**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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- Additional information on the DNA analysis of human bones
- Additional information on the radiocarbon dating
- Figures S1 to S5
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**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] |

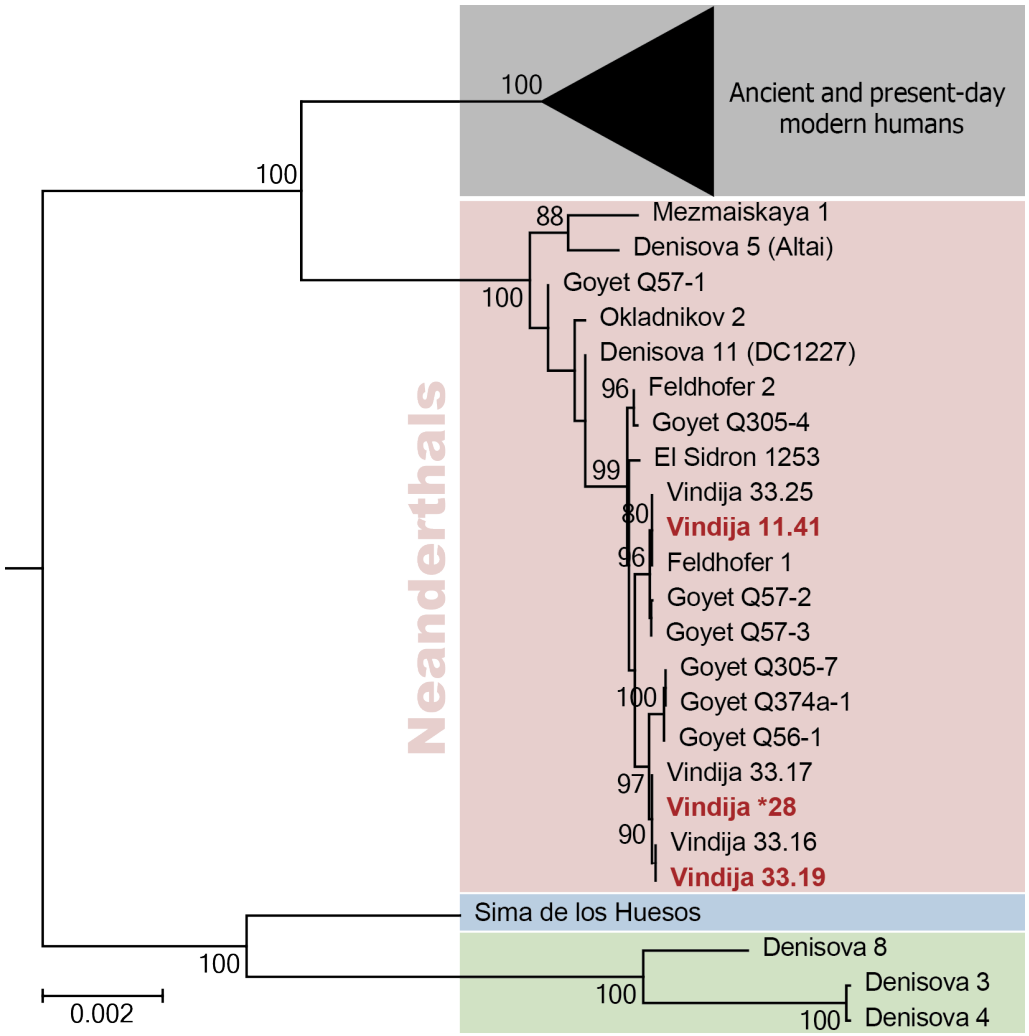


**Fig. S2:** Reconstructed phylogenetic tree based on full mitochondrial genomes of three out of four radiocarbon dated Neanderthals in this study. Full mitochondrial genomes were reconstructed for Vi-*28 and Vi-207 (Vindija 11.41), while Vi-33.19 was reconstructed as part of a previous study (1). Alignments 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.

