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A Complete mtDNA Genome of an Early Modern Human from Kostenki, Russia

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Extract	Specimen	PEC Nea	PEC Hum	Contamination Exact binomial test PEC					PCR Nea	PCR Hum	Contamination Exact binomial test PEC				
				x	n	mean	lower	upper			x	n	mean	lower	upper
1	Vi33.11	22	0	0	22	0.00	0.00	0.15	4	27	27	31	0.87	0.70	0.96
2	Vi33.22	24	0	0	24	0.00	0.00	0.14	517	49	49	566	0.09	0.06	0.11
3	Vi33.26	25	0	0	25	0.00	0.00	0.14	583	327	327	910	0.36	0.33	0.39
4	Vi33.26	25	0	0	25	0.00	0.00	0.14	761	161	161	922	0.17	0.15	0.20
5	Vi33.28	30	0	0	30	0.00	0.00	0.12	5	25	25	30	0.83	0.65	0.94
6	Vi33.26	37	1	1	38	0.03	0.00	0.14	0	321	321	321	1.00	0.99	1.00
7	Sid1435	40	0	0	40	0.00	0.00	0.09	324	1184	1184	1508	0.79	0.76	0.81
8	Sid1416	40	0	0	40	0.00	0.00	0.09	656	341	341	997	0.34	0.31	0.37
9	Vi33.16	44	0	0	44	0.00	0.00	0.08	1887	231	231	2118	0.11	0.10	0.12
10	Vi33.16	47	0	0	47	0.00	0.00	0.08	410	186	186	596	0.31	0.28	0.35
11	Sid1253	50	0	0	50	0.00	0.00	0.07	97	0	0	97	0.00	0.00	0.04
12	Vi33.25	94	1	1	95	0.01	0.00	0.06	251	94	94	345	0.27	0.23	0.32
13	Vi33.25	128	1	1	129	0.01	0.00	0.04	359	70	70	429	0.16	0.13	0.20
14	Vi33.25	152	1	1	153	0.01	0.00	0.04	161	61	61	222	0.27	0.22	0.34
15	Vi33.26	162	1	1	163	0.01	0.00	0.03	152	18	18	170	0.11	0.06	0.16
16	Vi33.16	174	1	1	175	0.01	0.00	0.03	477	81	81	558	0.15	0.12	0.18
17	Vi33.16	176	0	0	176	0.00	0.00	0.02	103	0	0	103	0.00	0.00	0.04
18	Vi33.16	272	0	0	272	0.00	0.00	0.01	243	48	48	291	0.16	0.12	0.21
19	Sid1253	405	0	0	405	0.00	0.00	0.01	388	42	42	430	0.10	0.07	0.13
20	Vi33.31	453	1	1	454	0.00	0.00	0.01	402	3	3	405	0.01	0.00	0.02
21	Vi33.16	465	0	0	465	0.00	0.00	0.01	111	1	1	112	0.01	0.00	0.05

Table S1 (related to Figure 1). Neandertal DNA extracts used for the PCR and PEC contamination assay. Contamination estimates based on exact bionomial tests are given as mean, maximum and minimum fractions of total recovered PEC or PCR clone sequences of the modern human type.

	diagnostic positions	diagnostic fragments	endogenous fragments	contaminating fragments
Feld2	134	1895	403	1492
Mez2	134	1063	529	534
Teshik Tash	134	225	5	220
Kostenki 14	4	77	76	1

Table S2 (related to Figure 4). For each sample, the number of mitochondrial positions which differ from >99% of a panel of 311 modern human mtDNA genomes as well as the number and identity of informative

fragments overlapping these positions is shown. Fragments were considered non-informative, if the difference could be due to cytosine deamination [1].

