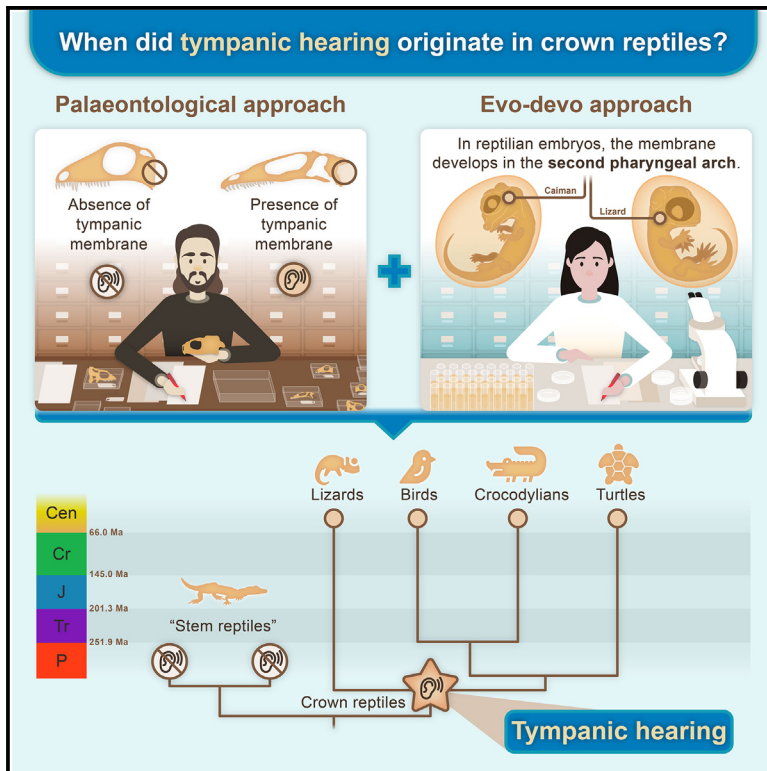


# Current Biology

## Deep-time origin of tympanic hearing in crown reptiles

### Graphical abstract



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### In brief

The tympanic ear is a key trait related to the evolutionary success of tetrapods on land, as it enhances the hearing capabilities of animals in this environment. Bronzati et al. use data from embryonic development and from the fossil record and provide evidence for a single origin of tympanic hearing in the group of living reptiles.

### Highlights

- Fossils reveal that the presence of tympanic membrane is ancestral to crown reptiles
- The eardrum of lizards and crocodylians develops within the second pharyngeal arch
- Fossils and embryos suggest a single origin of the tympanic ear in crown reptiles

Report

# Deep-time origin of tympanic hearing in crown reptiles

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## SUMMARY

The invasion of terrestrial ecosystems by tetrapods (c. 375 million years [Ma]) represents one of the major evolutionary transitions in the history of life on Earth. The success of tetrapods on land is linked to evolutionary novelties. Among these, the evolution of a tympanic ear contributed to mitigating the problem of an impedance mismatch between the air and the fluid embedding sound-detecting hair cells in the inner ear.<sup>1–3</sup> Pioneering studies advocated that similarities in the tympanic ear of tetrapods could only result from a single origin of this structure in the group,<sup>4,5</sup> an idea later challenged by paleontological and developmental data.<sup>4,6–8</sup> Current evidence suggests that this sensory structure evolved independently in amphibians, mammals, and reptiles,<sup>1,6</sup> but it remains uncertain how many times tympanic hearing originated in crown reptiles.<sup>9,10</sup> We combine developmental information with paleontological data to evaluate the evolution of the tympanic ear in reptiles from two complementary perspectives. Phylogenetically informed ancestral reconstruction analyses of a taxonomically broad sample of early reptiles point to the presence of a tympanic membrane as the ancestral condition of the crown group. Consistently, comparative analyses using embryos of lizards and crocodylians reveal similarities, including the formation of the tympanic membrane within the second pharyngeal arch, which has been previously reported for birds. Therefore, both our developmental and paleontological data suggest a single origin for the tympanic middle ear in the group, challenging the current paradigm of multiple acquisitions of tympanic hearing in living reptiles.

