Journal of Industrial Microbiology & Biotechnology (1998) 21, 121–127

http://www.stockton-press.co.uk/jim

Mammalian species identification by interspersed repeat PCR fingerprinting

JB Buntjer[1,2] and JA Lenstra[1]

[1]*Institute of Infectious Diseases and Immunology;* [2]*Department of the Science of Food of Animal Origin, Utrecht University, PO Box 80.165, 3508 TD Utrecht, The Netherlands*

Most DNA methods for species identification of animal tissues test the presence/absence of one species per assay, requiring several tests for a complete analysis and prior knowledge of the species that are potentially present in the sample. Here we demonstrate that PCR with fluorescently labeled MIR (mammalian-wide interspersed repeat) primers generate fingerprints that are suitable for rapid identification of known and unknown species on an automatic sequencing apparatus and with computer-assisted data processing. The method allows the analysis of processed meat samples and offers a convenient alternative to sequencing of mitochondrial DNA.

Keywords: species identification; animal tissues; PCR; DNA fingerprinting

Introduction

Species identification of animal tissues in food is important for economic and public-health reasons. Meat of different species may vary in price and susceptibility to microorganisms pathogenic for man. Further, consumption of meat from such species as pigs and cows may be disallowed for religious reasons.

The most common meat species are identified by immunochemical detection of specific proteins [11,12,20,21,29], by comparison of electrophoretic protein patterns [22,24,30,32,34] or by a combination of both [7,17]. Immunochemical detection allows an easy and sensitive analysis of species mixtures, but requires a specific serum and separate assays for each species. Electrophoretic patterns discriminate between several species in a single test, but interpretation becomes difficult when mixtures are analyzed. These methods are less suitable for heated meat samples since their specificity is decreased by heat denaturation of the proteins.

DNA-based tests allow both the analysis of highly processed samples and the differentiation of closely related species. DNA identification comprises either hybridization with specific probes [2–5,15,17,18] or PCR-RFLP [10,25,27]. However, this again requires a separate assay for each species.

Law enforcement in wildlife management as well as the increasing consumption of exotic animals demand tests with a broader species range. If there is little information about the species, identification may require assays with several probes or restriction-enzyme combinations. This may be circumvented by the forensically informative nucleotide sequencing (FINS) of amplified mitochondrial DNA [1,35], but this is relatively expensive and protected by a patent [6].

Correspondence: JA Lenstra, Institute of Infectious Diseases and Immunology, Utrecht University, PO Box 80.175, 3508 TD Utrecht, The Netherlands
Received 19 December 1997; accepted 15 June 1998

Recently, we described the use of mammalian-wide interspersed repeat (MIR) fingerprints [19] in phylogenetic reconstruction [4]. Patterns can be generated by PCR amplification with radioactively labeled MIR-specific primers (MS-PCR) and polyacrylamide gel electrophoresis. The resulting fingerprinting patterns are species-specific, phylogenetically informative at the level of taxonomic tribes and have low intra-species variation. Here we demonstrate the application of MS-PCR to forensic multi-species identification and its implementation in a fluorescent detection system with automated data processing.

Methods

DNA isolation

Species used in this study are summarized in Table 1. Chromosomal DNA was isolated from peripheral blood [9] or from solid tissues and meat products [31]. DNA concentrations were estimated after agarose gel electrophoresis and ethidium bromide staining.

Meat preparation

Meat products were prepared under industrial conditions. Fresh meat was obtained from local stores, cut into small pieces of approximately 1 g, heated at 120°C for 20 min in portions of ten pieces and frozen until use.

MS-PCR

MIR-specific PCR (MS-PCR) was performed with primers OmiR (5′ACCTTGAGCAAGTCACT, 5′Cy labeled, Pharmacia, Uppsala, Sweden) and OmiL (5′GATGAGGAA ACTGAGGC), derived from the MIR consensus [19]. PCR was performed in a volume of 20 μl containing standard *Taq* buffer (50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$ and 0.001% (w/v) gelatin), 0.2 mM dNTPs, 100 ng of both primers, 1 U *Taq* polymerase and 25 ng of chromosomal DNA. Thermocycling (30 cycles) was done at 94°C for 30 s, 50°C for 45 s and 72°C for 120 s. PCR products were detected by electrophoresis using an automated laser fluorescent sequencer (ALF-red, Pharmacia) in 6% Ready-

Table 1 Names and taxonomic position of species used in this study. Systematics according to Morris [26] and Webb *et al* [36]

