High-resolution X-ray computed tomography to understand ruminant phylogeny

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ABSTRACT

High-resolution X-ray computed tomography has become a vital technique to study fossils down to the true micrometer level. Paleontological research requires the non-destructive analysis of internal structures of fossil specimens. We show how X-ray computed tomography enables us to visualize the inner ear of extinct and extant ruminants without skull destruction. The inner ear, a sensory organ for hearing and balance has a rather complex three-dimensional morphology and thus provides relevant phylogenetical information what has been to date essentially shown in primates. We made visible the inner ears of a set of living and fossil ruminants using the phoenix|x-ray nanotom® m (GE Sensing & Inspection Technologies GmbH). Because of the high absorbing objects a tungsten target was used and the experiments were performed with maximum accelerating voltage of 180 kV and a beam current of 30 μA. Possible stem ruminants of the living families are known in the fossil record but extreme morphological convergences in external structures such as teeth is a strong limitation to our understanding of the evolutionary history of this economically important group of animals. We thus investigate the inner ear to assess its phylogenetical potential for ruminants and our first results show strong family-level morphological differences.

Keywords: Microtomography, laboratory X-ray source, fossil specimens, Ruminantia, phylogeny, inner ear

1. INTRODUCTION

Paleontology is the study of fossils. Fossilized remains of extinct animals are rare and these precious specimens give insights into past diversity or environments and testify to the evolution of life over millions of years. A task paleontologists are facing is the reconstruction of the tree of life. Understanding the relationships between the species, their origin and evolution through time in relation with their environment has bearings on conservation programs and definition of protected areas. In mammals, extinct species or genera are often recovered from tooth remains, which are composed of hard tissues (e.g., enamel) prone to fossilization. Those are highly convergent structures directly related to feeding habits and thus reach a limit in their discriminating power to differentiate the early divergence in several groups such as ruminants, the group of mouse deer, deer, musk deer, cattle, antelopes, giraffes and pronghorns. The bony labyrinth or inner ear is the organ of hearing and balance, its potential for discriminating primate species has recently been shown [1]. This structure has long been known [2–4] but from biological preparations or low-quality radiographs. It is virtually unknown in living and extinct ruminants. Maier increased our knowledge of parts of this organ but with the aim of looking at the development of the entotympanic bone [5]. He published sections of the inner ear of juvenile specimens in as most as 17 ruminant species. These sections give an idea of the shape of parts of the bony labyrinth in late fetal stages but do not allow reconstructing it in its entirety.

The position of the inner ear in the skull, embedded in the petrosal bone, is mostly the cause of this situation whereby very few is known. It is difficult to access, especially for rare fossil specimens where destruction is most of the time unimaginable. High-resolution X-ray computed tomography makes this structure available for three-dimensional representation and is the topic of this contribution.

The technique is not new to paleontologists. They have been investigating internal structures through CT-scanning for more than 20 years (e.g., [6–8]) but it became largely used in the past 5 to 10 years and is rapidly

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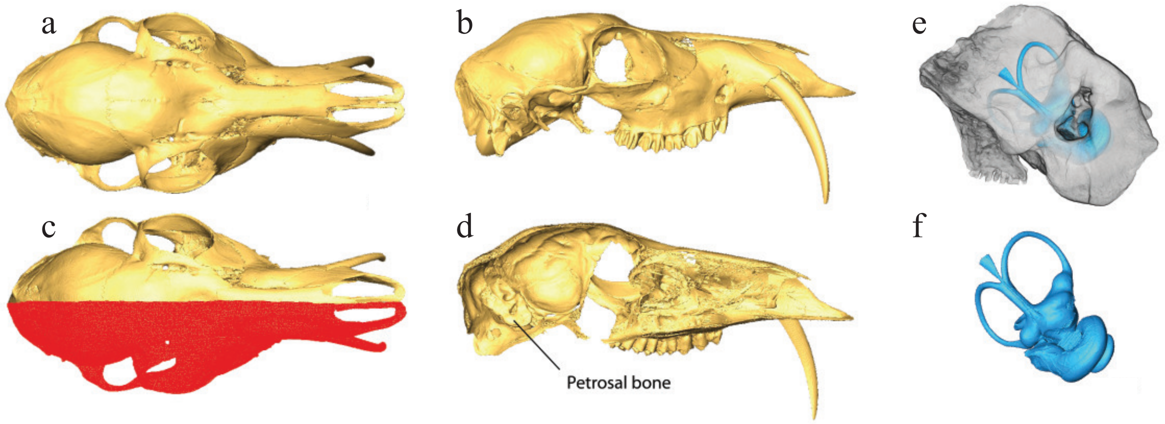

