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Species identification by means of the cytochrome b gene

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Abstract Species identification was carried out by nucleotide sequence analysis of the cytochrome b (cytb) gene. The aim of the study was to identify biological specimens from diverse vertebrate animals by extracting and amplifying DNA from 44 different animal species covering the 5 major vertebrate groups (i.e. mammals, birds, reptiles, amphibians and fishes). The sequences derived were used to identify the biological origin of the samples by aligning to cytb gene sequence entries in nucleotide databases using the program BLAST. All sequences were submitted to the GenBank including new species which were not observed in the databases. The applicability of this method to the forensic field is demonstrated by simulated casework conditions where different types of samples including problematic specimens such as hair, bone samples, bristles and feathers were investigated to identify the species.

Key words Species identification · Mitochondrial DNA · Cytochrome B gene · Sequence analysis · Forensic casework

Introduction

The identification of the biological origin of casework stains has commonly been performed by conventional methods (e.g. Schleyer and Oepen 1995) and immunological procedures have been widely applied in the forensic community. These techniques involve precipitation of the antigen fraction in a casework stain using a corresponding antibody raised against soluble proteins. These methods have several drawbacks for forensic casework analysis:

1. Analysis is restricted to a limited assortment of species which can be tested. A taxon can therefore only be identified when the corresponding antibody is included in the test. These antibodies are of limited stability and the commercial support is insufficient, rendering the maintenance of a representative set a costly task.
2. These techniques are unspecific at the species level and may only indicate the family to which the species belongs and proteins of related animals can cause cross-reactions.
3. These techniques are based on the characterization of proteins which may suffer environmental stress when a stain is exposed to casework conditions.
4. The analysis consumes a portion of the sample reducing the amount of evidential material for further investigations.

In routine casework analysis these methods have rarely been applied to identify the exact species of a stain sample and are mostly used for the general discrimination between human and non-human samples. In a number of cases this information usually meets the demand and non-human samples are therefore not investigated further. In particular cases however, detailed information on the species of the sample can be of importance, or even more, the aim of the investigation in for example, traffic accidents with animals, poaching, violation of animals and trading of animals.

In the current study sequence analysis of the cytochrome b (cytb) gene has been applied to the identification of vertebrate species. This method uses DNA to identify the biological origin of a casework sample, benefiting from an enhanced information content compared to proteins. The nucleotide sequence of the cytb gene contains species-specific information and has been used in phylogenetic as well as in forensic investigations in a number of studies (Kocher et al. 1989; Smith and Patton 1991; Carr and Marshall 1991; Bartlett and Davidson 1991, 1992; Irwin et al. 1991; Russo et al. 1996; Zehner et al. 1998;

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Bataille et al. 1999). Furthermore, the cytb gene is located on the mitochondrial genome taking advantage of the sensitivity of PCR-based mtDNA typing in the forensic context (Holland et al. 1993; Sullivan et al. 1992; Wilson et al. 1995 a, 1995 b).

Material and methods

Extraction

Biological specimens (e.g. blood, soft tissue, testicle, ovary, hair/bristle, bone, cartilage, liver and feathers) were obtained from 44 different vertebrate animals from the Alpenzoo Innsbruck and extracted using 5% chelex suspension (Walsh et al. 1991) in a total volume of 50–200 µl (depending on the size of the sample) for 2 h up to overnight at 56 °C. The samples were denatured at 95 °C for 10 min and centrifuged at 13200 rpm. Portions of the hairs and bones were also extracted by the phenol/chloroform method (Sambrook et al. 1989). Prior to extraction hairs were washed in sterile deionized water for 30 min, incubated and agitated in absolute alcohol for 30 min and dried at 56 °C. The exposed surfaces of the bone samples were removed with sandpaper, cleaned with absolute ethanol, dried and crushed in a mill (Retsch ZM 100) and the powder was then used for extraction.

