Letter to the Editor

Am. J. Hum. Genet. 68:287, 2001

Inadequate Use of Molecular Hybridization to Analyze DNA in Neanderthal Fossils

To the Editor:

Scholz et al. (2000) have recently claimed, in the *American Journal of Human Genetics,* to be able to discriminate, by means of Southern hybridization, between Neanderthal and Cro-Magnon fossils. Given the high profile of such studies and the value of the sample material, such work must meet, at the very least, the minimum standards of reporting and experimental design. However, the study raises a number of awkward questions that cannot be answered by the information provided in the article.

The authors state that, on the basis of three unadapted criteria, contamination of their ancient DNA extract with allochthanous DNA could be excluded. The first criterion is measurement of the D/L ratio for aspartic acid. The utility of racemization has recently been criticized because of the disparity in the fundamental process of racemization and depurination (Collins et al. 1999). Furthermore, this approach does not assess contamination with modern biological material. The second criterion is spectrometry at 240–500 nm, which cannot distinguish between modern and ancient DNA. The third criterion is viscosity, as assessed with a toothpick plunged into the extract (Scholz and Pusch 1997). This latter assay can only detect contamination by a high concentration of high molecular-weight DNA, a situation easily avoided by a thorough previous cleaning of the fossils. However, it is much more difficult to prevent and to assess contamination by a lower level of modern DNA, even though this modern DNA could still far exceed traces of ancient DNA.

Scholz et al. (2000) adopt an unusual approach to probing the samples, labeling material extracted from fossil samples that do not give rise to PCR products. PCR applications suffer from the problems of inhibitors present in the samples. The presence of such inhibitors will also interfere with other DNA enzymes. Indeed, up to now, I have always observed that the PCR inhibitors present in various fossil extracts also inhibit many other DNA-modifying enzymes—in particular, the Klenow fragment of DNA polymerase I used in the labeling step. What, indeed, are Scholz et al. labeling, if there is no material that can be amplified by PCR? There will be a preference for labeling undamaged DNA, most probably contamination from the soil or from the handling of samples. Contamination by soil DNA is a concern, since most fossils are buried in the soil and are infiltrated by microorganisms that release DNA during extraction (Sidow et al. 1991)

Scholz et al. (2000) have introduced small amounts of soil DNA (1.5 μg/ml) as a competitor in the hybridization medium, presumably to prevent hybridization of contaminants present in the fossil extract. However, the ecology of soil microorganisms is highly complex and is different from soil sample to soil sample (Copley 2000). It is thus very difficult to ensure that the competitor harbors all the sequence diversity of the DNA contaminating the fossil sample, particularly if the soil DNA used does not originate from the same site and layer as those containing the fossil. Furthermore, the concentration of competitor used is very low, and it is not clear whether it would saturate the DNA on the membrane (where very high local concentrations are achieved). Indeed, high concentrations (100–500 μg/ml) of salmon sperm DNA are commonly used in Southern hybridizations, and they do not prevent detection of highly conserved single-copy sequences that are present in the salmon genome (see, e.g., Grange et al. 1987).

To directly test the putative protective effect of such a low concentration of competitor, using *Escherichia coli* DNA spotted on a membrane and as a probe, in the presence of 100 μg/ml salmon sperm DNA containing or not containing 1.5 μg/ml of *E. coli* DNA as competitor, I performed parallel hybridization experiments under the conditions described by Scholz et al. (2000). The results show that the presence of such a homeopathic dose of homologous competitor decreases the signal obtained by $\leqslant$10% under these conditions (fig. 1). Thus, the conditions used cannot prevent interference by contaminating microorganismal DNA.

The ability to discriminate chimpanzee, Neanderthal, and human DNA by Southern hybridization is very surprising, given the low percentage of sequence difference between the human and chimpanzee genome. The global single-copy sequence divergence has been estimated to be 1.1% (King and Wilson 1975; Sibley and Ahlquist

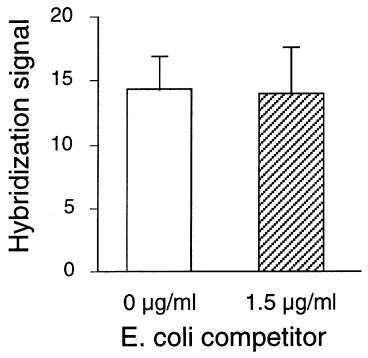

Figure 1 Quantitation of competitive hybridization of *E. coli* DNA (250 ng per slot), with *E. coli* DNA as a probe under the conditions described by Scholz et al. (2000) via PhosphorImager analysis. As indicated, the hybridization solution contained or did not contain 1.5 µg/ml of *E. coli* DNA as competitor.