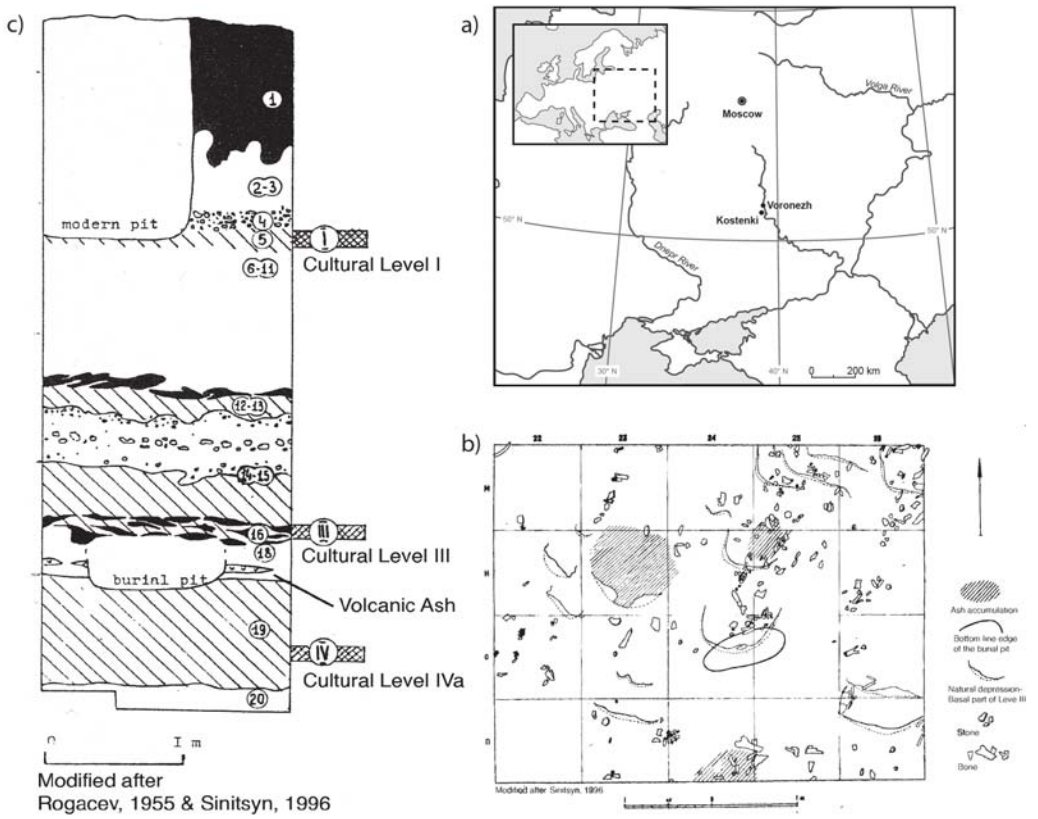

Figure S1 (related to description of the Kostenki 14 - Markina Gora site). a) Location of the Kostenki site, b) section showing stratigraphic location of the burial and c) Kostenki 14 level III plan view. The outline of the bottom of the burial pit is marked by the oval circle. A natural depression filled with burned sediment belonging to level III was found above the burial pit. This depression extends over the edges of the pit, providing a minimum stratigraphic age of the skeleton corresponding to the age of level III.

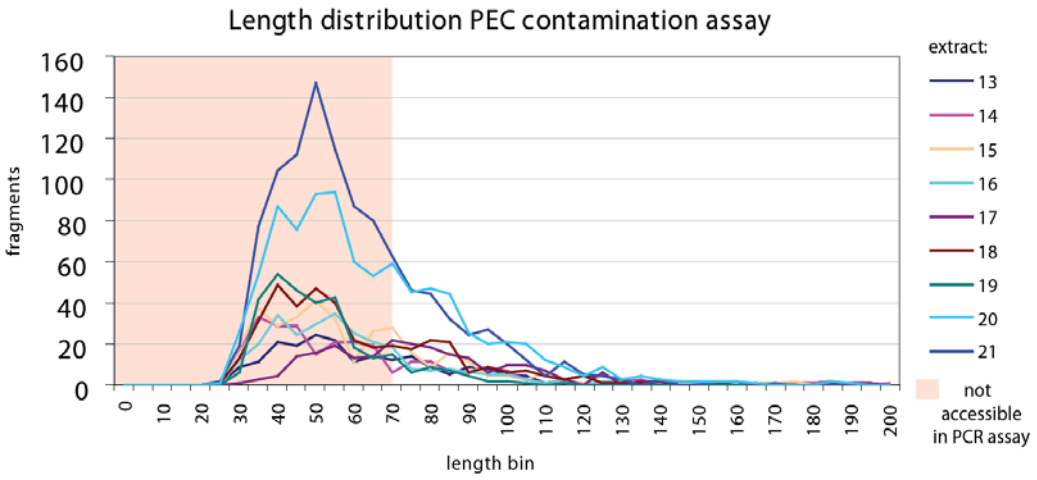

Figure S2 (related to Figure 1B). Length distribution of mtDNA fragments captured from 9 of the 21 Neandertal extracts, in which more than 200 unique mtDNA fragments could be recovered. Fragments below 67bp could not be amplified in the PCR-based contamination assay.

