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Fast Track

Shanan S. Tobe
Adrian M. T. Linacre

Centre for Forensic Science, WestCHEM, Department of Pure and Applied Chemistry, University of Strathclyde, Glasgow, UK

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A multiplex assay to identify 18 European mammal species from mixtures using the mitochondrial cytochrome *b* gene

A novel species-specific multiplex to identify 18 common European mammalian species (badger, cat, cow, dog, donkey, fox, goat, guinea pig, harvest mouse, hedgehog, horse, house mouse, human, pig, rabbit, rat, red deer and sheep), many of which are often associated with forensic investigations, has been developed. The assay is based on the mitochondrial cytochrome *b* gene, which is commonly used in species identification and phylogeny studies. Areas of homology and variation were identified and were used to create universal and species-specific primers. The species-specific primers were designed such that they will only react with the species for which they were designed. Two primer sets were designed for each species making the test self-confirmatory. All primer sets produced the expected results. The multiplex was balanced at template concentration of 40 000 copies (approximately 1.36 pg). Validation was accomplished by analysing the same sample ten times to determine run variation and several samples for each species to determine between-sample variation. Twenty-eight additional mammalian species were reacted with the multiplex. The multiplex provides, for the first time, a definitive method for identification of species in a forensic context.

Keywords:
Cytochrome *b* gene / Forensic science / Mixtures / Species identification / Trace samples
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1 Introduction

Non-human evidence is routinely encountered in forensic science and is often left unanalysed due to lack of a standard method, especially when extremely low levels of DNA are present or a mixture is suspected. Forensic scientists may have to investigate an apparent animal attack or instances where animal interference after death can be confused with pre-mortem injuries that can arouse suspicion of violence prior to death [1]. In this context, rodents [2] and canids [3] frequently alter remains. Other needs include determining the species of origin of bloodstains [4], identification of the components of meats (for religious purposes or to identify poached species which are out of season, endangered or where a quota has been exceeded) [4] and identification of human and animal components of commingled remains from mass disasters, fires, cremations and domestic crimes [5]. Customs officers require the ability to identify species (or products derived from those species) being poached or traded contrary to national and international legislation [4].

If gross morphological characteristics are present, it may simply be a case to identify the remains by microscopy or osteology [5–9]. Skins or pelts can be identified from microscopic analysis of the hairs, as the hairs of many animal species have distinct morphological characteristics. However, identification based on hair analysis is both subjective and rarely species specific. In Moore's key [10] dog appears in over ten categories and most identifications finish in a group of organisms and not a specific species. If an animal is killed for food or sport, identifying characteristics may be intentionally removed [4], making morphological methods unsuitable [11] and therefore requiring other methods of evaluation [5]. Forensic science laboratories often get powdered samples with no morphological characteristics for identification [12] also rendering morphological analysis unusable.

DNA-based tests that require no prior sequence information can be used, such as RAPD, amplified fragment length polymorphism (AFLP), or RFLP. RAPD, AFLP and

Correspondence: Dr. Shanan S. Tobe, Centre for Forensic Science, WestCHEM, Department of Pure and Applied Chemistry, University of Strathclyde, 204 George Street, Glasgow, G1 1XW, UK
E-mail: shanan.s.tobe@strath.ac.uk
Fax: +44-141-548-2532

RFLP have been applied to identification of animal components [13–17]. The problem and limitation with these whole-genome techniques is that there are always issues with reproducibility and when complex mixtures of two or more species are to be detected, interpretation of results may be difficult due to overlapping restriction patterns that may be generated [18].

Rastogi *et al.* [19], in their analysis of several techniques of species identification, found mitochondrial markers to be better suited for species identification and authentication than were nuclear markers. This coupled with the mitochondrial DNA (mtDNA) high copy number per cell makes it the method of choice for species identification, especially when analysing samples with little or no nuclear DNA [20, 21]. Currently, the standard tool in species identification is mtDNA amplification and sequencing [12, 22–28]. For species identification, any loci used must show interspecies variation but little intraspecies variation [6, 28]. Loci used are generally the cytochrome *b*, cytochrome oxidase (I-III), 12S and 16S rRNA genes.

