ELSEVIER

Meat Science 54 (2000) 369–376

MEAT SCIENCE

www.elsevier.com/locate/meatsci

Evaluation of a DNA fingerprinting method for determining the species origin of meats

L. Partis, D. Croan*, Z. Guo, R. Clark, T. Coldham, J. Murby

Australian Government Analytical Laboratories, Research & Development, 1 Suakin Street, Pymble, NSW, 2073, Australia

Received 1 March 1999; received in revised form 8 August 1999; accepted 11 August 1999

Abstract

This study describes an investigation into the use of a PCR-RFLP technique as a routine analytical tool for species testing. The technique was used to generate DNA fingerprints for 22 animal species by amplifying a 359 bp region within the cytochrome b gene and digesting the amplified product using *Hae* III and *Hinf* I. All species could be discriminated using the two restriction enzymes with the exception of kangaroo and buffalo. Cooking the tissues did not affect the DNA extractions or the profiles generated. When mixtures were investigated, pig was preferentially amplified and dominated over all species tested, even at levels of 1%. Another set of cytochrome b primers which amplified 464 bp, was also tested for the analysis of these mixtures. Beef was found to be favourably amplified over the other species. Anomolous results where the digested products exceeded 359 bp was also investigated. Co-amplification was found to occur in the species investigated. Results of this study suggest that the CytB PCR-RFLP method shows promise for the identification of both cooked and uncooked tissues, although the method is unsuitable for analysing meat mixtures.

1. Introduction

Recently, DNA rather than protein has been exploited for species identification due to its stability at high temperatures and its structure being conserved within all tissues of an individual. This has resulted in the development of, species specific DNA probes (Chikuni, Ozutsumi, Koishikawa & Kato, 1990; Ebbehøj & Thomsen, 1991), polymerase chain reaction (PCR) assays (Chikuni, Tabata, Kosugiyama & Monma, 1994; Meyer, Candrian & Lüthy, 1994;) random amplified polymorphic DNA (RAPD) (Welsh & McClelland, 1990; Williams, Kubelik, Livak, Rafalski & Tingley, 1991) and polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP).

PCR-RFLP allows the amplification of a conserved region of DNA sequence using PCR, and the detection of genetic variation between species by digestion of the amplified fragment with restriction enzymes. This technique has been used for speciation by exploiting DNA sequence variation within the mitochondrial D-loop region (Murray, McClymont & Strobeck, 1995) and cytochrome B (CytB) gene (Meyer, Höfelein, Lüthy & Candrian, 1995). In the latter study, DNA fingerprints were generated for 18 animal species using 20 restriction enzymes and included DNA templates derived from heat-treated and fermented products as well as sausages. We evaluated the potential for the CytB PCR-RFLP method, as developed by Meyer et al., to be used as a routine analytical tool for species identification. The results of this evaluation are described herein.

2. Materials and methods

2.1. Species tested

The 22 animal species studied are listed in Table 1.

2.2. Preparation of mixtures and cooked samples

For pure samples, 50 mg of homogenised tissue was analysed. For cooked samples, tissue portions (0.5 g) were cooked in a microwave oven on high for 30 s and the tissue homogenised. The following mixtures were also prepared and analysed:

* Corresponding author. Tel.: +61-2-9449-0120; fax: +61-2-9449-1653.

E-mail address: david.croan@agal.gov.au (D. Croan).

0309-1740/00/$ - see front matter
PII: S0309-1740(99)00112-6

Table 1
Species studied and their origin

Species	DNA source	Obtained from
1. *Bos taurus* (cow)	Tissue	Retail
2. *Bubalus bubalus* (buffalo)	Blood	AQIS[a]
3. *Sus scrofa domesticus* (pig)	Tissue	Retail
4. *Ovis ovis* (sheep)	Tissue	Retail
5. *Capra hircus* (goat)	Tissue	AQIS
6. *Cervus elaphus hippelaphus* (red dear)	Tissue	DPIE QLD[b]
7. *Equus caballus* (horse)	Tissue	DPIE QLD
8. *Macropus robustus* (red kangaroo)	Tissue	DPIE
9. *Lagomorpha oryctolagus* (rabbit)	Tissue	Retail
10. *Gallus domesticus* (chicken)	Tissue	Retail
11. Duck (species not documented)	Tissue	Retail
12. *Dromaius novaehollandiae* (emu)	Tissue	AQIS
13. *Malagris gallopavo* (turkey)	Tissue	Retail
14. *Homo sapiens* (human)	Saliva	In house
15. *Felis domestica* (cat)	Blood	Pathology
16. *Canis familiaris* (dog)	Blood	Pathology
17. *Crocodylus porosus* (crocodile)	Tissue	AQIS
18. *Lates calcarifer* (barramundi)	Tissue	Retail
19. *Zeus faber* (John Dory)	Tissue	Retail
20. *Lates niloticus* (Nile perch)	Tissue	Retail
21. *Salmo salar* (salmon)	Tissue	Retail
22. Tuna (species not documented)	Tissue	Retail

[a] AQIS = Australian Quarantine and Inspection service.
[b] DPIE QLD = Queensland Department of Primary Industries and Energy.

i. Emu and goat, seven mixtures ranging from 5 to 100%.
ii. Lamb, beef, pork, goat, 12 mixtures of two to four components ranging from 5 to 100%.
iii. Chicken, beef, lamb, pork, 15 mixtures of two to four components ranging from 25 to 100%.
iv. Binary mixtures of beef, pork, lamb and chicken, 72 samples at 1, 2, 5, 10, 20 and 50%.