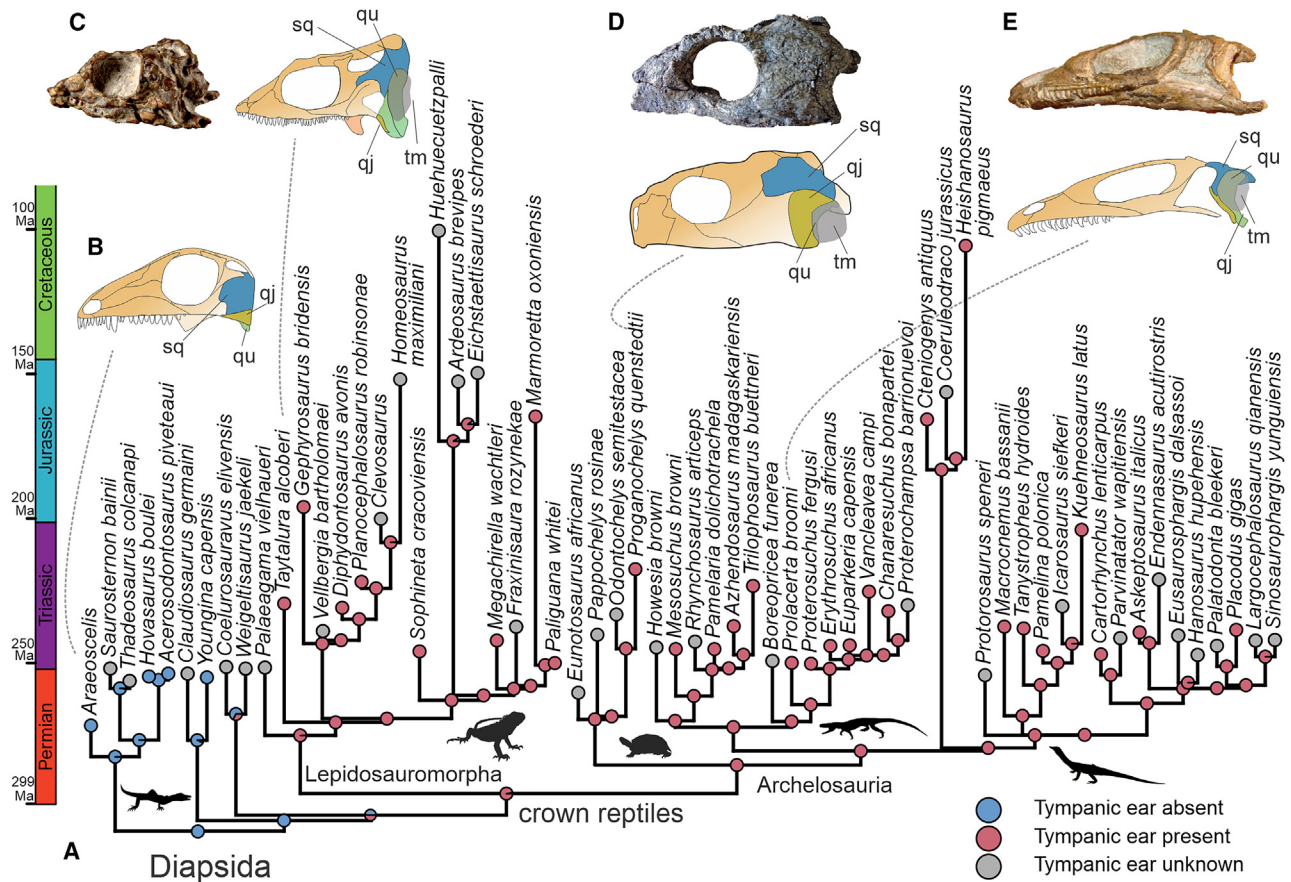
## RESULTS AND DISCUSSION

### The tympanic membrane in early crown reptiles

Crown reptiles compose an incredibly speciose group, encompassing about 20,000 species of birds, crocodylians, lizards, snakes, the tuatara, and turtles. This clade originated around 20–50 Ma after the beginning of the water-to-land transition in tetrapods and, along its evolution, includes forms such as the iconic non-avian dinosaurs, flying pterosaurs, and marine reptiles. Data from the rich reptilian fossil record might suggest that the tympanic middle ear is a derived feature that appeared more than once within the crown group (6), a paradigm that remains unchallenged until now.<sup>11</sup> Here, using the presence of a

tympanic membrane as a proxy for the presence of a tympanic middle ear in fossil taxa, we reassess the evolution of tympanic hearing in the reptilian fossil record (Figure 1) in the light of striking recent discoveries<sup>11</sup> and reinterpretations of key reptilian fossils.<sup>12,13</sup>

Soft tissues are rarely preserved in the fossil record, and so far, no evidence of a preserved tympanic membrane in Paleozoic and early Mesozoic reptiles has been reported—the tympanic membrane is, however, recognizable in Cretaceous lizards preserved in amber.<sup>14</sup> Therefore, the presence of the tympanic membrane (and hence the presence of a tympanic middle ear) in the origin of crown reptiles can only be inferred based on its osteological correlates (*sensu* Witmer<sup>15</sup>) on the morphology of



**Figure 1. Early evolution of tympanic hearing in stem and crown reptiles by ancestral state reconstruction on a timescaled phylogeny with the inferred morphology of the otic region for selected taxa, showing an estimated probability of >95% for the presence of a tympanic ear at the node of crown reptiles**

(A) One of the six phylogenetic hypotheses used in the analyses of ancestral trait reconstructions (STAR Methods), with pie charts indicating the probability for each possible state (legend at bottom right) for the nodes and tips of the tree.

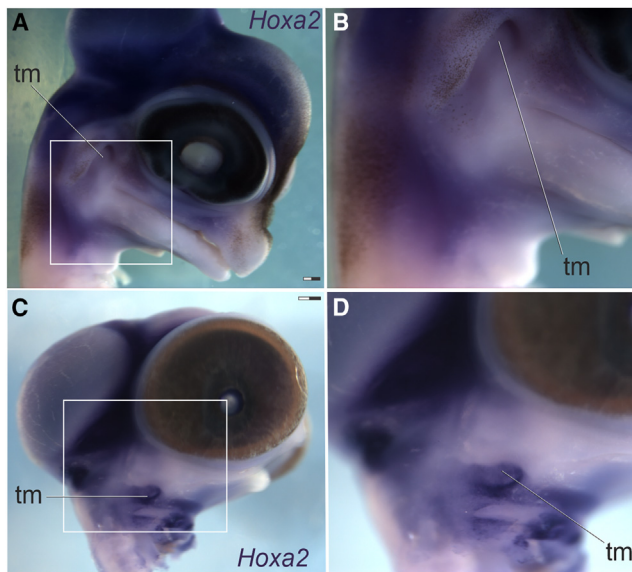
(B–E) (B) Schematic drawing of the skull of *Araeoscelis*, (C) photograph (left) and schematic drawing (right) of the skull of the specimen PVSJ 698 of *Taytalura alboberi*, (D) photograph (top) and schematic drawing (bottom) of the skull of the specimen SMNS 16980 of *Proganochelys quenstedtii*, and (E) photograph (top) and schematic drawing (bottom) of the skull of the specimen BPI-1-2576 of *Prolacerta broomi*. The position of the tympanic membrane in (B)–(D) was inferred from comparative morphological analyses of extant and extinct reptiles. The term “crown reptiles” is used to refer to the minimal clade, including all living lineages of reptiles.

Ma, million years; qj, quadratojugal; qu, quadrata; sq, squamosal; tm, tympanic membrane. For institutional abbreviations, see data and code availability. See also Figures S3–S8.

the otic region of the skull. Unambiguous osteological correlates of the tympanic membrane (in the sense that all living animals possessing this structure have a tympanic membrane associated with it) are, however, difficult to trace in the fossil record of early stem and crown reptiles, given the disparate morphology of their otic region compared with that of living reptiles (Figures S1 and S2). The oldest evidence of an unambiguous osteological correlate in crown reptiles is the presence of a tympanic conch and crest (a recessed surface in the lateral surface of the quadrata delimited by a lateral expansion of the anterior margin of the bone<sup>16</sup>) in the lepidosauromorph *Megachirella wachtlei* from the Middle Triassic (c. 240 Ma) of Europe.<sup>12</sup> The evolution of the tympanic crest in lepidosauromorphs is likely linked to the reorganization of the otic region of the skull due to the loss of a ventral process of the squamosal and a dorsal process of the quadratojugal contacting the anterolateral margin of

the quadrata. Differently, in stem taxa of all the three main living lineages of reptiles, the anterior margin of the quadrata is bordered by either one or both processes of the quadratojugal and squamosal bones (Figure 1).

