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RFLP and sequence analysis of the cytochrome b gene of selected animals and man: methodology and forensic application

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Abstract To identify common animal species by analysis of the cytochrome b gene a method has been developed to obtain PCR products of a large domain of the cytochrome b gene (981 bp out of 1140 bp) in humans, selected mammals and birds using the same specifically designed primers. Species-specific RFLP patterns are generated by co-restriction with the restriction endonucleases ALU I and NCO I. The RFLP patterns obtained are conclusive even in mixtures of two or more species. The results were confirmed by sequence analysis which in addition explained intraspecies variations in the RFLP patterns. The method has been applied to forensic casework studies where the origin of roasted meat, stomach contents and a bone sample has been successfully identified.

Key words Species differentiation · Cytochrome b · PCR · RFLP · Sequence analysis · Forensic samples

Introduction

The cytochrome b gene, localized in the mitochondrial genome, has been found to be a powerful marker for identifying species with DNA analytical techniques [1–5]. Moreover, mtDNA is present in a much higher copy number compared to nuclear DNA which makes it a useful tool in forensic casework, as also shown in D-loop analysis for discriminating human individuals [6–8] or for age analysis [9].

In the present paper a method is described which produces RFLP patterns of PCR products of nearly the complete cytochrome b gene (981 out of 1140 bp) to differentiate humans and several animal species i.e. cattle, pig, sheep, dog, cat, European hare, rabbit, chicken and turkey. This is of particular interest in forensic casework, when bloodstains, bone or meat samples, or stomach contents have to be analysed.

Database screening (EMBL, outstation EBI, Hinxton, UK) of the cytochrome b gene of these species indicates that commonly used PCR primers for cytochrome b amplification produce only a PCR product of 307 [1] or 357 bp [4], which is inappropriate for RFLP analysis with only one restriction enzyme. Other cytochrome b primers [3] lead to a 703 bp fragment which is useful for discriminating most of the species mentioned above, but here RFLP patterns exhibiting fragments with sometimes small differences in length are generated, which are difficult to analyse, especially in cases with mixed samples. On the other hand, the primers described by Kocher et al. [2] which produce a 1247 bp fragment are not applicable to chicken and to all other galliformes, duck and goose because of their divergent order of genes on the mtDNA [10]. These primers will hybridize outside the cytochrome b gene which in mammals is within the genes of t-RNA_{Thr} and t-RNA_{Gln}, respectively. In the case of the birds mentioned, between the genes for t-RNA_{Thr} and t-RNA_{Gln} a part of the gene for NADH dehydrogenase, but not for cytochrome b is localized. Therefore, it is not possible to analyse cytochrome b with these primers. Moreover, they will not hybridize in European hare because of the low nucleotide sequence homology. To overcome these problems new primers were designed to meet the requirements for successful sample identification.

Materials and methods

Samples

Muscle or liver samples from the following animal species were investigated: cattle (*Bos primigenius f. taurus*, n = 9) , pig (*Sus scrofa f. domestica*, n = 8), wild boar (*Sus scrofa*, n = 9), sheep (*Ovis ammon f. aries*, n = 7), dog (*Lupus canis f. familiaris*, n = 7), cat (*Felis silvestris f. catus*, n = 8), European hare (*Lepus europaeus*, n = 8), rabbit (*Oryctolagus cuniculus*, n = 1), chicken (*Gallus gallus gallus*, n = 8) and turkey (*Meleagris gallopavo domestica*, n = 2) were investigated. The samples were purchased from local butcher shops (cattle, pig, boar, sheep, European hare,

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rabbit, turkey, chicken) or kindly provided by the Institute of Veterinary Pathology, University of Giessen, Germany (dog, cat).

EDTA blood samples from eight Caucasian humans and DNA from the human tumor cell line K 562 were used.

For casework studies two roasted meat samples of unknown origin, three stomach content meat samples from corpses, collected during autopsy and a bone sample found in a forest area were investigated.

Meat and liver samples were used fresh or stored at –20 °C prior to examination, blood samples were used fresh or stored at 4 °C prior to examination. The bone sample had been stored at room temperature after collection.

DNA extraction and amplification

Total DNA was extracted from slices of meat or liver (1 × 1 cm, about 0.5 mm thick) using standard proteinase K digestion, phenol/chloroform extraction and ethanol precipitation [11] and dissolved in water. DNA from blood samples was extracted using 1 µl of blood in 200 µl 5% Chelex 100 [12]. For DNA extraction from bone 1 g was grounded into fine powder and then subjected to DNA extraction [13].