Amplification and then sequencing of the products for species testing is a laborious procedure. Sequence alignments show that there are a limited number of bases that are variable and therefore much of the sequence data are redundant. Many samples collected are mixed with DNA from humans or from another mammal. Sample mixtures pose a large problem in that the resulting sequence mixtures are too complicated to decipher. There is a rationale for developing species-specific primer sets.

Species-specific primers can be designed based on interspecies SNPs provided those SNPs have little or no intraspecies variation [29]. There have also been a number of multiplex tests developed to separate a small number of species based on this premise [7, 21, 30, 31]. These tests are being developed as they are needed, identify a small number of species and are used independently of each other. We describe a multiplex PCR that can separate and identify components of a mixture and obtain consistent results from as little as 10 000 copies (approximately 340 fg) of template DNA. Two separate primers sets were designed to amplify a species specific section of the cytochrome *b* gene for each of the 18 species tested. The result would be two PCR products of known size, both of which would be different to any other species tested. Any one of 18 common mammalian species can be identified, even within mixed samples, with this fast and straightforward test.

2 Materials and methods

2.1 Sequence information, primer design and DNA extraction

Cytochrome *b* sequence information was downloaded from NCBI (http://www.ncbi.nlm.nih.gov/) and aligned using Clustal W (http://www.ebi.ac.uk/clustalw/). The species used in this study and the accession numbers of the sequences used are listed in Table 1. Universal primers were designed such that they will react with all mammalian species. Two of the universal primers used are modified versions of those given by Pääbo *et al.* [32]. Species-specific primers were designed to only react with the species for which they were designed [three of which are from a previous study by Panvisavas (unpublished thesis)] and such that the size of the fragment could not be confused with any other fragments (Table 2). Two species-specific reverse primers for each species were designed to react with one of three labelled universal forward primers except for hedgehog, which has only one reverse primer (Fig. 1). The primers were ordered from Sigma Genosys® (Sigma-Aldrich, Haverhill, UK). Primers arrived lyophilized and were re-suspended with sterile water to a concentration of 100 μM. A dilution of each primer was made to 10 μM in volumes of 100 μL.

DNA was extracted from tissue, hair or buccal cells using the QIAamp® Micro Kit (Qiagen, Crawley, UK). Buccal swabs were obtained by rubbing a sterile swab against the inside of the cheek several times and then placing into a sterile tube and storing at 4°C until extraction of DNA. Swabs from meats were taken by rubbing the sterile swab directly onto the raw meat and then placing them into a sterile tube and storing at 4°C until extraction of DNA. Hairs were either plucked, so a root could be obtained, or loose hairs were

Table 1. List of the mammals used for sequence alignment and their accession numbers

Common name	Latin name	Accession number	Common name	Latin name	Accession number
Cat	*Felis catus*	NC_001700	Hedgehog	*Erinaceus europaeus*	NC_002080
Cow	*Bos taurus*	NC_001567	Horse	*Equus caballus*	NC_001640
Dog	*Canis lupus familiaris*	NC_002008	House mouse	*Mus musculus*	AY675564
Donkey	*Equus asinus*	NC_001788	Human	*Homo sapiens*	NC_001807
Badger	*Meles meles*	AB049808	Pig	*Sus scrofa*	AY337045
Fox	*Vulpes vulpes*	X94929	Rabbit	*Oryctolagus cuniculus*	NC_001913
Goat	*Capra hircus*	NC_005044	Rat	*Rattus norvegicus*	NC_001665
Guinea pig	*Cavia porcellus*	NC_000884	Red Deer	*Cervius elaphus*	AY244491
Harvest mouse	*Micromys minutus*	AB033697	Sheep	*Ovis aries*	NC_001941