Order	Family	Subfamily	Species	
Artiodactyla	Bovidae	Bovinae	Cattle	*Bos taurus*
			Bison	*Bos bison*
			Water buffalo, river type	*Bubalus bubalis*
			Water buffalo, swamp type	*Bubalus bubalis*
		Caprinae	Sheep	*Ovis aries*
			Goat	*Capra hircus*
		Antilopinae	Blackbuck	*Antilope cervicapra*
		Hippotraginae	Wildebeest	*Connochaetes taurinus*
	Cervidae	Cervinae	Hog deer	*Axis porcinus*
			Fallow deer	*Dama dama*
			Red deer (wapiti)	*Cervus elaphus*
		Muntiacinae/Cervinae[a]	Muntjac	*Muntiacus* sp
		Odocoileinae	Reindeer	*Rangifer tarandus*
			Moose	*Alces alces*
			Roe deer	*Capreolus capreolus*
			Whitetail deer	*Odocoileus virginianus*
			Mule deer	*Odocoileus hemionus*
	Suidae		Pig	*Sus scrofa*
Perissodactyla	Equidae		Horse	*Equus caballus*
			Donkey	*Equus asinus*
Carnivora	Felidae		Cat	*Felis catus*
	Canidae		Dog	*Canis familiarus*
	Ursidae		Brown bear	*Ursus arctos*
Primates			Human	*Homo sapiens*
Galliformes	Phasianidae	Phasianinae	Chicken	*Gallus gallus*
		Meleagridinae	Turkey	*Meleagris gallopavo*
Anseriformes	Anatidae	Anserinae	Goose	*Anser anser*
		Anatinae	Mallard duck	*Anas platyrhynchos*
			Muscovy duck	*Carina moschata*
Struthioniformes	Strutionidae		Ostrich	*Struthio camelus*

[a]The subfamily status of the muntjak is disputed [14].

Mix gel (Pharmacia) for 10 h at 1200 V, 34 W, 50°C. Fluorescent signals were sampled every 2 s. The first of four lanes was loaded with chicken MS-PCR products as markers to correct for variable mobilities across the gel.

Data analysis

ALF computer files were analyzed by the Biologist II software package ([4]; available on request). For visual inspection, the average values of five or ten sampling intervals were represented in a graphic format of a virtual electrophoresis image. Peaks were identified by a peak calling algorithm [4]. Peak retention times were corrected for gel-shift effects by double interpolation of differences in retention time of the chicken marker fragments (see above). A fragment is considered to be shared by two samples if retention times are within a range of ±20 sampling points. As measure of the similarity of two patterns, we used Jaccard's index (number of common bands divided by the total number of bands of two patterns; [16]) or, for the identification of degraded DNA samples, a pattern identification index (proportion of the bands of the pattern with the lowest amounts of bands that are shared with the reference pattern).

Results

Intraspecies and interspecies variability of MS-PCR fingerprints

MS-PCR fragments of mammalian and avian species were generated and detected by an automatic sequencer. The test set included domestic farm animals as well as wild species. An average of 34 mammalian DNA fragments of up to 400 bp were detected. Typical avian patterns contain fewer bands [19] and are easily distinguished by eye (Figure 1).

Previous studies showed a low intra-species polymorphism in bovid and cervid MS-PCR patterns with Nei's similarities in the range of 0.85–1.0 [4]. Independent interpretations by three persons of enlarged versions of the electrophoresis patterns of cattle, horse, pig and water buffalo (Figure 1) gave average intra-specific similarity of about 0.95. Automatic interpretation of the same patterns gave a lower value (0.85), mainly because weak bands were not detected by the automatic peak calling. Differences in peak intensity may be caused by the quality of the template (see below), the yield of the PCR and lane-to-lane variation.

The number of bands shared between species correlates with the taxonomic level of relatedness (Figure 2) and intra-species similarities are significantly higher than interspecies indices. Only with closely related species, such as the American deer species white tail deer (*Odocoileus virginianus*) and mule deer (*Odocoileus hemionus*) are the intra- and interspecies Jaccard indices in the same range.

Species recognition by matching of MS-PCR patterns

To test the feasibility of automatic species recognition, MS-PCR fingerprints of two different, unrelated individuals of 12 domestic and wild species, were generated and loaded on both halves of a gel. The electrophoretic mobilities were corrected for uneven migration across the gel by inter-

