The genome of the offspring of a Neandertal mother and a Denisovan father

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Neandertals and Denisovans are extinct groups of hominins that separated from each other more than 390,000 years ago[1,2]. Here we present the genome of *"Denisova 11"*, a bone fragment from Denisova Cave (Russia)[3], and show that it comes from an individual who had a Neandertal mother and a Denisovan father. The father, whose genome bears traces of Neandertal ancestry, came from a population related to a later Denisovan found in the cave[4-6]. The mother came from a population more closely related to Neandertals who lived later in Europe[2,7] than to an older Neandertal found in Denisova Cave[8], suggesting that migrations of Neandertals between eastern and western Eurasia occurred sometime after ~120,000 years ago. The finding of a first-generation Neandertal-Denisovan offspring among the small number of archaic specimens sequenced to date suggests that mixing between Late Pleistocene hominin groups was common when they met.

Neandertals and Denisovans inhabited Eurasia until they were replaced by modern humans around 40,000 years ago (40 kya)[9]. Neandertal remains are found in western Eurasia[10]; while physical remains of Denisovans have thus far been found only in Denisova Cave[4-6,11,12], where Neandertal remains have also been recovered[8]. Although little is known about the morphology of Denisovans, their molars lack derived traits typical of Neandertals[5,11].

DNA recovered from individuals of both groups suggests that they diverged from each other more than 390 kya[1,2]. The presence of small amounts of Neandertal DNA in the genome of "*Denisova 3*", the first Denisovan individual identified[4-6], indicates that the two groups mixed with each other at least once[8]. It has also been shown that Neandertals mixed with the ancestors of present-day non-Africans around 60 kya[2,8,13], and possibly with earlier ancestors of modern humans[1,14,15]; and that Denisovans mixed with the ancestors of present-day Oceanians and Asians[5,16,17]. Denisovans may furthermore have received gene flow from an

archaic hominin that diverged more than a million years ago from the ancestors of modern humans[8].

A fragment of a long bone, "*Denisova 11*" (Fig. 1), was identified among over 2,000 undiagnostic bone fragments excavated in Denisova Cave as being of hominin origin using collagen peptide mass fingerprinting[3]. Its mitochondrial (mt) DNA was found to be of the Neandertal type and direct radiocarbon dating showed it to be >50,000 years old[3]. From its cortical thickness, and under the conservative assumption that it comes from the thickest part of a femur or a tibia, the *Denisova 11* individual was at least 13 years old at death (Extended Data Figure 1, Supplementary Information [SI] 1). To sequence its genome, we performed six DNA extractions[18] from bone powder that we treated with 0.5% sodium hypochlorite solution prior to extraction to reduce microbial and present-day human contamination[19]. We produced ten DNA libraries[20] from the extracts (Extended Data Table 1, SI 2 and 3) and sequenced the *Denisova 11* genome to an average coverage of 2.6-fold. The coverage of the X chromosome is similar to that of the autosomes, indicating that *Denisova 11* was a female. Using positions where the previously reconstructed mtDNA of *Denisova 11* (ref. [3]) differs from all mtDNA sequences in a world-wide panel of 311 present-day humans[21], we estimate the extent of present-day human mtDNA contamination to be 0.3% (95% CI: 0.1-0.7%) in the entire dataset, and 0.4% (95% CI: 0.1-1.6%) among the DNA fragments carrying terminal cytosine to thymine substitutions (SI 2). Such substitutions arise from cytosine deamination and can be used to enrich for authentic ancient DNA fragments[22,23]. Based on the proportion of fragments from *Denisova 11* matching the derived allele present in at least 99% of present-day humans at positions where archaic genomes sequenced to date carry the ancestral allele[1,2], we estimate the contamination by present-day human DNA to 1.4% (95% CI: 1.3-1.6%) in the whole dataset; and 1.3% (95% CI: 0.9-1.7%) among DNA fragments with evidence of deamination.

We note that these are likely over-estimates, as some of the derived alleles used are likely to have also been present in archaic populations (SI 2).

