Discrimination Between Human and Animal DNA

Application of a Duplex Polymerase Chain Reaction to Forensic Identification

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Abstract: Identification of a report's species is one of the basic analyses in forensic laboratories. The authors report the case of 6 bone fragments recovered in a wooded area, which were not attributable to 1 animal species on the basis of morphologic examination. The aim of this study was to develop a duplex polymerase chain reaction (PCR) to discriminate human and animal origin of bone fragments. The method is based on the PCR amplification of cytochrome *b* and a 16S ribosomal mitochondrial DNA fragment, which has never been tested up to now. Our protocol combines a single-round PCR with direct visualization of amplicons in agarose gel, without sequencing analysis of the PCR products. The presence of a single band (359 bp) indicates a nonhuman origin of the sample, whereas 2 bands (157 and 359 bp) indicate a human biologic sample.

This method revealed to be useful for forensic purposes because the 16S ribosomal mitochondrial DNA is a small human-specific fragment that is easily amplifiable even with degraded DNA from biologic materials such as old bones.

Key Words: cytochrome *b*, 16S rRNA, DNA extraction, bone fragments, species identification

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Species determination of skeletal specimens is an important component of forensic and anthropological analyses. The problem is often referred to the differentiation of human bone material from nonhuman one. In most such cases, diagnosis based on anatomic features is possible. However, when remains are fragmentary or poorly preserved, species identification can be difficult or unachievable.

For the last few years, the analysis of mitochondrial markers has shown a good feasibility for species determination and individual human identification.[1,2] The mitochondrial genome is made up of a small, circular, double-stranded DNA molecule that is present in up to several thousand copies per cell. In animals, the mitochondrial genome is approximately 16,500 bp long and possesses 13 genes coding for proteins, 2 genes coding for ribosomal RNA (rRNA), 22 genes coding for transfer RNA, and 1 major noncoding region named d-loop, which contains the origin of replication.[3]

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The analysis of cytochrome *b* (cyt *b*) is a well-established method for animal species identification.[4–6] Other methods have been proposed for forensic purposes to distinguish human from nonhuman specimens, involving the amplification of different regions of mitochondrial DNA (mtDNA) in 1 round of polymerase chain reaction (PCR).[7,8] Among mtDNA genes used for species detection, the cyt *b* (alone or associated with other markers) has been successfully used in PCR-based method, even if sequence analysis and comparison in BLAST have made this analysis troublesome.[4–8] Another mtDNA marker commonly used for phylogenetic studies is the 16S rRNA gene.[9]

In a recent case, it was necessary for our laboratory to attempt species identification of 5 small bone fragments (Fig. 1). These tiny fragments were recovered slightly buried by a hunter in a hilly wooded zone in North Italy. The primary concern was whether they were of human origin. The small size of the pieces precluded positive recognition of human versus nonhuman origin based on their morphology and cortical thickness. For the purpose of establishing the species origin of these fragments, we have developed a method based on a duplex PCR products amplified from mtDNA detected in agarose gel electrophoresis. These amplicons correspond to cyt *b* (359 bp) and to a 16S rRNA (157 bp) fragment that is human specific and which has never been used for this purpose so far. Because 16S rRNA is a small mtDNA fragment that amplifies easily, this method could be very useful for forensic purposes in case of human species identification.

MATERIALS AND METHODS

The first step to determine the 16S rRNA primer specificity for human species was to check for minimum primer-dimer and hairpin secondary structures. Then we verified by BLAST analysis the primer sequences and subsequently tested them by PCR amplification.

DNA was extracted from the bone of each of the 5 fragments and from bone belonging to human and other species (cattle, sheep, horse, pig, chicken, rabbit, and cat).

The 5 fragments of unknown species were treated for 30 minutes with bleach.

Each fragment weighed between 1 and 2 g and was pulverized to a fine powder after immersion in liquid nitrogen. Then, it was decalcified and DNA extracted according to the methods of Crainic et al.[10]

We designed primers for 16S rRNA, considering of prime importance the melting and annealing working temperatures of the already existing cyt *b* primers.[11] The sequences of primers used in a single PCR are reported in Table 1.

