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ORIGINAL PAPER

P. D. Brodmann · G. Nicholas · P. Schaltenbrand
E. C. Ilg

Identifying unknown game species: experience with nucleotide sequencing of the mitochondrial cytochrome b gene and a subsequent basic local alignment search tool search

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Abstract Game meat is often a target for fraudulent labelling. Polymerase chain reaction (PCR) analysis on the mitochondrial cytochrome b sequence with subsequent restriction fragment length polymorphism is the most widely used method of identifying meat species. The lack of reference meat species and the possibility of point mutations affecting the typical restriction pattern of a species sometimes results in an analytical uncertainty. Nowadays, sequencing of the PCR fragment with a subsequent search in an internet-accessible database can avoid these problems. The database search results in a list of sequences in order of the highest percentage of correspondence. In this work it is shown what correspondences within different vertebrate classes, orders, families and species can be expected. Classing with the correct genus is possible, at least for game meat.

Keywords Species identification · Cytochrome b · Polymerase chain reaction · Sequence identity · Cervidae

Introduction

The consumer wants to know what kind of meat he eats for religious, dietary, sensorial, ecological, financial or other reasons. He has to rely on the labelling. Routine controls by government laboratories have shown incorrectly labelled meat in more than 10% of the tested samples (annual reports from Swiss authority laboratories, 1998 and 1999). In most cases a cheaper or less valuable meat of another species is added to the labelled game meat.

P.D. Brodmann (✉) · G Nicholas · P. Schaltenbrand · E.C. Ilg
Kantonales Laboratorium Basel-Stadt, Kannenfeldstrasse 2, 4056 Basel, Switzerland
e-mail: peter.brodmann@bs.ch
Tel.: +41-61-2852527
Fax: +41-61-2852509

To detect such frauds, methods are used which detect species-specific proteins or genetic material. Isoelectric focusing (IEF) and immunoassays [1, 2] are the most commonly used protein-based methods. It has been reported [3–5] that IEF is not suitable for processed (heated or marinated) meat products, because most soluble muscle proteins degrade very rapidly under such conditions. Nevertheless, Etienne et al. [6] have shown the possibility of species identification using IEF in heat-processed fish. Certain immunoassays have been shown to work in heated proteins [7, 8]. The disadvantage of this method is the non-availability of antibodies or cross-reactions of these to proteins from related organisms [9]. Furthermore the presence of proteins is a function of the cell type in which they are expressed.

For the identification of species, it is preferable to detect the rather stable DNA. DNA carries an organism's total genetic information. It is the same in all cell types of an organism, therefore it doesn't matter whether the DNA is extracted from blood, muscle, liver or any other tissue. The information content of DNA is greater than that of proteins, due to the degeneracy of the genetic code. For these reasons, protein-based methods are being replaced more and more by nucleic acid-based methods. DNA hybridisation [10–12] and polymerase chain reaction (PCR) [13] offer nucleic acid-based analytical approaches to identifying meat species.

Kocher et al. [14] have shown that some highly conserved regions on the mitochondrial (mt) cytochrome b gene are suitable for species identification in vertebrates: The mt-DNA accepts mutations fast enough to allow a differentiation between closely related species. mt-DNA is generally of maternal inheritance and therefore free of heterocygosity. The fact that there is a high copy number per cell [15] is an additional advantage.

Bossier [16] has reviewed different PCR methods for species identification. Most of the methods use the cytochrome b gene as a target sequence. PCR restriction

fragment length polymorphism (RFLP) is the most widely used method of identifying different fish [13,17–20] and meat-species [21–23]. Besides PCR-RFLP, sequencing of cytochrome b gene PCR fragments [24], the random amplified polymorphic DNA (RAPD)-PCR technique (in fish [25] and meat products [26]), and PCR single strand conformation polymorphism [27] are used to identify species in meat and food products.

In the last few years PCR-RFLP according to Meyer et al. [28] or Wolf et al. [23] has been applied. Endonuclease digestion with two or more restriction enzymes enables the most closely related animal species to be distinguished. However, if the digestion pattern doesn't look like the expected species or the known digestion patterns, a final judgement isn't possible. Such results have two possible reasons: either the corresponding reference material is not available or an intraspecies mutation has occurred in a restriction site by chance [23,29]. Such uncertainty would probably be difficult to argue in a court case, as a varying pattern need not conclusively demonstrate that a fraud has taken place.