Table 2. Primers used in the multiplex

Animal	Pos[b]	5'————Sequence————3'	Len (bp)	Conc (nM)	T_m (°C)
Universal	−50	FAM6-GACCAATGATATGAAAAACCATCGTTGT[c]		1000.00	68.72
Universal	400	HEX-TGAGGACAAATATCATTYTGAGGRGC[c]		1000.00	67.52
Universal	832	TET-TTTTTTTTTTTTCGVTCHATYCCHAAYAAACTAGG		2500.00	65.91
C. porcellus	85	GAGGGAGCCGAAGTTTCATCACGT	156	650.00	72.00
C. lupus familiaris	99	CAAGCATACTCCTAGTAAGGATCCG	170	750.00	64.67
O. cuniculus	115	GTGAAAATTTGAATTATAAGGCACAG	184	650.00	62.38
S. scrofa	147	TCTGATGTGTAATGTATTGCTAAGAAC	219	177.00	61.11
C. hircus	198	GCCATAATTTACATCTCGACAAATGTGAGTT	273	139.50	69.36
H. sapien	208	TTCAGCCATAATTTACGTCTCGAGT	277	71.00	65.49
C. elaphus	210	CGAATAATTCAGCCATAATTGACATCTCGA	285	85.00	70.95
M. minutus	260	GAAGGAATAAGCAGATAAAAAATATGGATG	340	123.00	65.02
F. catus	471	TTTCCCTCAGATYCATTCTACTAGTTCAGTC	89	187.50	66.98
C. porcellus	471	CCAGATTCACTCTACAAGGGTTGTC	94	600.00	65.71
E. europaeus	499	GTTAGAGTAGCTTTGTCAACTGAAAATGA	120	382.50	64.40
C. elaphus	567	AGTAAGTGTACTATAGCGAGTGCTGCG	188	31.30	66.24
O. cuniculus	571	AAAGAGGAGGTGAATTAAGACTAAAGT	192	225.00	60.72
V. vulpes	572	GGAGAAATAAGAGATGAACCATCGCTAATG	196	78.00	69.31
S. scrofa	580	CGTGCAGGAATAGGAGATGTACGGC	199	26.50	71.85
M. meles	624	TTTGTCAGAATTRGAGGGGATWCCAGAG	241	540.00	71.83
H. sapiens	624	ATCGGAATGGGAGGTGATTCCTAGG[d]	248	39.00	71.23
B. taurus	666	TAAGATGTCCTTAATGGTATAGTAG[d]	287	150.50	54.30
R. norvegicus	682	GGAATAATAGTAATATAAATACACCTAGGAGGTC	310	100.00	62.22
C. lupus familiaris	684	GAGTAGGAGTAAGGCTCCTAGGATA[d]	303	77.50	60.94
C. hircus	693	TTAGAACAAGAATTAGTAGCATGGCG	313	351.00	64.92
E. asinus	704	AATACTAGGGTTAGTAGGAGTAGGACTA	327	100.00	58.15
E. caballus	705	AGAATAATACTAGAGTTAGTAGGAGCAAGATC	333	133.50	60.97
O. aries	710	GGCGTGAATAGTACTAGTAGCATGAGGATGA	336	112.50	69.78
M. musculus	746	GTAGTTGTCTGGGTCTCCTAGTATATC	382	74.00	60.22
B. taurus	903	GTGTGTAGTAGGGGGATTAGAGCA	93	120.00	64.06
O. aries	906	GCTTTGATGTATGGAGGAGGGGTATAATT	98	260.00	68.62
V. vulpes	939	TAAGGGGYCGGAATATTATCCCA	125	91.50	65.75
M. minutus	960	TACAAGGATTCAGTAAAGTGTTTGGGAG	151	40.75	66.63
M. meles	985	GTTARGGTGARGAGGTCTGCAAC	173	186.50	64.41
F. catus	990	GATTCATGTTAGGGTTAGGAGATCC	180	35.00	63.57
M. musculus	996	CTCCAATTCAGGTTAAGATAAGT	186	234.00	57.02
R. norvegicus	999	GGTTGGCCTCCGATTCATGTTAAGACT	192	62.00	71.81
E. asinus	1014	TTCTACTGGTTGGCCACCA	198	111.50	64.17
E. caballus	1023	TACGTATGGGTGTTCCACTGGC	208	110.00	67.75

The species for which they were designed, their position on the cytochrome *b* gene, sequence, product length, concentration in the multiplex and T_m. Position is given as the 3' base for species-specific primers. Length is the observed[a] product size in base pairs as determined on the genetic analyser and includes the length of the primers.

a) Observed length was slightly different from the calculated length. This is attributed to the dye labels and differing pyrimidine/purine ratios within the fragments and the variation in fragment length remained consistent between individuals of the same species.

b) Position is in relation to the start of the cytochrome *b* gene, which was given a designation of 1.

c) Modified from Pääbo *et al.* [32].

d) Modified from Panvisavas (unpublished thesis).

collected. Hair was placed into a sterile bag or Eppendorf tube until extraction of DNA. Tissue samples were frozen at −20°C until the extraction of DNA. Hair samples were allowed to digest in proteinase K and DTT for up to 48 h until completely dissolved. DNA extracts were quantified by determining the total mammalian mtDNA and total human mtDNA present in each sample and subtracting the human fraction from the total mammalian mtDNA. This gave an accurate quantity of the non-human mammalian mtDNA present in each sample, which was then diluted to a quantity of 40 000 copies/μL. All weight values are given as equivalent to the number of copies of template in relation to a human mitochondrial genome of 16 569 bp.

