the other studied taxa (Figure 4b). In all species in which the anterior process of the columella contacts the pterygoid plate or membrane, pressing against the auditory area at the side of the head caused displacement of the columella.

3.2 | Ancestral state reconstruction

Posterior probabilities from ancestral state reconstruction strongly favor multiple independent acquisitions of a

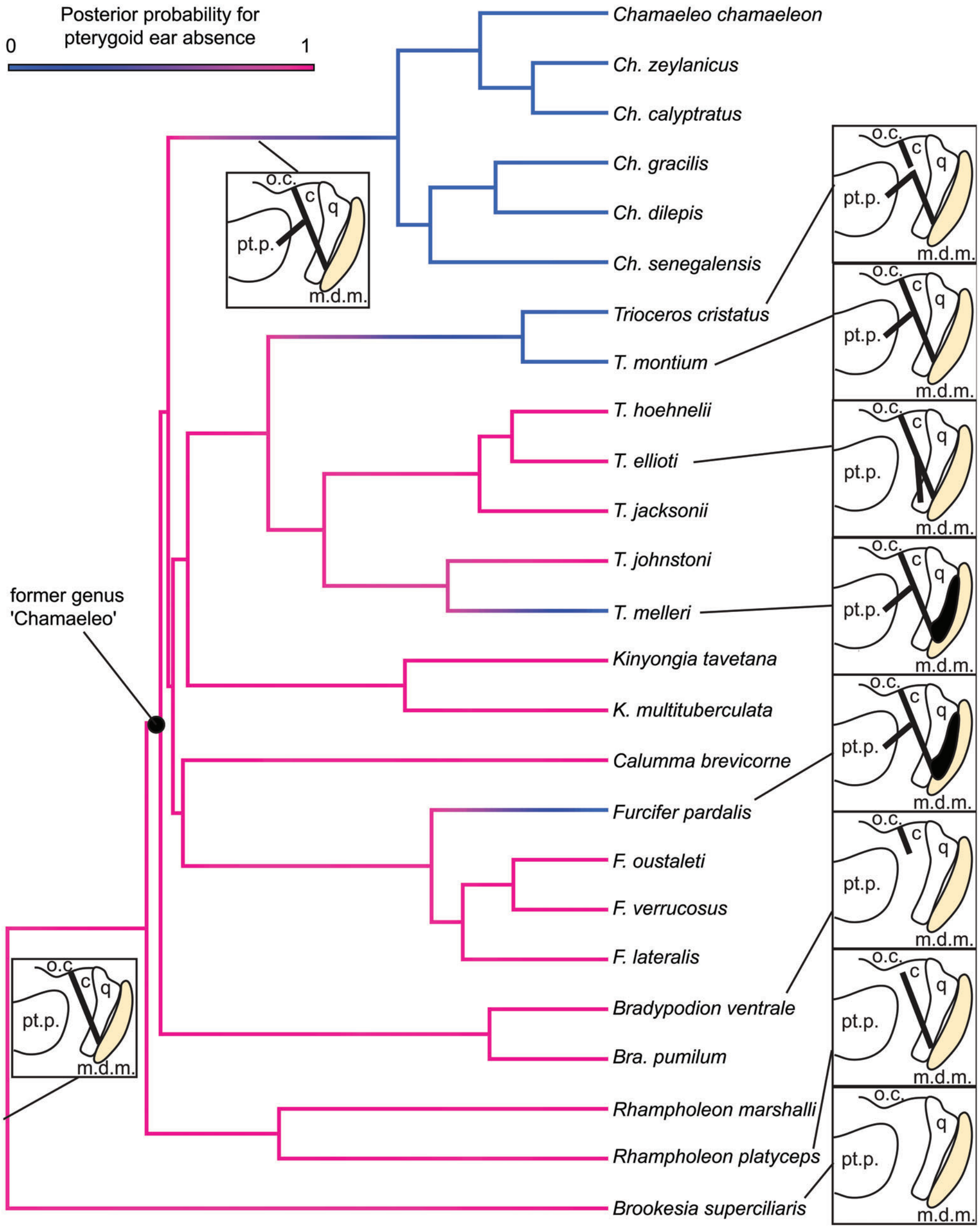

FIGURE 5 Phylogenetic tree of Chamaeleonidae with posterior probabilities for the presence or absence of a pterygoid ear. Schematics summarize differences in middle ear morphology. Tree based on Tolley et al., 2013. Abbreviations: c, columella; m.d.m., depressor mandibulae muscle; o.c., otic capsule; pt.p., pterygoid plate; q, quadrate

pterygoid-based hearing system (Figure 5). This system was likely present in the common ancestor of all modern *Chamaeleo* species. It also probably arose at least twice in the genus *Trioceros* and once in *Furcifer*.

3.3 | DiceCT scans

Only the muscles near the middle ear cavity were segmented. The different slips of the adductor mandibulae externus are not readily distinguishable in the scans, but the size and position of the muscles in these groups is similar between species (Figure 6). Deep to these muscles are the adductor posterior and the two slips of the pseudotemporalis. In *T. jacksonii*, the adductor posterior inserts on the dorsomedial surface of the surangular, just anterior to the jaw joint, but in *Ch. senegalensis* this insertion is farther anterior near the coronoid process (Figure 6). The insertion areas for the pseudotemporalis superficialis and pseudotemporalis profundus are also

