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Differentiation of cattle species in beef by PCR-RFLP of mitochondrial and satellite DNA

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Abstract

Methods currently used for the identification of the species origin of meat or tissue samples have not been validated for other bovine species than taurine cattle or water buffalo. These methods also do not discriminate between the different bovine species that are used as source of beef. Here, we describe two complementary methods for detection and differentiation of bovine species, which are based on mutations in mitochondrial DNA and centromeric satellite DNA, respectively. The analysis of satellite DNA is especially relevant for the identification of animals that are of hybrid origin.

Keywords: Species identification; PCR-RFLP; Mitochondrial DNA; Satellite DNA; Bovini; Beef

1. Introduction

Identification of the species origin in meat samples is relevant for economical, religious or public health concerning reasons (Buntjer, Lenstra, & Haagsma, 1995; Lenstra & Bradley, 1999). Species detection is also relevant for wildlife management, which so far has not received the level of attention as food inspection of human forensic science (Dove, 1999).

Modern methods for meat identification are based on DNA analysis. Hybridization of DNA extracted from meat to probes recognising the species-specific satellite repeats can be used to discriminate related species, e.g. sheep versus cattle; chicken versus turkey (Buntjer, Nijman, Zijlstra, & Lenstra, 1998; Hunt, Parkes, & Davies, 1997). Alternatively, PCR-RFLP analysis of mitochondrial DNA (Chikuni, Tabata, Kosugiyama, Momma, & Saito, 1994; Dickinson, Kroll, & Grant, 1995; Guglich, Wilson, & White, 1994; Wolf, Rentsch, & Hubner, 1999; Partis, Croan, Guo, Clark, Coldham, & Murby, 2000) is used. In most cases both methods are adequate to identify the species origin of meat in processed meat samples.

However, these methods are only partially suitable for the analysis of beef samples. First, the commonly used PCR-primers (Kocher et al., 1989; Meyer, Hofelein, Luthy, & Candrian, 1995) are based on the human mitochondrial cytochrome *b* sequence and have several mismatches relative to the bovine sequence. As a consequence, these primers are less suitable for analysis of mixed samples in which most of the DNA is degraded. Tartaglia (Tartaglia, Saulle, Pestalozza, Morelli, Antonucci, & Battaglia, 1998) designed a dedicated assay for a sensitive but exclusive detection of bovine material. Secondly, the standard PCR-RFLP test (Matsunaga et al. 1999; Meyer et al., 1995) have been validated only for the two most common bovine species, taurine cattle and water buffalo. Meat from other bovine species like zebu, banteng, gaur, bison or yak is consumed in several parts of the world, either as a substitute of taurine beef or as a high-quality beef variant. Thirdly, no method is available to discriminate between meat from other bovine species. PCR tests for differentiation of mitochondrial DNA from cattle and bison have been described for the purpose of wildlife conservation (Murray, McClymont, & Strobeck, 1995; Ward, Bielawski, Davis, Templeton, & Derr, 1999)

Finally, hybridization of different bovine species is not uncommon and invalidates any test based on the maternally transmitted mitochondrial DNA. Several African zebu breeds are taurine-indicine hybrids (Nijman,

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Bradley, Hanotte, Otsen, & Lenstra, 1999), while taurine–yak, banteng–zebu as well as gaur–zebu hybridization occurs in various regions in Asia (Bradley, MacHugh, Loftus, Sow, Hoste, & Cunningham, 1994; Lenstra & Bradley, 1999). Cattle introgression has occurred in the American bison population (Polziehn, Strobeck, Sheraton, & Beech, 1995; Ward et al., 1999). The American Beefalo cattle is bred for its beef quality and resulted from crossings of bison with cattle.

Here, we describe two complementary methods for bovine species identification. The first method is based on species-specific mutations on mtDNA of cytochrome *b* and cytochrome oxidase II, extending the range of applications of the mitochondrial PCR-RFLP assay. Secondly, as confirmatory method, we describe SFLP (satellite fragment length polymorphism), a PCR-RFLP procedure on centromeric satellite DNA (Nijman et al., 1999; Nijman & Lenstra, 2001), which offers the additional advantage of detecting interspecies hybridization.

2. Material and methods

2.1. Samples and DNA isolation

Blood or tissue (muscle or liver) from bovine species was collected from taurine cattle (*Bos taurus*), zebu (*Bos indicus*), banteng (*Bos javanicus*), gaur, gayal (*Bos gaurus*), yak (*Bos grunniens*), bison (*Bison bison*), wisent (*Bison bonasus*), water buffalo (*Bubalus bubalis*) and African buffalo (*Syncerus caffer*). Bison beef was purchased at a local supermarket. DNA from blood was isolated using the guanidium-isothiocyanate protocol as described (Ciulla, Sklar, & Hauser, 1988). DNA from tissue was isolated by proteinase-K/SDS extraction (Sambrook, Fritsch, & Maniatis, 1989).