2.2 Multiplex PCR

All PCRs were assembled in a sterile hood using sterile pipettes and tips. This was ensured by positive airflow from the

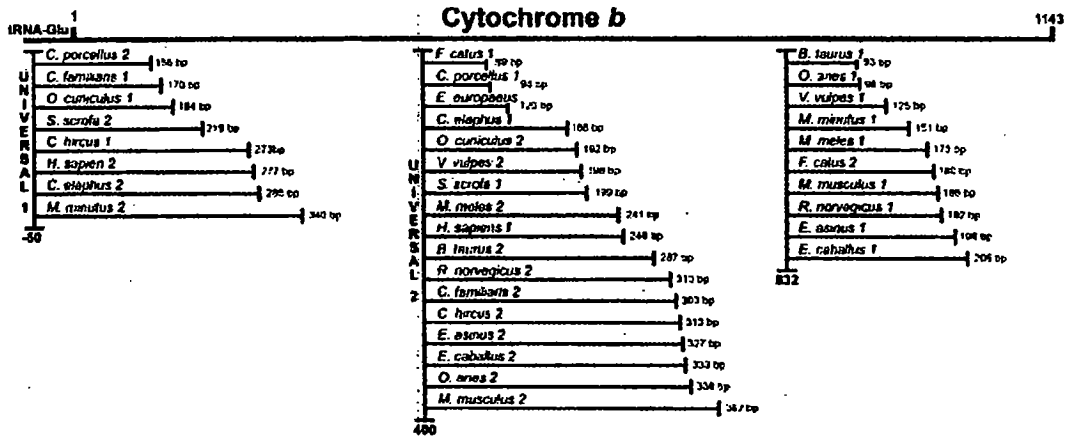

Figure 1. The end of the tRNA-Glu gene and the entire cytochrome *b* gene of the mtDNA showing the placement of the universal and species-specific primers. Numbers in bold indicate the position according to the start of the cytochrome *b* gene which was given a designation of 1. The names indicate for which species the primer will react and a designation of 1 is given to the smaller fragment of the pair designed for each species, the larger fragment is given the designation of 2. Fragments are shown according to the position of the 3′ end of the primer. Size in base pairs (bp) of each fragment (including primer length) is shown. Each universal primer is labelled with a fluorescent dye: universal 1 with FAM6 (blue); universal 2 with HEX (yellow) and; universal 3 with TET (green). This figure is an approximation.