1987). In addition, repeated sequences are similarly conserved. This is true for microsatellites (Deka et al. 1994) and for highly repetitive alphoid DNA sequences, which constitute nearly one-quarter of the genome (Maio et al. 1977) and are subjected to stringent conservation (Musich et al. 1980). Moreover, it has been proposed that most repeated sequences were already integrated in the genome before the "great ape" radiation and that few have changed positions since (Sawada et al. 1985). These global results have been recently confirmed by the sequence of 38.6 kb of the long arm of the X chromosomes in humans, chimpanzees, and gorillas. The overall sequence divergence between human and chimpanzee is 0.8% on the entire region, and this divergence is homogeneously distributed between nonrepeated and repeated sequences, the latter accounting for 61% of the total sequence (Bohossian et al. 2000). The repeated sequences within this gene-free region comprise the vast majority of all repeated sequences (Bohossian et al. 2000; analysis available upon request). Thus, it seems highly unlikely that molecular hybridization of fossil samples fixed on a membrane and probed with fossil extracts could be used as a method to discriminate such related genomes as those of human and chimpanzee, even more to discriminate human from Neanderthal DNAs that are expected to be even closer.

On the basis of details given in the article and information provided by the manufacturer of the hybridization solution (Oncor/Appligene), I calculate that the hybridization conditions would only distinguish sequences diverging by at least 30%–40% (6 × standard saline citrate buffer, 55°C; 40% mole fraction of guanosine plus cytosine in DNA; Thiery et al. 1975; Meinkoth and Wahl 1984; Springer et al. 1992). In my hands, using these conditions and modern probes, the difference in relative intensity between chimpanzee and human was ~10% (fig. 2*A*). Raising the stringency and increasing the number of washes did not help (fig. 2*B*). If it is not possible to distinguish chimpanzee and human, how can we distinguish Neanderthal and Cro-Magnon?

If we assume that the probe contained labeled DNA from organisms in the soil—as well as, eventually, ancient DNA from the fossil itself—is it possible to replicate the results obtained by Scholz et al. (2000)? Different mixtures of bacterial, fungal, plant, and human DNA were both labeled and spotted onto membranes. The mixture produced a pattern of cross-reactivity identical to that obtained by Scholz et al. (2000), despite the fact that only one sample contained any human DNA (fig. 3). Thus, the procedure of Scholz et al. (2000) allows for the identification of the presence of common microorganismal DNA in the sample far better than it allows for the distinguishing of closely related species. The question of why chimpanzee DNA, with 99% homology to modern human DNA, fails to hybridize with any of the extracts in the Scholz et al. (2000) study remains unresolved.

In conclusion, probing filter-immobilized fossil DNA with fossil DNA extracts is a very misleading approach, and the procedure described by Scholz et al. (2000) is not appropriate, not even for modern samples, to analyze sequence diversification along the primate lineage. Precious fossils should not be destroyed to be analyzed in such an unreliable manner.

Genomic Southern hybridization is a potentially valuable approach, capable of revealing phylogeny, since it assesses the average of the entire information content of the genome (Sibley and Ahlquist 1987). It can be used as a tool to identify ancient DNA in fossil samples and to make a rough classification of the taxa of origin, provided a number of control experiments are performed (Geigl 1997). In the light of the paper by Scholz et al. (2000), Cooper and Poinar (2000) see the need to state again the minimum criteria necessary to report PCR studies of ancient DNA. In the case of the use of genomic hybridization, I recommend some additional criteria:

1. Stringency conditions should be reported.
2. The use as probes of well-characterized modern DNA that can be tested with a wide range of DNA extracts (zoo blots), to find the optimal hybridization conditions that limit cross-hybridization to the desired degree of homology.
3. Possible microbial contamination sources, including the sediment surrounding the fossil should be tested for.
4. Labeled fossil extracts should not be used as probes in these experiments. Unlike PCR, for which it is possible to use sequencing to reveal the amplification of contamination, with hybridization the only control is to test the widest possible range of control samples. Since fossils are always contaminated with soil microbes, the

vast majority of which are unknown, the use of fossil extracts as probes does not allow the testing of their hybridization properties, because of the lack of reference DNA. Furthermore, to test extensively aDNA probes demands the unnecessary sacrifice of material.

