Direct dating of Neanderthal remains from the site of Vindija Cave and implications for the Middle to Upper Paleolithic transition

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Previous dating of the Vi-207 and Vi-208 Neanderthal remains from Vindija Cave (Croatia) led to the suggestion that Neanderthals survived there as recently as 28,000–29,000 B.P. Subsequent dating yielded older dates, interpreted as ages of at least ∼32,500 B.P. We have redated these same specimens using an approach based on the extraction of the amino acid hydroxyproline, using preparative high-performance liquid chromatography (Prep-HPLC). This method is more efficient in eliminating modern contamination in the bone collagen. The revised dates are older than 40,000 B.P., suggesting the Vindija Neanderthals did not live more recently than others across Europe, and probably predate the arrival of anatomically modern humans in Eastern Europe. We applied zooarchaeology by mass spectrometry (ZooMS) to find additional hominin remains. We identified one bone that is Neanderthal, based on its mitochondrial DNA, and dated it directly to 46,200 ± 1,500 B.P. We also attempted to date six early Upper Paleolithic bone points from stratigraphic units G_1, Fd/d+G_1 and Fd/d, Fd. One bone artifact gave a date of 29,500 ± 400 B.P., while the remainder yielded no collagen. We additionally dated animal bone samples from units G_1 and G_1–G_3. These dates suggest a co-occurrence of early Upper Paleolithic osseous artifacts, particularly split-based points, alongside the remains of Neanderthals is a result of postdepositional mixing, rather than an association between the two groups, although more work is required to show this definitively.

Vindija Cave (Croatia) | single-compound AMS dating | DNA analysis | zooarchaeology by mass spectrometry | Middle to Upper Paleolithic transition

The period between ∼45,000 and 35,000 cal B.P. in Europe witnessed the so-called biocultural transition from the Middle to early Upper Paleolithic, when incoming anatomically modern humans displaced Neanderthal groups across the continent (1, 2). Significant questions still remain regarding the precise nature of this transition, the humans responsible for the various transitional early Upper Paleolithic industries, the degree of overlap between Neanderthals and modern humans, and the timing of the disappearance of the former. The European record for the transition retains its interest because it is the best-documented sequence for the disappearance of a hominin group available (3). The latest data, both radiometric and genetic, suggest Neanderthals and modern humans coexisted or overlapped for up to several thousand years in Europe until Neanderthal disappearance at around 40,000 cal B.P. (4, 5). Ascertaining the spatial attributes of Neanderthal and modern human populations in Europe is an area of active research, and a reliable chronology remains essential.

Our understanding of the biocultural processes involved in the transition have been greatly influenced by improved accelerator mass spectrometry (AMS) dating methods and their application to directly dating the remains of late Neanderthals and early modern humans, as well as artifacts recovered from the sites they occupied. It has become clear that there have been major problems with dating reliability and accuracy across the Paleolithic in general, with studies highlighting issues with underestimation of the ages of different dated samples from previously analyzed sites (6). We have been working on redating some of the purported late-surviving Neanderthal sites from around Europe, which have included human and archaeological remains from sites such as Mezmaiskaya (Russia), where a previous directly dated Neanderthal infant yielded a radiocarbon age of ∼29,000 B.P. (7), and Zafarraya (Spain), which was thought to contain Neanderthal remains clustering in age around a small group of U-series–dated animal bones between 33,400 and 28,900 B.P. (8). At Mezmaiskaya, the AMS dates obtained for the Neanderthal excavated above the previously dated individual were substantially older (9). This, along with other AMS dates from cut-marked fauna from the same archaeological horizons, suggested the original date of 29,000 B.P. could not be correct. At Zafarraya, Wood et al. (10) showed that, when redated using ultrafiltration methods, the bones that produced ages of ∼33,000 B.P. were in fact beyond the radiocarbon limit, suggesting the

Significance

Radiocarbon dating of Neanderthal remains recovered from Vindija Cave (Croatia) initially revealed surprisingly recent results: 28,000–29,000 B.P. This implied the remains could represent a late-surviving, refugial Neanderthal population and suggested they could have been responsible for producing some of the early Upper Paleolithic artefacts more usually produced by anatomically modern humans. This article presents revised radiocarbon dates of the human bones from this site obtained using a more robust purification method targeting the amino acid hydroxyproline. The data show that all the Neanderthal remains are from a much earlier period (>40,000 cal B.P.). These revised dates change our interpretation of this important site and demonstrate that the Vindija Neanderthals probably did not overlap temporally with early modern humans.

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Neanderthal remains were unlikely to be as young as previously thought. In both cases, revised radiocarbon dates produced with more robust chemical pretreatment methods have illustrated significant underestimates in the previous dates that cannot be reconciled with a hypothesis of late-surviving refugial Neanderthals.

The Neanderthal fossil remains from level G_1 of Vindija Cave in northern Croatia have remained in the literature as potentially late individuals. Given the evidence from the Peştera cu Oase specimen, which demonstrates a recent Neanderthal ancestry in a 40,000 cal B.P. modern human from the Danube corridor (5), the renewed dating of the Vindija remains is overdue.

Two specimens, Vi-207 and Vi-208, were originally directly AMS dated in the late 1990s at the Oxford Radiocarbon Accelerator Unit (ORAU). Vi-207 is a right posterior mandible and Vi-208 is a parietal fragment, both showing Neanderthal-specific morphology (11, 12). The initial radiocarbon results were 29,080 ± 400 B.P. (OxA-8296) and 28,020 ± 360 B.P. (OxA-8295) (13). Higham et al. (14) attempted to redate these specimens by taking the very small amounts of collagen remaining from the original sample pretreatment and ultrafiltering the product before AMS dating. The revised measurements were 32,400 ± 1,800 B.P. (Vi-207: OxA-X-2089-07) and 32,400 ± 800 B.P. (Vi-208: OxA-X-2089-06), which indicated the previous dates were indeed too young. For sample Vi-208, after ultrafiltration, the C/N atomic ratio was 3.4, which indicates collagen of acceptable quality. However, for Vi-207, the >30-kDa fraction obtained produced a C/N ratio of 4.3, which indicates the presence of a high molecular weight contaminant. The radiocarbon date for this sample could therefore include a higher molecular weight noncollagenous contaminant, possibly cross-linked to the collagen. On the basis of the potential problems associated with the small size of the redated samples and the potential for remaining contaminants, OxA-X-2089-06 was considered to be a minimum age (14). If the dates are even approximately correct, however, it makes them the most recent known Neanderthals. This would imply a more extensive temporal overlap between Neanderthals and early modern humans in central Europe than has recently been documented (4).

