Deeply divergent archaic mitochondrial genome provides lower time boundary for African gene flow into Neanderthals

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Ancient DNA is revealing new insights into the genetic relationship between Pleistocene hominins and modern humans. Nuclear DNA indicated Neanderthals as a sister group of Denisovans after diverging from modern humans. However, the closer affinity of the Neanderthal mitochondrial DNA (mtDNA) to modern humans than Denisovans has recently been suggested as the result of gene flow from an African source into Neanderthals before 100,000 years ago. Here we report the complete mtDNA of an archaic femur from the Hohlenstein–Stadel (HST) cave in southwestern Germany. HST carries the deepest divergent mtDNA lineage that splits from other Neanderthals \( \sim 270,000 \) years ago, providing a lower boundary for the time of the putative mtDNA introgression event. We demonstrate that a complete Neanderthal mtDNA replacement is feasible over this time interval even with minimal hominin introgression. The highly divergent HST branch is indicative of greater mtDNA diversity during the Middle Pleistocene than in later periods.
In recent years, an increasing number of mitochondrial DNA (mtDNA) and nuclear genome (nDNA) data from archaic human remains have reshaped the understanding of evolutionary relationships among various hominin groups. Mitochondrial genomes provided evidence for at least two distinct mtDNA branches associated with Neanderthals and Denisovans, respectively, suggesting a sister group relationship between modern humans and Neanderthals with Denisovans as a basal mtDNA outgroup. However, nDNA data revealed that Neanderthal and Denisovan populations separated only after their divergence from the lineage leading to modern humans.

The estimate for the population split time between the two archaic hominin groups and modern humans was calculated to 765,000–550,000 years ago (765–550 ka) based on a recent estimate of the genome-wide human mutation rate. Furthermore, analyses of Y-chromosome data from a male Neanderthal returned an age of 806–447 ka for the divergence of Neanderthal and modern human Y-chromosome lineages. These time intervals largely overlap, suggesting that the Neanderthal Y chromosome differentiated through the population split from the most recent common ancestor (MRCA) of modern humans and Neanderthals. In contrast, the corresponding divergence time for mtDNA has been dated to ~400 ka (95% highest posterior density (HPD), 498–295 ka) and was thus found to be considerably younger compared to the time estimates obtained from autosomal and Y-chromosome data.

In addition, nDNA analyses of the Middle Pleistocene hominins from the Sima de los Huesos site in northern Spain confirmed their closer affinity to the Neanderthal lineage, suggesting that at least by ~430 ka, Neanderthals and Denisovans had already diverged. However, in contrast to genome-wide data, the Sima de los Huesos mtDNA was found to branch off with the deeply divergent Denisovan mtDNA lineage. The phylogenetic discrepancies could be reconciled if the mtDNA of early Neanderthals was indeed Denisovan-like and was subsequently replaced by a more derived mtDNA lineage. Therefore, a genetic introgression event from African hominins into the early Neanderthal population that gave rise to the ‘Late Pleistocene’ Neanderthal mtDNA lineage has been proposed. This event must have occurred after archaic and modern human populations diverged. However, the exact timing of the proposed gene flow is unknown and merely based on possible archaeological evidence that could provide a basal mtDNA outgroup for each of the four references was reconstructed with endoCaller implemented in the software schmutzi, followed by visual inspection to confirm the called polymorphisms (see Methods section). Using the RNRS as reference sequence resulted in the highest number of mapped reads and ~35-fold average mtDNA coverage. Around 50% of mtDNA fragments were damaged at the molecule termini with an average length of ~43 bp, both displaying the degradation patterns typical for ancient DNA (aDNA) (Supplementary Table 2 and Supplementary Fig. 3).

### Results

#### Archaeology and stable isotopes

The HST specimen is a right femur shaft circa 25 cm long displaying archaic hominin morphology, affected by heavy mineralization and gnawing by a large carnivore on both sides. During excavations in 1937, it was found in a black clayey layer with Middle Paleolithic artefacts known as the Black Mousterian based on the sediment colour and the cultural assignment of the technocomplex retrieved in the stratigraphic unit, which is associated throughout Europe with Neanderthals. The femur is the sole archaic human fossil originating from a Mousterian context, not only at the site but in the entire Swabian Jura region (Supplementary Note 1).