Based on our morphological analyses of fossil reptiles and comparisons with living forms (STAR Methods), we infer that a tympanic membrane was majorly anchored on the lateral surface of the quadrata, at the contact with the ventral ramus of the squamosal and/or the dorsal ramus of the quadratojugal, in crown reptiles (Figure 1). This interpretation is congruent with a morphological shift of the otic region observed in these taxa. Early crown reptiles exhibit a concave posterior margin of the quadrata shaft that is not completely overlapped by either the squamosal and/or by the quadratojugal and possess a slender columella that is freed from the basicranium. This combination of traits is only observed in living reptiles with tympanic ears or



**Figure 2.** *In situ* hybridization of *Hoxa2* expression in embryos of the lizard *Tropicurus catalanensis* and the crocodylian *Caiman yacare*

(A) Whole-mount *in situ* hybridization of *Hoxa2* expression in the head of an 18-dpo embryo of *C. yacare*.

(B) Close-up of the region within the rectangle in (A).

(C) Whole-mount *in situ* hybridization of *Hoxa2* expression in the head of a 23-dpo embryo of *T. catalanensis*.

(D) Close-up of the region within the rectangle in (C).

tm, tympanic membrane.

See also [Figure S9](#).

in lepidosaurs for which the lack of a tympanic membrane represents secondary losses within their lineage—as exemplified by the limbless lizards of the genus *Aprasia*.<sup>17</sup> Differently, a columnar quadrate shaft that is overlapped by either the squamosal and/or the quadratojugal is observed in members of the stem lineage<sup>18</sup>—excluding “parareptiles” that developed a tympanic ear.<sup>19</sup> Whereas the change in the morphology of the posterior margin of the quadrate created an aperture on the otic region of the skull, providing an anchorage point for the attachment of the tympanic membrane anteriorly and space for the columella (or extra columella) to contact the tympanic membrane, the loss of bracing between the columella with the basicranium allowed this ossicle, freed in the tympanic cavity, to participate in the transmission of the acoustic signal from the tympanic membrane to the inner ear.<sup>1,3,6</sup> This morphological shift in early crown reptiles is congruent with that reported for other amniote lineages where a tympanic membrane also evolved, including synapsids (the mammal lineage of amniotes) and parareptiles.<sup>19</sup>

We phylogenetically reconstructed the evolution of the tympanic membrane in stem and crown reptiles using the most extensive sample of fossil and extant specimens to date ([STAR Methods](#)). The results of our stochastic reconstructions ([Figures 1](#) and [S3–S8](#)) estimated a probability of more than 95% that a tympanic membrane, and hence a tympanic ear, was present at the origin of crown reptiles, between 300 and 250 mya (see [supplemental information](#) for more details). Additional analyses using alternative topologies ([Figures S3–S8](#)) support this result, regardless of recent phylogenetic controversies in

respect of early crown reptiles.<sup>11–13,20–24</sup> The recovered ancestral morphology, with a tympanic membrane anteriorly attached to the junction of the quadrate with the quadratojugal and/or the squamosal, can be confidently inferred for taxa such as the recently discovered lepidosauromorph *Taytalura alboberi* from the Late Triassic of Argentina, which corresponds to the sister group of all other lepidosauromorphs<sup>11,12</sup>; *Sophineta cracoviensis* from the Middle Triassic of Poland, a sister taxon of all squamates<sup>14</sup>; the stem-archosaur *Prolacerta broomi* from the Middle Triassic of South Africa<sup>25</sup>; and the stem turtle *Proganochelys quenstedti*<sup>26</sup> from the Late Triassic of Laurasia ([Figure 1](#)).

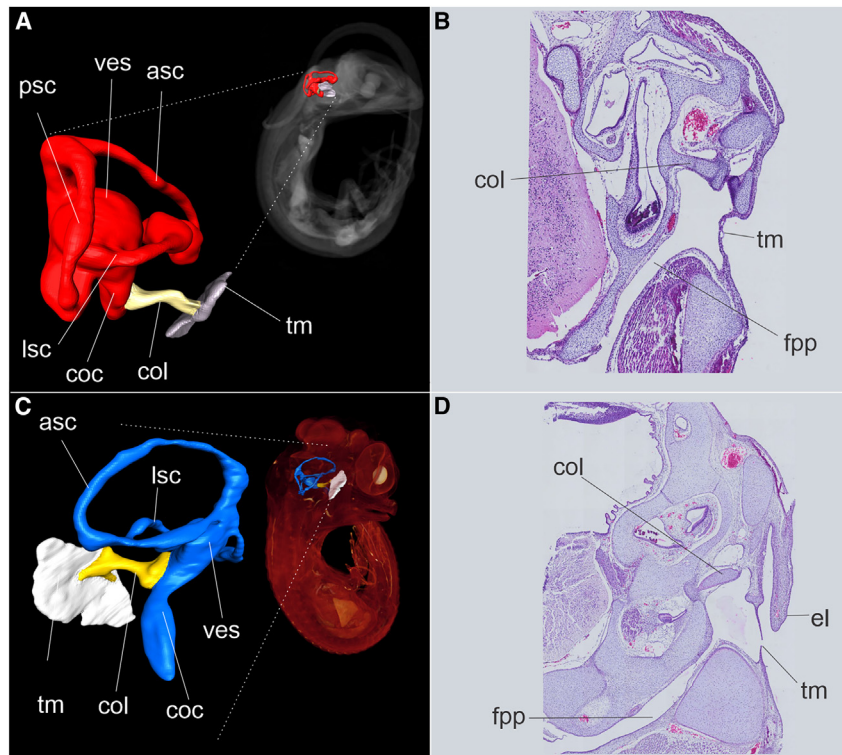
### Embryonic development of the reptilian tympanic membrane

We used *in situ* hybridizations, diffusible iodine-based contrast-enhanced computed tomography (diceCT) scans, and traditional histology to investigate the embryonic development of the tympanic middle ear in squamates and crocodylians, with a focus on the tympanic membrane. Thus, we fill a gap in the currently available data for the development of the tympanic middle ear in reptiles, which was previously mostly based on information from chicken embryos.<sup>7,8</sup> We analyzed the spatial development of the tympanic membrane in relation to the pharyngeal arches in the lizard *Tropicurus catalanensis* and the crocodylian *Caiman yacare* ([Figure 2](#)), biological representatives of Lepidosauromorpha and Archelosauria, respectively, the two main crown reptilian lineages. For that, we used whole-mount *in situ* hybridizations to detect the expression of *Hoxa2* (a marker for the second pharyngeal arch) in 23-days-post-oviposition (dpo) embryos of *Tropicurus catalanensis* and in 18 dpo embryos of *C. yacare* ([Figures 2](#) and [S9](#)). In whole-mount hybridization of *C. yacare* embryos, labeling is strongest at the neck region caudal to the lower jaw and at the tympanic membrane ([Figures 2A](#) and [2B](#)). *Hoxa2* labeling is absent at the posterior portion of the neck ventral to the otic region. It is also absent in both lower and upper jaws (although a small amount of expression was detected on the epidermis adjacent to the ventral limit of the eyes), which derive, respectively, from the frontonasal prominence and first pharyngeal arch ([Figures 2A](#) and [2B](#)). Whole-mount hybridization of *T. catalanensis* ([Figures 2](#) and [S9](#)) shows that *Hoxa2* is absent in the distal regions of the lower and upper jaws but becomes progressively stronger near the otic region ([Figures 2C](#) and [2D](#)). *Hoxa2* labeling is also absent in the posterior region of the neck ventral to the otic region. In this scenario, the most parsimonious explanation for the presence of *Hoxa2* in the otic region is that the tympanic membrane arises from the second pharyngeal arch, similarly to what has been reported for birds and in contrast with the condition in mammals in which the tympanic membrane is a derivative of the first pharyngeal arch.<sup>8</sup> Our data thus corroborate the independent developmental origins of the tympanic membrane in mammals and reptiles (including birds) and also indicate a topological equivalence of the membrane of living reptiles regarding its spatial organization within the pharyngeal arches early in ontogeny.

