

Comparative Anatomy of the Middle Ear Ossicles of Extant Hominids – Introducing a Geometric Morphometric Protocol

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Abstract

The presence of three interconnected auditory ossicles in the middle ear is a defining characteristic of mammals, and aspects of ossicle morphology are related to hearing sensitivity. However, analysis and comparison of ossicles are complicated by their minute size and complex three-dimensional shapes. Here we introduce a geometric morphometric measurement protocol for 3D shape analysis based on landmarks and semilandmarks obtained from μ CT images and apply it to ossicles of extant hominids (great apes and humans). We show that the protocol is reliable and reproducible over a range of voxel resolutions, and captures even subtle shape differences. Using this approach it is possible to distinguish the hominid taxa by mean shapes of their malleus and incus ($p < 0.01$). The stapes appears less diagnostic, although this may in part be related to the small sample size available. Using ancestral state estimation, we show that, within hominids, *Homo sapiens* is derived with respect to its malleus (short manubrium, long corpus, head anterior-posterior flattened, articular facet shape), incus (wide intercrural curvature, long incudal processes, articular facet shape) and stapes (high stapes with kidney-shaped footplate). *Homo sapiens* also shows a number of plesiomorphic shape traits whereas *Gorilla* and *Pan* possess a number of autapomorphic characteristics. The *Pongo* ossicles appear to be close to the plesiomorphic hominid condition. The malleus shows little difference in size among hominids, and allometry is thus of little importance. In contrast, the incus and stapes are more variable in size, and their shape is more strongly related to size differences. Although the form-function relationships in the middle ear are not fully understood, some aspects of ossicle morphology suggest that interspecific differences in hearing capacities are present among hominids. Finally, the results of this study provide a comparative framework for morphometric studies analyzing ossicles of extinct hominids, with a bearing on taxonomy, phylogeny and auditory function.

Keywords: malleus, incus, stapes, humans, great apes, 3D shape

Introduction

The presence in the middle ear of three interconnected auditory ossicles – malleus, incus, and stapes – is a defining character of mammals. The ossicular chain connects the tympanic membrane and the inner ear, and plays an important role in amplifying and regulating sound waves. It thus helps to overcome the impedance mismatch between air and the fluid of the inner ear, transferring high-frequency sound better than the single ossicle system of non-mammalian tetrapods (Zwislocki, 1965; Coleman and Ross, 2004; Puria and Steele, 2010; Schmidt et al., 2011). As such, the evolution of three auditory ossicles is a crucial adaptation allowing mammals to hear high-frequency sound (Rosowski, 2013). The transmission of sound energy from the tympanic membrane to the oval window involves two lever arms: one formed by the manubrium of the malleus and the other by the long crus of the incus. Furthermore, mass of the ossicles, moments of inertia, the morphology of the connecting joint and the resulting differences in rotational motion are important factors influencing the nature of sound transfer through the middle ear (Puria and Steele, 2010). Indeed, metrics expressing such morphological differences correlate with variation in frequency range of hearing across mammals (Hemilä et al., 1995; Coleman and Ross, 2004; Coleman and Colbert, 2010; Rosowski, 2013).

Ossicle morphology shows a wide range of variation within and between groups of mammals (Hyrtl, 1845; Doran, 1878; Fleischer, 1973; Hemilä et al., 1995; Nummela, 1995; Schmelzle et al., 2005; Masali and Cremasco, 2006; Mason, 2013; Quam et al., 2014), and can thus be used for taxonomic discrimination of extant and fossil species in, for example, primates

(Masali et al., 1992; Quam and Rak, 2008; Quam et al., 2013a,b, 2014). However, studying ossicles is methodologically challenging because of their small size and highly complex three-dimensional shape. Analyses of ossicle morphology have therefore focused on their mass or two-dimensional measurements, often to address questions related to middle ear physiology (Hemilä et al., 1995; Nummela, 1995; Coleman and Ross, 2004; Coleman and Colbert, 2010), although some quantify ossicle shape in more detail (Arensburg et al., 1981; Siori and Masali, 1983; Masali et al., 1992; Quam and Rak, 2008; Quam et al., 2013a, b, 2014). Such studies frequently employed size-calibrated photographs to obtain measurements, a method potentially prone to inaccuracies because of parallax errors and inter-observer differences in specimen orientation (Flohr et al., 2010). Furthermore, complex shape characteristics, such as manubrial curvature and head shape of the malleus and articular facets, cannot be represented in two dimensions (Schmidt et al., 2011).

Computed tomography with a spatial resolution well below 100 μm (micro CT) yields three-dimensional (3D) image data sets that provide completely new opportunities for the comparative and functional study of the auditory ossicles. Detailed CT-based surface reconstructions can be quantified accurately in 3D using landmarks (Schmidt et al., 2011). Subsequently, geometric morphometrics (GM) can be used for a full 3D analysis of size and shape (Bookstein, 1991; Slice, 2007; Mitteroecker and Gunz, 2009).

The purpose of this paper is twofold. First, we introduce a measurement protocol, based on 3D landmarks and semilandmarks, which comprehensively describes the size and shape of the ossicles, including functionally important aspects. Second, using the newly introduced methods we quantitatively describe and compare the ossicular chain of extant *Homo*, *Pan*, *Gorilla* and *Pongo*. To assess the phylogenetic polarity of morphological changes within hominids we also

included the ossicles of *Symphalangus syndactylus* – the largest hylobatid species – and apply phylogenetic comparative methods. In addition to providing a detailed exploration of extant hominid ossicle morphology, this study also aims to provide a framework for the comparative and functional interpretation of the fossil record of hominid ossicles (Angel, 1972; Arensburg and Nathan, 1972; Rak and Clarke, 1979; Heim, 1982; Arensburg and Tillier, 1983; Arensburg et al., 1996; Moggi-Cecchi and Collard, 2002; Spoor, 2002; Martínez et al., 2004; Lisonek and Trinkaus, 2006; Crevecoeur, 2007; Quam and Rak, 2008; Quam et al., 2013a, b).

Previous comparative work on hominid ossicle morphology has particularly highlighted how modern humans and extant great apes differ (Masali, 1968; Siori and Masali, 1983; Masali and Cremasco, 2006; Quam, 2006; Quam et al., 2013a, b, 2014), with some reference to differences among the latter. Great ape species show large differences in their vocal behavior and repertoire, even between closely related species like *Gorilla gorilla* and *Gorilla beringei* (Hohmann and Fruth, 1995; Hedwig et al., 2014), and they inhabit a large range of environments. Hence, given the correlations between hearing sensitivity and ossicle morphology (Hemilä et al., 1995; Coleman and Ross, 2004; Coleman and Colbert, 2010), distinct differences in ossicle shape may be expected between all hominid species.

Materials and methods

Sample and imaging the ossicles

The sample used in this study comprised mallei ($n=93$), incudes ($n=89$) and stapes ($n=45$) of *Homo sapiens*, *Pan troglodytes*, *Pan paniscus*, *G. beringei*, *G. gorilla*, *Pongo* sp. and *S. syndactylus*. We pooled both species of *Pongo* because the provenance (Borneo or Sumatra) of

these specimens was not known. Table 1 summarizes the information about provenance, sex, and age at death; these data (also including image spatial resolution) are provided for each specimen in the Supplementary Online Material (SOM) Table S1. Whenever possible, ossicles were extracted from the temporal bone and subsequently CT scanned with a spatial resolution in the range of 0.010 – 0.020 mm. In those cases where ossicles could not be removed without the risk of damage, or if the specimen was from a soft tissue collection, the temporal bone or, in some cases the entire skull, was CT scanned with the highest spatial resolution possible (0.022 – 0.091 mm). Subsequently, the ossicles were segmented manually (see below). Whenever possible, specimens were selected based on the presence of at least the malleus and incus. The majority of the modern human ossicle sample comes from the University of Leipzig anatomy collection (Germany), removed during cadaver dissections and from a collection of human skeletal remains from an early medieval cemetery at Greding, Germany, dated to the late 7th and early 8th century (Flohr et al., 2010). The majority of the non-human samples are wild specimens obtained from museum collections. CT images of specimens housed in the American Museum of Natural History were kindly provided by Rolf. M. Quam. All other specimens were scanned with the BIR ACTIS 225/300 or the Skyscan 1173 housed at MPI-EVA in Leipzig. Whenever possible, the right ossicles were analyzed, but when left ones were used, these were treated as right ones by mirror-imaging the image stack. Avizo 7.1 (Visualization Science Group) was used to create 3D digital surface models of the ossicles and place the landmarks and semilandmarks. In the case of isolated ossicles, Avizo's Isosurface module was employed using a single threshold value. In order to test for accuracy of threshold values, half maximum height levels were calculated periodically (Spoor and Zonneveld, 1995). Ossicles scanned inside the temporal bone were isolated and visualized using the Segmentation Editor. Surface models were saved in PLY

format. Landmark coordinates were exported from Avizo 7.1 and analyzed using Mathematica 8 (Wolfram Research, Inc.), with software routines developed by Gunz and Mitteroecker (2013).

