

PALEONTOLOGY

A fourth Denisovan individual

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The presence of Neandertals in Europe and Western Eurasia before the arrival of anatomically modern humans is well supported by archaeological and paleontological data. In contrast, fossil evidence for Denisovans, a sister group of Neandertals recently identified on the basis of DNA sequences, is limited to three specimens, all of which originate from Denisova Cave in the Altai Mountains (Siberia, Russia). We report the retrieval of DNA from a deciduous lower second molar (*Denisova 2*), discovered in a deep stratigraphic layer in Denisova Cave, and show that this tooth comes from a female Denisovan individual. On the basis of the number of “missing substitutions” in the mitochondrial DNA determined from the specimen, we find that *Denisova 2* is substantially older than two of the other Denisovans, reinforcing the view that Denisovans were likely to have been present in the vicinity of Denisova Cave over an extended time period. We show that the level of nuclear DNA sequence diversity found among Denisovans is within the lower range of that of present-day human populations.

INTRODUCTION

Genetic analyses of the remains of archaic hominins have yielded insights into their population history and admixture with each other and with modern humans [for example, (1–12)]. DNA retrieved from fossils also allows their attribution to a hominin group in the absence of clear archaeological context or informative morphology [for example, (13–15)]. One example is a hominin phalanx (*Denisova 3*) excavated in Denisova Cave (Altai, Russia) in 2008. Although its mitochondrial DNA (mtDNA) was found to fall outside the range of variation of both present-day humans and Neandertals (16), nuclear sequences retrieved from the specimen showed that it came from a member of a previously unknown sister group of Neandertals, thenceforth named “Denisovans” (2). The population split time between Neandertals and Denisovans has been estimated to be at least 190 thousand years ago (ka) and perhaps as much as 470 ka (7).

While Neandertals inhabited Europe and West Asia, Denisovans, who have been identified only from Denisova Cave to date (2, 3, 16, 17), inhabited Asia (2) where they overlapped geographically with Neandertals in the Altai region and possibly elsewhere. The two groups must have interacted, as analyses of their genomes have shown that Denisovans interbred with Neandertals and with an unknown archaic hominin group that diverged earlier from the human lineage (7). Denisovans, or a group related to them, have also contributed genetically to present-day populations in Southeast Asian islands and Oceania and at lower levels to populations across mainland Asia and the Americas (2, 3, 7, 18–22). Denisovan admixture has contributed to several traits in present-day humans (20, 23, 24), including, for example, the adaptation of Tibetan populations to life at high altitude (25).

In addition to *Denisova 3*, two permanent molars (*Denisova 4* and *Denisova 8*) have been identified as originating from Denisovans on the basis of DNA sequence data (2, 17); and mtDNA fragments of the

Denisovan type were identified in sediments deposited at Denisova Cave (26). Here, we present analyses of DNA sequences retrieved from a tooth (*Denisova 2*) that, on the basis of the stratigraphy of the site, is one of the oldest hominin remains discovered at Denisova Cave (27, 28).

RESULTS

The *Denisova 2* specimen

A worn deciduous molar (figs. S1 and S2) was discovered in 1984 in layer 22.1 of the Main Gallery of Denisova Cave and was initially described as a right lower first deciduous molar (dm_1) (29). However, Shpakova and Derevianko (30) believed that the tooth was more likely a lower second deciduous molar (dm_2), and we concur with their opinion on the basis of the lack of a *tuberculum molare* and the large size. The crown of the tooth is almost completely worn away, and only a thin rim of enamel is preserved buccally, mesially, and lingually. The only feature of crown morphology preserved is a small remnant of the buccal groove. The roots are mostly resorbed, with only short stumps remaining mesiobuccally and mesiolingually. The exposed pulp cavity shows five diverticles entering the crown. The resorption of the roots and the fact that the specimen exfoliated naturally indicate an age equivalent to about 10 to 12 years in modern humans (for details, see section S1). The strong wear makes most morphological comparisons impossible. However, the cervical mesiodistal and buccolingual diameters are very large, falling outside of the range of variation seen in modern humans and in the range of Neandertals (table S1 and fig. S3).