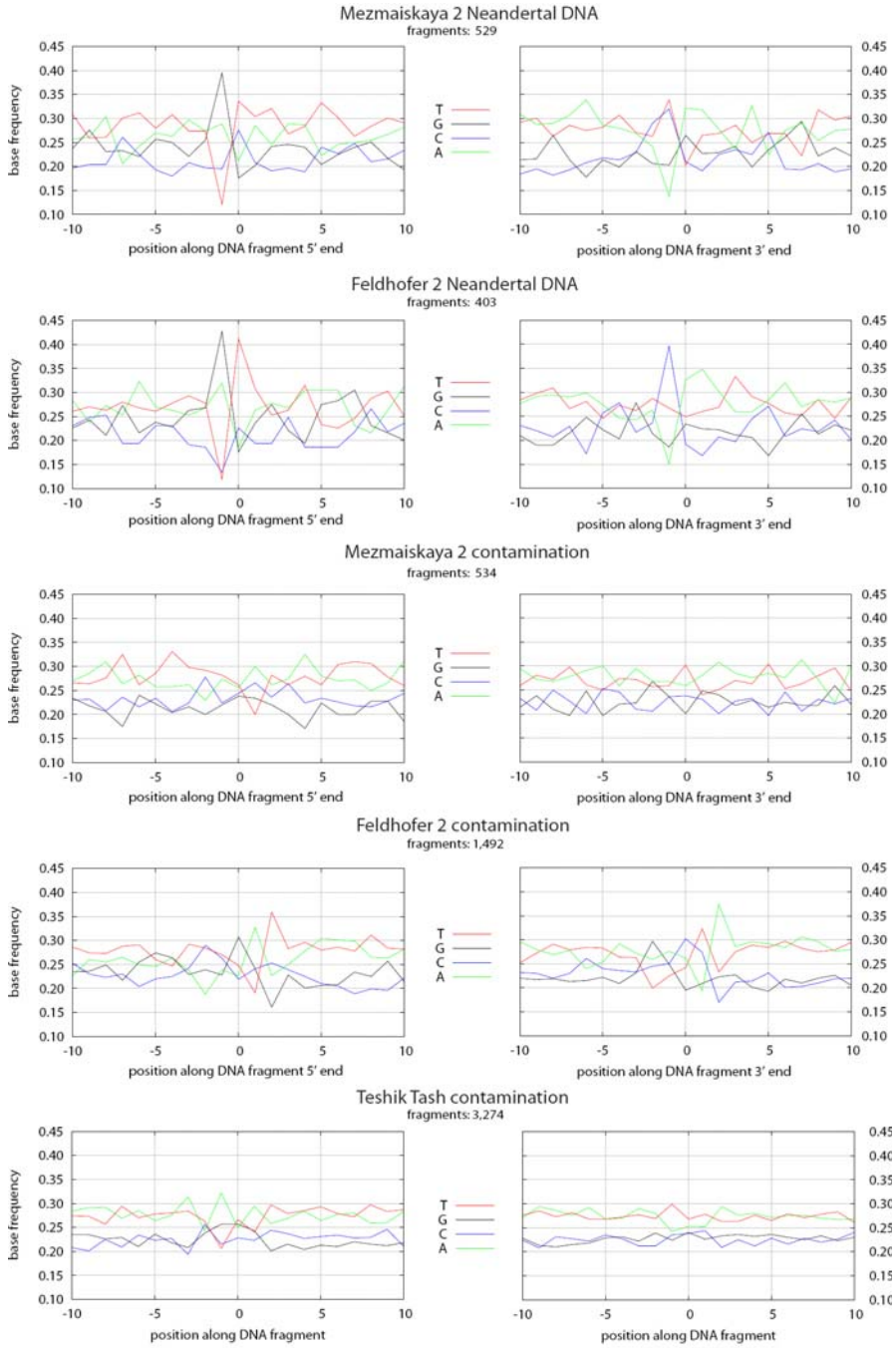

Figure S3 (related to Figure 2). Nucleotide base frequency for mtDNA fragments captured by PEC from the three contaminated Neandertals. Negative numbers on x-axis refer to positions in the aligned reference sequence outside of the fragment.