2.3. DNA isolation

Total cellular DNA was extracted from tissue, blood or saliva from 12 individuals of each species using the Wizard™ Genomic DNA Purification Kit (Promega). Pellets derived from tissue homogenates were suspended in 0.6 ml of Nuclei Lysis Buffer (PT# A794A, Promega) and DNA extraction carried out as per the manufacturer's protocol for tissue samples. For DNA extraction from saliva, epithelial cells were pelleted from a 1 ml sample aliquot by centrifugation (13,000×*g*, 5 min) and the cell pellet used for DNA extraction. DNA was then extracted according to the manufacturer's instructions for tissue culture cells. For blood samples, genomic DNA was extracted from a 300 μl blood aliquot as per the protocol described by the manufacturer for whole blood.

2.4. Primers

The PCR primers used were CYT b1 (5′-CCATCCAACATCTCAGCATGATGAAA-3′) and CYT b2 (5′-GCCCCTCAGAATGATATTTGTCCTCA-3′) as reported previously by (Carr & Marshall, 1991). The second set of PCR primers C1 and C2, which amplified 464 bp were C1(5′-CGAAGCTTGATATGAAAAACCATCGTTG-3) and C2 (5′-AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA-3′) (Unseld, Beyerman, Brandt & Hiesel, 1995). All primers were synthesised by Bresatac (Australia).

2.5. PCR amplification of the CytB gene

Each PCR amplification reaction was set in a volume of 50 μl with approximately 25 ng of template DNA, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 200 μM of each dNTP, 2.5 mM $MgCl_2$ and 1 unit Taq DNA polymerase (Perkin–Elmer). The reactions were each performed within 500 μl microtubes and were overlaid with one volume of mineral oil. PCR amplification was carried out using a thermal cycler (Hybaid Omnigene) as follows: 1 cycle of 94°C for 1 min, and 30 cycles of 94°C for 20 s, 55°C for 30 s, 72°C for 40 s and a final extension of 72°C for 2 min. For detection on the 373 DNA sequencer, 0.4 M of [R110] dUTP (ABI Biosystems) was included in the PCR amplification reaction.

2.6. Restriction fragment length polymorphism analysis (RFLP) analysis

Approximately 500 ng of each PCR amplicon was digested overnight with 10 U of *Hinf* I or *Hae* III (Promega) in a 15 μl reaction volume at 37°C.

2.7. Polyacrylamide gel electrophoresis

PCR amplicons and PCR-RFLP products were separated on 9% polyacrylamide gels and were visualised by staining with ethidium bromide.

2.8. 373 DNA sequencer and Genescan analysis

DNA fingerprints of seven individuals of each species were also analysed on a 6% polyacrylamide gel using the 373 DNA Sequencer and Genescan analysis software (Vs. 1.2.2-1). One microlitre of each PCR-RFLP reaction product was combined with 1 μl of 2500 ROX DNA marker (Applied Biosystems) and 3 μl of loading buffer (Applied Biosystems). The samples were loaded onto a 6% PAGE gel (19:1 acrylamide:bis solution, Bio-Rad) and were run at 600 V for 14 h on a 24 cm well to read plate. The fragments were sized using the Local Southern method, and a fragment size consensus for each species was determined. Fragment sizes less than

55 bp were not sized as they were often masked by unincorporated dUTPs and primers at the gel concentration used.

2.9. DNA cloning

Twenty nanogrammes of PCR product was ligated overnight at 4°C with 50 ng of the plasmid vector pGEM-T (Promega) and using 3 U of T4 DNA ligase. A 2 μl aliquot of the ligation reaction was then added to 50 μl of high efficiency competent cells (Promega) and incubated for 2 min at 0°C. The entire competent cell/plasmid DNA mixture was then plated out directly onto LB agar plates containing 100 μg/ml ampicillin, 0.5 mM IPTG, 40 μg/ml X-Gal and prewarmed to 37°C. The plates were incubated overnight at 37°C and recombinants identified by blue/white colour selection. Well isolated recombinant (white) colonies were picked and grown overnight in 5 ml of LB medium containing 50 μg/ml ampicillin. Bacterial cells were pelleted by centrifugation (15 000×*g*, 1 min) and the plasmid DNA was column purified using a plasmid DNA mini preparation kit (QIAGEN). Inserts were removed from the vector by overnight digestion of 1 μg of vector DNA with 10 U of the restriction enzyme *Eco* R1. The inserts were separated from the vector by electrophoresis through a 0.8% agarose gel, excised using a sterile scalpel blade and purified using DNA spin columns (QIAGEN). Purified inserts were then digested with the enzymes *Hae* III and *Hinf* 1 for restriction fragment analysis.