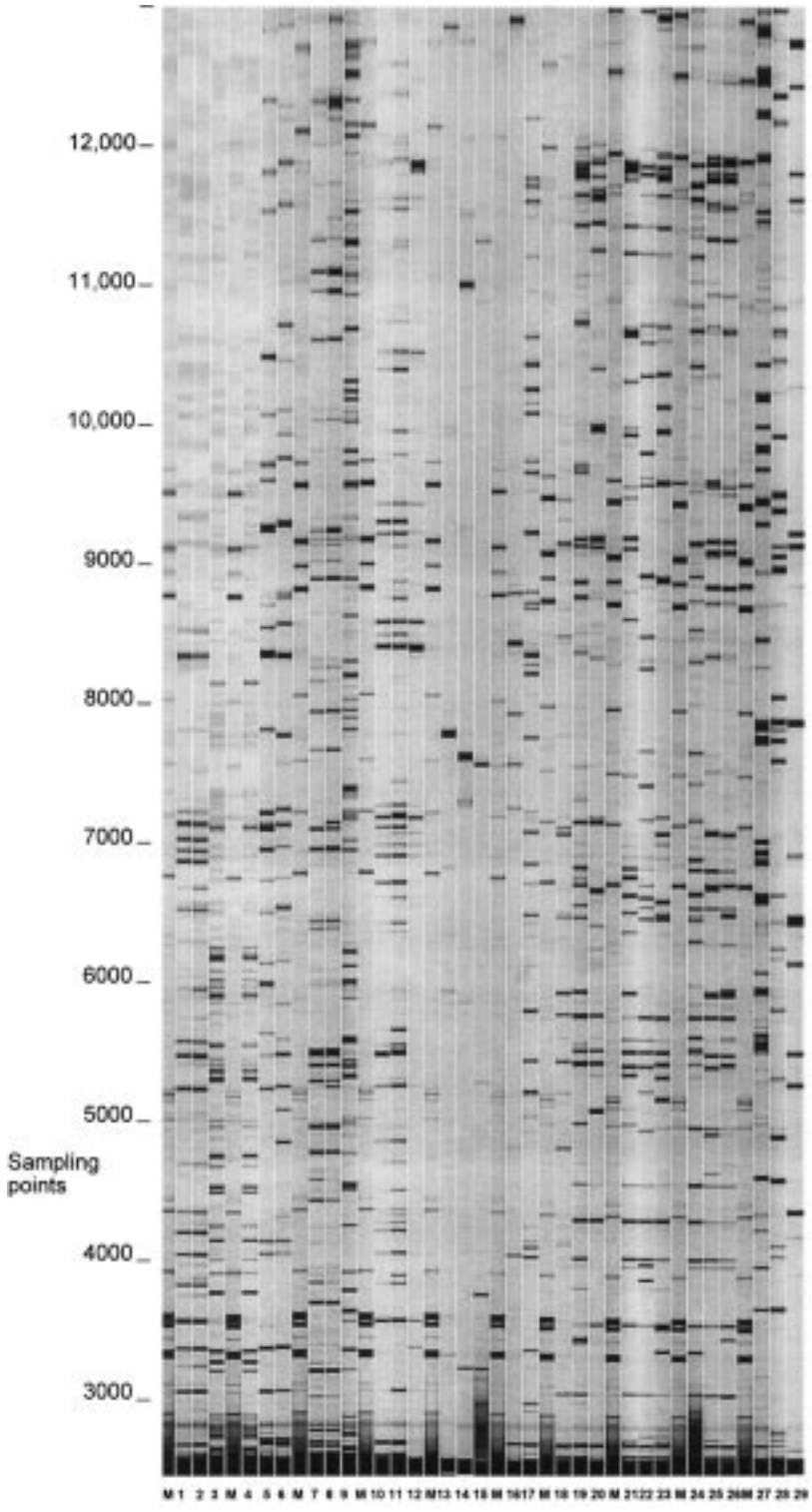

Figure 1 Virtual electrophoresis image of MS-PCR fragments, amplified with template DNA of several species. M, molecular marker (chicken); 1–2, cattle; 3–4, horse; 5, goat; 6, sheep; 7–8, pig; 9, ass; 10–11, water buffalo; 12, bison; 13, goose; 14, mallard duck; 15, muscovy duck; 16, ostrich; 17, blackbuck; 18, wildebeest; 19, reindeer; 20, moose; 21, roe deer; 22, fallow deer; 23, wapiti; 24, muntjac; 25, white tail; 26, mule deer; 27, brown bear; 28, cat; 29, dog.

polation of the mobilities of marker fragments (MS-PCR of chicken DNA) every fourth lane (Figure 3). Jaccard indices as measures of similarity between the patterns of the same species were calculated (Table 2). Using the highest similarities as criterion led in all cases to a correct species identification. Predictably, the difference between the correct species and the second best matching species was lower if species of short evolutionary distance were present