After an initial denaturation step of 1 minute at 94°C, samples were amplified in a thermocycler (9700; Applied Biosystems, Foster City, Calif), for 40 cycles of 5 seconds at 94°C, 30 seconds at 50°C, and 40 seconds at 72°C, followed by a final elongation step of 3 minutes at 72°C. The PCR mix was as follows: 0.4 μM each of cyt *b* primers, 0.6 μM each of 16S

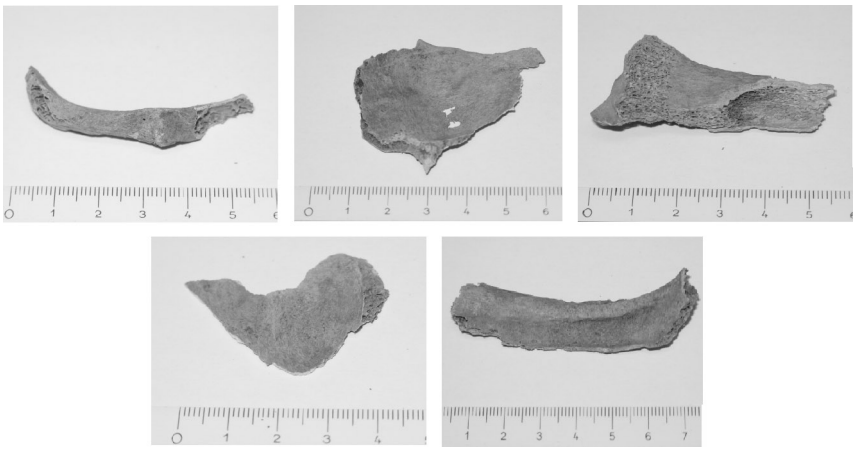

FIGURE 1. Five bone fragments recovery in the wooded zone.

rRNA primers, 1.25 U of Taq DNA polymerase (Applied Biosystems), 0.2 μM each dNTPs, 2.5 μL of 10× Taq buffer, and 5 to 10 ng of DNA in a final volume of 25 μL. Water instead of DNA was used as a negative PCR control.

To verify the efficiency of the PCR amplification, 10 ng of human DNA obtained from whole blood was used as a positive control. Control DNA was stored in Tris-Ethylenediaminetetraacetic acid buffer at −20°C at a final concentration of 100 ng/μL for up to a year.

Electrophoresis of 10 μL of PCR was performed in 2.0 % agarose gel in 1× Tris-borate-Ethylenediaminetetraacetic acid buffer with 1 mg/mL of ethidium bromide to directly visualize amplicons under ultraviolet.

Furthermore, we performed automated DNA sequencing of the different animal amplicons using BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing products were analyzed on ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

RESULTS

We analyzed 5 bone fragments (Fig. 1) with the aim to establish if they were of human origin.

DNA was extracted from a bone fragment of human origin as control, from samples belonging to different animals, and then from the 5 fragments of unknown origin. The DNAs obtained were amplified in a duplex PCR of cyt *b* (359 bp) and a 16S rRNA (157 bp) mtDNA fragments. Multiplex amplification showed a band of 359 bp for the cyt *b* and a second band of 157 bp for the 16S rRNA fragment for human biologic samples. Under the same conditions, only the cyt *b* fragment was evidenced for samples of animals, whereas the 16S rRNA fragment was completely absent (Fig. 2).

The 5 fragments of unknown species show all the 2 bands showing a human origin. Primer concentrations and number of PCR cycles were optimized to avoid preferential amplification; hence, under the reported condition, we did not see marked differences in the intensity of the 2 bands with the 5 bone samples of unknown origin that we have tested (Fig. 3).

TABLE 1. Oligonucleotide Sequences for PCR

Gene	Primer Sequence
cyt.*b* forward	5′-CCATCCAACATCTCAGCATGATGAAA-3′
cyt.*b* reverse	5′-GCCCCTCAGAATGATATTTGTCCTCA-3′[9]
16S rRNA forward	5′-CAATTGGACCAATCTATCACC-3′
16S rRNA reverse	5′-GTGAGGGTAATAATGACTTGT-3′

DISCUSSION

In situations where small fragments of bone are found, it may be impossible by morphologic features alone to confirm or exclude the human origin, and histologic or molecular methods have to be used. Here, we report the case of 5 bone fragments retrieved, whose origins were undetectable.