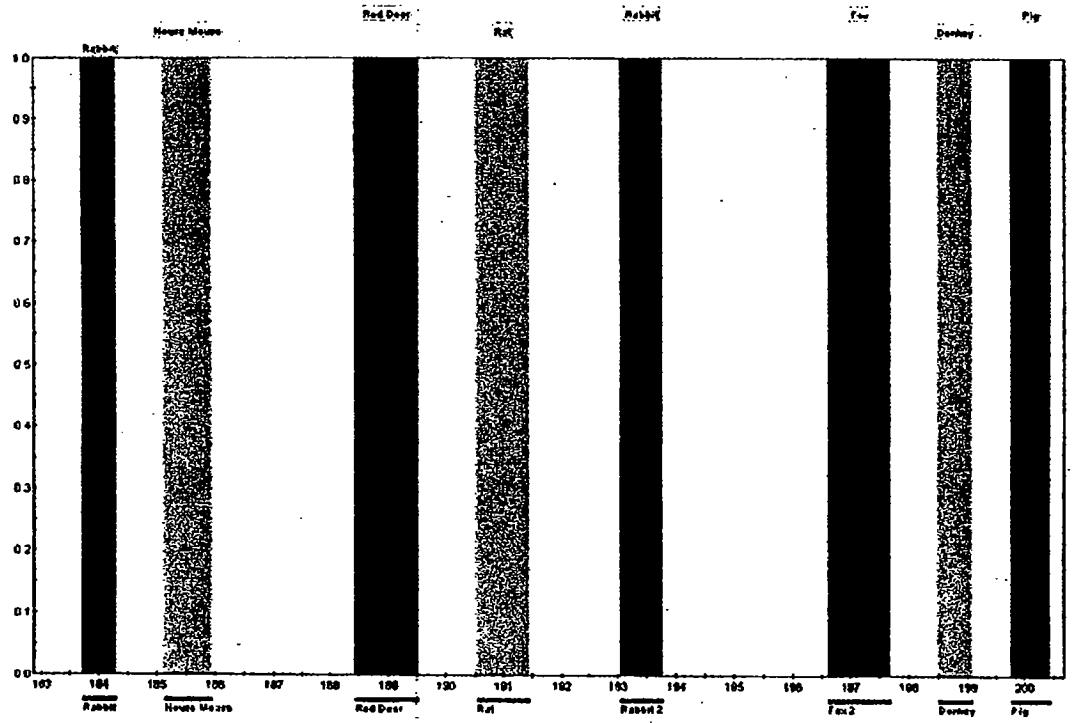

Figure 2. A section of the panel and bin set used to identify peaks present. Eight potential peak positions are shown which correspond to rabbit, house mouse, red deer, rat, rabbit, fox donkey and pig, respectively. All peaks are easily distinguished from each other.

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hood and sterilization with ultra-violet light before and after each use (approximately 60 s exposure at 200 W to destroy any DNA present).

The primers were multiplexed together into one PCR. Final PCR volume was 20 μL containing GeneAmp® 10X PCR Gold Buffer (100 mM TRIS-HCl, 15 mM $MgCl_2$, pH 8.0), dNTP (200 μM), 1.5 units AmpliTaq Gold® (Applied Biosystems, Foster City, CA), universal and species-specific primers (final concentrations ranging from 26.5 nM to 2.5 μM, see Table 2), sterile H_2O, and 40 000 copies of template DNA (1.36 pg). The PCR cycle proceeded for 35 cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 45 s followed by a final extension step of 20 min at 72°C.

All samples were analysed on an ABI 310 Genetic Analyser (Applied Biosystems, Foster City, CA) on filter set C using ROX500 size standard (Applied Biosystems). Preparation of samples involved placing 2 μL of PCR products in 16 μL of formamide with 0.5 μL of size standard. The threshold for each dye was set to 100. GeneMapper® ID v3.2 (Applied Biosystems) was used to visualize the electropherograms.

2.3 Validation and creating a ladder

For validation, control samples were analysed ten times each to determine run variation. In addition, multiple individuals (approximately ten) of each species were obtained and analysed with the multiplex to determine sample variation. Based on these results a panel and bin set were created to identify all peaks when analysed using GeneMapper™ (Fig. 2).

Control samples were further diluted to 10 000 (340 fg) copies, 1000 (34 fg) copies and 500 (17 fg) copies and tested with the multiplex under standard conditions and an additional dilution of 500 copies was tested with an additional five cycles of PCR (40 cycles total). Twenty-eight other animals were also tested with the multiplex (addax, American bison, Asiatic black bear, bank vole, Canadian black bear, chicken, chimpanzee, chinchilla, clouded leopard, duck, field vole, gerbil, hare, jaguar, koala, llama, margay, meerkat, muntjac deer, Père David deer, Siberian tiger, sika deer, snow leopard, serow, turkey, white tailed deer, wolf and wood mouse) to determine if any of the primers would cross react.

To test the accuracy of the multiplex reaction 20 blind trial samples were created using the 18 mammals of the multiplex and the 28 other animals that were tested. Blind trial samples included mixtures at varying concentrations of DNA.