5. Analysis of hominid and great ape fossils in this manner should be avoided, since it does not allow discrimination with modern human contamination.

Acknowledgments

I wish to thank Thierry Grange and Matthew Collins for their critical discussions and highly valuable comments on the manuscript.

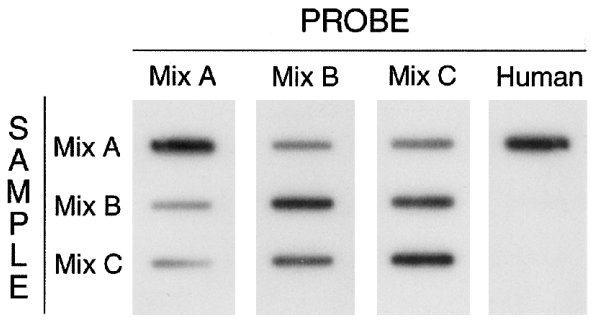

Figure 3 Hybridization of DNA mixtures, composed of DNA from different species, spotted on a membrane and hybridized according to the conditions of Scholz et al. (2000). Mix A: 10% human DNA, 50% plant (*Pisum sativum*) DNA, 20% yeast (*S. cerevisiae*) DNA, 10% bacterial (*E. coli*) DNA, and 10% fungus (*Ascobolus immersus*) DNA. Mix B: 80% *S. cerevisiae* and 20% *E. coli* DNA. Mix C: 20% *E. coli*, 40% *S. cerevisiae*, and 40% *A. immersus* DNA. These filter-immobilized mixes were hybridized with labeled Mix A, Mix B, Mix C, and human DNA, as indicated.

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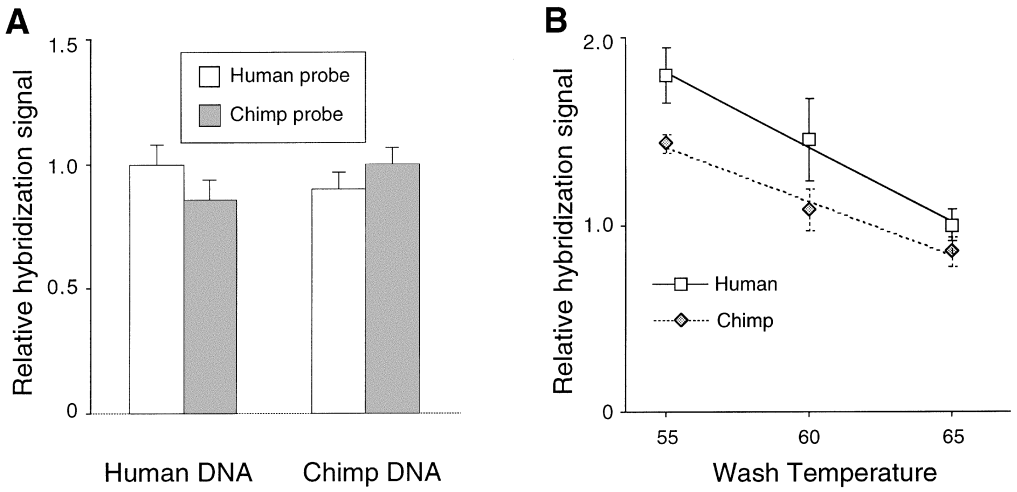

Figure 2 Stability of human-chimpanzee DNA hybrids. 250 ng of human and chimpanzee (*P. troglodytes*) DNAs were applied on a nylon membrane and were hybridized with human and chimpanzee DNA as probes. Hybridization was performed exactly according to the conditions of Scholz et al. (2000). The intensity of the hybridization signal was directly measured using a PhosphorImager. The relative values, corrected for variations in the amount of material loaded and the specific activity of the probe, are shown. *A*, Comparison of the signal intensities obtained by hybridization of human and chimpanzee DNA against each other. *B*, Discrimination between human and chimpanzee DNA is not achieved with increasing stringency of the washes. Results of the hybridization experiment in which human DNA was used as a probe are shown. Washes were done at 55°C, 60°C, and 65°C. The reverse hybridization, with the use of chimpanzee DNA as a probe, gave similar results.

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