Direct radiocarbon dating attempts have resulted in inconsistent results (Supplementary Note 2), suggesting that the bone may be suffering from modern C14 contamination and is possibly beyond the detection limit of this dating method. Isotopic analyses performed on the collagen of the femur revealed considerably lower 13C and 15N values than those reported for late Neanderthals from western and central Europe (Supplementary Table 1 and Supplementary Fig. 1). Moreover, collagen from two faunal remnants recovered from the same stratigraphic unit of HST was analysed. ZooMS analyses confirmed the morphological assignment to red deer and radiocarbon dating resulted in an age range beyond this dating method (Supplementary Table 1 and Supplementary Note 2). Both deer specimens provided notably lower 13C values compared to cervids from open steppe environment (Supplementary Fig. 2). The ecological background of the HST femur and deer specimens are, therefore, equivalent and indicate a more forested and closed environment compared to the habitat of late Neanderthals in northwestern Europe.

#### Ancient DNA retrieval and consensus reconstruction

The femur shaft was sampled from the proximal diaphysis longitudinally to the cortical bone, at the opposite site of the previous sampling for radiocarbon dating. DNA was extracted from 130 mg of bone powder, immortalized in a double-stranded library and hybridized to modern human mtDNA probes. The enriched library was sequenced and between 12,750 and 12,848 DNA reads were successfully aligned to the four reference sequences with the same mapping parameters (see Methods section): the reconstructed MRCA's of Neanderthal and Homo sapiens mtDNA (reconstructed Neanderthal reference sequence (RNRS) and reconstructed Sapiens reference sequence (RSRS))  and the Neanderthal-type specimen mtDNA (Feldhofer1) and the present-day human mtDNA reference (revised Cambridge reference sequence (rCRS)) , respectively. A consensus sequence for each of the four references was reconstructed with endoCaller implemented in the software schmutzi, followed by visual inspection to confirm the called polymorphisms (see Methods section). Using the RNRS as reference sequence resulted in the highest number of mapped reads and ~35-fold average mtDNA coverage. Around 50% of mtDNA fragments were damaged at the molecule termini with an average length of ~43 bp, both displaying the degradation patterns typical for ancient DNA (aDNA) (Supplementary Table 2 and Supplementary Fig. 3).

When comparing the four consensus sequences obtained by mapping against the different references we observed the influence of reference biases in reconstructing the HST mtDNA (Supplementary Fig. 4a). After manual inspection of the inconsistent positions, we identified RNRS as the reference producing a consensus sequence closest to the endogenous mtDNA (see Methods section). However, mapping bias disappeared when excluding from the alignment the highly variable D-loop.
Following a more conservative approach subsequent Bayesian and phylogenetic analyses were performed using the reconstructed HST mtDNA coding region. The phylogenetic comparison with 54 modern humans, three Denisovan and an extended dataset of 17 Neanderthal mtDNA sequences revealed a closer relationship of the femur’s mtDNA to Neanderthals. However, the HST mtDNA revealed a short phylogenetic branch length and fell basal to all other Neanderthal individuals, representing the deepest diverging lineage among Neanderthal mtDNAs discovered to date (Fig. 1c, Supplementary Fig. 5 and Supplementary Fig. 6).

The same HST genetic library before mtDNA capture was also sequenced through a shotgun approach. Only 0.46% of the over half million reads were aligned to the human reference genome (hg19) despite choosing a highly sensitive mapping parameters to account for aDNA damage and divergence from the reference sequence (Supplementary Table 3 and Methods section).

Contamination estimates. Three measurements were performed to estimate the level of present-day human contamination in the isolated mtDNA reads. The first approach is based on the assumption that aDNA is damaged, whereas contaminant DNA is less affected by this chemical modification (contDeam28). One molecule end is conditioned to exhibit damage while deamination levels are measured at the opposite end of the fragment. The discrepancy between the unfiltered and conditioned damage levels implied a contamination of 9.5–11.5% from modern human fragments (Supplementary Table 2).

This estimate is used as prior in an iterative likelihood approach in which mtDNA reads are compared to a data set of 256 Eurasian modern mtDNA sequences to refine the level of contamination (mtCont28). According to this second method, 9–11% of the bases aligned to the rCRS turned out to be of contaminant origin.

