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LETTERS

Neanderthals in central Asia and Siberia

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Morphological traits typical of Neanderthals began to appear in European hominids at least 400,000 years ago[1] and about 150,000 years ago[2] in western Asia. After their initial appearance, such traits increased in frequency and the extent to which they are expressed until they disappeared shortly after 30,000 years ago. However, because most fossil hominid remains are fragmentary, it can be difficult or impossible to determine unambiguously whether a fossil is of Neanderthal origin. This limits the ability to determine when and where Neanderthals lived. To determine how far to the east Neanderthals ranged, we determined mitochondrial DNA (mtDNA) sequences from hominid remains found in Uzbekistan and in the Altai region of southern Siberia. Here we show that the DNA sequences from these fossils fall within the European Neanderthal mtDNA variation. Thus, the geographic range of Neanderthals is likely to have extended at least 2,000 km further to the east than commonly assumed.

The partial skeleton of an 8–10-year-old child discovered in the late 1930s in Teshik-Tash Cave, Uzbekistan, is generally accepted to represent the easternmost extent of the Neanderthal range[3]. However, its Neanderthal affinities have been disputed[4,5]. Further to the east in the Altai region of Siberia, human remains have been found in association with Mousterian lithic technology, which is usually associated with Neanderthals in Europe but is also found in association with modern humans in the Near East and northern Africa[6]. For example, teeth found at Okladnikov Cave in the Altai Mountains, which are dated between 37,750 ± 750 (1σ) and 43,700 +1,100/−1,300 years BP[7] (see also Supplementary Table 1), have been suggested to stem from Neanderthals[8]. However, others have suggested that they come from modern humans with some Asian *Homo erectus* traits[9]. Okladnikov Cave has also yielded four postcranial bones: a middle phalanx and a distal humerus fragment of adults and the distal halves of the humerus and femur from what is likely to be a single subadult individual[10]. Although the fragmentary adult humerus cannot be assigned to either Neanderthals or modern humans, the subadult remains and the adult phalanx have been suggested not to be of modern human origin[10].

To determine whether the Teshik Tash and Okladnikov individuals are genetically affiliated with European Neanderthals, we attempted to retrieve mtDNA from the left femur of Teshik Tash and the three fragmentary long bones from Okladnikov. So far, mtDNA sequences have been determined from 13 Neanderthals in Europe[11–20]. Comparison of these DNA sequences with those of mtDNAs from contemporary humans shows that the Neanderthal mtDNA gene pool was distinct from that of modern humans[16,17,21].

We extracted DNA from samples (about 200 mg) from each of the four bones and amplified DNA with the use of three different primer pairs. Each product was cloned and multiple clones were sequenced. From the Teshik Tash specimen, 88 of 90 clones from a 63-base-pair (bp) amplification product generated by primers that amplify modern human as well as Neanderthal mtDNA[22] had sequences identical to those of modern human mtDNAs, whereas two were similar to previously determined Neanderthal sequences with one additional substitution at position 16,242 (C→T) when compared with the revised Cambridge reference sequence (RCRS)[23]. A 119-bp product[22] revealed 156 clones identical to those from modern humans and no clones with similarity to Neanderthal mtDNAs, whereas a Neanderthal-specific primer pair[16] retrieved Neanderthal-like mtDNA sequences (including the substitution at position 16,242). For the subadult humerus from Okladnikov, sequences from 2 out of 104 clones from the 63-bp product were found to be identical to previously amplified Neanderthal sequences, whereas none of 103 clones from the 119-bp product was Neanderthal-like. For the subadult femur and the adult humerus, neither the shorter nor the longer products yielded any Neanderthal-like sequences. By contrast, the Neanderthal-specific primer pair retrieved products from the two subadult remains but not from the adult humerus (Supplementary Table 3).

These results show that both the subadult individual from Okladnikov Cave and the Teshik Tash individual carried mtDNA of the Neanderthal type, whereas there is no indication that the adult individual from Okladnikov did so. The high ratio of modern human DNA to Neanderthal DNA for the subadult Okladnikov and the Teshik Tash specimens are in agreement with previous observations that modern human mtDNA occurs in most fossil bones[16,24], where it often outnumbers endogenous mtDNA[22]. Next, we directly dated the adult and subadult humerus from Okladnikov. Although the adult bone yielded an uncalibrated ^{14}C date of 24,260 ± 180 years BP, the subadult bone yielded uncalibrated dates ranging from 29,990 ± 500 years BP to 37,800 ± 450 years BP (see discussion in Supplementary Information, and Supplementary Tables 1 and 2), indicating that the latter bone is old enough to be of Neanderthal origin.

