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TECHNICAL NOTE

Terry Melton, Ph.D. and Charity Holland, M.P.H.

Routine Forensic Use of the Mitochondrial 12S Ribosomal RNA Gene for Species Identification

ABSTRACT: Since July 2004, Mitotyping Technologies has been amplifying and sequencing a ~150 base pair fragment of mitochondrial DNA (mtDNA) that codes for 12S ribosomal RNA, to identify the species origin of nonhuman casework samples. The ~100 base pair sequence product is searched at http://www.ncbi.nlm.nih.gov/BLAST and the species match is reported. The use of this assay has halved the number of samples for which no mtDNA results are obtained and is especially useful on hairs and degraded samples. The availability of species determination may aid forensic investigators in opening or closing off lines of inquiry where a highly probative but challenging sample has been collected.

KEYWORDS: forensic science, mitochondrial DNA, species identification, 12S ribosomal RNA, DNA sequencing, mammals, hair analysis

Hairs and skeletal remains constitute the majority of Mitotyping Technologies' mitochondrial DNA (mtDNA) forensic specimens. For skeletal samples, unless anatomically recognizable bones are recovered from a burial site, species may be unknown. In the case of hairs, a general but unfortunate trend of reduced training of and availability of experienced hair examiners sometimes results in the submission of nonhuman or indeterminable hairs, although our laboratory recommends hair microscopy and photodocumentation for each sample submitted. In addition, if the mtDNA is very degraded, even our routine use of control region human-specific mini-primers may not yield results. In this situation, one question remains unanswered: is this a highly degraded human sample with unrecoverable mtDNA or a nonhuman sample? For many cases, determination that a highly probative hair or bone believed to be human is actually from another species can be helpful to the investigation. In this study, we report the routine forensic use of mtDNA sequencing analysis that is both species-specific and useful in degraded remains.

Several useful DNA sequencing approaches to species determination exist, all of which use conserved mtDNA regions such as cytochrome b and ribosomal DNA for primer annealing (1–9). Simplicity, ease of validation, and utility on degraded samples are critical in a forensic context. We routinely and successfully apply polymerase chain reaction (PCR) and sequencing of a short fragment of mtDNA that codes for mitochondrial 12S ribosomal RNA (12S rRNA; human mtDNA rCRS nucleotide positions 650–1603) to our forensic casework. The advantage of this assay, beyond the obvious one of using the naturally abundant mtDNA genome, is that the amplicon size is ~150 base pairs (bp), therefore rendering it useful for all but the most degraded samples. In this respect, it is similar to the mini-primer set approach used by this lab and others for degraded but abundant control region mtDNA. The region amplified corresponds to nucleotide positions 1095–1198 in humans and the primers correspond to nucleotide positions 1071–1094 and 1199–1221 per the rCRS numbering scheme (similar to primers in [6] and [7]), shown most recently to be highly conserved among 30 mammalian species (6). In addition, an online resource exists for search and identification of the many thousands of organisms with homology to this region, thereby making species identification very straightforward. A February 2007 search of the http://www.ncbi.nlm.nih.gov/BLAST site (10) showed that using the search term "12S ribosomal" returned 34,809 entries, using the search term "12S ribosomal + vertebrata" returned 23,717 entries, and using the search term "12S ribosomal + mammalia" returned 7619 entries. Although some of these entries are duplicates within species, these results indicate that the 12S gene is substantially represented in the database.

Materials and Methods

Hairs, bones, teeth, and tissue samples are extracted and amplified according to previously described methods (11,12). In our routine mtDNA control region analysis, a set of human-specific primers is first used to attempt amplification of the second half of hypervariable region 1 (16,162–16,400). If that amplification fails, mini-primer sets are used to attempt amplification of positions 16,131–16,218 and 16,209–16,356. If these amplifications fail also, we proceed to species identification. We amplify 10–15 μL of extraction product with 12S forward (12SF: 5′-ACTGGGATTAGATACCCCACTATG-3′) and 12S reverse (12SR: 5′-ATCGATTATAGAACAGGCTCCTC-3′) primers in a hot start for 12 min at 96°C followed by 40 cycles of denaturation at 95°C for 15 sec, annealing at 53°C for 30 sec, and extension at 72°C for 45 sec. The extraction reagent blank, a known human DNA and a negative PCR control are amplified in parallel. Resulting PCR products are run on a 2% agarose yield gel with a 1 kb DNA ladder, photographed for results, and then a portion of the purified product is cycle-sequenced with the same primers used for amplification. Just as in mtDNA control region analysis, if any band appears on the yield gel, the associated amplicon is sequenced; no quantification is attempted prior to cycle sequencing. The products are purified and

Mitotyping Technologies, 2565 Park Center Boulevard, Suite 200, State College, PA 16801.

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TABLE 1—*Samples used in casework validation of 12S rRNA.*

Common Name of Known Sample	GenBank Species Closest Match 12S rRNA	Product Size Trimmed of Primers (bp)	Percentage Homology of Lab Product to GenBank BLAST Search 12S rRNA (%)
Human	*Homo sapiens*	104	100
Mountain lion	*Felix concolor*	105	100
Mouse	*Mus musculus*	105	100
Kangaroo rat	*Dipodomys ordii*	102	100
Squirrel	*Spermophilus lateralis*	103	100
Mule deer	*Odocoileus hemionus*	104	100
Chipmunk	*Tamias sibiricus*	103	96 (closest match)
Dog	*Canis familiaris*	103	100