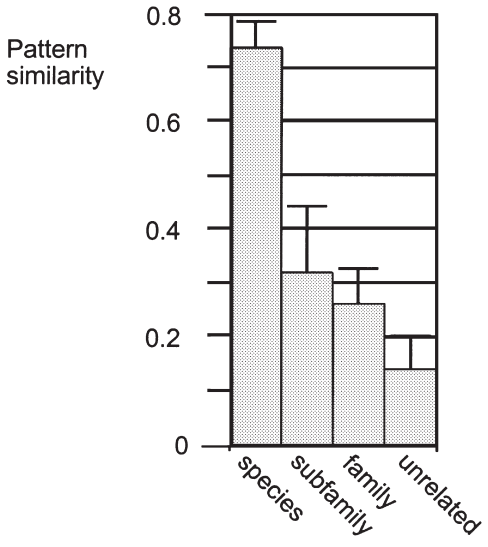

Figure 2 Average Jaccard's indices of mammalian MS-PCR patterns depending on taxonomic relatedness.

(mule deer and whitetail deer). The similarity of the two wildebeest patterns was only 0.31 because one of the patterns mainly contained bands corresponding to the shortest amplification products. This indicates degradation of DNA (see below), but did not preclude correct identification.

Identification of meat samples

Heating of meat samples at temperatures higher than 100°C leads to fragmentation of DNA and decreases the amount of template available for amplification of large fragments. To test whether identification is still possible, we carried out MS-PCR on DNA isolated from meat of five species and four commercial meat products.

DNA isolated from frozen raw beef showed a pattern similar to that of intact genomic DNA from blood, but with a lower signal (Figure 4). DNA of autoclaved mammalian meat gave fragments up to 300 bp in agreement with the average size of DNA isolated from heated meat [13]. The weak bands in the pattern of autoclaved chicken deviated from the bands obtained with intact chicken DNA. DNA isolated from a smoked ring sausage (meat ingredients declared as pork and poultry) gave a pattern resembling that of pig DNA without any avian fragments, although the presence of chicken meat was clearly demonstrated by hybridization experiments [3]. A similar dominance of the mammalian patterns was found with artificial mixtures of pig DNA and chicken or turkey DNA (not shown). Surprisingly, we found cattle-like bands with two out of three corned beef samples. In previous studies we found that extreme DNA degradation in these products interfered even with species identification by oligonucleotide hybridization [5].

Figure 5 shows a plot of the number of bands shared by the meat and genomic DNA samples from the gel in Figure 4. This depended not only on a common species origin, but also on the meat sample. An algorithm for automatic identification must take into account that the number of fragments of samples and references are unbalanced. Instead of using the Jaccard's index, which is an average