2.10. Computer analysis

Published CytB gene or mitochondrial genome sequences were extracted from the GenBank database which was accessed through the Australian National Genomic Information Service (ANGIS). Identification of CYT b1/CYT b2 binding sites, truncation of the extracted sequences and computer simulated restriction enzyme digestion were carried using the programs GAP, ASSEMBLE and MAP of the GCG software package.

3. Results

3.1. PCR-RFLP species profiles in cooked and uncooked tissues

Using the CYT b1/CYT b2 primers, a single PCR amplicon, corresponding in size to the predicted 359 bp, was observed following electrophoresis for 21 species. A additional larger fragment of approximately 600 bp was amplified from dog DNA. Unsuccessful attempts were made to selectively inhibit amplification of the larger fragment through the use of higher annealing temperatures and shorter extension times.

The DNA fingerprints were found to be conserved among the 12 individuals tested for each species and were identical between cooked and uncooked tissues (not shown). Furthermore, the fingerprints were sufficient to differentiate among all of the species with the exception of buffalo and kangaroo. Sheep and chicken had very similar PAGE profiles although the presence of an additional larger *Hinf* I band in sheep, also reported by Meyer et al. (1995), enabled one to distinguish between these species. Some examples of profiles obtained based on the analysis of cooked tissues (Fig. 1) and uncooked tissues (Fig. 2) are shown. Genescan analysis was performed on seven individuals of each species to enable more accurate sizing of *Hinf* I and *Hae* III restriction fragments and as an attempt to discriminate between buffalo and kangaroo which appeared indistinguishable by PAGE analysis. The fragment sizes from the Genescan profiles generated were averaged for each species and the standard deviation, % coefficient of variation and the 99% confidence limits determined. The results were precise with the coefficient of variation not exceeding 0.6% for nearly all of the restriction fragments analysed, although, one must interpret the results with caution as the sample size ($n=7$) was quite small. The most notable exceptions were in pig where *Hae* III (band 2) was sized at 129 bp in two individuals and 123 bp in the other five analysed, and in emu where *Hinf* I ranged in size from 313 to 325 bp among the seven individuals analysed. The Genescan size data revealed the size of the sheep *Hinf* I fragment, which enables its distinction from chicken, to be 298 bp. It is also worth noting a distinct size difference in other *Hinf* I fragments of these species, 184 bp (chicken) and 196 bp (sheep), which appeared indistinguishable by PAGE analysis. In comparison, the DNA fragments of buffalo and kangaroo are very similar and, as the 99% confidence limits overlap, an additional enzyme would be required to differentiate between these species. The fragment sizes for cattle, pig, sheep and chicken, clearly demonstrate that these four major commercial species can be identified using PCR-RFLP of the CytB locus and Genescan software. The five fish species studied also exhibited variant DNA fingerprints. For example, using the enzyme *Hinf* I, barramundi (*Lates calcarifer*) is clearly distinguished from the closely related nile perch (*Lates niloticus*) with respective fragment sizes of <55 bp + 130 bp + 150 bp and <55 bp + 285 bp. Figs. 3 and 4 give a schematic comparison of all species for both restriction enzymes, based on the Genescan size data.

A computer generated map of human CytB/*Hae* III yielded fragments of 233 bp + 105 bp + 21 bp (Table 2). These are considered the 'expected' fragment sizes since they are based on DNA sequence data which is absolute. In contrast, the Genescan size data, or 'inferred' fragment sizes, indicated the presence of only two restriction fragments (232 bp + 123 bp). Similarly, comparison of our