We then designed primers that amplify Neanderthal mtDNA preferentially, to determine larger parts of the hypervariable region I (HVRI) from the Teshik Tash and Okladnikov specimens found to carry Neanderthal-like mtDNA. We reconstructed 190 bp of the HVRI from the Teshik Tash specimen (corresponding to positions 16,130–16,319 of the RCRS) by eight overlapping fragments in which each fragment was independently amplified at least twice, and all products were sequenced from multiple clones (Supplementary Table 6). The resulting sequence shows 22 substitutions relative to the RCRS (Supplementary Table 4). Of these, 13 have been found in all previously studied Neanderthals, six are known Neanderthal polymorphisms, and three have not previously been observed among Neanderthals (positions 16,242, 16,274 and 16,319). The remaining

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HVRI segments from the Teshik Tash specimen could not be determined because it was not possible to design Neanderthal-specific primers spanning the entire HVRI given the short length of amplifications possible from this extract.

From the Okladnikov subadult humerus, the complete HVRI sequence between positions 16,035 and 16,378 was determined by using 16 overlapping fragments (Supplementary Table 7). It carries 22 substitutions relative to the RCRS (Supplementary Table 4), 16 of which are shared with all previous sequenced Neanderthals, four are known polymorphisms among Neanderthals, and two (positions 16,172 and 16,263) have not been seen before.

To ensure the reproducibility of the DNA sequences, bone samples of the Teshik Tash child and the Okladnikov subadult were sent to a DNA laboratory in Lyon that specializes in the analysis of ancient DNA and that did not have access to the results obtained in Leipzig. There, extracts were prepared and 48 bp of the HVR1 spanning positions 16,231–16,277 were amplified in two overlapping fragments. This HVR1 segment contains sequence positions that are common to all Neanderthals analysed so far, for example a Neanderthal-specific insertion at position 16,263b, as well as positions unique both to the Teshik Tash individual (16,242 and 16,274) and the Okladnikov individual (16,263), respectively. The consensus sequences of 67 clones for Teshik Tash and 51 clones for Okladnikov sequenced in Lyon (Supplementary Table 8) were identical to the corresponding sequences determined in Leipzig.

The HVR1 sequences from Okladnikov together with seven other Neanderthal sequences that span at least positions 16,078 to 16,378 (see Methods) were used to estimate the mean pairwise mtDNA sequence difference between Neanderthals across their entire currently known geographical range. Whereas for Neanderthals this difference is found to be 5.5 substitutions, it is 8.1 substitutions for 1,467 contemporary Africans, 6.3 substitutions for 3,217 Asians and 4.0 for 2,667 contemporary Europeans (Supplementary Table 5). Thus, mtDNA diversity in Neanderthals seems to have been within the range of present-day Europeans and Asians but significantly lower ($P = 0.0255$) than the diversity of present-day Africans (Supplementary Fig. 2).

The similarity of the Okladnikov and Teshik Tash mtDNA sequences to mtDNA determined from Neanderthals in Europe and the Caucasus, in conjunction with the absence of Neanderthal-like mtDNA from the more than 10,000 modern humans studied so far as well as from early modern humans[16,25], indicates that the Teshik Tash and Okladnikov individuals belonged to a population related to European and western Asian Neanderthals. This agrees with morphological evidence that the Teshik Tash hominid is of Neanderthal origin[3] and the suggestion that the subadult Okladnikov individual is related to Neanderthals on the basis of the morphology of teeth found in association with the bones[8]. The geographical range of Neanderthals therefore seems to have extended at least 2,000 km farther east into southern Siberia than has generally been assumed (Fig. 1).

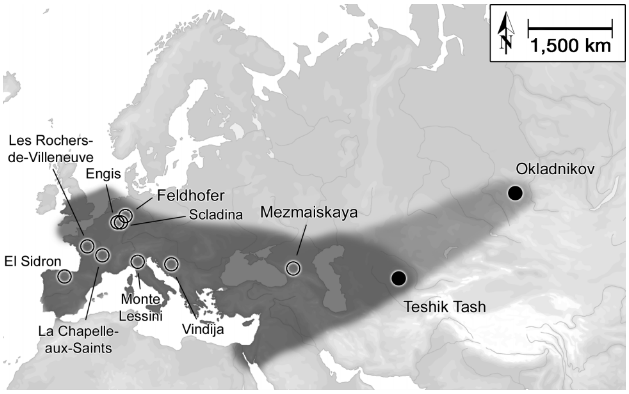

Figure 1 | Geographical range of Neanderthals. The previously known Neanderthal range based on the morphology of fossils[30] is indicated in dark grey; the Neanderthal range based on mtDNA is indicated in light grey. Sites where mtDNA sequences of the Neanderthal type were detected previously are shown as open circles, and the two sites presented in this study are indicated by black dots.

