The Neanderthal in the karst: First dating, morphometric, and paleogenetic data on the fossil skeleton from Altamura (Italy)

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ABSTRACT

In 1993, a fossil hominin skeleton was discovered in the karst caves of Lamalunga, near Altamura, in southern Italy. Despite the fact that this specimen represents one of the most extraordinary hominin specimens ever found in Europe, for the last two decades our knowledge of it has been based purely on the documented on-site observations. Recently, the retrieval from the cave of a fragment of bone (part of the right scapula) allowed the first dating of the individual, the quantitative analysis of a diagnostic morphological feature, and a preliminary paleogenetic characterization of this hominin skeleton from Altamura. Overall, the results concur in indicating that it belongs to the hypodigm of *Homo neanderthalensis*, with some phenetic peculiarities that appear consistent with a chronology ranging from 172 ± 15 ka to 130.1 ± 1.9 ka. Thus, the skeleton from Altamura represents the most ancient Neanderthal from which endogenous DNA has ever been extracted.

Introduction

The Lamalunga cave opens in the limestone of the Murgia plateau at an elevation of 508 m a.s.l., near the town of Altamura (Puglia, Italy; Agostini, 2011). It constitutes the upper part of a larger karstic complex where stalactites, stalagmites, and flowstones occur together with "coralloid" formations, which mostly represent the last phase of calcite precipitation caused by spray/aerosol phenomena. This complex consists mainly of a sub-horizontal gallery that had developed at a shallow depth from the surface, intercepted by pits that had originally opened to the surface but which have subsequently been clogged by detritus. In this context, the discovery of a virtually complete fossilized hominin skeleton in an excellent state of preservation gives rise to interesting taphonomic considerations. Particularly, faunal remains found in some of the galleries are often isolated bony elements accumulated in depressed areas of the cave, suggesting that they

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were transported and dispersed by water. This was not the case with the human skeleton, given that it is largely represented and concentrated in a small area. Thus, we may hypothesize that, after death and decomposition of the body, the skeleton collapsed where it has been found. Thus far, no lithic tools have been found in the cave.

Even though the skeleton is partly incorporated into calcite concretions and is covered by coralloid formations, most of the bones are visible (see Fig. 1; see also Supporting Online Material [SOM] Fig. 1), including the cranium (upside down), the mandible, and several postcranial elements. From the photographs available and direct observations made in situ by one of us (GM), the skeleton appears to exhibit a mixture of archaic and derived features, which fit the range of variation typical of European hominins of the late Middle/early Late Pleistocene (Manzi et al., 2011). In fact, even though a number of Neanderthal traits can be seen—particularly in the face and in the occipital bone—there are features that distinguish this specimen from the more typical morphology of Homo neanderthalensis, such as the shape of the brow ridges, the relative dimension of the mastoids, and the general architecture of the cranial vault.

Nevertheless, for many years after its discovery, the only information we had on this extraordinary fossil skeleton was based primarily on on-site photographs and observations (Pesce Delfino and Vacca, 1993), which were biased by the presence of calcite formations. More recently, a survey in the cave was carried out as part of a new project commissioned by the local authorities, with the aim of carefully removing an isolated skeletal fragment. Subsequently, in February 2011, other samples were taken, including calcite material suitable for U/Th dating.

Therefore, for the first time, we are able to report quantitative data for the skeleton from Altamura, including its first dating, the morphometric analysis of an aspect of its post-cranial morphology (part of the right scapula), and a preliminary paleogenetic characterization.

Material and methods

The sample

The skeleton from Altamura is in an excellent state of preservation with virtually every bone belonging to a single adult individual preserved in the rather small space in which it was found. In fact, all bones were concentrated at the end of a narrow corridor known as the "ramo dell'uomo" ("branch of man") and generally do not appear to be damaged or distorted, with the exception of a few elements identified in a smaller chamber behind the area where the main assemblage was found (SOM Fig. 1).