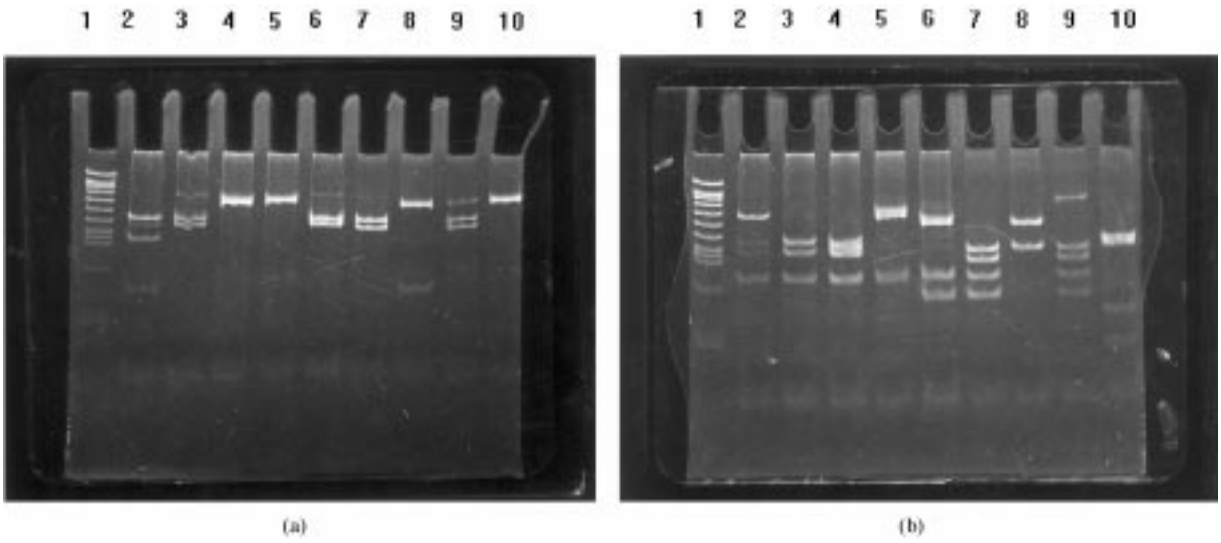

Fig. 1. *Hinf* I (a) and *Hae* III (b) restriction profiles obtained from PCR-RFLP analysis of cooked tissues from nine animal species. Lane numbers 1 = molecular weight marker VIII (Boehringher Mannheim); 2 = beef; 3 = lamb; 4 = pork; 5 = buffalo; 6 = goat; 7 = turkey; 8 = emu; 9 = John Dory; 10 = barramundi.

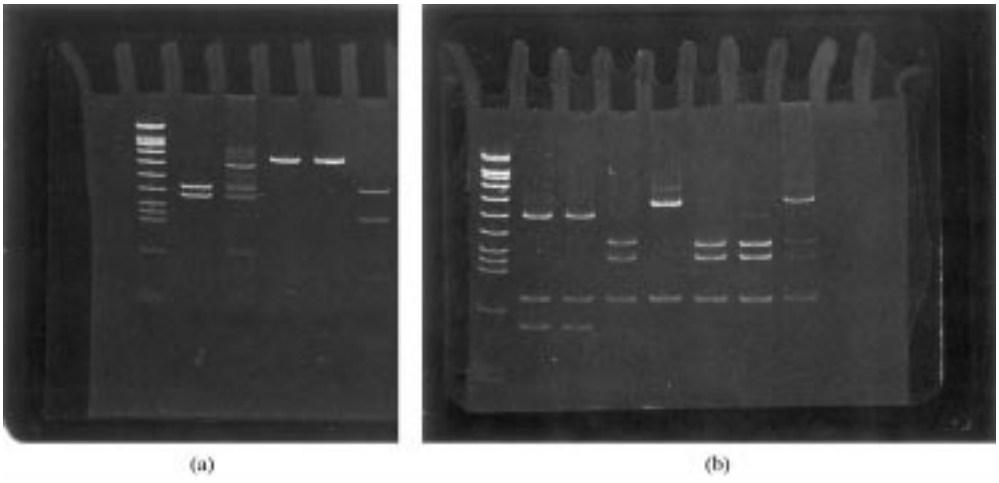

Fig. 2. *Hinf* I (a) *Hae* III (b) restriction profiles obtained from PCR-RFLP analysis of raw tissues from five animal species. Lane numbers *Hinf* I: 1 = molecular weight marker VIII (Boehringher Mannheim); 2 = goat; 3 = lamb; 4 = buffalo; 5 = pork; 6 = beef. Lane numbers *Hae* III: 1 = molecular weight marker VIII (Boehringher Mannheim); 2, 3 = goat; 4 = lamb; 5 = buffalo; 6, 7 = pork; 8 = beef.

tuna CytB/*Hinf* I fragment size data with a computer simulated DNA fingerprint for bluefin tuna indicated that our sample contained an additional restriction site. This was suggested by the apparent cleavage of the expected 250 bp fragment into smaller fragments of 194 bp and < 55 bp.

3.2. Anomalous results

PCR-RFLP analysis of deer, sheep, rabbit, horse and dog revealed that for these species, the sum size of restriction digestion products was greater than the 359 bp PCR amplicon from which they were derived (Table 2). These observations must be explained by the occurrence of either partial enzyme digestion, where not all restriction sites are fully cleaved by a restriction enzyme, or the simultaneous amplification of different DNA sequences. For the species studied, the size distribution of restriction fragments was clearly incompatible with partial restriction enzyme digestion having occurred and, consequently, the latter explanation is favoured. With the exception of dog where two amplicons of different size were observed, the presence of a single size amplicon for the other species suggests the co-amplicons were indistinguishable on the basis of size in our system. Consequently, the deer amplicon was cloned and individual cloned amplicons analysed separately by restriction enzyme analysis. Using *Hae* III, two distinct amplicons were identified from a population of five, and the sum of restriction fragments observed for these clones accounted for all bands observed in the deer CytB fingerprint (Fig. 5).