### A paleo-evo-devo perspective on the origin of the tympanic ear in crown reptiles

Our developmental and paleontological data introduce a new scenario to the evolution of tympanic hearing in the group,





**Figure 3. Embryonic development of the tympanic ear in the lizard *Tropidurus catalanensis* and the crocodylian *Caiman yacare***

(A) 3D reconstruction of the elements of the middle and inner ear (in posterolateral view) of a 31-dpo (stage 38) embryo of *T. catalanensis* based on diceCT data.

(B) Histological sections in coronal plane of the right side of the skull of a 31-dpo (stage 38) embryo of *T. catalanensis*.

(C) 3D reconstructions of the elements of the middle and inner ear (in anteromedial view) of a 32-dpo embryo of *C. yacare* based on diceCT data.

(D) Histological sections in coronal plane of the right side of the skull of a 32-dpo embryo of *C. yacare*. asc, anterior semicircular canal; coc, cochleae; col, columella; el, earlid; fpp, first pharyngeal pouch; lsc, lateral semicircular canal; psc, posterior semicircular canal; tm, tympanic membrane; ves, vestibule.

including all lineages of living reptilians. From the perspective of developmental biology, our data for lizards and caimans, which, together with birds, bracket all the crown reptilian lineages according to most recent phylogenetic hypotheses based on morphological and molecular data,<sup>11–13,20–23</sup> indicate topological equivalence regarding the development of the tympanic membrane within the second pharyngeal arch. The homology of other components of the tympanic middle ear, such as the columella and the tympanic cavity (Figure 3), across all tetrapod lineages is well established.<sup>1,5,6</sup> However, the exact contribution of the germ layers to the formation of the tympanic membrane in reptiles remains unknown.<sup>1</sup> From a paleontological standpoint, the fossils of early crown reptiles show similarities in the otic region that are compatible with the presence of a tympanic membrane. Assuming that this similar morphology is not homologous among crown reptiles and that it does not represent the ancestral condition of the group as supported by our analyses (Figure 1) would require several *ad hoc* explanations implying multiple evolution of the tympanic ear in the different lineages. Thus, when combined, our results indicate a single origin of tympanic hearing at the origin of the reptilian crown group (Figure 1). An ostensible singular origin for the tympanic middle ear in reptiles raises further questions regarding the evolution of hearing in reptiles, such as the timing of origin of traits enhancing the perception and localization of airborne sounds, including internally coupled middle ears and the development of an external acoustic meatus,<sup>3</sup> and also the timing of loss of a tympanic membrane and reorganization of middle ear components in several lepidosauromorph lineages that do not possess tympanic hearing, such as the tuatara, amphisbaenians, snakes, and chameleons, among others.<sup>27</sup>

the development of the tympanic membrane in mammals and reptiles,<sup>8</sup> at least two features of the tympanic ear are homologous in these two groups of amniotes: the tympanic cavity formed by the expansion of the first pharyngeal pouch and the columella (Figure 3). Taken together, the present data for the evolution of the tympanic ear in amniotes highlight that, for structures composed of multiple parts, such as the tympanic middle ear, whereas some of the parts might be homologous in different groups, as is the case for the columella and for the tympanic cavity of mammals and reptiles, other parts may not, as is the case for the tympanic membrane (see also Tucker<sup>1</sup>). This demonstrates that similar developmental routes can lead to the appearance of non-ancestrally related but anatomically akin complex structures such as the tympanic ear in different lineages.

In light of the results presented in this study and based on the current literature on the evolution of the tympanic ear, the most likely scenario for the evolution of tympanic hearing in amniotes is that this feature appeared at least three times, independently, in mammals, parareptiles (but see Müller and Tsuji<sup>19</sup>), and crown reptiles (see also Tucker,<sup>1</sup> Lombard and Bolt,<sup>4</sup> Gaupp,<sup>5</sup> Clack et al.,<sup>6</sup> Kitazawa et al.,<sup>7</sup> Sobral et al.,<sup>9</sup> Müller et al.,<sup>10</sup> and Clack<sup>28</sup>). Despite recent phylogenetic studies pointing out the affinity of some “parareptilian” lineages to the reptilian crown group,<sup>29</sup> a single origin of the tympanic ear in a clade including parareptiles and crown reptiles can be so far discarded, as the fossil record shows that the immediate sister groups of the lineages in these groups with a tympanic ear lacked this structure (Figure 1; see also Müller and Tsuji<sup>19</sup>). Our analyses indicate that the tympanic ear of crown reptiles might have already originated in the Paleozoic, but the known fossil record of crown reptiles extends back only as far as the first stages of the

Mesozoic.<sup>20</sup> So far, the oldest evidence for the presence of a true tympanic ear in the fossil record (based on the analysis of osteological correlates) is for Paleozoic parareptiles from the Late Carboniferous of the USA<sup>30</sup> and Middle Permian (c. 270–250 Ma) of Russia.<sup>19</sup> Regardless of the timing of the origin of the tympanic ear in crown reptiles, we suggest that the evolution of a tympanic ear represented one of the evolutionary novelties that is associated with the successful radiation of this group early in the Mesozoic, in the aftermath of the greatest mass extinction of all time, the Permo-Triassic event. As this sensory structure enables a better perception of the environment and enhances the ability of animals to detect predators and capture prey,<sup>1–3</sup> it likely provided an advantage for animals' survival during a period marked by drastic environmental changes.

### RESOURCE AVAILABILITY

#### Lead contact

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#### Materials availability

Plasmids generated in this study will be made available upon request.