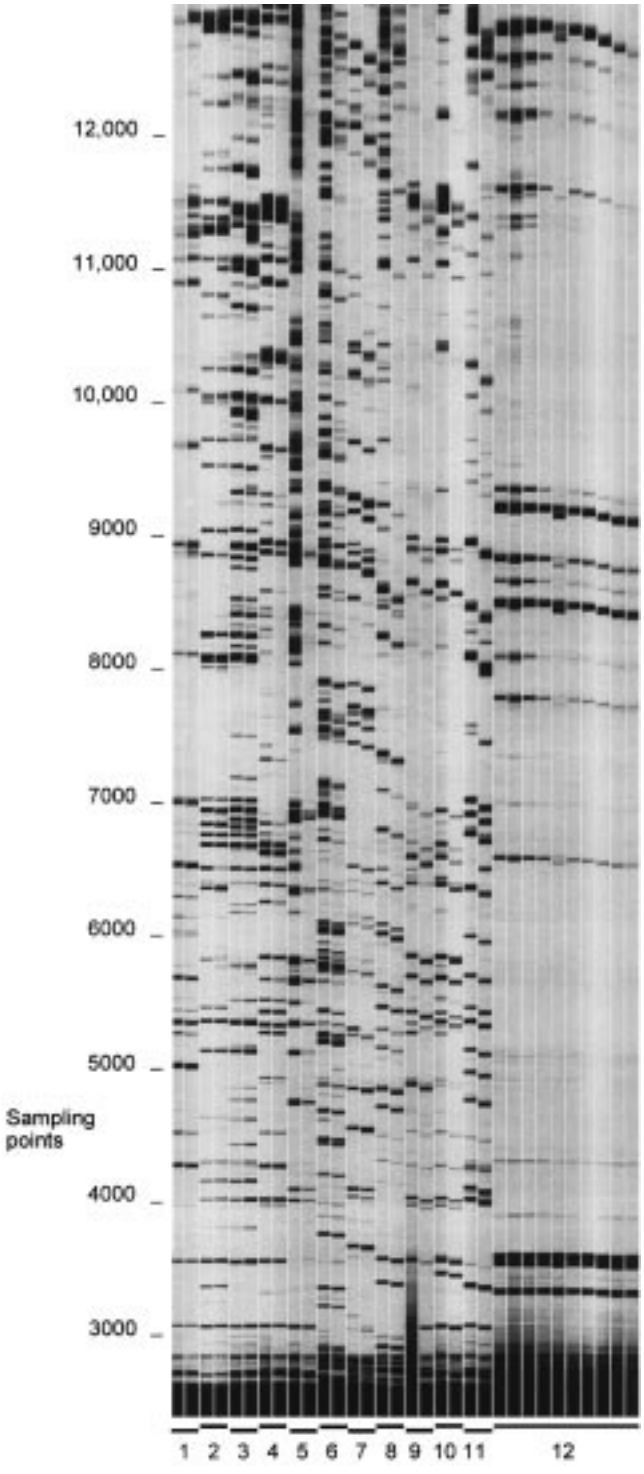

Figure 3 Comparison of MS-PCR patterns on one gel. MS-PCR fragments from two individuals of one species were loaded on two halves of the gel. In the virtual electrophoresis image, lanes were electronically reordered to facilitate comparison of the patterns. The mobility shift between the left and the right side of the gel was compensated by interpolation of the shift in marker fragments (electronically grouped together on the right side of the image). Templates: 1, moose; 2, ox; 3, water buffalo; 4, roe deer; 5, wildebeest; 6, horse; 7, cat; 8, human; 9, white tail; 10, mule deer; 11, sheep; 12, chicken (marker).

Table 2 Jaccard similarities of MS-PCR patterns from the right and left side of the same gel, respectively. Bold printing indicates the matching of individuals of the same species

		Right side											
		moose	cattle	chicken	water buffalo	roe deer	wildebeest	horse	cat	men	whitetail deer	mule deer	sheep
Left side	moose	**0.71**	0.25	0.03	0.24	0.35	0.17	0.14	0.19	0.19	0.28	0.29	0.20
	cattle	0.21	**0.74**	0.05	0.46	0.22	0.27	0.22	0.19	0.32	0.24	0.25	0.32
	chicken	0.02	0.05	**0.69**	0.10	0.09	0.04	0.10	0.00	0.14	0.08	0.04	0.09
	water buffalo	0.23	0.40	0.08	**0.83**	0.28	0.27	0.17	0.14	0.23	0.24	0.25	0.28
	roe deer	0.26	0.24	0.02	0.26	**0.68**	0.27	0.18	0.24	0.19	0.30	0.34	0.24
	wildebeest	0.15	0.24	0.02	0.26	0.19	**0.31**	0.23	0.26	0.04	0.16	0.18	0.27
	horse	0.14	0.16	0.08	0.29	0.24	0.20	**0.75**	0.21	0.18	0.20	0.21	0.14
	cat	0.20	0.14	0.04	0.12	0.15	0.17	0.20	**0.80**	0.13	0.13	0.15	0.13
	man	0.10	0.30	0.08	0.29	0.19	0.16	0.16	0.17	**0.74**	0.09	0.10	0.21
	whitetail deer	0.38	0.19	0.11	0.28	0.44	0.24	0.21	0.15	0.12	**0.62**	0.62	0.16
	mule deer	0.39	0.25	0.07	0.27	0.48	0.26	0.20	0.16	0.08	0.56	**0.82**	0.15
	sheep	0.16	0.25	0.09	0.25	0.13	0.25	0.20	0.15	0.22	0.15	0.19	**0.76**

representational figure of both patterns, we calculated the similarity of patterns by a pattern identification index, which corresponds to the proportion of fragments of the test sample shared with the reference sample. Using this index, all patterns from meat samples were correctly recognized by the algorithm, even if the recognition of the corned beef is based on a few matching bands only.

Discussion

We conclude that MS-PCR with fluorescence detection is a useful contribution to the methodology of species identification. Tissue samples can be typed without prior knowledge by comparing patterns with reference samples on the same gel. Computer processing allows automation of analysis and storage of data. Band sharing by related species allows the use of species of the same genus or tribe as reference material when identification on a higher taxonomic level than the species is sufficient.