DNA from meat samples was quantified photometrically and 1 ng of total DNA was used for PCR. From the DNA of the bone sample a dilution series was performed. Primers with the following sequences were designed to amplify a 981 bp segment of the cytochrome b gene: forward: 5′ CATCGACCTTCCAGCCCCATCAAACAT 3′; reverse: 5′ TGTTCTACTGGTTGGCCTCCAATTCA 3′ (Eurogentec, Belgium). The foward primer corresponds to the bovine gene, the reverse primer is an degenerated primer and both exhibit sequence homology high enough to allow hybridisation is all species investigated.

For amplification the following conditions were used: 10 mM Tris HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 µM of each dNTP, 160 µg/ml bovine serum albumin, 0.2 U Amplitaq polymerase, pH 8.3 in a reaction volume of 50 µl. Primers were used at a concentration of 20 pmol each per reaction. A Perkin Elmer 2400 Thermocycler was used applying 30 s at 94 °C, then 30 cycles with 30 s for denaturation at 94 °C, 30 s annealing at 55 °C and 30 s extension at 72 °C followed by a final extension step of 7 min at 72 °C. The PCR products were separated on a 1% agarose gel and visualized after ethidium bromide staining. Because of possible unspecific PCR products, the cytochrome b specific fragments were routinely excised and eluted using Micropure separators in combination with Microcon 100 microconcentrators (Amicon, Witten, Germany). The eluted fragments were reamplified using 1 µl of the eluate, applying the conditions for reamplification as for the first round PCR. However, the reamplification step can be omitted, when the first PCR product is visualized on a silver stained polyacrylamide gel and no unspecific products are detected, because of its higher sensitivity in detecting DNA fragments.

Unspecific fragments may sometimes occur in the analysis of forensic specimens even in evaluated PCR systems. In the present study they were larger than the specific 981 bp fragments and present in lower amounts, but in the presence of ALU I- or NCO I restriction sites this may lead to problems in the interpretation of RFLP patterns, therefore unspecific fragments have been omitted.

Defined mixtures of cattle and pork DNA in ratios 1:1, 1:2, 1:10, 1:20, 1:50 and 1:100 with pork as major component were also made prior to amplification.

RFLP analysis

Reamplified PCR products were codigested with ALU I and NCO I (Pharmacia, Uppsala, Sweden) according to the manufacturers recommendations, separated on a horizontal 6% denaturing polyacrylamide gel (Promega Acryl-a-mix 6, Promega, Madison, USA) and detected after silver staining.

Preliminary reviewing of the gene bank sequences of the investigated species indicated, that out of a large pool of restriction enzymes only ALU I would produce RFLP patterns which in mammals are highly discriminative as well as easy to interprete, because not more than three detectable fragments occur. To discriminate the birds species, which do not posess ALU I sites, NCO I was chosen because this enzyme has no restriction sites in mammals and therefore would not alter their ALU I pattern when applied in a corestriction analysis.

Sequence analysis

Reamplified PCR fragments were purified using Centricon 100 concentrators (Amicon, Witten Germany). Cycle sequencing was performed using the Dye Terminator Cycle Sequencing Kit FS (Perkin Elmer, USA) according to the manufacturers recommendations. The annealing temperature was 60 °C and sequencing primers were the same described for the PCR reaction with 10 pmol per sequencing reaction. The sense strand as well as the antisense strand was subjected to sequence analysis.

Electrophoresis and detection of of the sequencing reaction products was done on the capillary electrophoresis system ABI Prism 310 Genetic Analyzer using POP (Performance Optimized Polymer) 6, with a capillary length of 61 cm and diameter of 50 µm.

Sequence homology was evaluated using the software BLASTN 2.0 and FASTA 3.0 together with the EMBL-nucleotide sequence database (EMBL, outstation EBI, Hinxton, UK) via internet.

Results

Species identification

The evaluation of sequence data from the EMBL gene bank from cattle (accession number J 01394), pig (accession number X 56295), sheep (accession number L 56284), dog (accession number X 94920), cat (accession number X 82296), rabbit (accession number U 07566), chicken (accession number L 08376), turkey (accession number L 08381), and man (accession number X 62996) as well as the sequencing results for European hare (unpublished own data) lead to the deduction of fragment patterns shown in Table 1. The patterns are easy to differentiate even when a mixture of two different species is present, although due to running conditions and staining sensitivity in cases with minute amounts of PCR product small fragments can barely be detected.

