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Genomic Differentiation of Neanderthals and Anatomically Modern Man Allows a Fossil–DNA-Based Classification of Morphologically Indistinguishable Hominid Bones

Michael Scholz,[1] Lutz Bachmann,[5] Graeme J. Nicholson,[2] Jutta Bachmann,[3] Ian Giddings,[6] Barbara Rüschoff-Thale,[7] Alfred Czarnetzki,[1] Carsten M. Pusch[4]

[1]Osteological Collection, [2]Institute of Organic Chemistry, and [3]Institute of Cell Biology, University of Tübingen; and [4]University Eye Hospital II, Molecular Genetics Laboratory, Tübingen, Germany; [5]Pritzker Lab for Molecular Systematics and Evolution, The Field Museum, Chicago; [6]Institute of Cancer Research, Haddow Laboratories, Sutton, United Kingdom; and [7]Westfalian Museum of Archeology, Münster, Germany

Southern blot hybridizations of genomic DNA were introduced as a relatively simple fossil–DNA-based approach to classify remains of Neanderthals. When hybridized with genomic DNA of either human or Neanderthal origin, DNA extracted from two Neanderthal finds—the *Os parietale*, from Warendorf-Neuwarendorf, Germany, and a clavicula, from Krapina, Croatia—was shown to yield hybridization signals that differ by at least a factor of two compared to the signals obtained with the use of fossil DNA of an early *Homo sapiens* from the Vogelherd cave (Stetten I), Germany. When labeled chimpanzee DNA was used as a probe, Neanderthal and human DNA, however, revealed hybridization signals of similar intensity. Thus, the genome of Neanderthals is expected to differ significantly from the genome of anatomically modern man, because of the contrasting composition of repetitive DNA. These data support the hypothesis that Neanderthals were not ancestors of anatomically modern man.

Introduction

Neanderthals (*Homo neanderthalensis*) represent typical middle-Paleolithic hominids in Europe and in the Middle East who existed for ⩾200,000 years. The proximity in time to anatomically modern man has raised questions about the coexistence of these two hominid forms (Shreeve 1995). It is controversial as to whether Neanderthals (1) should be regarded as direct ancestors of anatomically modern man, (2) actually contributed by hybridization to the gene pool of *H. sapiens* before becoming extinct, or (3) evolved totally independent of anatomically modern man (Trinkaus and Shipman 1993, pp. 46–90; Bräuer and Stringer 1997).

Skeletal remains hold the only key to our understanding of the evolution of these two hominid taxa. Significant differences between Neanderthals and anatomically modern man can be observed in some aspects of bone morphology (Henke and Rothe 1994, pp. 483–500; Schwartz and Tattersall 1996), but individual variability in morphology may result in similarities of bones or bone fragments from the two lineages. A correct classification is therefore an essential prerequisite for any interpretation of a find.

Recently, Krings et al. (1997, 1999) showed that it is possible to extract and analyze DNA from Neanderthal fossils—in particular, from the Neanderthal found near Düsseldorf in 1856. They succeeded in inferring 333 bp of the hypervariable region I and 340 bp of the hypervariable region II of mitochondrial DNA (mtDNA). The divergence of mtDNA lineages of modern humans and the Neanderthal was estimated to date from 465,000 years before the present (BP), with confidence limits of 317,000 and 741,000 years. Their data indicate that it is highly unlikely that Neanderthals contributed to the mtDNA pool of modern humans.

The application of specific DNA analyses now allows scientists to enlarge the methodological repertoire in the classification of skeletal remains, which until now was based to a large extent on morphological and anthropological description. In the present study, we refrained from using the elegant but time-consuming method used by Krings et al. (1997, 1999). Instead, we use an approach based on membrane-bound fossil DNA (fDNA), as proposed elsewhere (Houde et al. 1995) and introduce a modified Southern hybridization technique that uses fDNA as an alternative tool to classifying morphologically indistinguishable hominid skeletal remains.