DNA extraction and sequencing

We extracted DNA (31) from ~10 mg of powder removed from the *Denisova 2* specimen (fig. S2). One aliquot of the extract was used to produce a single-stranded DNA library, as previously described (32, 33). Another aliquot was converted into a single-stranded DNA library using a modified version of a protocol that enriches the library for DNA molecules carrying uracil residues (34), which result from deamination of cytosine bases in ancient DNA (35–37). This protocol (“mini-U-selection”) enriches for uracils only at the 3′ ends of fragments, but it requires fewer reaction steps and allows for a simpler library preparation than the original method (34). Out of a total of 701 million and 604 million DNA fragments sequenced from the two libraries, 0.06 and 0.46% of

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all sequences could be mapped to the human genome and exhibited a cytosine (C)-to-thymine (T) substitution at the first or last alignment position (table S2). These substitutions, especially when they occur close to the ends of sequences, are highly indicative of the presence of uracils, which are read as thymines by DNA polymerases (36). The percentage of fragments carrying C-to-T substitutions at the unselected 5' ends was 9.4% in the former library and 7.3% in the library enriched for uracils. These percentages were 11.1 and 53.8% for the 3' ends of fragments, respectively (table S3 and figs. S4 and S5), suggesting that authentic ancient DNA is present in both libraries (section S2).

Mitochondrial DNA

We used oligonucleotide probes matching a modern human mtDNA sequence to enrich for mtDNA fragments from the library that was not selected for uracil residues (4, 38). Initial inspection of the sequences suggested that the library contained a mixture of contaminating present-day human mtDNA and endogenous sequences that are more similar to a Denisovan mtDNA than to modern human or Neandertal mtDNAs (section S3 and table S4). We therefore aligned the sequences from the mitochondrial capture as well as DNA fragments sequenced without enrichment from all libraries to the *Denisova 3* mtDNA genome (16) and identified 86,788 unique mtDNA fragments (table S2).

To mitigate the influence of contamination by present-day human mtDNA (section S2), we filtered the 21,537 fragments (table S2) that carried C-to-T differences relative to the *Denisova 3* mtDNA genome near the start or end position of sequence alignments (first three or last three positions for sequences from the standard library and first two or last two positions for sequences from the mini-U-selection protocol) (39). Using these fragments, we reconstructed the *Denisova 2* mtDNA genome with an average mtDNA coverage of 51-fold (fig. S6). When a given position was required to be covered by at least three fragments and when at least two-thirds of fragments overlapping a position were required to carry an identical base (39), all but 14 positions in the mtDNA genome were resolved (section S3).

A maximum likelihood phylogenetic tree shows that the mtDNA of *Denisova 2* clusters with the three previously determined Denisovan mtDNAs, to the exclusion of Neandertal and modern human mtDNAs (Fig. 1). It carries 29 nucleotide differences from *Denisova 8*, 70 nucleotide differences to *Denisova 4*, and 72 nucleotide differences to *Denisova 3* (table S5).

Relative mtDNA dating

The tree in Fig. 2 shows the relationships of the four currently known Denisovan mtDNAs using the Middle Pleistocene hominin mtDNA from Sima de los Huesos (39) as an outgroup. A total of 22.5, 33.5, 49.5, and 51.5 substitutions are inferred by parsimony (fractions reflect ambiguous character reconstructions) to have accumulated between the common ancestor and the mtDNAs of *Denisova 2*, 8, 4, and 3, respectively. In agreement with previous observations (17), *Denisova 8* appears to be substantially older than *Denisova 3* and 4. The mtDNA of *Denisova 2* is more closely related to that of *Denisova 8* than to the other two mtDNAs. Notably, 20 substitutions are inferred to have accumulated on the terminal edge leading to *Denisova 8*, whereas only 9 substitutions are estimated to have done so on the terminal edge leading to *Denisova 2*, indicating that *Denisova 2* is older than *Denisova 8*.

Using a mutation rate of 2.53×10^{-8} substitutions per site per year (95% highest posterior density, 1.76×10^{-8} to 3.23×10^{-8}) (6), we estimate that the *Denisova 2* individual is between 54.2 and 99.4 thousand years

(ky) older than the *Denisova 3* individual and between 20.6 and 37.7 ky older than the *Denisova 8* individual, whereas the *Denisova 3* and 4 individuals were roughly contemporaneous (between 3.7- and 6.9-ky difference). Although the absolute time estimates are dependent on whether the mutation rate of Denisovan mtDNA differs from that of modern human mtDNA, the difference in the number of substitutions between these individuals indicates that *Denisova 2* is likely to be older than *Denisova 8* and substantially older than *Denisova 3* and 4.

Nuclear DNA and sexing

For nuclear DNA analyses, DNA sequences from the *Denisova 2* specimen were aligned to the human reference genome. We determined the sex of the *Denisova 2* individual by counting the number of putatively deaminated DNA fragments that map to the X chromosome and the autosomes. The ratio of sequence coverage per base between the X chromosome and the autosomes is 1.06, indicating that *Denisova 2* was a female (fig. S7).

