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Identification of Contaminating Elements in Ancient DNA Research*

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Abstract: We obtained two mitochondrial 12S rDNA-like sequences from Pleistocene bones of horse and deer. Based on sequence comparison, these two sequences were neither close to that of human mitochondrial 12S rRNA gene, nor from that of horse or deer. Phylogenetic analysis shows that these two sequences are most close to the human nuclear DNA recently identified as the 12S rRNA gene counterpart. This result is important for identifying contamination from human nuclear DNA in ancient DNA research.

Key words: Ancient DNA, contamination, nuclear 12S rRNA-like gene

INTRODUCTION

The study of DNA sequence data obtained from specimens of fossil organisms offers a window into the evolutionary past. However, recent attempts to amplify DNA from some fossils have either failed or shown extant species contamination.

Mitochondrial genes have been widely used in ancient DNA studies because they are characterized by having high copy number and fast evolutionary rate. However, copies

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of mitochondrial genes have been observed in the nuclear genome of many species, including humans. Nomiyama *et al.* (1985) and Kaminura *et al.* (1989) reported some mitochondrial gene sequences in human nuclear clones which have >80% and 92% sequence identity with contemporary human mitochondrial DNA respectively. In ancient DNA studies, Van der Kuyl *et al.* (1995) found many 12S rRNA gene sequences from monkey mummy bone and teeth and from skin of mangabeys, which were identical or closely related to human nuclear DNA sequences resembling mitochondrial 12S rDNA. This result indicates that the nuclear counterparts with high sequence identity to mitochondrial genes from ancient species and contemporary humans may lead to false interpretation of ancient DNA analytical results.

In this paper, we report two 12S rRNA gene sequences we obtained from Pleistocene mammal bones which are regarded as human nuclear gene contamination.

MATERIALS AND METHODS

Samples

A total of 7 samples of teeth and bones from three species of Pleistocene mammals were analyzed, including horses, deers and rabbits. Samples were obtained from the Dalian Natural Museum, approximately 20,000 aBP based on characteristics of the associated faunal assemblage.

DNA Extraction

Total DNA was extracted from 0.2 g of ancient teeth or bone powder by a CTAB procedure modified from Yang *et al.* (1997). Appropriate precaution was taken to prevent contamination from contemporary DNA. Entire experiment was done in ultra-clean environment. UV irradiation was performed to all reagents used in DNA extraction procedures. Extract control containing no tissue was included to supervise the contamination from extract reagents.

PCR Amplification and Sequencing

PCR reaction solutions were exposed to UV light for 30 min before adding template DNA and enzyme. Primary PCR amplifications were performed with primers of L1091 and H1478 (Kocher *et al.*, 1989) in 30 μl volume containing 2 μl DNA extract. This primer pair can amplify approximately 390 bp of mitochondrial 12S rRNA gene. PCR reaction was done on a PE 9600 thermocycler with temperature settings of 94°C (40 sec), 50°C (1 min), and 72°C (1 min) for 35 cycles. Then 0.2 μl PCR product without further purification was added to a new tube to perform a nested PCR amplification with the primer pair of L1373