In May 2009, in agreement with the Soprintendenza per i Beni Archeologici della Puglia, we obtained permission from the Direzione Regionale per i Beni Culturali e Paesaggistici della Puglia to remove a piece of bone from the Lamalunga skeleton. We chose a bone from the smaller chamber behind the skeleton for the following reasons: 1) to obtain a bone with minimum contamination, in view of the paleogenetic investigations to be carried out; 2) to avoid interfering directly with the main assemblage of bones before a full and thorough 3D laser survey could be performed; and 3) to avoid bones with extensive calcite concretions. Thus, with the aid of the speleologists of the Centro Altamurano Ricerche Speleologiche (CARS) of Altamura, the sample was recovered by one of us (MM) in July 2009, following sterile collection procedures and according to a procedure inspired by laparoscopic surgery (SOM Fig. 2).

The sample consists of the articular portion of the right scapula, in which the glenoid fossa, the neck, part of the spine (without the acromion), and the root of the coracoid process were preserved. In contrast to most of the bones of the main assemblage, it was free from major concretion apart from a superficial film of calcite. When discovered, the scapula was fractured in two main parts—the articular portion that was extracted and a large part of the body visible on the cave floor—while other small fragments were also present and scattered in an area of about 40 cm^2 (SOM Fig. 1). It is
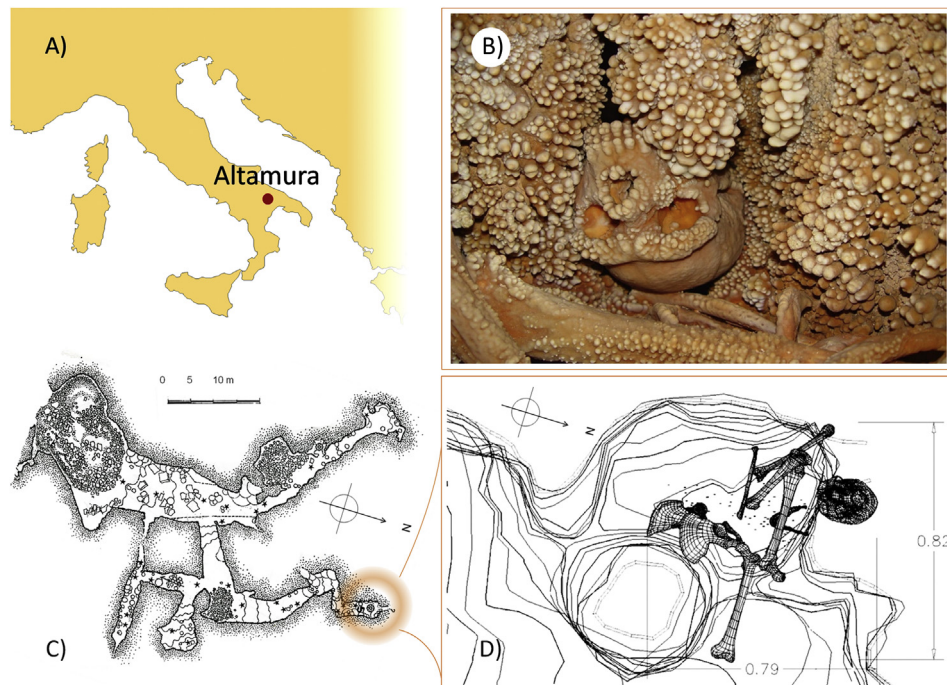
Figure 1. A) Position of Altamura within the Italian peninsula; B) hominin bones and calcite formations around the cranium (part of the mandible and right femur are visible); C) general topography of the northern part of the Lamalunga karstic system; note on the left the accumulation of detritus that represents the infilling of the probable main original access point from the external surface; and D) distribution of the main bones of the skeleton at the end of the so-called "ramo dell'uomo" (compare SOM Fig. 1). Drawing and data of Fig. 1D are from Vacca and Pesce Delfino (2004).

reasonable to infer that the scapula is part of the main assemblage of the skeleton, given that only various bony elements belonging to a single individual are documented in the cave and that the right scapula is missing from the inventory of bones in the main assemblage; moreover, in the small chamber behind the skeleton, the humerus of the same side of the body is visible.

