The makers of the Protoaurignacian and implications for Neandertal extinction

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Title: The Makers of the Protoaurignacian and Implications for Neandertal Extinction

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Abstract: The Protoaurignacian culture is pivotal to the debate about the timing of the arrival of modern humans in Western Europe and the demise of Neandertals. However, which group is responsible for this culture remains uncertain. Here we investigate dental remains associated with the Protoaurignacian. The lower deciduous incisor from Riparo Bombrini is modern human based on its morphology. The upper deciduous incisor from Grotta di Fumane contains ancient mitochondrial DNA of a modern human type. These teeth are the oldest human remains in an Aurignacian-related archeological context, confirming that by 41,000 cal BP, modern humans bearing Protoaurignacian culture spread into Southern Europe. Since the last Neandertals date to 41,030-39,260 cal BP, we suggest that the Protoaurignacian triggered the demise of Neandertals in this area.

One Sentence Summary: The Protoaurignacian culture is a modern human industry that overlaps in time with the last Neandertals.

Main Text: The timing and pattern of the biological and cultural shifts that occurred in Western Europe around 45,000-35,000 calendar years ago (cal BP) fuel continuing debates among paleoanthropologists and prehistorians *(1-3)*. During this period, Neandertals were replaced by anatomically modern humans (AMH) *(4)*, and a variety of "transitional" and early Upper Paleolithic cultures emerged. Among them, the Protoaurignacian is crucial to current interpretations regarding the timing of arrival of AMH and their interaction with Neandertals *(5-9)*.

The Protoaurignacian appeared around 42,000 cal BP *(8, 10)* in Southwest and South-Central Europe (Fig. S1). In addition to the presence of personal ornaments, such as perforated shells and worked bones, the Protoaurignacian is characterized by a dominance of bladelets with typical retouched standardized implements like Font-Yves points and Dufour bladelets produced from unipolar cores *(5)*. This techno-complex has been tentatively linked to the Ahmarian industry of the Levant *(6, 9)*. Since the Ahmarian has been attributed to modern humans *(11)*, it has been suggested that the Protoaurignacian reflects a westward population movement of AMH from the Near East *(1, 7)*. However, because only three non-diagnostic human remains are associated with this culture, it is still uncertain who the makers of the Protoaurignacian were *(9, 12)*. The fossil remains associated with the Protoaurignacian available for study consist of the undiagnostic skeletal fragments of a fetus retrieved from Le Piage rock-shelter (France) *(13)*, for which the stratigraphic integrity of the Châtelperronian/Aurignacian sequence has been questioned *(5)*, and of two deciduous incisors from two Northern Italian sites: Riparo Bombrini (Western Ligurian Alps, Italy) and Grotta di Fumane (Western Lessini Mountains, Italy). The lower left lateral deciduous incisor (Ldi_2; Fig. 1) found in 1976 in Riparo Bombrini *(14, 15)* (Figs. S2-S5) and the upper right lateral deciduous incisor (Rdi^2, Fig. 1) labelled Fumane 2, which was retrieved in

1992 from the Protoaurignacian deposit of Grotta di Fumane *(15, 16)* (Figs. S6, S7), have to date not been conclusively attributed to modern humans or Neandertals.

The crown diameters of deciduous incisors are undiagnostic for Neandertals and modern humans, as is the case also for other tooth classes *(2)*. However, on the basis of the buccolingual crown diameter, the Bombrini specimen is close to the mean of Upper Paleolithic modern humans, whereas Fumane 2 is closer to the Neandertal mean (Table S1). Other than that, the worn deciduous lower incisors do not provide any morphologically diagnostic information.
To establish the identity of the makers of the Protoaurignacian, we analyzed the 3D enamel thickness components of the Bombrini Ldi_2 using a digital approach *(17)*, and we were able to investigate DNA from the Fumane 2 specimen *(15)*.

The relative enamel thickness (RET) index has been recognized as an effective taxonomic discriminator between Neandertals and modern humans. Neandertal deciduous and permanent teeth are characterized by significantly thinner enamel relative to dentine volume *(18)*.
To facilitate comparisons with the Bombrini specimen, which is affected by wear stage 4, the Neandertal and recent modern human (RMH) di_2 samples were divided into sub-groups based on their degree of wear (from wear stage 1/2 to wear stage 4) *(15, 19)* (Table 1, Table S2). The Neandertal di_2 RET indices are lower than those of RMH at similar wear stages, and no overlap in the range of variation is observed between the two groups. The RET index of Bombrini is higher than any values obtained for Neandertals (Table S2), despite the missing portion of the enamel cap, and its computed Z-score is close to the modern human mean in wear stage 4 (Table 1).