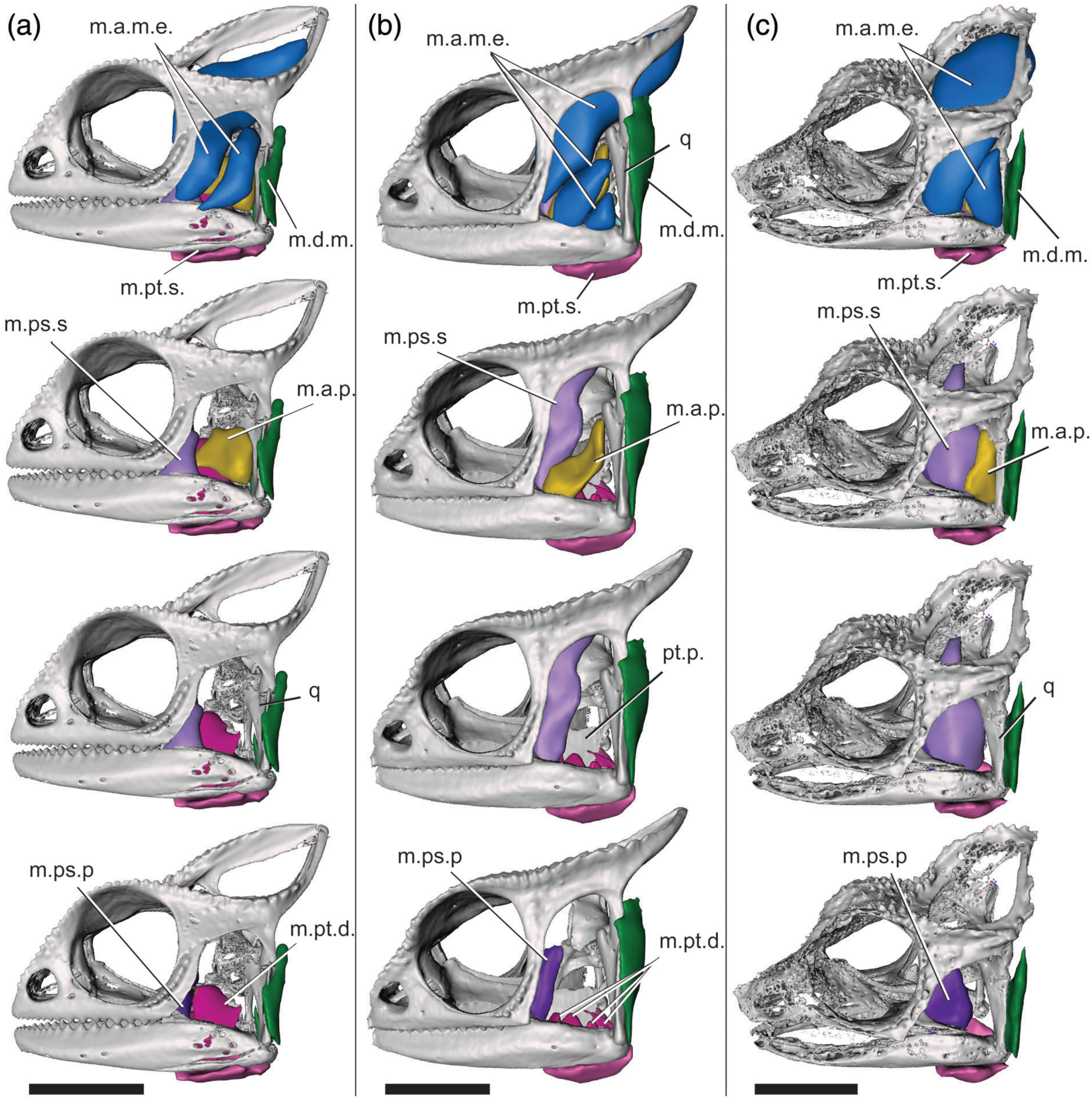

FIGURE 6 Segmented diceCT scans in left lateral view showing muscles in close proximity to the middle ear cavity in chameleons. (a) *Chamaeleo chamaeleon*, (b) *Ch. senegalensis*, and (c) *T. jacksonii*. Superficial muscles are successively removed down the page to reveal deeper muscles. Scale bars 1 cm. Abbreviations: m.a.m.e., adductor mandibulae externus muscle; m.a.p., adductor posterior muscle; m.d.m., depressor mandibulae muscle; m.ps.p., pseudotemporalis profundus muscle; m.ps.s., pseudotemporalis superficialis muscle; m.pt.d., pterygoideus muscle deep head; m.pt.s., pterygoideus muscle superficial head