Sequencing of PCR fragments results in the highest amount of information. Although the use of sequencing would be favourable, it has been judged to be too time-consuming and too expensive. In recent years, however, progress in the nucleotide sequencing process has been made. The method is nowadays a simple, fast and rather cheap method. We have tested the reliability of sequencing a PCR fragment together with a subsequent basic local alignment search tool (BLAST) search in a public database for characterisation of meat probes. The application of sequencing proved to be useful for species identification. Firstly, sequencing is actually an ideal confirmation test. Secondly, sequencing results allow an assignment to a species if there is no reference material present. The differentiation between different game species were investigated.

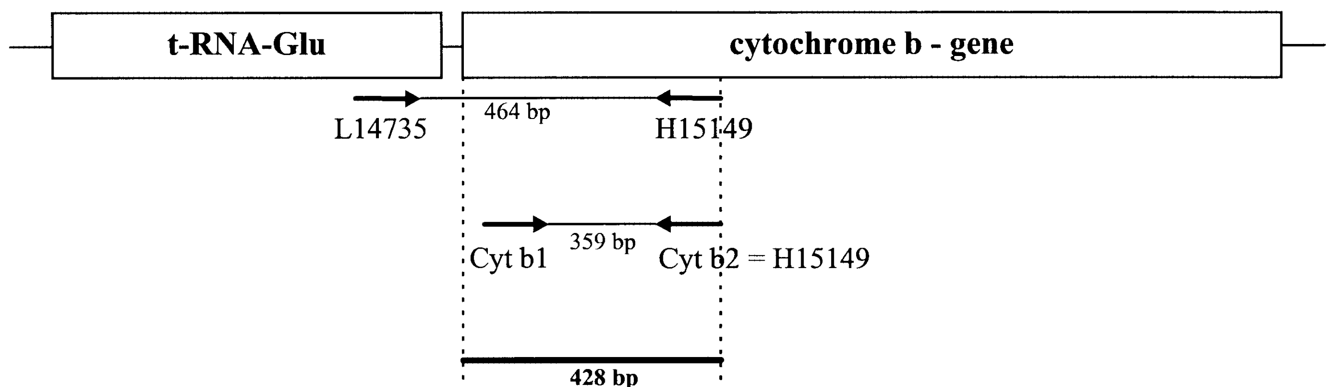
Material and methods

Reference species. The reference species were mostly bought in food stores or butchers in a condition whereby the species was easy to identify (whole chicken, whole seabass, etc.). Some of the reference species were obtained from the state veterinarian. These animals were either hunted (for example red deer or roe deer) or killed by a car accident (for example wild boar). Twelve different reference materials were obtained. With the exception of two chamois samples, one sample per species was examined.

Isolation of nucleic acids. The extraction of DNA was performed according to the procedure described by Meyer et al. [28] using the Wizard DNA Clean Up System (Promega, Madison, Wis.). Marinated game was washed to remove the sauce. The meat pieces were minced with sterile surgical blades and 0.3 g of the mince was transferred into a sterile 2 ml Eppendorf tube, to which was added 860 μl extraction buffer [10 mM Tris-HCl (pH8.0), 150 mM NaCl, 2 mM EDTA and 1% (w/v) sodium dodecyl sulphate (SDS)], 100 μl of 5 M guanidine hydrochloride and 40 μl of 20 mg/ml proteinase K (Merck, Darmstadt, Germany). The mixture was mixed by inversion and incubated at 57 °C for 3 h. After digestion, samples were centrifuged for 10 min at 20,000 *g*. Then, 500 μl of the aqueous phase was added to 1 ml Wizard DNA purification resin (Promega) and mixed by gentle inversion. The mixture was transferred to a syringe plugged into a Wizard column, which was attached to a vacuum manifold. Vacuum was applied and the column was washed with 2 ml of 80% isopropanol followed by centrifugation at 20,000*g* for 1 min. After drying at 70 °C for 10 min the DNA was eluted with 50 μl of elution buffer [10 mM Tris-HCl (pH 9,0)] and stored at –20 °C.

