RESEARCH ARTICLE SUMMARY

PALEOGENOMICS

Unearthing Neanderthal population history using nuclear and mitochondrial DNA from cave sediments

Benjamin Vernot*, Elena I. Zavala, Asier Gómez-Olivencia, Zenobia Jacobs, Viviane Slon, Fabrizio Mafessoni, Frédéric Romagné, Alice Pearson, Martin Petr, Nohemi Sala, Adrián Pablos, Arantza Aranburu, José María Bermúdez de Castro, Eudald Carbonell, Bo Li, Maciej T. Krajcarz, Andrey I. Krivoshapkin, Kseniya A. Kolobova, Maxim B. Kozlikin, Michael V. Shunkov, Anatoly P. Derevianko, Bence Viola, Steffi Grote, Elena Essel, David López Herráez, Sarah Nagel, Birgit Nickel, Julia Richter, Anna Schmidt, Benjamin Peter, Janet Kelso, Richard G. Roberts, Juan-Luis Arsuaga, Matthias Meyer*

INTRODUCTION: The study of hominin history has progressed through both archaeological and genetic insights. Although DNA sequencing from hominin skeletal remains allows the association of ancient populations with specific places in time and space, many archaeological sites lack associated hominin remains, limiting the scope of genetic analyses. Even when ancient hominin remains are found, they often do not cover the full time span of a site or sampling them for DNA may not be possible. The fossil record is particularly sparse for Pleistocene hominins, leaving large gaps in our understanding of the genetic histories of archaic and early modern humans.

RATIONALE: Recent work has demonstrated the feasibility of sequencing ancient mammalian mitochondrial DNA (mtDNA), including that of hominins, from Pleistocene cave sediments. However, mtDNA represents only the maternal lineage and thus provides limited data for the resolution of population relationships. It is therefore desirable to complement mtDNA analysis with the retrieval of nuclear DNA, but no strategies are in place to enrich hominin nuclear DNA from a background of related sequences from other mammals present in most sedimentary deposits. To close this gap, we developed a set of probes for hybridization capture that targets 1.6 million ancestry-informative positions in the hominin nuclear genome, specifically at loci with high mammalian sequence divergence. We then developed computational methods to deplete residual microbial and faunal DNA sequences, along with methods to account for such non-hominin DNA in population genetic analyses.

RESULTS: We applied these methods to explore the history of Neanderthal populations in western Europe and southern Siberia using sediment samples from three Pleistocene caves: Galería de las Estatuas, a site in northern Spain with 40 thousand years of Neanderthal occupation but that is genetically unexplored, and Chagyrskaya and Denisova Caves, which have previously yielded high-coverage genomes of two Neanderthals and one Denisovan hominin. In total, we recovered Neanderthal or Denisovan mtDNA from >60 sediment samples and nuclear DNA from 30 of these. For Chagyrskaya and Denisova Caves, our phylogenetic results from sediment DNA were consistent with previously published results from skeletal remains, confirming the accuracy of our approach. At Galería de las Estatuas, we recovered Neanderthal DNA from layers spanning nearly the entire stratigraphy, and identified a population turnover ~100,000 years ago accompanied by a loss of mtDNA diversity. By incorporating genetic data from previously published skeletal samples, we associated this turnover with two putative radiations in Neanderthal history.

CONCLUSION: We developed methods for the effective retrieval and analysis of ancient hominin nuclear DNA from sediments and used them to uncover previously unknown events in Neanderthal history. This work demonstrates that detailed genetic analyses are now possible for many more archaeological sites than previously thought, with DNA from abundant sediments allowing dense time-series studies that are independent of the fossil record. ■

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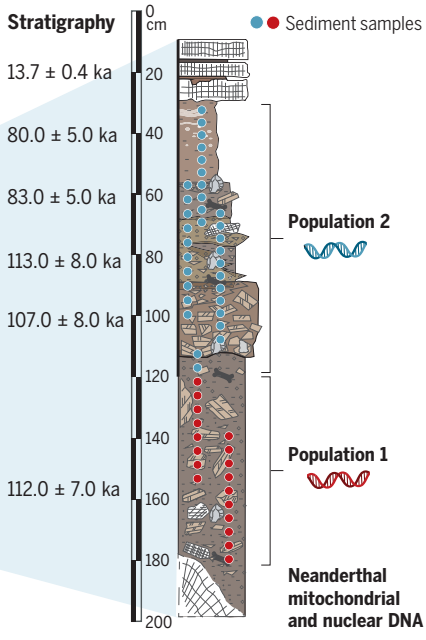

Sediments from Pleistocene caves contain hominin mitochondrial and nuclear DNA that can be enriched, sequenced, and analyzed to reveal the genetic histories of past occupants even in the absence of their skeletal remains. Shown is a view of pit I at the Galería de las Estatuas, Spain, and stratigraphic column with ages in thousands of years (ka).

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RESEARCH ARTICLE

PALEOGENOMICS

Unearthing Neanderthal population history using nuclear and mitochondrial DNA from cave sediments

Benjamin Vernot[1]*, Elena I. Zavala[1], Asier Gómez-Olivencia[2,3,4], Zenobia Jacobs[5,6], Viviane Slon[1,7,8], Fabrizio Mafessoni[1], Frédéric Romagné[1], Alice Pearson[1], Martin Petr[1], Nohemi Sala[4,9], Adrián Pablos[4,9], Arantza Aranburu[2,3], José María Bermúdez de Castro[9], Eudald Carbonell[10,11], Bo Li[5,6], Maciej T. Krajcarz[12], Andrey I. Krivoshapkin[13,14], Kseniya A. Kolobova[13], Maxim B. Kozlikin[13], Michael V. Shunkov[13], Anatoly P. Derevianko[13], Bence Viola[15], Steffi Grote[1], Elena Essel[1], David López Herráez[1], Sarah Nagel[1], Birgit Nickel[1], Julia Richter[1], Anna Schmidt[1], Benjamin Peter[1], Janet Kelso[1], Richard G. Roberts[5,6], Juan-Luis Arsuaga[4,16], Matthias Meyer[1]*

Bones and teeth are important sources of Pleistocene hominin DNA, but are rarely recovered at archaeological sites. Mitochondrial DNA (mtDNA) has been retrieved from cave sediments but provides limited value for studying population relationships. We therefore developed methods for the enrichment and analysis of nuclear DNA from sediments and applied them to cave deposits in western Europe and southern Siberia dated to between 200,000 and 50,000 years ago. We detected a population replacement in northern Spain about 100,000 years ago, which was accompanied by a turnover of mtDNA. We also identified two radiation events in Neanderthal history during the early part of the Late Pleistocene. Our work lays the ground for studying the population history of ancient hominins from trace amounts of nuclear DNA in sediments.