3 Results

Each species for which the multiplex was designed produced the expected size of PCR fragment and each fragment could be unambiguously distinguished from all other PCR fragments generated from the other 17 species (Fig. 3). Testing

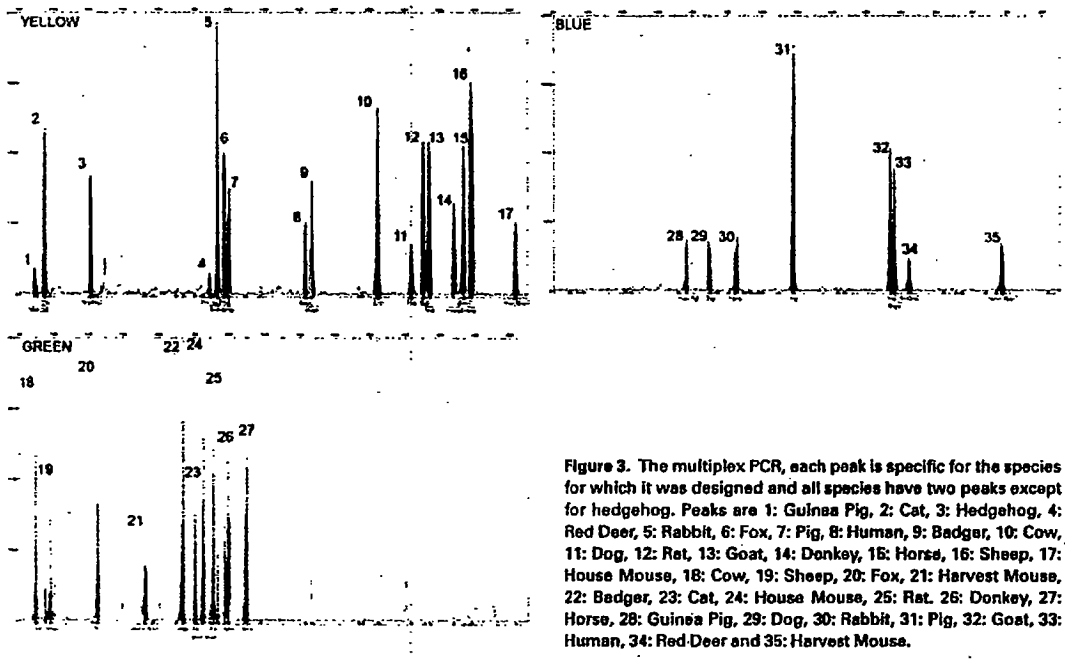

Figure 3. The multiplex PCR, each peak is specific for the species for which it was designed and all species have two peaks except for hedgehog. Peaks are 1: Guinea Pig, 2: Cat, 3: Hedgehog, 4: Red Deer, 5: Rabbit, 6: Fox, 7: Pig, 8: Human, 9: Badger, 10: Cow, 11: Dog, 12: Rat, 13: Goat, 14: Donkey, 15: Horse, 16: Sheep, 17: House Mouse, 18: Cow, 19: Sheep, 20: Fox, 21: Harvest Mouse, 22: Badger, 23: Cat, 24: House Mouse, 25: Rat, 26: Donkey, 27: Horse, 28: Guinea Pig, 29: Dog, 30: Rabbit, 31: Pig, 32: Goat, 33: Human, 34: Red Deer and 35: Harvest Mouse.

with multiple members of the same species showed that peak sizes remained consistent between individuals. Based on these results a panel and bin set were designed for use with GeneMapper™ to automatically identify the peaks (Fig. 2).

Sensitivity studies showed that at 10 000 copies all peaks for all species were detected except for both guinea pig peaks and the rabbit peak expected at 184 bp. At 1000 copies of template DNA the observed peaks with peak size in bp in brackets were badger (241), cat (89 and 180), cow (93), donkey (198), fox (125 and 196), goat (273), harvest mouse (340), horse (208 and 333), human (246), pig (199 and 219) and, rat (192 and 310). At 500 copies with the standard 35 PCR cycles the observed peaks were goat (273), horse (333) and, pig (219). At 500 copies with the extended 40 cycles of PCR the observed peaks were: badger (173 and 241), cat (180), cow (93), donkey (198 and 327), horse (333), house mouse (186), human (246 and 277) and, rabbit (192).

In addition, the multiplex was reacted with 28 related and unrelated mammals. Eighteen of the tested species did not produce any fragments or produced fragments indicative of human contamination only. Ten of the species tested produced PCR products but none (except wolf) could be confused as being from one of the species, which the multiplex was designed to detect. Products either were off ladder or were indicative of only one fragment of the expected two for any given species.