To determine from which hominin group *Denisova 11* originated, we compared the proportions of DNA fragments that match randomly drawn derived alleles from a Neandertal genome ("*Altai Neandertal*", also known as "*Denisova 5*") or a Denisovan genome (*Denisova 3*), both determined from bones discovered in Denisova Cave[6,8], as well as from a present-day African genome (Mbuti)[6]. When using all fragments from *Denisova 11* which overlap such informative sites[1], 38.6% carried alleles matching the Neandertal genome and 42.3% carried alleles matching the Denisovan genome (Fig. 2a), suggesting that both archaic groups contributed to the ancestry of *Denisova 11* to approximately equal extents (SI 4). To exclude that this surprising finding was due to an accidental mixing of DNA or sequencing libraries in the laboratory, we analysed each of the ten libraries separately and in each case found approximately equal sharing of alleles with the Neandertal and the Denisovan genomes (SI 3). In contrast, the percentage of DNA fragments mapping to the human genome differs more than ten-fold among the libraries (Extended Data Table 1). It seems highly unlikely that an accidental mixture of DNA would result in approximately equal proportions of Neandertal and Denisovan DNA in all libraries, while the total proportion of hominin DNA would vary drastically. We also note that we detect only one ancient mtDNA type in the dataset, arguing against a mixture of DNA from different individuals (SI 3). Finally, we note that libraries from other projects that were prepared, sequenced and processed in parallel with the ones from *Denisova 11* do not display mixt ancestries, excluding the possibility of a systematic error in data processing.

To estimate the heterozygosity of *Denisova 11*, we restrict the analysis to transversion polymorphisms to prevent deamination-derived substitutions from inflating the estimates, and find 3.7 transversions per 10,000 autosomal base pairs. This is over four times higher than the

heterozygosity of the two Neandertal (*Altai Neandertal* and "*Vindija 33.19*") and one Denisovan (*Denisova 3*) genomes sequenced to date, and similar to the heterozygosity seen in present-day Africans. In fact, the heterozygosity of *Denisova 11* is similar to what would be expected if this individual carried one set of chromosomes of Neandertal origin and one of Denisovan origin, as estimated from the number of differences between randomly sampled DNA fragments from either the *Vindija 33.19* or the *Altai Neandertal* genome and the *Denisova 3* genome (Fig. 2b, SI 5).

To explore whether *Denisova 11* had approximately equal amounts of Neandertal and Denisovan ancestry because she belonged to a population with mixed Neandertal and Denisovan ancestry, or because her parents were each from one of these two groups, we considered alleles carried by two randomly drawn DNA fragments from *Denisova 11* that cover sites where the genomes of the *Altai Neandertal* and *Denisova 3* carry a transversion difference in a homozygous form. Note that when two fragments covering such sites are sampled, heterozygous sites have a 50% chance of appearing as homozygous because the two fragments come from the same chromosome. Thus, the expected proportion of heterozygous sites is 50% for a first-generation (F1) offspring while it is 25% in a population at Hardy-Weinberg equilibrium with mixed ancestry in equal proportions (SI 6). We find that in 43.5% of cases, one fragment from *Denisova 11* matches the Neandertal genome and the other matches the Denisovan genome, while in 27.3% and 29.2% of cases both fragments match the state seen in the Neandertal or the Denisovan genome, respectively (Fig.2c). When a low-coverage Neandertal genome ("*Goyet Q56-1*")[7] is analysed in the same way, the two fragments match different states in 2.1% of cases, while they both match the Neandertal state in 90.3% of cases and the Denisovan state in 7.5% of cases (Fig. 2c).