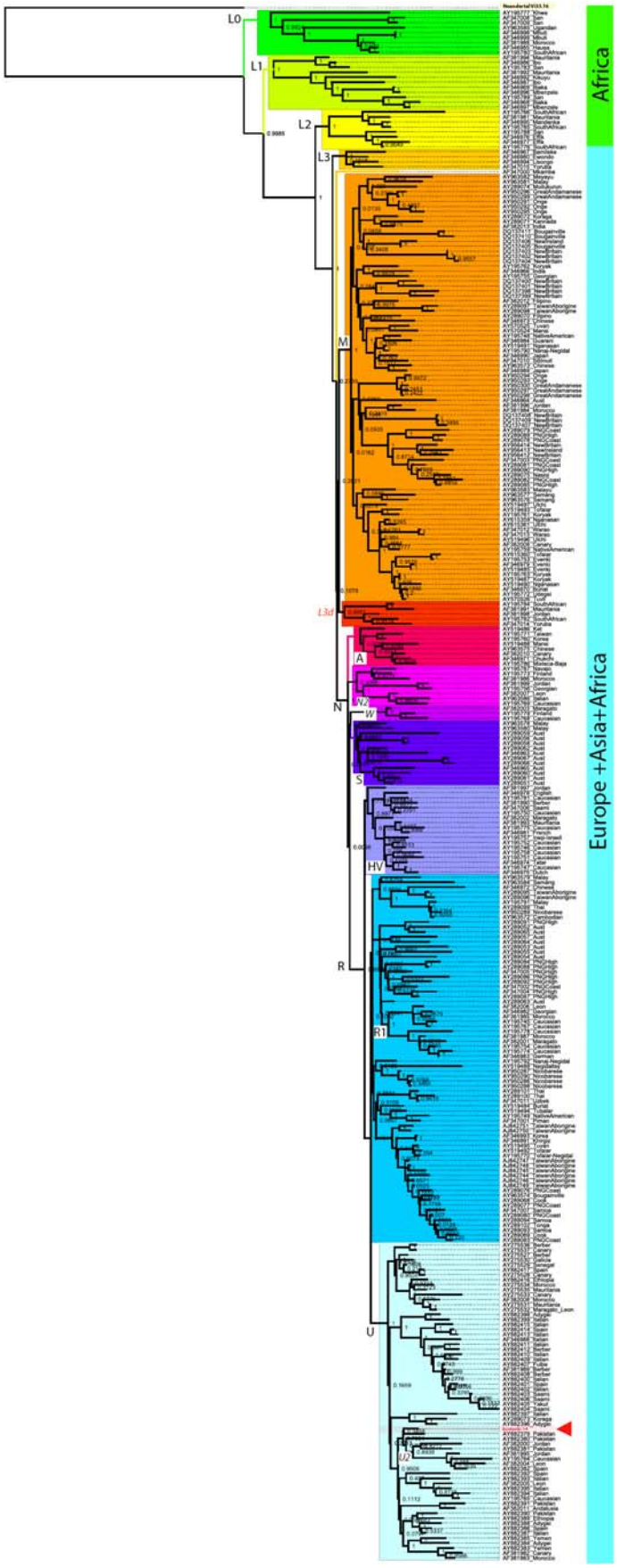
L0
L1
L2
L3
M
L3d
A
N
W
S
HV
R
R1
U
U2
Africa
Europe +Asia+Africa

Figure S4 (related to Figure 3D). Phylogenetic tree of the 311 mtDNAs and the Kostenki 14 sequence using the Neandertal Vi33.16 as an outgroup. The phylogeny was estimated with a Bayesian approach under a GTR+I+Γ model of sequence evolution. Major haplogroups indicated by different colours are given beside each major node. The posterior probability can be found next to each node.