Table 1 Fragment length (basepairs) of the species investigated deduced from genebank data (EMBL)

	Cattle	Pig	Sheep	Dog	Cat	Hare	Rabbit	Turkey	Chicken	Man
ALU I	453	580	517	463	655	450	834	–	–	978
	322	243	450	387	206	374	157			3
	206	131	14	101	120	157				
		27		30						
								623	623	
NCO I	–	–	–	–	–	–	–	349	349	–

RFLP analysis of all specimens from cattle, sheep, dog, cat, European hare, rabbit, chicken and turkey showed the expected fragment patterns (Table 1).

Gene bank (EMBL) screening indicated that chicken and turkey do not possess a cleavage site for ALU I but for NCO I. Therefore, to differentiate chicken and turkey from man (the ALU I pattern seems to be the same on the gel, because in human two fragments of 978 and 3 bp length are produced) digestion with NCO I is necessary. By cleavage with this enzyme only chicken and turkey samples exhibited new fragments whereas DNA from human as well as from the other mammals investigated remained undigested.

Chicken and turkey exhibited NCO I cleveage sites at the same positions but sequence comparisons indicated homology of only 86%. Both species can, therefore, be differentiated by sequence analysis.

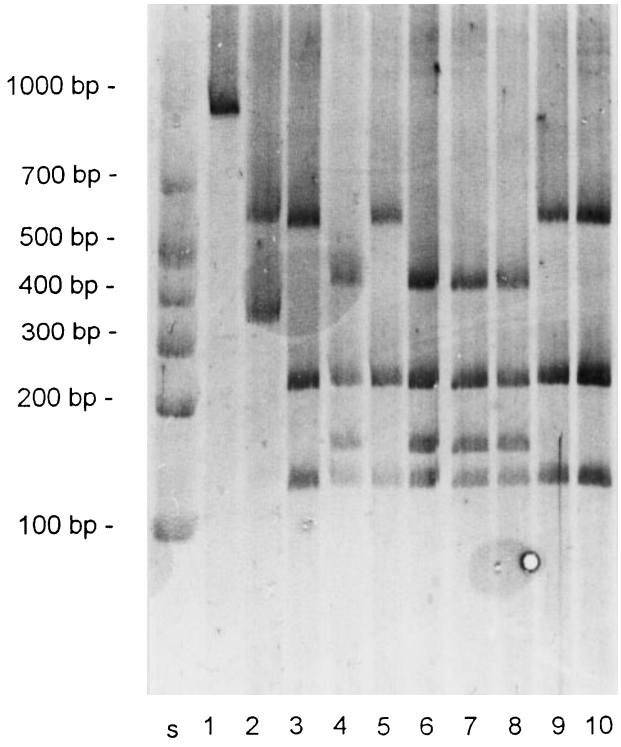

Fig. 1 RFLP pattern of PCR products from samples of eight different pigs and one boar. Lane 1 undigested sample, lane 2-9: digested pig samples, lane 10 digested wild boar sample, s: size standard

In eight meat samples from pig, three different types of RFLP pattern were obtained (Fig. 1). One type, shown in lane 3, 5 and 9 corresponds to the pattern shown in Table 1, but two others were different. Note that the 27 bp fragment is not detectable on the gel. Sequence analysis confirmed correct species identification (pig) of all samples, but sequence differences led to different restriction sites in the samples (Fig. 2). The three variant pork sequences exhibited overall sequence homology of 98.8%, 99.40% and 99.95%, when compared to the gene bank data (accession number X 56295) and are therefore pig specific.

All nine boar samples showed restriction patterns identical to pig type 1 (Fig. 1, lane number 10, as one example). Sequence analysis revealed identical sequences when compared to this type of pig.

Out of ten human samples nine resulted in the expected fragment pattern, which cannot be distinguished from an undigested sample because of the size of the fragments with one very small (3 bp) and the other very large (978 bp). One sample (cell line K562) showed an additional cleavage site leading to three fragments with a length of 3, 170 and 808 bp. Sequence analysis confirmed that a substitution of A to G had occurred at position 807, changing the pattern AACT to AGCT (data not shown).

A mixture of cattle and pork DNA resulted in the expected fragment patterns. The minor component of this mixture (cattle) was detectable only when present as at least 5–10% the total amount of template DNA (Fig. 3).

Casework studies

Roasted meat, stomach contents and a bone sample were examined. RFLP analysis of two samples of roasted meat presumed to be turkey and pork produced the expected pattern. For the turkey sample a sequence analysis was necessary to distinguish the sample from chicken which has the same RFLP pattern.

Three samples of stomach contents were examined and could be sucessfully identified. Two samples, presumed to be meat and a sample of brown liquid exhibited a cattle pattern, another presumed to be sausage, showed a mixed pork-cattle pattern (Fig. 4).