--- *Table 1 approximately here* ---

Measurement protocol

The measurement protocol was designed so that the landmarks and semilandmarks represent the overall size and shape of the ossicles and quantify features known to be important functionally (Figure 1). Several of the anatomical landmarks follow the protocol of Schmidt et al. (2011).

--- *Figure 1 approximately here* ---

Malleus

Four landmarks were placed on the surface of the malleus (Figure 1). Landmark 1 represents the apex of the manubrium, positioned furthest away from the corpus of the malleus (Schmidt et al., 2011), and landmark 2 the center of the apex of the lateral process (*processus lateralis*).

Landmark 3 was placed on the deepest point of the posteriorly facing part of the articular facet of the malleus (Schmidt et al., 2011). Although easily recognized on an isolated malleus, the placement of the landmark can be aided by calculating a best-fit plane through landmarks placed on the margin of the posteriorly facing surface ('Slice' module in Avizo 7.1). Anterior translation

of this plane will identify a single, deepest point of the surface. Landmark 4 was directly placed on the joint wall of the medio-inferior part of the articular facet. As shown in Figure 1, it was located at the anteriormost aspect of the medio-inferior part and coincided with a narrow but distinct indentation of the joint wall of the articular facet. On the easily recognizable anterior process no landmark was placed since it was intraspecifically highly variable, often changing form during ontogeny of single individuals (Rosowski, 1994).

The shape of the manubrium was quantified by 20 semilandmarks positioned along the attachment of the tympanic membrane, going from landmarks 1 to 2 (Figure 1). On the broadened and flattened inferior part (spatula), the landmarks were placed along the anterior attachment ridge. The margin of the articular facet was quantified using 30 semilandmarks, running counterclockwise for a right malleus seen in posterior view, with landmark 4 as start and end point. The head of the malleus was quantified using semilandmarks that were placed automatically, following the procedure described in Gunz et al. (2005) and Gunz and Mitteroecker (2013). A mesh of surface semilandmarks was placed on the malleolar head of a template specimen (ULAC-M3). This mesh was warped from the template to each specimen, using a thin-plate spline (TPS) interpolation function computed from the four landmarks and the semilandmarks of the two curves. The warped mesh points “floated” in the vicinity of the actual surface of the receiving malleus specimen and was subsequently projected onto its surface (Gunz and Mitteroecker, 2013).

Incus

Four landmarks were placed on the surface of the incus. Following Schmidt et al. (2011), landmark 1 was placed on the center of the apex of the short crus, and landmark 2 on the most

inferior point of the long crus. Landmark 3 was placed on the deepest point of the laterally facing part of the articular facet. Landmark 4 was located in the deepest indentation of the margin of the articular facet's lateral side, where the latter was narrowest mediolaterally.

The shape of the intercrural arch was quantified using 20 semilandmarks placed along the curve between landmarks 1 and 2 (Figure 1). Semilandmarks were placed on the convex rounding furthest away from the articular facet. The articular facet was described by placing 30 semilandmarks on its margin, starting from landmark 4 and in a counterclockwise direction for a right incus seen in anterior view.

For the head of the malleus, semilandmarks were used to quantify the surface relief of the articular facets of the malleus and incus in shape analyses. However, our sample included a substantial number of specimens with ossicles still in articulation which required manual segmentation of the facet surfaces. In these cases, surface representation proved to be more irregular than when using threshold-based segmentation and, to avoid substantial differences in landmark accuracy, the analyses presented here use the outlines but not the surfaces of the facets. Nevertheless, in studies addressing specific questions regarding function it is advisable to extend the protocol with semilandmarks representing the facet surfaces.

Stapes

Three landmarks were placed on the surface of the stapes. Landmark 1 was placed on the posteriormost corner of the footplate, whereas landmark 2 was located at the anteriormost point of the footplate (Figure 1). Landmark 3 was placed in the center of the surface of the articular facet of the articulation with the lenticular process of the incus (Figure 1).

The shape of the arch formed by the anterior and posterior crura and the head of the stapes was quantified using 20 semilandmarks placed along the outermost (convex) surface of the two crura starting from landmark 1 and ending at landmark 2 (Figure 1). The shape of the stapes footplate was described by placing 30 landmarks along its margin, starting from landmark 1, in a clockwise direction for the right stapes seen in lateral view (Figure 1).

Geometric morphometrics & statistical analysis

Geometric morphometric methods require all specimens to have the same number of coordinates in homologous locations (Slice, 2007; Mitteroecker and Gunz, 2009). Following Gunz et al. (2005, 2012) the semilandmarks were therefore allowed to slide along their respective curves and surfaces to minimize the thin-plate spline bending energy between each specimen and the average shape, computed as the mean of all Procrustes coordinates (see below). This sliding step removes the effect of point spacing prior to the statistical analysis and thus ensures geometric correspondence of the semilandmarks among individuals; for technical details about semilandmark sliding see Bookstein (1997), Gunz et al. (2005), and Gunz and Mitteroecker (2013). All landmark and slid semilandmark data were subsequently converted into shape variables using Procrustes superimposition (Rohlf and Slice, 1990). Procrustes superimposition standardizes position and orientation and scales all specimens to unit centroid size — the square root of the sum of squared distances between a specimen's centroid and every (semi)landmark, respectively.

The Procrustes coordinates were analyzed using principal component analysis (PCA). To further consider differences in ossicle size we also computed a PCA in Procrustes form-space by analyzing the Procrustes shape coordinates with the natural logarithm of centroid size together

(Gunz and Mitteroecker, 2013). The statistical significance of shape differences between groups was tested using a permutation test (Good, 2013) based on the Procrustes distance between group means. To this end, we compared the length of the Procrustes distance between two group means with average differences computed after randomly reshuffling group affiliations 5000 times (Mitteroecker and Gunz, 2009). We acknowledge that estimation of mean shapes based on small sample sizes is problematic. However it is worth noting that the majority of the interspecific differences are significant in the permutation tests despite small sample sizes. Therefore, only *P. paniscus* ($n=1$) was excluded. Importantly, any reference to size in descriptions and comparisons based on shape space-based PCAs given below (e.g. the manubrium is long) concerns relative size, because absolute size has been removed.

To visualize subtle changes in ossicle shape, thin plate spline (TPS) interpolation (Bookstein, 1989, 1991), based on landmark and semilandmark coordinates, was used to warp a template surface according to the Procrustes shape variables along the first three principal components. Thin plate spline interpolation was also used to compute group mean shapes for visualization and comparison of species, by averaging the respective Procrustes shape coordinates, and by applying TPS interpolation based on the landmark and semilandmark coordinates a template surface of each species was warped to the (species) mean (Gunz and Harvati, 2007; Gunz et al., 2012).

To assess intra-observer error and the influence of differences in spatial resolution of the CT images, a malleus, an incus and a stapes of a modern human (Greding-41A) with a spatial resolution of 0.013 mm were resampled to 0.030 mm and 0.080 mm. Subsequently, one observer (AS) applied the landmark protocol three times for each bone and each resolution with an interval of an hour between each attempt (Figure 2).