To minimize the effect of present-day human contamination (section S2 and table S3), we retained only sequences carrying a C-to-T substitution to the human reference genome at their first or last position (39), leaving 1.08 million DNA sequences (table S2) spanning 47 Mb of the human genome for further analysis.

Attribution to a hominin group

Genetic, archaeological, and anthropological evidence indicate that Denisovans, Neandertals, and anatomically modern humans were present in Denisova Cave (2, 3, 7, 16, 17, 28, 40, 41). We computed the proportion of DNA fragments from the *Denisova 2* specimen that share derived alleles specific to each branch in a phylogenetic tree relating the high-coverage genomes of a Denisovan (3), a Neandertal (7), and a present-day human from Africa (7). A total of 69,315 fragments overlapped phylogenetically informative positions at which a randomly drawn allele from at least one of these high-coverage genomes is derived (12). Of fragments that overlap positions where both the Denisovan and the Neandertal genomes are derived, 84% (1888 of 2246) share the derived allele. Of fragments that overlap positions where only the Denisovan genome is derived, 49% (2051 of 4160) carry the Denisovan-like allele, whereas the corresponding values for the sharing of Neandertal- and modern human-specific alleles are 6% (252 of 4231) and 5% (307 of 5924), respectively (Fig. 3A). We thus conclude that the *Denisova 2* specimen originated from a Denisovan individual.

The corresponding values for two previously sequenced Denisovan teeth, *Denisova 4* and *Denisova 8* (17), are 92 and 93% of sequences sharing derived alleles with the Neandertal-Denisovan branch, 72 and 60% with the Denisovan branch, 5% with the Neandertal branch, and 2% with the modern human branch, respectively (fig. S8). Thus, *Denisova 2* shares fewer derived alleles with the high-coverage *Denisova 3* genome than the other two Denisovan genomes ($\chi^2 = 7.6257$, $P = 0.003$ and $\chi^2 = 43.015$, $P = 2.717 \times 10^{-11}$ for *Denisova 4* and 8, respectively), showing that *Denisova 2* is more distantly related to *Denisova 3* than *Denisova 4* and *Denisova 8*.

Denisovan DNA sequence diversity

To gauge the average sequence divergence between the DNA fragments sequenced from *Denisova 2* and the high-coverage *Denisova 3* genome, we calculated how many of the substitutions on the lineage leading from the human-chimpanzee ancestor to *Denisova 3* have occurred after the split from the *Denisova 2* genome, that is, the fraction

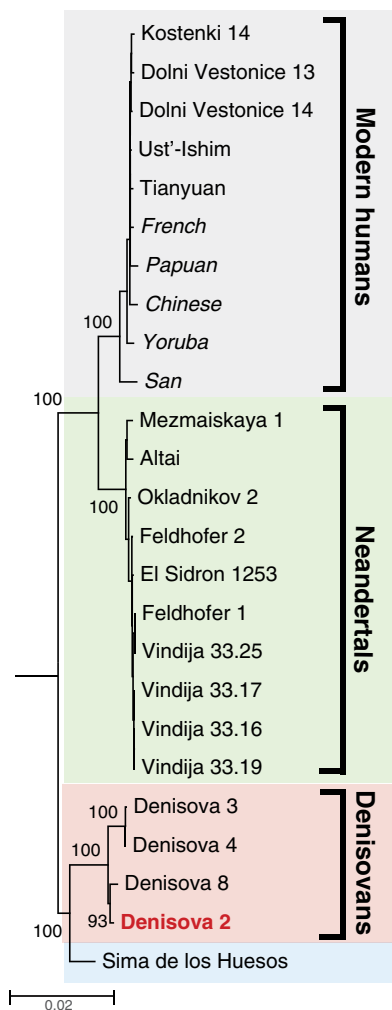


Fig. 1. Maximum likelihood tree relating the *Denisova 2* mtDNA to other ancient and present-day mtDNAs. The *Denisova 2* mtDNA (in red) clusters with the three previously determined Denisovan mtDNAs, to the exclusion of Neandertals and modern humans. Present-day human mtDNA sequences are noted in italics. The tree was rooted using a chimpanzee mtDNA sequence (not shown). Support for each branch is based on 500 bootstrap replications. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Accession codes for the comparative data and the geographical origins of ancient individuals are presented in table S5.