3.3. Analysis of meat mixtures

Initially, a mixture of goat and emu tissue was studied since their distinct DNA fingerprints suggested that these species would be easy to detect and differentiate between in a mixture. Binary mixtures of these species with each at levels of 0, 5, 10, 50, 90, 95 and 100% were tested and revealed that both species could be detected to the minimum level tested. Binary mixtures of the commercially important species cattle, chicken, pig and sheep were also analysed at levels ranging from 1 to 99%. Pork was always detected at 1% and often

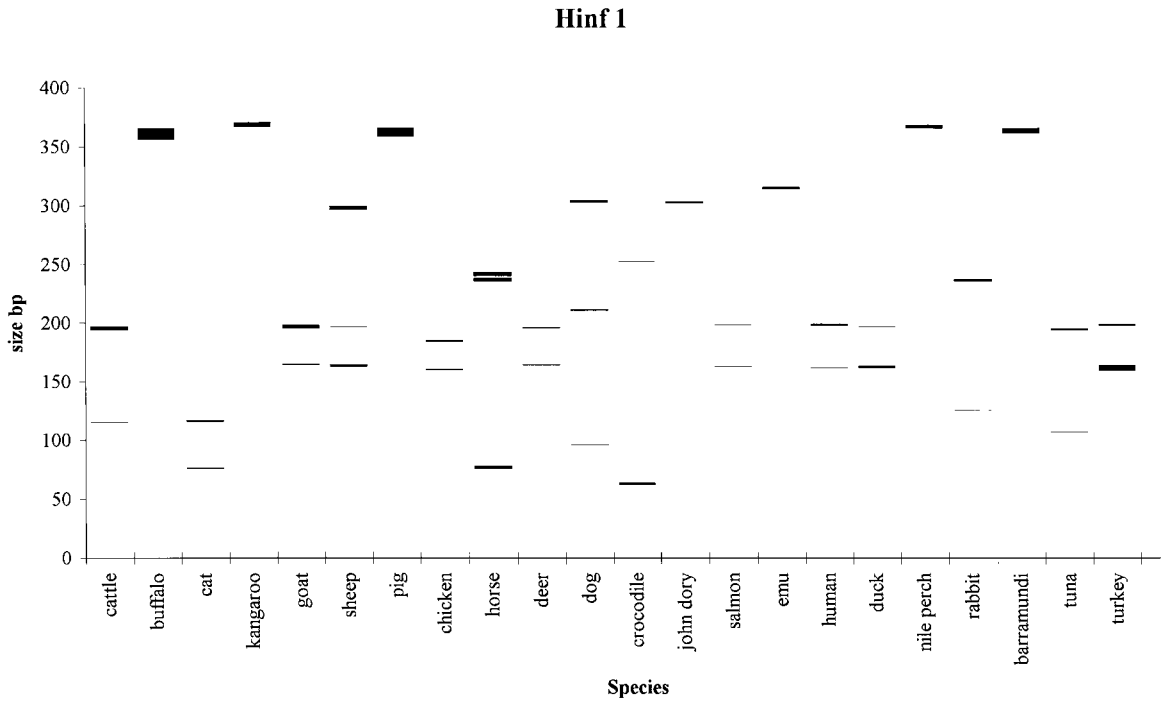

Fig. 3. Schematic representations of *Hinf* I digests for 22 species.

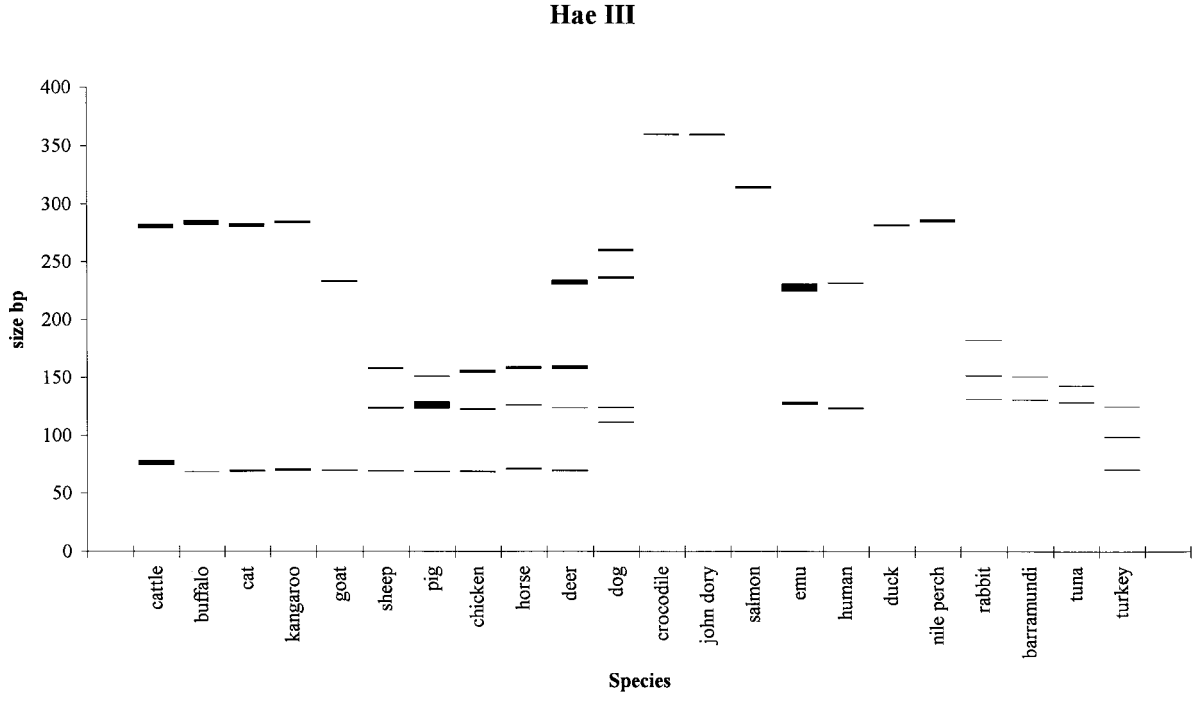

Fig. 4. Schematic representations of *Hae* III digests for 22 species.