Figure 1. Skull of the Siberian musk deer *Moschus moschiferus* (NMB 8874). a: dorsal view; b: lateral view; c: dorsal view of the skull showing digitally removed half in red to indicate the petrosal bone in d; d: lateral view of halved skull showing the internal part; e: transparent left petrosal bone (NMB 4201) with the same orientation as in d showing the inner ear (blue); f: isolated left inner ear of same specimen showing its orientation in the skull.

developing (e.g., [9–14]). Morphological variability and ontogenetic development of this organ are only starting to be investigated in most mammals [12, 15]. The first results of the latter studies indicate that post-natal development may be limited since the petrosal bone ossifies before birth in many mammals. Large variability may only occur in species with very specific behaviours such as very slow locomotion. Both preliminary observations confirm the high potential of this structure for functional and phylogenetical analyses.

We show two examples, in deer and musk deer, where we trace back the inner ear morphology at the origin of the two families. We indicate how this structure relates living and extinct species of the same families and how it helps understanding their phylogenetical relationships, in the context of the debated phylogenetic positions of both families within ruminants. We show how the specimens were scanned and how CT scanning improves our knowledge of internal structures in ruminants.

2. MATERIALS AND METHODS

2.1 Specimen preparation

Four specimens from the collections of the Natural History Museum Basel (NMB) are studied in this paper. Two living ruminants from two families are studied. The muntjak deer *Muntiacus muntjak* (family *Cervidae*, specimen number NMB C.2408) and the musk deer *Moschus moschiferus* (family *Moschidae*, specimen number NMB 4201). Another skull of the musk deer (NMB 8874) is presented in Fig. 1 and shows the orientation of the petrosal bone and the bony labyrinth within the brain cavity. Two fossil ruminants belonging to the same families were also investigated; they both come from the fossil-bearing locality Steinheim (Germany, dated to ca. 14.5 Myrs [16]): the fossil musk deer *Micromeryx flourensianus* (family *Moschidae*, specimen number NMB Sth.865) and the fossil deer *Heteroprox* sp. (family *Cervidae*, specimen number NMB Sth.2390a).

2.2 X-Ray microtomography

The X-ray microtomography measurements were performed using a nanotom® m (phoenix|x-ray, GE Sensing & Inspection Technologies GmbH, Wunstorf, Germany) which is equipped with a 180 kV / 15 W nanofocus X-ray source. During 1440 aquiangular radiographs were taken over 360° the specimens were fixed on the precision rotation stage. In order to image the high absorbing specimens an accelerating voltage of 180 kV and a beam current of 30 μA were used where the mean photon energy was increased by adding a 0.25 mm *Cu* filter. The whole scanning time of one specimen took about 2 hours. The camera readout (3072 × 2400 pixels) resulted in a pixel length of 39.2 μm for *Muntiacus muntjak*, 35.0 μm for *Heteroprox larteti*, 18.5 μm for *Moschus moschiferus*