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Address for correspondence and reprints: Dr. Lutz Bachmann, Pritzker Lab for Molecular Systematics and Evolution, The Field Museum, 1400 South Lake Shore Drive, Chicago, IL 60605-2496. E-mail: bachmann@fmppr.fmnh.org

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Material and Methods

Samples

Five skeletal remains were selected for DNA analyses (table 1). Two samples were classified as being undoubtedly of Neanderthal origin—the *Os parietale,* excavated at Warendorf-Neuwarendorf (W-NW), Germany, and a clavicula, from Krapina, Croatia. The third sample is a humerus from a *H. sapiens,* discovered in the Vogelherd cave (Stetten I), Germany. A mammoth humerus from Neuwied, Germany, and a reindeer femur from Banks Island, Canada, were selected as nonhominoid controls.

Amino Acid Racemization

Pulverized bone (1 mg) was hydrolyzed at 110°C for 24 h in 200 μl 6N ^{2}HCl in ^2H_2O. After evaporation to dryness, the sample was first esterified (200 μl 2N ^{2}HCl in CH_3O^2H/15 min at 110°C) and then trifluoroacetylated (100 μl trifluoracetic anhydride/10 min at 110°C). The acylation reagent containing the amino acid derivates was decanted off, leaving the bulk of the inorganic salts adhering to the walls of the reaction vial. After evaporation of excess trifluoracetic anhydride, the amino acid derivatives were dissolved in 20 μl of toluene and analyzed by selected-ion gas chromatography–mass spectrometry (SIM–GCMS), on a Chirasil-Val column. The ions m/z 140, 182, and 156, corresponding to nonlabeled alanine, leucine, and aspartic acid, respectively, were selectively monitored. The D/L ratio measured with these ions reflects the true enantiomeric composition of the sample—that is, before hydrolysis and derivatization.

DNA Techniques

Fossils were handled under aseptic conditions in the laminar flow cabinet of a laboratory dedicated to fDNA work. Standard precautions—such as, for example, the use of plugged pipette tips and UV-irradiated solutions—were taken to avoid contamination with recent human DNA during bone handling and extraction procedures (Scholz and Pusch 1997). fDNA was extracted according to the protocols of Scholz and Pusch (1997) and Pusch and Scholz (1997) (Elsevier Trends Journals Technical Tips Online). A further purification was introduced by means of three additional extractions; once with 4M guanidinium isothiocyanate, 0.5 vol phenol, and twice with 1 vol chloroform. A subsequent repair reaction of the extracted DNA was performed, as described by Pusch et al. (1998). Most of the DNA molecules of the obtained fDNA extracts were <200 bp (fig. 1). Contemporary genomic DNA of high molecular weight from chimpanzee and a human was extracted according to the instructions of the "QIAamp DNA Blood Kit" (Qiagen).

To test all components used and the blank extractions for contamination with human contemporary DNA (Pusch et al. 1998), a PCR amplification was performed of a 255-bp mitochondrial D-loop region-specific product. As expected, an amplification product was only obtained in the positive control (contemporary DNA isolated from human blood). Contamination of the fDNA with DNA from the edaphon could be excluded according to the procedure proposed by Pusch and Scholz (1997; Elsevier Trends Journals Technical Tips Online).

DNA was separated on 1.8% low-melting agarose gels in 1 × TBE buffer and subsequently transferred onto Qiabrane plus membranes by capillary transfer in 0.5M NaOH; 0.025M KOH; 1M NaCl. The membranes were soaked in 2 × SSC and prehybridized overnight in 10 ml HybrisolXR™ (Oncor) at 45°C. Unlabeled sonified salmon sperm DNA (100 μg/ml), denatured soil DNA (1.5 μg/ml), heparin (300 μg/ml) and dextran-sulfate (10%) were added. Probe DNA was labeled by means of α-[^{32}P]dCTP, α-[^{32}P]dATP, and the Random Primed Labeling Kit (Biolabs). The labeling reaction was re-