The MS-PCR patterns with 20–60 bands are clearly more informative than patterns generated by iso-electric focusing or fatty acid analysis. Unlike protein patterns, the MS-PCR patterns are independent of tissue type or diet. A major advantage of the MS-PCR over RAPD reactions is the high annealing temperature (50–55°C), leading to specific and reproducible patterns that are not influenced by the amount and quality of the DNA template. The technique probably has the same discriminative power as sequencing of mitochondrial DNA, but MS-PCR has fewer reaction steps, does not require sequencing reagents and is not protected by a patent [6]. On the other hand, the amplification of the abundant mtDNA requires less template DNA, which is relevant if highly processed samples are tested. Although MS-PCR may be more suitable than FINS for the detection of admixtures, hybridization assays [3,15,18] or PCR-RFLP [10,25,27] are the most suitable to analyze the composition of samples of mixed origin.

Identification of heated samples is partly limited by degradation of the template DNA (Figures 4 and 5). This resulted in a loss of longer amplification products and a few additional bands, but in all cases a clear match with intact DNA of the same species was found. The MS-PCR identification can be further optimized by using multiple primer combinations to generate more data per sample. In particular with degraded template DNA, this would compensate for the loss of long DNA fragments.

Maximum advantage of the automatic pattern recognition is taken if data of previous analysis could be used as a reference database. This requires gel-to-gel transfer of data and accurate determination of the length of fragments. Preliminary results showed that size estimation with one fluorescent dye was disturbed by mobility shifts between lanes. This can be eliminated by using instruments that allow the use of internal markers with a second dye [33].

Few clear bands are found in MS-PCR patterns of birds, while the weaker bands are not reproducible with DNA from heated samples. MS-PCR of mixtures of porcine and avian DNA only amplified the porcine fragments. Presumably, the less robust amplification of avian fragments is caused by either a low sequence similarity of the avian MIRs with the mammalian consensus or by random priming [23]. In the current procedure, identification has to rely on two or three intense bands that are not affected by heating. An interesting option may be the use of primers derived from the avian Cr1 SINE [8,23,28] for phylogenetic and analytic fingerprinting.

Acknowledgements

We thank for kindly providing DNA or tissue samples: JJ Beintema (Groningen University), PA Dratch (National Fisheries and Wildlife Forensic Laboratory, Ashland), MH Van der Hage (Utrecht University), ER Mahfour (National Research Centre, Cairo), S Mikko (Swedish University of Agricultural Sciences, Uppsala), WS Modi (NCI-Frederick Cancer Research and Development Centre, Frederick), A Rando (Instituto Produzione Animale, Potenza), K Roed (Norwegian College of Veterinary Medicine, Oslo), Antwerpen Zoo (Antwerp) and Blijdorp Zoo (Rotterdam). We thank Dr A Ruiter (Utrecht University) for reading the manuscript. This research was financially supported by the