Obviously, the *Altai Neandertal* and *Denisova 3* are unlikely to be identical to the genomes of the individuals that contributed ancestry to *Denisova 11*. To take this into account,

we used coalescent simulations to estimate the expected proportions of DNA fragments matching a Neandertal or a Denisovan genome in populations with demographic histories similar to those of the *Altai Neandertal* and *Denisova 3* (SI 6). The proportion of cases where one of the two DNA fragments sampled from *Denisova 11* would match the Neandertal state and the other the Denisovan state under this scenario fits the expectation for an F1 Neandertal-Denisovan offspring, but not an offspring of two F1 individuals, an offspring of an F1 parent and a Neandertal or a Denisovan parent, nor an individual from a population of mixed ancestry at Hardy-Weinberg equilibrium (Extended Data Figure 2, SI 6). We conclude that *Denisova 11* did not originate from a population carrying equal proportions of Neandertal and Denisovan ancestry. Rather, she was the offspring of a Neandertal mother, who contributed her mtDNA, and a Denisovan father.

We next plotted the distribution of sites across the genome, where *Denisova 11* carries an allele matching the *Altai Neandertal* genome and a different allele matching the *Denisova 3* genome. Such sites are distributed largely uniformly (Fig. 3), as would be expected for an F1 offspring of Neandertal and Denisovan parents. To explore the ancestry of the parents of *Denisova 11*, we looked for regions in the genome that deviate from a pattern consistent with *Denisova 11* being an F1 offspring (Extended Data Figure 3). Using four tests for enrichment of Denisovan or Neandertal ancestry, we identify at least five ~1 Mb long (0.72-0.95 Mb) regions, all of which are homozygous for Neandertal ancestry. This suggests that the Denisovan father of *Denisova 11* had some Neandertal ancestry. Given conservative estimates of the size and number of these regions, it is likely that there was more than one Neandertal ancestor in his genealogy, possibly as far back as 300-600 generations beforehand (SI 7). Interestingly, the heterozygosity in the regions of Neandertal ancestry in *Denisova 11* is higher than in the same regions in the genomes of *Vindija 33.19* or the *Altai Neandertal*, suggesting that the

Neandertals that contributed to the ancestry of *Denisova 11*'s father were from a different population than her mother (SI 5).

To explore how the mother of *Denisova 11* was related to the two Neandertals that have been sequenced to high quality to date, we evaluated the proportions of fragments from *Denisova 11* that match randomly drawn derived alleles from either of these two Neandertal genomes. *Denisova 11* shares derived alleles seen in the *Altai Neandertal* genome in 12.4% of cases and those present in the *Vindija 33.19* genome in 19.6% of cases, showing that the Neandertal mother of *Denisova 11* came from a population that was more closely related to *Vindija 33.19* than to the *Altai Neandertal* (SI 8). We estimate the population split times of *Denisova 11*'s Neandertal mother from the ancestors of the *Altai Neandertal* to ~20,000 years (20 ky) prior to the time when the *Altai Neandertal* lived, and her split time from the ancestors of *Vindija 33.19* to ~40 ky prior to *Vindija 33.19*. The population split between the Denisovan father of *Denisova 11* and *Denisova 3* is estimated to ~7 ky prior to the latter individual (SI 8). In Fig. 4 we present a population scenario that is compatible with these observations as well as with the population split times and molecular estimates of the ages of the three high-coverage archaic genomes[2]. We caution that the age estimates are associated with uncertainties, *e.g.*, regarding mutation rates and generation times, and that additional gene flow events are likely to have affected the population split times. Nevertheless, that Neandertals in Siberia ~90 kya shared at least partial ancestry with Neandertals that lived at least ~20 ky later in Europe[2,7] seems to suggest either that eastern Neandertals spread into Western Europe sometime after ~90 kya or that western Neandertals spread to Siberia before that time, two hypotheses that could be tested by sequencing the genomes of early Neandertals from Western Europe.

In conclusion, the genome of *Denisova 11* provides direct evidence for genetic mixture between Neandertals and Denisovans on at least two occasions: once between her Neandertal mother and her Denisovan father, and at least once in the ancestry of her Denisovan father.

Therefore, of the six individuals from Denisova Cave from whom nuclear DNA is available[5,6,8,11,12], two (*Denisova 3* and *Denisova 11*) show evidence of gene flow between Neandertals and Denisovans. We note that of the three genomes[24-27] retrieved from modern humans who lived at a time when Neandertals were present in Eurasia (*i.e.*, at least ~40 kya)[9], one individual, "*Oase 1*", had a Neandertal ancestor four to six generations back in his family tree[26].