In addition to the Neanderthal remains, level G_1 has yielded a small archaeological assemblage that contains techno-typologically Middle and Upper Paleolithic lithic artifacts plus several distinctively early Upper Paleolithic osseous points (12). It has been argued that the mix of Neanderthals, Middle Paleolithic tools, and Upper Paleolithic technology was the result of cryoturbation and *Ursus spelaeus* activity in level G_1, with elements mixing into level G_1 from both the Upper Paleolithic unit F above and the Middle Paleolithic level G_3 below (15, 16). Zilhão (17) has suggested that the G_1 lithic assemblage has parallels with the Szeletian technocomplex, and further, that there is a mixture of elements of Szeletian and Aurignacian I and II within the level [see also Svoboda (18)]. Karavanić and Smith (19) have suggested that the mixture of elements may represent the interaction and possible acculturation between modern humans and late Neanderthals. These alternatives are testable by selecting human and organic osseous points, as well as animal bones, for renewed AMS dating. This is what we have undertaken and describe here.

Materials

A total of 10 samples from Vindija Cave were selected for AMS radiocarbon dating (Table 1). These included three previously dated Neanderthal specimens (Vi-207, Vi-208, and Vi-33.19), as well as a fourth Neanderthal bone (Vi-*28) discovered using zooarchaeology by mass spectrometry (ZooMS) screening. In addition, to test the reality of the co-occurrence of earlier Upper Paleolithic bone and antler point artifacts with the Neanderthal remains, we selected six osseous points for dating (*SI Appendix*, Fig. S1). To test collagen preservation, we took ∼3–5 mg bone powder, using tungsten carbide drills, and measured the %N content. This is an indicator of collagen preservation (20). The results show that only one bone point sample (Vi-3446) had sufficient levels of nitrogen to warrant full sampling for collagen extraction and AMS dating; the remainder failed and therefore were not sampled further (*SI Appendix*, Table S1). We also included the sample from a split-based bone point (Vi-3437) that had been analyzed in the laboratory previously, producing only a small collagen yield. We decided to attempt to redate it, using a larger starting mass of bone powder. Unfortunately, there was insufficient collagen remaining from this sample after pretreatment. We also selected four animal bones of *Cervidae* (Vi-*17), *Panthera* sp. (Vi-*6), *Ursus* sp. (Vi-*7), and *Bovidae* (Vi-*60), identified by ZooMS from stratigraphic units G_1 and G_1–G_3 to explore the potential issue of postdepositional mixing in the deposits further.

Results and Discussion

ZooMS Collagen Fingerprinting. We used ZooMS to identify potential hominin bone fragments among the unidentified faunal remains from the G_1 and G_3 levels, as well as the stratigraphic unit G_1–G_3. The majority of the 383 samples we analyzed yielded poor collagen preservation, which prevented any identification to genus or taxon. Only 101 samples produced identifiable spectra; a summary of all taxa identified by ZooMS is shown in *SI Appendix*, Table S2. This assemblage is dominated by *Ursus*, and only six of the 27 taxa identified by morphological study of the bones in Miracle et al. (21) could be identified here. We identified a single hominin specimen (Fig. 1 *A* and *B*), which again highlights the use of applying such techniques to groups of unidentified Paleolithic bone samples. The bone was analyzed using ancient DNA techniques to enable a formal species identification.

DNA Analysis of the Human Bones. Genomic analysis based on mitochondrial DNA revealed that all four human specimens fall into Neanderthal mitochondrial variation. Full mitochondrial genomes of Vi-207 and Vi-*28 were reconstructed with an average coverage of 103-fold and 257-fold, respectively. The mitochondrial DNA sequence of Vi-207 was identical to Vi-33.25 and Feldhofer 1 mitochondrial genomes, whereas Vi-*28 had an identical mitochondrial

Table 1. Samples selected for AMS dating

ORAU P Number	Sample Reference	Archeology Level	Species
39039	Vi-33.19 (SP2756)	G_3	Neanderthal
41681	Vi-*28 (SP4162)	G_1	Neanderthal
41415	Vi-208/Vi-11.29 (SP3563)	G_1	Neanderthal
41416	Vi-207/Vi-11.41 (SP3562)	G_1	Neanderthal
41417	Vi-3446	Fd/d+G1	Not known (point)
41687	Vi-3437	G_1	Not known (split-based point)
41803	Vi-*17	G_1	*Cervidae*
41801	Vi-*6	G_1	*Panthera* sp
41802	Vi-*7	G_1	*Ursus* sp
41804	Vi-*60	G_1–G_3	*Bovidae*

All samples produced enough collagen for radiocarbon dating with the exception of P41687, which failed to produce any collagen. See *SI Appendix*, Fig. S1 for images of the bone points included in this study.

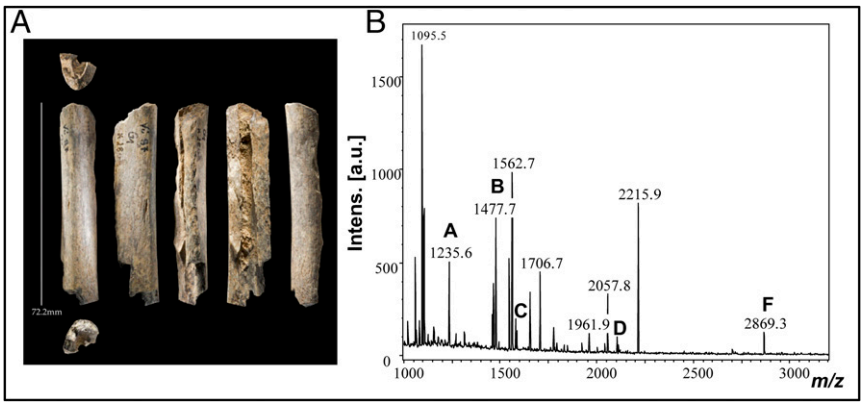

Fig. 1. (*A*) High-resolution photographs of the Vi-*28 Neanderthal bone found using ZooMS. The bone yields evidence for a probable cut and gauge marks (right upper part of the bone). The picture was taken after the bone had undergone sampling for ZooMS and before sampling for aDNA, radiocarbon, and stable isotope analysis. (*B*) MALDI-TOF mass spectrum of digested collagen from the Vi-*28 bone. All tagged peaks (A, B, C, D, and F) denote sequence-matched peptides observed in human collagen (27, 28).

sequence to Vi-33.17 (*SI Appendix*, Fig. S2). Both Vi-33.25 and Vi-33.17 were found in layer I of Vindija Cave. As previously published, Vi-33.19 has the same mitochondrial sequence as Vi-33.16 (22). Because of lower endogenous DNA content in Vi-208, a full mitochondrial genome could not be reconstructed for the sample. However, from the limited amounts of mitochondrial sequences, we were able to trace most of the observed variants to variations found in previously sequenced Neanderthal mitochondrial genomes (*SI Appendix*, Fig. S3).