of derived sites that *Denisova 2* does not share with *Denisova 3* (1, 3, 7). This value is 5.9% [95% confidence interval (CI), 5.6 to 6.2%]. In comparison, it is 9.4% (95% CI, 9.0 to 9.9%) for derived alleles not shared with the Neandertal genome and 10.9 to 11.6% for 12 present-day human genomes (Fig. 3B and table S6). The corresponding values for *Denisova 4* and *Denisova 8* relative to the high-coverage *Denisova 3* genome are 4.3% (95% CI, 2.3 to 6.7%) and 4.3% (95% CI, 4.0 to 4.7%), respectively. Excluding two Neandertal specimens yielding very little genetic data (0.1 Mb or less), the lower and upper boundaries of the 95% CIs for the fraction of substitutions occurring on the branch leading to the high-coverage Neandertal genome after the split from four low-coverage Neandertal genomes range between 2.6 and 4.2% (table S6). Comparable estimates calculated among 12 humans from various parts of the world range between 5.1 and 9.2% (table S7). Thus, the estimated sequence diversity among Denisovans,

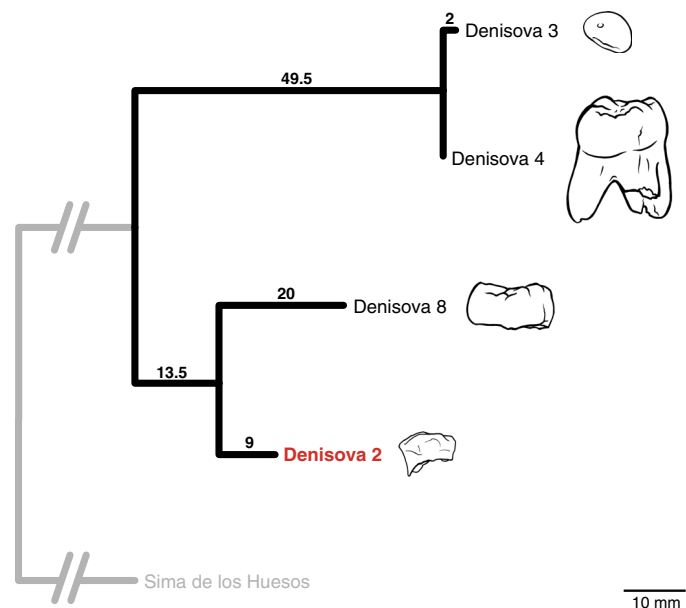


Fig. 2. Phylogenetic tree relating the *Denisova 2* mtDNA to other Denisovan mtDNA sequences. The number of substitutions on each branch was inferred by maximum parsimony, and the Middle Pleistocene mtDNA from Sima de los Huesos was used as an outgroup. The schematic representations of the specimens are drawn to scale, shown in the lower right corner.

which all originate from a single cave, is comparable to that of Neandertals sampled in several locations and within the lower range of the diversity of present-day human populations worldwide.

DISCUSSION

The *Denisova 2* specimen adds a deciduous molar to the meager *Denisova* fossil record, which so far included one distal manual phalanx (*Denisova 3*) (2, 3, 16) and two permanent molars (*Denisova 4* and 8) (2, 17).

The fact that only 47 Mb of nuclear sequences could be retrieved from ~10 mg of tooth powder removed from *Denisova 2* precludes many analyses. For example, subsampling DNA sequences from *Denisova 3* shows that the power to detect gene flow from Denisovans into modern humans is limited (section S4, table S8, and fig. S9). Sequence errors in the low-coverage data also hinder our ability to ask whether the gene flow from an unknown archaic hominin into Denisovans detected in the *Denisova 3* genome (7) also affected the ancestors of *Denisova 2* (section S4 and fig. S10).

Denisova 2 was found in layer 22.1 of the Main Gallery of Denisova Cave, which has been dated to between 128 and 227 ka by radiothermoluminescence (42, 43). *Denisova 3* was found in layer 11.2 of the East Gallery (16). Its age has been estimated to be between 48 and 60 ka on the basis of “missing substitutions” in its nuclear genome relative to present-day humans (7). This is consistent with the direct dating of associated finds, which are beyond the range of radiocarbon dating (2). Using nucleotide substitutions inferred to have occurred in the mtDNAs of *Denisova 2* and *Denisova 3* after their divergence from a common ancestor (Fig. 2), we estimate that the *Denisova 2* individual lived approximately 50,000 to 100,000 years earlier than *Denisova 3*. Even given the uncertainty about the substitution rate in Denisovans, these results