The fragment patterns of the PCR product obtained from the morphologically unidentified bone sample allowed the identification of pig origin (Fig. 5). Additional sequence analysis of this PCR product indicated a 99.9% homology to the pig gene.

Fig. 2 Analysis of the three variant pig sequences. Bases at positions 120–140 and 610–630 of the PCR product are shown compared to the gene bank data. Arrows indicate sequence variations producing the ALU I restriction site AG↓CT. Type 1 corresponds to lanes 3, 5, 9, 10, type 2 to lane 2 and type 3 to lanes 4, 6, 7, 8 in Fig. 1

	Position 120–140	Position 610–630
EMBL X 56295	ACA ACA AC**A** **GCT** TTC TCA TCA	CCC ATA CTA CAG CTA TTA AAG
pig type 1	ACA ACA AC**A** **GCC** TTC TCA TCA	CCC ATA CTA CAG CTA TTA AAG
pig type 2	ACA ACA AC**A** **GCT** TTC TCA TCA	CCC ATA CTA CAG CTA TTA AAG
pig type 3	ACA ACA AC**A** **GCT** TTC TCA TCA	CCC ATA CTA G**AG** **CT**A TAA AAG

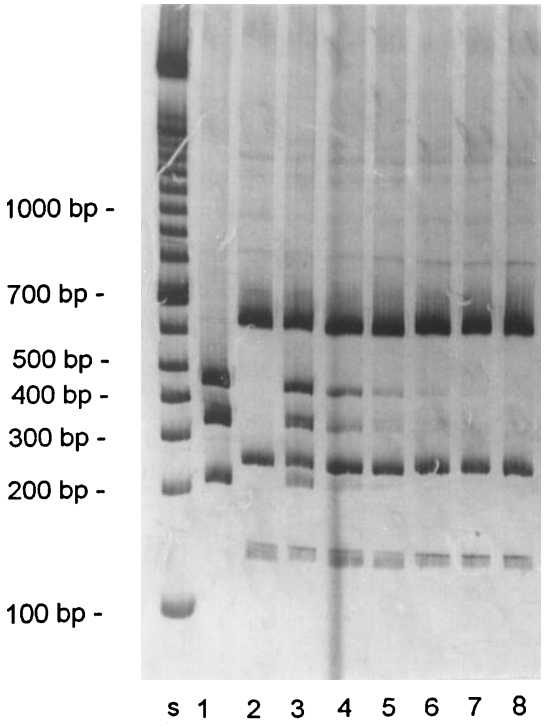

Fig. 3 RFLP pattern of PCR products from a mixture of cattle and pig DNA. Lane 1: cattle (reference), lane 2: pig (reference), lane 3–8: mixtures of cattle and pork in ratios 1:1, 1:2, 1:10, 1:20, 1:50, 1:100

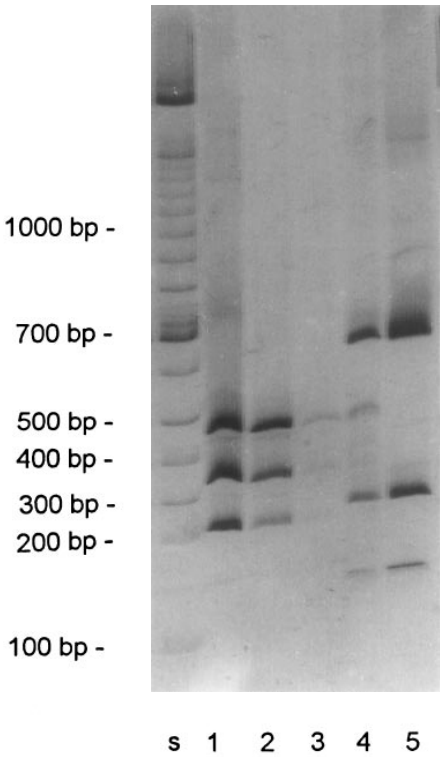

Fig. 4 RFLP patterns of PCR products from stomach contents identified as cattle and mixed cattle/pig samples. Lane 1: cattle (reference), lane 2: stomach content meat, lane 3: brown liquid, lane 4: stomach content sausage, lane 5: pig (reference): s: size standard