The analysis of ancient DNA from Pleistocene hominins has greatly enhanced our understanding of the evolutionary history of archaic humans and their interactions with early modern humans. To date, complete or partial nuclear genome sequences have been recovered from the skeletal remains of 23 archaic hominin individuals: 18 Neanderthals from 14 sites across Eurasia (mostly in Europe), four Denisovans, and the offspring of a Neanderthal mother and a Denisovan father (*Denisova 11*) (*1*) recovered from Denisova Cave in the Altai Mountains of southern Siberia. Although numerous Paleolithic sites have been excavated, relatively few have yielded skeletal remains of hominins, which are often concentrated in one or a few strata. Attempts to reconstruct the genetic history of archaic hominins are therefore constrained by an uneven temporal and spatial sampling, limited largely by the availability of specimens.

In 2017, it was found that hominin mitochondrial DNA (mtDNA) can be recovered from Pleistocene sediments (*2*), indicating that it may be possible to overcome the dependency on the scarce fossil record in the quest for hominin DNA. However, mtDNA only carries information about the maternal lineage and does not always reflect the complete population history [e.g., (*3*)]. Nuclear DNA contains far more information, but its retrieval from sediments presents substantial challenges: It is present in fewer copies than mtDNA, and many loci are not informative for population genetic analyses. Additionally, by far most mammalian DNA in sediments is non-hominin, which may be difficult to distinguish from hominin DNA because of sequence homology at many loci. These characteristics, as well as the dominance of microbial DNA (*2*), hamper attempts to retrieve nuclear sequences in sufficient number and quality for population genetic analyses by simple shotgun sequencing. To overcome these challenges, we set out to retrieve hominin nuclear genomic sequences from sediments using hybridization capture to target regions in the nuclear genome with high mammalian sequence diversity, and used these sequences to explore the history of Neanderthal populations in western Europe and southern Siberia.

Archaeological sites

We focused our analyses on sediments from three Paleolithic sites. Denisova Cave (*4*) and Chagyrskaya Cave (*5*), both located in the Altai Mountains (Fig. 1A), were included for their known mtDNA preservation in sediments (*2*) and to enable comparisons with three high-coverage nuclear genomes generated previously from bones from these sites: *Denisova 5* [the *Altai Neanderthal* toe bone, dated to 90.9 to 130.0 thousand years (ka) ago (*6*, *7*)], *Denisova 3* [a Denisovan finger bone, 51.6 to 76.2 ka ago (*6*, *8*)], and *Chagyrskaya 8* [a Neanderthal finger bone, 49.0 to 92 ka ago (*5*, *9*)]. All age ranges include the 95% confidence interval (CI) of the dating method(s). Whereas Denisova Cave has evidence for at least 250 millennia of archaic human occupation (*4*), the Neanderthal-bearing deposits at Chagyrskaya Cave (layers 5 and 6; Fig. 1B and fig. S1) accumulated in <10 millennia (*5*).

The third site, Galería de las Estatuas (hereafter "Estatuas"), is part of the Atapuerca archaeo-paleontological complex in northern Spain (Fig. 1C and fig. S2). Almost 500 stone artifacts with clear Mousterian affinities, combined with single-grain optical dating of the associated sediments, indicate Neanderthal occupation from at least 113 ± 8 to 70 ± 5 ka ago [total uncertainty at 1σ; (*10*, *11*)], yet only a single Neanderthal foot phalanx has been recovered (*12*) (fig. S3). Initial screening of the Estatuas sediments indicated the presence of ancient mammalian mtDNA, including that of hominins [figs. S4 to S6 and tables S10 and S11 (*13*)]. Analysis of sediment DNA may therefore be the only viable approach for reconstructing the population genetics of the occupants of this site during a time period not currently well represented in the genetic record of European Neanderthals.

For Denisova Cave, we retrieved nuclear DNA from three existing sediment samples with hominin mtDNA preservation, from: layers 11.4 and 15 in the East Chamber and layer 14.3 in the Main Chamber (*2*, *4*). At Chagyrskaya Cave and Estatuas, we extensively sampled across the Paleolithic layers, collecting 76 samples from two pits at Estatuas and 73 samples from Chagyrskaya Cave [Fig. 1, B and C, and figs. S4 and S5 (*13*)], and targeted both hominin mtDNA and nuclear DNA.

Retrieval of hominin mtDNA

We enriched hominin mtDNA from Chagyrskaya Cave and Estatuas samples using protocols for automated DNA extraction (*14*), library preparation (*15*), and hybridization capture