Polymerase chain reaction. Vertebrates were normally examined using the method described by Wolf et al. [23]. The only exception was the investigation of birds (chicken, duck and turkey) because in birds the tRNA-Glu fragment is not located upstream of the cytochrome b gene [30] (Fig. 1). Therefore the PCR system described by Carr et al. [13] amplifying only parts of the cytochrome b sequence was used. DNA amplification was carried out in a final volume of 100 μl in 0.5 ml microcentrifuge tubes containing 1x reaction buffer [10 mM Tris-HCl (pH9.0), 50 mM KCl, and 0.1% Triton X-100; Promega]; 2.0 μg/ml bovine serum albumin (Sigma, St. Louis, Mo.); 2.0 mM magnesium chloride; 0.2 mM each of dATP, dCTP, dGTP and dTTP; 0.5 μM of the primers L14'735 (5′-aaa aac cac cgt tgt tat tca act a-3′) and H15′149 (5′-gcc cct cag aat gat att tgt cct ca-3′) [23], respectively, and of CYTb1 (5′-cca tcc aac atc tca gca tga tga aa-3′) and CYTb2 (5′-gcc cct cag aat gat att tgt cct ca-3′), and 2 units of *Taq* DNA polymerase (Promega). Forty cycles of amplification (first denaturation at 94 °C for 1 min, 94 °C for 5 s, 55 °C for 30 s, 72 °C for 40 s, and final extension at 72 °C for 3 min) were performed with a Techne Genius thermal cycler (Techne, Princeton, N.Y.). Primers L14'735 and H15′149 produce a 464 base pair (bp) fragment, and the primers CYTb1 and CYTb2 a 359 bp fragment.

Fig. 1 Amplification range of the polymerase chain reaction systems and the 428 bp-range used for database comparison



DNA sequencing. PCR products were purified using the QIA-quick PCR purification kit (Qiagen, Hilden, Germany) according to the producer manual. The purified PCR products were sequenced on a DNA sequencer (ABI Prism 377, Perkin-Elmer) using fluorescence dye-labelled dideoxynucleotides (Microsynth, Balgach, Switzerland).Where the software programme of the sequencing reaction was not able to class the sequencing result with a defined bp and resulted with a N instead, the sequencing chromatogram has been interpreted by eye and the N′s were possibly replaced by a defined bp.

Sequence comparison against a public database. The sequences were subjected to a BLAST search (www.ncbi.nlm.nih.gov/blast/blast.cg). This service is made available by the National Centre for Biotechnology Information. As a result, a list of database sequences ordered by the highest percentage of correspondence is displayed. The identity is stated as a percentage and in the number of identical bps per total number of bps. The phylogenetic terms used in the European Molecular Biology Laboratory (EMBL) database were chosen for this publication. They can vary from one publication to another.

Sequence comparison of 428 bp fragments against each other. DNA sequences from the EMBL database were used to compare cytochrome b gene sequences of different vertebrates. For that purpose 428 bp of 1143 bp of the total cytochrome b genes were compared to each other. These 428 bp do include the whole 359 bp amplification fragment of the *cytb*-PCR, and the biggest part of the 464 bp fragment of the *cytb*-tRNA-Glu-PCR (Fig. 1). The EMBL identification numbers of the sequences used are listed in Tables 2–5. The sequences were compared with the "Compare Maximum Match" function from the software programme DNAsis (Hitachi Software , San Francisco, Calif.). The identity is stated as a percentage of the correspondence.

Results and Discussion

Sequence comparison with sequences from reference materials

To check the reliability of sequencing a PCR fragment for species identification, we used 12 different reference samples. The lengths of the sequences we got were between 317 and 323 bp for the *cytb*-PCR [13] and between 394 and 425 bp for the *cytb*-tRNA-Glu PCR [23]. Sequence comparison with a BLAST search resulted in correspondences with homologies from the sequence of the tested species to the sequence in the database of between 99.4% and 100% (Table 1). All 12 samples were correctly assigned. Related animals such as red deer (*Cervus elaphus*) and fallow deer (*Cervus dama*) or red deer and roe deer (*Capreolus capreolus*) could easily be distinguished. The sequences of pig (*Sus scrofa domestica*) and wild boar (*Sus scrofa fera*) can't be distinguished using the cytochrome b gene, as this part of the genome is identical for both subspecies belonging to the species *Sus scrofa*.