To test how much the loss of enamel on the mesial side of the Bombrini tooth affects the computed RET value, two RMH specimens were digitally worn and damaged to simulate the condition observed in Bombrini *(15)* (Fig. S8). The results confirm that tooth wear, at least up to wear stage 4, decreases the RET index by about 10%, while the mesial loss of enamel affects the index by less than 1.6% (much less than the values considered acceptable for intra- and inter-observer error) *(2)*. Therefore, the RET value for the unworn Bombrini Ldi_2 was certainly much higher (Table S3), further supporting its attribution to modern humans.

DNA was extracted from the Fumane 2 tooth, which yielded few mitochondrial DNA (mtDNA) sequences (Table S4). With respect to 63 ‘diagnostic’ positions at which ten Neandertal mitochondrial genomes differ from 311 present-day humans *(20)*, these sequences were of modern human origin (Table S5). To further explore this, we prepared a second DNA extract and two DNA libraries from this specimen, which yielded a total of 335,628 unique mtDNA fragments (Table S4).

The frequencies of cytosine (C) to thymine (T) substitutions at the ends of these fragments (34-37%, Fig. S9), which reflect the deamination of cytosine residues typical of ancient DNA *(21, 22)*, are consistent with results from other specimens of similar age *(23-26)*. Among the fragments carrying terminal C to T substitutions, we estimated the residual present-day DNA contamination to be 3.8% (*15*).

Using these fragments, we reconstructed a mitochondrial genome of 157-fold coverage (Fig. S10). This mtDNA sequence was aligned to the mtDNAs of 54 present-day humans, ten ancient modern humans, ten Neandertals, two Denisovans, a hominin from Sima de los Huesos (Spain) and a chimpanzee (*Pan troglodytes)*. The Fumane 2 mitochondrial genome falls within the variation of modern humans (Fig. 2) and basally in haplogroup R (Table S6), as also observed for the ~45,000 year old AMH specimen from Ust'-Ishim (in western Siberia *(26)*), a major group of related mtDNAs in Eurasia *(27)* into which most pre-agricultural mtDNAs in Europe fall *(28)*.

As expected for an ancient specimen *(23, 25)*, the Fumane 2 mtDNA has accumulated fewer nucleotide substitutions than present-day mtDNA (Fig. 2). Using 10 directly dated ancient modern humans *(25, 26)* as multiple calibration points, we estimated the age of the Fumane 2 terminal node to be 44,599 (95% highest posterior density: 19,755-72,070) years BP.
We thus conclude that the Fumane 2 individual carried a mitochondrial genome of a modern human type. This shows that this individual was a modern human, or had at least some ancestors who were modern humans.

Based on recent chronometric data for the Protoaurignacian deposit of Grotta di Fumane, the specimen Fumane 2 is dated to 41,110-38,500 cal BP *(10)*, as recalibrated with IntCal13 *(29)*. Among modern humans in Western Europe, it is currently predated only by the contested Kent's Cavern maxilla *(30, 31)* and by the 45,000-43,000 cal BP AMH specimens from the Uluzzian levels of Grotta del Cavallo *(2)*, for which a recent taxonomic reassessment is stimulating intense debate *(32, 33)*.

New radiocarbon dates of the Protoaurignacian layers of Riparo Bombrini were obtained from faunal bones recovered during the G. Vicino excavation (five samples) and the more recent 2002-2005 excavations (three charcoals and eight animal bones) *(15)* (Tables S7-S10). The new dates confirm the integrity of the cemented deposit explored in 1976 by Vicino (which yielded the Ldi_2 tooth), but suggest that some stratigraphic disturbance affected a restricted area explored during the 2002-2005 excavations (supplementary text; Table S7, Fig. S11). The ^{14}C dates of the Vicino 1976 excavation (Table S7) were incorporated into a Bayesian model for the distribution of ages *(34)* (Fig. 3). The Protoaurignacian levels (level III to level II) are dated between 40,710 and 35,640 cal BP (68.2% probability), corresponding to a cold phase that marks the onset of Heinrich Stadial 4 *(35)*.