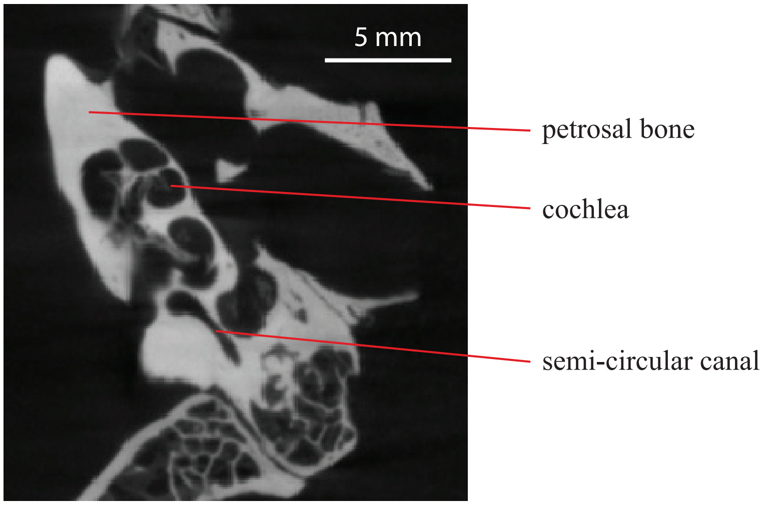

Figure 2. Slice through the petrosal bone of the Siberian musk deer *Moschus moschiferus* including the cochlea and the semi-circular canal.

and for *Micromeryxflourensianus*. For the reconstruction of the bony labyrinths the pixel size was chosen to be below 40 μm since even fine details are largely visible under that spatial resolution resolution.

2.3 Data treatment

The projections were reconstructed using a cone beam filtered back-projection algorithm using phoenix datos|x 2.0.1 - RTM (GE Sensing & Inspection Technologies GmbH, Wunstorf, Germany). Before further data treatment, the data sets were converted into DICOM format using VGStudio MAX 2.1 (Volume Graphics GmbH, Heidelberg, Germany).

3D representation of the bony labyrinth is achieved through a segmentation process. This was done using software AVIZO® 7.0. It requires loading the slices produced by the micro-CT scanner and filling in the gaps corresponding to structures of the targeted bony labyrinth on each slice (cp. Figs. 1 and 2). This is an easy and rapid process when contrast between bone and voids is strong. As far as fossils are concerned, fossilisation has led to a bone being embedded in sediment and often being filled with sediment. During fossilisation, the bone and the sediment is hardened. This can lead to a situation where bone and voids, filled with sediment, may show a very close contrast impeding an automatized segmentation. In this case, manual selection of the structures belonging to the bony labyrinth is needed and dramatically increases segmentation time. In our case, only minor sediment deposition occurred within the bony labyrinth rendering segmentation easier, hence the quality of the fossil bony labyrinths as high as in the living species.

3. RESULTS

3.1 The bony labyrinth of deer

Muntiacus muntjak The bony labyrinth of the muntjac (Fig. 3 top left) has a cochlea showing 2.5 turns with a large but not massive basal whorl. All turns are not overlapping leading to a not tightly coiled cochlea. The cochlear aqueduct jetting out of the cochlea is rather long and not very large, its diameter is slightly larger than that of the semicircular canals. Both fenestra face posteriorly and the fenestra cochleae is much larger than the fenestra vestibuli (where the stapes articulates with the inner ear). The vestibule shows a well visible utricule and saccule, the latter being larger. The semicircular canals are round in shape and their ampulla at their base are bulbous. The posterior limb of the lateral semicircular canal enters the vestibule in the ampulla of the posterior canal, very close to it building a junction between both canals at their base. Both the anterior and posterior canals are extended above the level of the common crus linking them. The vestibular aqueduct is aligned with the common crus but it originates slightly rostrally, not directly in line with it. The endolymphatic sac at its end is small and triangular in shape.