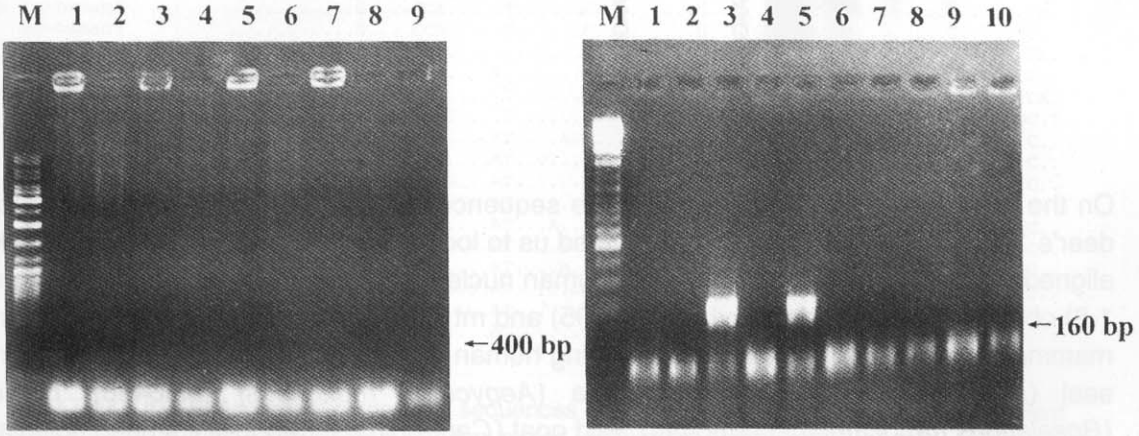
(Thomas *et al.*, 1989) and H1478. This pair of primers can amplify approximately 104-bp mitochondrial 12S rRNA gene, originally designed to amplify 12S rRNA gene from marsupial wolf. Wu *et al.* (1998a, 1998b) used them for identification of Chinese traditional medicine of turtle and soft-shell turtle shells. The condition of the nested PCR amplifications is the same as primary PCR. Extraction and negative PCR controls were carried through the secondary amplification to monitor contamination. Positive PCR products were purified using Wizard™ PCR Preps DNA Purification System (Promega), then sequenced in both directions ug Silver Staining Sequencing System (Promega), following the manufacturer's protocols.

PHYLOGENETIC ANALYSES

Alignment of the sequences was done using Clustal X (Higgins *et al.*, 1996). Phylogenetic analysis was done using the neighbor-joining (Saitou and Nei, 1987) and parsimony method (Fitch, 1971) in PHYLIP package (Felsenstein, 1993). The distance matrix was made using Kimura 2-parameter distances (Kimura, 1980).

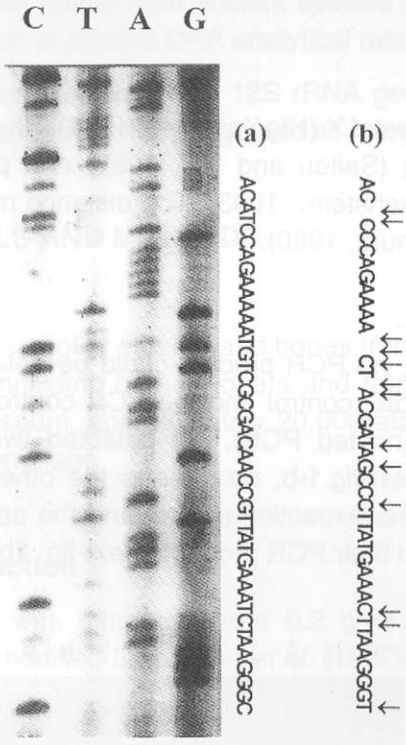
RESULTS

When the primer pair L1091/H1478 was used, no PCR product could be detected with agarose gel electrophoresis, including the extract control and the PCR control (text-fig. 1a). However, in the second amplifications (nested PCR), we detected two positive products, one from an ancient horse teeth (text-fig.1-b, no. 3) and the other from an ancient deer femur (text-fig.1-b, no. 5), while the extraction control and the primary and second amplification blanks were all negative in their PCR products (text-fig. 1b).



TEXT-FIGURE 1. Agarose gel electrophoresis of PCR products. a. primary PCR products with 1% agarose gel; b. secondary PCR with 2% agarose. M: DNA size marker; 1-7: extracts from ancient bone; 8: extraction control; 9: primary PCR blank; 10: secondary PCR blank.