The Bombrini Ldi_2 and potentially the Fumane 2 Rdi^2 thus represent the oldest AMH remains in an Aurignacian-related (i.e. Protoaurignacian or Early Aurignacian) archaeological context, confirming that by around 41,000 cal BP (68.2% probability), AMH populations bearing Protoaurignacian culture had spread into Europe along the Mediterranean coast. They are similar in age to, or slightly older than, the modern human remains from: Peștera cu Oase (Romania, ca. 40,000 cal BP), which lacks archaeological context; Kostenki 14, Layer III (Russia, ca. 38,000 cal BP), which is possibly Aurignacian; Kostenki 1, Layer III (ca. 38,000 cal BP) which is associated with diagnostic Aurignacian artifacts; Kostenki 14, Layer IVb and Kostenki 17, Layer II that underlie the Campanian Ignimbrite tephra and are of comparable age to the Protoaurignacian in Italy, are tentatively assigned to AMH and also associated with an assemblage that includes bladelets; La Quina-Aval and Brassempouy (France), which are Early Aurignacian and more recent than 40,000 cal BP (for a review see *(9)*).

The Protoaurignacian dispersal overlaps in time with late Neandertal populations, as indicated by the 41,030-39,260 cal BP age of the last Mousterian sites *(4)*, and the ~45,000-40,000 cal BP age of the Châtelperronian culture *(3)*, which is currently attributed to Neandertals *(36)*. The Protoaurignacian dispersal may therefore have been a cause (either directly or indirectly) for the extinction of the Neandertals, at least in Northern Italy.

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Fig. #:

Fig. 1. Three-dimensional digital models of the Protoaurignacian human remains. The Bombrini tooth is a lower left lateral deciduous incisor (Ldi_2), while Fumane 2 is an upper right lateral deciduous incisor (Rdi^2). B, buccal; D, distal; L, lingual; M, mesial; O, occlusal. Scale bar, 1 cm.

Fig. 2. Phylogenetic analysis of the Fumane 2 mtDNA genome, inferred using the Neighbor-Joining method. The Fumane 2 mitochondrial genome falls within the variation of modern humans, and outside the variation of Neandertals, Denisovans and a hominin from Sima de los Huesos. The insert shows the branches closest to Fumane 2. Other ancient modern humans are noted in italics. Branch lengths represent the evolutionary distance between individuals, reflected by the number of inferred substitutions per sequence.

Fig. 3. Bayesian model of dates from the 1976 excavation by Vicino at Riparo Bombrini. Bombrini AMS results compared with Grotta di Fumane boundaries created in ref. *(4)*. Bone samples treated with ultrafiltration in grey; shell samples from the Mousterian level *(4)* in blue. Radiocarbon dates are calibrated in IntCal13 *(29)* and Marine13 *(29)* for shell samples. The model and boundaries were calculated using OxCal 4.2 *(34)* including the performance of the General t-type Outlier Model *(34)*. The results are linked with the (NGRIP) $\delta^{18}O$ climate record *(15)*. The grey shaded bar denotes Heinrich event 4 (HE4) *(35)*.

Table #:

Table 1. Three-dimensional enamel thickness. Bombrini (Ldi_2) is standardized to Z-scores (for RET index) of the Neandertal and recent modern humans (RMH) di_2 sample in different wear stages

Taxon	Wear stage*	*n*	AET (mm)		RET (scale free)		Z-scores for RET index
			Mean	Range	Mean	Range	
Bombrini	4		0.29		9.22		
Neandertals	1/2	3	0.29 (0.01)	0.28-0.30	7.88 (0.33)	7.54-8.20	4.06
Neandertals	3	2	0.26 (0.007)	0.26-0.27	6.95 (0.55)	6.56-7.34	4.13
RMH	2	3	0.35 (0.006)	0.35-0.36	11.41 (0.41)	10.97-11.77	-5.34
RMH	3	11	0.31 (0.04)	0.24-0.35	9.98 (1.17)	8.01-11.85	-0.65
RMH	4	4	0.26 (0.04)	0.22-0.32	8.67 (1.4)	6.98-10.40	0.39

Standard deviation is indicated in parenthesis. AET, average enamel thickness index; RET, relative enamel thickness index. * Based on ref. *(19)*.