AMS Dating. Nine of the samples selected produced enough collagen (or hydroxyproline) to be dated by AMS. All dates obtained on the four Neanderthal specimens at the ORAU are reported in Table 2. We also list the dates obtained on two other hominin samples: Vi-75-G3/h-203, analyzed at the Uppsala Radiocarbon Laboratory (Sweden) (23), and Vi-2291-18 (level G, sublayer unknown), prepared at the Max Planck Institute, Leipzig, and dated at the ORAU (24). The different sample pretreatments are also indicated in Table 2.

Vi-208 and Vi-207 produced hydroxyproline dates of 42,700 ± 1,600 and 43,900 ± 2,000 B.P., respectively. These ages are significantly older than any of the dates obtained previously for these specimens using the AG (gelatinized filtered collagen) and AF (ultrafiltered collagen) procedures, and this strongly suggests that

Table 2. Radiocarbon dates of the Vindija Neanderthal remains

P no.	OxA/OxA-X	CRA	±	P code	Used, mg	Yield, mg	%Yield	%C	δ^{13}C, ‰	δ^{15}N, ‰	C/N
Sample Vi-208 (SP3563)											
9663	8295	28,020	360	AG	233.9	15.2	6.5	37.1	−19.5	10.6	3.2
9663	2082-09	29,200	360	AG	229.9	10.9	4.7	42.7	−19.8	11.4	3.6
9663	2089-06	32,400	800	AF	n/a	n/a	n/a	42.3	−20.2	10.3	3.4
41415	X-2689-09	42,700	1,600	HYP	626.0	35.1	5.6	45.2	−26.1	10.2	5.6
Sample Vi-207 (SP3562)											
9665	8296	29,080	400	AG	229.2	9.7	1.5	36.6	−20.5	11.3	3.6
9665	2082-10	29,100	360	AG	128.8	6.7	5.2	41.6	−22.8	12.2	4.1
9665	2089-07	32,400	1,800	AF	n/a	n/a	n/a	39.0	−24.6	11.1	4.3
41416	X-2689-10	43,900	2,000	HYP	629.0	37.4	6.0	42.2	−24.7	11.5	5.6
Sample Vi-33.19 (SP2756)											
39039	32278	45,300	2,300	AF	560	62.2	11.1	45.2	−18.6	10.7	3.4
39039	X-2717-11	44,300	1,200	HYP	n/a	n/a	n/a	46.5	−24.4	12.3	4.9
Sample Vi-*28 (SP4162)											
41681	X-2687-57	46,200	1,500	HYP	1,000.0	54.0	5.4	34.3	−25.5	11.7	5.0
Sample Vi-2291–18 (level G, sublayer unknown)											
22966	V-2291-18	44,450	550	XB	/	/	/	39.3	−18.7	10.8	3.3
Sample Vi-75-G3/h-203											
/	Ua-13873	>42,000							−19.4	15.2	3.3

Radiocarbon dates of the Vindija Neanderthal remains. CRA is conventional radiocarbon age, expressed in years B.P. (49). Stable isotope ratios are expressed in per mil (‰) relative to Vienna Pee Dee Belemnite with a mass spectrometric precision of ±0.2‰ (50). PCode refers to pretreatment code; AG is gelatinized filtered collagen, AF is ultrafiltered collagen, XB denotes collagen extracted at a different laboratory than the ORAU, and HYP denotes the extraction of hydroxyproline from hydrolyzed bone collagen (51, 52). Collagen yield represents the weight of gelatin or ultrafiltered gelatin in milligrams. %Yld is the percent yield of extracted collagen as a function of the starting weight of the bone analyzed. When further purification (AF or HYP) was performed on previously extracted collagen, the yields are not reported. %C is the carbon present in the combusted sample (gelatin or hydroxyproline). C/N is the atomic ratio of carbon to nitrogen and is acceptable if it ranges between 2.9–3.5 in the case of collagen or ~5.0 in the case of hydroxyproline. All samples were prepared and dated at the ORAU with the exception of Vi-2291-18, which was extracted at the Max Planck Institute in Leipzig and dated in Oxford (24) and Vi-75-G3/h-203, which was prepared and dated at Uppsala in Sweden (23). The background carbon derived from the HPLC separation has been accounted for by using a correction where appropriate (details can be found in the *SI Appendix*). Slash indicates data not available.

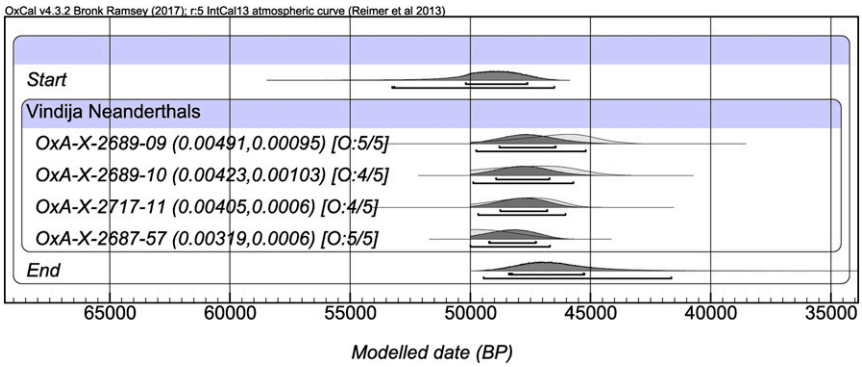

Fig. 2. Bayesian age model showing the calibrated HYP ages of the four Neanderthal samples from Vindija Cave. The model is a simple phase model in OxCal 4.3 (47), in which all F14C determinations are assumed to have no relative order. "Start" and "End" correspond to the boundaries calculated by the model. The calibration curve of Reimer et al. (48) was used to calibrate the results. Details can be found in the *SI Appendix*.

noncollagenous high molecular weight contaminants, probably crosslinked to the collagen, were still present in the sample previously dated. It is only by hydrolyzing the collagen and selecting the hydroxyproline that we were able to successfully remove these contaminants.