After extraction, the specimen was kept in sterile conditions and was submitted to professional photographic documentation and medical tomography (SOM Fig. 3).

Dating of the scapula

The first attempt to directly date the scapula with AMS-C14 was made immediately after its recovery in 2009. This analysis was conducted on three separate fragments at the Centro di Datazione e Diagnostica (CEDAD) of the University of Salento, Lecce. Unfortunately, however, the pervasive calcite overgrowth within and around the fragments made the collagen extraction ineffectual and too little C was recovered for a proper analysis (Lucio Calcagnile, pers. comm.).

Additional sampling to establish the date of the skeleton was subsequently carried out in February 2011 when four calcite specimens directly covering the hominin bones were carefully removed along with a small naturally broken stalagmite encrusted with a thick coralloid overgrowth. All of the samples were cleaned with ultrasonic processes, embedded in resin, and sliced along the axial growth direction. Optical microscopy observation of the samples revealed several phases of growth in the coralloid overgrowths, and enabled us to select three of the best samples for U/Th dating. These were: 1) a thin calcite crust coating the underside of a long bone (ABS2, fibula); 2) a 5 mm-thick coralloid overgrowth covering the end of a short bone (ABS3); and 3) a 10 mm-thick coralloid overgrowth covering the naturally broken stalagmite (ABS5).

The U/Th analyses were conducted with a Multicollector Ion Coupled Plasma Mass Spectrometer (MC-ICP-MS) at the Laboratory of Isotope Geochemistry, University of Melbourne. The analytical procedures for chemical preparation and subsequent analysis by mass spectrometry are described in detail by Hellstrom (2003).

Morphometrics

Morphological analyses were performed on a digital replica, both by recording metrical variables and using a geometric morphometrics approach. For the former analysis, comparative data are reported in the SOM, while the latter analysis was performed on the outline of the scapular glenoid fossa (SGF) of 68 fossil and recent adult hominins, grouped in 10 OTUs (comparative data in SOM Table 1). A total of 60 landmarks and sliding semi-landmarks (see Fig. 4A) were digitized along the profile of sufficiently well-preserved SGFs, following the methodology described by Di Vincenzo and colleagues (Di Vincenzo et al., 2012) and reported in the SOM. All data were collected and analyzed using the TPS geometric morphometrics package (http://life.bio.sunysb.edu/morph) and MorphoJ version 1.05c (Klingenberg, 2011); additional metrical and statistical analyses were performed using PAST 2.17 (Hammer et al., 2001) and SPSS 13.0 (SPSSInc., Chicago, IL).

Paleogenetics

All DNA extractions and PCR reactions were carried out in exclusively dedicated ancient DNA facilities, which were physically separated from the facilities where PCR cycling and post-PCR analysis were performed. The two laboratories involved (one in Florence and one in Barcelona; SOM) adhered to standard criteria and precautions for the analysis of ancient samples (Handt et al.,

1994; Cooper and Poinar, 2000). For DNA extractions, three powder aliquots were globally used, as described in Rohland and Hofreiter (2007). Multiple PCR amplifications with multiple primer pairs that cover the entire hypervariable region I (HVR-I) of mitochondrial DNA (mtDNA; Caramelli et al., 2006) were performed as described in the SOM. In the second laboratory (Barcelona), a set of nine primers (Lalueza-Fox et al., 2011), together with blocking primers designed to prevent the amplification of possible modern human contaminant DNA (Gigli et al., 2009), were used. PCR amplicons obtained were cloned and multiple clones were sequenced as reported in Lari et al. (2010). Phylogenetic analyses were generated with all of the already available Neanderthal HRV-I sequences and the Denisova sequence (see details in SOM Tables 2 and 3).