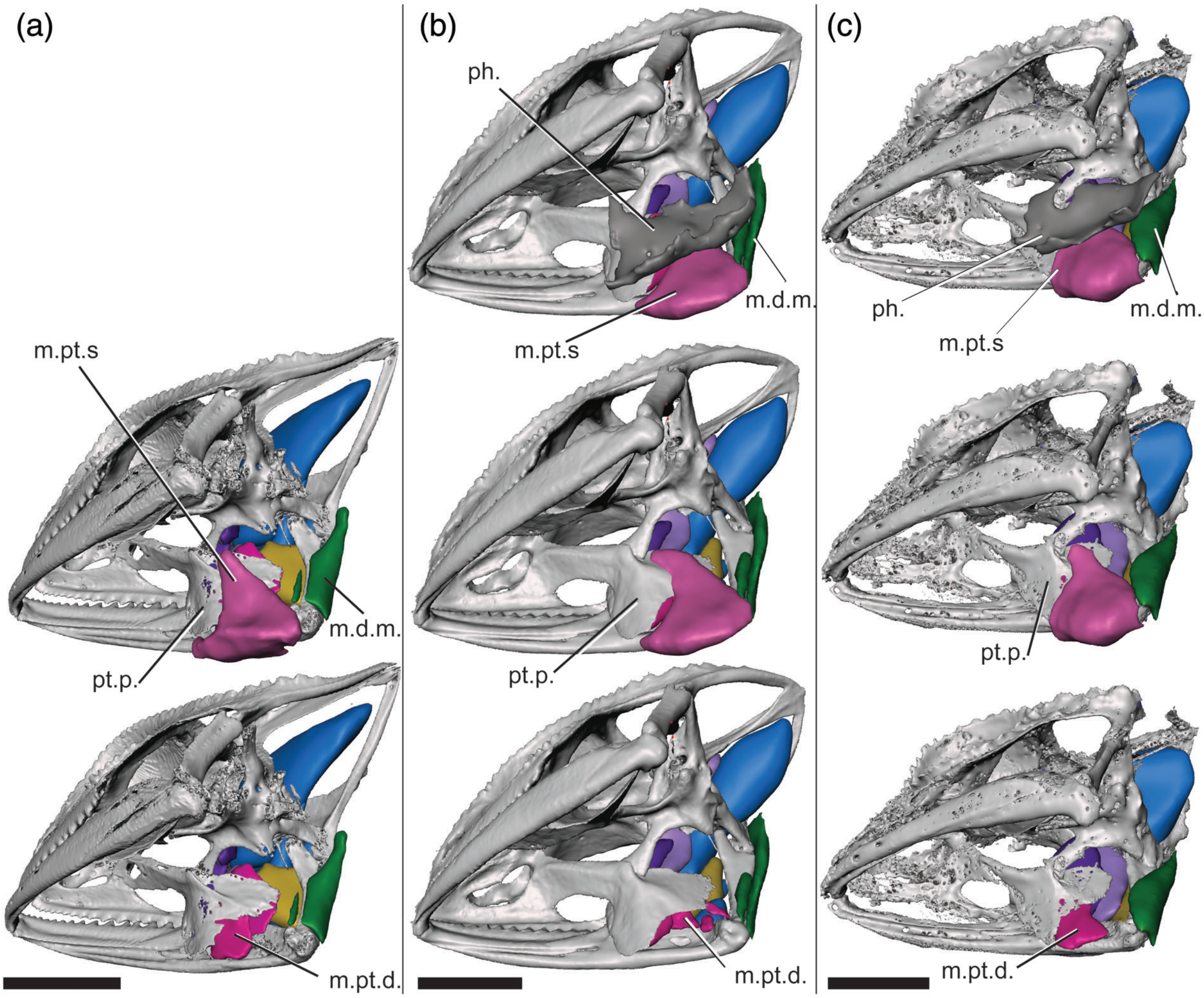

FIGURE 7 Segmented diceCT scans in oblique ventromedial view showing pharyngeal wall and muscles in close proximity to the middle ear cavity in chameleons. (a) *Chamaeleo chamaeleon*, (b) *Ch. senegalensis*, and (c) *Trioceros jacksonii*. Superficial structures are successively removed down the page to reveal deeper muscles. Scale bars 1 cm. Anterior is to the left. Abbreviations: m.d.m., depressor mandibulae muscle; m.pt.d., pterygoideus muscle deep head; m.pt.s., pterygoideus muscle superficial head; ph., pharyngeal wall

restricted to a more anterior position in *Ch. senegalensis*, whereas they have a broad insertion in *T. jacksonii*. The result of these two differences is that in *Ch. senegalensis*, there are fewer layers of muscle lateral to the pterygoid plate toward its posterior end, providing a more open cavity surrounding the pterygoid plate. *Ch. chamaeleon* has a broad insertion of the adductor posterior, but the insertions for the pseudotemporalis superficialis and pseudotemporalis profundus are anteriorly restricted as in *Ch. senegalensis*. The superficial head of the pterygoideus is virtually identical between the species examined here (Figure 7). The deep head of this muscle is also similar between species. The scan of *Ch. senegalensis* indicates four separate slips of this muscle (Figures 6 and 7), but it is unclear whether this reflects wear and tear on the specimen or an actual subdivision of the muscle. The contralateral muscle also appears to be subdivided in this specimen. Muscles directly attached to the pterygoid plate are the adductor posterior and both heads of the pterygoideus. The wall of the pharynx also lies against the dorsomedial portion of the pterygoid plate (Figure 7).