Table 2
Inferred and expected restriction fragment sizes following PCR-RFLP analysis of the CytB gene with two restriction enzymes

Species	*Hae* III fragment size (bp)		*Hinf I* fragment size (bp)	
	Inferred	Expected	Inferred	Expected
Cattle	76, 281	–	< 55, 115, 195	–
Buffalo	68, 284	–	uncut	–
Cat	69, 281	–	< 55, 76, 116, 116	–
Kangaroo	70, 284	74, 285	uncut	uncut
Goat	< 55, 69, 233	55, 74, 230	164, 196	161, 198
Pig	68, (123/129), 151[a]	74, 132, 153	uncut	uncut
Sheep	69, 124, 157	74, 126, 159	163, 196, 298	63, 296
Chicken	69, 122, 155	74, 126, 159	160, 184	10, 161, 188
Horse	71, 126, 158	74, 126, 159	< 55, 77, 241, 320	44, 81, 234
Deer	< 55, 69, 123, 159, 232	74, 126, 159	164, 196	161, 198
Dog	111, 124, 236, 260	126, 233	53, 96, 211, 303	9, 54, 296
Crocodile	uncut	–	< 55, 62, 252	–
John Dory	uncut	–	302	–
Salmon	< 55, 314	–	162, 198	–
Emu	128, 228	–	> 55, (323/313)[a]	–
Human	123, 232	21, 105, 233	161, 198	161, 198
Duck	< 55, 281	–	162, 196	–
Nile perch	< 55, 285	–	uncut	–
Rabbit	131, 151, 182	30, 44, 132, 153	125, 236	126, 233
Barramundi	< 55, 130, 150	–	uncut	–
Tuna	< 55, 128, 142	37, 44, 132, 146	< 55, 107, 194	109, 250
Turkey	< 55, 70, 98, 125	–	161, 198	–

[a] Denotes intraspecies variation.

appeared to be the dominant component even when present at this level. The detection of beef, however, was variable in the presence of pork and at times could not be detected at levels of 80%. In the presence of chicken, beef could only be detected at a level of 50% or more while with lamb the limit for detection was 5%. Based on the presence of the 298 bp *Hinf* I fragment in sheep, this species could be detected to a level of 1% in a mixture with chicken by PAGE analysis otherwise they cannot be differentiated. Lamb was also detectable to a level of 1% with beef and 50% in the presence of pork. Chicken could be detected to levels of 10 and 20%, respectively, in the presence of beef and pork. The same mixtures were also tested with another set of cytochrome b primers which amplified 464 bp (Unseld et al., 1995). Beef was easily detected at low levels (1%). These primers appeared to favour the detection of beef, however both pork and chicken were difficult to detect in mixtures (results not shown).

A blind study was conducted in which unknown mixtures containing beef, pork, lamb and goat were analysed. Pork was easily detected to the lowest level tested (5%) in each mixture. Furthermore, as pork DNA was preferentially amplified, the presence of as little as 5% pork in a mixture resembled a level of 80% if only the intensity of fluorescence of the DNA bands were considered. Goat could also be detected to 5% while lamb could be detected to 10%. Beef was often not detected in the presence of pork and, in some cases, lamb.

An additional blind study consisting of 15 combinations of chicken, beef, lamb and pork was also carried out. The number of species present varied from one to four and were present in equal amounts. Five of each of these mixtures were tested by three analysts using Genescan analysis. Two-thirds (10/15) of the samples, which included unadulterated tissues as well as mixtures, were correctly interpreted. However, analysis of mixtures which contained at least three species revealed that usually, one of the species was not detected. It is notable that in the latter mixtures, pork was always detected while in some cases chicken, lamb and beef were not.

4. Discussion

The Cytochrome B locus has been well characterised among different vertebrate groups (Hatefi, 1985; Irwin, Kocher & Wilson, 1991). These studies have revealed that the level of CytB gene sequence variation is suitable for addressing general questions on inter-specific diversity. However, since DNA sequence analysis is costly, the PCR-RFLP approach as applied by Meyer et al. (1995) provides a more practical approach for detecting genetic variation between species. This method was therefore evaluated in the current study to assess its suitability as a routine analytical method for determining the species origin of food.