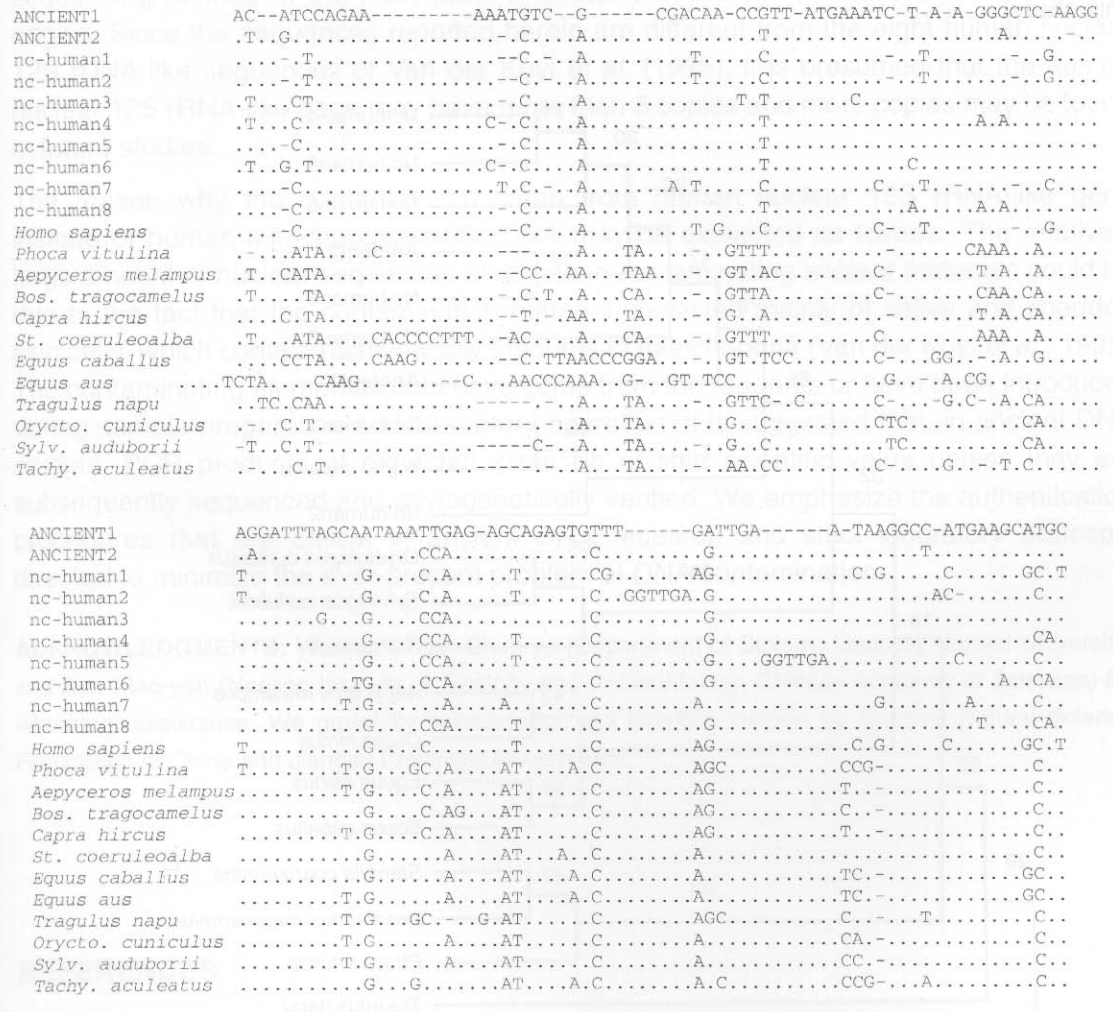
The result of a sequence analysis shows that the length of these two sequences is 108 bp and 106 bp respectively and there are 15 nucleotide position differences between them. These two sequences were significantly different from human mitochondrial (mt) 12S rDNA sequence (text-fig. 2), so we suggest that they are not derived from laboratory or other environmental contamination by modern human mtDNA.



TEXT-FIGURE 2. Direct sequencing reaction of secondary amplification product from ancient bone (no. 3 in text-fig. 1-b).