4 | DISCUSSION

4.1 | The evolutionary history of the chameleon middle ear

To my knowledge, the chameleon middle ear has not been formally described in the literature since Wever's (1978) work. Since that time, chameleon phylogeny has undergone substantial changes. The genus *Chamaeleo* once comprised the majority of chameleon diversity, but detailed morphological research and application of molecular phylogenetics led to its subdivision into the genera *Bradypodion*, *Calumma*, *Chamaeleo*, *Furcifer*, *Kinyongia*, *Nadzikambia*, and *Trioceros* (Klaver & Böhme, 1986; Tilbury et al., 2006; Tilbury & Tolley, 2009). These genera form a monophyletic clade, in which *Bradypodion* is the earliest diverging genus (Tolley et al., 2013; Figure 5). There are only a few genera outside this clade, including the "pygmy chameleons" *Brookesia* and *Rhampholeon* (Tilbury, 2013).

The presence of a well-developed middle ear cavity and columella in all the specimens dissected here is interesting because these structures are commonly reduced or absent in chameleons (Brock, 1940; Frank, 1951; Siebenrock, 1893; Toerien, 1963). However, these studies only documented the reduction of middle ear structures in the genera *Bradypodion*, *Brookesia*, and *Rhampholeon*, all of which lie outside the clade encompassing the sample in this study (Figure 5). It is thus likely that the common ancestor of this clade retained the middle ear cavity and columella. It is possible that these middle ear structures were retained because this ancestor used the depressor mandibulae hearing system, as this hearing system is known to occur in at least some members of *Chamaeleo*, *Trioceros*, and *Kinyongia* (Wever, 1978). The expanded extracolumellar plate overlapping or embedded in the depressor mandibulae in *Ca. brevicorne*, *F. pardalis*, and *T. melleri* suggests the use of this structure as an additional sound receptor by these species. The depressor mandibulae has no major role in hearing in *Ch. dilepis* and *T. jacksonii* (Wever, 1978), but that may represent a secondary loss of this feature. This study focused on the pterygoid hearing system, so the depressor mandibulae was not thoroughly investigated. Further research into the distribution of the depressor mandibulae hearing system would help clarify the evolutionary history of the chameleon middle ear.

A connection between the extracolumella and the pterygoid does not necessarily mean that the latter was used for sound reception. However, physiological experiments have demonstrated that this connection transmits sound to the inner ear, where cochlear potentials are generated in response (Wever, 1978), and there is no reason to expect this to differ in the species examined here. Mechanical stimulation of the auditory area during dissection was observed to cause displacement of the columella in all specimens with a connection to the pterygoid, an observation that has been made for other chameleons with a pterygoid ear (Wever, 1968, 1978), so vibrations in the pterygoid would certainly have been transmitted to the inner ear. It is reasonable to assume that these vibrations could stimulate a response from the inner ear, as has been seen in Wever's work.

Ancestral state reconstruction indicates that the pterygoid plate hearing system almost certainly evolved independently at least four times within Chamaeleonidae (Figure 5). The variation in morphology of the extracolumella and its interactions with the pterygoid and depressor mandibulae may reflect different anatomical or developmental constraints governing the evolution of this feature in each lineage. The forked extracolumella of *T. ellioti* bears a striking similarity to those of chameleons with a pterygoid hearing system. This could indicate that the ancestor of *T. ellioti* had a pterygoid ear, but the connection between the anterior process and the pterygoid was lost. However, this scenario is unlikely because none of the close relatives of *T. ellioti* use their pterygoid for hearing (Wever, 1969), and ancestral state reconstruction does not support the presence of the pterygoid ear in its ancestor (Figure 5). It is more likely that the well-defined anterior process in this species arose to allow stabilization of the columella against the quadrate while the posterior process transfers vibrations from the depressor mandibulae. The presence of an anterior process of the extracolumella in this species may indicate that this morphology results from simple changes in the development of the extracolumella, which could explain why this morphology was able to evolve independently in *Chamaeleo*, *Trioceros*, and *Furcifer*.