Results and discussion

U/Th dating and petrography

A previous series of 25 U/Th dating was carried out by alpha spectrometry on a series of stalactites, flowstones, and coralloids (Fig. 2) by Branca and Voltaggio (2011). These revealed an ancient phase of speleothem formation, dating to between 189 ± 29 and 172 ± 15 ka, and a second phase, indicated by some of the flowstones, between 45.9 ± 1.7 and 34.4 ± 1.5 ka, while the ages of the coralloids (13 analyses in all) were distributed continuously between 43.3 ± 1.6 and 29.1 ± 1.0 ka and between 17.1 ± 0.7 ka and 13.4 ± 0.5 ka. Outside of these age groups, two additional single dates were recorded: 133 ± 9 ka for a stalactite and 98.7 ± 4.4 ka for a flowstone.

The microstratigraphy of the newly collected calcite samples ABS3 and ABS5 highlights four growth phases separated by three discontinuities (Fig. 3). The oldest growth phase, directly coating the hominin bone in ABS3 and the broken stalagmite in ABS5, is characterized by micrite laminae grading into microsparite and columnar calcite. This small similar pattern suggests that the two layers could be correlated and have a similar age. The other layers consist of elongated open and compact columnar calcite (cf. Frisia et al., 2000; Frisia and Borsato, 2010). On the basis of the identical microstratigraphy in the two samples, a similar micro-sampling strategy was performed in order to double check the age of the calcite overgrowth on the hominin bone. Moreover, since the overgrowth on ABS5 is considerably thicker than that on ABS3, this approach permitted avoidance of possible contamination from
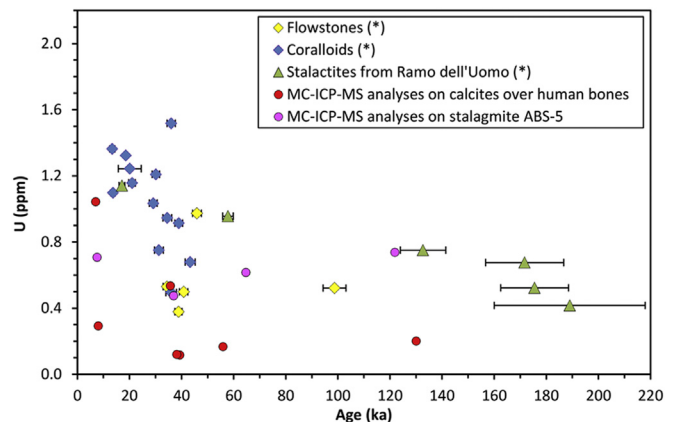

Figure 2. Synthesis of the Alpha spectrometry ages on speleothem from the Lamalunga cave and the new MC-ICP-MS analyses versus their U content; the error bars of the MC-ICP-MS analyses (2σ) are always smaller than the plotted symbols. (* Data from Branca and Voltaggio, 2011).
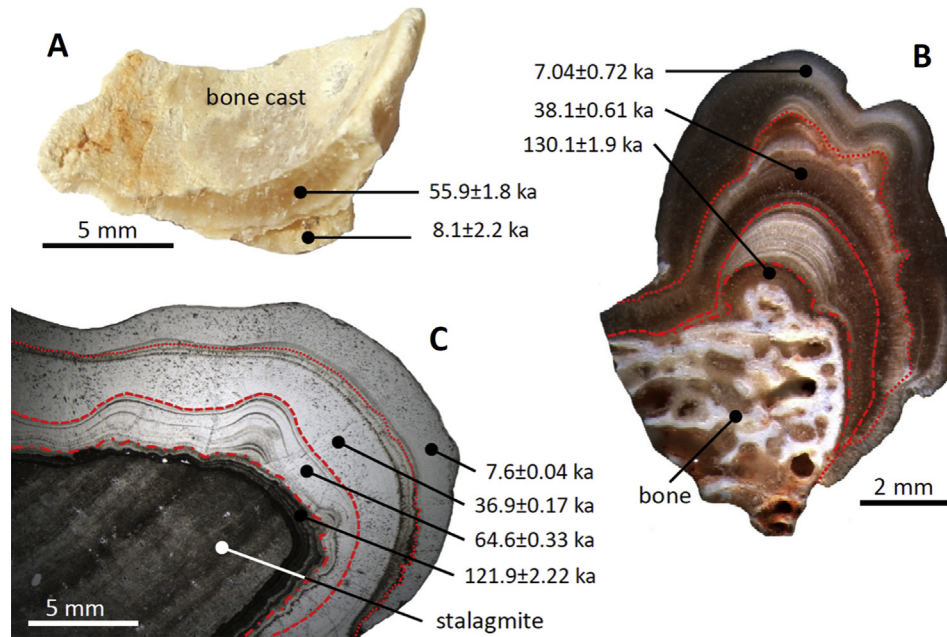
Figure 3. Selected calcite crusts and coralloid overgrowths with the calculated U/Th ages: A) thin calcite crust coating the underside of a long bone (fibula; ABS2); B) polished slab of mm-thick coralloid overgrowth covering the termination of a short bone (ABS3); and C) thin section of coralloid mm-thick overgrowth covering a naturally broken stalagmite (ABS5). The dotted, dashed, and dotted-dashed red lines on ABS3 and ABS5 visualize similar discontinuities that define the different growth phases identified by the U/Th ages. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