On the other hand, the two 12S rDNA-like sequences are neither close to horse nor to deer's 12S rDNA sequences. This prompted us to look for an alternative explanation. We aligned these two sequences with eight human nuclear 12S rDNA sequences (nc-human 1-8) obtained from Van der Kuyl *et al.* (1995) and mt 12S rRNA gene sequences of other mammals retrieved from GenBank, including human (*Homo sapiens*) (X62996), harbour seal (*Phoca vitulina*) (X63726), impala (*Aepyceros melampus*) (M86496), nilgai (*Boselaphus tragocamelus*) (M86494), wild goat (*Capra hircus*) (M55541), striped dolphin (*Stenella coeruleoalba*) (X78169), wild horse (*Equus caballus*) (U02581), African ass (*Equus asinus*) (X97337), Greater Malay chevrotain (*Tragulus napu*) (M86494), common rabbit (*Oryctolagus cuniculus*) (AJ001588), desert cottontail (*Sylvilagus auduborii*) (AF038019) and Australian echidna (*Tachyglossus aculeatus*) (U02579) (text-fig. 3).

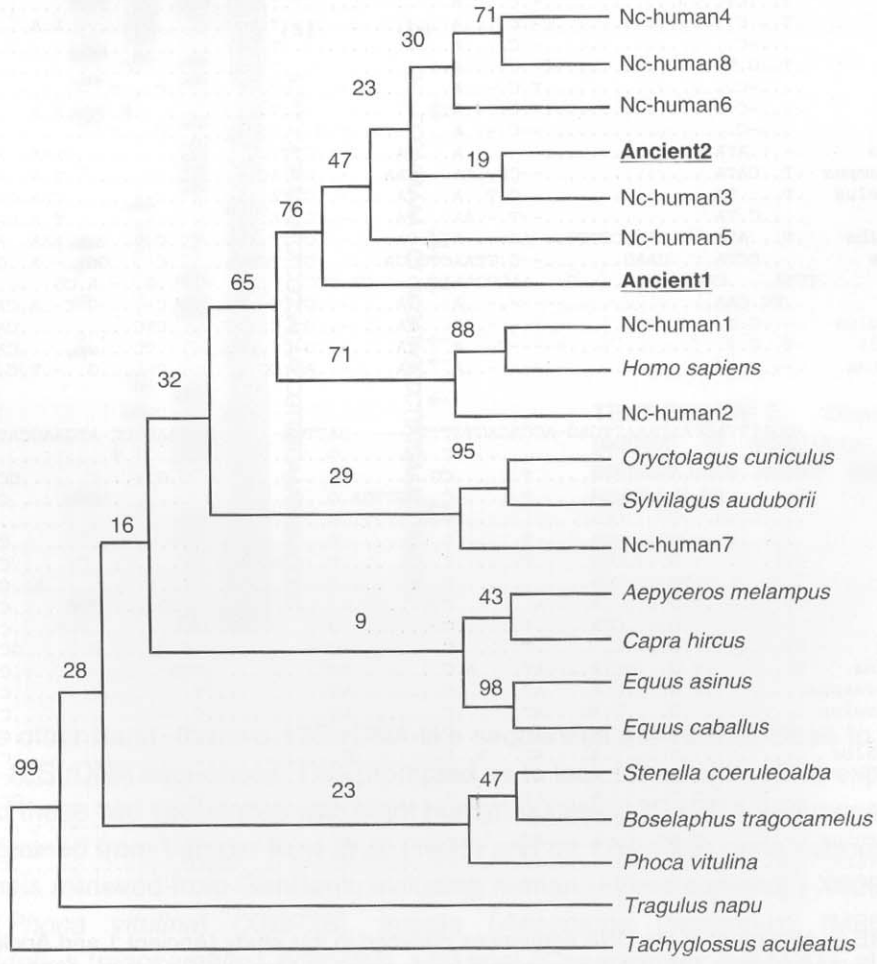
Phylogenetic analysis using neighbor-joining method (text-fig. 4) shows that the two sequences from ancient mammals obtained through this study are most close to human nuclear 12S rDNA-like sequences. One sequence (Ancient 2) is most close to nc-huamn 3, although the bootstrap value is low, while the other (Ancient 1) is the sister group of nc-human 4, 8, 6, 3, 5 and Ancient 2. The phylogenetic result using parsimony method shows that the sequence of nc-human 3 is a sister group of Ancient 1 plus Ancient 2 (cladogram not shown).