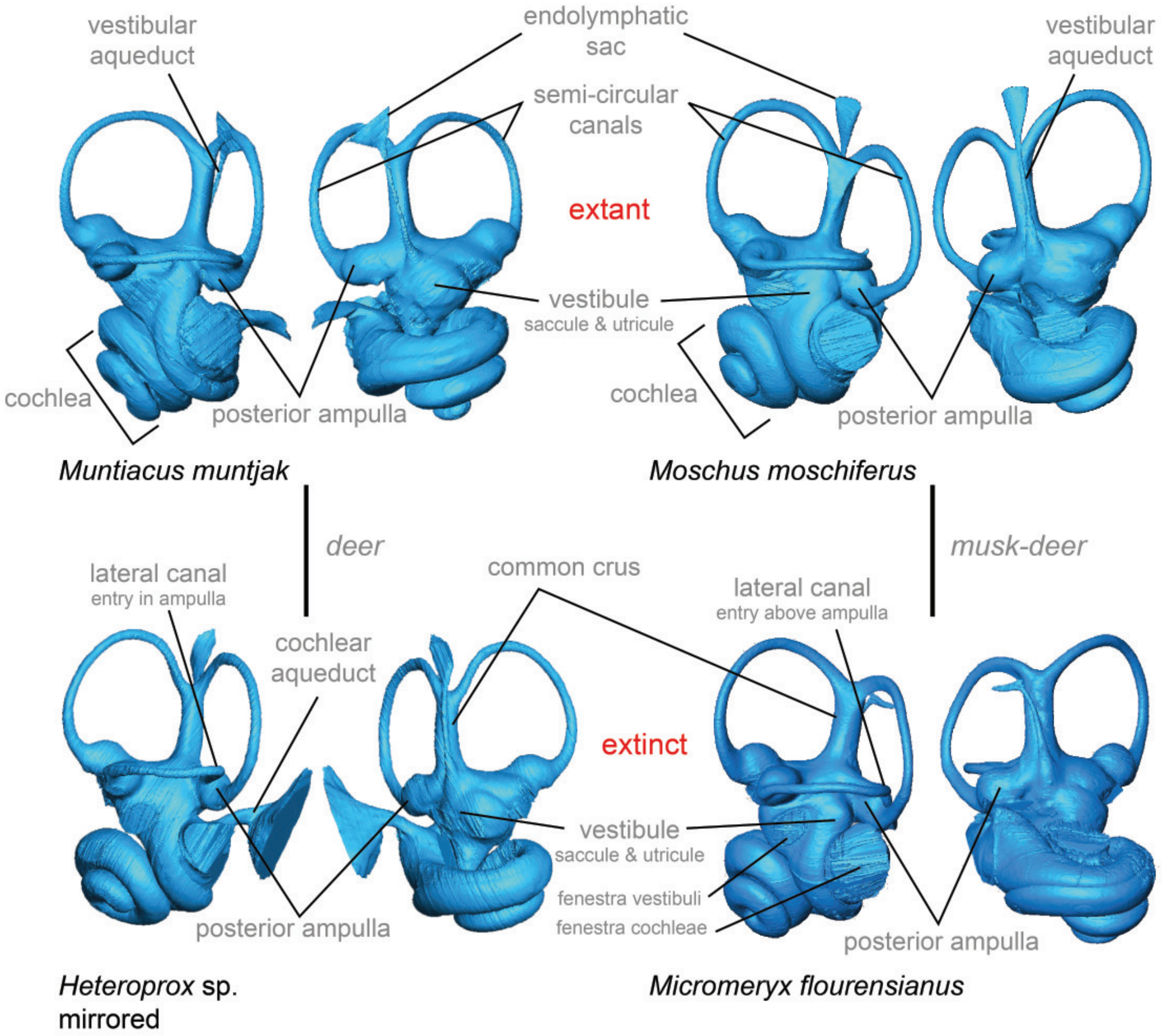

Figure 3. 3D representations of the inner ears in two ruminant lineages, that of deer (left) and of musk-deer (right). On the top: living animals illustrated by pictures, on the bottom: fossil species.

***Heteroprox* sp.** The cochlea of *Heteroprox* sp. (Fig. 3 bottom left) only shows 2 turns. The basal whorl is more massive than in *Muntiacus* and the cochlea is slightly more tightly coiled. The cochlear aqueduct is long, its diameter is about the size of a semicircular canal but it ends in a very large opening within the petrosal bone. As usual in most ruminants, the fenestra cochleae is larger than the fenestra vestibuli. Both chambers of the vestibule, the utricule and saccule are round and well visible; the saccule is larger. The semicircular canals extend largely above the common crus, more than in *Muntiacus*. The vestibular aqueduct runs along the common crus but on its rostral side. It originates rostrally at its base. The posterior limb of the lateral semicircular canal enters the vestibule in the ampulla of the posterior canal but rostrally, no junction between both canals is evidenced. The lateral canal does not extend enough to reach the posterior canal. The endolymphatic sac is very small at the end of the aqueduct.