Third, we identified mtDNA diagnostic positions, as the nucleotides where the reconstructed HST complete sequence
MtDNA Neanderthal diversity. In a previous study\(^2\), the mtDNA diversity among seven Neanderthals, three Denisovans and 311 modern humans were compared through the Watterson’s estimator \(\theta_w\) resulting in the lowest mtDNA distance within Neanderthals. The value decreased even further when 10 additional Neanderthal mtDNAs available in the literature were included (1.37 \(\times\) 10\(^{-3}\)), which confirms the small population size of late Neanderthals\(^{26}\) (Supplementary Table 4). However, by adding the HST mtDNA in the Neanderthal group the \(\theta_w\) estimation almost doubled to 2.50 \(\times\) 10\(^{-3}\). Although the value is still below the results obtained from the three Denisovan sequences (3.46 \(\times\) 10\(^{-3}\)), the HST mtDNA exhibits an average pairwise nucleotide distance to the other Neanderthal mtDNAs of 104 (89–111) positions (Fig. 2 and Supplementary Table 5). These values are greater than among any Denisovan mtDNA pair and are in the upper range of the modern human worldwide pairwise distance distribution (Fig. 2). This shows that HST belongs to a mtDNA branch highly divergent from the one represented in other Neanderthals (Altai branch) and overall Neanderthal mtDNA diversity was larger than that assumed previously.

The Neanderthal mtDNA effective population size (\(N_e\)) through time was estimated in a Bayesian statistical framework\(^{33}\) under the simplified assumption they belonged to a panmictic population with a fixed mutation rate previously calculated with ancient modern human mtDNAs as calibration points\(^{10}\) (Supplementary Note 4). The reconstructed skyline plot describes a \(N_e\) reduction through Middle and Late Pleistocene, reaching the lowest mean value at around 42 ka (Supplementary Fig. 7). Subsequently, a steep population expansion appears to have occurred before the Neanderthal extinction, in accordance with the reported analyses of chromosome 21 of the Vindija late Neanderthal\(^{14}\).

**Molecular dating analyses.** To estimate the molecular age of HST and other undated Neanderthal mtDNAs as well as the temporal range of MRCAs (TMRCAs) on the mtDNA tree, we performed a Bayesian dating analysis as implemented in BEAST v.1.8.1 (ref. 33). A multiple genome alignment of the coding region from 54 modern humans, 18 Neanderthals and 1 Denisovan mtDNA were tested for a strict and uncorrelated lognormal relaxed clock under both a constant size and a Bayesian skyline tree prior (see Methods section). As reported above, a fixed mutation rate was selected for the coding region\(^{10}\) with the addition of eight dated Neanderthal mtDNAs as time anchors on the Neanderthal branch (Supplementary Table 6). The four model combinations were compared by stepping-stone and path sampling (PS) methods\(^{34}\). This analysis indicated that a skyline model associated with a strict rate variation among branches is the model that most adequately fits the data (Supplementary Table 7). In Table 1 we report the TMRCAs between Neanderthal and modern human mtDNAs and among modern human mtDNAs itself, which largely overlap with previously published studies\(^{10,11}\). We further estimate the divergence time between HST and all other Neanderthals to \(~\)270 ka (95% HPD 316–219 ka), while the TM RCA for the Altai branch was inferred to be \(~\)160 ka (95% HPD 199–125 ka).

Based on phylogenetic branch shortening, we then molecularly dated 10 Neanderthal sequences that had not been radiocarbon dated previously or were considered beyond the radiocarbon dating detection limit (Table 1). The two oldest mtDNAs were HST with an age of 124 ka (95% HPD 183–62 ka) and Altai Neanderthal with an age of 130 ka (95% HPD 172–88 ka). Notably, the mean value for the latter individual largely overlaps with the inferred age of 136–129 ka from its high coverage nuclear genome analyses, when applying recent estimates of the human mutation rate\(^5\).

**Exploration of putative Neanderthal mtDNA replacements.** The probability that the initial Denisovan-like Neanderthal mtDNA present in Eurasia was totally displaced by an incoming lineage\(^6\) is dependent not only on the admixture rate but also on
the size of the introgressing population compared to the local one (Supplementary Fig. 8). When considering the effective population size history estimated with the Bayesian skyline method (Supplementary Fig. 7), the probability that all Neanderthal mtDNA originated from an introgression event is almost directly proportional to the admixture rate (Supplementary Fig. 9 and Supplementary Note 5). Moreover, assuming that a complete mtDNA replacement took place, we estimated under neutrality\(^3\) (see Methods section) the mean time period a complete mtDNA would be necessary to measure the total mtDNA distance between HST and Valdegoba, this finding might have occurred later but most likely before 160 ka, our estimated date for the start of the Altai branch diversification (Fig. 1c and Table 1).

