Development of a Multiplex DNA Test To Identify Mammalian Species Present in Forensic Samples

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INTRODUCTION:

Ongoing research into the identification of non-human trace evidence, and the identification of that evidence, is being undertaken throughout the forensic community. These tests rely primarily on the mitochondrial genome due to its high copy number per cell. The use of the cytochrome *b* gene (cyt*b*), located on the mitochondrial genome and well established for taxonomic purposes, is often used in specie identification. The full cyt*b* gene is 1140 base pairs (bp) in size and is well known to show a high degree of interspecies variation, but little intraspecies variation (1). Usually all or part of the cyt*b* gene is amplified, sequenced and compared to an online database, such as NCBI (http://www.ncbi.nlm.nih.gov) for positive identification. Problems with this technique are that it is time and labour intensive, mixtures cannot be separated and degraded samples may not generate sufficient sequence data.

Based on the variation in the sequences it is possible to develop species specific primers that will only react for the species for which they were designed. This would decrease the time and cost of the test by removing the sequencing step, allow separation of mixtures and allow identification of highly degraded samples since large fragments of intact DNA would not be required.

MATERIALS AND METHODS:

Sequence Information and Primer Design

Cytochrome *b* sequence information for the six animals (*F. catus, B. taurus, C. lupus familiaris, V. vulpes, E. europaeus,* and *O. aries*) were downloaded from the NCBI website and aligned using Clustal W (http://www.ebi.ac.uk/clustalw/). Sequences were then inspected for variations that could be useful for primer design. Once an potential species specific priming site had been found, the sequence for that site was entered into the Basic Local Alignment Search Tool (BLAST) which compared it to every other sequence in the database. In this way, it could be insured that the species specific primers should not react with any other animal but the one for which they were designed. Universal primers from Hsieh *et al.* (2) were altered slightly and a new universal priming site was found to react with the species specific reverse primers.

DNA extraction and PCR amplifications

DNA was extracted from blood, tissue, hair, or buccal cells using the QIAamp® Micro Kit (Qiagen). Hair samples were allowed to digest in Proteinase K and DTT for up to 48 hours. PCR amplification was performed in a 20 µl reaction mixture, which contained 2-5 ng genomic DNA, reaction buffer (200 mM Tris-HCl (pH 8.4), 500 mM KC), 50 mM $MgCl_2$, 1.5 unit of Platinum *Taq* DNA polymerase (Invitrogen) and primers (14 total). Amplification was conducted on an ABI 2720 Thermal Cycler with the following conditions: 32 cycles of 95°C for 45 sec, 60°C for 45 sec and 72°C for 60 sec, then 72 °C for 20 min for further extension. Analysis was conducted on an ABI PRISM 310 Genetic Analyser.

Table 1: Universal and species specific primers, their location on the cytochrome *b* gene and fragment length

Animal	Location*	5'----------Sequence----------3'	Fragment Length (bp)
Universal-50†	-50	FAM6-GACCAATGATATGAAAAACCATCGTTGT	N/A
Universal+400†	400	FAM6-TGAGGACAAATATCATTYTGAGGRGC	N/A
Universal+832	832	TET-TAYGCHATYYTHCGNTCHATYCCHAAYAAA	N/A
V. vulpes	39	AAGGTCGATGAATGAGTCGTTTACG	107
E. europaeus	178	GTGAGTAATGGATGAAAATGCTGTAAG	248
O. aries	495	TAGCTTTGTCTACTGAGAATCCTCCC	113
E. europaeus	499	GTTAGAGTAGCTTTGTCAACTGAAAATGA	120
F. catus‡/*O. aries*	615	GGATGTAATTCCTGAGGGGTTGTTA	233
B. taurus‡	666	TAAGATGTCCTTAATGGTATAGTAG	283
C. lupus familiaris‡	684	GAGTAGGAGTAAGGCTCCTAGGATA	303
C. lupus familiaris	708	TAAAACTAGTGATATTAGGATTAGG	328
B. taurus	903	GTGTGTAGTAGGGGGATTAGAGCA	89
V. vulpes	1104	TTTAGGAGATTGTTTTCGATAATGCTAATG	301
F. catus	1123	TACAAAGACTCTTCATTTGAGTAGACG	317

† From Pääbo *et al.* (1988) (2) ‡ From Panvisavas (2004) (3)
* in relation to the start of the cytochrome *b* gene which was given the designation of 1
bp = base pairs, A = adenine, T = thymine, C = cytosine, G = guanine, R=A/G, Y=C/T, H=A/T/C, N=A/T/C/G

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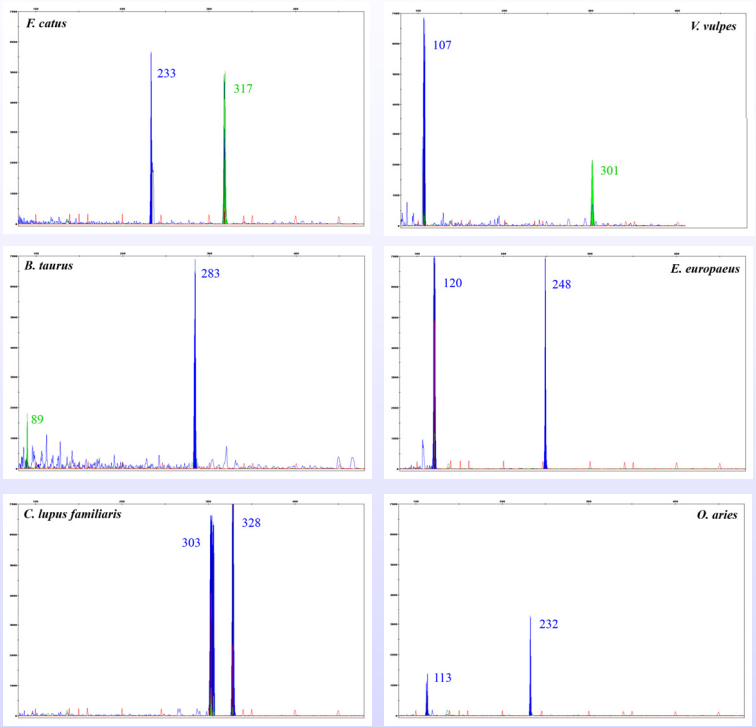
RESULTS:



RESULTS AND CONCLUSIONS:

Primers reacted only with the animal(s) for which they were designed resulting in peaks of expected size (in base pairs) that were easily distinguishable from the other species tested. The different colours of the resulting peaks (due to the different dye labels attached to the universal primers) also aid in the clarity of the results. Similarly sized peaks such as *F. catus* (317 bp) and *C. lupus familiaris* (328 bp) would be easily distinguishable even in the case of a mixture. The test is simple, fast, cheap and the results are easily interpreted making the test ideal for use in forensic casework. The test also has the potential for more species to be added at a later time.

REFERENCES:

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3. Panvisavas N. Species Testing by DNA Analysis. Unpublished Thesis. Centre for Forensic Science, Strathclyde University. 2004.