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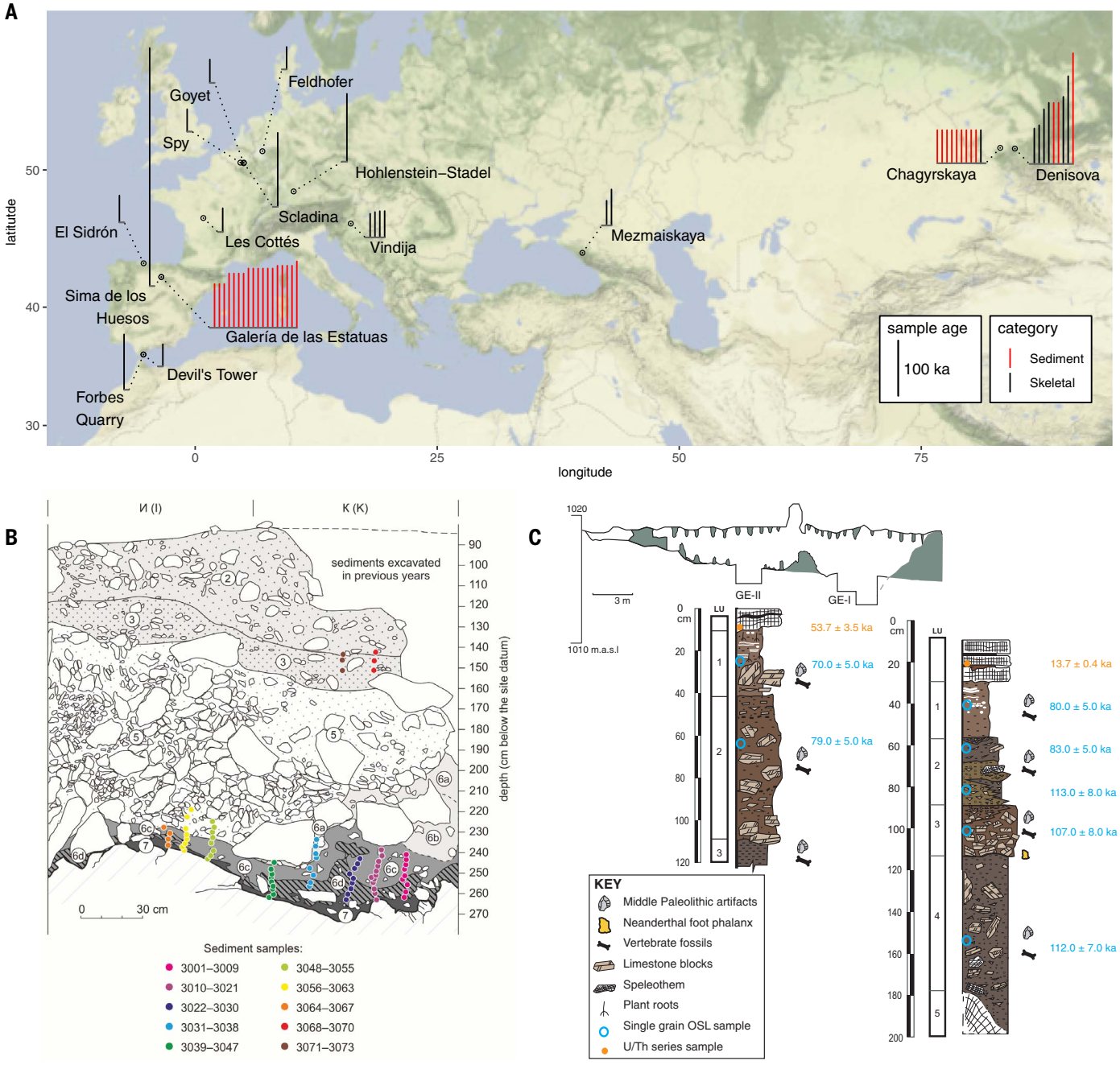

Fig. 1. Samples and stratigraphies. (**A**) Geographic locations of all skeletal (black) and sediment (red) samples with >0.0001× coverage. Each bar represents one sample; height is the age of the sample (ka, thousands of years). (**B**) Stratigraphic profile of the sampled section at Chagyrskaya Cave. Sediment samples were collected in 10 vertical columns and numbered from bottom to top. Colored circles denote individual sample locations. (**C**) Cross section of Estatuas showing the locations of the two test pits, GE-I and GE-II, and detailed stratigraphic columns showing the lithostratigraphic units (LUs) and ages obtained.

(*2*). To maximize the number of libraries containing sufficient amounts of hominin DNA for analysis, multiple subsamples were taken from some of the Estatuas samples and several libraries produced from some subsamples, for a total of 369 libraries. After assigning sequences to mammalian families using MEGAN (*16*), we found that 74% of samples (n = 54) from Chagyrskaya and 56% (n = 43) from Estatuas yielded at least one library containing hominin mtDNA fragments with significantly elevated frequencies of cytosine (C) to thymine (T) substitutions at their 5′ and 3′ ends, compatible with the presence of deaminated ancient DNA [binomial CI ≥ 10% at both ends; table S10 (*13*)]. Of the 223 libraries containing ancient hominin mtDNA, 182 (82%) yielded sufficient fragments to allow their assignment to a hominin group based on "diagnostic" positions in the mtDNA genome that are derived in one hominin group (modern humans, Neanderthals, Denisovans, or the Sima de los Huesos hominins) and ancestral in the others (*3*, *13*). All such assignments were to Neanderthal mtDNA, consistent with archaeological evidence for the presence of Neanderthals in all layers, with exception of the upper portions of layer 7 in Chagyrskaya Cave, which is archaeologically sterile and dated to >300 ka ago. The detection of Neanderthal

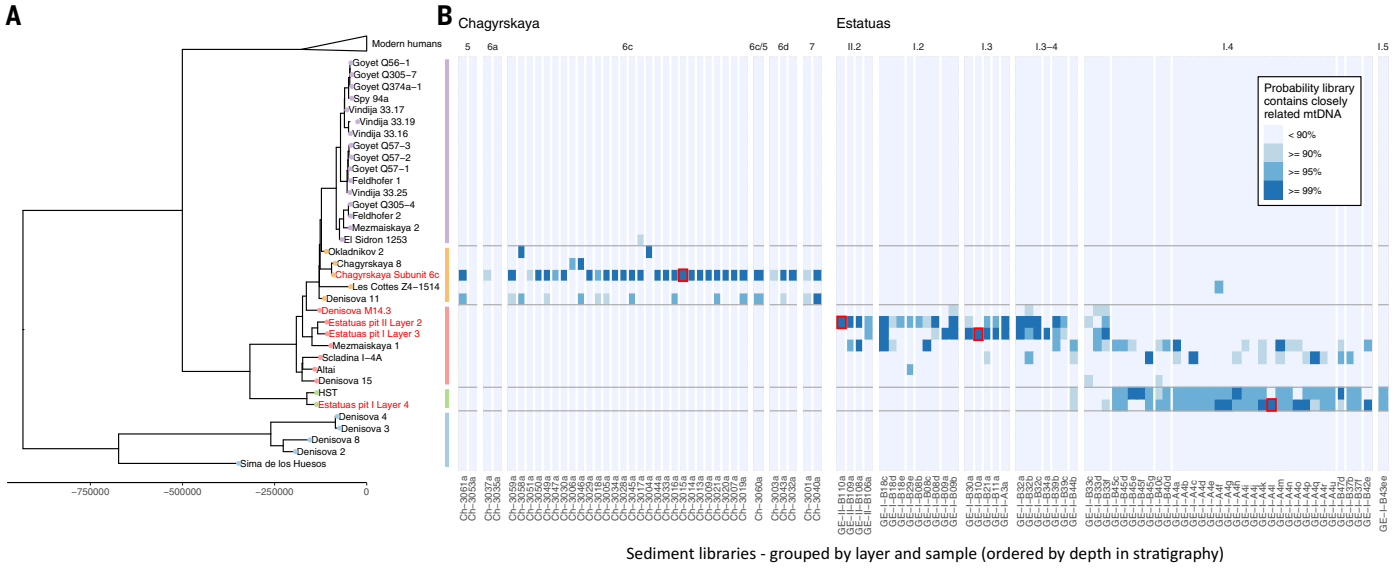

Fig. 2. mtDNA from sediments. (**A**) Mitochondrial phylogeny, including five haplotypes inferred from sediment samples (red labels). Five mtDNA groupings are labeled (colored dots and vertical bars). (**B**) Probabilistic phylogenetic placement of mtDNA from 97 sediment subsamples from 63 sediment samples from Chagyrskaya Cave (left) and Estatuas (right). Samples are divided by white lines. Subsamples from which mtDNA haplotypes were inferred are denoted with red boxes.

mtDNA near the top of layer 7 may be due to its being a former living floor and/or a consequence of post-depositional mixing with sediments from the overlying subunit 6c, resulting in subunit 6d (*5*, *13*).