adjacent layers. We performed six MC-ICP-MS analyses on the three calcite overgrowths over the hominin bones and four analyses on the corresponding growth phases identified on the overgrowth on stalagmite ABS5 (Fig. 3). The results (Table 1) revealed four growth episodes dated to 7.04 ± 0.72—7.6 ± 0.04 ka, 36.9 ± 0.17—38.1 ± 0.61 ka, 64.6 ± 0.33 ka, and 121.9 ± 2.22—130.1 ± 1.9 ka, respectively corresponding to the warm Marine Isotope Stages (MIS) 1.0, 3.1, 5.1, and 5.5 (cf. Martinson et al., 1987) and matching coeval speleothem growth phases in Mediterranean caves (Bar-Matthews et al., 2003; Badertscher et al., 2011). The correlation between speleothem growth phases in Altamura cave and warm MIS gives rise to an

important consideration: the warm Marine Isotope Stage 7.1, between ca. 185 and 200 ka (Martinson et al., 1987), was not recorded in the coralloid overgrowths over the hominin bones, although it was recorded in two other stalactites from the same cave chamber (growth phase between 189 ± 29 and 172 ± 15 ka; cf. Fig. 2), as well as in speleothems from other Mediterranean caves (Bar-Matthews et al., 2003; Badertscher et al., 2011). Given the fact that all the other growth phases are represented in overgrowth ABS3, this suggests that the hominin bones could be more recent than 172 ± 15 ka.

Therefore, on the basis of these considerations, the Altamura skeleton is very likely older than 130.1 ± 1.9 ka and possibly

Table 1
Results of Multicollector ICP Mass Spectrometry U/Th analyses on calcites.[a]