Within the Late Pleistocene mtDNA clade, we explored if the HST mtDNA branch might have survived long after the estimated molecular age of the HST femur. All complete Neanderthal mtDNAs were combined with sequences from published hypervariable regions (HVRI) of four additional Neanderthal individuals. We identified the Valdegoba sequence (JQ670672) sharing three derived mutations with HST and falling on the same branch in a HVRI tree (Supplementary Fig. 10 and Methods section). This specimen was found on the Iberian Peninsula and dates to 48,400 ± 3,300 ^14^C years BP\(^3\). Although a complete mtDNA would be necessary to measure the total mtDNA distance between HST and Valdegoba, this finding might suggest that the HST branch was found during the Late Pleistocene as far as western Europe. Based on the geographical and temporal distributions of HVRI sequences, it was proposed that the Neanderthal population in western Europe underwent a demographic turnover followed by a subsequent recolonization\(^3\). Under that scenario, the HST lineage would have been largely replaced towards the end of the Neanderthal temporal range by mtDNAs descendants on the Altai branch.

### Discussion

The African introgression hypothesis suggests that Late Pleistocene Neanderthal mtDNAs originated through gene flow from an African source\(^5\), which we constrain taking place more than ~270 ka (Table 1). Our analytical calculations (Supplementary Table 8 and Supplementary Note 5) show that this event is plausible even if the introgressing lineage represented a minimal proportion of the initial gene pool. This scenario reconciles the discrepancy in the nDNA and mtDNA phylogenies of archaic hominins and the inconsistency of the modern human–Neanderthal population split time estimated from nDNA and mtDNA (Fig. 1d). Under this demographical model, the Denisovan mtDNA type was common among early Neanderthals in Eurasia (for example, Sima de los Huesos) and was then largely replaced by an introgressing African mtDNA that evolved into the Late Pleistocene Neanderthal mtDNA type. While the upper bound for the time of this putative gene flow event would be the divergence time between Neanderthal and modern human mtDNAs, here dated to 413 ka (95% HPD 468–360 ka), the lower temporal limit was represented so far by the ~160 ka TMRCA of all published Neanderthal mtDNAs (Table 1). However, the finding of the deeply diverged HST lineage splitting from the Altai branch, ~270 ka, sets an older lower boundary for the time of this admixture event. An alternative but less parsimonious scenario is that both HST and Altai mtDNA lineages reached Eurasia independently after diverging inside Africa. In that case the suggested introgression event might have occurred later but most likely before 160 ka, our estimated date for the start of the Altai branch diversification (Fig. 1c and Table 1).

The presence of modern human admixture into archaic humans has already been detected in the high coverage Neanderthal genome from the Altai region but not in sequences of chromosome 21 of two Neanderthals from Spain and Croatia\(^1\). The authors therefore suggested that a genomic contribution estimated between 0.1 and 2.1% occurred after the divergence of Altai from other late Neanderthals. However, there is a high level of uncertainty around the time of the inferred gene flow event since only one high coverage Neanderthal nuclear genome has been analysed so far. Moreover, the divergence time of the introgressing African population was estimated to date before or right after the TMRCA of modern-day humans (~200 ka)\(^1\), while the mtDNA coalescence time between Neanderthals and modern humans is calculated at least twice as old (~400 ka). The evolutionary scenario responsible for providing the mtDNA to the Late Pleistocene Neanderthals might have been an even earlier Middle Pleistocene gene flow from Africa, occurring in a time interval that we date between 413 and 268 ka (460–219 ka including upper and lower 95% HPD).