Table 1

Fossil Material Used in the Present Study

Species	Bone Sample	Site	Common Name	Stratigraphical Age (years BP)	Catalog Number/Repository of Fossils
H. neanderthalensis	*Os parietale*	W-NW	Neanderthal	>50,000	MKZ 4013, 123; Westfalian Museum of Archaeology, Münster, Germany
H. neanderthalensis	Clavicula	Krapina	Neanderthal	110,000–100,000	Krapina Fe. 1.si/213 Geological and Paleontological Museum, Zagreb, Croatia
H. sapiens	Humerus	Stetten I	Human	35,000 (^{14}C-dating)	OSUT 5829a; Osteological collection, University of Tübingen, Tübingen, Germany
Mammuthus primigenius	Humerus	Neuwied	Mammoth	>40,000	AB 25a; Private collection, M.S.
Rangifer tarandus	Femur	Banks Island	Reindeer	>10,000	RA-E 278; Institute of Archaeobiology, University of Tübingen, Tübingen, Germany

peated after precipitation of the DNA in 3 vol 90% EtOH; 10% NaOAc (pH 5.3); 0.5mM $MgCl_2$, for 30 min at –20°C. After the second labeling, the probe was purified by four passages through polymer cotton/sephadex G-50 columns at room temperature. The hybridization was performed at 55°C for 20 h. After hybridization, the filters were washed twice for 30 min in 4 × SSC; 0.15% SDS; 10 μg glycogen and twice for 10 min in 0.1 × SSC; 0.1% SDS; 50 μg glycogen. The intensities of hybridization signals were quantified by a Bio-Print CCD camera and the Bio-Profile Bio-1D V.97 analysis software (Vilber Lourmat).

Results

Prior to starting DNA analyses, DNA survival in the five bone samples was tested by measuring the rate of amino acid racemization of aspartic acid, alanine, and leucine. The obtained values are listed in table 2. In all samples, the D/L Asp values are $<80 \times 10^{-3}$ and are greater than those of D/L Ala and D/L Leu. According to Poinar et al. (1996), the data indicate that (1) there is no contamination of the sample with exogenous DNA and (2) the extracted DNA might be suitable for PCR amplification. The absence of exogenous DNA was verified by a spectrophotometrical wave-scanning analysis (240–500 nm) that could not detect any impurities. Furthermore, contamination of the fDNA with DNA from the edaphon could be excluded according to the procedure proposed by Pusch and Scholz (1997; Elsevier Trends Journals Technical Tips Online).

Table 2

Racemate Content (D/L Ratio) of the Bone Samples Investigated

	Alanine	Leucine	Aspartic Acid
H. neanderthalensis:			
W-NW	2×10^{-3}	3×10^{-3}	47×10^{-3}
Krapina	2×10^{-3}	2×10^{-3}	39×10^{-3}
H. sapiens, Stetten I	2×10^{-3}	3×10^{-3}	40×10^{-3}
M. primigenius, Neuwied	2×10^{-3}	3×10^{-3}	42×10^{-3}
R. tarandus, Banks Island	4×10^{-3}	2×10^{-3}	15×10^{-3}
Modern bone	3×10^{-3}	2×10^{-3}	10×10^{-3}

fDNA extracted from the Neanderthal specimen from W-NW and from Krapina was electrophoretically separated, along with DNA extracted from bone fragments of (1) the *H. sapiens* from Stetten I, (2) the mammoth, (3) the reindeer, and (4) a blank extraction, and subsequently were transferred to nylon membranes. The immobilized DNA was subsequently hybridized to labeled DNA of the samples from (1) Stetten I, (2) W-NW, (3) Krapina, (4) human leukocytes, and (5) a contemporary chimpanzee. The amount of radioactivity bound by each lane was quantified and compared to that of the W-NW find (fig. 1 and table 3). DNA from