The AMS measurement of the third human bone from level G_1 (Vi-*28), identified using the ZooMS method, gave a date of 46,200 ± 1,500 B.P. For these three HYP (extraction of hydroxyproline from hydrolyzed bone collagen) dates obtained on the Neanderthal bones from level G_1, we performed a χ^2 test using the modern fraction F14C and its error. The error weighted mean is 0.0038 ± 0.0005 with a t value of 2.57. If t is <5.99, the value for χ^2, the error weighted mean is not significant. This shows that the results obtained on the three Neanderthal bones from level G_1 are statistically in agreement. Vi-33.19 (level G_3) was also dated using the hydroxyproline method and produced a date of 44,300 ± 1,200 B.P., which is a more precise date than the one obtained using the AF procedure (45,300 ± 2,300 B.P.). The AF determination had a low target current in the AMS (85% of normal current) and was imprecise for this reason. The fact that the two dates overlap suggests no significant contamination in this bone. Taken together, these dates show a significantly older occupation of the site by Neanderthals, suggesting the site cannot be considered to be a refugium for late-surviving Neanderthals (Fig. 2, code in *SI Appendix*). The two other dates obtained on samples Vi-33.26 and Vi-75, even if obtained with a method that can be less efficient in removing contamination, confirm the dating of level G is more than 42,000 B.P.

Only one bone point (Vi-3446–P41417) produced enough nitrogen during prescreening to warrant pretreatment chemistry. Less than 500 mg bone was available, so we applied an AG (gelatin) treatment to maximize the collagen yield. We obtained a date of 29,500 ± 400 B.P., which we consider to be a minimum age, as this pretreatment has been shown before to underestimate the age of ancient bones such as this (Table 3). To test possible perturbation in level G_1, we also dated four faunal samples identified as originating from levels G_1 and G_1–G_3, using ultrafiltration (AF). These samples produced dates that were all greater than or very close to the radiocarbon limit (Table 3). These samples are less likely to have been treated with conservation materials than the human bones, and therefore were not prepared using the HPLC method. Three of these faunal bones are from level G_1 (Table 1). We see more variation that would be expected statistically if we combine the three Neanderthal bones and three faunal bones from level G_1 for a χ^2 test (error weighted mean, 0.0027 ± 0.0004; $t = 22.52$; $\chi^2 = 11.07$). This shows there is bone of variable radiocarbon age in the G_1 level and suggests the possibility of postdepositional mixing and movement of material. In consequence, the bone tools cannot be associated with the Neanderthal remains unless further direct radiocarbon dating using the HYP approach is undertaken. Clearly, the low collagen content of the points appears to preclude this at this time.

Conclusions

Single-amino acid AMS dating of the Vindija Neanderthals has yielded results that are substantially older than the previous ages that were initially obtained. We have shown that the Neanderthals predate ~44,000 cal B.P. The results suggest this group was not a late-surviving refugial Neanderthal population, as previously thought, and means the group almost certainly did not overlap with early anatomically modern humans in this part of Europe. Despite our best attempts, we were not able to date the bone industry associated with the archaeology of level G_1. The one date we obtained from a later stratigraphic unit was younger than 30,000 B.P., but because the bone was not treated with the most rigorous pretreatment chemistry methods, it could potentially be older. The dating of other faunal materials from level G_1 highlighted a significant range in age, which could indicate a perturbation of the general sequence. The question, then, of whether some of the points could have been produced by Neanderthals remains open; however, it is parsimonious to conclude that the split-based point at least must have a maximum age of 32,000–34,000 B.P. based on evidence for its association with the Aurignacian in other regions, and so it likely postdates the Vindija Neanderthals significantly.

Table 3. Radiocarbon determinations and analytical data from Vindija faunal remains and bone point

OxA/OxA-X	CRA	±	PCode	Species	Used, mg	Yield, mg	%Yield	%C	δ^{13}C, ‰	δ^{15}N, ‰	C/N
34458	29,500	400	AG	Not known (bone point)	450	6.58	1.5	46.0	−19.4	3.5	3.4
2695-21	>46,700		AF	*Cervidae*	960	2.60	0.3	35.9	−20.7	5.6	3.3
34471	>48,400		AF	*Panthera* sp.	1,100	9.09	0.8	39.1	−18.9	10.1	3.2
34472	>49,000		AF	*Ursus* sp.	1,000	4.53	0.5	37.1	−23.0	8.8	3.2
34473	47,200	2,900	AF	*Bovidae*	950	40.41	4.3	42.2	−20.8	4.4	3.2

See *SI Appendix*, Table S2 caption for details of the terms used.

Bone points have been recovered throughout Eurasia with dates as early as ~37,000 B.P. (e.g., from the Aurignacian site of Pes'ko and the Châtelperronian site of Arcy-sur-Cure) (6, 25, 26). It is therefore clear that both anatomically modern humans and Neanderthals produced bone points, with only split-based bone/antler points being diagnostic of the earlier facies of the former.

Our perception of the biological transition between Neanderthals and modern humans has changed radically during the last decade. Evidence suggests interbreeding and a significant temporal overlap between the two from ~44,000–40,000 cal B.P. On the basis of our hydroxyproline dates and the DNA results, the Vindija Neanderthals date before the period when the first modern humans arrived into Europe and interbred with Neanderthals.

Methods

Bone Screening by ZooMS. We used collagen peptide mass fingerprinting or ZooMS to analyze preserved type 1 collagen (COL1) from small unidentified bone fragments from the Vindija site. COL1 is characterized by three polypeptide (alpha) chains, and within these chains there are small differences in the amino acid sequences among different species. These offer the possibility of identification of species-specific amino acid markers. We used matrix-assisted laser desorption/ionization mass spectrometry to determine the relative mass-to-charge (*m/z*) values of the tryptic digest peptides in each bone sample. By comparing these values with a library of known fauna, we identified bones to genus or species in some instance (27–31). We screened a total of 383 unidentified bone samples from Vindija Cave (levels G1, G3, and stratigraphic unit G1–G3).

Radiocarbon Dating. We used two different methods to prepare the samples for AMS dating. First, samples from artifacts were pretreated following the routine ORAU procedure, comprising a decalcification, base wash, reacidification, gelatinization, and ultrafiltration (coded AF in the ORAU), as described by Brock et al. (20). Sample P41417 produced too little collagen for ultrafiltration, so the treatment was halted after gelatinization (coded AG). The four human bone samples were dated using the single amino acid radiocarbon dating method developed at ORAU (32). This method involves separation of the underivatized amino acids from hydrolyzed bone collagen samples using preparative HPLC. Details of the method are described in the *SI Appendix*. Hydroxyproline (HYP), was isolated by Prep-HPLC, combusted, graphitized, and AMS-dated. This pretreatment approach is the most efficient technique to remove contaminants, including conservation materials (unless collagen-based glue has been applied).

DNA Analysis.