### Table 1 | Divergence times and molecular ages estimated in BEAST.

| Mitochondrial lineages                             | Mean value | 95% HPD interval |
|----------------------------------------------------|------------|------------------|
| Modern humans—Neanderthals                         | 412,930    | 467,720–360,230  |
| HST—Altai branch Neanderthals                      | 267,770    | 316,080–218,980  |
| Altai—rest of Altai branch Neanderthals            | 160,480    | 198,800–125,410  |
| San—rest of modern humans                          | 146,730    | 169,520–123,650  |
| Altai age                                           | 130,010    | 171,600–88,010   |
| HST age                                             | 123,800    | 182,560–62,013   |
| Mezmaiskaya 1 age                                   | 89,075     | 126,700–91,648   |
| Denisova 11 age                                     | 88,244     | 113,760–63,840   |
| Okladnikov 2 age                                    | 81,446     | 109,290–56,213   |
| Vindija 33.17 age                                   | 48,809     | 57,157–40,532    |
| Vindija 33.19 age                                   | 43,939     | 51,029–35,336    |
| Vindija 33.25 age                                   | 42,996     | 52,305–34,450    |
| Goyet Q374a-1 age                                   | 40,867     | 46,942–32,697    |
| Goyet Q305-7 age                                    | 40,832     | 47,057–33,134    |

HST, Hohlenstein–Stadel. Reported values derive from the skyline tree prior and strict molecular clock model that best fits the data (see Methods section).
It should be highlighted that this additional genomic contribution might have already been accounted for in ref. 14, which effectively measures the total amount of African introgression into Neanderthals after their split from Denisovans (473–381 ka; ref. 5).

The phylogenetic branch length of mtDNA sequences from 10 non-dated Neanderthal individuals was considered in BEAST, to assess individual molecular ages spanning from 130 to 40 ka. Although it is not known if the mtDNA mutation rate in modern humans is comparable to that of Neanderthals (Supplementary Note 4), molecular dating can at least be used to provide relative ages when the radiocarbon absolute chronometric method is not applicable. After the Altai mtDNA, HST is estimated to be the second oldest mtDNA with an age of 124 ka (95% HPD 183–62 ka). This wide temporal interval largely overlaps with the Marine Isotope Stage 5 (MIS 5: ~130–73 ka)37. After its initial interglacial period (MIS 5e), central Europe was characterized by climatic fluctuations resulting in forestation phases (MIS 5c/5a) alternated with the development of steppe-tundra biomass (MIS 5d/b)38. The stable isotopic δ13C and δ15N values of the archaic femur collagen and associated faunal remains suggest a more temperate, forested rather than a colder, steppe environment and is therefore consistent with an ecological context during the early warm phases of the last glaciation17.

Despite having only a single complete mtDNA on the HST lineage, the two highly differentiated Neanderthal mtDNA branches suggest higher mtDNA diversity during the Middle Pleistocene, which then declined during the Late Pleistocene (Supplementary Table 4). This observation is also supported by the steady decline in mtDNA effective population size displayed in the skyline plot before a steep growth in late Neanderthal population sizes (Supplementary Fig. 7). Studies focusing on the demographic patterns of late Neanderthals who overlapped with the earliest modern humans in Europe are of key importance to understand population dynamics and interactions between archaic and modern humans. In conclusion, the HST mtDNA provided insights into the mtDNA diversity of Neanderthal populations inside the Middle and Late Pleistocene. Its deep divergence time allowed us to further constrain the lower boundary for the time of the proposed African mtDNA gene flow into Neanderthal populations. The temporal corridor for this introgression event between 460 ka and 219 ka is compatible with the evidence of archaeological similarities between Africa and western Eurasia during the Lower to Middle Paleolithic transition39 and potentially may explain the dissimilarities in Middle Paleolithic industries between eastern and western Eurasia. Environmental changes across this time span might have facilitated a hominin expansion out of Africa and potentially spread cultural innovations such as the Levallois technology into Eurasia40. Alternatively, other scenarios such as multiple inventions of similar technologies by various hominin groups may explain the complex tapestry of technological variability during the late Middle Pleistocene.

Nuclear data from the HST femur would be pivotal in assessing its genomic relationships with Neanderthals, Denisovans and modern humans. However, the scarce preservation of HST endogenous DNA in combination with high level of modern human contamination challenge the retrieval of its complete genome. Analyses of high-quality nDNA from more than one well-preserved Neanderthal individual are necessary to detect the consequences of African admixture into archaic human populations.