In the EMBL database more than 12,000 entries of cytochrome b sequences from vertebrates are found. Naturally, a lot of animals are present with two or more sequences. It is possible to find the total cytochrome b sequence from 13 different species of the deer (Cervidae) family including all important representatives of the deer family, although Burgener [31] has stated that a lot of fish of economic interest are missing from the gene bank. For meat species that are commercially important, our investigation shows that the opposite is true.

Table 1 Correspondence between sequenced reference samples and sequences from the European Molecular Biology Laboratory database

Reference species	Latin name	Identical bp/total bp
Chamois 1	*Rupicapra rupicapra*	414/415 (99.8%)
Chamois 2	*Rupicapra rupicapra*	392/394 (99.5%)
Chicken	*Gallus gallus*	319/320 (99.7%)
Duck	*Cairina moschata*	316/317 (99.7%)
Fallow deer	*Cervus dama*	416/416 (100%)
Pig	*Sus scrofa domestica*	420/420 (100%)
Red deer	*Cervus elaphus*	412/413 (99.8%)
Roe deer	*Capreolus capreolus*	423/425 (99.5%)
Seabass	*Dicentrarchus labrax*	378/380 (99,5%)
Sheep	*Ovis aries*	394/395 (99.7%)
Turkey	*Meleagris gallopavo*	321/323 (99.4%)
Wild boar	*Sus scrofa fera*	410/412 (99.5%)

Sequence comparison with sequences from the database

If sequences of the cytochrome b gene of different animal species should be compared, it is necessary to know what differences can be expected. Therefore EMBL-database sequences of the cytochrome b gene from different species were compared to each other. Where it was possible, animals that have a certain importance as food were taken for that purpose. As game meat is often a target for fraud, the comparison was made using the deer family (Cervidae) as a model system.

For the vertebrates, comparing sequences from species of six different classes showed that the correspondence between different classes is at the most, 80% (Table 2). For roe deer the correspondence was even lower, at 75% or less. When six species of different orders from the class Mammalia were compared to each other (Table 3), the same result was obtained. The highest correspondence was 81%.

These results (and further unpublished comparisons) have shown that it is almost impossible to mistake a species from one order for a species from another order using sequencing of *cytb*-PCR fragments. To investigate the situation within an order, the order Cetartiodactyla was chosen to compare different families. In the order Cetartiodactyla (even-toed ungulates) the most important meat species are included. Comparing nine species from four different families, correspondences between 78% and 89% were found (Table 4). Species from the families Suidae and Tylopoda corresponded with 78–84% to species from another family. Species from the families Bovidae and Cervidae resulted in correspondences of 78–88%. Again, however, a wrong

Table 2 Degrees of relationship within the vertebrates (different classes) as percentages. *EMBL* European Molecular Biology Laboratory

Scientific name	*Thunnus thynnus*	*Negaprion brevirostris*	*Rana nigro-maculata*	*Gallus gallus*	*Python sebae*	*Capreolus capreolus*	Common name	Class	EMBL-ID-Nr.
Thunnus thynnus	100						Bluefin tuna	Acanthopterygii	MTTTCYTB
Negaprion brevirostris	75	100					Lemon shark	Chondrichthyes	MINBCYBA
Rana nigromaculata	73	75	100				Black-spotted frog	Amphibia	AF205087
Gallus gallus	76	77	80	100			Chicken	Aves	MIGGCYTBA
Python sebae	62	64	65	65	100		Rock python	Lepidosauria	U69863
Capreolus capreolus	74	77	74	75	65	100	Roe deer	Mammalia	MICCYB24

Table 3 Degrees of relationship within the class Mammalia (different orders) as percentages

Scientific name	*Planigale ingrami*	*Equus caballus*	*Homo sapiens*	*Lepus comus*	*Ursus arctos*	*Capreolus capreolus*	Common name	Order	EMBL-ID-Nr.
Planigale ingrami	100						Long-tailed Planigale	Dasyuromorphia	MIPIIU103
Equus caballus	75	100					Horse	Perissodactyla	ECCYTB
Homo sapiens	72	76	100				Human	Homides	MIHSSU095
Lepus comus	74	80	74	100			Yunnan hare	Lagomorpha	LCA279414
Ursus arctos	74	81	73	78	100		Brown bear	Carnivora	MIUAAU110
Capreolus capreolus	75	81	73	81	81	100	Roe deer	Cetartiodactyla	MICCYB24

classing with species from the order Cetartiodactyla is very unlikely.