DNA extraction and library preparation. Genomic analysis of the four human bones was performed at the Max Planck Institute for Evolutionary Anthropology in Leipzig, Germany. Three samples (Vi-207, Vi-208, and Vi-*28) were prepared specifically for this project, whereas Vi-33.19 was previously analyzed as part of a separate study (22). Bones were sampled using a sterile dentistry drill, and between 15 and 30 mg bone powder was used for DNA extraction, with the exception of Vi-*28, in which three separate parts of the bone were sampled (*SI Appendix*, Table S3). DNA extraction was performed using a method for ultrashort DNA fragment retrieval (33) with modifications (34). The bone powder of Vi-207 and Vi-208 was treated with 0.5% hypochlorite solution before DNA extraction (34) to remove microbial and modern human DNA contamination. Next-generation sequencing libraries were prepared from 10 μL of each extract, using two single-stranded library preparation schemes: Vi-207 and Vi-208 with a previously published method (35), with modifications from Korlević et al. (34), and Vi-*28 with an automated library preparation scheme (36). Libraries were amplified and labeled with a pair of unique index sequences (37, 38).

Enrichment of the mitochondrial DNA and sequencing. Amplified libraries were enriched for human mitochondrial DNA, using a bead-based hybridization capture (39) with modifications (40). Enriched libraries were sequenced on an Illumina MiSeq platform with a double index configuration (41). Overlapping paired-end reads were merged (42) and mapped to the revised Cambridge Reference Sequence mitochondrial genome (rCRS; NC_0120920), using Burrows-Wheeler Aligner (43) with parameters "-n 0.01 -o 2 -l 16500" (44). Analyses were restricted to sequences with perfect index combinations. PCR duplicates were removed using bam-rmdup (https://bitbucket.org/ustenzel/biohazard-tools), and mapped sequences longer than 35 bases with a mapping quality of at least 25 were retained for subsequent analyses. To assess whether the results could be affected by present-day modern human contamination, sequences with ancient DNA specific cytosine to thymine substitutions (C to T) at the 5′ or 3′ molecule end were selected and analyzed separately.

Phylogenetic analyses. To reconstruct the mitochondrial genomes of the three previously unanalyzed hominin samples, DNA sequences were realigned to the Vindija 33.16 mitochondrial genome (AM948965) (45). Data were processed as described earlier. Data analysis was performed as previously published (ref. 28 for full mitochondrial genomes, ref. 46 for partial mitochondrial data).

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PNAS Supporting Information for:

Direct dating of Neanderthal remains from the site of Vindija Cave and implications for the Middle to Upper Paleolithic transition

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This PDF includes:

- Additional information on the DNA analysis of human bones
- Additional information on the radiocarbon dating
- Figures S1 to S5
- Tables S1 to S6

Fig. S1: Pictures of the bone points from Vindija Cave analysed in this study. From left to right; Vi-3437 (split-based point), Vi-3439, Vi-3445 (not sampled), Vi-3446, Vi-3450, Vi-3449 (not sampled), Vi-3454 and Vi-3455.

Table S1: % nitrogen and C/N atomic ratios for the bone points from Vindija Cave. To be acceptable the ORAU must have a %N>0.7%, C/N atomic ratios ought to be >4-5. In the case of the Vindija samples only one (Vi-3446) was above this threshold. The remainder were too low in nitrogen and so were not sampled for AMS dating. Vi-3437 was not tested for %N; it had previously been analysed in the ORAU in 1998. 300 mg from the point was treated using the AG method, producing 0.8% collagen (2.5 mg of collagen) but the determination was failed due to suspected remaining contamination.

Sample code	**%N**	**C/N atomic ratio**
Vi-3446 (P41417)	1.08	5.18
Vi-3455	0.10	31.40
Vi-3439	0.05	52.01
Vi-3450	0.06	43.99
Vi-3454	0.06	42.91

Table S2: Taxonomic identification achieved using the ZooMS method on 383 samples.

Taxa	**Number of samples**
Hominin	1
Panthera sp.	1
Cervidae	12
Bovidae	14
Equus sp.	1
Ursus sp.	72
Unidentified	282

Additional information on the DNA analysis of human bones

Table S3: DNA summary for the three human samples prepared specifically for this study.

Sample reference	Library ID	Bone material used (mg)	№ of molecules in library (qPCR)	Total № of generated sequences	% of sequences mapped to rCRS (≥35bp, MQ25)	№ of unique mtDNA sequences	Sequencing duplication rate	№ of sequences with a C to T substitution at molecule ends (5' or 3')	All sequences		Sequences with a C to T substitution at the opposing end	
									5' C to T (%) [95% CI]	3' C to T (%) [95% CI]	5' C to T (%) [95% CI]	3' C to T (%) [95% CI]
Vi-207 (SP3562)	R5559	15.2	8.8E+07	1033271	83	30562	22.2	9799	46.6 [45.5-47.7]	52.0 [50.7-53.3]	45.6 [42.3-48.9]	49.6 [46.2-53.1]
Vi-208 (SP3563)	R5560	16.2	1.1E+08	731200	70.2	1283	278.7	296	32.6 [27.7-37.9]	58.6 [51.2-65.7]	25.9 [13.2-44.7]	53.8 [29.1-76.8]
Vi-*28 A (SP4162)	D5818	30.1	6.2E+09	431022	59.9	24576	7.4	4740	47.4 [46.2-48.6]	36.0 [34.7-37.3]	46.0 [41.9-50.1]	34.9 [31.6-38.4]
Vi-*28 B (SP4162)	D5819	32.2	4.5E+09	599769	59	26910	9.1	5310	49.4 [48.2-50.6]	36.1 [34.9-37.3]	50.3 [46.2-54.3]	34.4 [31.2-37.6]
Vi-*28 C (SP4162)	D5820	28.9	3.7E+09	847219	57.8	24278	13.7	4908	49.9 [48.7-51.1]	36.8 [35.5-38.1]	49.8 [45.7-53.9]	36.1 [32.8-39.5]