2.2. PCR-RFLP

PCR was performed in a total volume of 25 μl, containing 50 ng genomic DNA and 50 ng of both primers in Taq DNA polymerase buffer (Promega, Madison, USA) with 1.5 mM $MgCl_2$, 0.2 mM dNTP (Promega) and 1.25 U Taq DNA polymerase. The following program was used: predenaturation for 2 min at 95°C, followed 25 PCR cycles of 15 s at 92°C, 30 s at 38°C and 45 s at 72°C and by a final extension of 5 min at 72°C. For restriction endonuclease analysis with *Ava*III, *Bam*HI, *Eco*RI, *Hin*dIII, *Hin*fI, *Stu*I, *Taq*I, *Xba*I (Amersham-Pharmacia, Amersham, UK; Uppsala, Sweden), *Ban*II or its isoschizomer *Tru*9 (Fermentas, Vilnius, Lithuania) and *Mse*I (New England BioLabs, Beverley, USA). 5 μl of the PCR product was digested by addition of 5–10 U of restriction endonuclease and the recommended concentrated reaction buffer. Samples were digested for 3 h at 37°C, 60°C (*Taq*I) or 65°C (*Tru*9) and fractionated on a 2% agarose (SphaeroQ, Leiden, The Netherlands)/0.5×TBE gel. For sequencing, PCR products were separated on a 2% agarose/0.5×TBE gel, excised and purified with the QIAquick system (QIAGEN Inc., Valencia, CA, USA) following the manufacturers protocol. Sequencing was performed using the Cy5 Big Dye terminator kit (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) on an ABI Prism 310 sequencer (Perkin-Elmer).

Table 1
Genbank entries of mitochondrial sequences[a]

Species	Cytochrome b	Cytochrome oxidase II
Taurine cattle	D34635	M10544
Zebu	AF348597*	AF348595*
Banteng	D34636	U18821
Gaur	AF348596*	AF348592*
Gayal	AF348593*	U18818
Yak	Y16063	AF348594*
Bison	Y16060	U62568
Wisent	Y16061	U62567
Water buffalo	X78960	U18822
African buffalo	Y16056	U18825

[a] Asterisks denote new data generated in order to resolve discrepancies between predicted and observed restriction digests.

3. Results and discussion

3.1. Mitochondrial DNA

Sequences of cytochrome *b* (Hassanin & Douzery, 1999; Schreiber, Seibold, Notzold, & Wink, 1999) and cytochrome oxidase II (Janecek, Honeycutt, Adkins, & Davis, 1996) from bovine species were retrieved from Genbank (Table 1) and aligned (Corpet, 1988). For a few species the sequences were partially re-analysed to resolve discrepancies. The commonly used downstream cytochrome *b* primer (Meyer et al., 1995) has four mismatches with the bovine sequence, one of which is 3 nt from the 3′ end.

Therefore, new cytochrome *b* primers for the bovine species were designed (Table 2). These primers amplified the cytochrome *b* fragment from all artiodactyles tested (bovines, sheep, goat, nilgai, raindeer and pig) and from horse. A faint product was obtained with human DNA and chicken DNA was negative (results not shown). The cytochrome oxidase II primers (Janecek et al., 1996) have a narrower species range and generated a product only with DNA from bovine species or with DNA from the nilgai, also belonging to the Bovinae subtribe.

Meyer and co-authors identified two diagnostic restriction sites present in the cytochrome *b* genes from taurine cattle and water buffalo, *Hae*III and *Alu*I. The *Hae*III site is present in the sequence of all bovine species except the African buffalo, but this site is not within

Table 2
Oligonucleotides used as PCR primers

Target	Primer sequence 5′–3′ (left and right primer, respectively)	Amplicon length (bp)
Cytochrome b	ACAAATCCTCACAGGCCTATTC TAGGACGTATCCTATGAATGCT	271
Cytochrome oxidase II	ATGGCATATCCCATACAACTAG ACTTTAGTGGGACTAACTCAAG	651
Satellite IV	AAGCTTGTGACAGATAGAACGAT CAAGCTGTCTAGAATTCAGGGA	604
Satellite 1.711b	CTGGGTGTGACAGTGTTTAAC TGATCCAGGGTATTCGAAGGA	822

Table 3
Lengths of fragments generated by digestion of mitochondrial PCR products with the indicated restriction enzymes