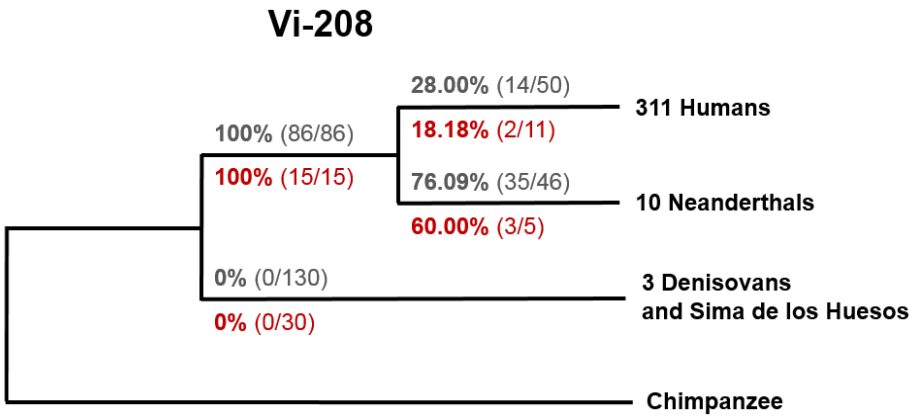


**Fig. S3:** Percentage of variants supporting the derived state at positions diagnostic for each branch of the hominin mitochondrial DNA tree for mitochondrial sequences obtained for Vi-208. Above each branch are the percentage of shared derived variants and the total number of observation for all mtDNA 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");
};
};
```

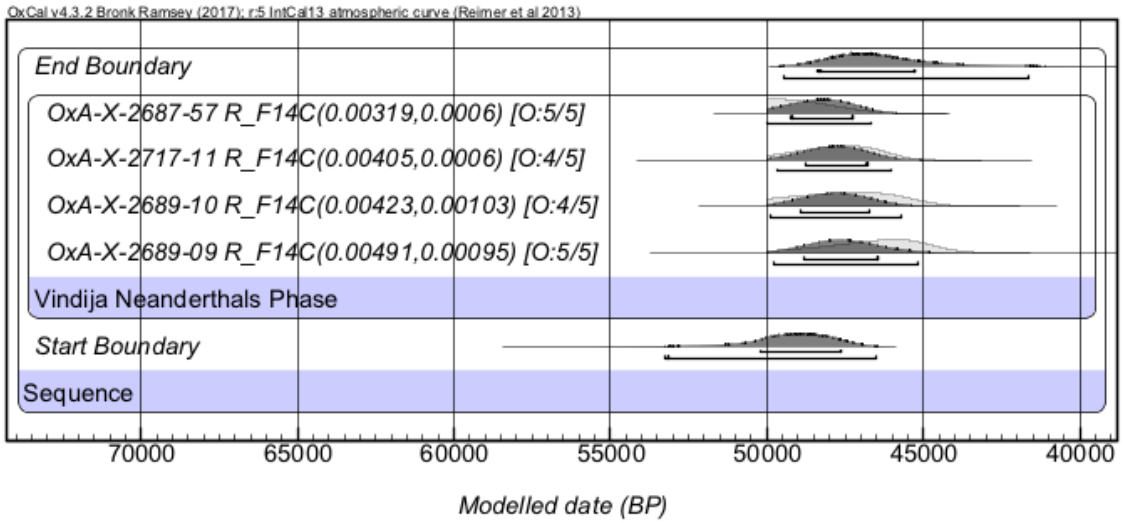


**Fig. S4:** Bayesian model for the HYP determinations obtained in this paper.

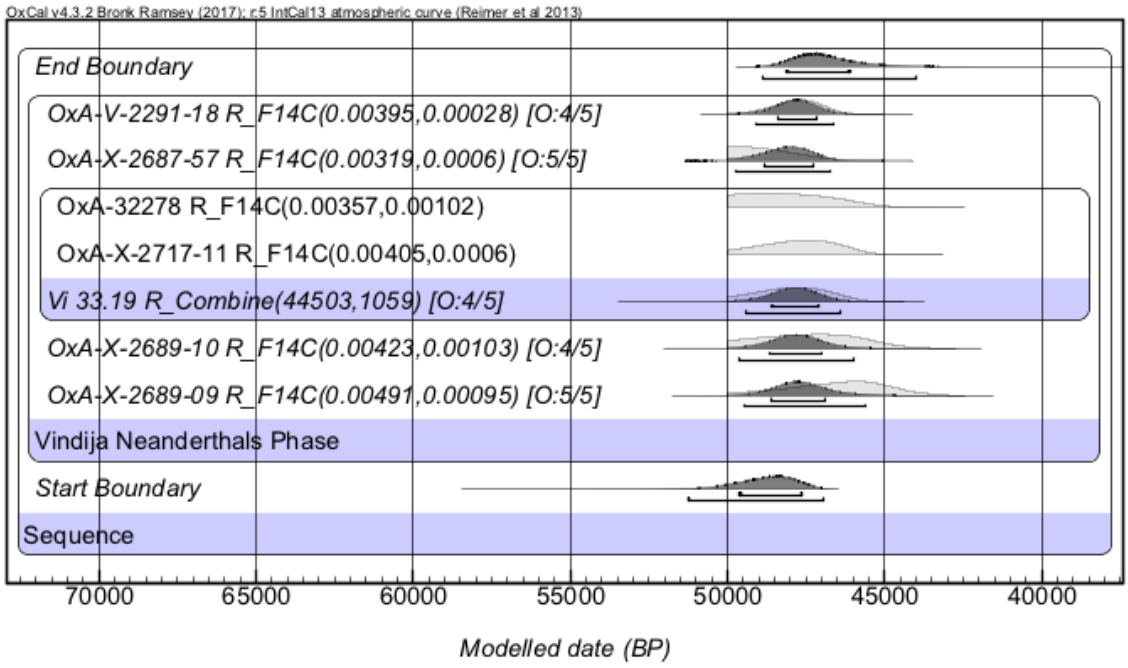


**Fig. S5:** Bayesian model for the HYP determinations and AF results obtained in this paper. Values for the Vi-33.19 sample are R_Combined.

**References**


1. Gansauge M-T & Meyer M (2014) Selective enrichment of damaged DNA molecules for ancient genome sequencing. *Genome Research* 24(9):1543-1549.
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