#### Data and code availability

R scripts for performing ancestral state reconstruction analyses are available in the Open Science Framework at <https://osf.io/6d4pg/>. CT data and three-dimensional (3D) models of the tympanic ear are deposited in the public repository MorphoSource at <https://www.morphosource.org/projects/000662006>. The sequences of *HOXA2* DNA templates used for synthesis of RNA probes are available at Genbank: PQ421447 (*Caiman yacare*) and PQ421448 (*Tropidurus catalanensis*).

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### AUTHOR CONTRIBUTIONS

M.B. and T.K. designed the study. M.B. processed and sampled CT data. M.B. and I.W. analyzed histological sections. M.B. and J.L. obtained embryos. M.B., F.M.V., V.S.B., and C.Y.I.Y. conducted the *in situ* hybridizations. M.B., F.C.M., J.P.M.N., and D.R. analyzed the osteological correlates in extant reptiles. M.B. and P.L.G. conducted the analysis of reconstructions of ancestral traits. M.B., F.M.V., P.L.G., and T.K. wrote the bulk of the manuscript. M.B. made figures. All authors collected data and contributed to the writing, discussion, and conclusion.

### DECLARATION OF INTERESTS

The authors declare no competing interests.

### STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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  - Inference of the ancestral morphology

### SUPPLEMENTAL INFORMATION

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## STAR★METHODS

### KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
Anti-Digoxigenin-AP, Fab fragments	Sigma-Aldrich	Cat#11093274910, RRID: AB_2734716
<b>Biological samples</b>		
<i>Acerosodontosaurus piveteaui</i>	Personal observation (MB)	MNHN 1908-32-57
<i>Araeoscelis</i>	Reisz et al. <sup>31</sup>	MCZ 4380
<i>Ardeosaurus brevipes</i>	Personal observation (MB)	SNSB-BSPG 1923. I. 501
<i>Askeptosaurus italicus</i>	Müller <sup>32</sup>	MSNM V456; PIMUZ T 4846
<i>Azhendosaurus madagaskariensis</i>	Flynn et al. <sup>33</sup>	FMNH PR 2751
<i>Boreopricea funerea</i>	Benton and Allen <sup>34</sup>	PIN 3708/1
<i>Cartorhynchus lenticarpus</i>	Motani et al. <sup>35</sup>	AGB6257
<i>Chanaresuchus bonapartei</i>	Personal observation (MB)	MCZ 4037
<i>Claudiosaurus gemaini</i>	Personal observation (MB)	MNHN 1978-6-1
<i>Clevosaurus hudsoni</i> & <i>Cl. cambrica</i>	Chambi-Trowell et al. <sup>36</sup>	NHMUK PV R36832; NHMUK PV R37013-37014
<i>Coelurosauravus elivensis</i>	Personal observation (MB)	MNHN.F.MAP325a; 317 a,b; 327 a,b
<i>Coeruleodraco jurassicus</i>	Personal observation (MB)	IVPP V 23318
<i>Ctenioagenys antiquus</i>	Evans <sup>37</sup>	NHMUK R.11753-4
<i>Diphyodontosaurus avonis</i>	Whiteside <sup>38</sup>	BRUG 23782-23783
<i>Eichstaettisaurus schroederi</i>	Personal observation (MB)	SNSB-BSPG 1937 I
<i>Endennasaurus acutirostris</i>	Personal observation (MB)	MBSN 5170
<i>Erythrosuchus africanus</i>	Gower <sup>39</sup>	BP/1/5207
<i>Eunotosaurus africanus</i>	Bever et al. <sup>40</sup>	CM777; SAM-PK-K7909
<i>Euparkeria capensis</i>	Personal observation (MB)	SAM-PK-5867
<i>Eusaurosphargis dalsassoi</i>	Scheyer et al. <sup>41</sup>	PIMUZ A/III 4380
<i>Fraxinisaura rozynekae</i>	Personal observation (MB)	SMNS 91547
<i>Gephyrosaurus bridensis</i>	Evans <sup>42</sup>	UCL T769; UCL T1251
<i>Hanosaurus hupehensis</i>	Personal observation (MB)	IVPP V3231
<i>Heishanosaurus pygmaeus</i>	Personal observation (MB)	IVPP V25324
<i>Homoeosaurus maximilliani</i>	Personal observation (MB)	SNSB-BSPG 1887 VI 502
<i>Hovasaurus boulei</i>	Personal observation (MB)	MNHN 1925-5-34
<i>Howesia browni</i>	Personal observation (MB)	SAM-PK-5884
<i>Huehuecuetzpalli mixtecus</i>	Personal observation (MB)	Cast of IGM 7389 at SMNS-BSPG
<i>Icarosaurus siefkeri</i>	Colbert <sup>43</sup>	AMNH 2101
<i>Kuehneosaurus latus</i>	Robinson <sup>44</sup>	BMNH R.8172
<i>Largocephalosaurus qianensis</i>	Personal observation (MB)	IVPP V 15638
<i>Macrocnemus bassanii</i>	Personal observation (MB)	PIMUZ T 2477
<i>Marmoretta oxoniensis</i>	Griffiths et al. <sup>45</sup>	NMS G1992.47.1a; CAMSM X9991
<i>Megachirella wachtleri</i>	Personal observation (MB)	PZO 628
<i>Mesosuchus browni</i>	Personal observation (MB)	SAM 6536
<i>Odontochelys semitestacea</i>	Li et al. <sup>46</sup>	IVPP V 15639
<i>Palaeagama vielhaueri</i>	Carroll <sup>47</sup>	MGM 3707
<i>Palatodonta bleekeri</i>	Neenan et al. <sup>48</sup>	TW480000470
<i>Paliguana whitei</i>	Ford et al. <sup>13</sup>	AM 3585
<i>Pamelaria dolichotrachela</i>	Sen <sup>49</sup>	ISIR 316/1