In order to estimate the ancestral state of ossicle shape at successive nodes of the hominoid tree, as approximated by the ancestral coordinates along the first three principal components axes, we applied Phylogenetic Generalized Least-Squares (PGLS) methods (Martins and Hansen, 1997; Garland Jr and Ives, 2000; Rohlf, 2001). Determination of plesiomorphic/autapomorphic shape traits was done using Mesquite (Maddison and Maddison, 2007). Here, we first automatically portioned each PC axis into three equivalent bins. Subsequently, we mapped the distribution of these bins onto a phylogenetic tree of the hominoid species analysed using the linear parsimony reconstruction option of Mesquite. Finally, we interpreted the mapping of the PC scores onto the tree in order to reconstruct Figure 21.

Interspecific relationship and divergence dates of the species analyzed here were obtained from Perelman et al. (2011) and Langergraber et al. (2012). Names of the hominid clades follow Shoshani et al. (1996), and are defined as follows: [*Pongo/Gorilla/Pan/Homo*] = Hominidae (hominids); [*Gorilla/Pan/Homo*] = Homininae (hominines or African hominids); [*Homo/Pan*] = Hominini (hominins). Using this classification implies that the human clade should be named at a subtribal level as Hominina or homininans (Andrews & Harrison., 2005; Harrison, 2010a; Wood, 2010). In contrast, most current studies name the human clade at the tribal level as Hominini or hominins, but such classifications do not name either the *Gorilla/Pan/Homo* clade (Wood and Richmond, 2000) or the *Pan/Homo* clade (Harrison, 2010b) and cannot be used here. Finally, we use ‘great ape’ to describe non-human hominids, and not as a taxonomic term implying monophyly.

Linear and area measurements

Several functionally important dimensions of the ossicles were calculated to provide a record of our sample that can be compared easily with that of other studies (SOM Tables S2 – S4). For the malleus we computed the length of the manubrium as the direct distance between landmark 1 and landmark 2 and the maximum length as the direct distance between landmark 1 and an additional landmark (asterisk in Figure 1A; following landmark ‘HEA’ in Schmidt et al., 2011). For the incus we measured the direct distance between landmark 1 and landmark 2 as the intercrural distance. The functional length of the incus was measured between landmark 2 and 3 and the maximum length between landmark 2 and an additional landmark on the incus (asterisk in Figure 1B; following landmark ‘MAH’ in Schmidt et al., 2011). The surface area of the stapes footplate was computed as the area enclosed by landmark 1 and the semilandmarks of the footplate. Stapes height was measured between the calculated center of the area enclosed by the footplate landmarks and landmark 3. Of these measurements, manubrium length of the malleus was compared to stapes footplate area to get an estimation of the areal convergence ratio of the middle ear. Manubrium length was also compared to incus functional length to obtain the lever arm ratio of the malleus/incus complex. The software to compute the linear measurements can be obtained from the communicating author (AS).

Results

Measurement error

Figure 2 visualizes how differences in voxel size affect the surface reconstruction of the ossicles. Increase of the voxel size from 0.013 to 0.030 mm results in surface reconstruction which depicts all of the original morphological detail, but when using a voxel size of 0.080 mm a clear reduction in surface detail is apparent. Nevertheless, even the latter surface reconstructions preserve sufficient information to apply the measurement protocol used here. Repeated measurements were included with the rest of the sample in a PCA (Figure 3). For the incus and stapes these tightly cluster, showing that shape differences between the repeated measurements of this specimen are distinctly smaller than the differences to the specimen that is most similar (Greding-162). For the malleus, all but two of the 0.080 mm-based data points tightly cluster, but even the two relative outliers are closer to the cluster than to any other specimen.

--- *Figure 3 approximately here* ---

Malleus shape space

--- *Figure 4 approximately here* ---

The first three principal components (PCs) of the Procrustes shape variables account for 56% of the total sample variation. In the shape space defined by these three PCs all taxa form distinct non-overlapping clusters (Figure 4). The subsequent three PCs account for 18% of the

total sample variance (PC 4: 8%; PC 5: 6%; PC 6: 4%), but these and higher PCs will not be considered in detail because they do not discriminate between any of the taxa.

--- *Figure 5 approximately here* ---

--- *Figure 6 approximately here* ---

Principal component 1 explains 24% of the variation found. Going from negative to positive values, it is related to a decrease in overall size of the manubrium but increase in corpus size, an increase mainly of the medio-inferior part of the articular facet leading to distinct shape changes of the articular facet, a more flattened head (medio-lateral to anterior-posterior), a less lateral inflection of the spatula, and a more superiorly directed lateral process (Figures 5, 6, 7). Principal component 2 accounts for 18% of total sample variation (Figure 5). Positive values are related to an overall more curved manubrium, a more open angle between the long-axes of the manubrium and the corpus, and a less globular but more extended head (anterior-superior to posterior-inferior). Changes in size and orientation of the articular facet follow changes of the corpus of the malleus. Principal component 3 explains 14% of the variation found (Figure 6). Going from negative to positive values along the axis, shape changes relate to an increase in length and curvature of the manubrium (particularly in length and inflection of the spatula). It further involves shape and size changes in the articular facet, a reduction in the length of the neck of the corpus and the change from conically extending head to a more anterior-posteriorly broadened head (Figures 5, 6, 7).

--- *Figure 7 approximately here* ---

Malleus shape comparisons

All taxa investigated in this study show distinct differences in the shape of the malleus, the articular facet in particular (Figure 8). All interspecific differences of the mean shapes are statistically significant at $p < 0.05$ (Table. 2).

--- *Table 2 approximately here* ---

--- *Figure 8 approximately here* ---

The malleus of *Pongo* is characterized by a combination of a medium sized manubrium, a small articular facet with a more rectangular outline, a long corpus neck and a medio-superior extension of the corpus head (Figure 8).

Both species of *Gorilla* can be distinguished from the other taxa by shape characteristics associated with particularly low values for PC 2 (Figures 5, 6, 7). These include a long but straight manubrium including a broad spatula, a globular head and a long-axis of the corpus that almost forms a right angle relative to the manubrium (seen from posterior). The articular facet is small and facet shape is characterized by a straight superior-posterior outline. In *G. gorilla* all of these features are more pronounced than in *G. beringei*. Besides minor differences in articular facet shape *G. beringei* also exhibits a slightly larger corpus head than *G. gorilla* (Figure 8).

Both species of *Pan* are characterized by a strong lateral inflection of the spatula, strong curvature of manubrium, characteristic shape of the articular facet and a medio-laterally rounded but anterior-superior conically extending head, features expressed by high values for PC 3 (Figure 5, 6, 7). Other features characterizing *Pan*, not found along PC 3, are an open angle between the long-axes of the manubrium and the corpus, an anteriorly angled corpus relative to the manubrium and a strong extension of the posterior articular facet area. *Pan troglodytes* and the single *P. paniscus* are very similar in shape, but the latter shows a larger articular facet and wider angle between long-axes of manubrium and corpus than the mean shape of *P. troglodytes* (Figure 8).

Homo sapiens is most distinct among the hominoids, standing out by shapes associated with PC 1, including a short manubrium, a long corpus, an open angle between the lateral process and remaining manubrium, little lateral inflection of the spatula, and an enlarged medio-inferior part of the articular facet. A further characteristic is the distinct anterior-posteriorly flattening of the head (Figure 7, 8).

Symphalangus syndactylus can be distinguished from the hominids by shapes expressed by PC 3. These concern the articular facet and a very small spatula of the manubrium, as well as a conically extended head (Figures 7, 8).

Homo sapiens and *Pan* share a large but differently-shaped articular facet. *Pan* and *Gorilla* share the combination of a very long manubrium and short head. *Pan* and *Pongo* share a curved manubrium, whereas it is straighter in *H. sapiens* and *Gorilla*.