Sample	Laboratory number	U (ppb)	$^{230}Th/^{238}U$ (AR)	$^{234}Th/^{238}U$ (AR)	$^{230}Th/^{232}Th$ (AR)	Age corrected (ka)	Initial $^{234}Th/^{238}U$ (AR)
ABS-U1.1	UMD111024-207	116 (09)	0.3281 (0.0022)	1.0195 (0.0027)	11.8	39.316 (1.560)	1.0218 (0.0030)
ABS-U2.1	UMD111024-211	167 (13)	0.4252 (0.0044)	1.0186 (0.0027)	15.2	55.851 (1.764)	1.0218 (0.0032)
ABS-U2.2	UMD111024-214	292 (22)	0.1080 (0.0009)	1.0132 (0.0021)	2.8	8.055 (2.159)	1.0135 (0.0021)
ABS-U3.1	UMD111024-218	201 (16)	0.7143 (0.0051)	1.0203 (0.0029)	126.0	130.069 (1.937)	1.0293 (0.0041)
ABS-U3.2	UMD111024-231	120 (09)	0.3052 (0.0028)	1.0166 (0.0029)	39.8	38.118 (0.606)	1.0185 (0.0032)
ABS-U3.3	UMD120228-209	1044 (80)	0.0690 (0.0014)	1.0087 (0.0065)	16.1	7.035 (0.718)	1.0089 (0.0066)
ABS-U5.1	UMD111024-232	616 (46)	0.4858 (0.0015)	1.0799 (0.0021)	2015.2	64.654 (0.331)	1.0960 (0.0025)
ABS-U5.2	UMD111024-237	475 (36)	0.2997 (0.0010)	1.0427 (0.0020)	2793.5	36.901 (0.172)	1.0474 (0.0022)
ABS-U5.3	UMD111024-307	708 (53)	0.0737 (0.0004)	1.0979 (0.0022)	770.1	7.571 (0.045)	1.1000 (0.0022)
ABS-U5.4	UMD120228-216	738 (56)	0.7002 (0.0034)	1.0290 (0.0039)	68.0	121.904 (2.220)	1.0409 (0.0054)

[a] ABS-U1 to ABS-U3 represent calcite crusts on human bones, while ABS-U5 represents the sample of calcite coralloides on the broken stalagmite. The isotope analyses are reported as activity ratios (AR), and the errors are reported in brackets as ±2σ.

younger than 172 ± 15 ka. These dates should be confirmed with a second batch of analyses on similar calcite samples coating the skeleton, whereas the actual lower age limit can be identified by the dating of fossil speleothems underneath the hominin bones. Both of these topics will be addressed in the near future as soon as the sampling authorization is granted.

Morphometric analysis

Metrical variables show that the scapulo-humeral joint of the Altamura skeleton is similar to those of European samples from the Middle and Upper Pleistocene with respect to the diameter and depth of the SGF (as reported in the SOM Fig. 5).

Geometric morphometrics may be used to better characterize the shape of the SGF, which has diagnostic significance (Di Vincenzo et al., 2012). When 2D landmark data are analyzed by Principal Component Analysis (PCA; Fig. 4B), the distribution of the samples reveals a signal of taxonomic and phylogenetic significance. Notably, the linear regression of the centroid size values on the 1st PC ($r = -0.23$; $p = 0.057$), as well as the multivariate regression of the centroid sizes on all the PC scores ($p = 0.522$) are

not significant; thus, our results are not significantly influenced by allometry.

Specimens are aligned along PC1 and show a progression of shapes from plesiomorphic to more derived morphologies, with modern humans mostly in the range of positive values and *Australopithecus* at the opposite extreme. In particular, the "early Neanderthal" (i.e., pre-Würmian) European sample from Krapina, Croatia, is interposed between the European Middle Pleistocene sample from Atapuerca Sima de los Huesos and the Neanderthals (both Würmian-European and Levantine). The variation in shape captured along PC1 (65.1% of variance explained) deals with changes from elongated SGFs (negative values) to more rounded shapes, while along PC2 (only 10.8%) the variation is from oval (negative) to pyriform morphologies, due to an enlargement of the coracoid component of the SGF, which is probably related to functional demands (Di Vincenzo et al., 2012).