For amplification 1 µl of the extract (or up to 5 µl for hair and bone samples) was used for PCR in a 9600 GeneAmp (Perkin Elmer) and performed according to the protocol described by Bartlett and Davidson (1992) in a total volume of 25 µl consisting of 1 × PCR reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl and 1.5 mM $MgCl_2$), 250 µM each dNTP, 0.25 µM each primer (L14816: 5′-CCATCCAACATCTCAGCATGATGAAA-3′ and H15173: 5′-CCCCTCAGAATGATATTTGTCCTCA-3′) and 2 U AmpliTaq Gold polymerase (Perkin Elmer) for 30 cycles (35 cycles for hair and bone samples) at 94 °C for 30 s, 50 °C for 45 s, and 72 °C for 45 s including one initial denaturation step at 95 °C for 11 min. The primer sequences are based on the numbering system of a human reference (Anderson et al. 1981) referring to the 5′ end of the primer. Amplification products were loaded on a non-denaturing polyacrylamide gel, run at constant power of 5 W for 30 min and visualized using silver staining (Goldman and Merril 1982).

Amplicons displaying artifacts on the amplification control gel were loaded on a discontinuous agarose gel (stacking gel, 0.8% standard agarose, separation gel 2% LMP agarose, 10 mM TA, pH 8.3, 0.1 mM EDTA; 1 µg ethidium bromide/ml) and subjected to electrophoresis at 90 V for 1 h. Bands were visualized with UV light (254 nm) prior to excising from the gel using autoclaved pipette tips cut at the tip. DNA was extracted from the gel using GenElute agarose spin columns (Supelco, Bellefonte, Pa.). A 1 : 1000-dilution of the purified amplicon was re-amplified applying the above described protocol. Amplified DNA was quantified on a Hitachi F 2000 spectro-fluorometer using the Picogreen dsDNA quantitation kit (Molecular Probes Europe, Leiden, The Netherlands).

Sequencing

Sequencing reactions were performed as described previously by Parson et al. (1998) except that the cytb PCR primers were used as sequencing primers. Analysis was performed using ABD Prism Sequencing Analysis software, version 3.0. Sequences were aligned and analysed with Sequence Navigator software, version 1.0.1 (Perkin Elmer).

Database search

A 300 bp fragment of the consensus sequences (from nucleotide position 14846 to 15145, nomenclature according to the human reference sequence of Anderson et al. 1981) obtained in the laboratory was imported into the BLAST query window (Altschul et al. 1990, 1997). A non-redundant search was launched applying default settings of the software package (Smith et al. 1996).

Results and discussion

Commonly used forensic typing systems including STRs, VNTRs and reverse dot-blot techniques such as HLA-DQalpha are known to produce human-specific results because the amplification primers usually do not amplify non-primate DNA or at least do not produce results in the expected fragment size-range (Baechtel et al. 1995; Cosso and Reynolds 1995; Sparkes et al. 1996; Taylor et al. 1997). Since the DNA profile obtained indicates the human origin of the sample, these cases do not require further species determination. However, species identification of a case stain becomes an issue if non-human material is present.

In this study, a short fragment (358 bp) of the cytb gene was investigated for species identification because this fragment has by far the widest taxonomic representation in nucleotide databases. It is therefore likely to find a sequence entry of an unknown sample or at least of a related species. Currently, there are more than 8000 cytb gene sequences of vertebrate animals available in the GenBank/EMBL/DDJB and this data set is constantly growing. Within 1 week in April 1999 the addition of more than 100 additional sequence entries was observed. A list of the distribution of cytb gene sequences in the five major vertebrate classes is given in Table 1. For more detailed information, nucleotide sequence search programmes can be used to find the sequence of a particular species. As an example, we used Entrez from the National Center of Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/Entrez/ nucleotide.html). This programme also provides a taxonomy browser which gives information on the number of sequence entries that can be found for a particular species of interest or any other animal group (http://www.ncbi.nlm.nih.gov/Taxonomy/tax.html).