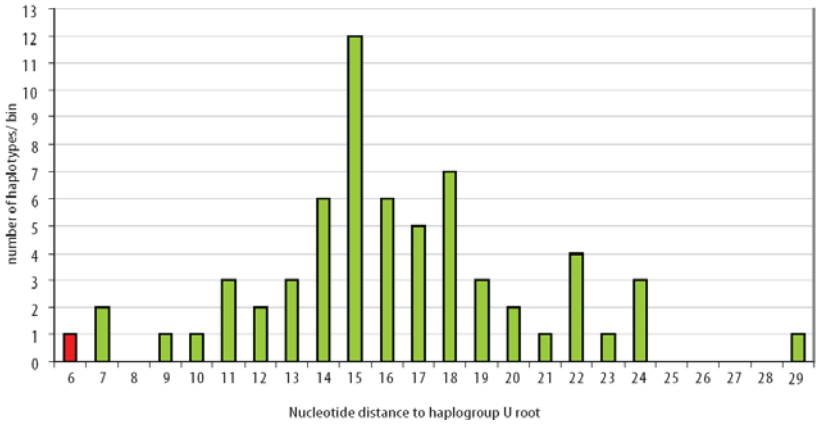

Figure S5 (related to Figure 3D). Histogram of the nucleotide distances to the root of the U haplogroup for all 63 mtDNAs falling into haplogroup U in the worldwide dataset of 311 contemporary mtDNAs (green), as well as the nucleotide distance of the Kostenki 14 consensus sequence (red).

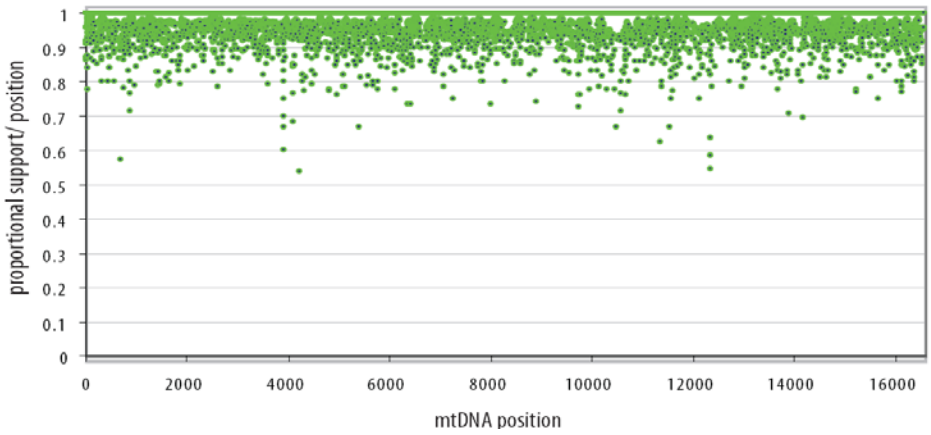

Figure S6 (related to Figure 3E). Support for the Kostenki 14 assembly. At each mtDNA position, the fraction of bases agreeing with the consensus base is shown.

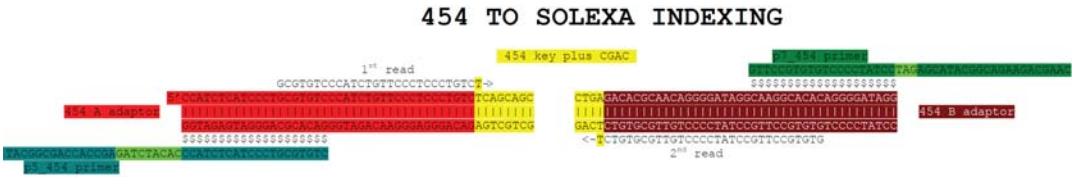

Figure S7 (related to Illumina/Solexa conversion and sequencing). Adapter design. The initial adapter ligation was done using 454 A-adapter (red) and B-adapter (brown). The 454 to Solexa conversion was done by PCR using the p5_454 and p7_454 custom designed primers. For sequencing primers were used that match the 454 A-adapter for the forward read and the 454 B-adapter for the reverse read.

Supplemental Experimental Procedures

Description of the Kostenki 14 - Markina Gora site and burial

The Kostenki 14 skeleton was discovered in 1954, in a pit of oval form (99 cm length and 39 cm width) about 35 cm below the cultural level III at the site of Kostenki, Russia (Figure S1). Only small flint and animal bones splinters were found in the pit fill, together with a hare vertebra and scapula, and a mammoth phalange. The skeleton was found lying on its left side, in an extreme crouched position, with the long axis aligned east-west [2]. The face was oriented downward with fists reaching the mandible. The bottom of the pit, the skeleton and especially the skull were covered by red ochre [2, 3]. The red ochre covering as well as the unusual position suggests an intentional burial [3].