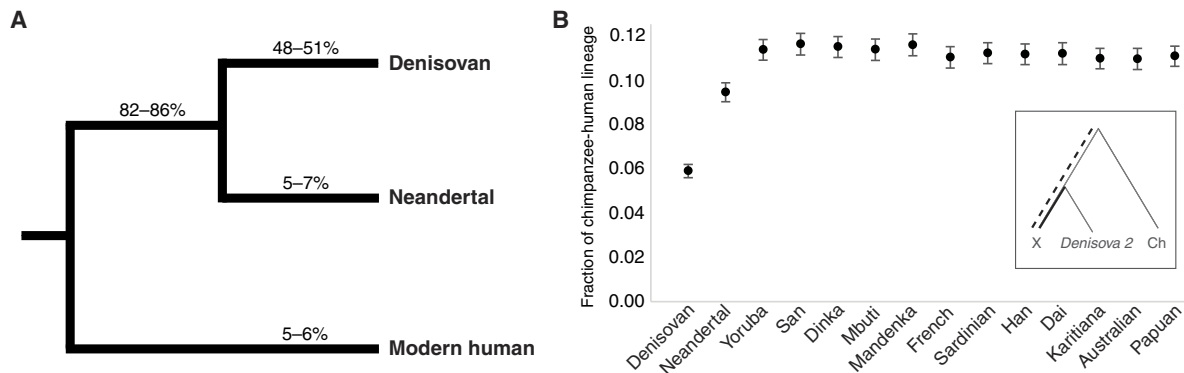


Fig. 3. Attribution of *Denisova 2* to a hominin group. (A) For each branch of a phylogenetic tree relating the high-coverage genomes of a Denisovan, a Neandertal, and a present-day human from Africa, the 95% binomial CIs of the proportion of DNA fragments from the *Denisova 2* specimen that share a derived allele with that branch are given. (B) The fraction of substitutions inferred to have occurred after the split from the *Denisova 2* genome along the branch from the human-chimpanzee (Ch) ancestral sequences to the high-coverage genomes of a Denisovan, a Neandertal, and 12 present-day humans (“X” in the schematic phylogenetic tree shown in the inset) is given. Error bars denote 95% CIs.

suggest that *Denisova 2* lived at least 100 ka, making it one of the oldest hominin remains discovered in Central Asia to date. Beyond reinforcing the idea that both Neandertals and Denisovans lived in the cave or its vicinity (7, 17), our findings indicate that Denisovans were present over an extended period in the Altai region, where the two archaic groups may have met and mixed.

The seemingly great difference in age between the Denisovan individuals is congruent with previous indications that Denisovans inhabited the Altai region over tens of thousands of years (17). Despite the wide age range of Denisovan individuals, their DNA sequence diversity is in the lower range of diversity among contemporaneous humans today. This low estimate of diversity among the Denisovans is consistent with the longtime small population size inferred from the high-coverage *Denisova 3* genome (3). We note that age differences between the high-coverage archaic individuals and present-day modern humans can affect the comparability of our diversity estimates. However, this effect is expected to be minor, given that these branch length differences are far shorter than the common branch leading back to the human-chimpanzee ancestor. Moreover, as all four Denisovan individuals originate from a single location, it is possible that they represent an isolated population and that the genetic diversity of Denisovans across their entire geographical range was greater than that seen in these geographically restricted samples. Additional Denisovans from other locations are needed to more comprehensively gauge their genetic diversity across space and time.

MATERIALS AND METHODS

Morphological analysis

The *Denisova 2* specimen was discovered in the Main Gallery of Denisova Cave (Russia), in sector 4, square B-8, in layer 22.1, the deepest horizon of this gallery. Description of the specimen is based on the original material as well as later analyses of casts and photographs. The specimens used for comparison were as follows: prehistoric and historic Central and Southern European (Natural History Museum Vienna and Department of History and Methods for the Conservation of Cultural Heritage, University of Bologna), prehistoric Siberian (Institute of Archaeology, Siberian Branch, Russian Academy of Sciences, Novosibirsk), historical Khoisan individuals (Department of Anthropology, University of

Vienna), Neandertals (Okladnikov 1; Roc de Marsal 1; Krapina D62, D63, D64, D65, D66, and D68; Scladina; Archi 1; Abri Suard S14-5, S37, and S42; Couvin; Engis 2; and La Ferrassie 8), and Upper Paleolithic modern humans (Strashnaya 1b; Lagar Velho; and Pavlov 7, 8, 9, and 10). Okladnikov 1, Strashnaya 1b, *Denisova 2*, and most of the recent comparative sample were measured using a Paleo-Tech dental caliper, whereas the measurements on the rest of the Neandertal sample as well as the recent Southern European subsample (housed in Bologna) were taken in Avizo 8 (FEI Visualization Sciences Group) on surface models based on micro-computed tomography (μ CT) scans. Cervical measurements of Lagar Velho are from Hillson and Trinkaus (44), and those of Pavlov 7 to 10 are from Sládek *et al.* (45).