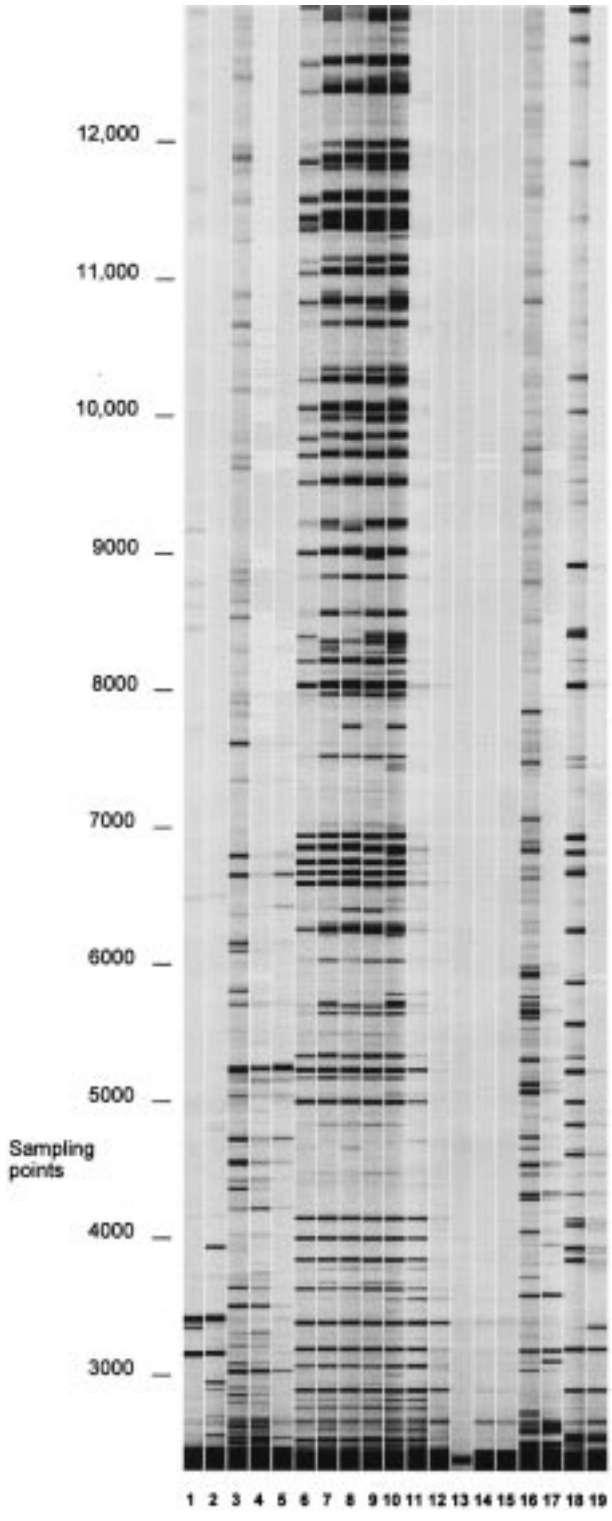

Figure 4 Comparison of MS-PCR patterns obtained with lymphocyte DNA and with DNA isolated from heated and/or processed meat products, respectively. Templates: 1, chicken DNA; 2, chicken meat (autoclaved); 3, pig DNA; 4, pork (autoclaved); 5, sausage; 6–10, cattle DNA; 11, beef (raw); 12, beef (autoclaved); 13–15, corned beef; 16, horse DNA; 17, horse meat (autoclaved); 18, sheep DNA; 19, mutton (autoclaved).

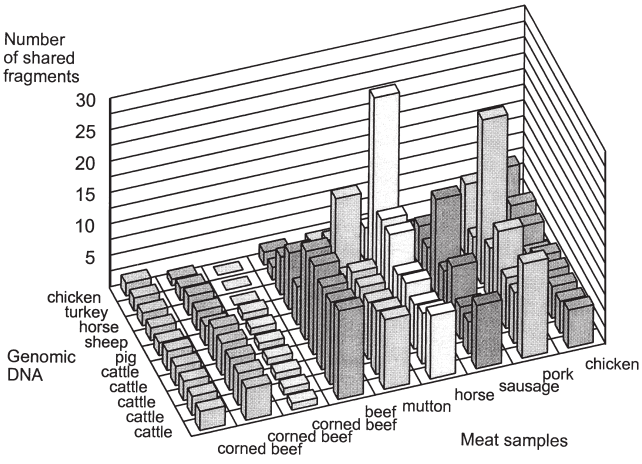

Figure 5 Sharing of MS-PCR fragments by meat samples and purified genomic DNA. Data are from Figure 4.

Inspectorate for Health Protection, Rijswijk, The Netherlands.

References

1 Bartlett SE and WS Davidson. 1992. FINS (forensically informative nucleotide sequencing): a procedure for identifying the animal origin of biological specimens. Biotechniques 12: 408–411.
2 Blackett RS and P Keim. 1992. Big game species identification by deoxyribonucleic acid (DNA) probes. J Forensic Sci 37: 590–596.
3 Buntjer JB, JA Lenstra and N Haagsma. 1995. Rapid species identification by using satellite DNA probes. Z Lebensm Unters Forsch 201: 577–582.
4 Buntjer JB. 1997. DNA repeats in the vertebrate genome as probes in phylogeny and species identification. Academic thesis, Utrecht University, The Netherlands.
5 Buntjer JB, A Lamine, N Haagsma and JA Lenstra. Species identification by oligonucleotide hybridization: the influence of processing of meat products. J Sci Food Agric (in press).
6 Butler D. 1995. Patent row splits fish identity researchers. Nature 377: 9.
7 Casa C, J Tormo, PE Hernandez and B Sanz. 1985. The detection and partial characterization of horse muscle soluble proteins by immunoelectrophoresis in agarose gels. Meat Sci 12: 31–37.
8 Chen ZQ, RG Ritzel, CC Lin and RB Hodgetts. 1991. Sequence conservation in avian CR1: an interspersed repetitive DNA family evolving under functional constraints. Proc Natl Acad Sci USA 88: 5814–5818.
9 Ciulla TA, RM Sklar and SL Hauser. 1988. A simple method for DNA purification from peripheral blood. Anal Biochem 174: 485–488.
10 Cronin MA, DA Palmisciano, ER Vyse and DG Cameron. 1991. Mitochondrial DNA in wildlife forensic science: species identification of tissues. J Forensic Sci 19: 94–105.
11 Cutrufelli ME, RP Mageau, B Schwab and RW Johnston. 1993. Development of a multispecies identification field test by modified agar-gel immunodiffusion. J Assoc Off Anal Chem 76: 1022–1026.
12 Dincer B, JL Spearow, RG Cassens and ML Greaser. 1987. The effects of curing and cooking on the detection of species origin of meat products by competitive and indirect ELISA techniques. Meat Sci 20: 253–265.
13 Ebbehøj KF and PD Thomsen 1991. Species differentiation of heated meat products by DNA hybridization. Meat Sci 30: 221–234
14 Groves CP and P Grubb. 1987. Relationships of the living deer. In: Biology and Management of the Cervidae (Wemmer CM, ed), pp 21–59, Smithsonian Institution Press, Washington DC.
15 Hunt DJ, HC Parker and ID Lumley. 1997. Identification of the species of origin of raw and cooked meat products using oligonucleotide probes. Food Chem 60: 437–442.
16 Jaccard P. 1908. Nouvelles recherches sur la distribution florale. Bull Soc Vaud Sci Nat 44: 223–270.