In this study the cytb fragment was amplified and sequenced from 44 different vertebrate animals (15 mammal, 22 bird, 1 amphibian, 2 reptile and 4 fish species) in order to determine whether the species can be re-identified by the information content of the nucleotide sequence derived (see Table 2). This was achieved by searching a homologous 300 bp fragment of the sequences obtained in the laboratory against nucleotide sequence databases accessible via internet. Furthermore, different biological specimens including problematic samples like hair shafts, bones, bristles and feathers were investigated using this technique in order to simulate casework conditions.

Extraction, amplification and sequencing

Only one pair of PCR primers was used to amplify a 358 bp of the cytb gene of all investigated animals (Table 2). Am-

Table 1 Overview of vertebrate cytochrome b gene sequence entries in nucleotide databases *(April 1999)*. Families, which include a majority of sequence entries are presented next to the corresponding order (e.g. 654 sequences were found for primates, 502 of which are *Hominidae*)

Class/Order	Entries	Family	Entries
Mammalia			
Primates	654	Hominidae	502
Lagomorpha	22		
Rodentia	1448	Murinae	725
Artiodactyla	50	Bovidae	38
Cetacea	96		
Carnivora	390	Canidae	111
		Felidae	54
		Ursidae	95
Chiroptera	123	Microchiroptera	95
		Macrochiroptera	28
Insectivora	156	Soricidae	118
Others	24		
Aves			
Apterygiformes	24		
Porcellaniformes	154	Diomedeidae	25
		Porcellariidae	126
Pelecaniformes	32		
Ciconiiformes	46		
Anseriformes	157	*Anatidae*	156
Falconiformes	56		
Galliformes	90		
Gruiformes	47		
Charadriiformes	182	Alcidae	44
		Stercorariidae	55
		Scolopacidae	37
		Charadriidae	45
Psittaciformes	45		
Cuculiformes	46		
Strigiformes	10		
Caprimulgiformes	20		
Apodiformes	58		
Trochiliformes	56		
Trogoniformes	43		
Coraciiformes	10		
Piciformes	28		
Passeriformes	779	Fringillidae	190
		Pyconotidae	83
		Sylviidae	41
		Timaliidae	37
		Corvidae	27
		Turdidae	33
		Paridae	24
		Rhinocryptidae	38
		Vireonidae	69
Others	36		
Reptilia			
Rhynchcephalia	1		
Squamata	847	Lacertilia	587
Chelonia	8		
Crocodilia	5		
Amphibia			
Anura	99		
Urodelia	143		
"Pisces"		Order	
Chondrichthyes	59		
Euteleostei	1333	Cyprinodontiformes	328
		Perciformes	522
		Cypriniformes	230
		Gadiformes	79
		Salmoniformes	40

plification of the chelex extracts generally led to the expected PCR products except for the bone samples, which failed to amplify. However, phenol/chloroform extraction of the bone samples subsequently resulted in successful amplification. Hair samples and bristles generally performed better with the organic extraction method.

Amplification yielded a single band in most of the species samples investigated (Fig. 1), as the PCR primers anneal in conservative regions of the cytb gene (Kocher et al. 1989). However, variations in the base composition of the annealing sequence between variant species can influence the performance of the PCR. In one case the amplification resulted in a pattern of several bands, including the 358 bp fragment. This phenomenon was especially obvious in the cat sample and confirmed by multiple amplifications using other specimens from the same animal. The nucleotide sequence of the reverse primer contains several substitutions compared to the annealing sequence of the cat mtDNA, two of them at the 3′ end of the primer (see also GenBank accession numbers X82296, U20753). This incompatibility might have led to non-stringent PCR conditions and to the formation of additional unspecific amplification products. The 358 bp band was purified by excising from a 2% LMP agarose gel and re-amplified, which resulted in a single amplification product. The amplicons were directly sequenced using the PCR primers. As described previously, the nucleotide sequence of the forward primer gave a clearer signal-to-noise ratio than the reverse primer, a phenomenon which was also observed when analysing other mitochondrial sequences (Parson et al. 1998). Nevertheless, the nucleotide sequences of both strands were determined and the consensus sequence was analysed and served as a basis for the identification of the biological origin of the sample.