Fourteen samples produced libraries with high coverage of the Neanderthal mtDNA genome (>17-fold) and point estimates of present-day human contamination <10% (*13*). Four of these (from Chagyrskaya subunit 6c, Estatuas pit II/layer 2, and Estatuas pit I/layers 3 and 4) appeared to contain a single mitochondrial sequence on the basis of the consistency of nucleotides observed at each position [fig. S7 and table S3 (*13*)]. These were used for generating near-complete consensus sequences and building a phylogenetic tree with BEAST2 (*17*), along with previously published hominin mtDNA sequences derived from skeletal remains or from present-day humans and a sequence reconstructed from the layer 14.3 sediment sample from the Main Chamber in Denisova Cave (*2*) [Fig. 2A, fig. S8, and table S4 (*13*)].

Most notably, we found that the consensus mtDNA genome from Estatuas pit I/layer 4 was most similar to the mtDNA of the ~120 ka old Neanderthal from Hohlenstein-Stadel (*HST*), Germany, which falls basal to all other known Neanderthal mitochondrial genomes (*18*, *19*). The Estatuas pit I/layer 4 sediments (dated to 112 ± 7 ka ago) are broadly contemporaneous with *HST*. The mtDNA genomes from Estatuas pit II/layer 2 (79 ± 5 ka ago) and pit I/layer 3 (107 ± 8 ka ago) group with mtDNA from the 60 to 70 ka old *Mezmaiskaya 1* individual from the northern Caucasus (*7*, *20*), whereas the sequence for Chagyrskaya subunit 6c groups with the mtDNA from *Chagyrskaya 8* (*9*). We note that ages estimated from the branch lengths of the sediment mtDNA sequences in the tree, such as 136 ka ago (95% CI: 75 to 200 ka ago) for the Estatuas pit I/layer 4 sample, match previously published ages for the respective sites and layers [table S5 (*13*)].

To further investigate mitochondrial diversity in the sediments from Chagyrskaya Cave and Estatuas, we developed a method to probabilistically place libraries with as few as 250 ancient fragments in the known Neanderthal mtDNA diversity [figs S9 to S12 (*13*)]. This method allowed phylogenetic assignments of mtDNA from 38 libraries from Chagyrskaya Cave and 59 from Estatuas, spanning most layers sampled in each cave (Fig. 2B). We found that Estatuas pit I/layers 4 and 5 contain both *HST*-like and non–*HST*-like Neanderthal mtDNA, often in the same subsample, with the latter largely grouping with *Mezmaiskaya 1* and *Scladina I-4a*, an ~130 ka old Neanderthal from western Europe (*19*, *21*). *HST*-like mtDNA then disappears from the upper layers of Estatuas, leaving mtDNA predominantly related to the *Mezmaiskaya 1*–like consensus sequences from those layers (Fig. 2B). Simulated mixing of DNA from the upper layers and pit I/layer 4 does not generate the observation of *Mezmaiskaya 1*– and *Scladina I-4a*–like DNA in pit I/layer 4, indicating true heterogeneity of mtDNA in the lower layers, consistent with the previously observed integrity of the Estatuas stratigraphy [fig. S13 (*11*, *13*)]. In Chagyrskaya Cave, we found remarkable homogeneity: All samples from layers 5 to 7 grouped with the subunit 6c consensus sequence, *Chagyrskaya 8* or *Okladnikov 2* (also from the Altai region) (*22*, *23*), with occasional support for *Denisova 11*–like sequences.

Nuclear DNA enrichment method

To extend the study of hominin DNA from sediments to the nuclear genome, we designed a probe set targeting 1.6 million informative single nucleotide polymorphisms (SNPs) in the nuclear genome and enriched for DNA fragments overlapping these sites through hybridization capture (*2*, *13*). We used several techniques to both reduce and measure the extent of mismapping of non-hominin faunal DNA, and evaluated these measures using simulated ancient brown bear (*Ursus arctos*) DNA, with fragment sizes and deamination profiles taken from an ancient DNA library (*13*). We found that faunal misalignment was substantially reduced in regions of high mammalian diversity (e.g., Fig. 3, A versus B), by at least 48-fold where the human genome differed by eight or more base pairs from at least one non-primate genome (in a 52–base pair region centered on a target SNP; Fig. 3C). We therefore restricted our design to SNPs in these regions. We additionally assigned each DNA fragment to National Center for Biotechnology Information (NCBI) taxonomy using the metagenomics software Kraken (*24*) and restricted our analyses to fragments classified as primate (*13*). This metagenomic filtering step substantially enriches for hominin DNA, reducing the alignment of simulated ancient bear DNA to the human reference genome by a factor of 140, versus 1.8-fold for Neanderthal DNA (*13*). However, these approaches may not eliminate all faunal misalignment, particularly if the DNA originates from a species that is