The position of the SGF from Altamura in Figure 4B is within the Neanderthal range of variation, though peripheral with respect to the Neanderthal cluster along the PC2; while looking at PC1, it is clearly separated from both the Atapuerca Sima de los Huseos and Krapina samples, on one side, and from the modern range of
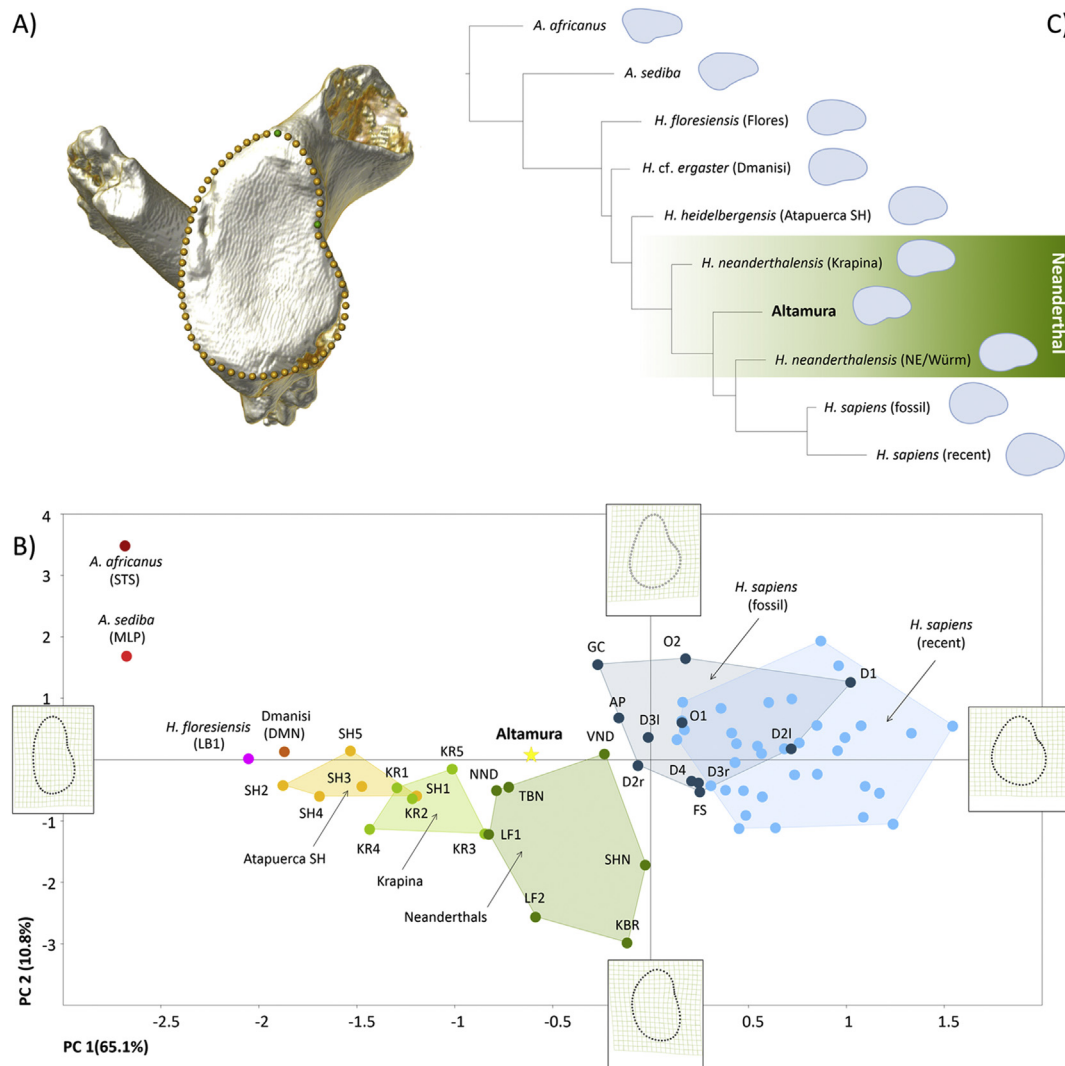
Figure 4. A) The articular portion of the right scapula from Altamura, viewed from the glenoid fossa (SGF), with landmarks (darker/green points in the online version of the paper) and semilandmarks (lighter/yellow points); B) Variance along PC1 and PC2 of the whole sample; OTUs are bounded by lines, while labels of the specimens are as in SOM Table 1; deformation grids represent extreme shape variations. C) Neighbour Joining of the phenetic relationships between the averaged OTUs and the specimen from Altamura. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

variation, on the other. The Neighbour Joining obtained from the Euclidean distances calculated for all of the PC scores (Fig. 4C) further demonstrates the phenetic position of the scapula from Altamura, interposed between the "early Neanderthals" from Krapina and the other Neanderthals.