17 Janssen FW, GH Hägele, AMB Voorpostel and JA De Baaij. 1990. Myoglobin analysis for determination of beef, pork, horse, sheep and kangaroo meat in blended cooked products. J Food Sci 55: 1528–1530.
18 Janssen FW, GH Hägele, JB Buntjer and JA Lenstra. 1998. Species identification in meat by using PCR-generated satellite probes. J Ind Microbiol Biotechnol 21: 115–120.
19 Jurka J, E Zietkiewicz and D Labuda. 1995. Ubiquitous mammalian-wide interspersed repeats (MIRs) are molecular fossils from the Mesozoic era. Nucleic Acids Res 23: 170–175.
20 Kang'ethe EK, SJ Jones and RLS Patterson. 1982. Identification of the species origin of fresh meat using an enzyme-linked immunosorbent assay procedure. Meat Sci 7: 229–240.
21 Kang'ethe EK and JM Gathuma. 1987. Species identification of autoclaved meat samples using antisera to thermostable muscle proteins in an enzyme immunoassay. Meat Sci 19: 265–270.
22 King NL 1984. Species identification of cooked meats by enzyme-staining of isoelectric focusing gels. Meat Sci 11: 59–72.
23 Levin I, LB Crittenden and JB Dodgson. 1994. Mapping DNA polymorphisms using PCR primers derived from the sequence of an CR1 element. J Hered 85: 73–78.
24 McCormick RJ, DA Collins, RA Field and TD Moore. 1992. Identification of meat from game and domestic species. J Food Sci 57: 516–517.
25 Meyer R, C Hofelein, J Lhthy and U Candrian. 1995. Polymerase chain reaction-restriction fragment length polymorphism analysis: a simple method for species identification in food. J Assoc Off Anal Chem 78: 1542–1551.
26 Morris D. 1965. The Mammals. A Guide to the Living Species. Hodder and Stoughton, London.
27 Murray BW, RA McClymont and C Strobeck. 1995. Forensic identification of ungulate species using restriction digests of PCR-amplified mitochondrial DNA. J Forensic Sci 40: 943–951.
28 Okimoto R, HH Cheng and JB Dodgson. 1997. Characterization of CR1 repeat random PCR markers for mapping the chicken genome. Anim Genet 28: 139–145.
29 Patterson RM and TL Spencer. 1985. Differentiation of raw meat from phylogenically related species by enzyme-linked immunosorbent assay. Meat Sci 15: 119–123.
30 Prasad VSS and DS Misra. 1981. Differentiation of meats of different species of animals by muscle esterase pattern in different age and sex groups. Indian J Anim Sci 51: 211–214.
31 Sambrook J, EF Fritsch and T Maniatis. 1989. Molecular Cloning. A Laboratory Manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
32 Slattery WJ and AJ Sinclair. 1983. Differentiation of meat according to species by the electrophoretic separation of muscle lactate dehydrogenase and esterase isozymes and isoelectric focusing of soluble proteins. Aust Vet J 60: 47–51.
33 Sullivan KM, S Pope, P Gill and JM Robertson. 1992. Automated DNA profiling by fluorescent labeling of PCR products. PCR Meth Appl 2: 34–40.
34 Ukishima Y, M Kino, H Kubota, S Wada and S Okada. 1991. Identification of whale species by thin-layer isoelectric focusing of sarcoplasmic proteins. J Assoc Off Anal Chem 74: 943–950.
35 Unseld M, B Beyermann, P Brandt and R Hiesel. 1995. Identification of the species origin of highly processed meat products by mitochondrial DNA sequences. PCR Meth Appl 4: 241–243.
36 Webb JE, JA Wallwork and JH Elgood. 1979. Guide to Living Animals, 2nd edn. Macmillan Press, London.