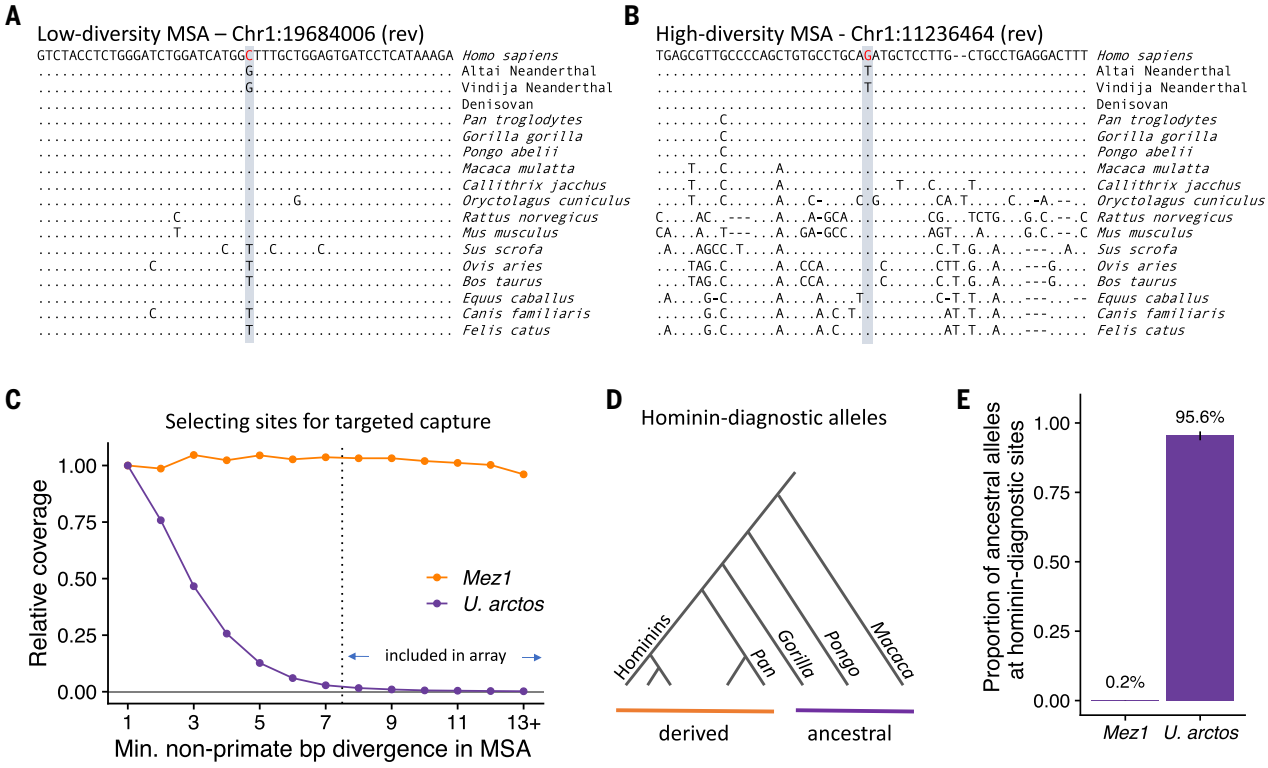

Fig. 3. Nuclear DNA capture design. (**A**) Multiple sequence alignment (MSA) of 15 mammalian species, plus the sequences of two Neanderthals, and one Denisovan. This region has low sequence diversity between hominins and non-primate mammals. (**B**) High-diversity MSA. (**C**) Faunal misalignment (purple) in the human genome as a function of mammalian diversity (the minimum base pair divergence between *Homo sapiens* and nine non-primate mammalian sequences) compared with the alignment of DNA fragments from the *Mezmaiskaya 1* Neanderthal (orange; *Mez1*). The *y*-axis is relative coverage compared with the first bin. (**D**) Ascertainment of hominin-diagnostic alleles; ancestral alleles are indicative of faunal misalignment. (**E**) Proportion of ancestral alleles at hominin-diagnostic sites in *Mezmaiskaya 1* and simulated *U. arctos* ancient DNA.

not well represented in the NCBI taxonomy. Therefore, we included in our probe design 98,887 "hominin diagnostic" sites that are fixed derived in hominins, chimpanzees, and bonobos (Fig. 3D). At these sites, ancestral alleles in the captured fragments are strongly indicative of the presence of non-hominin mammalian DNA [0.2% ancestral in Neanderthal compared with 95.6% in *U. arctos* DNA; Fig. 3E (*13*)] and can be used to estimate faunal misalignment proportions.

Nuclear DNA recovery and sexing

We first applied these methods to previously prepared libraries from three Denisova Cave sediments, two with Neanderthal mtDNA and one with Denisovan mtDNA (*2*). All showed significant levels of C-to-T substitutions in DNA fragments overlapping our targeted SNPs, consistent with the presence of ancient nuclear DNA [fig. S14 and table S13 (*13*)]. One sample showed evidence of moderate (~15%) non-hominin faunal misalignment before metagenomic filtering, but filtering reduced this to <1% (table S13). After filtering, 1764, 27,923, and 162,508 DNA fragments (459, 8698, and 42,103 with evidence of deamination, respectively) were retained from the three samples, representing up to ~0.1× coverage of our targeted sites.

Having confirmed the presence of ancient hominin nuclear DNA, we next investigated whether we could assign each sample to a hominin group. By examining deaminated DNA fragments at sites where the high-coverage Denisovan and *Altai Neanderthal* genomes are homozygous and differ from each other, we found that in the two samples containing Neanderthal mtDNA, ~90% of DNA fragments carried the Neanderthal derived state, versus 2% carrying the Denisovan derived state (Fig. 4A, top left, red points). By contrast, the nuclear DNA in the sample containing Denisovan mtDNA carried the Denisovan derived allele in 65% of cases but no Neanderthal derived alleles (Fig. 4A, top left, blue point). These results are consistent with those obtained from low-coverage Neanderthal and Denisovan genomes from skeletal remains [Fig. 4A, bottom right, red and blue points (*1*, *13*, *19*, *25*–*27*)], suggesting that the nuclear DNA in the three sediment samples is either of Neanderthal or Denisovan origin, but not both.

We next captured nuclear DNA from Chagyrskaya Cave and Estatuas sediment samples. Twenty-nine samples yielded at least one library with significant evidence for deamination and <5% faunal misalignment [tables S12 and S13 (*13*)]. Four libraries showed evidence for >5% faunal misalignment and were excluded from further analyses, highlighting the importance of per-library misalignment estimates [table S14 (*13*)]. In total, we retrieved nuclear DNA from Chagyrskaya Cave subunits 6a to 6d and 7 [the latter likely intrusive from subunits 6c and 6d (*13*)], and Estatuas pit II/layer 2 and pit I/layers 2 to 5. Recovery of hominin DNA was lower than for the Denisova Cave samples, with our best libraries yielding 134,497 fragments (33,594 with evidence of deamination) at target sites for Chagyrskaya Cave and 47,667 for Estatuas (16,678 deaminated). In a plot of Neanderthal versus Denisovan alleles, these samples clearly contained Neanderthal nuclear DNA and clustered together closely with Neanderthal skeletal samples (Fig. 4A, top right and bottom left). Equivalent plots considering sites that differ among the *Altai Neanderthal*, *Vindija 33.19*, or *Chagyrskaya 8* genomes lack the resolution to resolve their relationship to these Neanderthal genomes, given the small amounts of data per sample at these SNPs [figs. S15 and S16 (*13*)].