With the aim of distinguishing human from nonhuman bone fragments, we proposed a method involving the amplification of the cyt *b* region and the never-used 16S rRNA human-specific fragment, without sequencing analysis of the PCR products. The goal of detecting the human origin of the sample can be easily achieved by direct visualization on agarose gel of 2 bands for human biologic samples or only 1 band for all other animal species. The presence of the cyt *b* band is a demonstration of the animal origin of the specimen, whereas the second band is specific only for the human. No faint or unspecific bands appear in the animal samples tested: cattle, sheep, horse, pig, chicken, rabbit, and cat. This aspect is particularly important because another study reported the presence of faint nonspecific bands

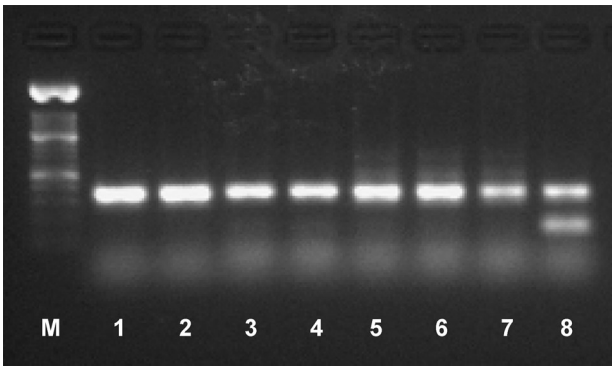

FIGURE 2. mtDNA amplification of cyt *b* (359 bp) and 16S rRNA (157 bp) fragments from different animals. Lane 1 indicates cattle; lane 2, sheep; lane 3, horse; lane 4, pig; lane 5, chicken; lane 6, rabbit; lane 7, cat; lane 8, human; M, 100-bp molecular weight marker.

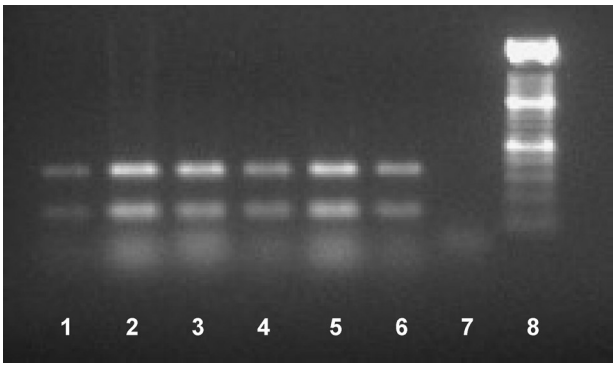

FIGURE 3. mtDNA amplification of cyt *b* (359 bp) and 16S rRNA (157 bp) of the 5 bone fragments of unknown origin analyzed from lane 1 to 5. Lane 6 indicates human positive control; lane 7, negative control; lane 8, 100-bp molecular weight marker.

in animal samples using other mtDNA fragments for human identification.[7]

Moreover, to verify the occurred PCR, we suggest the routine use of positive human control DNA to discriminate negative results for the 16S rRNA band. In this case, the lack of 16S rRNA amplicon will be surely due to the presence of biologic material from animals rather than to an error in the experimental conditions. However, special attention must be paid to avoid contamination by carryover of other samples.

In our experiment, we did not find differences in the intensity of the 2 bands, which could allow us to identify the sample of human origin.

In the case report, all 5 bone fragments result as being of human origin: all 5 DNA samples obtained and amplified show the same pattern of the human bone sample used as a positive control.

This assay demonstrates as being useful for identifying nonhuman species by means a simple comparative analysis in BLAST of the cyt *b* DNA sequence.

On the basis of the results obtained in the case reported, we consider the method proposed as straightforward and reliable, which permits the discrimination of human and animal DNA with a single-round PCR. Because recovery of information from degraded samples is often enhanced by the use of smaller PCR products, we believe that the analysis of the new small 16S rRNA fragment could represent an improvement over the multiplex PCR methods proposed in literature so far.

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