Malleus ancestral shapes within the Hominidae

Table 3 contains the values of the species mean scores of the first three principal components and Table 4 refers to the computed ancestral coordinates of the principal components of the successive nodes within Hominidae. The absolute difference and the polarity of the difference (negative or positive) of the taxon-specific PC mean scores (Table 3) compared to the estimated ancestral coordinates (Table 4) provide an approximation of the way and degree the taxa differ from the ancestral states of the successive nodes of the hominoid phylogeny when looking at the first three principal components. The short manubrium, long corpus and large medial articular facet of *H. sapiens*, associated with PC 1, represent the most derived morphology from the maximum likely ancestral state of the Hominidae, the Homininae and the Hominini. However, modern human mean scores on PC 2 and PC 3 are close to the ancestral states of these clades. Here, *Gorilla* is strongly derived from the condition found at the ancestral node of the Hominidae and the Homininae. *Pan* is the most derived from the ancestral state of Hominidae and Homininae for shapes associated with PC 3.

--- *Table 3 approximately here* ---

--- *Table 4 approximately here* ---

Incus shape space

--- *Figure 9 approximately here* ---

The first three principal components account for 56% of the total sample variation. In Procrustes shape space, most taxa form non-overlapping clusters (Figure 9). The African hominids are distinctly separated, but particularly *Pongo* sp. and *S. syndactylus* broadly overlap. The subsequent three PCs account for 19% of the total sample variance (PC 4: 7%; PC 5: 7%; PC 6: 5%), but these and higher PCs will not be considered in detail because they do not discriminate between any of the taxa.

--- *Figure 10 approximately here* ---

--- *Figure 11 approximately here* ---

The scores along PC 1 explain 28% of the total sample variation (Figures 10, 11). Going from negative to positive values, PC 1 is related to an increase in narrowing (more deeply rounded) of the intercrural curvature together with a more parallel orientation and size reduction of the short and long processes. It also involves distinct shape differences marked by an increase of the anterosuperior articular facet area but a narrowing of the inferolateral articular facet area (Figure 12).

Principal component 2 explains 18 % of the variance (Figure 10). When going from negative to positive values shape changes relate to a narrowing of the intercrural curvature. However, in contrast to PC 1, the size of the two processes is not affected. Principal component 2 further relates to an increase in height and to a decrease in width of the articular facet (Figure 12).

Principal component 3 accounts for 10% of total sample variation (Figure 11). The associated shape variation, when going from negative to positive values, ranges from a continuously rounded intercrural curvature and similar sized processes (when seen from a lateral view) to an almost rectangular intercrural curvature combined with a long and straight long process and small short process. Positive values are further related to an increasingly medially oriented short process relative to the long process and to a broadened anterosuperior articular facet area and a more symmetrically shaped facet outline (Figure 12).

--- *Figure 12 approximately here* ---

Incus shape comparisons

The mean shape differences between all species investigated here are statistically significant at $p < 0.05$, except between *G. beringei* and *S. syndactylus* (all represented by small sample size; Tables 1, 5). The shape differences that are most diagnostic relate to variation in the articular facet shape and rounding of the intercrural curvature (Figure 13).

--- *Table 5 approximately here* ---

--- *Figure 13 approximately here* ---

The incus of *Pongo* is particularly variable in shape, especially as expressed by PC 2. Nevertheless, it can be easily separated from the other hominid genera (except for the single *P.*

paniscus specimen) by an almost rectangularly-shaped intercrural curvature that is associated with high values on PC 3. Moreover, *Pongo* combines an extended and straight long process that is laterally deflected with a small short process that points more medially (Figure 13). The high values on PC 3 also reflect a more symmetrically shaped outline of the articular facet than in the African hominids, marked by a less prominently developed superoanterior portion. Orientation of the entire articular facet is more lateral relative to the main body of the incus (Figure 13). *Pongo* shares the majority of these features with *S. syndactylus* (see below).

Gorilla can be distinguished from the other genera by shapes showing low values along PC 2 (Figure 10, 12). Both species of *Gorilla* share a shallow intercrural curvature and a distinctly shaped articular facet with a mediolateral and inferosuperior extension of similar size (Figure 13). The articular facet is the smallest of all the species analyzed. On PC 2 (Figure 10), *G. gorilla* is more distinct from the other hominid species than is *G. beringei*.

The morphology of the *Pan* incus is diagnostic in features associated with positive values of PC 2. The intercrural curvature is narrow and the articular facet is supero-inferiorly elongated. The *Pan* incus also possesses a number of shape characteristics associated with high values of PC 1 (Figures 10, 11, 12). These shape characteristics comprise a narrow intercrural curvature including a more inferiorly directed short process in combination with a very large articular facet and small incudal processes. Enlargement of the articular facet comes from a size increase of the anterior facet which extends notably superiorly (Figure 13). The shape characteristics of *P. paniscus* along PC 1 and PC 2 are less pronounced than in *P. troglodytes*. For shapes associated with PC 3 *P. paniscus* does not overlap with the range of variation found in *P. troglodytes*.

Homo sapiens is mainly characterized by shape features related to negative values of PC 1 (Figures 10, 11). In contrast to *Pan*, the large articular facet of *H. sapiens* is particularly associated with lateral expansion. *H. sapiens* is further characterized by a wider intercrural curvature with a more open angle between both processes (Figure 13). Since the short and long processes are both elongated, *H. sapiens* shows the longest intercrural curve of all species analyzed.

Symphalangus syndactylus can be distinguished from the African hominids by shapes linked with high values on PC 3 (Figure 11). Since *S. syndactylus* also overlaps with *Pongo* along PC 1 to PC 3 it is almost indistinguishable from *Pongo* based on Procrustes shape variables alone (Figure 10, 11). However, in form space the two taxa can be easily separated because of the distinct size differences (see below).

Compared to the malleus the amount of intraspecific variation in the incus shape variables is larger, and the distinction between *H. sapiens* and the great apes is not as pronounced. However, among all hominids the mean shape differences are more pronounced. The African hominids (including *H. sapiens*) share a more continuously rounded intercrural curvature with a more symmetric length ratio between the two processes (Figure 13). They also share similarities in the orientation of the articular facet relative to the main body of the incus and a more laterally directed short process (same orientation as the long process) when seen from an anterior view. In contrast, *Pongo* and *S. syndactylus* share the opposite shaping of the characters (small short process and asymmetric ratio between the two processes, more angularly shaped intercrural curvature, shape and orientation of articular facet, more medially directed short process [opposite orientation to the long process] when seen from anterior). *Pan* and

Gorilla share a narrow curvature between the two almost equally sized but short processes of the incus. *Pan* and *H. sapiens* share large articular facets, but these are differently shaped.

Incus ancestral shapes within Hominidae

For PC 1, the negative mean value shown by *H. sapiens* is derived relative to the hominid ancestral condition while *Pongo* falls with the ancestral condition (Tables 6, 7). The positive values found for *P. troglodytes* and *G. gorilla* are also derived from the primitive hominid condition.

For PC 2, *H. sapiens* is similar to the maximum likely ancestral state of the Hominidae, the Homininae, and also the Hominini. In contrast, *Pan* (positive values) is derived from the ancestral condition of these three nodes. The negative values of both species of *Gorilla*, *G. gorilla* in particular, are also derived from the ancestral state of Hominidae and Homininae.

For PC 3, the more negative values of the African hominids (with the exception of *P. paniscus*) relative to the ancestral hominid condition show the opposite character state compared with the positive values of *Pongo*. Except for *P. paniscus*, the African hominids more or less retain the ancestral condition of the Homininae and the Hominini.

--- *Table 6 approximately here* ---

--- *Table 7 approximately here* ---

Stapes shape space

--- *Figure 14 approximately here* ---

The first three PCs account for 68% of the total sample variation of the Procrustes shape variables. The hominid species are arranged along a linear trend from *H. sapiens*, *P. troglodytes*, *Gorilla* to *Pongo*, with limited interspecific overlap (Figure 14). *Symphalangus syndactylus* is most similar to *Pongo*, with one specimen suggesting that its species mean may fall away from the hominid trend. Although sample size is small, the amount of intraspecific variation captured in this small sample is large, particularly on PC 1. Principal components 4 – 6 account for 11% of the total sample variance (PC 4: 6%; PC 5: 5%; PC 6: 4%), but these and higher PCs will not be considered in detail because they do not discriminate between any of the taxa.