It is striking that one direct offspring of a Neandertal and a Denisovan (*Denisova 11*) and one modern human with a close Neandertal relative (*Oase 1*) have been identified among the few individuals from whom DNA has been retrieved and who lived at the time of overlap of these groups (Fig. 1). In conjunction with the presence of Neandertal and Denisovan DNA in ancient and present-day people[2,5,8,13,16,17,28-30], this suggests that mixing among archaic and modern hominin groups may have been frequent when they met. However, Neandertals inhabited western Eurasia[10] while Denisovans inhabited as yet unknown parts of eastern Eurasia[5,17]. Thus, their zones of overlap may have been restricted in space and time. This, as well as possibly reduced fitness of individuals of mixed ancestry, may explain why Neandertals and Denisovans remained genetically distinct from each other. In contrast, when modern humans spread across Eurasia after ~60,000 years ago, spatial and perhaps temporal overlap may have been more extensive. This may have allowed archaic populations to become partly absorbed into what were probably larger modern human populations[6,8].

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Contributions: V.S. and S.N. performed the laboratory work; V.S., F.M., B.Ve., C.d.F., S.G., M.H., S.Pe., J.K., M.M., K.P. and S.Pä. analyzed the genetic data; B.Vi. carried out the morphological analysis; S.B., K.D., T.H., M.B.K., M.V.S. and A.P.D. discovered *Denisova 11* and provided archaeological data; V.S., K.P. and S.Pä. wrote the manuscript with input from all authors.

Author Information: Sequences generated from *Denisova 11* have been deposited in the European Nucleotide Archive under study accession number PRJEB24663. Reprints and permissions information are available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to viviane_slon@eva.mpg.de (V.S.) or paabo@eva.mpg.de (S.Pä.).