Reconstruction of the biological identity

The sequences obtained from all 44 samples were used to identify the corresponding species, were aligned applying the BLAST software package (Smith et al. 1996) and searched against non-redundant nucleotide sequence databases (GenBank, EMBL). As a result, high-scoring se-

Table 2 List of vertebrate animals investigated in this study. The nucleotide sequence derived is available under the corresponding accession number. The biological specimens used in this study are given in abbreviations (*B* blood *Br* bristle *Bo* bone *Ca* cartilage *Fe* feather *H* hair *Li* liver *Ov* ovary *ST* soft tissue *T* testicle) (Searching DNA databases on the internet, the most homologue sequence entry referred to as maximum scoring segment pair is depicted by accession number. If the corresponding species was not found in the databases, the name of the MSP was added to the accession number)

Investigated species	Accession no.	Specimen	MSP (similarity %)
Mammalia			
Rabbit *(Oryctolagus cuniculus)*	AF172354	ST	U07566 (99%)
Common hare *(Lepus europaeus)*	AF172355	ST	AF010161 (100%)
Mouse *(Mus musculus)*	AF172356	Bo, Ca	Z96069 (100%)
Dog *(Canis familiaris)*	AF172357	Te	AF064587 (100%)
Badger *(Meles meles)*	AF172358	B, H	X94922 (99%)
Cat *(Felis catus)*	AF172359	Ov, B, Te, H	X82296 (100%)
Lynx *(Felis lynx)*	AF172360	H	D285902 (100%)
Pig *(Sus scrofa)*	AF172361	ST	Z50089 (100%)
Wild boar *(Sus scrofa)*	AF172362	Br	Z50089 (100%)
Roe deer *(Capreolus capreolus)*	AF172363	B	Y14951 (99%)
Cow *(Bos taurus)*	AF172364	ST	D34635 (100%)
Sheep *(Ovis aries)*	AF172365	ST	X56284 (100%)
Chamois *(Rupicapra rupicapra)*	AF172366	H	AF034725 (100%)
Ibex *(Capra ibex)*	AF172367	H	AF034735 (100%)
Man *(Homo sapiens sapiens)*	AF172368	H, B	Z96069 (100%)
Aves			
Ostrich *(Struthio camelus)*	AF172369	ST	Y12025 (99%)
Little grebe *(Tachybaptus ruficollis)*	AF172370	ST	U43625 *Grus antigone* (90%)
Sacred ibis *(Threskiornis aethiopicus)*	AF172371	Fe	U08941 *Platalea alba* (89%)
Waldrapp ibis *(Geronticus eremita)*	AF172372	Fe	AF076054 *Fregetta grallaria* (89%)
Bald ibis *(Geronticus calvus)*	AF172373	Fe	U08941 *Platalea alba* (89%)
Common buzzard *(Buteo buteo)*	AF172374	ST	X86741 (100%)
Golden eagle *(Aquila chrysaetos)*	AF172375	Fe	X86740 (99%)
Marsh harrier *(Circus aeruginosus)*	AF172376	ST	X86745 *Circus cyaneus* (93%)
Peregrine *(Falco peregrinus)*	AF172377	ST	X86746 (100%)
Kestrel *(Falco tinunculus)*	AF172378	ST	U83310 *Falco femoralis* (92%)
Capercaillie *(Tetrao urogallus)*	AF172379	ST	AF068191 *Tymp. phasianellus* (92%)
Ptarmigan *(Lagopus mutus)*	AF172380	ST	AF068191 *Tymp. phasianellus* (92%)
Chicken *(Gallus gallus)*	AF172381	ST	AF028795 (100%)
Water rail *(Rallus aquaticus)*	AF172382	ST	U77172 (99%)
Lapwing *(Vanellus vanellus)*	AF172383	ST	U70489 *Pterodroma baraui* (89%)
Woodcock *(Scolopax rusticola)*	AF172384	ST	U08940 *Phoenicopterus ruber* (88%)
Swift *(Apus apus)*	AF172385	ST	U27545 *Anthropoides virgo* (88%)
Eagle owl *(Bubo bubo)*	AF172386	ST	U27545 *Anthropoides virgo* (88%)
Green woodpecker *(Picus viridis)*	AF172387	ST	U83283 *Colaptes auratus* (90%)
Kingfisher *(Alcedo atthis)*	AF172388	ST	D38329 (99%)
Coal tit *(Parus ater)*	AF172389	B	M88590 *Parus major* (95%)
Carrion crow *(Corvus corone)*	AF172390	ST	U86032 (99%)
Amphibia			
Afr. clawed frog *(Xenopus laevis)*	AF172391	ST	M10188 (99%)
Reptilia			
Grass snake *(Natrix natrix)*	AF172392	ST	U69846 *Micrurus fulvius* (84%)
Aesculapian snake *(Elaphe longissima)*	AF172393	ST	AF036010 *Elaphe taeniura* (88%)
Pisces			
Eel *(Anguilla anguilla)*	AF172394	Li	D28775 (99%)
Rainbow trout *(Onchorhynchus mykiss)*	AF172395	B	L29771 (100%)
Brown trout *(Salmo trutta)*	AF172396	B	D58400 (100%)
Danube salmon *(Hucho hucho)*	AF172397	ST	D58396 *Hucho perryi* (90%)