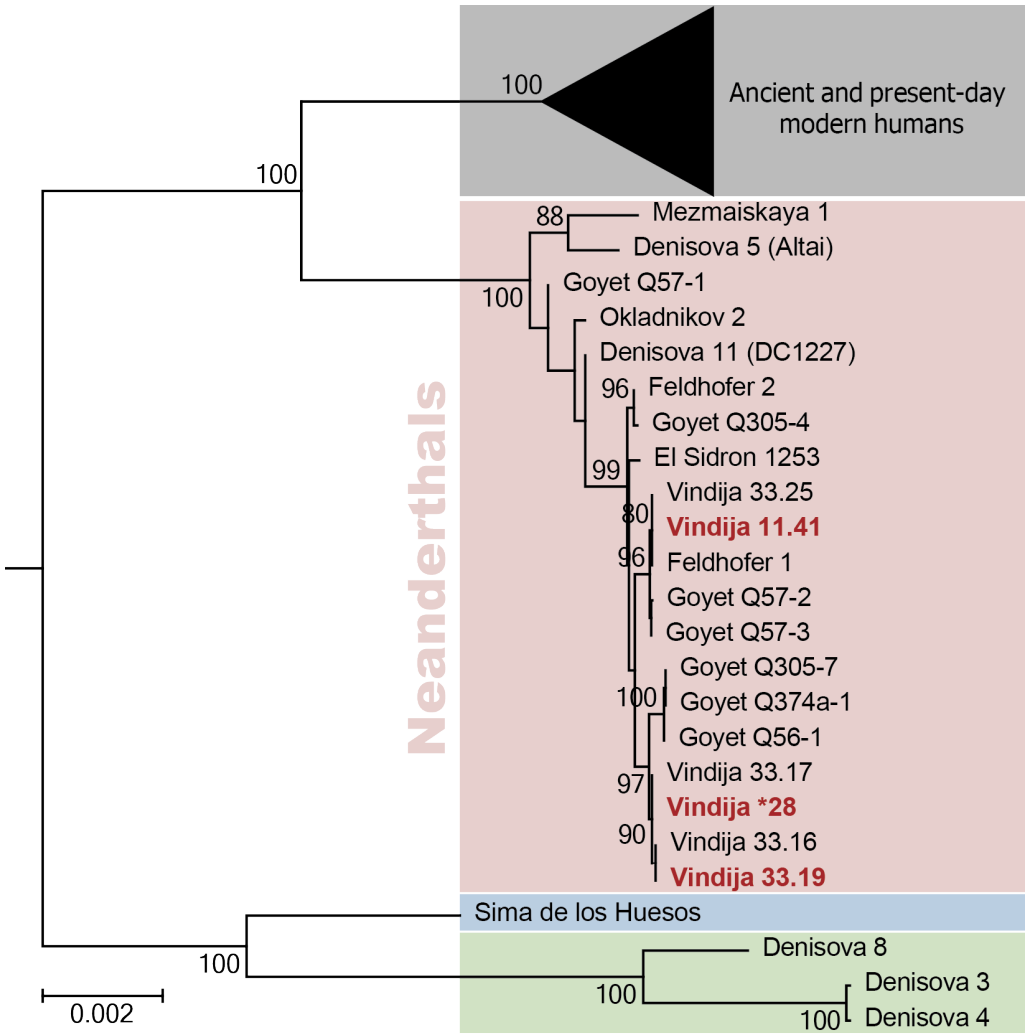

Fig. S2: Reconstructed phylogenetic tree based on full mitochondrial genomes of three out of four radiocarbon dated Neanderthals in this study. Full mitochondrial genomes were reconstructed for Vi-*28 and Vi-207 (Vindija 11.41), while Vi-33.19 was reconstructed as part of a previous study (1). Alignments were created using mitochondrial genomes from 65 ancient and present day modern humans, 17 Neanderthals, 3 Denisovans, one Sima de los Huesos individual, and one chimpanzee. Presented here is the Neighbor-joining tree of the reconstructed mitochondrial genomes of Vindija Neanderthals, previously published Neanderthals and ancient and present-day modern human mitochondrial genomes. The optimal nucleotide substitution model was TrN+I+G, branch support was computed from 500 bootstrap replications and bootstrap values >80% are shown at roots. The chimpanzee mitochondrial genome was used for rooting the tree.

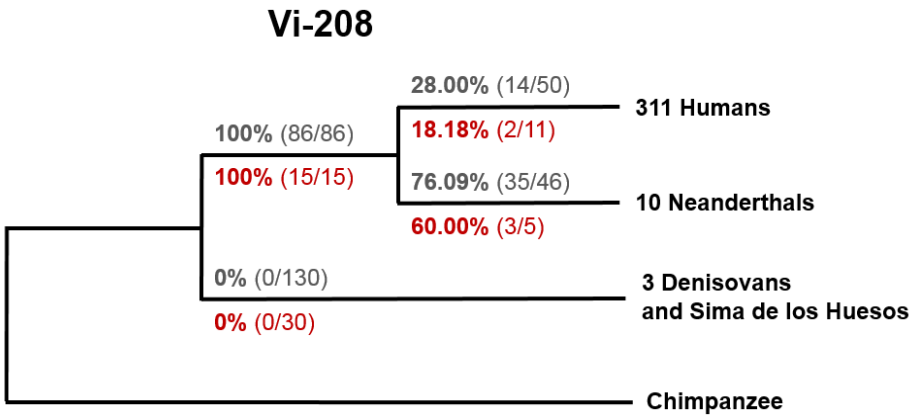

Fig. S3: Percentage of variants supporting the derived state at positions diagnostic for each branch of the hominin mitochondrial DNA tree for mitochondrial sequences obtained for Vi-208. Above each branch are the percentage of shared derived variants and the total number of observation for all mtDNA sequences (grey) and only sequences with terminal C to T substitutions (red).

Additional information on the radiocarbon dating

Method for the sample preparation of human bone samples by HPLC

The three human bone samples followed the initial treatment as outlined by Brock et al. (2). Bone samples were sandblasted with aluminium oxide to clean the surfaces and crushed using a steel pestle and mortar. They were then demineralised with three 0.5M hydrochloric acid treatments, the first two for 2 hours and the third one overnight. Following the demineralisation, the organic fraction was rinsed 3 times with ultrapure MilliQ™ deionised water. Samples were then treated with 0.1M sodium hydroxide for 30 min and rinsed 3 times in MilliQ™ deionised water. A final 0.5M hydrochloric acid wash was used to eliminate any atmospheric carbon dioxide dissolved during the base treatment. Once more, this was followed by three MilliQ™ deionised water rinses. After each acid or base treatment and water rinse, samples were centrifuged and the supernatant discarded. The resulting collagen was then gelatinised at 75°C for 20 hours in a solution of pH 3 water (10mL, 1mM hydrochloric acid) and filtered using Ezee-filters™ (60-90µm). Finally, samples were freeze-dried using a VaCo 5 freeze-dryer (Zirbus, Germany) for approximatively 24 hours.

Freeze-dried collagen samples (35-50mg) were accurately weighed into 11.5 mL screw top test tubes and concentrated hydrochloric acid added via micropipette at a ratio of approximately 1 mL of 6M HCl per 10 mg of collagen. The tubes were flushed with N_2 gas for 5 minutes to provide an inert atmosphere, then capped and set in a heating block at 110°C for 24 hours. After hydrolysis, the samples were evaporated to dryness in a Genevac EZ-2 vacuum evaporator (Genevac Ltd, Ipswich, UK). 900µl of 0.1M NaOH was

added to re-dissolve the sample. This was then loaded into a 2 mL BP Plastipak™ syringe fitted with a Thermo Scientific 0.2 µm PTFE syringe filter to remove any insoluble matter and filtered into a Waters® HPLC 1 mL total recovery vial (Agilent Technologies). 200 µL of MilliQ™ water was added to the amino acid residue and filtered into the same HPLC vial to recover as much sample as possible.