Species	Cytochrome *b*						cytochrome oxidase II
	*Alu*I	*Xba*I	StuI	*Bam*HI	*Hin*fI	*Taq*I	EcoRI
Taurine cattle	131, 140	198,73	271	271	101, 170	271	244, 407
Zebu	131, 140	198, 73	271	271	101, 170	271	244, 407
Banteng	131, 140	271	171, 100	271	271	108, 163	651
Gaur, gayal	131, 140	271	171, 100	271	271	271	651
Yak	131, 140	271	271	271	271	271	244, 407
Bison	131, 140	271	171, 100	271	101, 170	271	244, 407
Wisent	131, 140	198, 73	171, 100	271	271	271	651
Water buffalo	131, 140	198, 73	271	271	271	108, 163	244, 407
African buffalo	131, 140	271	171, 100	184, 87	271	108, 163	244, 407

Table 4
lengths of fragments generated by digestion of satellite DNA PCR products with the indicated restriction enzymes[a]

Species	Satellite IV		Satellite 1.711b		
	*Ban*II	*Mse*I	*Hin*dIII	*Taq*I	*Sau* 3AI
Taurine cattle	604, 435, 169	604	822, 500, 322	809, 552, 257	822
Zebu	604, 435, 169	604	822, 500, 322	809, 552, 257	822, 741, 81
Banteng	604, 435, 169	604, 472, 132	822, 500, 322	822, 553, 269	822
Gaur, gayal	604, 435, 169	604, 472, 132	822, 500, 322	822, 553, 269	822
Yak	604, 435, 169	604, 529, 467, 137, 75, 63	822	822	822
Bison	604, 435, 169	604, 529, 467, 137, 75, 63	822, 500, 322	822	822
Wisent	604, 435, 169	604, 529, 467, 137, 75, 63	822, 500, 322	822	822

[a] Fragments that are underlined are the most intense.

the cytochrome *b* amplicon used in our assay. The *Alu*I site is conserved in all bovine species (Table 3). As reported previously (Meyer et al., 1995), a *Hin*fI site discriminates cattle from water buffalo. However, this site is lacking in other bovine species that are used as source of beef. For a complete discrimination of bovine species, other sites were identified in the alignments (Table 3). All cleavage patterns predicted from the sequence were checked experimentally.

By combining diagnostic restriction sites of two mitochondrial genes, the mitochondrial PCR-RFLP assay gives a positive identification of all bovine species via the presence of a restriction site, except that taurine cattle and zebu are not differentiated. In all cases except gayal versus banteng and gayal versus wisent the species identification can be based on two mutations.

Recently, a PCR-RFLP test on the mitochondrial 12S RNA was described that differentiated taurine cattle and Asian zebu (Meirelles et al., 2001). However, African zebu breeds emerged by introgression and have retained mitochondrial DNA of the taurine type.

3.2. Satellite DNA

A drawback inherent to any mitochondrial assay is that in cases of species hybridization only the maternal lineage is identified. Therefore, a confirmatory assay is needed that is based on nuclear DNA. Satellite DNA consists of centromeric tandem repeats and occupies up to 20% of the cattle genome (Jobse, Buntjer, Haagsma, Breukelman, Beintema, & Lenstra, 1995; Nijman & Lenstra, 2001; Skowronski, Pluciennicczak, Bednarek, &

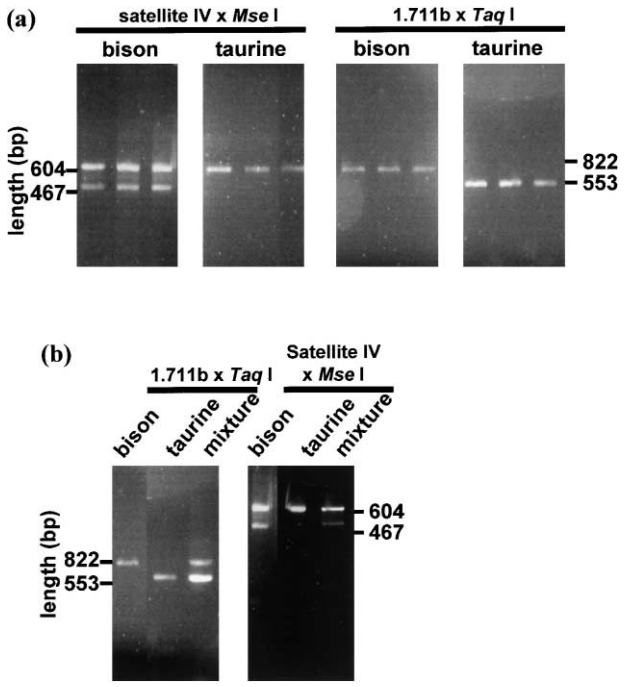

Fig. 1. (a) SFLP patterns of bison and different breeds of taurine cattle (from left to right; Holstein-Friesian, Meuse-Rhine-Yssel and Jersey, respectively). (b) Analysis of bison and taurine samples and 1:1 mixtures of both. Fragments of satellite DNA were amplified and cleaved with the indicated restriction enzymes.