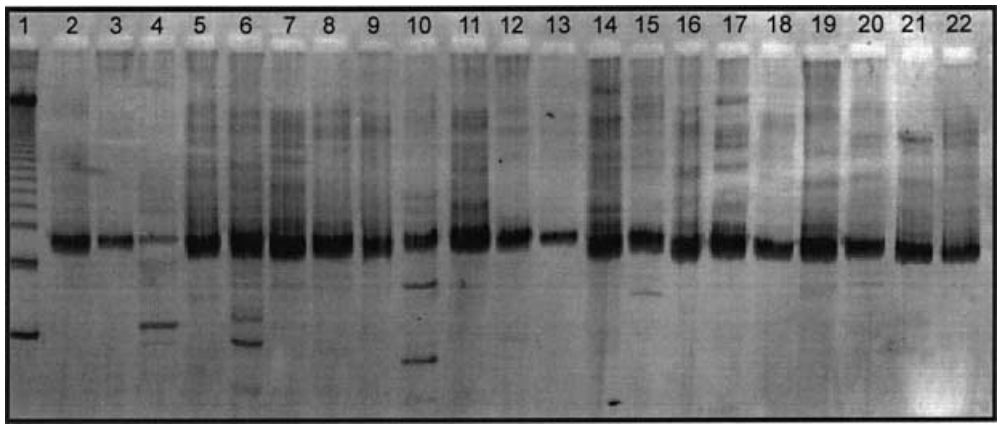

Fig. 1 Example of a silver stained PCR product gel (PAGE) depicting the 358 bp fragments from 21 vertebrate species. *Lanes1* 123 bp ladder (Life Technologies, Gaithersburg, Md.), *2* grass snake, *3* aesculapian snake, *4* danube salmon, *5* eagle owl, *6* carrion crow, *7* kestrel, *8* woodcock, *9* kingfisher, *10* swift, *11* lapwing, *12* water rail, *13* capercaille, *14* peregrine, *15* common buzzard, *16* marsh harrier, *17* little grebe, *18* ptarmigan, *19* green woodpecker, *20* golden eagle, *21* pig, *22* badger

quence pairs (HSP) were presented, consisting of the query sequence and a database entry in combination with a similarity value reporting the significance of the match (Altschul et al. 1990). The result of a sequence search contains a list of database entries which are sorted by decreasing sequence similarity, the first entry defined as maximum-scoring segment pair (MSP). The MSP displays the best concordance to the query sequence. The accession numbers of all MSPs obtained for the investigated species are given in Table 2, including the species name of the matching database entry, in cases where the corresponding species was not included in the database.