3.2 The bony labyrinth of musk deer

Moschus moschiferus *Moschus* (Fig. 3 top right) has a cochlea with 2 turns, both turns are massive and tightly joined. The cochlear aqueduct is short and rather large in diameter. The fenestra vestibuli is elliptical in shape, more than in the above-mentioned taxa. The utricule and saccule are well visible, and their sizes are almost identical with a slightly less expanded utricule. The anterior semicircular canal extends above the common crus more than the posterior canal. The posterior limb of the lateral semicircular canal ends above the posterior ampulla, a very different situation in both deer described above. The lateral semicircular canal is rather small in size. The posterior ampulla is low. The vestibular aqueduct runs along the common crus, it originates along its midline at its base and ends in a small triangular and tubular endolymphatic sac.

Micromeryx flourensianus The bony labyrinth of *Micromeryx* (Fig. 3 bottom right) looks very close to that of *Moschus*. The cochlea is only slightly more massive, especially the basal whorl. It is tightly coiled and closely packed against the vestibule. The cochlear aqueduct is also small and has a small diameter. Both chambers of the vestibule are well visible with a larger saccule. The ampulla are bigger and rounder than in *Moschus*. The posterior limb of the lateral semicircular canal ends above the posterior ampulla and the posterior ampulla is low, as it is also the case in *Moschus*. Both canals do not join. Only the anterior semicircular canal extends above the common crus. The vestibular aqueduct runs along the common crus up to half its length and then turns posteriorly ending in a small, flat and triangular in shape endolymphatic sac.

4. DISCUSSION

While the inner ear is the organ of hearing and balance leading to its study for functional and ecological aspects (e.g., [17, 18]), its potential for phylogeny has recently been shown in mammals [1, 13]. Debated questions regarding the phylogeny of ruminants led us to start investigating this organ in the group. We study ruminant phylogeny with a deep-time approach bringing the evolutionary history of closely related taxa into the debate. Living and extinct taxa are thus studied here and in a broader project. Large differences are evidenced between both ruminant families studied here. Musk-deer seem to exhibit a very conservative morphology of their inner ears with a massive and short cochlea (low number of whorls), a massive vestibule and a low-positioned posterior ampulla. The latter is higher in deer and their cochleas are less massive with broader and thinner whorls. The posterior limb of the lateral semicircular canal of deer enters the vestibule through the posterior ampulla whereas it enters the vestibule higher above the ampulla in musk-deer. Early artiodactyls show a secondary common crus linking the lateral and posterior semicircular canals (e.g., [11]). The junction of both canals in *Muntiacus* slightly mimics this basal condition but no real common crus is achieved since both canals are still visible over their whole length. Proximity in both canals only led to a partial junction. The functional impact of this structure is still unknown.

Deer and musk deer have long been phylogenetically associated, either based on morphological or molecular characters [19]. Recently, musk deer have been shown to be closer to bovids [20]. This question is still debated and the bony labyrinth shows how different deer and musk deer are, especially when considering the two fossil species situated deep in time, close to the origin of both families [21]. Comparison of the living musk deer bony labyrinth with a living bovid indicated some proximity especially in the way the posterior limb of the lateral semicircular canal enters the vestibule [22]. Such differences in animals that are almost 15 million years old show that the divergence of both taxa occurred earlier and were already well separated by the Middle Miocene. No early bovid inner ear is known so far. It could bring more information on this debate.

Both fossil taxa have more massive basal whorls of their cochlea than in the living species. This character seems to be plesiomorphic in ruminants since it also occurs in other fossil taxa. All four taxa exhibit cochleas with a low number of turns which seems to correlate to higher frequencies capacities [23, 24] but musk deer seem to have more tightly coiled cochleas than deer, a condition that has been associated to lower frequencies capacities [23]. Loosely coiled cochleas have indeed been shown to favor higher frequency abilities. This could apply to Muntiacus although its slight loose coiling does not reach the level seen in odontocetes [25].