Though the acquisition of a novel hearing system seems complex, the structure of the skull might make such sound conduction pathways relatively easy to establish. The contact between the columella/stapes and the otic capsule is deeply entrenched in vertebrate ancestry (Reichert, 1837; Gaupp, 1911), and it provides a conveniently direct pathway by which sound can reach the inner ear. Any link between the columella/stapes and a nearby structure with suitable acoustic properties (e.g., wide surface area, minimal mechanical loading) will result in amplified sound vibrations reaching the inner ear. After such a connection is established, the middle ear may gain basic functionality and become "visible" to selection, allowing further refinement. An example of this can be seen in the evolution of the mammalian middle ear. The sound conduction pathway seen in extant mammals was likely present in their ancestors, but the connection between the middle ear ossicles and the dentary likely hindered its ability to transmit vibrations (Allin and Hopson, 1992). A complete separation between these ossicles and the dentary was accomplished independently at least three times within crown Mammalia, probably requiring only a single genetic change (Martin and Luo, 2005; Urban et al., 2017). Chameleons now provide an additional example of simple evolutionary changes resulting in parallel acquisition of a novel hearing apparatus, as any connection between the extracolumella and the nearby pterygoid will unlock a potential pathway for sound conduction. If the association between the stapes/columella and the otic capsule indeed facilitates the assembly of a functional ear, we may expect to find parallelism in the evolution of other vertebrate hearing systems.

4.2 | Potential interactions between ear and jaw adductor anatomy

The arrangement of the jaw adductor musculature was overall similar among the scanned specimens, but the following differences may be related to specializations in the sound conduction pathway. The insertions for both slips of the pseudotemporalis are farther posterior in *T. jacksonii* than in *Ch. chamaeleon* and *Ch. senegalensis*. The latter also has an anteriorly restricted insertion for the adductor posterior. While this pattern could reflect variation in diet and/or jaw adductor function, it may be related to the ear anatomy in these species. A notable consequence of these differences is that there are fewer muscle layers overlying the posterior end of the pterygoid plate and its associated membrane in *Chamaeleo* than in *T. jacksonii*. The space immediately lateral to the sound receiver in *Chamaeleo* is more open, which may minimize the dampening of vibrations on the pterygoid by surrounding tissues. These muscle insertions may have shifted anteriorly in *Chamaeleo* to accommodate the unique hearing system in this genus. Muscle insertions in *T. jacksonii* would be under no such selective pressure because they lack a pterygoid ear. However, the sample examined here is insufficient to fully assess whether this was the case. Descriptions of chameleon adductor musculature in the literature do not provide details on the posterior extent of the pseudotemporalis insertions (Rieppel, 1981, 1987), so it is unclear which arrangement represents the ancestral condition in chameleons. The position of the adductor posterior in Rieppel's illustrations of *Bradypodion pumilum*, *Brookesia superciliaris*, and *Rhampholeon kerstenii* is similar to that seen here for *T. jacksonii* and *Ch. chamaeleon*, suggesting that this muscle was indeed shifted anteriorly in *Ch. senegalensis*. *Bra. pumilum* and *Bro. superciliaris* have nonfunctional middle ears (Siebenrock, 1893; Toerien, 1963), as do the only examined members of *Rhampholeon* (Toerien, 1963). Further investigation into the jaw adductor musculature of chameleons with and without a pterygoid ear would clarify the interaction between these two systems. If the arrangement of the jaw muscles did change in response to the adoption of the pterygoid ear, it would illustrate the impact that the ear can have on surrounding structures.