A 359 bp region within the CytB gene was successfully amplified from DNA which was extracted from both

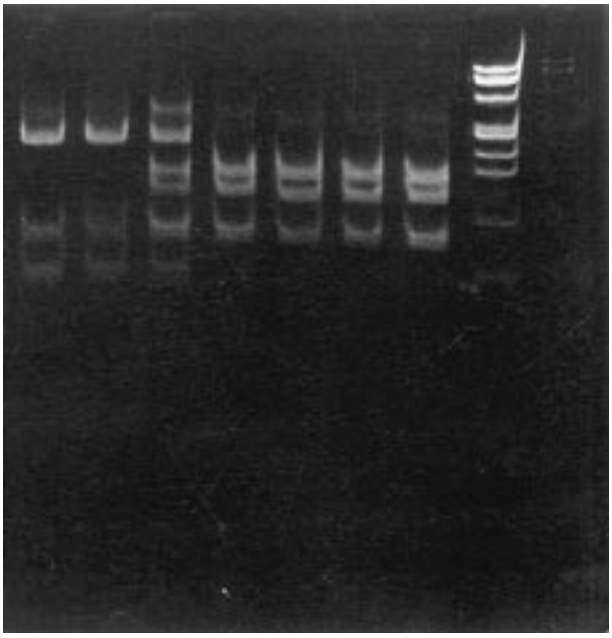

Fig. 5. *Hae* III restriction analysis of cloned venison PCR amplicons. Lane numbers 1 = molecular weight marker VIII (Boehringher Mannheim); 2–5 = *Hae* III digest of venison clones I–IV, respectively; 6 = *Hae* III digest of a venison PCR amplicon; 7, 8 = *Hae* III digest of venison clones 6, 7 respectively.

cooked and uncooked tissues of 17 animal species. It should be noted that the times used for cooking animal tissues in this study was quite short due to the small size of tissue portions, however, thermal denaturation of DNA is known to be time dependant (Ebbehøj & Thomsen, 1991). In order to confirm the utility of the method for analysing cooked tissues, it would be required that amplification be carried out on DNA derived from larger portions which were subject to longer cooking times more representative of commercial processes. The CytB gene was also amplified from DNA derived from either blood, saliva or uncooked tissue of five additional species. The CYT b1/CYT b2 primers employed are considered to be universal (Carr & Marshall, 1991) and were shown to amplify DNA from each species in the panel studied, which comprises a considerable span of vertebrate evolutionary diversity. An advantage in employing universal primers is that it obviates the requirement for an internal control, which is otherwise used to monitor the success of DNA amplification. In addition, since each cell may contain up to 1000 copies of the CytB locus, PCR assays based on its amplification should offer the advantage of increased sensitivity in comparison to single or low copy nuclear DNA targets.

Using only two restriction enzymes, we were able to distinguish between CytB amplicons from all but two of the 22 species studied. It is possible that as DNA profiles are obtained for more species, then additional examples of "profile convergence" will be found, although this might be overcome by the judicious choice of alternate restriction enzymes. Our analysis did, however, include most species of commercial interest and their documented or likely substitutes. Importantly, pig and emu were the only species tested which showed intra-specific CytB DNA fingerprint variation. In the case of pig we note that, for two individuals, the *Hae* III fragment in question (Table 1) is quite similar in size (129 bp) to that predicted by computer simulated digestion of the database sequence (132 bp) while those of the other five individuals, from which the mean was calculated, are significantly smaller (123 bp).

The most likely explanation is that these data are detecting intraspecific sequence polymorphism in pigs in which, for some individuals, an additional restriction site results in the cleavage of a small DNA fragment from the expected 132 bp fragment. This may also be the case for human and bluefin tuna. The computer generated map of the human database sequence suggested that it contained an additional *Hae* III site when compared with the 12 individuals we studied. Since there is no evidence for partial digestion (Table 1) this disparity is most likely attributable to either intraspecific polymorphism or an error in the database sequence. Although a similar situation was seen for bluefin tuna, in this instance, as well as the potential for intra-specific polymorphism and for errors to exist in database sequences, such disparity might also be explained by our sample being a tuna species other than *Thunnus thynnus* since the sample we obtained was not authenticated.

It is worth emphasising that intra-specific variation of the CytB gene has been previously reported (Meyer et al., 1995). Moreover, other workers have shown CytB sequence differences between taxa whose specific status remains questionable, for example, *Sus scrofa scrofa* (wild boar)/*Sus scrofa domesticus* (domestic pig) and the sheep species *Ovis ovis*/*Ovis aries*. (Meyer et al., 1995). The existence of such variation notwithstanding, the overall uniformity in species fingerprints suggests that the method remains a suitable tool for speciation purposes. It is likely that as more species are analysed, predominant fingerprints will emerge that will enable one to take into consideration intraspecific differences.

Some species gave unusual DNA fingerprints in which a greater than expected number of DNA fragments were obtained following restriction enzyme digestion of PCR amplicons. As partial digestion was shown to be improbable and contamination of tissue samples or DNA extracts unlikely, the most likely explanation is the co-amplification of nuclear CytB pseudogenes. The existence of mitochondrial sequences in mammalian nuclear genomes is not without precedent, since such fragments have been found in the human nuclear genome (Kamimura, Ishii, Liandong & Shay, 1989). As

pseudogenes are generally not translated, they are characterised by an increased number of nucleotide replacement substitutions, which in addition to altering the distribution of restriction enzyme sites, also change the amino acid sequence of the encoded protein. Nevertheless for the species we studied, despite the presence of additional bands, these were found to constitute a stable component of the species DNA fingerprints.