Based on metric analysis of the pelvis, the sex of the individual was determined to be male and the age was estimated according to the teeth and cranial sutures to be at least 20 years old [3]. One of the most atypical features is the low cranial capacity (1160-1170 cc), putting this specimen at the lower edge of the EMH range. It has one of the smallest brain cases in the European Upper Paleolithic record [4, 5].

Direct dating of the skeleton performed in two different labs has yielded incoherent results: 4,705±40 BP[1] (OxA-7126) and 3,730 ±40 BP[1] (GrA-9303) [6]. An additional attempt using AMS radiocarbon dating with a pre-treatment including the Longin [7]

[1] All dates mentioned in the text are uncalibrated radiocarbon dates

method and an additional ultrafiltration step [8] in Leipzig failed. The C/N ratio of this sample was 4.13 (Table S3). The C/N ratio comprises the ratio of the total carbon to the total nitrogen present in the collagen extracted from the bone. It has been suggested that ratios higher than 4 indicate the presence of large amounts of exogenous carbon [9]. In this case the exogenous carbon may result from the conservation measures that were taken to preserve the bone [6] and may, therefore, also explain the anomalously young radiocarbon dates. As a result, it seems impossible to get a reliable radiocarbon date directly from the Kostenki 14 skeleton.

Table S3. Isotope values for the Markina Gora – Kostenki 14 bone.

Material	$\delta^{13}C$	$\delta^{15}N$	%Coll	%C	%N	C:N
Ulna	*-20.97*	*11.81*	8.1	*49.70*	*14.05*	***4.13***

In the absence of direct dating of the skeleton, the stratigraphic position of the pit is the best indicator of the age of the specimen. The burial pit was found below cultural level III (Figure S2). The upper part of the pit was cut by a semi-circular depression. Several of these depressions are associated with level III and exhibit burned sediment at their bottom, suggesting that they were used as fireplaces. It can be seen from the original plan drawing of level III (Figure S3) that the semi-circular depression above the burial pit extends over the edge of the burial pit and must, therefore, post date it. This stratigraphic association provides a minimum age for the skeleton of cultural level III. Cultural level III yielded an Upper Paleolithic blade industry including bone tools with a sub-rectangular section (rods) and beads made from bird long bones (3,7). Despite the lack of very typical elements, this assemblage is usually attributed to the Gorodsovian tradition [6]. Radiometric dates for this level indicate an age of about 30 ka[1] [10] (Table S4). Cultural level II, dated to around 27-25 ka[1] [10] (Table S4), is absent in this part of the site. However, cultural level I, which is stratigraphically about 3 m above the burial and undisturbed, corresponds to a well defined recent Gravettian with shouldered points (Kostenki-Avdeevo group) dated to around 22-20 ka[1] [2, 11] (Table S4, Figure S2). Near its base the pit cuts through an ash layer making it clearly younger than this ash which has subsequently been identified as coming from the campanian ignimbrite eruption [12]. The CI tephra is dated to circa 33 ka[1] at Kostenki 14 and contains a cultural level attributed to Aurignacian [12, 13]. Thus, based its stratigraphic context, the

maximum age of the skeleton is circa 33 ka[1], while the skeleton's association with the Gorodsovian layer III provides a minimum age of around 30 ka.

Table S4. Uncalibrated radiocarbon dates from cultural layer I-III [10, 14-16].

Date	Lab #	Material
Cultural layer I		
19,700±1300	LE-5567	mammoth bone
19,900±850	GIN-8024	mammoth rib
20,100±1500	LE-5269	bone
22,500±1000	LE-5274	bone
22,780±250	OxA-4114	bone
Cultural layer II		
19,300±200	LE-1400	bone
25,600±400	GIN-8030	bone
26,400±660	LU-59a	bone (fr. A)
28,200±700	LU-59b	bone (fr. B)
26,700±190	GrA-10954	charcoal
27,860±270/260	GrA-13292	charcoal
29,240±330/320	GrA-13312	charcoal
28,380±220	GrN-12598	charcoal
28,580±420	OxA-4115	bone
Cultural layer III		
28,370±140	GrA-15960	charcoal
29,320±150	GrA-15955	charcoal
30,080±590/550	GrN-21802	charcoal
31,760±430/410	GrA-13288	charcoal
Cultural layer (volcanic ash)		
32,420±440/420	GrA-18053	charcoal