Paleogenetic analysis

Despite several PCR attempts, it was only possible to obtain positive results from one of the shortest amplicons (82 bp; see SOM Fig. 6). All sequence results, obtained in two different laboratories, were concordant and presented a typical Neanderthal mtDNA haplotype (Condemi et al., 2013; A16230G, G16244A, C16256A, A16258G; Fig. 5). The consensus sequence was deposited in Gen-Bank under the accesion number KJ888153. Despite its short length, the mtDNA fragment from Altamura falls within the known range of Neanderthal diversity, being clearly distinct from both modern humans and the Denisova sequences. The sample from Atapuerca Sima de los Huesos (Meyer et al., 2013) was excluded from the subsequent analysis because for the fragment considered here it is poorly covered and misses informative sites. The sequence from Altamura clusters together with other Neanderthals from Western Europe in the tree reported in Figure 6. This picture seems to confirm the existence of some degree of population structuring among Neanderthals, already present around 150 ka. Unfortunately, the genetic data used in the analysis are too scant to make robust inferences regarding patterns of temporal and/or geographic variation in Neanderthal genetic diversity.

Interestingly, these results concur in indicating that endogenous DNA is still present in the samples and is characterized by a high level of fragmentation, as expected given the geological age of this specimen. The fact that the longest fragments could not be amplified by PCR, combined with the typical Neanderthal sequence profile obtained in all the clones from the positive amplicons, points to very limited (if any) contamination of the sample from recent modern human DNA, at least at the mtDNA level.

Conclusions

Overall, the results of our morphometric and the paleogenetic analyses concur in indicating that the skeleton from Altamura

Figure 6. Neighbor joining tree showing the phylogenetic relationships among Neanderthals on the basis of the present analysis. The name of the samples are reported next to their accession number (if present); Denisova is used as an outgroup.

belongs to a Neanderthal. In addition, using U/Th dating we were able to provide the first range of dates for the specimen, between 130 ± 2 ka and 172 ± 15 ka.

Nevertheless, some features exhibited by the skeleton and observed in situ (on the cranium, in particular, as summarized in the Introduction) differ from the morphology known among the typical representatives of *Homo neanderthalensis*, while they appear consistent with the pre-Würmian age we obtained. Metrical variables show that the scapula-humeral joint is closer to the morphotype usually referred to the so-called "early Neanderthals," including specimens such as those from Saccopastore (e.g., Bruner and Manzi, 2006), Krapina (e.g., Monge et al., 2008), or Apidima (Harvati et al., 2011). In addition, geometric morphometric analysis of the SGF from Altamura suggests some peculiarities of this small piece of bone, while (consistent with the mtDNA data) the same analysis strengthens the notion that the Neanderthal morphology was essentially present in the late Middle Pleistocene.

Finally, it is of great interest that mtDNA was sufficiently preserved to permit paleogenetic analysis. The results of the explorative approach used here have shown that the sample contained endogenous DNA (although highly fragmented) with a typical Neanderthal haplotype; moreover, there was no evidence of modern human contamination in the bone fragment, at least not at the mtDNA level. For these reasons, the Altamura skeleton should be considered a good candidate for more innovative genomic analyses, like capture approaches or ultra-deep shotgun sequencing,

Figure 5. Genetic diversity among Neanderthals. Polymorphic sites of the available Neanderthals in comparison with the Cambridge reference sequence (CRS) and those of the Denisova and Atapuerca Sima de los Huseos fossil samples.

especially when we consider that Altamura represents the most ancient Neanderthal from which endogenous DNA has been retrieved so far.

Appendix A. Supplementary data

Supplementary data related to this chapter can be found at http://dx.doi.org/10.1016/j.jhevol.2015.02.007.

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