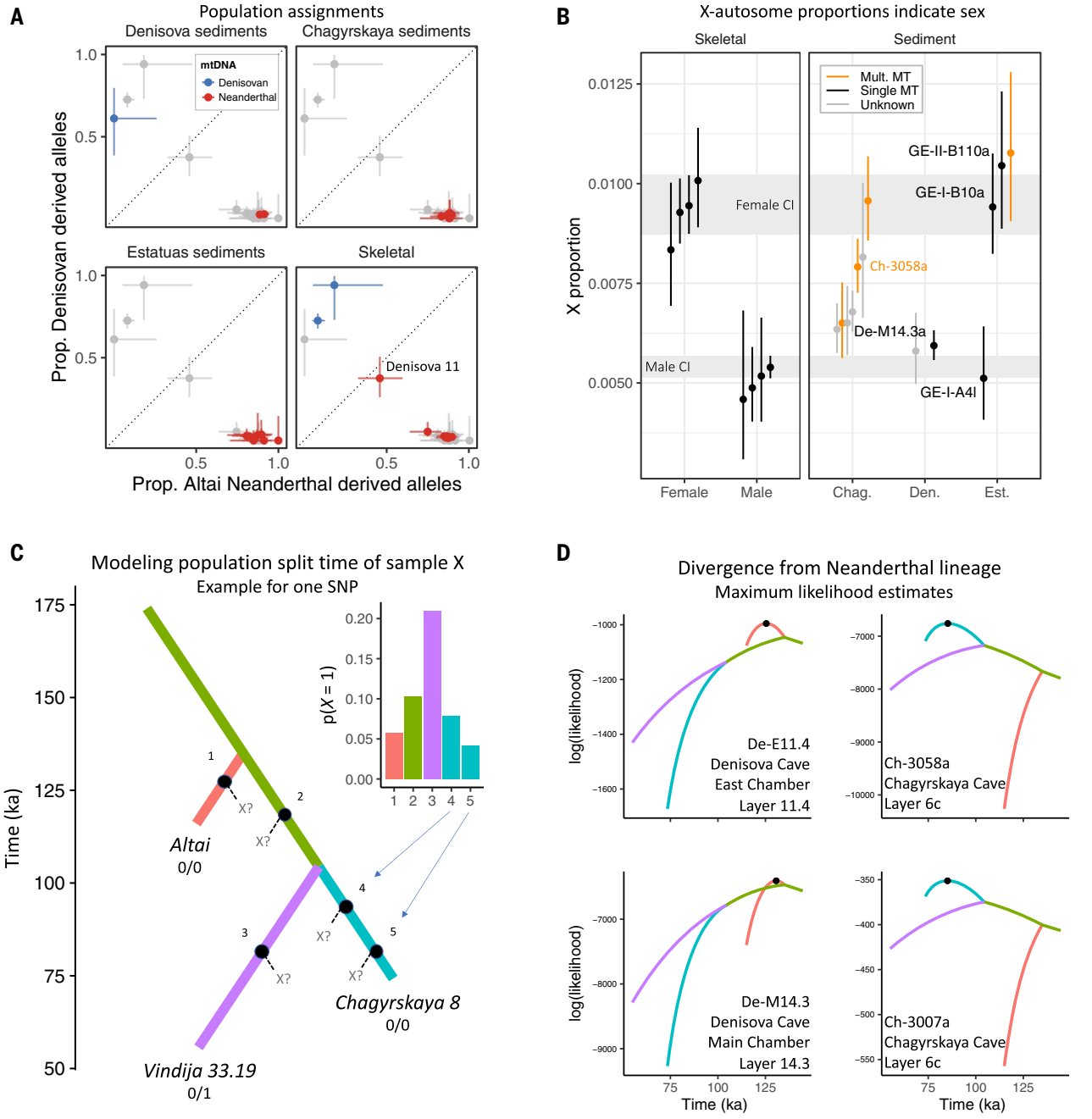

Fig. 4. Sediment nuclear DNA. (**A**) Neanderthal versus Denisovan alleles place sediment (top row and bottom left) and skeletal (bottom right) samples into broad population groups. All samples are shown in gray in all plots. CI, 95% binomial confidence interval. (**B**) X-autosome proportions for skeletal and sediment samples. Male and female CI bands (gray) denote male and female skeletal samples with the narrowest CI, respectively. Evidence of single (black) or multiple (orange) mtDNA haplotypes was taken from table S3. All samples are labeled in fig. S17. (**C**) Modeling $p(X = 1)$, the probability that a sediment sample X carries a derived allele at an example SNP in the genome, where *Vindija* is heterozygous (0/1) and *Chagyrskaya* and *Altai* are homozygous ancestral (0/0). $p(X = 1)$ depends on the time at which sample X diverges from the Neanderthal phylogeny [black dots are hypothetical split times; inset shows $p(X = 1)$ for each split time]. (**D**) Likelihood surfaces (lines) and maximum likelihood estimates of branching times for four sediment samples (black dots).

For skeletal specimens, the relative proportions of X and autosomal DNA have been used to determine sex (*3*). We applied this approach to sediment subsamples with <10% present-day human contamination and deaminated DNA fragments covering at least 5000 sites, and found that all such Denisova Cave and Estatuas samples showed X/autosome proportions consistent with hominin DNA originating primarily from a single sex (three male and three female; Fig. 4B and fig. S17). By contrast, most samples from Chagyrskaya Cave fell between the expected male and female proportions, suggesting that they contained DNA from multiple individuals of different sexes (Fig. 4B). All four libraries for which we identified a single mtDNA sequence had X/autosome proportions consistent with the DNA originating from a single sex, suggesting that they may contain DNA from individual Neanderthals (Fig. 4B, black-labeled sediment