Methods

**aDNA lab work.** aDNA work was performed in the dedicated facilities of the Institute for Archaeological Sciences in Tubingen, Germany. The HST femur was first irradiated with ultraviolet light on the selected sampling area and then drilled with a dentist drill along the cortical bone. A total of 130 mg of bone powder went through extraction following an established protocol. The DNA was eluted in 100 µl of TET and 20% of the extract (GXX5) was used to build a double-stranded genetic library (GA87)23. The total copies in the resulting library were measured with quantitative PCR (qPCR) (7.53 × 10^5 copies). They were split into four 100 µl indexing PCR reactions with 10 cycles where an individual index pair (8 bp each) was used to create a unique index (Supplementary Table 3). From the two reactions were amplified and indexed in each with AccuPrime Pfx DNA polymerase. The PCR products were purified over a single MinElute spin column and the concentration after amplification was quantified to 286 ng µl−1 on an Agilent 2,100 Bioanalyzer DNA 1,000 chip. Extraction and library negative controls were carried along the workflow and treated equally.

The amplified library was enriched for mtDNA using modern human baits as reported by Marić et al.24. This protocol has been previously used to successfully capture complete Neanderthal mtDNA genomes4. Four hundred nanograms of the amplified library were pooled with the same amount of four other libraries for a total of 2,000 ng and captured with 300 ng of mtDNA probes. After purification, the isolated molecules were quantified with qPCR (4.84 × 10^5 copies) and re amplified for 20 additional cycles as described above. The captured pool as well as the uncaptured GA87 library was quantified with Agilent 2,100 Bioanalyzer DNA 1,000 chip, diluted to 10 nM and sequenced with other equimolar libraries on an Illumina HiSeq2500 Rapid run via 2 × 100 + 8 + 8 cycles and on an Illumina NextSeq500 run via 2 × 75 + 8 + 8 cycles.

**Sequence processing and mtDNA consensus reconstruction.** Sequenced molecules were converted from bc1 to fastq files and reads containing the defining library indexes were binned in an individual folder. The EAGER pipeline was used for all subsequent data processing41. Initially adapter and index sequences were trimmed off. Only merged reads where forward and reverse reads overlapped at least 10 bp were retained. Shotgun sequences above 30 bp were aligned to the complete human genome (hg19) with Burrow–Wheeler Aligner (parameters –n 0.01 and seeding off) to calculate the percentage of human DNA. Duplicates and reads with mapping quality below 30 were discarded to estimate damage patterns and average fragment length (Supplementary Table 3). From the total of ~3 Ma paired-end reads sequenced after mtDNA capture, 89.31% were successfully merged and fragments below 30 bp length were further discarded for mapping. The resultant ~1.3 Ma merged reads were aligned to four reference mtDNA sequences: the RSRS2, the rCRS2, the Neanderthal Feldhofer 1 sequence42 and the RNRS originally proposed in Behar et al.25 and later updated when the more basal Altai mtDNA was published26. The rCRS and Feldhofer 1 references are two derived mtDNA sequences on the modern human and Neanderthal branch, respectively. Instead, RSRS and RNRS represent the MRCA mtDNA for modern humans and Neanderthals, respectively. Reads were mapped against the Wheeler Aligner (parameters identical to the above described) for all four references, in combination with a tool able to consider the variability of mtDNA as part of EAGER. The percentage of target DNA was calculated by dividing the total number of input reads by the reads mapping to each mtDNA reference. Duplicates with the same start and end coordinates were removed and the duplication factor was measured by dividing the total number of reads mapping before the total number of reads mapping after duplicate removal. All fragments with map quality below 30 were removed to estimate the average mtDNA coverage. The resulting molecules were also used to calculate average fragment length and deamination patterns43 (Supplementary Fig. 3). Statistics for each processing step of the four reference sequences are reported in Supplementary Table 4.

Consensus reconstruction was performed in a two-step approach. First, schmutz28 was used to infer the endogenous sequence. An internal program of the software package (contDeam) was first run to calculate the endogenous deamination rate and a contamination prior. To each nucleotide a base likelihood value was assigned incorporating damage, base quality and mapping quality information in a Bayesian framework24. The endogenous consensus was then determined by endoCaller after the first iteration of the program. No cutoff to the nucleotide posterior probability was selected resulting in base called even in positions covered with only one fragment. This produced a consensus sequence with three unassigned positions.