Cytochrome b sequences from 13 different Cervidae species could be found in the EMBL database. The comparison of these sequences showed correspondences of between 86% and 96% (Table 5). Animals of the same genus showed correspondence above 94%. Between *Capreolus capreolus* and *Capreolus pygarus*, between *Cervus elaphus* and *Cervus nippon* 96% and between the three Muntjac deers, correspondences of 96%, 96%, and 94 or 95% respectively were found. Comparison of species within the family Cervidae but not within the same genus resulted in only three cases with a correspondence above 90%. Between *Odocoileus hemionus* and *Mazama sp*. a correspondence of 93% was found and between *Hydropotes inermis* and the two *Capreolus* species correspondences of 92% and 93% respectively were found. Comparing sequences of different subspecies within the same species results in correspondences between 93% and 99% (unpublished data).

For the deer family at least, these results clearly show that with an experimentally found correspondence higher than 95%, it is certain that the database search results at least in the right genus. Correspondences of more than 99% indicate very often the right species.

For other vertebrate families these comparisons are expected to result in similar correspondences, but to be sure a careful verification should be done. At least for some mammalian families this verification could be made, as there is enough sequence information present. The latest work in the field of sequencing of mt-DNA will possibly lead to access to additional sequence information from all mammalian families [32]. In the case of the commercially interesting fishes the sequence information is probably not present.

Results from a market survey of game meat

Knowing this relationship, we started a market survey of game meat in November 1999. Thirty different samples were analysed. In particular, the expensive and popular roe deer meat was subject of the investigation. The labelled species were mostly roe deer, four chamois and one wild boar.

It was shown that some samples were labelled with the wrong meat species. In the marinated ragout probes we found in four cases not only the labelled meat but also meat from one or two other species. The PCR fragments of the samples were purified and sequenced. The sequences obtained were compared to the EMBL database using the BLAST search. The correspondences of the sample sequences with the database-sequences are shown in Table 6. Four samples were disputed, as some of the ragout meat pieces weren't correspondent to the labelling. With the help of sequencing it was shown that in sample A, red deer (*Cervus elaphus*) instead of roe deer (*Capreolus capreolus*) was present. In sample B, pieces from red deer (*Cervus elaphus*) and muntjak (*Muntiacus muntjak*) were detected besides that of the labelled roe deer. Sample C contained pieces from sheep (*Ovis aries*) and roe deer (*Capreolus capreolus*)

Table 4 Degrees of relationship within the order Cetartiodactyla (four different families) as percentages

Scientific name	*Antidorcas marsupialis*	*Bos taurus*	*Capra hircus*	*Ovis aries*	*Capreolus capreolus*	*Cervus elaphus*	*Rangifer tarandus*	*Sus scrofa*	*Camelus dromedarus*	Common name	Family	EMBL-ID-Nr.
Antidorcas marsupialis	100									Springbok	Bovidae	AF022054
Bos taurus	84	100								Cattle	Bovidae	MIBTCBA
Capra hircus	83	85	100							Goat	Bovidae	MICHCYTB
Ovis aries	86	85	89	100						Sheep	Bovidae	MIOACBE
Capreolus capreolus	86	87	87	88	100					Roe deer	Cervidae	MICCYB24
Cervus elaphus	85	86	87	87	88	100				Red deer	Cervidae	AB021096
Rangifer tarandus	86	85	88	87	89	89	100			Caribou	Cervidae	MIRTCYB29
Sus scrofa	80	82	83	83	84	83	83	100		Pig	Suidae	MISSCYTB
Camelus dromedarius	80	81	78	80	80	81	81	81	100	Arabian Camel	Tylopoda	MICDCYTB

Table 5 Degrees of relationship within the mammalian family Cervidae (different species)as percentages