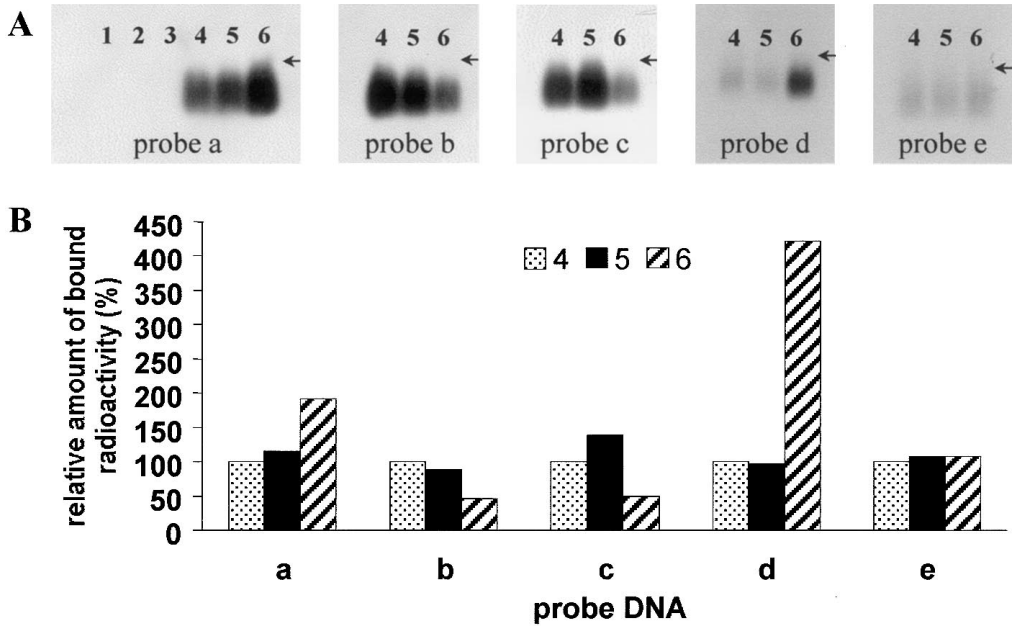

Figure 1 *A,* Autoradiograms of hybridizations of probes a–e to immobilized DNA of different specimens 1–6. The results of the control hybridizations to DNA in lanes 1–3 are shown only for probe a. *B,* Relative amount of radioactivity bound by the immobilized DNA. The amount of radioactivity bound to DNA extracted from the Neanderthal of W-NW is taken as 100%. 1, blank extraction; 2, reindeer; 3, mammoth; 4, Neanderthal from W-NW; 5, Neanderthal from Krapina; 6, human from Stetten I; probe a, human from Stetten I; b, Neanderthal from W-NW; c, Neanderthal from Krapina; d, contemporary human; e, present-day chimpanzee.

Table 3

Radioactivity Bound by Immobilized fDNA in Southern Hybridization Experiments

	Amount of Radioactivity Bound by Target DNA (%)			
	H. neanderthalensis		*H. sapiens,*	
Probe DNA	W-NW	Krapina	Stetten I	Controls[a]
H. neanderthalensis:				
W-NW	100	88	46	0
Krapina	100	139	49	0
H. sapiens:				
Stetten I	100	115	192	0
Recent individual	100	97	421	0
P. troglodytes, recent individual	100	107	107	0

[a] Mammoth, reindeer, or blank extraction.

neither the mammoth, the reindeer, nor the blank extraction bound any radioactivity and thus indicated the specificity of the probes for primate DNA.