Second, the four consensus sequences, one from each reference, were visually compared in Geneious 8.1.7 (http://www.geneious.com)45. A multiple genome alignment was produced and each of the 19 inconsistent positions between the four consensus was evaluated. We imported the bam files in Geneious and for each read covering those positions we inspected if they also overlapped in the other three alignments. Only reads with confidently assigned SNPs (for example, called in all four consensus). Fragments containing such SNPs were considered as endogenous, whereas reads containing the alternative allele were considered as contaminants. In every case, the consensus sequence reconstructed after mapping against the RNRS reference was found to exhibit the endogenous base. This confirms that mapping against a reference sequence that is phylogenetically closer to the consensus sequence increases mapping accuracy (see Supplementary Note 3). Using the same criterion described
above we then manually screened the RNRS mapped consensus and edited the positions following according to rCRS coordinates. Two mass-mapped insertions were removed (pos. 2474T↓E and 16184delA), two uncertain positions with low coverage were edited (A189G and A2496N) and two regions covered with only contaminant reads were masked (pos. 203–214Ns and 5486–5508Ns). We additionally removed the long questionable regions of poly-C (pos. 362–365) and poly-AC (518–524) stretches. Combining the two approaches resulted in a total of 39 unassigned positions in the final consensus sequence that was used for phylogenetic (Supplementary Fig. 5) and mtDNA diversity analyses. We then generated a more conservative consensus by setting a coverage cutoff to twofold. The resulting mtDNA sequence exhibits BNs, but none of the 22 additional unassigned bases overlapped with polymorphic positions within the known Neanderthal mtDNA diversity. Therefore, the tree topology and mtDNA diversity within Neanderthals was not affected.

**Mitochondrial DNA diversity.** The pairwise nucleotide distance among Neanderthals with (n = 18) and without HST (n = 17), Denisova (n = 3) and modern humans was calculated using MEGA6. As before, we used the complete mtDNAs sequences and the number of differences between them was counted with pairwise deletion where all unassigned positions were removed for each sequence pair. We plotted the pairwise nucleotide distance against their frequencies for each of the four data sets (two Neanderthals, Denisova and modern human and Neanderthals with the phylogenetic assignment. However, the nucleotide distances between HST and other Neanderthals are the largest observed among Neanderthals (89–111 nucleotides). These values are higher than between Denisova 3–Denisova 4 and Denisova 8 and around the uppermost edge among 311 worldwide mtDNAs (HST).

We further measured the mtDNA diversities of the enlarged Neanderthal mtDNA data set with the Watterson’s estimator, as reported in ref. 2. We first prepared a multiple genome alignment of Neanderthal mtDNAs both including HST (18 sequences) and excluding HST (17 sequences) using MUSCLE. Then, the number of segregating sites (Ks) was used with a variation in the nucleotide sequence (DnaSP) v.5.10.01 (ref. 52). Finally, we calculated Ks/16,595, where $\theta_w$ is $N_e \mu_1/11$ to take into consideration the number of mtDNA sequences in each data set. Adding HST to the 17 Neanderthal mtDNAs, the number of segregating sites almost doubled (from 78 to 145), whereas $\theta_w$ increased from 1.37 $\times$ 10^{-6} to 2.50 $\times$ 10^{-6} (Supplementary Table 4). The latter value is closer to the mtDNA diversity estimated within three Denisovan mtDNAs (3.46 $\times$ 10^{-2}).