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
<i>Pamelina polonica</i>	Personal observation (MB)	ZPAL RV/1029 and 1083
<i>Parvinatorator wapitiensis</i>	Nicholls and Brinkman <sup>50</sup>	TMP 1989.127.0008
<i>Placodus gigas</i>	Personal observation (MB)	SNSB-BSPG 1968 I 75
<i>Planocephalosaurus robinsonae</i>	Fraser <sup>51</sup>	AUP 11071, 11079, 11081
<i>Proganochelys quenstedtii</i>	Personal observation (MB)	SMNS 15759; MB 1910.45.1
<i>Prolacerta broomi</i>	Personal observation (MB)	BPI-1-2576
<i>Proterochampsia barrionuevoi</i>	Personal observation (MB)	PVSJ 77; MCZ 3408
<i>Proterosuchus fergusi</i>	Personal observation (MB)	SNSB-BSPG 1934 VIII 514
<i>Protosaurus speneri</i>	Personal observation (MB)	NMK S 180; WMsN P 47361
<i>Rhynchosaurus articeps</i>	Personal observation (FCM)	NHMK R1237
<i>Saurosternon bairdii</i>	Carroll <sup>47</sup>	NHMK R1234
<i>Sinosaurophargis yunguiensis</i>	Personal observation (MB)	IVPP V 17040 and 16076
<i>Sophineta cracoviensis</i>	Personal observation (MB)	ZPAL RV/974 and 245
<i>Tanystropheus hydroides</i>	Personal observation (MB)	PIMUZ T 2790
<i>Taytalura alcoberi</i>	Martínez et al. <sup>11</sup>	PVSJ 698
<i>Thadeosaurus colcanapi</i>	Personal observation (MB)	MNHN (PM) 1908-11-13/1908-11-19
<i>Trilophosaurus buettneri</i>	Personal observation (MB)	TMM-31025-040
<i>Vancleavea campi</i>	Nesbitt et al. <sup>52</sup>	GR 138
<i>Vellbergia bartholomaei</i>	Sobral et al. <sup>53</sup>	SMNS 91590
<i>Weigeltisaurus jaekeli</i>	Personal observation (MB)	SSWG 113/7
<i>Youngina capensis</i>	Gardner et al. <sup>54</sup>	AMNH 5561
<b>Chemicals, peptides, and recombinant proteins</b>		
BCIP	MilliporeSigma	Cat# 11383221001
DIG RNA Labeling Mix	MilliporeSigma	Cat# 11277073910
NBT	MilliporeSigma	Cat# 11383213001
Ncol	Thermo Fisher Scientific	Cat# ER0572
Normal Sheep Serum (sterile)	Abcam	Cat# ab7489
RNasin Ribonuclease Inhibitor	Promega	Cat# N2111
SP6 RNA Polymerase	MilliporeSigma	Cat# RPOLSP6-RO
<b>Critical commercial assays</b>		
Wizard SV Gel and PCR Clean-Up System	Promega	Cat# A9282
MicroSpin G-50 Columns	Cytiva	Cat# 28-9034-08
<b>Deposited data</b>		
Caiman yacare HOXA2 partial exonic sequence	<a href="https://www.ncbi.nlm.nih.gov/genbank/">https://www.ncbi.nlm.nih.gov/genbank/</a>	GenBank: PQ421447
Tropidurus catalanensis HOXA2 partial exonic sequence	<a href="https://www.ncbi.nlm.nih.gov/genbank/">https://www.ncbi.nlm.nih.gov/genbank/</a>	GenBank: PQ421448
Morphological data matrix (osteological correlates)	Open Science Framework: <a href="https://osf.io/6d4pg/">https://osf.io/6d4pg/</a>	<a href="https://doi.org/10.17605/OSF.IO/6D4PG">https://doi.org/10.17605/OSF.IO/6D4PG</a>
Time-calibrated trees	Open Science Framework: <a href="https://osf.io/6d4pg/">https://osf.io/6d4pg/</a>	<a href="https://doi.org/10.17605/OSF.IO/6D4PG">https://doi.org/10.17605/OSF.IO/6D4PG</a>
Ancestral state reconstruction analyses (supplementary plots and R objects)	Open Science Framework: <a href="https://osf.io/6d4pg/">https://osf.io/6d4pg/</a>	<a href="https://doi.org/10.17605/OSF.IO/6D4PG">https://doi.org/10.17605/OSF.IO/6D4PG</a>
Additional results of stochastic reconstructions	Open Science Framework: <a href="https://osf.io/6d4pg/">https://osf.io/6d4pg/</a>	<a href="https://doi.org/10.17605/OSF.IO/6D4PG">https://doi.org/10.17605/OSF.IO/6D4PG</a>
CT data and three-dimensional (3D) models of the tympanic ear	<a href="https://www.morphosource.org/projects/000662006/">https://www.morphosource.org/projects/000662006/</a>	MorphoSource: 000662006
<b>Experimental models: Organisms/strains</b>		
<i>Caiman yacare</i> (embryos)	This study	CHRP - unnumbered
<i>Tropidurus catalanensis</i> (embryos)	This study	CHRP - unnumbered

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Oligonucleotides</b>		
Primer: TcHOXA2 Forward: ACGGCTTACACCAACACGCAGC	This study	N/A
Primer: TcHOXA2 Reverse: TGGGCCATTGTTGACAGGCAGT	This study	N/A
Primer: CyHOXA2 Forward: AGTGGGAACGGTGGCTCCAGGA	This study	N/A
Primer: CyHOXA2 Reverse: CCAGCTGCACAGTTCTGGGCCA	This study	N/A
<b>Recombinant DNA</b>		
pGEM-T	Promega	Cat# A3600
Plasmid: pGEM-T(TcHOXA2 probe)	This study	N/A
Plasmid: pGEM-T(CyHOXA2 probe)	This study	N/A
<b>Software and algorithms</b>		
Avizo Lite 9.2.0	Thermo Fisher Scientific	N/A
R scripts for performing ancestral state reconstruction analyses	<a href="https://osf.io/6d4pg/">https://osf.io/6d4pg/</a>	<a href="https://doi.org/10.17605/OSF.IO/6D4PG">https://doi.org/10.17605/OSF.IO/6D4PG</a>
Mesquite V 3.81	<a href="https://www.mesquiteproject.org/">https://www.mesquiteproject.org/</a>	N/A
MrBayes V 3.2.7a	<a href="https://nbisweden.github.io/MrBayes/">https://nbisweden.github.io/MrBayes/</a>	N/A
R V 4.3.1	<a href="https://www.r-project.org/">https://www.r-project.org/</a>	N/A
R package paleotree V 3.3.25	<a href="https://github.com/dwbapst/paleotree">https://github.com/dwbapst/paleotree</a>	N/A
R package phytools V 1.0-1	<a href="https://github.com/cran/phytools">https://github.com/cran/phytools</a>	N/A
R package geiger V 2.0.7.1	<a href="https://github.com/cran/geiger">https://github.com/cran/geiger</a>	N/A
R package tibble V 3.1.6	<a href="https://github.com/tidyverse/tibble">https://github.com/tidyverse/tibble</a>	N/A
R package foreach V 1.5.1	<a href="https://github.com/cran/foreach">https://github.com/cran/foreach</a>	N/A
R package reshape V 0.8.9	<a href="https://github.com/hadley/reshape">https://github.com/hadley/reshape</a>	N/A
R package palaeoverse V 1.0.0	<a href="https://github.com/palaeoverse/palaeoverse">https://github.com/palaeoverse/palaeoverse</a>	N/A

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Embryos of *Tropidurus catalanensis***

Embryos of the lizard *Tropidurus catalanensis* were obtained from eggs of gravid females captured at the municipality of São Simão, São Paulo, Brazil (21° 28' 44" S 47° 33' 03" W). The females were transported in cloth bags and maintained in the animal facility 'Biotério de Vertebrados Silvestres' of the FFCLRP (see [supplemental information](#) for institutional abbreviations) at the University of São Paulo. Animal capture and maintenance were conducted according to the Brazilian legislation, with authorization from the Brazilian 'Ministério do Meio Ambiente' – SISBIO (*Sistema de Autorização e Informação em Biodiversidade* - Permit Number: 70079-1), and approval from the 'Comitê de Ética no uso de Animais' (Ethic Committee on the Use of animals in Research) of FFCLRP at the University of São Paulo. After oviposition, eggs were incubated at 30°C and embryos were extracted following previously documented developmental stages<sup>55</sup>: stage 35 (initial formation of the tympanic membrane – [Figure 2](#)) to stage 38 (tympanic membrane fully formed – [Figure 3](#)).