All cytb gene sequences of the mammalian species and the amphibians were found in the DNA databases. The MSPs displayed high similarity between the laboratory results and the database entry (99% and 100%). Single nucleotide substitutions between the query and the subject sequence can be attributed to individual variation. Similar polymorphisms of the cytb gene can be found in wild populations, especially within species demonstrating a large range of geographical distribution (Taberlet et al. 1992). This phenomenon was also observed in four different populations of brown trout studied from Austrian and Italian trout populations (data not shown). A total of 35 trout were investigated and 4 variable nucleotide positions were identified within the investigated sequences thus separating the populations into 2 groups. Both genotypes were present and nearly equally distributed in three of the four observed populations.

Within the fish species, three of the four taxa were found in the databases (i.e. eel and the two trout genotypes), again displaying high similarity (Table 2). The MSP matching the danube salmon is from another genus, *Hucho perryi*, which shows only a 90% sequence identity to the query sequence.

Only 8 (ostrich, common buzzard, golden eagle, peregrine falcon, chicken, water rail, kingfisher and carrion crow) of the 22 bird species investigated were found in the databases (Table 2). Three MSPs matching only the genus of the corresponding taxon (marsh harrier, kestrel and coal tit) were assigned with a sequence match between 92% and 95%. Another three MSPs, which were not found in the databases, are closely related to the species studied such as capercaille – *Tympanchus phasianellus* (pheasant) 92%, ptarmigan – *Tympanchus phasianellus* 92% and green woodpecker – *Colaptes auratus* (common flicker) 90%. In five other cases however, the MSP was not closely related to the query species (little grebe, lapwing, woodcock, swift and eagle owl), which is also illustrated by low similarity values in the range of 88%. The MSP *Fregetta grallaria* (white-bellied storm petrel) reported for the waldrapp ibis is not closely related to this bird, but the species listed below (with second-highest homology value) is a member of the same family (*Platalea alba*, african spoonbill). This result is surprising and can be misleading, as the other two ibis species used in this study (sacred ibis and bald ibis) were both assigned to *Platalea alba* as MSP. *Aves* is a very species-rich taxon and so far, only a low percentage of this biodiversity has been studied genetically. Furthermore, species included in the databases may be biased towards American specimens, as a majority of the studies has been published using species from the new world.

Only a distant biological relationship can be attested to the MSP of the grass snake *(Natrix natrix)* which was assigned to *Micrurus*, a coral snake, being a member of a different biological family. Again, this result is illustrated by a low homology value of 84%.

In conclusion, the information content within the nucleotide sequence of a 300 bp fragment of the cytb gene enabled the identification of all species used in this study to at least the level of the vertebrate class. The nucleotide sequences were identified correctly at the species level, when the corresponding species was included in the databases. In such cases, the observed similarity of the sequence pair exceeded 99%. If a species was not found in the databases, the biological significance of the matching result strongly depended on the availability of closely related species, sometimes matching only distantly related taxa. This result was then coupled with lower sequence similarity values. Principally, there is a wide range of cytb polymorphisms that could exist within different species. That is why a high sequence similarity value (e.g. 98%) can originate from both, two members of the same species for older, more diverse species or two closely related species which separated recently. The correct identifica-

tion of an unknown sample therefore depends on the availability of the sequence in the nucleotide database. The taxonomy browser can therefore give information on the representation of related taxa.

Sequence analysis of the cytb gene proved to be a very sensitive and powerful technique for forensic species identification demonstrated by the following features:

1. The determination of the biological origin of an unknown sample applying the cytb method is a process which is not dependent on physical material present from the animal taxon in question, but is achieved by comparison of the resulting sequence against large, and still growing, databases of cytb gene sequences.
2. This method uses DNA, therefore an unknown stain sample can be extracted in a straightforward manner without consideration of saving a portion of the material for conventional species identification methods. This may be helpful for the analysis of microstains.
3. The technique benefits from the advantages of mtDNA typing in conjunction with PCR, displaying a high sensitivity and a remarkable stability against environmental stress compared to proteins.

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