--- *Figure 15 approximately here* ---

--- *Figure 16 approximately here* ---

Principal component 1 explains 43% of the sample variation (Figure 15). Going from negative to positive values, PC 1 is related to a decrease in the height of the stapes (length from head to footplate) and a decrease of the level of development of the stapedial head. Principal component 1 is further related to size and shape changes of the stapes footplate changing from kidney-shaped to more oval-shaped (Figure 17).

Principal component 2 accounts for 14% of the total sample variation and relates to the length ratio between the anterior and posterior crus and the direction of the footplate and stapedial head. Negative values are associated with a short anterior and long posterior crus as

well as a more anteriorly directed footplate and more anteriorly directed joint surface of the head; positive values with a long anterior and short posterior crus, as well as a more posteriorly directed footplate and more posteriorly directed joint surface of the head when looking at the exaggerated shape changes of PC 2 (Figures 17).

Principal component 3 accounts for 11% of the sample variation (Figure 16). Going from negative to positive values, it is related to the decrease in convex rounding of the anterior and posterior crus leading to straight crura at the most positive values. Size of the footplate also decreases and shape becomes more narrow and regular (Figure 17).

--- *Figure 17 approximately here* ---

Stapes shape comparison

Statistically significant mean shape differences ($p < 0.05$) are found between most species (Table 8). While PC 2 and PC 3 are not informative regarding species-specific shapes, PC 1 shows a shape trend from a short stapes with a small and barely distinct head but a large and relatively regularly shaped footplate in *S. syndactylus* and *Pongo* via *Gorilla* and *P. paniscus* to a high stapes with a very distinct head and a small and kidney-shaped footplate in *P. troglodytes* and the majority of *H. sapiens* specimens in particular (Figures 15, 18).

--- *Table 8 approximately here* ---

--- *Figure 18 approximately here* ---

Stapes ancestral shapes within Hominidae

Since all species occupy almost the same range of variation of scores along PC 2 and PC 3, it is meaningful to look at ancestral states of only the first principal component (Tables 9, 10). Here, the negative mean value of *H. sapiens* associated with a high stapes with a distinct head and a kidney-shaped footplate is most derived from the ancestral condition of the Hominidae, the Homininae and the Hominini. Both species of *Gorilla* are close to the estimated ancestral condition of the Hominidae. Distinct differences exist between the two species of *Pan*. While *P. paniscus* is close to the ancestral condition of the Hominidae and the Homininae, *P. troglodytes* is more derived toward a shape similar to *H. sapiens*.

--- *Table 9 approximately here* ---

--- *Table 10 approximately here* ---

Comparing form space with shape space

--- *Figure 19 approximately here* ---

Figure 19 provides plots of the three ossicles showing in each case PC 1 against PC 2 in form space. For the malleus, the variance explained by the first three PCs remains almost similar

compared with shape space. Principal component 1 explains 23%, PC 2 16% and PC 3 16% in form space, and 24%, 18% and 14% in shape space, respectively. The correlation coefficient of the shape PC scores with centroid size is highest for PC 3 ($r=0.565$) followed by PC 1 ($r=0.260$) and PC 2 ($r=0.014$). While of similar size among all hominid species, the malleus of *S. syndactylus* is distinctly smaller.

For the incus, the variance explained by the first three PCs differs between form space and shape space. In form space, PC 1 explains 65% of the total variance followed by 10% for PC 2 and 7% for PC 3, whereas this is 28%, 18% and 10% in shape space, respectively. The correlations of the individual PC scores (shape space) with the logarithm of centroid size are $r=-0.360$ for PC 1, $r=-0.143$ for PC 2 and $r=-0.584$ for PC 3. In form space of the incus, *H. sapiens* and *G. beringei* are characterized by possessing the largest incudes of the sample, while *S. syndactylus* has a distinctly small incus. The incudes of *Pan* and *Pongo* are of similar size, while the *G. gorilla* incus is intermediate in size between those of *Pan/Pongo* and *H. sapiens/G. beringei*.

In the stapes, the disparity between form and shape space is greater than in the incus (Figure 19). In form space of the stapes variables, PC 1 explains 75% of the total variance, PC 2 7% and PC 3 4%, and in shape space 43%, 14% and 11%, respectively. The correlation coefficient of the score of shape PC 1 and centroid size is $r=-0.709$ (PC2 $r=-0.173$; PC3 $r=-0.120$). *H. sapiens* is characterized by a stapes that is larger in all dimensions, whereas the great apes possess similar sized stapes except for the *P. paniscus* specimen showing a distinctly smaller stapes. Hence, stapes size separates the stapes of *H. sapiens* from the great apes (Figure 19).

Metrics relevant for auditory function

Figure 20A provides a plot of manubrium length versus stapes footplate area, which can be used as an approximation of the areal convergence ratio (tympanic membrane area against stapes footplate area) across hominids. It shows that *H. sapiens* is characterized by a low ratio whereas the non-human African hominids have higher ratios. *Pongo* sp. is marked by an intermediate ratio between *H. sapiens* and the African apes, whereas *S. syndactylus* shows a similar ratio as *H. sapiens*.

The ratio between the manubrium length of the malleus and the functional length of the incus provides an estimate of the lever arm ratio. The plot of these two functional lengths (Figure 20B) and the ratio available for specimens which preserve both the malleus and incus, show that *H. sapiens* (1.24 ± 0.08) has a low lever arm ratio, whereas both *P. troglodytes* (1.73 ± 0.11) and *S. syndactylus* (1.59 ± 0.05) have high lever arm ratios. *Pongo* and *Gorilla* are intermediate, with *Pongo* (1.42 ± 0.12) having a slightly lower mean value than *Gorilla* (*G. beringei*, 1.50 ± 0.10 ; *G. gorilla*, 1.54 ± 0.14). Surprisingly, the one *P. paniscus* specimen is also intermediate (1.43), falling outside the range of variation of *P. troglodytes* (1.49 – 1.88).

--- *Figure 20 approximately here* ---

Discussion

In this study we introduce a geometric morphometric measurement protocol for analyzing the size and shape of the middle ear ossicles. It is reliable and reproducible over a range of image

spatial resolutions and allows the description and measurement of even subtle shape changes. Using this approach we show that it is possible to distinguish the hominid taxa assessed here, based on the mean shapes of their malleus and incus. The stapes appears less diagnostic, although this may in part be related to the small sample size available to us. After a brief evaluation of the approach used and a summary of taxon-specific morphology, the present discussion will interpret ossicle shape differences in the light of hominid phylogeny and variation in middle ear architecture and size. Finally, the results will be discussed with reference to the known relations between auditory capacities and middle ear morphology.

Measurement error and sample

Repeated measurements for the specimen (Greding-41A) based on image spatial resolution of 0.013 and 0.030 mm result in tightly defined clusters of the individual ossicles in Procrustes shapes space. While this applies also to surface reconstructions of the incus and stapes based on 0.080 mm image spatial resolution, two of the three 0.080 mm malleus specimens are slightly more distinct from the cluster. However, based on Procrustes distance these two data points are still more similar in shape to the other values of the specimen than to any other individual. Hence, the results of the repeated measurements allow two conclusions. First, the series of measurements demonstrates the reliability of the measurement protocol introduced here, similar to other geometric morphometric approaches using semilandmarks (Neubauer et al., 2009; Skinner et al., 2009; Gunz et al., 2012). Second, despite the minute size of the ear ossicles, surface reconstructions based on relatively low image spatial resolution (here 0.080 mm) still yield reliable results.

The small number of infant ossicles included in the sample plot well within the range of variation of individual species. This finding supports the notion that the ossicles are fully formed at birth, with the exception of the anterior process of the malleus (Rosowski, 1994; Scheuer et al., 2000; Quam et al., 2014), a structure which is therefore not landmarked here. Availability in museum collections restricted the sample sizes of *G. beringei*, *Pongo* and in particular *P. paniscus*, as well as the number of stapes examined overall. In future studies, a targeted effort should be made to correct for these imbalances, thus improving the ability to assess interspecific differences.