Figure legends

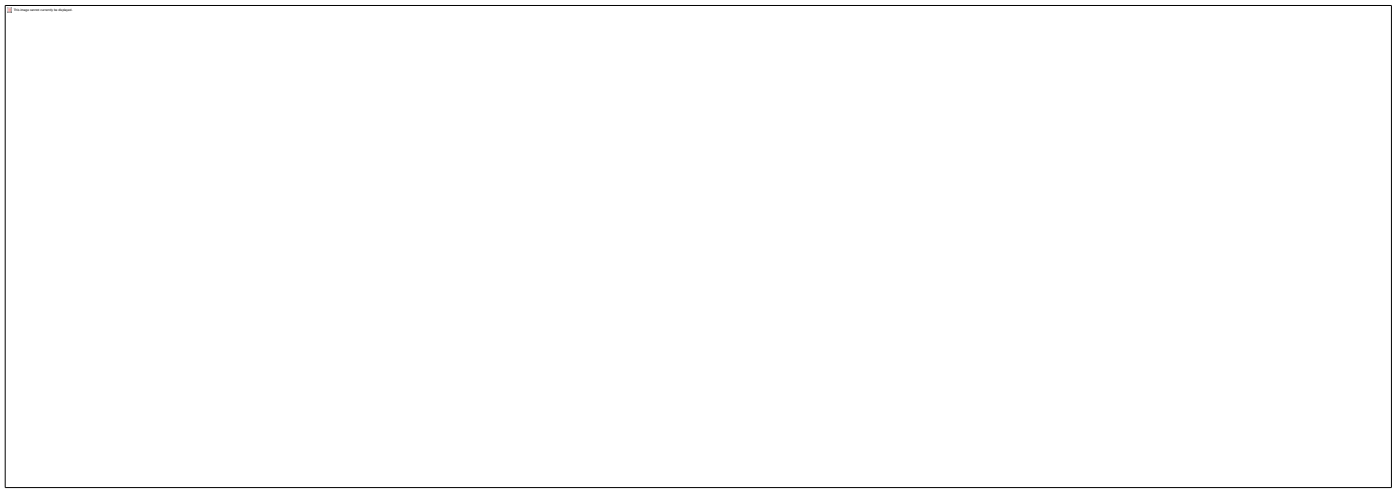

Figure 1. Location of Neandertals (blue), Denisovans (red) and ancient modern humans (yellow) dated to ~40 kya or older, from which sufficient nuclear DNA fragments have been recovered to enable their attribution to a hominin group. Full or abbreviated names of other specimens are shown below each figure. Asterisks indicate that the genome was sequenced to high-coverage, a question mark that the individual is of unknown sex. The blue outline of *Oase 1* and the blue dot in *Denisova 3* denotes Neandertal ancestry in their genomes. Data taken from [1,2,5-8,11-13,24-27].

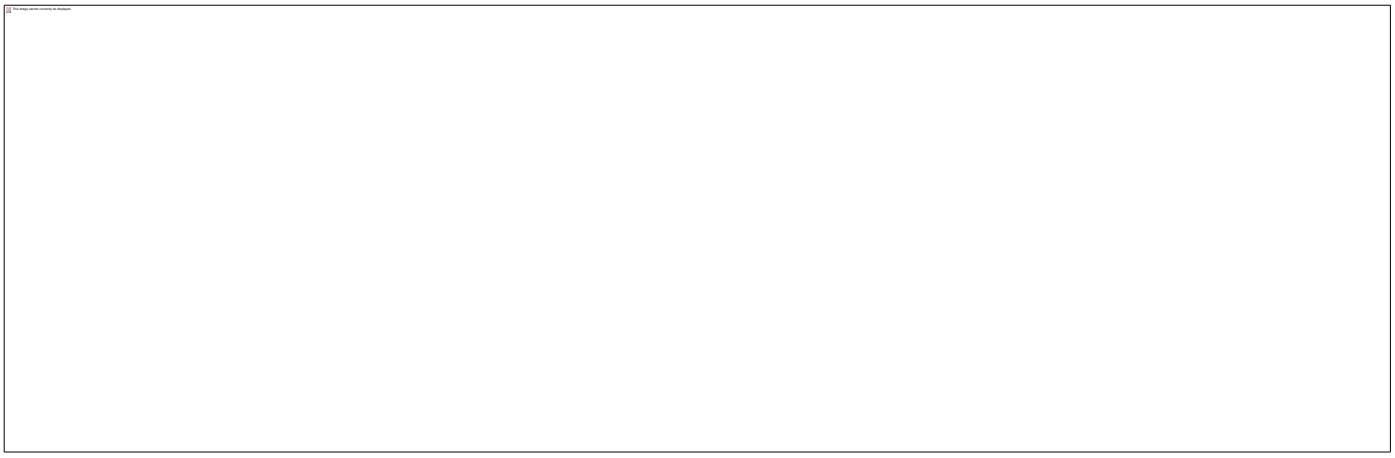

Figure 2. *Denisova 11* carries both Neandertal and Denisovan ancestry. a. Percentage of DNA fragments in *Denisova 11* carrying derived alleles seen on each branch of a tree relating a Neandertal, a Denisovan and a present-day human genome. **b.** Distribution of heterozygosity per chromosome in two Neandertals (blue), a Denisovan (red), *Denisova 11* (purple) and present-day humans (44 Africans [yellow] and 235 non-Africans [orange] from [34]), and the expectation for Neandertal-Denisovan F1 offspring (grey). White squares represent autosome-wide estimates for the archaic hominins, and the average of estimates across individuals for present-day humans. **c.** Percentage of sites at which two sampled DNA fragments both carry "Neandertal alleles" (blue), "Denisovan alleles" (red), and one allele of each type (purple); and the expectations for an offspring of a Neandertal and a Denisovan (F1), of two F1 parents (F2), and of an F1 and a Denisovan (F1xD).