Although the application of the CytB PCR-RFLP to unadulterated animal tissues is simple and straightforward, the analysis of meat mixtures is more problematic. This is the result of differential template amplification, a phenomena which also prevents a semi-quantitative analysis of meat mixture components. However, a more critical analytical limitation imposed by differential enzyme amplification is that, depending on the extent to which different DNA templates are differentially amplified, some tissues may go undetected even when present in large proportions. For example, in a mixture containing 5% pork and 95% beef, pig DNA was preferentially amplified to the extent that it appeared the major tissue component. In contrast, in a mixture of beef and pork, in some cases the former was detectable only to a level of 50%. Differential amplification generally results from primer mismatches which affect the stability of primer-DNA duplexes. It is interesting to note that CYT b2 contains a single identical mismatch with respect to both the bovine and porcine database sequences while CYT b1 contains three mismatches in both species at different sites. In this regard, the vastly different amplification efficiencies of the CytB primers which is apparent between these two species is remarkable. Also interesting to note is that analysis of the same mixtures using another set of CytB primers, C1 and C2, resulted in the preferential amplification of beef over pork. The use of adjuvants such as DMSO, glycerol or bovine serum albumin which may promote more even amplification of different DNA templates may be of value in this situation.

5. Conclusions

The CytB PCR-RFLP species identification assay was determined to be a suitable method for the identification of raw or cooked pure species. The method is versatile as it can be used as a general screen for all vertebrate species. The amplified target is also present in a high copy number in each cell which substantially increases the sensitivity of the PCR assay. The identification of species following restriction enzyme digestion was shown to be simple and straightforward by judicious choice of restriction enzymes.

The major limitation of the assay is the inconsistency between the quantity of product amplified, relative to the amount of target DNA present which precludes the semi-quantitation of tissue constituents in a mixture. This is most evident in the case of porcine DNA, which tends to mask other species even when pork is present as a minor tissue component. However, with the other primer set bovine DNA was found to be the dominant product. Therefore, the method is unsuitable for analysing mixtures, since the results may not be representative of the true components present in the mixture.

Finally the assay would also be highly useful in investigations of animal species as contaminants of plant products, as the targeted region is not amplified from plant DNA due to their greater genetic divergence.

References

Carr, S. M., & Marshall, H. D. (1991). Detection of intraspecific DNA sequence variation in the mitochondrial cytochrome b gene of Atlantic cod (*Gadus morhua*) by the polymerase chain reaction. *Canadian Journal of Fisheries and Aquatic Sciences, 48*, 48–52.

Chikuni, K., Ozutsumi, K., Koishikawa, T., & Kato, S. (1990). Species identification of cooked meats by DNA hybridisation assay. *Meat Science, 27*, 119–128.

Chikuni, K., Tabata, T., Kosugiyama, M., & Monma, M. (1994). Polymerase chain reaction assay for detection of sheep and goat meats. *Meat Science, 37*, 337–345.

Ebbehøj, K. F., & Thomsen, P. D. (1991). Species differentiation of heated meat products by DNA hybridisation. *Meat Science, 30*, 221–234.

Hatefi, Y. (1985). The mitochondrial electron transport and oxidative phosphorylation system. *Annual Review of Biochemistry, 54*, 1015–1069.

Irwin, D. M., Kocher, T. D., & Wilson, A. C. (1991). Evolution of the cytochrome b gene of mammals. *Journal of Molecular Biology, 32*, 128–144.

Kamimura, N., Ishii, S., Liandong, M., & Shay, J. W. (1989). Three separate mitochondrial DNA sequences are contiguous in human genomic DNA. *Journal of Molecular Biology, 210*, 703–707.

Meyer, R., Candrian, U., & Lüthy, J. (1994). Detection of pork in heated meat products by the polymerase chain reaction. *Journal of the AOAC International, 77*, 617–622.

Meyer, R., Höfelein, C., Lüthy, J., & Candrian, U. (1994). Polymerase chain reaction-restriction fragment length polymorphism analysis: a simple method for species identification in food. *Journal of the AOAC International, 78*, 1542–1551.

Murray, B. W., McClymont, R. A., & Strobeck, C. (1995). Forensic identification of ungulate species using restriction fragment digests of PCR-amplified mitochondrial DNA. *Journal of Forensic Sciences, 40*, 943–951.

Unseld, M., Beyerman, B., Brandt, P., & Hiesel, R. (1995). Identification of the species origin of highly processed meat products by mitochondrial DNA sequences. *Genome Research, 4*, 241–243.

Welsh, J., & McClelland, M. (1990). Fingerprinting genomes using arbitrary primers. *Nucleic Acids Research, 18*, 7213–7218.

Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A., & Tingley, S. V. (1991). DNA polymorphisms amplified by arbitrary primers are useful genetic markers. *Nucleic Acids Research, 18*, 6531–6535.