**Embryos of *Caiman yacare***

Embryos of *Caiman yacare* were obtained in collaboration with the caiman farm 'Caimasul', in the municipality of Corumbá (MS – Brazil). Due to the lack of a developmental table establishing the embryonic stages of *Caiman yacare*, we estimated the day of embryonic development based on comparisons with data published for the closely related species *Caiman latirostris*.<sup>56</sup> Eggs were incubated at 36°C. Embryos of *Caiman yacare* from 18 days (initial formation of the tympanic membrane – [Figure 2](#)) to 32 days (tympanic membrane fully formed – [Figure 3](#)) were collected.

**METHOD DETAILS**

**Nomenclature of reptilian groups**

The term "crown reptiles" is here used to refer to all members, extant and extinct, of the minimal clade including the most recent common ancestor of living turtles, squamates, rhynchocephalians, birds, and crocodylians. Additionally, the term 'reptiles' is here

used to refer to all taxa belonging to the most inclusive clade containing crown reptiles but not mammals (see also Simões et al. and Modesto and Anderson<sup>20,57</sup>). Note that under the phylogenetic hypotheses used in this work (see below), our informal definition of “crown reptiles” would be equivalent to that of Reptilia presented in the Phylonyms.<sup>58</sup> However, no total clade definition for Reptilia has been proposed so far. Hence, taxa traditionally considered as reptiles (or sauropsids<sup>20</sup>), such as some “parareptiles” would not be compose Reptilia. As pointed out in a recent study,<sup>20</sup> the definitions for the more inclusive clades of reptiles still require a substantial revision.

### Phylogenetic framework

Composite phylogenies combining trees from current literature were used as the framework for ancestral state reconstruction analyses of the presence/absence of a tympanic membrane (see further details below). The core of the topologies corresponds to phylogenetic hypotheses presented in two recent studies on the early evolution of lepidosauromorphs,<sup>11,13</sup> which in turn used a modified version of one of the most complete phylogenetic data matrices built for early reptiles so far.<sup>12</sup> To maximize information on earlier nodes of reptilian subgroups, we added taxa from poorly sampled groups following recent phylogenetic hypotheses proposed for each of them, namely: Archosauromorpha,<sup>24</sup> “marine reptiles” (the least inclusive clade including Ichthyopterygia and Sauropterygia,<sup>48</sup> and Choristodera.<sup>59</sup>

To accommodate major uncertainties in early reptilian phylogeny, we performed the analyses using six alternative topologies. Two of these correspond to the original results of Ford et al.<sup>13</sup> and Martínez et al.<sup>11</sup> with the additional taxa incorporated as described above. These topologies accommodate the uncertainties regarding the position of the gliding kuehneosaurids, with their position either as archosauromorphs<sup>11</sup> or as lepidosauromorphs.<sup>13</sup> Additionally, as both original topologies have turtles depicted as the sister group of ‘Lepidosauromorpha + Archosauromorpha’, two alternative phylogenetic arrangements for each of these trees were also tested, one with turtles as the sister group of Archosauromorpha and the other with turtles as the sister group of Lepidosauromorpha.<sup>20,22,23</sup>

### Defining the presence/absence of the tympanic membrane

In the lack of unambiguous correlates of the tympanic membrane (see Main Text), the presence of a tympanic membrane was inferred based on a combination of traits related to the otic region of the skull using two different approaches. In the first approach, a tympanic membrane was considered present for taxa exhibiting the character state ‘0’ for the characters A, B, and C (see below). To maximize the number of taxa for which the presence absence of a tympanic membrane could be scored, a second analyses was conducted with the presence of a tympanic membrane being inferred for those taxa scored with character state ‘0’ for characters A and B below. The exclusion of character C is due to the fact that the columella is more rarely preserved and usually only briefly described.

#### Character A

*Posterior Margin of the quadrate shaft: 0 – concave; 1 straight/convex* (Figure S1). This character captures the variation on the posterior margin of the quadrate that is observed in animals with tympanic and atympanic ears. Stem-saurians, which do not have an aperture in the otic region - and hence no tympanic membrane - have a quadrate with either a straight (e.g., *Araeoscellis*, *Coerulosauravus elivensis*) or slightly convex (e.g., *Youngina capensis*) posterior margin.<sup>31,54</sup> A columnar quadrate is also observed in living reptiles without a tympanic middle ear, such as chameleons and snakes (Table S1). On the other hand, the evolution of a tympanic middle ear is associated with the opening in the otic region of the skull, which is typically related to a quadrate with a concave posterior margin of the quadrate, establishing a space for the columella (or extracolumella) to attach to the tympanic membrane.

#### Character B

*Quadrate shaft: 0 – lateral and posterior surface exposed; 1 – lateral surface overlapped by either the squamosal or quadratojugal* (Figure S1). In living reptiles (Figure S2), excluding turtles, the anterior margin of the tympanic membrane is attached to the quadrate, either on the medial (e.g., some birds) or on the lateral (e.g., lizards and crocodylians) margin of the quadrate. In lizards and crocodylians, the lateral surface of the quadrate is completely exposed, in a way that the tympanic membrane covers the entire lateral flange of the bone. In extinct taxa, the ventral ramus of the squamosal and/or the dorsal ramus of the quadratojugal overlap parts of the lateral flange of the quadrate in taxa such as the early lepidosauromorph *Sophineta cracoviensis* (Figure S1) In this configuration, a tympanic membrane, when present, would only cover the lateral flange of the quadrate posterior to the region where the bone overlaps the quadratojugal and/or the squamosal. On the other hand, in stem-saurians such as *Petrolacosaurus kansensis* and *Youngina capensis*<sup>54</sup> the quadrate shaft is overlapped by either the squamosal and/or the quadratojugal.