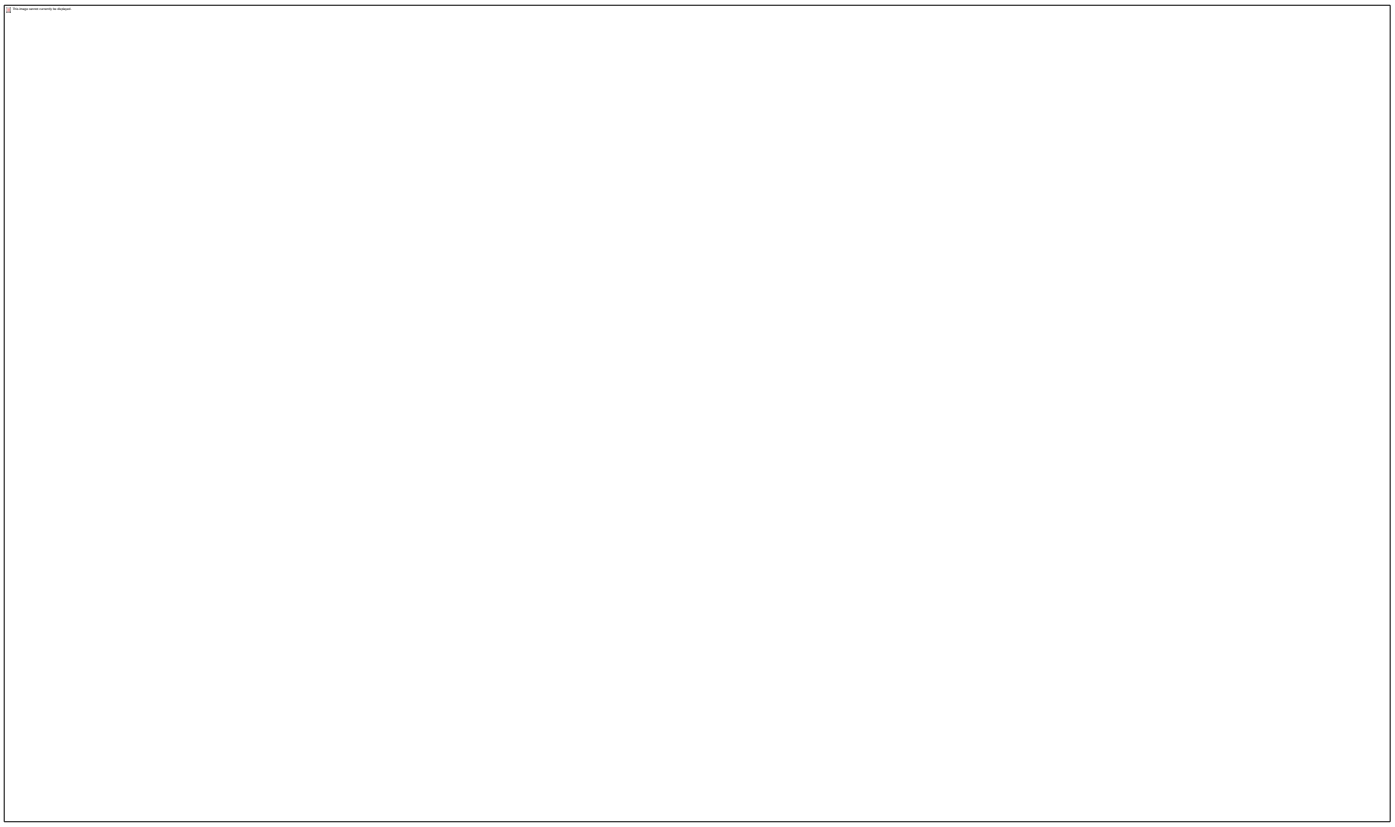

Figure 3. The distribution of Neandertal-like (blue) and Denisovan-like (red) alleles across the *Denisova 11* genome. Positions where randomly drawn DNA fragments match both archaic groups are marked in purple. Black lines indicate centromeres. The inset shows one region out of five (green boxes) where both chromosomes carry predominantly Neandertal-like alleles. For comparison, the distribution of alleles in this region is shown for a Neandertal genome (*Goyet Q56-1*).