A series of 20 blind trials were used to further validate the test. These all consisted of mixtures of two or more species at varying concentration. All species for which the multiplex was designed which were present were correctly identified even in the presence of other animal DNA. The most complicated sample consisted of a mixture of ten species (Fig. 4), all of which were identified.

4 Discussion

The developed multiplex provides, for the first time, a method to accurately identify up to 18 mammalian species simultaneously from mixtures at DNA template levels far below what has previously been reported in other multiplex reactions. The test is straightforward to set up, it does not require further sequencing of PCR products and the data are easily interpreted. The multiplex consists of 3 labelled universal forward primers and 35 species-specific reverse primers designed to identify 18 mammalian species. Using only 3 labelled primers decreases the cost of the test significantly. Primers amplified the expected PCR products successfully with unambiguous identification of the species for which they were designed; cross-reaction was minimal and no species showing cross-reactivity could be misidentified as one of the species in the multiplex (except wolf). Two products being obtained for each species (except for hedgehog) means

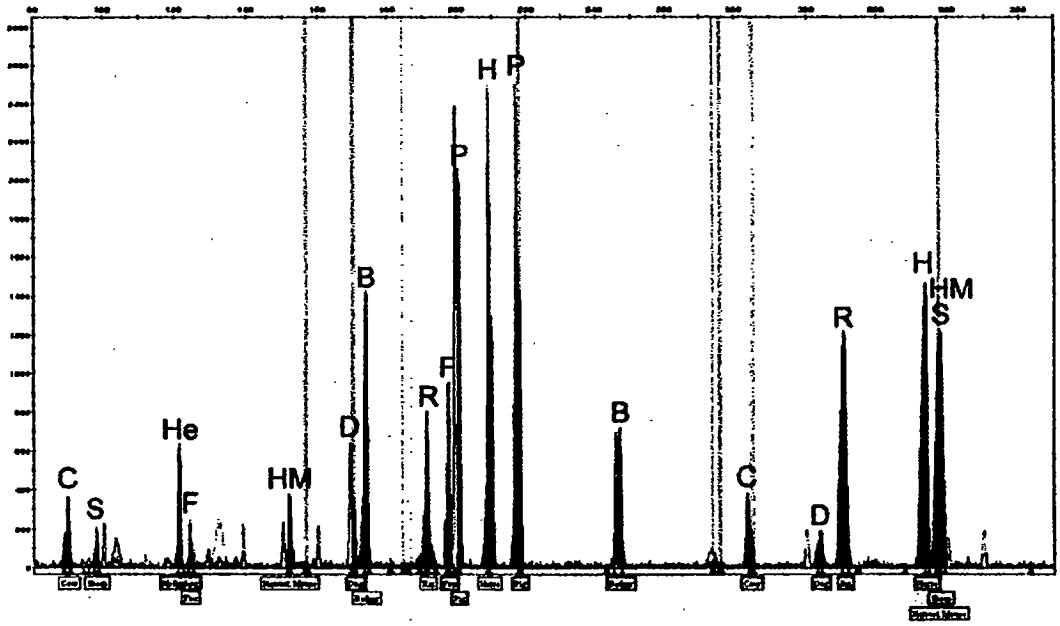

Figure 4. Blind trial mixture of 10 species, all of which were correctly identified. C: cow, S: sheep, He: hedgehog, F: fox, HM: house mouse, D: dog, B: badger, R: rat, P: pig, H: horse.

that the test is self-confirmatory. Our studies showed that primers were sensitive to approximately 340 fg.

Validation of the samples showed that the multiplex would produce consistent results between members of the species for which it was designed. Individual species were identified within complex mixtures of samples. This is necessary to be able to separate human and non-human DNA in cases where the samples are contaminated with human DNA.

The complete DNA sequence of the cytochrome b gene for a range of mammals including those used in the trial were aligned to illustrate the genetic relationship of the species so as to note potential cross species reactivity. This is illustrated in Fig. 5. Wolf reacted with both dog primers but not with any of the other primers. This was expected; as there is only about a 4-bp difference between the wolf and dog cytochrome *b* genomes although some may have even less variation. Apart from wolf, no other species reacted with the multiplex such that it could be confused with any of the species for which the assay was designed.

The benefit of the multiplex is that most of the species that can be identified are done so with at least two specific fragments. Even with the cross-reaction observed, no species (except wolf) produced fragments that could be confused with one of the detectable species. This means that none of the related and exotic species tested could ever be confused for another species using the multiplex analysis.