Summary of species-specific aspects of ossicle morphology

Our results demonstrate that the ossicles show strong species-specific morphology, useful for discriminating hominoid taxa. This is particularly true for the malleus and incus, whereas the stapes shows more morphological overlap between hominoid taxa. *Pongo* sp. is characterized by a malleus with a medium-sized manubrium, a small articular facet with a more rectangular outline, a long corpus neck and a medio-superior extension of the corpus head. The incus of *Pongo* sp. shows an almost rectangularly shaped intercrural curvature and a more simply shaped outline of the articular facet with a small superoanterior portion. All stapes belonging to *Pongo* sp. are short, possessing a small and barely distinct head but large and regularly shaped footplate. The malleus of *G. gorilla* is characterized by a long and straight manubrium with a broad spatula, a globularly shaped head and a close, almost rectangular angle between the head and the manubrium. The small articular facet of *G. gorilla* shows a distinctive outline that has a straight superior-posterior segment. The incus of *G. gorilla* has a shallow and symmetric intercrural curvature and a distinctly shaped articular facet with a mediolateral and inferosuperior extension of similar size. Its articular facet is the smallest of the taxa analyzed here. The shape of the

ossicles of *G. beringei* is most similar to *G. gorilla* but is not as distinctive and is therefore best characterized by comparing it to the latter species. The malleus head of *G. beringei* is slightly larger but less globular than in *G. gorilla*. Also, the angle between the slightly shorter manubrium and head is more open. The larger articular facet extends more superiorly and is also larger in its medial aspect. The articular facet of the incus is slightly larger than in *G. gorilla* and symmetry and rounding of the intercrural curvature is less pronounced. Characteristic for *G. beringei* is the narrow stapes footplate.

Morphology of the malleus of *P. troglodytes* and the single *P. paniscus* specimen in the sample is similar and will be summarized together. Characteristic for the *Pan* malleus is a strong lateral inflection of the spatula, a strong curvature of the manubrium, an open angle between the long-axes of the manubrium and the corpus, a unique shape of the articular facet and a medio-laterally rounded but anterior-superior conically extending head. The incus of *P. troglodytes* shows a narrow intercrural curvature, short incudal processes and a large supero-inferiorly elongated articular facet. The *P. paniscus* incus differs from *P. troglodytes* by a longer long process and more asymmetric and wider intercrural curvature. Stapes morphology is less diagnostic since it overlaps with both *G. gorilla* and *H. sapiens*. Morphology of the *H. sapiens* ossicles is most distinct among the hominoids, standing out by a malleus with a short manubrium, a long corpus, an anterior-posteriorly flattened head and an enlarged medio-inferior part of the articular facet. The *H. sapiens* incus is characterized by the possession of a large, laterally expanding articular facet and a wide intercrural curvature between the elongated short and long processes. The tall stapes of *H. sapiens* generally shows a distinct head and a small and kidney-shaped footplate.

Compared to the hominids, the ossicles of *S. syndactylus* are easily distinguishable by their small size. The malleus is diagnostic by the presence of a laterally arched manubrium with a very small spatula and a medio-laterally flattened head. Besides differences in articular facet shape the incus of *S. syndactylus* is similar to *Pongo* sp. but is distinctly smaller. Except for differences in size, the stapes is difficult to distinguish from that of *Pongo* sp.

Phylogenetic polarity of hominid ossicle morphology

Figure 21 maps the autapomorphic character states of ossicle shapes onto a hominid cladogram (with *S. syndactylus* as outgroup). This is intended to summarize unequivocal character changes among hominids obtained from ancestral state estimation. The ossicles of *H. sapiens* possess nine traits autapomorphic for this taxon, while *Gorilla* shows six and *Pan* has four. *Gorilla gorilla* generally displays a more pronounced expression of the traits specific for *Gorilla*. For *Pongo* sp. only one character was unambiguously identified as derived within hominids, which indicates a more plesiomorphic ossicle morphology in *Pongo*. However, inclusion of *S. syndactylus* as the only outgroup taxon may obscure a straight-forward identification of apomorphic traits of *Pongo* since it impedes the description of unequivocally derived features of hominids. In contrast, character polarity within the African hominids was consistently identified without ambiguity.

--- *Figure 21 approximately here* ---

Morphology of the malleus indeed shows that *H. sapiens* is more distinct from any great ape taxon than the great apes are from one another. *H. sapiens* is derived regarding the short manubrium and long corpus, the anterior-posterior flattening of the head and an increase of the medial part of the articular facet. Changes in this character complex also relate to the changes of

scores of PC1. These results are consistent with previous metric studies and further support the distinctiveness of the *H. sapiens* malleus morphology from a more general great ape (or African ape) pattern (Masali, 1968; Siori and Masali, 1983; Masali and Cremasco, 2006; Quam, 2006; Quam et al., 2013b, 2014). However, PC 1 explains only slightly more variance than PC 2 and PC 3 (+6% and +10% more, respectively). Morphological differences among the great ape taxa are reflected by the scores of PC 2 and PC 3 allowing for a clear distinction of all taxa in 3D shape space. All genera show a number of derived traits in malleus morphology, reflecting phylogenetic relationships within hominids (Figure 21). A more spherically shaped head and long spatula is shared by all hominids and an enlarged articular facet is shared by *Pan* and *Homo*. Morphology that is derived in *Gorilla* relates to the close angle between the straight manubrium and the globular head. *Pan paniscus* and *P. troglodytes* share, inter alia, an antero-superior extension of the head. Looking at the ancestral states of PC 2 and PC 3, *H. sapiens* is closer to the ancestral morphology of the Hominidae, the Homininae and the Hominini than are *Gorilla* and *Pan*. This points to the strong degree of mosaic evolution in the (African) hominid malleus.

In contrast to the malleus, a distinction between *H. sapiens* and great ape patterns is hardly possible based on the morphology of the incus. This has been also suggested by Quam et al. (2014) based on linear measurements. As shown in Figure 21, *Gorilla*, *Homo* and *Pan* are each individually supported by derived traits of the incus and each of the three genera also retains a distinct set of characters that are plesiomorphic for hominids. Distribution in Procrustes shape space reveals the incuses of the three genera as equally different. Hence, the *Gorilla* incus is not just an upscaled version of the *Pan* incus as suggested previously (Quam et al., 2014). The hominin clade is supported by an enlarged articular facet shared by both genera. Monophyly of the African hominids is supported by the presence of a symmetric length ratio between the

incudal processes and a continuously rounded intercrural curvature. The more asymmetric length ratio of the incudal processes and almost rectangularly shaped intercrural curvature that is characteristic for the incus of *Pongo* is the plesiomorphic condition in hominids. Its presence in *P. paniscus* is a case of homoplasy. In shape space, these findings are reflected by an African hominid cluster (except *P. paniscus*) on PC 3, which relates to the change from an asymmetric, almost rectangular shaped intercrural curvature to a continuously rounded and symmetric curvature (Fig. 11, 12). Aspects of these complex shape changes have already been captured by Masali (1968, p 83), showing discrimination between Asian and African apes when considering what the author calls the “the intercept of the intercrural arch.” In general, ancestral state estimation shifts *Pongo* most closely to the plesiomorphic incus shape of hominids.

Results for the stapes broadly correspond with previous analyses (e.g., Quam et al., 2014). Morphology of the stapes is the least species-specific of all the three bones although significant mean shape differences exist between most taxa. In shape space, only the scores of PC 1 show a taxon-specific pattern, and the African hominids are derived from the ancestral condition of hominids. However, diagnostic stapes characters are only found for *H. sapiens*. Compared to the African hominids, *Pongo* is oppositely derived from the ancestral state of hominids which indicates distinct evolutionary transformations in both the Asian and the African clades.