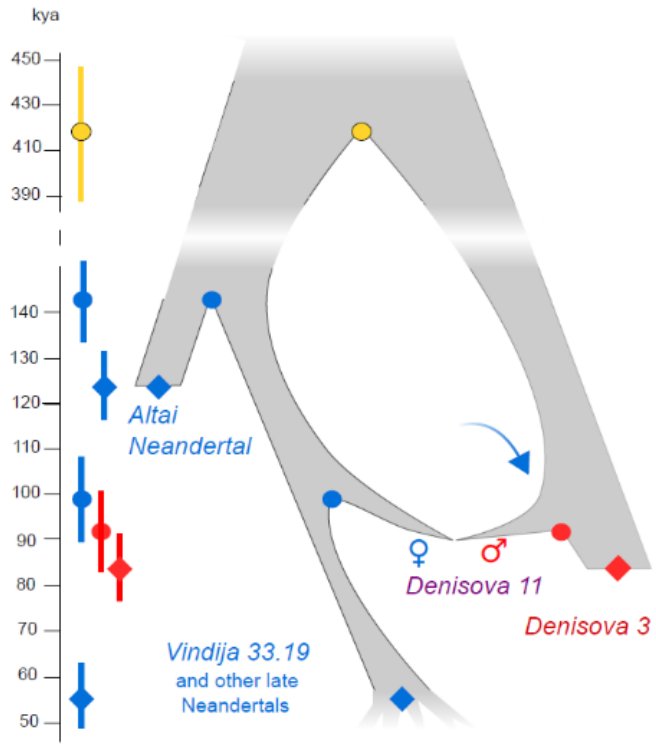

Figure 4. Relationships and gene flow events between Neandertal and Denisovan populations inferred from genome sequences. Diamonds indicate ages of specimens estimated via branch shortening[2]; circles indicate population split times estimated from allele sharing between *Denisova 11* and the high-coverage genomes (blue and red) and among the three high-coverage genomes (yellow, from [2]); the arrow indicates Neandertal gene flow into Denisovans. Ages before present are based on a human-chimpanzee divergence of 13 million years[25,35]. Error bars indicate 95% confidence intervals (CIs) based on block jackknife resampling across the genome. Note that the CIs do not take the uncertainty with respect to mutation rates or generation times into account.

Methods

Sampling and pre-treatment of bone powder

An overview of the laboratory experiments is in Extended Data Table 1. Bone powder was removed from the specimen using disposable sterile dentistry drills after the removal of a thin layer of surface material. Six samples were collected, each consisting of ~30mg of bone powder. Because a previous analysis of the bone revealed that it is contaminated with present-day human DNA[3], each sample of bone powder was incubated with 1ml 0.5% sodium hypochlorite solution as described[19] and as indicated in Extended Data Table 1, to reduce the amounts of present-day human and microbial DNA[7,19]. Residual sodium hypochlorite was removed by three consecutive 3-minute washes with 1ml water[19]. One extraction negative control (no powder) was included in each set of extractions.

DNA extraction and DNA library preparation

DNA was extracted using silica columns[18] as described[19], and eluted in 50µl 10mM Tris-HCl, 1mM EDTA, 0.05% Tween-20, pH 8.0. Ten µl of each DNA extract were used to prepare single-stranded DNA libraries as described[19,20]. Extraction negative controls were carried along, and a library preparation negative control was included in every experiment. Two additional 5µl aliquots from the extracts E3652 and E3655 were used to generate additional libraries (library preparation setup C in Extended Data Table 1), resulting in a total of ten DNA libraries. The number of DNA molecules in libraries was estimated by digital droplet PCR[36] or quantitative PCR[20]. Each library was amplified into plateau while incorporating a pair of unique indexes[37] using 1µM primers[19,37] and AccuPrime Pfx DNA polymerase (Life Technologies)[38]. Amplification products were purified using the MinElute PCR purification kit (Qiagen) or SPRI technology[39] on a Bravo NGS workstation (Agilent Technologies) as described[40]. Indexed DNA libraries were pooled with libraries from other projects.