DNA extraction and library preparation

Following the removal of surface material, 10.2 mg of powder was sampled from the apical end of a root of *Denisova 2* using a dentistry drill. DNA was extracted using a silica-based protocol (31) modified as in the study by Korlević *et al.* (33) and eluted in 50 μ l of TET [10 mM tris-HCl, 1 mM EDTA, and 0.05% Tween 20 (pH 8.0)]. Fifteen microliters of the DNA extract (E2084) was converted into a DNA library (A4881), as previously described (32), except that the primer extension and blunt-end repair reactions were performed in one step (33). The number of DNA molecules in the library was estimated by quantitative polymerase chain reaction (PCR) (Stratagene MX3005P, Agilent Technologies), as previously described (32). The library was barcoded with two unique indexes (46) using 1 μ M primer concentrations (33) and AccuPrime Pfx DNA polymerase (Life Technologies) (47). Amplification products were purified with the MinElute PCR Purification Kit (Qiagen) and eluted in 30 μ l of TE [10 mM tris-HCl and 1 mM EDTA (pH 8.0)]. After quantification using a NanoDrop ND-1000 (NanoDrop Technologies) photospectrometer, 1 μ g of the amplified library (A4891) was enriched for human mitochondrial sequences, as previously described (4, 38). The captured library (A4928) had a final volume of 20 μ l in EB [10 mM tris-HCl (pH 8.0)].

A 29- μ l aliquot of the same extract was converted into a DNA library enriched for DNA molecules carrying a uracil (U) at their 3' end using a modified version of a published protocol (34). This mini-U-selection protocol was carried out as follows: (i) The single-stranded adapter oligonucleotide (CL78) was decontaminated by treatment with *Escherichia*

coli exonuclease I (33). (ii) Pretreatment by uracil excision and DNA cleavage at abasic sites was carried out using 1 μ l of USER Enzyme mix (1 U/ μ l) (New England BioLabs) (34). (iii) Ligation of the first adapter and immobilization on beads were performed as described in steps 3 to 11 of the protocol by Gansauge and Meyer (32). (iv) Primer extension and blunt-end repair were carried out in one reaction (33). (v) Ligation of the second adapter was done as in steps 20 to 23 in the study by Gansauge and Meyer (32). (vi) Uracil excision, which releases library molecules with uracils close to the 3' end of DNA fragments, was performed by incubating the beads in an excision reaction mix of 1 μ l of USER Enzyme mix (1 U/ μ l) (New England BioLabs) and 49 μ l of EBT [10 mM tris-HCl and 0.05% Tween 20 (pH 8.0)] for 30 min at 37°C. (vii) Recovery of the residual library molecules was carried out as in the study by Gansauge and Meyer (34). The number of DNA molecules in the two library fractions was assessed by digital droplet PCR (Bio-Rad QX200) using an EvaGreen (Bio-Rad) assay with primers IS7 and IS8 (48, 49). The uracil-enriched library was split into four aliquots (A4944 to A4947), each of which was barcoded with a unique combination of two indexes, amplified and purified as above.

Before sequencing, 2 μ l of each library was amplified in one PCR cycle using Herculase II Fusion DNA polymerase (Agilent) (47) with primers IS5 and IS6 (48) to remove heteroduplicates. The libraries were purified using the MinElute PCR Purification Kit (Qiagen), and their concentration was assessed using a DNA 1000 chip (Bioanalyzer 2100, Agilent) (3). A DNA extraction blank and one library preparation blank were carried along each experiment.

Sequencing and processing of sequence data

Shotgun sequencing of library A4891 was first performed on 12% of an Illumina HiSeq lane and then sequenced deeper on four HiSeq lanes. Libraries A4944 to A4947 were sequenced on 90% of a MiSeq (Illumina) platform and then sequenced together on four HiSeq lanes. The captured library A4928 was sequenced on 12% of a MiSeq lane. Sequencing was performed using paired-end runs with double-index configuration (46).

Base calling was carried out using freeIbis (50) and Bustard (Illumina) for HiSeq and MiSeq runs, respectively. Adapter sequences were removed and overlapping forward and reverse reads were merged using leeHom (51). Sequences that did not perfectly match the expected barcode combinations were discarded. For each library, sequences originating from different sequencing runs were combined using SAMtools (52).

Mapping to a reference genome was performed using Burrows-Wheeler Aligner (BWA) (53), with parameters “-n 0.01 -o 2 -l 16500” (3). To recover nuclear DNA fragments, sequences originating from shotgun sequencing were aligned to the human reference genome [UCSC (University of California, Santa Cruz) version hg19]. For mtDNA analyses, sequences from all libraries were aligned to the Denisovan mitochondrial reference sequence [National Center for Biotechnology Information (NCBI) reference NC_013993.1] (16). Because BWA cannot use circularized references, the mitochondrial reference sequence was altered by copying the first 1000 bases to its end (39). After mapping, PCR duplicates were removed by collapsing sequences starting and ending at identical coordinates using bam-rmdup (<https://bitbucket.org/ustenzel/biohazard>).