Chromatography experiments were performed on a Varian ProStar HPLC system equipped with an autosampler (Model 410), two isocratic pumps with titanium heads (Model 210), a column oven set at 30°C containing a Primesep A preparative column (22 × 250 mm, particle size 5 µm; SIELC, IL, USA), a UV detector (Model 320) set at 205 nm and a fraction collector (Model 701). The system is controlled by Star workstation PC software (Version 6.0). The autosampler was modified with a 1 mL glass syringe and a 2 mL sample stainless steel loop, enabling up to 1 mL of sample to be injected. The separation was achieved using a gradient of MilliQ™ deionised water (eluent A) and 0.3% phosphoric acid diluted with MilliQ™ deionised water (eluent B), as described in Table S4 and at a total flow rate of 18 mL/min.

Table S4: HPLC gradient for the separation of underivatised amino acids on Primesep A column with MilliQ™ deionised water as eluent A and 0.3% phosphoric acid diluted with MilliQ™ deionised water as eluent B

Time (min)	% eluent A	% eluent B
0 - 20	100	0
20 - 21	Linear gradient	Linear gradient
21 - 320	0	100

For each sample, the collected water fraction containing hydroxyproline was concentrated using a Genevac EZ-2 Plus vacuum evaporator until totally dried. The hydroxyproline was then reconstituted in 25 µL of MilliQ™ deionised water and loaded on to 12 mg of pre-combusted Chromosorb™ in cleaned tin capsules. Stable isotopic measurement for carbon and nitrogen, combustion and graphitisation were performed as described in Brock et al. (2).

Correction of dates to include carbon contribution related to the HPLC procedure

All the dates reported in this paper are corrected for routine procedures such as pre-treatment chemistry, combustion and graphitisation. For the HYP dates it is necessary to also include a correction for the extraneous dead (fM=0) and modern (fM=100) carbon added during the chromatographic separation (Table S5).

In order to calculate this, we run background samples (which should not contain any ^{14}C) and modern sample of known age (bone form the Mary Rose ship sank in 1545 AD). The dating of the background

standards does show that there is some modern carbon contamination which needs to be accounted for (Table S6). The average of the Mary Rose dates is 315 ± 25 BP which is very close to the real value (311 ± 8 BP or 1545 AD) (3). The correction for the dead carbon contribution is therefore negligible. The two formulae applied to correct the AMS ages and account for the uncertainty on the age are reported below (Formula S1 and S2):

$$F^{14}C_{Hyp} = \frac{(AMS - MF_{Mod})}{MF_{Hyp}}$$

Formula S1: Formula for correcting AMS single amino acid ages where AMS is the measured $F^{14}C$; MF_{Mod} is the mass fraction of modern contamination ≈ (AMS_{Std} x C_{Std}) / C_T; MF_{HYP} is the mass fraction of sample hydroxyproline ≈ 1 – MF_{Mod}; AMS_{Std} is the measured $F^{14}C$ of the background standards; C_{Std} is the mass of carbon in the background standards measured on the mass spectrometer; C_T is the mass spectrometer measured mass of carbon in the sample.

$$\Delta F^{14}C_{Hyp} = \sqrt{\left(\left(\frac{1}{MF_{Hyp}} * \Delta AMS\right)^2 + \left(\frac{1}{MF_{Hyp}} * \Delta MF_{Mod}\right)^2 + \left(\frac{AMS - MF_{Mod}}{\left(MF_{Hyp}\right)^2} * \Delta MF_{Hyp}\right)^2\right)}$$

Formula S2: Formula for the corrected uncertainty on the AMS ages. See Formula S1 for details.

Table S5: HYP dates before and after correction. No correction was applied to OxA-X-2687-57 because the background standards run at the same time ($F^{14}C$ = 0.00006 +/- 0.00054 ; 0.00000 +/- 0.00071) indicated that no background ^{14}C was added during the sample preparation. C_T is the mass spectrometer measured mass of carbon in the sample.

			Original uncorrected values				Values corrected for background			
P Number	**OxA-X Number**	**C_T (mg)**	**CRA**	**±**	**AMS $F^{14}C$**	**±**	**corrected CRA**	**±**	**corrected $F^{14}C$**	**±**
41415	X-2689-09	1.493	38800	800	0.00797	0.00078	42700	1600	0.00491	0.00095
41416	X-2689-10	1.349	39200	900	0.00762	0.00084	43900	2000	0.00423	0.00103
39039	X-2717-11	2.141	43000	900	0.00472	0.00053	44300	1200	0.00405	0.0006
41681	X-2687-57	1.99	46200	1500	0.00319	0.0006	46200	1500	0.00319	0.0006

Table S6: AMS results of the background and modern standards run on the HPLC to evaluate extraneous dead and modern carbon added during the chromatographic separation. P Code HYP refers to pretreatment based on the extraction of hydroxyproline from hydrolysed bone collagen (2, 4). C_{Std} is the mass of carbon in the standards measured on the mass spectrometer. CRA is the conventional radiocarbon age, expressed in years BP (5). AMS $F^{14}C$ corresponds to the fraction modern carbon as measured on the AMS. Details of the independent ages for the background and modern standards are provided in Brock et al., 2010 (2).

P Number	Sample	P Code	C_{Std}	CRA	±	AMS $F^{14}C$	±
P18802.138	Fairbanks, Alaska bison	HYP	1.85	49200		0.00103	0.00057
P18802.152	Fairbanks, Alaska bison	HYP	1.35	45200	1800	0.0036	0.00082
P18802.153	Fairbanks, Alaska bison	HYP	1.76	48300	2100	0.0025	0.00064
P19651.141	Ash bend bison	HYP	1.67	50400		0.00063	0.00063
P19651.163	Ash bend bison	HYP	1.86	50500		0.00073	0.00057
P19651.148	Ash bend bison	HYP	2.13	54400		0.00006	0.00054
P40854.2	Mary Rose	HYP	2.14	337	24	0.95889	0.00292
P40854.2	Mary Rose	HYP	2.12	330	24	0.95972	0.00290
P40854.2	Mary Rose	HYP	1.98	303	26	0.96294	0.00314
P39840.31	Mary Rose	HYP	0.90	291	28	0.96442	0.00342