Heteroduplices, which confound DNA separation and concentration measurements in chromatography, were removed from the pools by a single cycle amplification using Herculase II Fusion DNA polymerase (Agilent Technologies)[38] with primers IS5 and IS6 (ref. [41]). Prior to deeper sequencing of libraries R5507, R5509, R9880, R9881, R9882, R9883 and R9873, heteroduplices were removed from each library separately. The concentration of DNA in each pool or each individual library, respectively, was determined using a DNA-1000 chip (Agilent Technologies).

Sequencing and data processing

Sequencing was performed on Illumina platforms (MiSeq or HiSeq 2500) using 76-cycle paired-end runs adapted to double-indexed libraries[37]. Bases were called using *Bustard* (Illumina). Adapter sequences were trimmed and overlapping paired-end reads were merged into single sequences using *leeHom*[42]. Demultiplexing was carried out using *jivebunny*[7]. Sequences generated from a given library were merged using *SAMtools*[43] and aligned to the human reference genome (hg19/GRCh37) with the decoy sequences as in [2] using BWA[44] with parameters adjusted to ancient DNA[6]. PCR duplicates were collapsed using *bam-rmdup* (https://bitbucket.org/ustenzel/biohazard) and DNA fragments of length $\geq$35 bases that map within regions of unique mappability (Map35_100% from ref. [8]) with a mapping quality of 25 or higher[7] were used for analyses. Further filtering criteria used for certain analyses are detailed in the supplementary sections.

Code availability

The computer code used for simulations is presented in SI 6.

Methods references:

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Extended Data Tables legends

Extended Data Table 1. DNA extracts and DNA libraries prepared from the *Denisova 11* specimen. Data are shown by DNA extraction set, and libraries prepared in the same setup are denoted by the same letter (A, B or C). Relevant negative controls are marked in grey. The number of molecules in each library was quantified by digital droplet PCR or quantitative PCR (denoted by *). The number of DNA fragments sequenced per library are for the combined data from all sequencing runs. Mapped fragments were counted if they were at least 35 bases long and mapped to the human reference genome with a mapping quality of 25 or higher; and their percentage was calculated out of sequenced fragments of length 35 bases or more. Following the removal of PCR duplicates, unique DNA fragments were retained if they mapped to the reference genome within the mappability track used. Such fragments were considered to contain a terminal C to T substitution relative to the human reference genome if a putative cytosine deamination was within the first three or last three bases of the strand. Extr. – extraction; Prep. – preparation; L – length; MQ – Mapping quality; Map35_100% - mappability track from [8]; bp – base pairs; C – Cytosine; T – Thymine; ENC – Extraction negative control; LNC – Library preparation negative control.

Extended Data Figures legends

Extended Data Figure 1. Maximum cortical thickness of femora, tibiae, humeri, radii and ulnae among humans from the Bronze Age and two Neandertals, compared to the minimum thickness of *Denisova 11* (dashed line).

Extended Data Figure 2. Percentage of sites at which *Denisova 11* and genomes simulated under the demographic model detailed in SI 6 carry two Neandertal alleles (NN, blue), two Denisovan alleles (DD, red), or one allele of each type (ND, purple). a. Percentages calculated for two random DNA fragments from *Denisova 11* (leftmost column) and from simulated F1, F2, Neandertal (NF0) or Denisovan (DF0) genomes (columns 2-5). **b**. Proportions of sites for the simulated genotypes, prior to sampling two fragments.

Extended Data Figure 3. Neandertal and Denisovan allele proportions from *Denisova 11* in 1 Mb windows (100 kb step). Y-axis shows –log(p-value) of the deviation of Neandertal and Denisovan allele counts from the genome-wide average (chi-square test of goodness-of-fit; see SI 7); color shows the proportion of alleles matching the Neandertal state (%N) within each 1 Mb window.