Analyses of mtDNA

Filtering

Analyses of the mtDNA data were restricted to sequences between 30 and 75 base pairs (bp) long, which present C-to-T substitutions to the

reference genome adjacent to their ends (39). Sequences from single-stranded DNA libraries (A4891 and A4928) were retained if they carried C-to-T substitutions at the first three or last three terminal positions, whereas for the libraries enriched for uracil-containing DNA fragments (A4944 to A4947), sequences with apparent C-to-T substitutions at their first two or last two positions were retained. Thymines at the abovementioned positions where the reference base is a cytosine were converted to N's.

Reconstructing the Denisova 2 mtDNA sequence

The mtDNA of *Denisova 2* was called by a majority vote using the “mpileup” command of SAMtools (52). A base was called only if a position was covered by at least three fragments, of which at least two-thirds carried an identical base (39).

Phylogenetic analysis

The *Denisova 2* mtDNA was aligned to the mtDNAs of 3 Denisovans (2, 16, 17), 5 present-day humans from a variety of geographical origins (54), 5 ancient modern humans (4, 6, 55, 56), 10 Neandertals (7, 34, 57–59), 1 Middle Pleistocene hominin (39), and 1 chimpanzee (*Pan troglodytes*, NC_001643) (60) using MAFFT (61). NCBI accession codes for the comparative sequence data and the geographical origin of the individuals are presented in table S5. A maximum likelihood phylogenetic tree with 500 bootstrap replications (62) was reconstructed in MEGA6 (63) using the Hasegawa-Kishino-Yano substitution model (64) with gamma distribution and allowing for invariable sites (HKY + G + I), as determined by jModelTest2 (65, 66). The numbers of pairwise base differences observed between mtDNAs were determined using MEGA6 (63).

Molecular dating

The *Denisova 2* mitochondrial genome was aligned to the other three Denisovan mtDNA sequences (2, 16, 17) and to the mtDNA of a Middle Pleistocene hominin from Sima de los Huesos (Spain) (39) using MAFFT (61). The number of substitutions occurring on each branch since the split from the most recent common ancestor of all Denisovan mtDNAs was inferred by maximum parsimony in the R package “phangorn,” using an accelerated transformation (“ACCTRAN”) algorithm to optimize ambiguous character reconstructions (67). The difference in the number of mutations inferred to have occurred on each branch was translated to differences in time based on a mitochondrial mutation rate for the modern human lineage of 2.53×10^{-8} substitutions per site per year (95% highest posterior density, 1.76×10^{-8} to 3.23×10^{-8}) (6).

Analyses of nuclear DNA

Filtering

Given the low amount of nuclear DNA retrieved from *Denisova 2*, nuclear DNA fragments were filtered using a scheme stricter than the one used for mtDNA analyses. Fragments shorter than 35 bp or with a mapping quality lower than 30 (Phred scale) were discarded, and an alignability track excluding nonunique sequences was applied (7). Because present-day contaminating DNA sequences may be more prevalent among longer DNA fragments (55), fragments longer than 75 bp were discarded (fig. S4). Nuclear analyses were restricted to sequences carrying a C-to-T substitution relative to the reference genome at one of their ends (39), as these nucleotide differences are the result of damage occurring over time and are thus indicative of ancient DNA (36, 55, 68). The base quality of terminal thymines was reduced to 2 regardless of the base in the reference genome (7), and subsequent processing was limited to bases with a quality higher than 30. Comparative data from previously sequenced low-coverage Denisovan (17) and Neandertal (1, 7) genomes were processed as above, except that the Neandertal

sequences were filtered for the presence of guanine (G)–to–adenine (A) substitutions at the 3' ends of fragments since the data were generated using a double-stranded DNA library preparation protocol (48).

Lineage attribution

To attribute the *Denisova 2* specimen to a hominin group, the state of sequences overlapping sites at which the genomes of a Denisovan (3), a Neandertal (7), and a present-day modern human from Africa (HGDP00982) (7) differ from those of other great apes (chimpanzee, bonobo, gorilla, and orangutan) and the rhesus macaque was investigated. The proportion of sequences that share the derived state with each of the hominin branches was calculated with 95% binomial CIs (12). A one-sided two-sample proportions test implemented in R (69) was used to test for significant differences in allele sharing with the high-coverage Denisovan genome among the three low-coverage Denisovans.