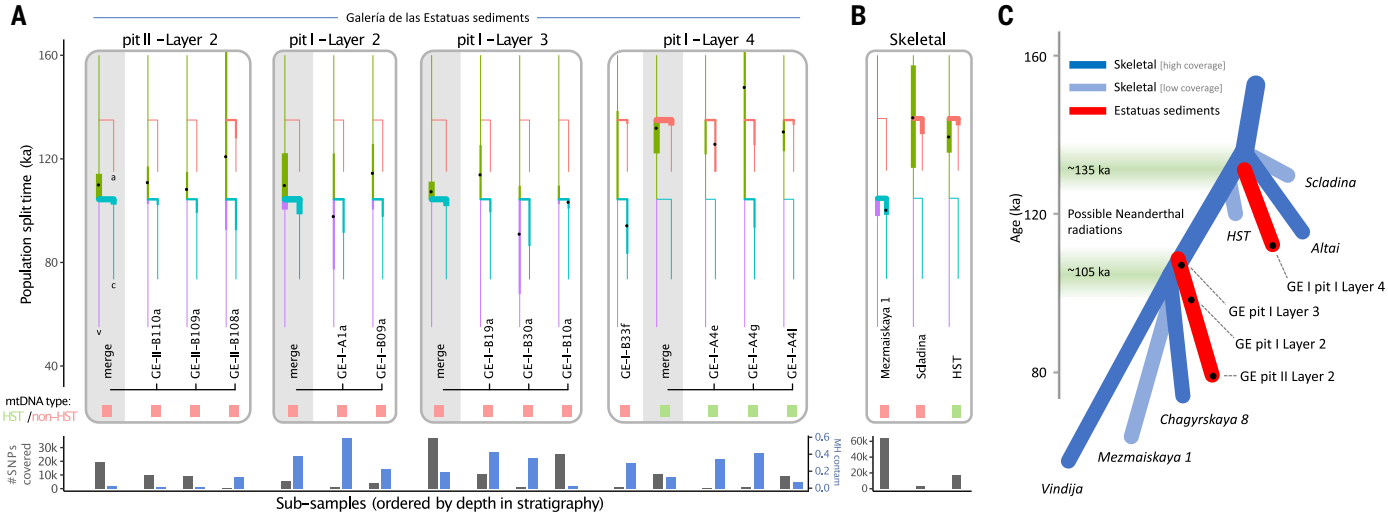

Fig. 5. Sediment samples placed on Neanderthal phylogeny. (**A**) Population split estimates for sediments across four layers of Estatuas (black dot is the maximum likelihood estimate; thick lines are 95% block bootstrap CI; CI clipped at 160 ka for GE-II-B108a and GE-I-A4g) plotted on the Neanderthal population tree with *Vindija 33.19* (v), *Chagyrskaya 8* (c), and the *Altai Neanderthal* (a). For each layer, subsamples were merged to produce per-layer estimates (gray blocks, "merge"). For pit I/layer 4, only samples with *HST*-like mtDNA (green squares) were merged. Bottom row shows the estimated number of Neanderthal SNPs used in the branch-time analysis (gray) and modern human contamination estimates (blue). (**B**) Same as (A) but for three skeletal samples. For *Mezmaiskaya 1*, a 1× coverage genome was down-sampled to ~60,000 informative reads. (**C**) Clustered split times in the Neanderthal phylogeny suggest successive radiations of Neanderthal populations ~105 and 135 ka ago.

points), although we cannot exclude the presence of identical mtDNA from multiple individuals of the same sex.

Nuclear phylogenetic analysis

To place each sample on the larger archaic phylogeny despite limited data, we developed a maximum likelihood framework that, for a sample *X*, co-estimates the point at which *X* branched from the archaic hominin tree—defined by the three Neanderthal and one Denisovan high-coverage genomes (e.g., Neanderthal phylogeny in Fig. 4C)—along with the proportion of fragments deriving from non-hominin faunal misalignment and present-day human contamination. This method makes use of the fact that all sites that are polymorphic in archaic hominins are informative for their population histories. For example, at a site that is heterozygous in one archaic genome but homozygous ancestral in the others, the probability of observing a derived allele in a sample *X* [$p(X = \text{der})$] varies based on the point at which *X* diverged from the overall tree (Fig. 4C, black points and bar plot). These probabilities are obtained from coalescent simulations for which effective population sizes and split times are inferred from the respective high-coverage genomes (*13*). Misalignment and contamination proportions are estimated independently for deaminated and nondeaminated fragments, allowing all fragments to be used in the analysis (*13*). Although some sediment samples may represent single individuals, this method operates on allele frequency expectations, making it equally applicable to samples representing single or multiple individuals from a population. When applied to previously published low-coverage genomes from skeletal samples, our method infers population split times and contamination proportions consistent with previous estimates (*13*). In a power analysis, we estimated accurate population split times in down-sampled low-coverage Neanderthal genomes (*25*) with up to 70% present-day human contamination [mean absolute error 10 to 16 ka with 500 Neanderthal DNA fragments; 4 to 7 ka with 4000 fragments; figs. S18 to S22 (*13*)] and accurately infer present-day human contamination proportions of up to 90% [fig. S20 (*13*)].

Applying this method to sediment libraries from Denisova and Chagyrskaya Caves, we found results consistent with previously published ancient DNA from skeletal elements. Specifically, we found that the two Neanderthal samples from Denisova Cave fall on the lineage leading to the *Altai Neanderthal* individual (Fig. 4D, De-E11.4 and De-M14.3, and table S14). This result is consistent with the first of these samples originating from the same layer in the East Chamber (layer 11.4) as the *Altai Neanderthal* and the second sample from a contemporaneous layer (layer 14.3) in the Main Chamber (deposited ~105 to 120 and 97 to 112 ka ago, respectively) (*4*). The Denisovan sample from layer 15 in the East Chamber (dated to ~200 ka ago) (*4*) falls on the Denisovan lineage, consistent with its mtDNA [fig. S23 (*2*)]. The sediment samples from all layers at Chagyrskaya Cave fall on the *Chagyrskaya 8* lineage (Fig. 4D, Ch-3058a and Ch-3007a, and fig. S24), consistent with a short-lived occupation of the site by Neanderthals associated with a distinctive Middle Paleolithic toolkit (*5*).