The amount of radioactivity bound to the DNA of the Neanderthal from W-NW was always similar to that bound by the DNA of the Krapina specimen. The amount of radioactivity bound by the fDNA of the *H. sapiens* from Stetten I exceeded significantly the amounts bound by the fDNA of the Neanderthal samples when probed with fDNA from *H. sapiens* from Stetten I. The excess of radioactivity bound by the fDNA of the *H. sapiens* from Stetten I, compared to those bound by the two Neanderthal samples, was even more pronounced (approximately fourfold) when probed with labeled contemporary human DNA. This certainly reflects a higher labeling efficiency of contemporary DNA, compared to degraded and chemically altered fDNA. The hybridization of genomic DNA from a contemporary chimpanzee, which served as an outgroup, produced equivalent results for both the DNA of Neanderthal and human origin. This indicates that the differences between Neanderthal and human DNA observed in the other experiments are not due to the find-specific preservation of different genomic components.

Discussion

The delineation of human evolution and hominid phylogeny is the end-point of a progression of events that begins with fossil finds. The most critical stage in this process is the morphological classification of the find, since an incorrect classification destroys the credibility of subsequent paleoanthropological interpretation. To date, researchers have had to rely upon the morphological description for this purpose. Such an approach tends, however, to be complex and insecure, especially when only small bone fragments are preserved, and, consequently, this has led to frequent and controversial debate. The verification of the morphological classification by an independent approach is, therefore, highly desirable.

To establish a reliable fDNA-based classification procedure, we worked on DNA extracted from the *Os parietale* found at W-NW, and the clavicula, from Krapina. The morphological characteristics of the *Os parietale* found at W-NW argue for it being of Neanderthal origin. The most convincing evidence is the archaic pattern of the course of the *Impressiones arteriosae,* which has never been observed in *H. sapiens* (Saban 1984, 1986). The clavicula from Krapina, is undoubtedly of Neanderthal origin, because it was dated to 100,000–110,000 years BP. It clearly predates the appearance of anatomically modern man in Europe.

The relatively simple Southern blot hybridization technique succeeds in telling apart the DNA from the two well-defined Neanderthal finds and the human remains from Stetten I. DNA extracted from both the W-NW find and the clavicula of a Neanderthal specimen from Krapina yield hybridization signals of the same order of magnitude that differ at least by a factor of two from the signals obtained with human DNA when hybridized with genomic DNA of either human or Neanderthal origin. Using labeled chimpanzee DNA as probe, we found that Neanderthal and human DNA produce hybridization signals of similar intensity. We can safely assume that it will also succeed in classifying morphologically indistinguishable hominid fossils (at least if fDNA survived in the find).

The data presented in this study indicate that the composition of the genomes of Neanderthals and anatomically modern man is significantly different, although they do not allow the specific characterization of these differences. It is, however, possible to differentiate between Neanderthals and anatomically modern man on the basis of genomic differences observed between humans and their closest living relative, the chimpanzee. Neanderthals and anatomically modern man both de-

scend from *H. erectus*. The split-off occurred within the last 1 million years. The time of divergence of humans and chimpanzees is assumed to be ~4–5 million years BP (Takahata et al. 1995). Genomic differences between Neanderthals and modern humans should therefore be smaller than those between modern humans and chimpanzees. We will consider three classes of DNA in this context.

(*1*) *mtDNA*

Krings et al. (1997) estimated that the fast-evolving hypervariable region I of mtDNA of modern humans and the Neanderthal from Düsseldorf diverged on average by 6.7%, although it differs on average by 14.5% between humans and chimpanzees. The greatest part of the mtDNA molecule is significantly less variable. Thus, an even lower level of differentiation between Neanderthals and humans is to be expected for mtDNA as a whole. mtDNA will, therefore, only slightly contribute to the significantly different hybridization signals observed for human and Neanderthal DNA.

(*2*) *Single-Copy DNA*

Provided that the estimate of 1%–2% sequence diversity between humans and chimpanzees is reliable (Sibley et al. 1984; Miyamoto et al. 1988), it is reasonable to assume that single-copy DNA sequences of Neanderthals and humans are nearly identical. It is thus very unlikely that the observed differences in the hybridization signals of human and Neanderthal DNA reflect diversity of single-copy DNA.