**BEAST analyses.** We used the software package BEAST v.1.8.1 (ref. 33) to both estimate the divergence times between and within modern and archaic humans as well as to track the changes in the maternal effective population size ($N_e$) of Neanderthal mtDNA through time. For the skyline analyses, we first created a multiple genome alignment with only the mtDNA coding region of 18 Neanderthal mtDNAs and the rCRS as outgroup. We then removed from the alignment all columns where at least one mtDNA presented a gap or missing data, resulting in 15,345 positions. We ran two models (MrBayes v.3.2.1 and MrPrior v.8.5 (ref. 48) on our data set to identify Tamura-Nei 93 with a fixed fraction of invariant sites as the best-supported model. We set a fixed mutation rate ($1.57 \times 10^{-8}$ sites $\times$ year$^{-1}$) calculated for the coding region of modern humans with ancient mtDNAs as calibration points (Supplementary Note 4). As tree prior we selected the Bayesian skyline coalescent with 10 as group number and piecewise linear as the skyline model. We tested both a strict clock and an uncorrelated lognormal-distributed relaxed clock. For both models three MCMC runs with 50,000,000 iterations were run, with 10,000 sampling frequency. We discarded 10% of the states from each run as chain burn-in and then combined the three independent runs for both models using LogCombiner v1.8.1 (included in the BEAST package) resulting in a total of 1.38 billion trees. The two models were compared with a marginal likelihood estimation using path sampling (PS) and stepping-stone sampling (SS) (ref. 34). The skyline tree prior in combination with a strict variation among tree branches performed better according to PS, while lognormal-distributed relaxed clock was best supported according to SS (Supplementary Table 7). The strict clock provided higher effective sample size (ESS) values because of earlier chain convergence; therefore, it was the chosen model to reconstruct a skyline plot for the 18 Neanderthal mtDNAs. We used Tracer v.1.6 selecting linear change as Bayesian skyline variant and a default of 100 as number of bins. In Supplementary Fig. 7 we report the mean $N_e$ (black line) and the 95% HPD interval (purple lines) of the Neanderthal mtDNAs in logarithmic value on the y axis and the temporal range from 350 to 32 ka on the x axis. We observe a $N_e$ reduction until around 42 ka followed by a rapid and short growth inversion, predating the Neanderthal disappearance.

For the dating analyses, we instead used a data set composed of 18 Neanderthal mtDNAs, including the branch length excluding HST. As described above, we removed the D-loop from the alignment and further excluded all positions containing gaps and missing data for a total of 15,334 positions. The best-supported model for this data set was again Tamura-Nei 93 with invariant sites as the best-supported model. We set a fixed mutation rate of $3.2 \times 10^{-8}$ sites $\times$ year$^{-1}$ calculated for the coding region of modern humans and modern mtDNAs and the rCRS using MUSCLE. We further measured the mtDNA diversities of the enlarged Neanderthal mtDNA data set with the Watterson’s estimator, as reported in ref. 2. We first prepared a multiple genome alignment of Neanderthal mtDNAs both including HST (18 sequences) and excluding HST (17 sequences) using MUSCLE. Then, the number of segregating sites (Ks) was used with a variation in the nucleotide sequence (DnaSP) v.5.10.01 (ref. 52). Finally, we calculated $K_s/16,595$, where $\theta_w$ is $N_e \mu_1/11$ to take into consideration the number of mtDNA sequences in each data set. Adding HST to the 17 Neanderthal mtDNAs, the number of segregating sites almost doubled (from 78 to 145), whereas $\theta_w$ increased from 1.37 $\times$ 10^{-6} to 2.50 $\times$ 10^{-6} (Supplementary Table 4). The latter value is closer to the mtDNA diversity estimated within three Denisovan mtDNAs (3.46 $\times$ 10^{-2}).

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Modelling the mitochondrial replacement. Under neutrality and assuming the Eurasian Neanderthal effective population size \((N_e)\) to be relatively small (that is, <10,000 \(N_e\) units), we calculated the mean time period necessary for an introgressing mtDNA lineage below 20% in frequency to reach fixation, when conditioning for that (Supplementary Table 8). This was computed using the following formula from Kimura and Ohta\(^{45}\), where \(N\) is the Eurasian Neanderthal \(N_e\) and \(p\) is the proportion of the introgressing mtDNA lineage. 

\[
T(p) = \frac{1}{1-p} \ln(1-p) 
\]

that was rederived from 40% for autosomes to 2N for mtDNA in ref. 35. Generations were converted into years assuming a generation time of 29 years (Supplementary Table 8). We also calculated the likelihood of a complete replacement as a function of the Neanderthal effective population size and the admixture rate from a branch basal to modern humans (Supplementary Note 5).

Data availability. The HST mtDNA consensus sequence reported in this paper is available in GenBank with the accession code KT751400. All other data are available on request to the corresponding authors.

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**Author contributions**

K.W., N.J.C. and C.J.K. provided archaeological material and related information. C.P., C.W. and K.K. performed laboratory work. C.W. and H.B. analysed isotopic data. C.P., L.P., L.v.H., F.R. and J.K. analysed genetic data. C.P., L.P. and J.K. wrote the manuscript with input from all co-authors.

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