Bayesian modelling

We built a simple single-phase model using OxCal 4.3 assuming that the directly dated Neanderthal bone samples have no relative age order (See Figure S4). The model was built using the INTCAL13 calibration curve (3). The CQL code for the model is shown below:

```
Plot()
 {
  Outlier_Model("General",T(5),U(0,4),"t");
  Sequence()
  {
   Boundary("Start");
   Phase("Vindija Neanderthals")
```

```
  {
   R_F14C("OxA-X-2689-09", 0.00491, 0.00095)
   {
    Outlier("General", 0.05);
   };
   R_F14C("OxA-X-2689-10", 0.00423, 0.00103)
   {
    Outlier("General", 0.05);
   };
   R_F14C("OxA-X-2717-11", 0.00405, 0.0006)
   {
    Outlier("General", 0.05);
   };
   R_F14C("OxA-X-2687-57", 0.00319, 0.0006)
   {
    Outlier("General", 0.05);
   };
  };
  Boundary("End");
 };
};
```

We also ran a second model including the non-conserved Neanderthal bone dated using AF pretreatment for sample Vi-33.19 as well as the OxA-V-2291-18 (Vi-33.26) determination. There were no outliers of significance or major differences in the overall results of the model in terms of the start and end boundaries (See figure S5). The CQL code is shown below:

```
Plot()
 {
  Outlier_Model("General",T(5),U(0,4),"t");
  Outlier_Model("SSimple",N(0,2),0,"s");
  Sequence()
  {
   Boundary("Start");
   Phase("Vindija Neanderthals")
   {
```

```
  R_F14C("OxA-X-2689-09", 0.00491, 0.00095)
  {
   Outlier("General", 0.05);
  };
  R_F14C("OxA-X-2689-10", 0.00423, 0.00103)
  {
   Outlier("General", 0.05);
  };
  R_Combine("Vi 33.19")
  {
   Outlier("General", 0.05);
   R_F14C("OxA-X-2717-11", 0.00405, 0.0006)
   {
    Outlier("SSimple", 0.05);
   };
   R_F14C("OxA-32278",0.00357,0.00102)
   {
    Outlier("SSimple", 0.05);
   };
  };
  R_F14C("OxA-X-2687-57", 0.00319, 0.0006)
  {
   Outlier("General", 0.05);
  };
  R_F14C("OxA-V-2291-18",0.00395,0.00028)
  {
   Outlier("General", 0.05);
  };
 };
 Boundary("End");
};
};
```

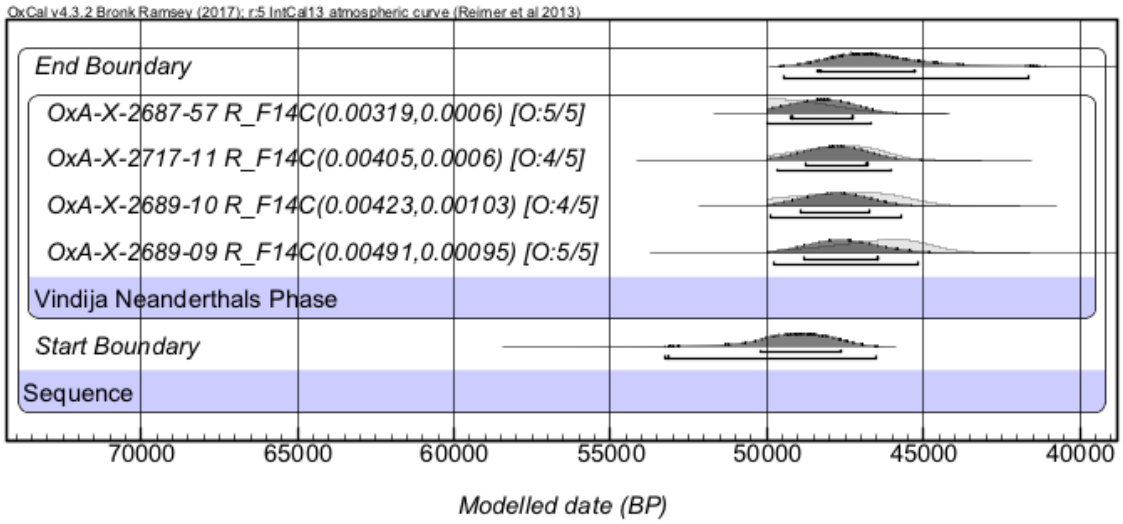

Fig. S4: Bayesian model for the HYP determinations obtained in this paper.

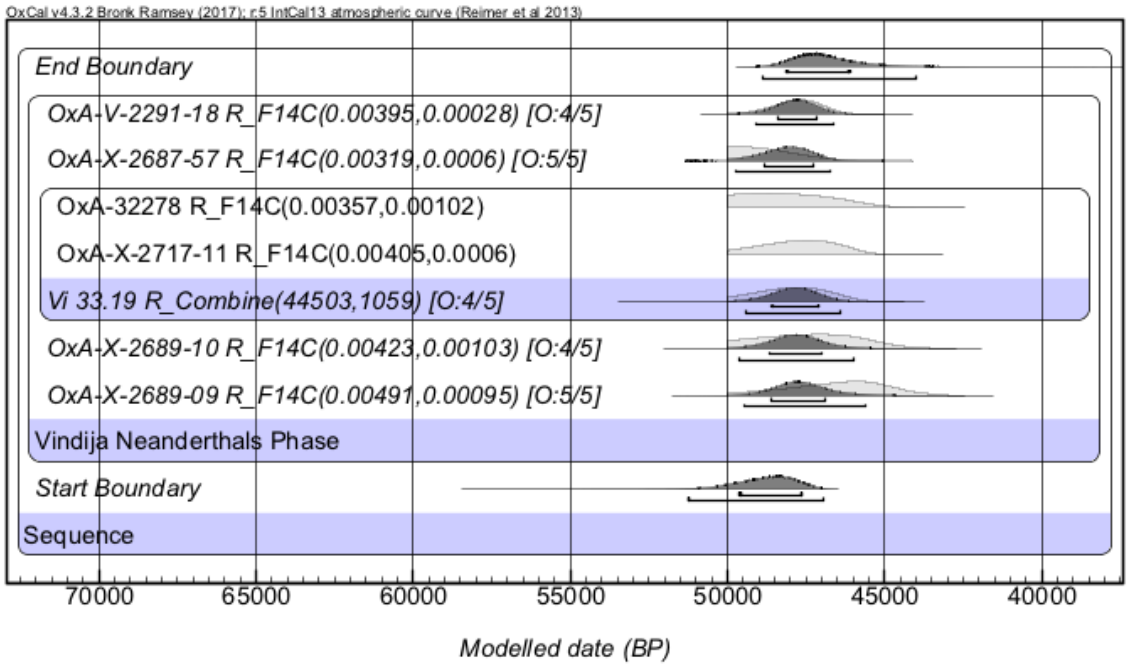

Fig. S5: Bayesian model for the HYP determinations and AF results obtained in this paper. Values for the Vi-33.19 sample are R_Combined.

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