Relative DNA sequence diversity

We estimated the divergence of the DNA fragments sequenced from *Denisova 2* along the lineage leading from the ancestor shared with the chimpanzee and the high-coverage genomes of a Denisovan (3), a Neandertal (7), or a modern human [panel B from the study by Prüfer *et al.* (7)]. We calculated how many of the substitutions inferred to have occurred from the human–chimpanzee ancestral sequences to the high-coverage genomes occurred after the split from the *Denisova 2* genome, that is, the fraction of derived sites that *Denisova 2* does not share with each high-coverage genome (1, 7). CIs were calculated by jackknife resampling in 5-Mb windows along the high-coverage genomes. For the high-coverage genomes, the homozygous genotype was selected at homozygous sites, and a random allele was selected at heterozygous sites, whereas for the *Denisova 2* data, a random allele was selected in the rare cases when more than one DNA fragment covered a site.

For comparative purposes, we repeated this calculation while replacing the *Denisova 2* data with low-coverage sequence data from two other Denisovans (*Denisova 4* and *Denisova 8*) (17) and six Neandertals (*Feldhofer 1*, *Mezmaiskaya 1*, *El Sidron 1253*, *Vindija 33.16*, *Vindija 33.25*, and *Vindija 33.26*) (1, 7). We reprocessed these sequences using the same filtering scheme that we applied to the *Denisova 2* data. For data generated with the single-stranded DNA library preparation protocol (*Denisova 4* and 8), a terminal C-to-T substitution to the reference genome at either end was required. For data generated with a double-stranded DNA library preparation scheme (*Feldhofer 1*, *Mezmaiskaya 1*, *El Sidron 1253*, *Vindija 33.16*, *Vindija 33.25*, and *Vindija 33.26*), putative deamination was identified as a C-to-T substitution at the 5' end of fragments or a G-to-A substitution at their 3' end (32, 36, 48). The base quality of terminal thymines or adenines was reduced to 2, and subsequent processing was limited to bases with a quality higher than 30.

To compute estimates of diversity among present-day humans that would be comparable to our estimates for the archaic genomes, we subsampled each of the 12 present-day high-coverage human genomes (7) to retain only positions covered by at least 1 of the low-coverage archaic genomes in our data set. The percentage of inferred substitutions occurring on the lineage to a high-coverage genome that are not shared with the subsampled DNA sequences was computed as described above.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at <http://advances.sciencemag.org/cgi/content/full/3/7/e1700186/DC1>

section S1. The *Denisova 2* specimen

section S2. Authenticating the DNA sequences

section S3. The mtDNA sequence of *Denisova 2*

section S4. Nuclear DNA sequences from *Denisova 2*

fig. S1. The *Denisova 2* specimen.

fig. S2. The *Denisova 2* specimen.

fig. S3. Biplot of cervical buccolingual (BL) and mesiodistal (MD) diameters.

fig. S4. Characteristic of DNA fragments from the two libraries prepared from the *Denisova 2* specimen, per size bin.

fig. S5. Frequency of nucleotide substitutions in the *Denisova 2* sequences.

fig. S6. Reconstructing the *Denisova 2* mitochondrial genome.

fig. S7. Sex determination of *Denisova 2*.

fig. S8. Lineage attribution of two other low-coverage Denisovan individuals.

fig. S9. Testing our power to detect the sharing of derived alleles between Denisovans and present-day humans.

fig. S10. Ancestral allele counts in Denisovan and Neandertal sequences at sites that are fixed or nearly fixed for a derived allele in present-day humans.

table S1. Metric comparisons of cervical and maximum mesiodistal and buccolingual diameters of *Denisova 2*, Neandertals, and recent and Upper Paleolithic modern humans.

table S2. Characteristics of the DNA libraries prepared from *Denisova 2*.

table S3. Frequencies of terminal C-to-T substitutions to the human reference genome.

table S4. Percentage and number of sequences matching the derived state of mitochondrial genomes from three hominin groups.

table S5. Number of base differences between the *Denisova 2* mtDNA and other mtDNAs.

table S6. Estimates of DNA divergence between low-coverage archaic genomes and the high-coverage genomes of a Denisovan, a Neandertal, and 12 present-day humans.

table S7. Estimates of DNA divergence between subsampled genomes and the high-coverage genomes of a Denisovan, a Neandertal, and 12 present-day humans.

table S8. Sharing of derived alleles between *Denisova 2* and present-day humans.

table S9. *Denisova*-specific nonsynonymous coding changes corroborated by sequences from *Denisova 2*, 4, or 8.

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A fourth Denisovan individual

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