The present study has impact and gets feedback from microtomography studies of the cochlea from currently living animal studies [26] and even post-mortem studies of the human inner ear with two and a half turns and with a resolution down to individual cells [27, 28]. Compared to the high-resolution data of Lareida et al., studies of fossils cannot incorporate staining procedures with materials such as osmium. While high-resolution images from human and porcine cochleas have to be based on rather sophisticated synchrotron radiation sources, fossils only require a moderate resolution available at advanced laboratory sources. The results of fossil studies, however, only include hard tissues and mineralised parts. Related structures of the soft anatomy are generally not present anymore.

5. CONCLUSIONS

Very little is known as to how reliable the inner ear can contribute to the ruminant phylogenetic tree. We briefly show here that access to this structure through high-resolution X-ray computed tomography is a source of previously unknown morphological information. We hope this will help resolving the still unresolved phylogenetic position of musk-deer, variously related to deer or bovids in morphological and molecular studies. Phylogenetic analysis of this morphological data constitutes the next step of this work together with increase in sampled taxa within living and extinct ruminants. Empirical data show that reconstructing ruminant inner ears requires a resolution of at least 70 μm, worse resolutions induce a loss of details that does not allow comparisons to be made, hence the uselessness of medical scanners. Likewise, for this purpose it is not necessary to scan at very high resolutions or below 20 μm. It can make the scanning process longer and most of the segmentation very time-consuming, for only a moderate quality gain. The original bone material in fossils is often recrystallized and empty space filled with sediments; this simply requires higher energy (up to 180 kV, depending on the specimen) to yield reasonable contrast data.

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REFERENCES

[1] Gunz, P., Ramsier, M., Kuhrig, M., Hublin, J. J., and Spoor, F., "The mammalian bony labyrinth reconsidered, introducing a comprehensive geometric morphometric approach," *J. Anat.* **220**, 529–543 (2012).

[2] Hyrtl, J., [*Verleichende-anatomische Untersuchungen über das innere Gehörorgan des Menschen und der Säugethiere*], Friedrich Ehrlich, Prague (1845).

[3] Gray, A. A., [*The labyrinth of animals: including mammals, birds, reptiles and amphibians, Volume 1*], J. and A. Churchill, London (1907).

[4] Gray, A. A., [*The labyrinth of animals: including mammals, birds, reptiles and amphibians, Volume 2*], J. and A. Churchill, London (1908).

[5] Maier, W., "The Entotympanic in Late Fetal Artiodactyla (Mammalia)," *J. Morphol.* **275**, 926–939 (2013).

[6] Zonneveld, F. W., [*Computed Tomography of the Temporal Bone and Orbit: Technique of Direct Multiplanar, High-resolution CT and Correlative Cryosectional Anatomy*], Urban and Schwarzenberg (1987).

[7] Luo, Z.-X. and Ketten, D. R., "CT scanning and computerized reconstructions of the inner ear of multituberculate mammals," *J. Vert. Pal.* **11**, 220–228 (1991).

[8] Spoor, F. and Zonneveld, F. W., "Morphometry of the primate bony labyrinth: a new method based on high-resolution computed tomography," *J. Anat.* **186**, 271–286 (1995).

[9] Luo, Z.-X., I., R., Schultz, J. A., and Martin, T., "Fossil evidence on evolution of inner ear cochlea in Jurassic mammals," *Proc. R. Soc. B* **278**, 28–34 (2011).

[10] Meng, J., Yuanqing, W., and Chuankui, L., "Transitional mammalian middle ear from a new Cretaceous Jehol eutriconodontan," *Nature* **472**, 181–185 (2011).

[11] Orliac, M., Benoit, J., and OLeary, M. A., "The inner ear of *Diacodexis*, the oldest artiodactyl mammal," *J. Anat.* **221**, 417–426 (2012).

[12] Billet, G., Hautier, L., Asher, R. J., Schwarz, C., Crumpto, N., Martin, T., and Ruf, I., "High morphological variation of vestibular system accompanies slow and infrequent locomotion in three-toed sloths," *Proc. R. Soc. B* **279**, 3932–3939 (2012).