Neandertal DNA extraction and contamination assays

In order to test the amount of human contamination 21 DNA extracts were made from 80 -100mg of ten different Neandertal bones (Table S1) as previously described [17]. The PCR contamination analysis was performed as described [1, 18] using up to three primer pairs in a Multiplex 2-step-PCR [19] targeting conserved mtDNA regions that show informative positions where humans and Neandertals differ in DNA sequence. The retrieved PCR products were tagged [20], and sequenced on the *Roche 454* platform.

For the PEC contamination test 23 µl from each Neandertal DNA extract were made into 454 sequencing libraries as described [21]. Before PEC, each library was amplified using the same primers used for 454 emulsion PCR (emPCR) [22]. The

PEC protocol was performed as described [21] using 6 biotinylated PEC primers that target informative differences between human and Neandertal mtDNA [1]. After the last spin column purification, PEC products were quantified with qPCR and used directly as templates for emulsion PCR [23].

Emulsion PCR and 454 sequencing

Emulsion PCR and 454 sequencing were carried out according to manufacturer's instructions on the *Roche 454 FLX* platform.

Kostenki 14 DNA extraction and PEC mtDNA enrichment

DNA was extracted from 100mg of bone sampled from the Kostenki 14 ulna as described [17], with one modification the DNA elution from the silica particles was done using TE buffer with 0.05% Tween 20. We used siliconized tubes for long-term storage of the DNA extract. For the sampling of the bone the surface of the bone was cautiously removed and, using a dentist drill, only interior bone material obtained. Before PEC, 50µl of extract were made into a 454 library using a modified protocol as described [21] with one modification that another A-adapter was used in the ligation step. The specific A-adapter was designed to avoid contamination from other 454 sequencing libraries [24, 25]. Unlike the A-adapter used for Neandertal libraries this adapter carried a standard 454 key sequence TCAG but was designed to have the additional four bases CGAC at the 3' end right after the key sequence. Fragments that did not show this specific tag were later on excluded from analysis. The PEC protocol was performed as described [21] using the exact same four 144-plex and 143-plex mixes used to retrieve the four complete Neandertal mtDNAs [21]. These primers are 5'-biotinylated and consist of a universal twelve-base 5' spacer sequence (CAAGGACATCCG) followed by a specific primer sequence. To design the specific primer sequences, Primer3 [26] was used to find all possible primer sites on the light strand of the Vindija 33.16 Neandertal mtDNA sequence [1]. Since the Neandertal mtDNA falls outside modern human variation this primer set will not preferentially target any specific modern human mtDNA variants.

Illumina/Solexa conversion and sequencing

After the final spin column purification into 50µl TE buffer, the PEC products were converted to *Solexa* libraries and sequenced on the *Illumina GA2* platform. For this purpose a PCR primer pair was constructed that is complementary to the 454 A and B primers on the 3'-ends and has a tail carrying the Solexa p5 and p7 adapter sequences. The 454 library was than amplified in a 100µl reaction containing 50µl

Phusion™ High-Fidelity Master Mix, 500nM of each 454-Solexa-conversion primer and 10µl PEC product template (Figure S7). Annealing temperature was 60°C and a total of 10 cycles of PCR were performed. The amplified products were spin column purified and quantified on an Agilent 2100 Bioanalyzer DNA 1000 chip. The PEC product was than diluted and sequenced according to *Illumina* GA2 protocols on a paired end run with a total of 102 cycles.

The sequencing run was analyzed starting from raw images using the Illumina Genome Analyzer pipeline 1.3.2. The first five sequencing cycles were used for cluster identification. After standard base calling, reads of the PhiX 174 control lane were aligned to the corresponding reference sequence to obtain a training data set for the base caller Ibis [27]. Raw sequences obtained from Ibis for the two paired end reads of each sequencing cluster were merged (including adapter removal) requiring at least 11nt overlap between the two reads. For bases in the overlap, quality scores were summed up. In cases where different bases were called, the base with the higher quality score was chosen.