Supplementary Materials:

Materials and Methods

Supplementary online text

Figures S1-S11

Tables S1-S10

References (37-127)

Author Contributions: S.B., F.N., M.P., M.A.M. and J.J.H. initiated and organized the project. S.B. collected the fossil and modern human comparative samples for enamel thickness analysis. D.P. and P.A.S. carried out the microCT scan of the teeth and reconstructed the digital models. S.B. and S.E.B. provided the morphological description of the fossil sample. S.B. carried out the digital morphometric analysis of the teeth. V.S., M.M. and S.S. carried out aDNA analysis. S.B., J-J.H., V.S., S.S., M.M. and S.P. analyzed the data. S.T. initiated and performed the radiocarbon dating project at Riparo Bombrini. S.B., V.S., S.T., F.N., M.P., S.E.B., G.V., M.A.M., M.M., S.P., J-J.H. discussed the results. S.B., V.S., S.T., F.N., M.P., S.E.B., D.P., E.S., M.A.M., M.M., S.P., J-J.H. wrote and edited the manuscript.

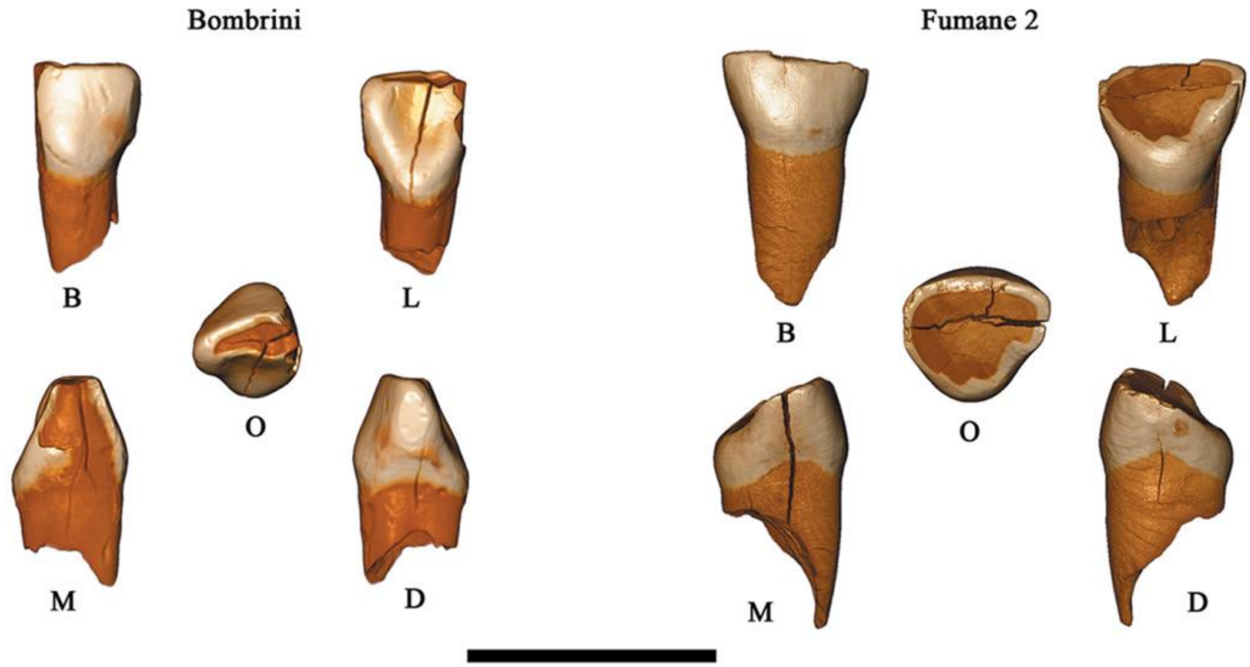
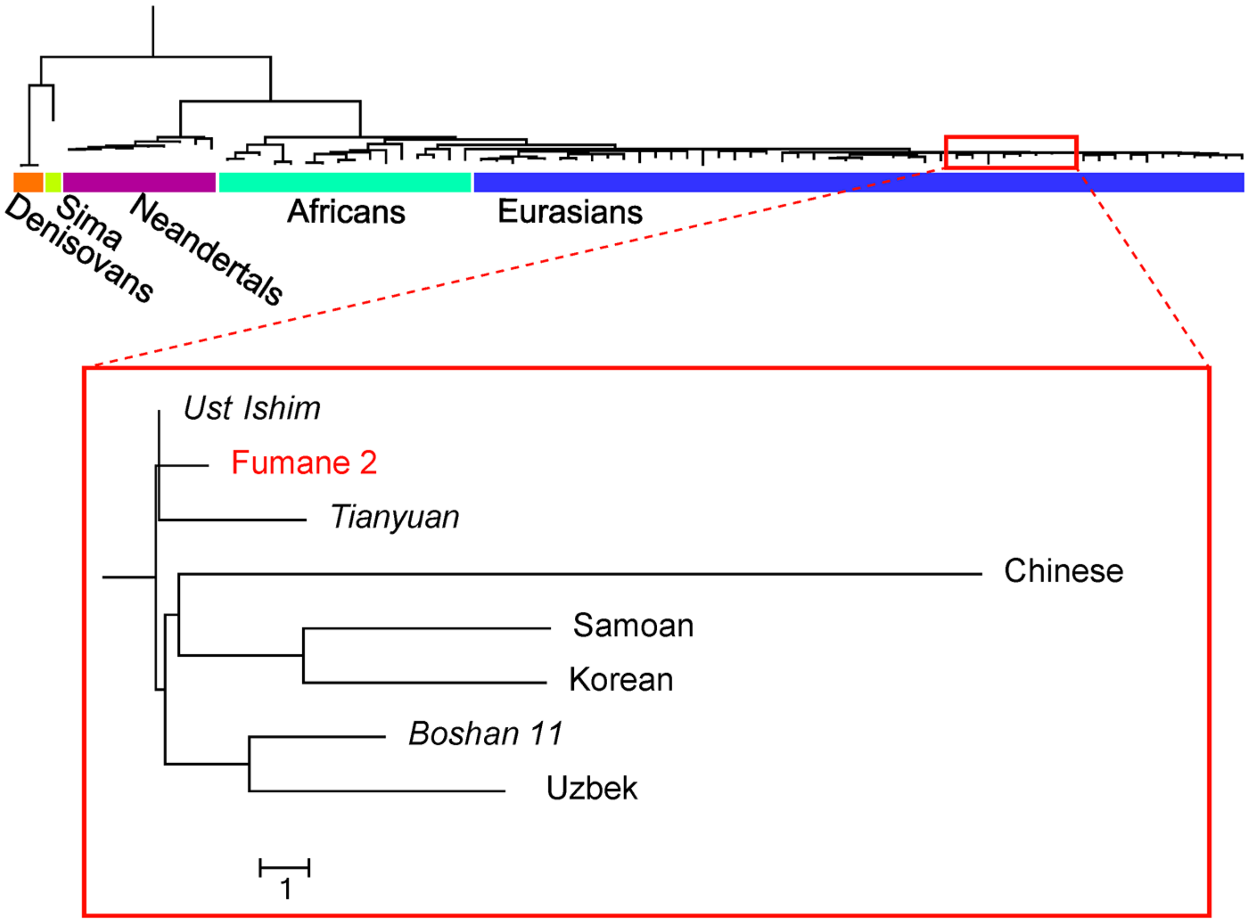
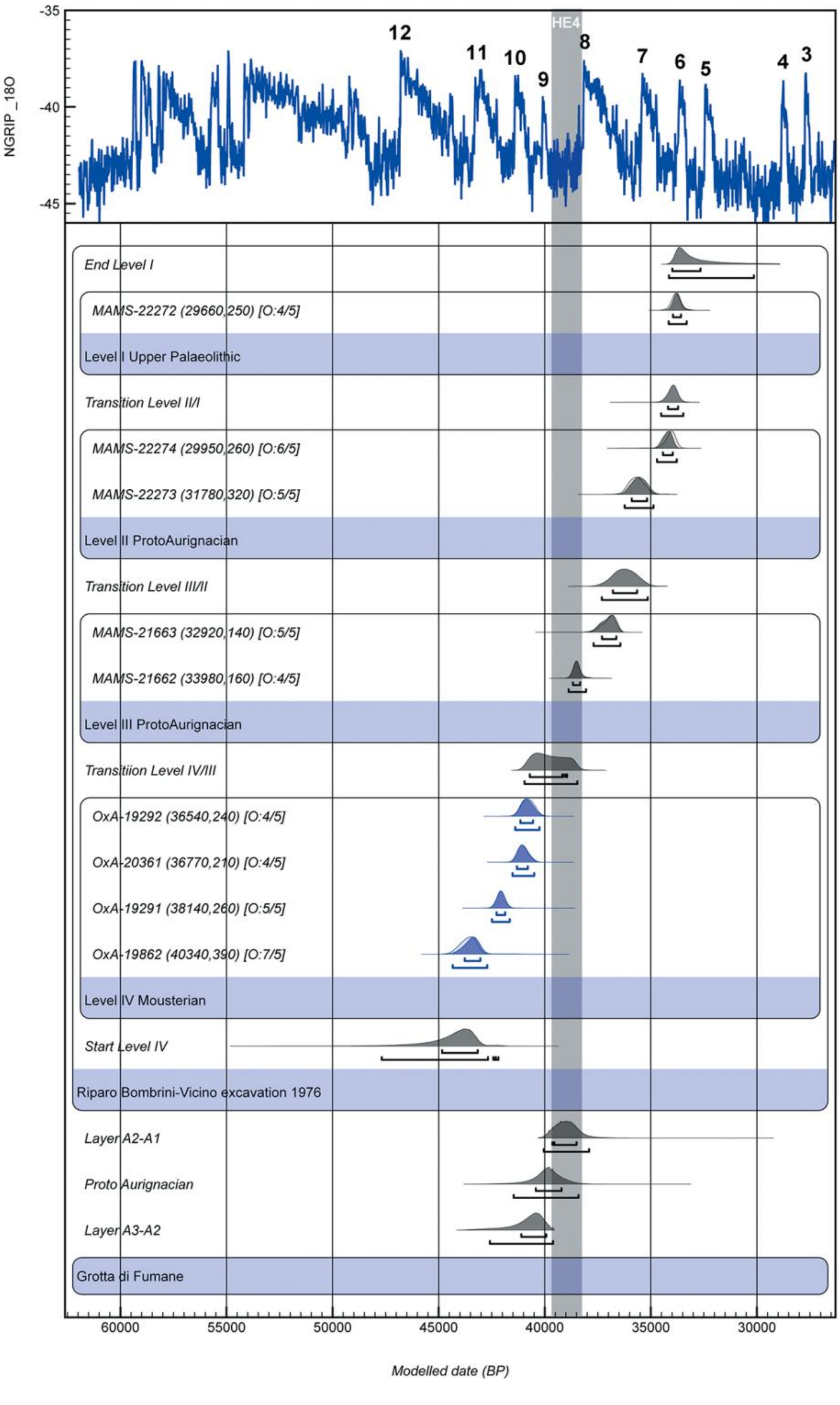