TEXT-FIGURE 3. Alignment of the DNA sequences obtained in this study (Ancient 1 and Ancient 2) with eight human nuclear 12S rDNA sequences (nc-human1-8, Van der Kuyl *et al.*, 1995) and 12S rDNA sequences of other mammals retireved from GenBank (their accession numbers are listed in the text above).

DISCUSSION

When we used L1373/H1478 or L1091/H1478 primer pair to directly amplify 12S rDNA fragment from the total bone extracts, no positive PCR product could be detected. But when we used the PCR product of L1091/H1478 as the template to further amplify using the L1373/H1478 primer pair (nested PCR), we obtained positive PCR products which, according to the phylogenetic analyses conducted here, appear to be close to human nuclear 12S rDNA-like segment. The result indicates that a second amplification is necessary in order to detect a minute amount of nuclear DNA (contaminating elements in this study).



TEXT-FIGURE 4. Phylogenetic tree reconstructed using neighbor joining (NJ) method with Kimura 2-parameter distance. Australian echidna (*Tachyglossus aculeatus*) was used as outgroup. Numbers are bootstrap value based on 1,000 replications.

Although appropriate precautions were taken to prevent contamination with contemporary DNA, and the extract and amplification control are negative, we could also detect contamination possibly from contemporary DNA. This may be explained by the carrier effect (Handt *et al.*, 1994), where a very low amount of contaminating molecules in extraction and amplification reagents may not yield positive PCR products because they may be adsorbed to the plastic ware; however, other molecules in tissue extract may act as carriers of the contaminating elements and thus allow the contamination molecules to be available for amplification. The two PCR products with different sequences using direct sequencing method of the PCR product shows that the contaminations are of multiple origins. Since the sequences reported herein are different from the eight human nuclear 12S rDNA-like sequences of Van der Kuyl *et al.* (1995), it is presumed that the human nuclear 12S rRNA-like gene may have more than 8 copies and more copies may be found in future studies.

The reason why the contamination came from human nuclear 12S rRNA-like gene instead of human mitochondrial 12S rDNA may be explained as follows. The relatively large amount of nuclear sequences amplified in contaminating ancient materials could be due to the fact that the contamination material consisted mainly of saliva and shedded skin cells, which contain relatively low amounts of mitochondria (Van der Kuyl *et al.*, 1995). The contaminating elements could have come from the reagents or have been introduced during sample preparation and laboratory operation. It is suggested that, in ancient DNA studies, PCR products of expected sizes be of little scientific value unless they are subsequently sequenced and phylogenetically verified. We emphasize the authentication procedures that are critical in ancient DNA research and strict laboratory protocols directed to minimize the ever-present problem of DNA contamination.

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古 DNA 研究中污染因子的鉴别

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提 要 污染问题是古 DNA 研究中的技术瓶颈，一些国际与国内重要学术刊物上发表的古 DNA 成果，最终被证实来自不同的污染源。本文详细分析了古 DNA 研究中的实验过程及不同污染产物，采用实际研究结果说明这些污染产物的导入过程和计算机判断方法，并以实例阐明人类核基因组中的似 12S rDNA 序列(12S rDNA-like)是古 DNA 研究中的常见污染源之一。本文报告的是一个古 DNA 研究中的负面结果及其分析、判断过程，它对于古 DNA 的甄别研究具有重要的现实意义与参考价值。