4.3 | Function of the pterygoid ear

Given that many chameleons have nonfunctional middle ears, what selective pressures led the chameleons in this study to independently evolve a novel hearing system multiple times? A sense of hearing can greatly facilitate the localization of resources, depending on the resources required by the taxon in question. The detection of sounds produced by insects, especially buzzing during flight, has been proposed as the main driver in the origin of the middle ear in many tetrapod clades (Clack, 1997; Müller & Tsuji, 2007). Chameleons are extremely specialized for visual hunting of invertebrates and occasional small vertebrates (Anderson & Higham, 2013; Measey et al., 2013), but a nonvisual means for prey detection would likely prove useful in the complex arboreal habitats where many chameleons reside. Insects produce a wide variety of sounds at a vast range of frequencies. The buzzing of insect wings has been recorded with median frequencies of 130–320 Hz for bees (Burkart et al., 2011; Gradišek et al., 2017), 200–400 Hz for large flies (Sueur et al., 2005), and 400–800 Hz for mosquitoes (Arthur et al., 2014). The mating stridulations of crickets, cicadas, and cockroaches range from 2 to 15 kHz (Hartman & Roth, 1967; Pringle, 1954; Walker, 1962). While high-pitched mating chirps would be undetectable to a chameleon, buzzing sounds of insect flight are well within the frequency range of the pterygoid ear, which is most effective at 200–600 Hz in most species and 600–900 Hz in *Ch. chamaeleon* (Wever, 1978). The pterygoid ear arrangement would be effective at detecting some insects, so it may have evolved for localizing prey. However, it should be noted that there are no consistent differences in prey or habitat type known to exist between chameleons with and without the pterygoid ear (Akani et al., 2001; Measey et al., 2013; Tolley et al., 2013).

Another potential function for the pterygoid ear is in intraspecific communication, which is hypothesized to be one of the major drivers of hearing evolution (Fay & Popper, 2000; Gans, 1992). Chameleons are quite social, employing complex visual displays to attract mates and ward off competitors (Ballen et al., 2014; Stuart-Fox, 2013). They also seem to communicate via substrate-borne vibrations (Barnett et al., 1999; Tilbury, 1992). It has been hypothesized that these vibrations are produced by muscle contractions amplified by the gular pouch, a diverticulum of the trachea (Huskey et al., 2020; Tegge et al., 2020; Tornier, 1905). Though airborne sound can be produced by these vibrations, only barely audible sounds have been documented thus far (Barnett et al., 1999; Tilbury, 1992). Still, it is worth noting that six species known to possess a pterygoid ear also have a gular pouch, while only *T. cristatus* has a pterygoid ear and no gular pouch (Huskey et al., 2020). Similarly, five species known to lack a pterygoid ear also lack the gular pouch, while only *F. lateralis*, *F. oustaleti*, and *F. verrucosus* lack a pterygoid ear and have a gular pouch (Huskey et al., 2020). This general pattern could indicate that the pterygoid ear plays some role in detecting vibrations produced by the gular pouch. It seems unlikely that the pterygoid is particularly useful in receiving substrate-borne vibrations, as all the studied chameleons have a direct connection between their extracolumella and quadrate that would provide a pathway for vibrations to travel from the substrate through the jaw and quadrate to the inner ear. The pterygoid plate has a membranous attachment to the quadrate, so an additional connection between the extracolumella and pterygoid would be redundant with the existing quadrate-based pathway. Another possibility is that the gular pouch is also involved in the production or amplification of airborne sounds such as the hissing that occurs during chameleon confrontations (Bustard, 1967). Such calls could aid in communication between animals on different trees rather than relying solely on substrate-borne vibrations.

Finally, the pterygoid ear could be used to detect predators. Chameleons are primarily preyed on by birds and snakes, although mammals, frogs, and large invertebrates are also known to prey on them (Measey et al., 2013). These predators produce a wide variety of sounds that may be informative for a chameleon with a suitable ear, which makes this hypothesis difficult to test.

The role of the ear in chameleon biology has received little attention because their ears are typically considered vestigial and barely functional. However, the widespread occurrence of a well-developed tympanic cavity, repeated evolution of a novel sound conduction pathway, and possible shifting of muscle insertions to accommodate the ear highlight the potential significance of the ear for many chameleons. Further research is needed to understand exactly how these ears are used. Such inquiries will likely reveal a complex and interesting evolutionary history that may inform the origin of the ear in other clades, as well.

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