Jaworski, 1984). By the process of concerted evolution, the sequences become species-specific (Elder & Turner, 1995). The bovine species have similar satellites, but the frequencies of sequence variants is variable, which can be detected by satellite fragment length polymorphism assays (Table 4: SFLP Nijman et al., 1999). As an example, Fig. 1a shows a differentiation of bisons (three animals) and cattle (three animals from different breeds) by SFLP. A sequence variant of satellite IV with a *Mse*I site is clearly more frequent in bison than cattle, while a *Taq*I variant of the same satellite predominates in cattle. So far, we have never observed intraspecies variation of SFLP patterns (Nijman et al., 1999). Both the bison and cattle satellites are also amplified in a mixture of DNA from both species (Fig. 1b).

Fig. 2 shows that SFLP patterns of a commercially obtained bison beef sample is identical to the patterns of purified bison DNA and confirms the mitochondrial *Stu*I PCR-RFLP. However, about 6% of the American bison populations carries mitochondria of taurine origin (Ward et al., 1999). For these animals a mitochondrial assay will be misleading, while satellite-based assays would still indicate the predominantly bison origin of the nuclear genome.

Another case of bovine species hybridization is shown in Fig. 3. Bali cattle is considered to be domesticated banteng, which is apparent from the satellite IV *Mse*I pattern. However, the Hin*fI* and *Taq*I digests of cytochrome *b* amplicons clearly show zebu-specific patterns,

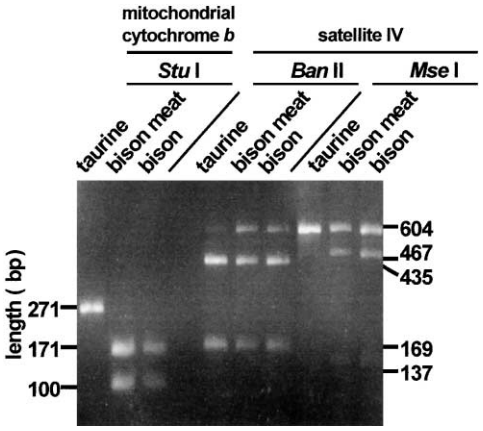

Fig. 2. Mitochondrial PCR-RFLP and SFLP patterns with the indicated restriction sites of commercially obtained bison meat sample compared with the ox and bison reference samples.

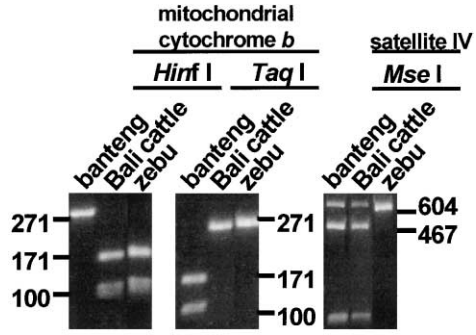

Fig. 3. Mitochondrial PCR-RFLP and SFLP with the individual restriction sites of a Bali cattle individual compared with banteng and zebu reference samples.

indicating that this Bali cattle individual is a hybrid of banteng and zebu. Nijman et al.(1999) has used a *Sau*3AI-1711b SFLP assay to analyse the various degrees of taurine-indicine (zebu) hybridization in African cattle breeds.

The SFLP test relies on the simultaneous amplification of several, slightly different repeated elements and is based on semi-quantative rather than absolute differences in the occurrence of restriction sites. Consequently, it has not been designed for the detection of admixtures or analysis of degraded DNA and is most useful for unprocessed samples that originate from a single animal.

We note that also transplantation of embryos from the endangered gaur species in taurine surrogate mother cows (Lanza, Dresser, & Damiani, 2000) results in animals for which the species origin can only be verified by analysis of nuclear DNA like the SFLP.

4. Conclusion

All bovine species, can be identified by convenient, sensitive and versatile PCR-RFLP assays. This may

serve as follow-up if an *Alu*I digestion (Meyer et al., 1995) indicates the presence of bovine material, but does not differentiate between bovine species. We propose the following recommendations in order to ensure validity of the assays described in this paper. Firstly, all relevant reference animals should be tested in parallel. Secondly, the differentiation should be based on at least two different restriction enzyme sites in order to exclude intraspecies polymorphism. Thirdly, at least one SFLP assay should be used if species hybridization has to be excluded. These considerations may be of general relevance for the discrimination of other closely related species.

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