Fig. 1



Denisovans
Sima
Neandertals
Africans
Eurasians
Ust Ishim
Fumane 2
Tianyuan
Chinese
Samoan
Korean
Boshan 11
Uzbek
1

Fig. 2



NGRIP_18O
-35
-40
-45
HE4
12
11
10
9
8
7
6
5
4
3
End Level I
MAMS-22272 (29660,250) [O:4/5]
Level I Upper Palaeolithic
Transition Level II/I
MAMS-22274 (29950,260) [O:6/5]
MAMS-22273 (31780,320) [O:5/5]
Level II ProtoAurignacian
Transition Level III/II
MAMS-21663 (32920,140) [O:5/5]
MAMS-21662 (33980,160) [O:4/5]
Level III ProtoAurignacian
Transitiion Level IV/III
OxA-19292 (36540,240) [O:4/5]
OxA-20361 (36770,210) [O:4/5]
OxA-19291 (38140,260) [O:5/5]
OxA-19862 (40340,390) [O:7/5]
Level IV Mousterian
Start Level IV
Riparo Bombrini-Vicino excavation 1976
Layer A2-A1
Proto Aurignacian
Layer A3-A2
Grotta di Fumane
60000
55000
50000
45000
40000
35000
30000
Modelled date (BP)

Fig. 3