[13] Ekdale, E. G., "Comparative anatomy of the bony labyrinth (inner ear) of placentals mammals," *PlosOne* **8**, 1–100 (2013).

[14] Ravel, A. and Orliac, M., "The inner ear morphology of the condylarthran hyopsodus lepidus," *Hist. Biol.* **doi:10.1080/08912963.2014.915823**, in press (2014).

[15] Sánchez-Villagra, M. R. and Schmelzle, T., "Anatomy and development of the bony inner ear in the wooly opossum, Caluromys philander (Didelphimorphia, Marsupiala)," *Mastozool. Neotrop.* **14**, 53–60 (2007).

[16] Heizmann, E. P. J. and Reiff, W., [*Der Steinheimer Meteorkrater*], Dr. Friedrich Pfeil, Munich (2002).

[17] Spoor, F., Bajpal, S., Hussaim, S. T., Kumar, K., and Thewissen, J. G. M., "Vestibular evidence for the evolution of aquatic behaviour in early cetaceans," *Nature* **417**, 163–166 (2002).

[18] Spoor, F., Garland, T., Krovitz, G., Ryan, T. M., Silcox, M. T., and Walker, A., "The primate semicircular canal system and locomotion," *Proc. Natl. Acad. Sci. USA* **104**, 10808–10812 (2007).

[19] Hernández Fernández, M. and Vrba, E. S., "A complete estimate of the phylogenetic relationships in Ruminantia: a dated species-level supertree of the extant ruminants," *Biol. Rev.* **80**, 269–302 (2005).

[20] Hassanin, A., Delsuc, F., Ropiquet, A., Hammere, C., Jansen van Vuuren, B., Matthee, C., Ruiz-Garcia, M., Catzeflis, F., Areskoug, V., Thanh Nguyen, T., and Couloux, A., "Pattern and timing of diversification of Cetartiodactyla (Mammalia, Laurasiatheria), as revealed by a comprehensive analysis of mitochondrial genomes," *C. R. Biol.* **335**, 32–50 (2012).

[21] Bibi, F., "A multi-calibrated mitochondrial phylogeny of extant Bovidae (Artiodactyla, Ruminantia) and the importance of the fossil record to systematics," *BMC Evol. Biol.* **13**, 1–15 (2013).

[22] Costeur, L., "The petrosal bone and inner ear of *Micromeryx flourensianus* (Artiodactyla, Moschidae) and inferred potential for ruminant phylogenetics," *Zitteliana* **B32**, in press (2014).

[23] Coleman, M. N. and Colbert, M. W., "Correlations between auditory structures and hearing in non-human primates," *J. Morphol.* **271**, 511–532 (2010).

[24] Coleman, M. N., Kay, R. F., and Colbert, M. W., "Auditory morphology sensitivity in fossil new world monkeys," *Anat. Rec.* **293**, 1711–1721 (2010).

[25] Ketten, D. R., "The Cetacean ear: form, frequency, and evolution," in [*Marine Mammal Sensory Systems*], Thomas, J., Kastelein, R. A., and Supin, A. Y., eds., Plenum Press, New York (1992).

[26] Richter, C.-P., Liddy, W., Vo, A., Young, H., Stock, S., Xiao, X., and Whitlon, D., "Evaluation of neural cochlear structures after noise trauma using X-ray tomography," *Proc. SPIE* **9212**, in press (2014).

[27] Müller, B., Lareida, A., Beckmann, F., Diakov, G. M., Kral, F., Schwarm, F., Stoffner, R., Gunkel, A. R., Glueckert, R., Schrott-Fischer, A., Fischer, J., Andronache, A., and Freysinger, W., "Anatomy of the murine and human cochlea visualized at the cellular level by synchrotron-radiation-based microcomputed tomography," *Proc. SPIE* **6318**, 631805 (2006).

[28] Lareida, A., Beckmann, F., Schrott-Fischer, A., Glueckert, R., Freysinger, W., and Müller, B., "High-resolution X-ray tomography of the human inner ear: synchrotron radiation-based study of nerve fiber bundles, membranes, and ganglion cells ," *J. Microsc.* **234**, 95102 (2009).