Underlying aspects of hominid ossicle shape variation

The consistent and distinct differences in ossicle shape imply differences in the articulation of the ossicular chain or overall architecture of the tympanic cavity between all hominids (Quam et al., 2014). The variability observed in the angle of the corpus of the malleus

relative to the manubrium indicates either variation in the angle of the tympanic membrane relative to ossicular chain and/or oval window or differences in the angulation of the ossicular chain relative to an unvarying tympanic membrane/oval window complex. Shape changes associated with this malleus angle are found among the scores of PC 2. Here, Procrustes mean shape distance is largest between *Pan* and *Gorilla*, pointing at distinct differences in middle ear morphology between the two taxa. The large intraspecific variation of malleus shape variables found along PC 2 is also consistent with considerable variation in tympanic cavity architecture within the individual hominid taxa. Some aspects of the shape differences found for the hominid incus and stapes are probably also related to tympanic cavity architecture. In particular, the variation in intercrural curvature of the incus and angulation of the stapes footplate relative to the crura are likely related to slight differences in orientation between tympanic membrane, oval window and the ossicular chain.

The few published data on tympanic membrane area size of great apes indicate that *H. sapiens* is characterized by smaller tympanic membrane dimensions particular when compared relative to body mass (Masali et al., 1991; Martínez et al., 2004; Quam et al., 2014). In the small comparative sample published by Masali et al. (1991), the absolute tympanic membrane area of *Gorilla* is largest among all hominids, whereas tympanic membrane area relative to body mass is largest in *Pan*. The differential development of tympanic membrane area among hominids seems to be reflected in malleus shape. *Homo sapiens* has the shortest manubrium, and that of *Pan* is larger than in *Pongo* and at least of similar (relative) length as in *Gorilla*. Given the tight correlation of manubrium length and tympanic membrane size (Coleman and Ross, 2004) this pattern implies that the smaller tympanic membrane dimension is a derived feature characteristic for *H. sapiens*. The phylogenetic polarity of this feature in the other hominid genera cannot be

resolved based on this limited sample, and future research should assess whether the relatively enlarged tympanic membrane of *Pan* is an autapomorphy of this taxon.

Our data support the assumption of Quam et al. (2014) that *H. sapiens* can be characterized by a slightly larger tympanic cavity size when compared to the great apes. The larger corpus of the malleus, larger intercrural curvature and intercrural distance and the greater stapes height imply at least a wider tympanic cavity between centers of the tympanic membrane and the oval window in *H. sapiens*. In this view, the larger tympanic cavity dimensions represent a derived characteristic of the *H. sapiens* ear region. The limited data on tympanic cavity volume of *H. sapiens* and *P. troglodytes* seem to be in accordance with a slightly larger tympanic cavity in *H. sapiens* (Ikui et al., 2000; Martínez et al., 2013). While beyond the scope of this paper, more work on comparative tympanic cavity size in hominids is necessary, particularly since it is an important parameter in auditory function (Coleman and Ross, 2004).

Hominid mallei and incudes show marked differences in articular facet shape, and these will likely relate to the angulation of the ossicular chain. For example, *H. sapiens* differs from *Pan* in having an enlarged medio-inferior part of the malleolar articular facet and a laterally expanding articular facet of the incus. In *Pan* the posterior part of the articular facet of the malleus is enlarged and the incudal articular facet is supero-inferiorly elongated. This morphology suggests a slightly larger angle between the long axes of the malleus and incus in *Pan*. Similar assumptions can be made for other differences in the articular facet shape across hominid taxa, but their validity will have to be tested based on articulated ossicular chains.

Size of the ossicles

The striking congruity between shape and form space of the malleus variables reflects the minimal size differences between the mallei of hominids (but not *S. syndactylus*). This finding is likely related to the differences in middle ear architecture in hominids outlined above, including a reduction of tympanic membrane area and manubrium length combined with concomitant widening of the tympanic cavity and lengthening of the malleolar corpus in *H. sapiens*. Data from more diverse mammalian samples indicate that the minimal differences in overall size among hominid mallei are specific for the latter group and cannot be generalized (Hemilä et al., 1995; Nummela, 1995; Coleman and Ross, 2004). In contrast to the malleus, allometry has a strong influence on the overall morphology of the incus and the stapes of hominids. Here, pronounced size-related shape differences exist between most taxa. This observation means that previously published comparisons of raw linear and angular measurements of different hominid species (Masali et al., 1991; Quam et al., 2014) can be more reliably compared for the malleus than for the incus and stapes. Of all hominids, *H. sapiens* has the largest intercrural curvature length and the largest stapes height despite the significantly larger body mass of *Gorilla*. Hence, body mass is not the only factor driving allometric shape differences of the incus and stapes, and differences in tympanic cavity width are likely a more specific influence on shape.

Size differences (but shape differences as well) between the malleus, incus and stapes could further correspond to developmental differences between the three ossicles. Differences between the stapes and the other two ossicles could be due to the exclusive developmental origin of the stapes as a derivative of the second branchial arch, whereas the malleus and incus develop from the mandibular or maxillary part of the first branchial arch, being homologous to the articular and quadrate of non-mammalian vertebrates (reviewed in Anthwal et al., 2013). Therefore, the malleus and incus are expected to strongly co-vary and the differences in

allometry reported here are surprising. However, the manubrium of the malleus seems to be a novel mammalian structure, and fate-mapping experiments have further shown that the lateral process of the malleus is actually second arch derivate (O’Gorman, 2005; Anthwal et al., 2013). Knock-out experiments in mice have shown that several transcription factors (such as *Dlx2*, *Emx2*, and *Prx1*) and signaling molecules (such as *Endothelin1* and *Fgf8*) affect the development of the malleus and incus differently and underline the importance of the tympanic membrane for manubrium development (reviewed in Mallo, 2001; Chapman, 2010; Anthwal et al., 2013). Development of the manubrium needs a functioning external acoustic meatus and a temporally coordinated growth of the external acoustic meatus, tympanic ring, tympanic membrane and manubrium (Chapman, 2010). Developmental integration of the tympanic membrane and manubrium is tight, and in the absence of the tympanic membrane the manubrium is malformed or lacking in an otherwise normal looking malleus. On the other hand, the manubrium develops in the presence of the tympanic membrane even in complete absence of the malleus (Martin et al., 1995; Mallo et al., 2000; Mallo, 2001). Hence, differences between the malleus and incus, observed when comparing shape and form space, could also be related to developmental constraints specific to each ossicle. It shows that the formation of the ossicular chain is complex, reflecting the multifarious structure, function and evolution of the entire middle ear region.

Aspects relevant for auditory function

Most of the aspects outlined above are also relevant when considering the morphological variation of the middle ear in relation to hearing. Auditory function is complex and depends on a number of parameters that cannot be considered here, including tympanic membrane shape,

middle ear muscle and ligament properties and ossicle joint stiffness. Nevertheless, some aspects of the morphology studied here are functionally important.

Frequency transduction of the middle ear strongly depends on the inertial properties of the ossicles. Inertial properties relate to features such as the muscles, ligaments and stiffness of the joints, but differences in ossicle mass are particularly important, and will consequently affect the frequency range of hearing (Sim et al., 2007; Puria and Steele, 2010; Heldmaier and Neuweiler, 2013). Data on hominid ossicle masses are rare, but it can be assumed that the observed differences in size also relate to differences in mass of the hominid ossicles (Nummela, 1995). Particularly the larger dimensions and concomitant larger masses of the *H. sapiens* incus and stapes suggest a more restricted high-frequency hearing range in *H. sapiens* when compared to the great apes. Published hearing data of *H. sapiens* and *P. troglodytes* seem to support this notion (Elder, 1934; Kojima, 1990, 2003; Heffner, 2004).

The areal convergence ratio between the tympanic membrane area and the stapes footplate area is the most important parameter in impedance matching (sound energy amplification) of the middle ear (Heldmaier and Neuweiler, 2013). Although tympanic membrane size was not measured directly in this study, some inferences about the ratio can be made by looking at manubrium length because it strongly correlates with tympanic membrane size (Hemilä et al., 1995; Nummela, 1995; Coleman and Ross, 2004). Particularly, the distinctly lower aerial convergence ratio found for *H. sapiens* is consistent with differences in hearing abilities compared to other primate species, as suggested by audiograms (e.g., Kojima, 1990; Heffner, 2004). The functional implications of diverse aerial convergence ratios among great apes are difficult to discern using the available evidence. For this, future research will have to

consider direct measurements of the tympanic membrane area and a larger sample including more haplorrhine species.