#### Character C

*Stapes, contact between basicranium and quadrate: 0 – absent; 1 – present*. In stem reptiles such as *Youngina capensis*, the stapes is a massive bone that serves as a bracing structure between the braincase and the quadrate.<sup>31,54</sup> Differently, the stapes of crown reptiles is a slender bone that does not function as support for the basicranium.

### In-situ hybridization

In-situ hybridizations were performed in embryos at initial stages of the formation of the tympanic membrane: stage 35 of *Tropidurus catalanensis* and 15-18 dpo embryos of *Caiman yacare*. We designed primers for PCR amplification of *Tropidurus catalanensis* and *Caiman yacare* *Hoxa2* DNA templates used for synthesis of *in situ* hybridization RNA probes based on Genbank sequences of *Anolis carolinensis*-*Pogona vitticeps* and *Alligator mississippiensis*-*Alligator sinensis*, respectively. Products with the expected length (~0.5 kb) were amplified from genomic DNA and cloned into pGEM-T vector (Promega), confirmed by sequencing, and used as



templates for *in vitro* transcription with digoxigenin-labelled UTP (Roche). Whole embryo heads ( $n=5$  for embryos of *T. catalanensis* in stage 35 and  $n=5$  for 15–18 dpo *C. yacare* embryos) were fixed in 4% PFA, dissected from extra embryonic membranes and dehydrated in methanol prior to storage at  $-20^{\circ}\text{C}$ . Before rehydration, embryos were decapitated with a scissor and the heads were permeabilized with 10  $\mu\text{g}/\text{ml}$  Proteinase K for 30 min and *in situ* hybridized at  $67\text{--}69^{\circ}\text{C}$ , following procedures previously described for chicken whole-mount embryos.<sup>60</sup> The washes and buffer switching steps were extended to 30 min each to enable efficient diffusion into tissue. The reaction was developed with an NBT/BCIP mix.

### Histological sections and diceCT

Histological sectioning and diffusible iodine-based contrast-enhanced computed tomography (diceCT<sup>61</sup>; [Figure 3](#)) were implemented using embryos at different stages that encompass the complete development of the tympanic membrane, from its initial formation (stage 35 [21–23 dpo] for *Tropidurus catalanensis* and 15–18 dpo for *Caiman yacare* embryos) until it was almost fully formed (stage 38 [30–38 dpo] for *Tropidurus catalanensis* and 32 dpo for *Caiman yacare*), which corresponds to a morphology mostly similar to that observed in the adults. For the processing of histological sections and computed tomography, embryos were fixed using 4% paraformaldehyde (PFA) in PBS. Early-stage embryos were fixed overnight at  $4^{\circ}\text{C}$ , whereas embryos at later stages were fixed overnight at room temperature. Afterwards, embryos were stored in 70% ethanol (EtOH) solution. For histological sections, embryos were dehydrated in an ethanol-rising series, cleared with xylol, and then impregnated and embedded in paraffin. For the diceCT procedure, embryos were stained with Lugol solution (1% w/v). Embryos were maintained in the Lugol solution (1%) for 1–2 hours. The scans were conducted using a Nanotom Scan machine—GE Sensing & Inspection Technologies GmbH (Wunstorf, Germany) at the Centro para Documentação da Biodiversidade, Universidade de São Paulo (Ribeirão Preto, Brazil). We processed the 3D models of the developing ear using the software Amira (version 5.3.3, Visage Imaging, Berlin, Germany).

## QUANTIFICATION AND STATISTICAL ANALYSIS

### Time calibration

To account for eventual effects of differences in branch lengths, we time-calibrated these six initial topologies using two different approaches: the probabilistic ‘*a posteriori*’ time-scaling method *cal3*,<sup>62</sup> and a Bayesian tip-dating analysis using a fossilized birth–death (FBD) model.<sup>63–66</sup> Temporal information for each taxon (i.e., geological age ranges) was obtained from the literature ([data and code availability](#)). For the *cal3* method, speciation and extinction rates (assumed to be the same) were estimated using the function *Rate2sProb*<sup>67</sup> in R version 4.1.0<sup>68</sup> and dividing them by the interval length, whereas the sampling rate was randomly drawn from a uniform distribution of rates previously estimated for tetrapods.<sup>69,70</sup> Time calibration was performed using the R package *paleotree*<sup>67</sup> scripts made available by Stubbs et al.<sup>71</sup> resulting in 100 trees for each initial topology to account for temporal uncertainty. For the FBD analyses, we used the R package *paleotree*<sup>67</sup> to create an “empty” character matrix, with the initial topologies considered as topological constraints, which was used as a MrBayes (version 3.2.6<sup>72</sup>) command file. Uniform priors were placed on taxon ages (using taxon age ranges) and on the root age (with maximum and minimum ages set to 315.2 and 307 Ma, respectively). For each initial topology, we performed two independent Markov Chain Monte Carlo (MCMC) runs, with four chains each, for 20,000,000 generations. Convergence was assessed using PSRF (potential scale reduction factor) and average standard deviation of split frequency values (with values approaching 1.0 and below 0.01, respectively). After the runs converged, 30% of sampled trees were discarded as burn-in. As for *cal3*, 100 trees were randomly selected for each initial topology and used in downstream analyses, resulting in 1,200 trees in total. AIC scores ([data and code availability](#)) indicate that trees calibrated with the fossilized birth–death (FBD) had the strongest support in 10 out of 12 different combinations of reconstructions (i.e., six initial topologies and two morphological characters).

### Inference of the ancestral morphology

We used the 1,200 time-calibrated trees to run stochastic ancestral state reconstruction analyses using the R function *make.simmap* from the package *phytools*,<sup>73</sup> which fits a continuous-time reversible Markov model to simulate character history. Given that the morphology was unknown for certain taxa (i.e., tips with missing data), we provided specified prior probabilities on the tip states (following Revell<sup>74</sup>), based on the number of states for each character. We estimated the ancestral states of these three characters, which provided us robust evidence for the presence or absence of a tympanic middle ear at the base of the crown group. To do that, we used two different models of evolution: an all-rates-different model (ARD model), which assumes that different states evolved freely and had different rates parameters, and an equal rates model (ER model), which assumes a single rate for all branches in the topology. We ran 100 stochastic simulations for each tree. Results from all 100 trees of each initial topology and each time-calibration method were summarized using the R function *describe.simmap*.<sup>73</sup> For each of the two approaches and six initial topologies, relative support for the evolutionary models and time-calibration methods was assessed using AIC (Akaike’s information criterion<sup>75</sup>) scores to determine the best fit. AIC scores ([data and code availability](#)) indicate that the ARD model was preferred in all of the different combinations of reconstructions (i.e., six initial topologies and two morphological characters).