TABLE 2—*Casework results using 12S rRNA.*

Case Number	Sample	Species Designation
1	1.2 cm hair	*Canis familiaris* (dog)
1	0.9 cm hair	*Sus scrofu* (feral pig)
1	2 cm hair	*Procyon lotor* (raccoon)
2	7 cm hair	*Canis familiaris* (dog)
2	6 cm hair	*Canis familiaris* (dog)
2	8 cm hair	*Canis familiaris* (dog)
3	4.5 cm hair	*Canis familiaris* (dog)
3	2 cm hair	*Canis familiaris* (dog)
3	4 cm hair	*Canis familiaris* (dog)
4	4 cm hair	*Felis catus* (cat)
5	2.5 cm hair	*Canis familiaris* (dog)
6	3 cm hair	*Capra hircus* (goat)
7	0.6 cm hair	*Ovis aries* (sheep)
8	2 cm hair	*Canis familiaris* (dog)
9	clump tissue	*Rattus norvegicus* (rat)
9	clump tissue	*Rattus norvegicus* (rat)
10	2 cm hair	*Canis familiaris* (dog)
11	1.3 cm hair	*Canis familiaris* (dog)
12	10.8 cm hair	*Bos grunniens* (yak)

sequenced on an ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). Two qualified examiners edit the resulting DNA sequence according to standard protocol. The consensus sequence is copied and pasted into the nucleotide–nucleotide BLAST function at the website http://www.ncbi.nlm.nih.gov/BLAST (Entrez, National Center of Biotechnology Information public databases; 10). Following the prompts generates a report showing the degree of sequence homology with organisms in the database. The most likely species match is reported.

Prior to implementation in casework, the assay was validated using a variety of samples from known species (Table 1). A single sample was used for each test. For every validation sample, the correct match to species was observed in a BLAST search.

Results/Discussion

Since validation in June 2004, species identification using the 12S assay has been used in 12 cases on a total of 19 samples (Table 2), all but two of them hairs. In almost all these cases, the samples were submitted without having had the benefit of experienced microscopic examination, or a nonhuman origin was suspected based on sample morphology, and a species confirmation was desired. In many cases, the hair was small or fragmented, meaning that hair microscopy was more difficult for an inexperienced examiner.

In Case 12, beauty salon owners wished to know if their hairpiece supplier was providing them with guaranteed human hair as promised. A microscopic hair examination performed by an outside consultant revealed the hair to be animal hair, and the 12S assay showed the species to be *Bos grunniens* (yak). Upon further investigation, we learned that yaks are common sources of human-appearing hairs that are frequently incorporated into hair weaves and wigs in the beauty industry.

In Case 9, investigators reopened an unsolved 50-year-old homicide in which tissue had been collected from an automobile undercarriage. The tissue was sent for mtDNA analysis and comparison with a maternal relative of the missing victim. Attempts to amplify HV1 and HV2 with standard and mini-primer sets were unsuccessful. When the 12S assay was performed, the tissue was determined to belong to *Rattus norvegicus* (common rat). This result resulted in steering the investigation away from homicide by vehicle. In addition, this seemingly odd result was explained by the investigator who related that the vehicle's owner was an animal trapper.

Other cases turned up hairs from domestic animals such as dog, cat, sheep, and goat as well as from wild animals such as raccoon and feral pig. As the majority of our casework involves hairs, and most of our 12S assays have been applied to hairs, by definition a physical characteristic of mammals, this assay is highly useful in our laboratory. Because we already perform DNA sequencing, the addition of a 12S assay has required no change in our general sequencing or data editing routines.

Of interest might be the extent of 12S homology within and between species, or whether there would be a likelihood of spurious matches. While it would be difficult to investigate systematically the homologies for all possible species, we examined the 100 most homologous species returns for the *Canis familiaris* search. All 100% homologies ($N = 41$) were either *Canis familiaris* (domestic dog), *Canis latrans* (coyote), or *Canis lupus* (wolf), and there were other *Canis* entries at 99% ($N = 7$), 98% ($N = 1$), 97% ($N = 1$), and 94% ($N = 1$) homologies. There was no intromission of non-*Canis* species into this cluster, but species falling with the range of 94–91% homology included the bushbuck, bat, red fox, giant eland, weasel, giraffe, musk deer, spotted linsang, seal, and cow. Keeping in mind that most forensic cases will not involve animals like seals or bats, it appears that there can be a high degree of confidence in those species returned in the BLASTn search. Although there is no guarantee that a recovered animal hair or tissue sequence will find a perfect BLAST match, we note that the profiles of most common North American mammals are present in the database and the assay as designed is sufficient for general forensic species identification.

Investigation of primate homologies within the NCBI database revealed that *Pan troglodytes* (chimpanzee) shares a 98% homology with the human 12S region used here, while *Gorilla gorilla* and *Macaca mulatta* (rhesus macaque) share 97% and 90% homologies with humans, respectively. Therefore, it is unlikely that a nonhuman primate hair could be easily confused with a human hair using this system.

We previously reported that 7.2% of our hair samples give no profile due to either a nonhuman origin or absence of recoverable mtDNA due to degradation (11). Since application of the 12S assay, our frequency of no-result hairs has dropped to 4.5%. A test yielding no results is now most likely due to the complete absence of mtDNA due to degradation rather than to a nonhuman source. The size of the 12S amplicon, 150 bp, is the same as the average size of our mini-primer set amplicons used for the control region, meaning it has the same efficacy in recovery of degraded mtDNA for nonhuman samples as our mini-primer set approach does for human samples.

Although this method has so far been applied only to hairs and tissue in our laboratory, it can be applied to any other kind of biological material such as bone, blood, or saliva. Once species type is identified, a species-specific STR or mtDNA control region analysis could be applied to the remaining DNA extraction product for further animal individualization.

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Additional information and reprint requests:
Terry Melton, Ph.D.
Mitotyping Technologies, LLC
2565 Park Center Boulevard, Suite 200
State College, PA 16801
E-mail: twm107@mitotyping.com