The lever arm ratio between the functional lengths of the malleus and incus is another important variable in modelling audition (Martínez et al., 2004; Quam et al., 2014). Comparing our results for hominoids with data for 19 non-hominoid haplorrhine primates presented by Coleman and Ross (2004) shows that all hominids except for *P. troglodytes* have mean lever arm ratios that are close to, or well below, the minimum values found in other haplorrhines. *Homo sapiens* is marked by a particularly low lever arm ratio, which seems to be derived within hominids. Coleman and Colbert (2010) have shown that in non-hominoid primates, higher lever arm ratios are correlated with a decrease in the low, middle and total audible areas. Although the complex interplay between malleus and incus is far from being fully understood, the drastic decrease of the lever ratio in *H. sapiens* seems to be consistent with human hearing capabilities when compared to other primates, as suggested by audiograms (e.g., Kojima, 1990; Heffner, 2004). Finally, recent work has pointed to a functional incudomalleolar joint allowing actual mobility between the malleus and incus in large mammals including humans (Puria and Steele, 2010; Huber and Eiber, 2011). This mobility sets in when frequencies increase. It is hypothesized that mobile incudomalleolar joints allow for ossicle moments of inertia about the superior-inferior axes of the ossicles that are smaller than about the anterior-posterior axes. This could enable efficient high-frequency sound transmission despite the relatively large ossicle masses in large-sized mammals (Puria and Steele, 2010). Thus, it can be hypothesized that the distinct shape differences between the articular facets of the ossicles also have implications for auditory capacities of hominids and will be a focus of research in the future.

Conclusions

The malleus, incus and, to a lesser degree, the stapes show a strong taxon-specific shape among extant hominid taxa. Hence, the morphology of the ossicles are a valuable taxonomic marker which can be used when interpreting the fossil record, including that of human evolution (Quam et al., 2013a,b). Autapomorphic features could be identified for the hominid genera, the African ones in particular. Overall, *H. sapiens* and *Pongo* sp. are the most and least derived, respectively. Few autapomorphic features were identified for the hominids, and for the malleus only, but this result may well be affected by the use of *S. syndactylus* as outgroup.

We suggest that the differences in ossicle morphology are likely related to differences in the overall middle ear architecture and size. Here, variation in orientation of the tympanic membrane, oval window, and ossicular chain, as well as relative tympanic cavity width, are important factors influencing ossicle morphology. How the morphology of the ossicles, and the middle ear more generally, relate to auditory function is not fully understood, but several differences between hominid taxa are probably linked to hearing characteristics. Future research should increase the samples of *P. paniscus*, *G. beringei* and the two *Pongo* species. Functional analyses would benefit from more detailed comparative analyses of the incudomalleolar joint, and a focus on articulated ossicular chains, including the associated soft tissue. Finally, the results of this study will provide the comparative framework for morphometric studies analyzing ossicles of extinct hominids, particularly in the context of questions related to taxonomy, phylogeny and auditory function. Thus far the hominid fossil record includes relatively few ossicles (e.g. Quam, 2013a, b), but the increasing application of μ CT holds the prospect that more specimens will be found, trapped within the tympanic cavity. For example, over the past decades several ossicles have been found by CT examination of Neandertal temporal bones (Ponce de Leon and Zollikofer, 1999; Stoessel et al., 2014; Gomez-Olivencia et al., 2015).

Ossicle morphology may help in distinguishing taxa coming from sites with mixed assemblages, such as Sterkfontein or Swartkrans in South Africa and sites in the Turkana Basin in eastern Africa. What is more, correlations between middle ear morphology and hearing characteristics found in extant primates (e.g. Coleman and Colbert, 2010) can be used to interpret hominid fossils, providing information related to paleoenvironment and behavior (Quam et al., 2015).

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Figure captions

Figure 1. Set of landmarks (blue), curve semilandmarks (red) and surface landmarks (green) for statistical shape analysis of the malleus (A), incus (B), and stapes (C). The asterisk (*) on A and B shows the placement of an additional landmark for computation of linear measurements.

Figure 2. The ossicles of a modern human (Greding-41A) in original spatial resolution of 0.013 mm (A), and resampled to 0.030 mm (B) and 0.080 mm (C) to show the effect of different spatial resolutions on surface reconstruction of ear ossicles.

Figure 3. Principal component analysis of the (semi)landmark set of malleus (A), incus (B) and stapes (C) including modern human specimen Greding-41A (marked by black-bordered circles) with spatial resolution 0.013 mm, 0.030 mm, and 0.080 mm, measured three times each.

Figure 4. Principal component analysis of the malleus (semi)landmark set in 3D shape space defined by the first three principal components. The single *P. paniscus* specimen is marked by a bright blue circle.

Figure 5. Principal component analysis of the malleus (semi)landmark set in shape space of principal component 1 and 2. The single *P. paniscus* specimen is marked by a bright blue circle.

Figure 6. Principal component analysis of the malleus (semi)landmark set in shape space of principal component 1 and 3. The single *P. paniscus* specimen is marked by a bright blue circle.

Figure 7. Exaggerated shape changes supported by principal component 1 (blue), 2 (red) and 3 (green) of the malleus seen from the posterior. The black arrows indicate the anatomical orientation of the malleus in the diagram.

Figure 8. Species mean shapes of the malleus seen from the posterior. The black arrows indicate the anatomical orientation of the malleus in the diagram. Scale has been standardized by centroid size.

Figure 9. Principal component analysis of the incus (semi)landmark set in 3D shape space defined by the first three principal components. The single *P. paniscus* specimen is marked by a bright blue circle.

Figure 10. Principal component analysis of the incus (semi)landmark set in shape space of principal component 1 and 2. The single *P. paniscus* specimen is marked by a bright blue circle.

Figure 11. Principal component analysis of the incus (semi)landmark set in shape space of principal component 1 and 3. The single *P. paniscus* specimen is marked by a bright blue circle.

Figure 12. Exaggerated shape changes supported by principal component 1 (blue), 2 (red) and 3 (green) of the incus seen from medial and anterior, respectively. The black arrows indicate the anatomical orientation of the incus in the diagram.

Figure 13. Species mean shapes of the right incus seen in medial and anterior view, respectively. The black arrows indicate the anatomical orientation of the incus in the diagram. Scale has been standardized by centroid size.

Figure 14. Principal component analysis of the stapes (semi)landmark set in 3D shape space. The single *P. paniscus* specimen is marked by a bright blue circle.

Figure 15. Principal component analysis of the stapes (semi)landmark set in shape space of principal component 1 and 2. The single *P. paniscus* specimen is marked by a bright blue circle.

Figure 16. Principal component analysis of the stapes (semi)landmark set in shape space of principal component 1 and 3. The single *P. paniscus* specimen is marked by a bright blue circle.

Figure 17. Exaggerated shape changes supported by principal component 1 (blue), 2 (red) and 3 (green) of the stapes seen from medial and inferior, respectively. The black arrows indicate the anatomical orientation of the stapes in the diagram.

Figure 18. Species mean shapes of the stapes seen from medial and inferior, respectively. The black arrows indicate the anatomical orientation of the stapes in the diagram. Scale has been standardized by centroid size.

Figure 19. Principal component analysis of the malleus (A), incus (B), and stapes (C) (semi)landmark set in form space of principal component 1 and 2. The single *P. paniscus* specimen is marked by a bright blue circle.

Figure 20. A). Bivariate plot of manubrium length against the stapes footplate area. Circles represent specimens that have both values, and squares represent the species mean based on all specimens available. Both values have been log-transformed. The dashed line represents an areal convergence ratio of approximately 30:1 when computing tympanic membrane area from manubrium length. B). Bivariate plot of manubrium length against incus functional length. Symbols as described for Fig. 20A.

Figure 21. Character mapping of autapomorphic shape traits of the malleus, incus and stapes onto a cladogram reflecting hominid phylogeny with *S. syndactylus* as outgroup.