When the relationship of the Teshik Tash and Okladnikov mtDNA sequences to other Neanderthal mtDNA sequences is estimated (Supplementary Fig. 3), the Teshik Tash mtDNA sequence seems to be more closely related to the mtDNA sequence from Scladina in western Europe than to the sequence from Okladnikov. Further Neanderthal mtDNA sequences from across their range and from different time horizons will obviously be necessary to permit insights into how Neanderthals colonized both western and central parts of the Old World. However, the fact that no deep mtDNA divergence is seen between the central Asian Neanderthals and European and Caucasian Neanderthals shows that they were not separated for a long time. This supports the view that central Asia was colonized relatively recently by Neanderthals[26]. In fact, it has been suggested that Neanderthals did not colonize most of the Russian plains before an exceptionally warm episode 125,000 years ago[27], during which the Caspian Sea was drastically reduced in size. This may have facilitated the expansion of Neanderthals into central Asia and Southern Siberia[26]. Intriguingly, their presence in southern Siberia raises the possibility that they may have been present even farther to the east, in Mongolia and China. Further work will be necessary to address this possibility.

METHODS SUMMARY

DNA extraction was performed in a laboratory dedicated exclusively to work on ancient DNA. About 200 mg of bone was powdered and extracted as described[28]. To minimize the loss of material, a two-step multiplex polymerase chain reaction (PCR)[29] in a total volume of 20 µl containing up to nine primer pairs was performed. In total, 60 cycles of PCR were completed, 27 in the first step and 33 in the second. All reaction conditions were as described except for the annealing temperature, which was 55 °C for all primer pairs used. Amplification products of the correct size were cloned with the TOPO TA cloning kit (Invitrogen), and 3–17 clones were sequenced with an ABI3730 capillary sequencer (Applied Biosystems) for each product. In total, nine primer pairs in overlapping fragments with a length of 61–85 bp were used to reconstruct the 192 bp of the Teshik Tash sequence (Supplementary Table 6). For the 345 bp of the Okladnikov subadult humerus HVR1 sequence, 17 primer pairs were used, ranging from 61 to 109 bp (Supplementary Table 7). Every position was amplified independently at least twice, and for a third time where a difference between all clones from one amplification and all clones from another amplification was observed. Amplification and sequencing procedures in the Lyon laboratory are given in Supplementary information.

Several previously published Neanderthal HVR1 sequences that span at least positions 16,078–16,378 of the RCRS and Okladnikov (subadult humerus) were used to calculate the mean pairwise difference as a measure of mtDNA diversity within Neanderthals. Sequence alignments of the 2,667 European, 3,217 Asian and 1,467 African modern human HVR1 sequences were retrieved from the HvrBase++ site (www.HVRbase.org) and restricted to positions 16,078–16,378. The mean pairwise difference for the eight Neanderthal and three modern human HVR1 sequence alignments was calculated with the software MEGA3.1. From each of the three modern human populations, eight sequences were randomly extracted without replacement and the mean of their pairwise differences was calculated 10,000 times (Supplementary Table 5). The mean pairwise difference among the Neanderthal sequences falls in the lower 5% ($P = 0.0255$) of the values observed for Africans (Supplementary Fig. 2).

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions A.D. provided Neanderthal samples and palaeontological information; S.P. and D.S. collected the samples; B.V. and J.J.H. provided palaeontological and archaeological information; M.P.R. performed dating; J.K., L.O. and D.S. extracted ancient DNA; J.K. and L.O. amplified and sequenced DNA; J.K. performed the phylogenetic analyses and the statistical analysis in cooperation with K.P.; C.H. coordinated the work in Lyon; S.P. initiated, planned and coordinated the study; J.K. and S.P. wrote the paper.

Author Information The Teshik Tash and Okladnikov Neanderthal sequences are deposited in GenBank under accession numbers EU078679 and EU078680, respectively. Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to S.P. (paabo@eva.mpg.de).