In cases where only one product of the expected two is observed the test should only be considered as a presumptive test. The failure of the second product could be due to one of three reasons: less than 340 fg of template was present; the sample contained highly degraded DNA or, unexpected cross-reaction with another species. For a more definitive identification, the product obtained can be sequenced and compared to known sequences.

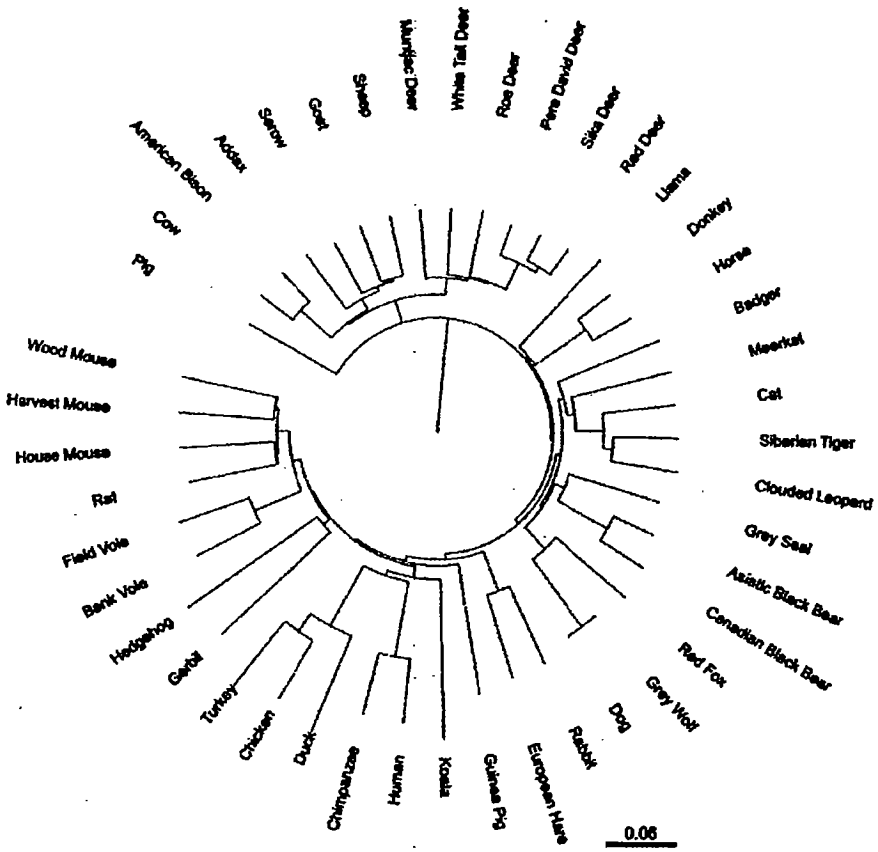

Figure 5. Phylogenetic tree of the species in the multiplex and the other species tested for which a complete cytochrome *b* sequence could be obtained. The tree was created using the Interactive Tree of Life [33]. Table 1. A list of the mammals used for sequence alignment and their accession numbers

The multiplex has provided for the first time an effective method for simultaneous identification of mammalian species from trace samples and mixtures. The ability to identify animal components in a mixture and at levels of mtDNA as low as the femtogram level (3.40×10^{-13} g according to human mtDNA weight) is a major advantage over current techniques. Little validation is required to allow this test to be used in different laboratories. The test is accessible to any laboratory with similar equipment and inter-laboratory sharing of data is possible.

Samples were kindly obtained from Dudley Zoo; the Isle of Wight Zoo; Edinburgh Zoo; Auchingarrich Wildlife Park; The City of Glasgow City Council; Stuart Becker at Liverpool University; Linda Horan at University of Strathclyde; Trevor at The Fox Trust; Hez Hird at Central Science Laboratory UK; Eleanor McMillan; Kathryn Tobe; Sarah Reid; Sarah Cresswell; Mr. Gilchrist; Tracey Nielson; Ainsley Dominick; Joanne Kemp; Niamh Nic Daeid; Erin Harvey; Frances Newman; Lynn Curran; Lindsey Dixon; Pamela Allan; Jennifer Moore and Bryan Evans.

The authors have declared no conflict of interest.

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