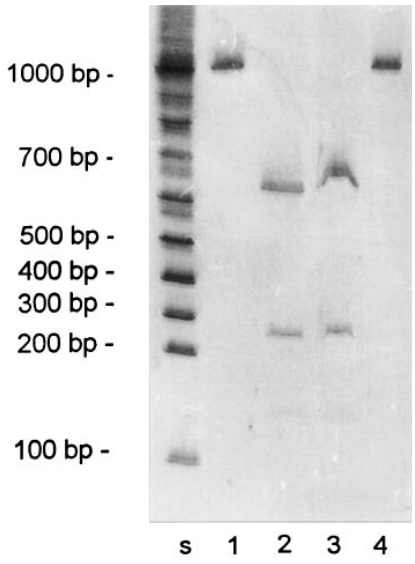

Fig. 5 RFLP pattern of a PCR product from a bone sample compared to human and pig samples. Lane 1: human, digested, lane 2: bone sample, digested, lane 3: pig (type 1) digested, lane 4: pig undigested, s: size standard

Discussion

The present study demonstrates that it is possible to identify various animals by analysing the cytochrome b gene of mtDNA through RFLP of one PCR product of nearly the complete gene. Only one restriction enzyme ALU I is necessary to reach conclusive results in mammals. However, corestriction with NCO I is recommended to obtain RFLP patterns for chicken and turkey, because these have no cleavage site for ALU I. An undigested sample would lead to a pattern which is not distinguishable from humans, where commonly only a 3 bp fragment is cut by ALU I. Humans as well as the other mammals investigated have no NCO I cleavage site.

Typing and identification of animal species by cytochrome b analysis has also been described by other authors [1–5] but the strategy presented here is the first which uses only one pair of primers to amplify nearly the whole cytochrome b gene of a selection of mammals and of at least two birds, chicken and turkey.

Because human samples commonly show no detectable digestion with ALU I or NCO I, a mixture of one or two animal DNAs with human DNA do not produce patterns which are too complex to analyse, when fragments of all species occur due to resolution and sensitivity of the detecting system. This is of particular importance when stomach contents have to be analysed where contamination with human tissue has occurred.

Although RFLP typing represents a technique leading to reproducible and conclusive results, RFLP patterns provide information only for the position of cleavage sites, which may vary because of intraspecies variation or mutations, as demonstrated in the samples from pig and human. Therefore in cases of exclusion additional sequence

analysis should be performed for unambiguous species typing.

Attention should be paid to the problem of possible undigested PCR fragments. In the present procedure this may lead to misinterpretation, because undigested animal PCR products could resemble a sample of human origin. However, due to the fact that PCR products do not posess methylated nucleotides which occur in plasmids and genomic DNA, cleavage inhibition due to methylation cannot occur. Cleavage inhibition can be mediated by substances which originate from the DNA source and are transferred together with the template DNA into the PCR tube.

In samples where human patterns occur (apparently undigested) a further test is recommended where the unknown sample is mixed with a PCR product of known origin e.g. cattle or pork. If the expected pattern occurs, it indicates digestion. Furthermore, the human fragment in question can be eluted from the gel and subjected to sequence analysis.

Species with only a short evolutionary distance might not be differentiated by the RFLP technique, although they have different base sequences. This has been demonstrated in the case of chicken and turkey, which share the same NCO I - RFLP pattern, but their cytochrome b gene sequence exhibits marked differences (sequence homology of 86% only).

The RFLP analysis has its main advantages in cases of mixed samples, because analysis of those samples by sequence analysis (data not shown) indicated that unambiguous typing is often not possible. Only if the DNA of one component is present in a ratio of more than 4:1, is it possible to perform a reliable sequence analysis of the major component, because the minor component is not detectable. However, the present study shows that in RFLP patterns of mixed cattle and pork DNA both species are detectable, also when cattle DNA is present as the minor component only in a ratio of 1:10. It should be considered that the detection limit of the minor component in a mixture is dependant on the homology of primer and the primer binding site as well as the apparent fragment length, longer fragments exhibit a higher amount of DNA than smaller fragments. The samples analysed in casework studies provided conclusive results. The fragment patterns of the roasted meat and the samples of the stomach content allowed the identification of the meat origin confirming that this method can be successfully applied to forensic examinations. The results obtained with bone material demonstrated that this method is also applicable to material other than meat.

Because forensic specimens could be contaminated with fungal species, the impact on the PCR has to be considered. The fact, that the fungal mtDNA and therefore their cytochrome b gene is organized in exons and introns will prevent the synthesis of a 981 bp fragment with primers hybridizing near the ends of the gene. Furthermore, EMBL database screening with FASTA 3.0 indicated, that the primers described should not produce fragments in species other than vertebrates, especially not in fungi or bacteria.

Further studies are necessary to determine the extent of the intra-species variability in animals. The analysis of pig samples clearly demonstrates that different individuals of the same species can produce different RFLP patterns, although the sequence homology of the cytochrome b gene from various pig types is very high. For general conclusions the number of samples may be too low to detect all possible types of cytochrome b sequences.

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