(*3*) *Repetitive DNA*

A variety of repeated sequences makes a significant contribution to primate genomes in which many are known to evolve rapidly. These genome components can provide most of the informative hybridization signal, since not only will sequence similarity diverge in time, but so will sequence copy numbers. It is reasonable to assume that diversity in the repetitive components of the genome leads to the different hybridization signals of Neanderthal and human DNA. Data available on the genomes of chimpanzees and humans support this assumption: for example, alpha satellite DNA repeats, which are present at the centromers of primate chromosomes, differ significantly between species (Waye and Willard 1989), and a prominent 32-bp satellite DNA occurs adjacent to the telomers of chimpanzees but is absent in humans (Royle et al. 1994). Unfortunately, it is almost impossible to verify the assumption that the genomes of Neanderthals and modern humans differ significantly in the composition of repetitive DNA. Even if DNA sequences of repetitive DNA of Neanderthals can be obtained that differ from similar ones that are known to occur in the genome of modern humans, it will be almost impossible to prove (1) their authenticity and (2) their specificity for Neanderthals.

In the past century, *H. neanderthalensis* was regarded as a discrete hominid species, different from *H. sapiens* (King 1864). Later, both taxa were regarded as subspecies, *H. s. neanderthalensis* and *H. s. sapiens* within *H. sapiens* (Campbell 1964). Although this research rests on a number of assumptions, the mtDNA sequences provided by Krings et al. (1997, 1999) and the data presented here indicate a clear differentiation of Neanderthals and anatomically modern man and support the assumption that both taxa are discrete species. This also means that Neanderthals cannot be the direct ancestor of anatomically modern man.

Technical Considerations

Recent improvements in molecular approaches allow us to add DNA analyses to the numerous techniques used in the classification of fossil remains. The data so far available are restricted to nucleotide sequences generated via PCR and are thus subject to an extremely high standard of verification when compared to standard analyses. Such an effort is certainly not justified for classification purposes.

The application of Southern blotting and subsequent hybridization techniques for identification of fossil materials has several advantages over PCR-based strategies. The data obtained do not only pertain to a specific sequence but also to the genome as a whole. As already stated by Houde et al. (1995), such a membrane-bound hybridization approach makes more frugal use of the limited resources. Furthermore, minor contamination with contemporary human DNA can be tolerated for two reasons. First, contemporary DNA is of high molecular weight and can therefore be identified as such on autoradiographs. Second, it will increase the hybridization signal of fDNA of low molecular weight to a negligible degree only. In addition, there is no need to optimize amplification reactions. One also does not have to worry about the initial number of template molecules for purposes of reproducibility or about artifacts produced by jumping PCR (Krings et al. 1997, 1999).

The major argument in favor of hybridization experiments relates to the limitations in retrieving undamaged fDNA from fossils (Höss et al. 1996; Lindahl 1997). A threshold of 100,000 years BP has been proposed before which time it is believed that hydrolytic and oxidative processes damage fDNA to such an extent that it is usually unsuitable for PCR. Our data suggest that hybridization experiments may work equally with DNA extracted from older material. The Krapina collection is relatively dated from 86,000 to 130,000 years BP. Cooper et al. (1997) extracted DNA from animal bones of

the Krapina collection but, as expected, failed to amplify mtDNA via PCR. They therefore concluded that bone material from Krapina is not suitable for any fDNA analysis. In our hybridization experiments, however, DNA from the Krapina fossil find proved to be just as suitable as DNA of the much younger W-NW find.

Should the hybridization technique permit the routine analysis of DNA extracted from material >100,000 years old, it might then allow the classification of numerous important finds, such as the skull of Steinheim (Gieseler 1974, pp. 129–143), the mandible of Mauer (Bilsborough 1992, pp. 170–171), and the Eritrean skull (Abbate et al. 1998), which are still of controversial chronological and taxonomic position.

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