Scientific name	*Alces alces*	*Capreolus capreolus*	*Capreolus pygarus*	*Mazama sp*	*Odocoileus hemionus*	*Rangifer tarandus*	*Cervus dama*	*Cervus elaphus canadensis*	*Cervus nippon*	*Hydropotes inermis*	*Megamuntiacus vuquangensis*	*Muntiacus muntjak*	*Muntiacus reevesi*	Common name	EMBL-ID-Nr.
Alces alces	100													Moose	MIAACYB26
Capreolus capreolus	88	100												Roe deer	MICCYB24
Capreolus pygarus	86	96	100											Roe deer	MICPCYB25
Mazama sp	88	87	86	100										Spike-antlered brockets	MIMSCYB27
Odocoileus hemionus	88	89	88	93	100									Mule deer	AF091630
Rangifer tarandus	90	89	89	90	89	100								Caribou	MIRTCYB29
Cervus dama	88	89	87	89	88	89	100							Fallow deer	MIDDCYTB
Cervus elaphus	89	88	87	90	87	89	90	100						Red deer	AB021096
Cervus nippon	89	89	88	90	88	89	90	96	100					Sika deer	AB021093
Hydropotes inermis	88	93	92	87	87	88	89	87	88	100				Chinese water deer	MIHICYB28
Megamuntiacus vuquangensis	86	89	88	90	88	89	89	88	88	88	100			Large-antlered Muntjac	AF042720
Muntiacus muntjak	86	89	89	89	87	89	89	88	90	89	94	100		Indian Muntjac	AF042715
Muntiacus reevesi	87	89	89	89	87	89	89	90	90	89	94	95	100	Reeves's Muntjac	AF042719

Table 6 Wrongly labelled samples in a market survey. *BLAST* Basic local alignment search tool

No.	Labelled name	Best score from BLAST search	Homology of best score	Homology to labelled
A	*Capreolus capreolus*	*Cervus elaphus*	99.5% (420/422)	89.1% (376/422)
B	*Capreolus capreolus*	*Muntiacus muntjak*	99.8% (421/422)	90.4% (378/418)
		Cervus elaphus	99.3% (406/409)	88.9% (364/409)
C	*Rupicapra rupicapra*	*Ovis aries*	100% (421/421)	90.1% (374/415)
		Capreolus capreolus	99.3% (415/418)	88.7% (361/407)
D	*Sus scrofa fera*	*Ovis aries*	98.2% (390/397)	86.4% (343/397)
E	*Capreolus capreolus*	*Nemorhaedus caudatus*	94.9% (394/415)	87.2% (360/413)
F	*Capreolus capreolus*	*Gazella gazella*	91.7% (355/387)	88.3% (340/385)

instead of the labelled chamois (*Rupicapra rupicapra*). Sample D was labelled as dried wild boar meat (*Sus scrofa fera*). The sequencing results showed that it was actually sheep (*Ovis aries*). In the samples A to D the homology of the best score was between 98.2% and 100%. Regarding the formerly mentioned relationship it is very likely that we have found the right species. In Table 6 the homology of the sequencing results from the sample to the sequence of the labelled species (data from the EMBL database) was shown to be between 86.4% and 90.4%. Therefore the finding that the labelling was wrong is additionally supported.

In sample E, the best score from the BLAST search was found with an Asian wild goat (*Nemorhaedus caudatus*). The homology was 94.9%. Therefore the meat present is possibly not from *Nemorhaedus caudatus* (an endangered species!) but from another closely related species. Certainly it is not meat from roe deer. In four out of six pieces of sample F, the label of roe deer was certainly wrong. The homology from the sequence results to the sequence of the labelled species was 88.3%. This indicates that it was certainly not roe deer. The BLAST search resulted in the best homology with *Gazella gazella*. The result of 91.7% indicated that the meat was from another species than the best fit indicates; a species that is not present in the EMBL database. Looking at the results from the sequence comparisons it is very likely that the meat found in sample F comes from another species of the family Bovidae.

These results help us to interpret results from sequence comparisons as long as we have free access to internet databases. Sequencing can therefore be used to prove frauds, even if we do not have reference material of the fraudulent meat and even if the species is not present in the EMBL database. In the case of a court case, the analytical results would provide strong evidence of a fraud.

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