Assembly procedure

The 4,066,269 merged sequences were used as input for a custom iterative mapping assembler (MIA, Green unpublished, available from the authors upon request). In the first assembly round, sequences were aligned to the revised Cambridge Reference Sequence (NC_012920.1, [28]). The mapper uses a position-specific scoring matrix designed to capture the most relevant features of nucleotide misincorporations affecting ancient DNA sequences. In total 938,882 (23%) of the merged sequences aligned to the reference mtDNA. Since several amplification steps were performed, the aligned sequences were filtered for uniqueness by grouping sequences with the same direction, start and end coordinates. From each such cluster a consensus sequence was made by taking for each position the base with the highest quality score. That resulted in a total of 10,664 distinct sequences. These were then used to call a consensus, after that a second round of alignment to this consensus reference was performed, after which a new consensus was called. This process was iterated until convergence on the final consensus sequence after 3 rounds. The assembly covers all positions in the mtDNA at least twice with an average coverage of 31.2 for each position. The 70 positions that are only covered twice are all consistent. In order to check if the sequence coverage was high enough to overcome nucleotide misincorporations, the proportion of reads that matched the consensus base at each position was calculated (Figure S6). Among a total of 16,566 positions the average

fraction of sequences that agreed with the consensus sequence was 98%. For 23 positions support was less than 75% for the consensus base. In 22 of these cases, coverage varied between 3- and 19-fold and the minority base could be attributed to nucleotide misincorporations. One position (pos. 4,216) differed from the other 22 positions in that it had had 39-fold coverage and 17 sequences carried a T while 22 carried a C (resulting in a total overage of 39). Since positions where the consensus sequence is unique (compared to the present-day human mitochondrial genomes analyzed) suggest that mtDNA from a single individual was present in the sample, we argue that this position is either due to heteroplasmy in this individual or due to an unusually high rate of deamination.

We compared the assembly obtained by MIA to the fast mapper bwa [29] which was used in an iterative manner with parameters -n 0.01 -o 2 in conjunction with the consensus caller in the samtools package [30] with heterozygosity parameters r and G set to zero. We mapped all reads to the revised Cambridge Reference Sequence (extended by 10 nt at the beginning copied from the end of the sequence), filtered for reads with identical strand, start and end position, retained the sequence with the highest sum of quality score and called a consensus. The new consensus was used for mapping in the next iteration until the process converged in the third iteration. We observe four differences to the MIA assembly – all of which can be explained by deamination-induced nucleotide misincorporations which are not considered by bwa and samtools.

Evolutionary analysis

The Kostenki consensus sequence was manually added to the alignment of 311 mtDNAs and the Vindija 33.16 Neandertal mtDNA [9]. A Phylogenetic tree was constructed in a Bayesian framework using MrBayes 3.1.2 [31], with a GTR+I+Γ model of substitutions and default parameters for the MCMC, with 5,500,000 generations sampling every 100 generations and a burn-in of 1,000,000 generations. We observed stationarity (using Tracer 1.4 [32]) after 1,000,000 generations. A consensus tree from all 45,000 generated trees was calculated using TreeAnnotater V.1.4.8 [32] (Figure S4). The major haplogroups were annotated manually based on the information available from Phylotree.org-mtDNA tree build 5 [33].

The most recent common ancestral sequence (MRCA) of haplogroup U was inferred by taking the 63 sequence that according to Phylotree.org [33] carry the substitutions that define haplogroup U (11,467, 12,308, 12,372), identifying the sequence that carries the fewest additional substitutions (AY275530) and reverting these back to

the ancestral state for haplogroup U. For each of the 63 sequences as well as the Kostenki sequence the number of observed nucleotide differences to the ancestral sequence was then determined using MEGA 4.1 [34] (Figure S5).

We observe on average 16.34 nucleotide differences between the 63 human mtDNAs and the inferred MRCA. Assuming a Poisson distribution of substitutions, it is unlikely to observe only 6 substitutions if the Kostenki mtDNA lived now ($p<0.005$). The average length of the lineage from the MRCA to the Kostenki mtDNA is 36.7% of the entire distance to the present, with a 97.5% confidence interval of 79.9% to 13.5%. Given an age of 51,700 ± 6,200 years for the MRCA [35], this corresponds to an age of 32,730 y (CI: 10,400 - 44.700 y).

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