For Estatuas, where less nuclear DNA was recovered from the sediment samples and no previous genetic data exist from skeletal remains, we estimated population split times for individual subsamples with at least 500 Neanderthal DNA fragments and <70% present-day human contamination. We also merged samples to obtain per-layer estimates, with ~5000 to 36,000 fragments per layer (Fig. 5A). Samples from pit II/layer 2, pit I/layer 2, and pit I/layer 3 (*10*) diverged from the Neanderthal tree ~100 to 115 ka ago (block bootstrap 95% CIs: 102 to 114, 99 to 122, and 102 to 112 ka ago, respectively; Fig. 5A), similar to the split times of *Vindija 33.19*, *Chagyrskaya 8*, and *Mezmaiskaya 1* from each other (~104 ka ago; Fig. 5, B and C). The time of deposition of pit I/layer 3 [107 ± 8 ka ago; (*10*, *13*)] is indistinguishable from this date of divergence, suggesting that the Neanderthals from pit I/layer 3 were closely related to the ancestors of *Vindija 33.19* and *Chagyrskaya 8*. We were unable to determine whether the aforementioned layers represent a repeated occupation of the cave by the same Neanderthal population, but the split times and the mtDNA data (Fig. 2B) are consistent with this hypothesis.

In contrast, sediments from pit I/layer 4 carrying the *HST*-like mtDNA diverged from the Neanderthal tree ~122 to 135 ka ago (Fig. 5A, block bootstrap 95% CIs). This divergence

time is similar to that of the *HST* Neanderthal itself, as well as of the *Scladina Neanderthal* and the *Altai Neanderthal* (Fig. 5, B and C). The latter two carry the more common non–*HST*-like Neanderthal mtDNA type (Fig. 5B), consistent with mitochondrial diversity in the ancestral Neanderthal population, which was also observed in pit I/layer 4 (Fig. 2B). GE-I-B33f, the only sample of the common Neanderthal mtDNA type from pit I/layer 4 that yielded nuclear DNA, was collected from near the boundary with layer 3 and produced a divergence time similar to those of the layer 3 samples, albeit with a large CI (83 to 139 ka ago) because of the small dataset (867 ancient hominin DNA fragments). Taken together, these observations suggest that a population replacement occurred at Estatuas toward the end of the time of deposition of layer 4, which was accompanied by a loss of mtDNA diversity. Similar results were obtained when using only deaminated fragments (fig. S25), highlighting the robustness of the method to the effects of present-day human contamination.

Discussion

The apparent clustering of branching times suggests two distinct radiations of Neanderthal populations: *Mezmaiskaya 1*, *Vindija 33.19*, *Chagyrskaya 8*, and Estatuas pit II/layer 2 and pit I/layers 2 and 3 diverged from each other ~100 to 115 ka ago, whereas the *Altai*, *HST*, *Scladina*, and Estatuas pit I/layer 4 Neanderthals and the lineage leading to *Vindija 33.19* and *Chagyrskaya 8* diverged from each other ~135 ka ago (Fig. 5C). These radiation events therefore occurred during the early part of the Late Pleistocene and may be associated with changes in climate and environmental conditions during the last interglacial. In addition, it has been noted that the typical Neanderthal morphology evolved in several stages (*28*, *29*), with the last stage, the "classic" Neanderthals, appearing ~100 ka ago. Despite the uncertainty in dating these events, it seems plausible that the latter transition could be linked to the younger population radiation that we detected. However, determining whether such factors played a key role in the population dynamics of Neanderthals and other Pleistocene hominins (*30*, *31*) would require time-series data from additional sites and more precise estimates of the timing of these genetic events of interest. The methods presented here open the possibility of obtaining such data independently of the fossil record, limited only by biochemical constraints on long-term DNA preservation.

Our results also show that the recovery of hominin DNA from sediment may not be limited to population samples, as DNA that putatively derived from individual Neanderthals (i.e., sediment samples with a single mtDNA sequence and sex) was identified in sediment samples from all three study sites. This observation suggests that it may be possible in the future to also assess heterogeneity in the genetic composition of past populations based on the analysis of sediment DNA. In light of the substantial variation in the quantity of hominin DNA observed among sediment samples taken in close proximity (and within single samples), and considering the low abundance of hominin DNA versus non-hominin faunal DNA, it seems unlikely that the analysis of hominin DNA from Pleistocene sediments is substantially affected, if at all, by leaching of DNA through archaeological layers. However, the presence of Neanderthal DNA in layer 7 of Chagyrskaya Cave highlights the need to evaluate evidence for post-depositional mixing of sediments when assigning DNA sequences from sediment to specific layers, as is common practice when interpreting finds of artifacts, skeletal remains, and other archaeological materials. Finally, our work also highlights the value of high-coverage archaic human genomes, even if generated only in small numbers, as scaffolding for defining the past genetic landscape onto which less complete genome-wide sequence data from sediments and bones can be mapped.

Materials and Methods

Sediment samples were taken from the exposed sections of Chagyrskaya Cave and Estatuas, and subsamples with weights between 21 and 128 mg were used for DNA extraction (*14*) and single-stranded DNA library preparation (*15*). All libraries were enriched for hominin mtDNA using hybridization capture (*32*), and a subset for mammalian mtDNA (*33*) to evaluate the preservation of ancient faunal DNA. Based on the content of ancient hominin mtDNA, and aiming to recover hominin nuclear DNA from all relevant layers, libraries from this study and a previous study on DNA preservation in the sediments of Denisova Cave (*2*) were selected for hybridization capture using probes targeting phylogenetically informative positions in the nuclear genome. Mitochondrial sequences were identified on the biological family level using a previously established analysis pipeline (*2*), which includes an evaluation of the presence of deamination patterns typical for ancient DNA and assignments of the Hominidae component to specific hominid groups. Further assignments to specific branches of the hominin mtDNA tree were performed using a method based on the software kallisto (*13*, *34*). Hominin nuclear DNA sequences were identified, and phylogenetic analyses were performed as summarized in fig. S26 and described in full detail in (*13*), which also contains details on sample collection, sample preparation, and data processing. Software and scripts were written in Python (*35*) and R (*36*), and are publicly available as described in (*13*). Plots were created with ggplot2 (*37*), cowplot (*38*), RColorBrewer (*39*), and ggmap (*40*).

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SUPPLEMENTARY MATERIALS

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Science

Unearthing Neanderthal population history using nuclear and mitochondrial DNA from cave sediments

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The value of dirty DNA

Environmental DNA can identify the presence of species, even from the distant past. Surveying three cave sites in western Europe and southern Siberia, Vernot *et al.* identified nuclear DNA and confirmed that it is from the close relatives of anatomically modern humans—Neanderthal and Denisovan individuals. A phylogenetic analysis and modeling show that the DNA in sediment samples from several layers corresponds to previously studied skeletal remains. These results demonstrate that environmental data can be applied to study the population genetics of the extinct Neanderthal and Denisovan lineages, identifying a turnover of Neanderthal populations ~100,000 years ago.
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