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Paternally inherited markers in bovine hybrid populations

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The genetic integrity of crossfertile bovine- or cattle-like species may be endangered by species hybridization. Previously, amplified fragment length polymorphism, satellite fragment length polymorphism and microsatellite assays have been used to analyze the species composition of nuclear DNA in taurine cattle, zebu, banteng and bison populations, while mitochondrial DNA reveals the origin of the maternal lineages. Here, we describe species-specific markers of the paternally transmitted Y-chromosome for the direct detection of male-mediated introgression. Convenient PCR–restriction fragment length polymorphism and competitive PCR assays are shown to differentiate the Y-chromosomes of taurine cattle, American bison and European bison, and to detect the banteng origin of Indonesian Madura and Bali cattle bulls.
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Introduction

Domestication of several bovine species has made the tribe of the *Bovini* one of the numerically most important mammalian taxa with 11 extinct species: ox (*Bos taurus*), zebu (*Bos indicus*), gayal (*Bos frontalis*), gaur (*Bos gaurus*), banteng (*Bos javanicus*), bison (*Bison bison*), wisent (*Bison bonasus*) or European bison, yak (*Bos grunniens*), water buffalo (*Bubalus bubalis*), African buffalo (*Syncerus caffer*) and anoa (*Bubalus depressicornis*) (Lenstra and Bradley, 1999). Speciation of the *Bos* and *Bison* species is incomplete and hybridization of these species occurs worldwide (Felius, 1985; Bongso *et al*, 1988; Lenstra and Bradley, 1999; Nijman *et al*, 1999; Ward *et al*, 1999; Kikkawa *et al*, 2003). Female progeny as well as the male taurine–zebu, gaur–gayal and bison–wisent hybrids are fertile, while fertility of other male hybrid offspring can be restored by repeated backcrossings. Introgression in the wild species may compromise their genetic integrity. Conversely, organized crossing of wild bovines in domestic populations may create animals or even new breeds with unique properties. In Africa, the introgression of Indian zebu bulls in taurine herds has occurred (Bradley *et al*, 1996). The Chinese yakow is a yak–ox hybrid, which is held at altitudes between the habitat ranges of the parent species (Felius, 1985). The South-East Asian Selembu dairy and beef cattle result from gayal–zebu crossings (Felius, 1985). Taurine mitochondria have been found in wild North American bison populations (Ward *et al*, 1999), while the beefalo may be regarded as a domestic hybrid breed of bison and taurine cattle.

Several types of molecular markers have been used for the detection of bovine species hybridization. Mitochondrial DNA is informative as a marker of the maternal lineage (Bradley *et al*, 1996; Janecek *et al*, 1996; Ward *et al*, 1999; Verkaar *et al*, 2002). Amplified fragment length polymorphisms (AFLP), satellite fragment length polymorphisms (SFLP) and species-specific autosomal microsatellite alleles may serve as autosomal markers (Nijman and Lenstra, 2001; Buntjer *et al*, 2002). Y-chromosomal markers are especially relevant because hybridization in species that live in herds occurs mostly via male introgressions. As a consequence, Y-chromosomal alleles of the male lineage may have a broader geographical range than alleles informative for the female mitochondrial lineage (Van Hooft, 2001). However, few Y-chromosomal markers are available (Edwards *et al*, 2000; Liu *et al*, 2002; Kikkawa *et al*, 2003). A btDYZ-1/*Hae*III restriction fragment length polymorphism (RFLP) and the Y-chromosomal microsatellites, INRA124 and INRA008, have been used in studies on the African bovine populations (Bradley *et al*, 1994; Hanotte *et al*, 2000; Van Hooft *et al*, 2002). Ward *et al* (2001) described bison-specific alleles of the Y-chromosomal microsatellite BYM1.

Here, we describe assays based on mutations in *SRY* (sex-determining region Y-chromosome) and in the multicopy *TSPY* (testis-specific protein, Y encoded, Jakubiczka *et al*, 1993; Vogel *et al*, 1997). The detection of these mutations by convenient competitive PCR or RFLP) analysis has been applied for the analysis of bulls from American bison populations in Belgium and to the

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paternal origins of Indonesian Bali and Madura cattle, respectively.

Material and methods

Blood samples

Samples from American bison (*Bison bison*) were obtained from Artis Zoo, Amsterdam or La Ferme des Bisons, Recogne, Belgium. Wisent (*Bison bonasus*) and yak *(Bos grunniens* samples were obtained from Artis Zoo, banteng (*Bos javanicus*) from Blijdorp Zoo, Rotterdam, and taurine cattle (*Bos taurus*) were obtained from the Faculty of Veterinary Medicine, Utrecht. Blood samples from five Bali cattle (*Bos javanicus*) bulls and two Madura cattle (*Bos indicus*) bulls were collected at breeding stations (Malang, Indonesia). Two samples from zebu (*Bos indicus*) bulls were donated by Dr DG Bradley (Trinity College, Dublin) and a sample from a beefalo bull by Dr B Morris (Stormont Laboratories, Woodland, USA). DNA was isolated from the whole blood by using standard SDS/proteinase K extraction (Sambrook *et al*, 1989), the GuITC protocol (Ciulla *et al*, 1988) or the Qiagen blood extraction method (Qiagen).

SRY and TSPY typing

The typings have been developed on the basis of recently submitted *SRY* nucleotide data from banteng (AY079146), bison (AY079141) and wisent (AY079142), respectively (unpublished results). The numbering of primer binding sites and restriction sites refer to the Genbank entry AB039748 for bovine *SRY* (Kato *et al*, 1995).

For differentiation of bison and cattle Y-chromosomes, PCR was performed in a total volume of 25 μl, containing 50 ng genomic DNA and 50 ng of primers SRY-1 (5′-GTT GAT GGG TTT GGG CTG ACT) and SRY-3 (5′-AAA TTG AGA TAA AGA GCG CCT) in *Taq* DNA polymerase buffer with 1.5 mM $MgCl_2$, 0.2 mM dNTP and 1.25 U *Taq* DNA polymerase (Promega, Madison, WI, USA). The program consisted of an initial denaturation of 2 min at 95°C followed by 30 cycles of 15 s at 92°C, 30 s at 60°C and 45 s at 72°C, and by a final extension of 5 min at 72°C. PCR products were fractionated on a 1% agarose/ 0.5 × TBE gel (SphearoQ, Leiden, The Netherlands), excised and extracted with the QiaexII gel extraction kit (QIAGEN Inc., Valencia, CA, USA). A measure of 5 μl of the PCR product was digested by the addition of 5–10 U of *Btr*I (New England Biolabs, Beverley, MA, USA) and the recommended concentrated reaction buffer. The samples were digested for 3 h at 37°C and fractionated on a 2% agarose/0.5 × TBE gel.

For differentiation of bison and wisent, a competitive PCR was performed as the normal PCR (see above) with primer SRY-3 (50 ng), SRY-1 (10 ng) and an internal primer SRY-bison (5′-ACA GCA ACA AAC TAC TCT CT, 40 ng). The nucleotide of this primer at the 3′-end of SRY-bison only matches the sequence of the American bison.

For detection of the banteng Y-chromosome, PCR was performed as described above with 50 ng of primers SRY-4 (5′-GCC TGG ACT TTC TTG TGC TTA) and SRY-5 (5′-ACA GTG GGA ACA AAA GAC TAT). After purification (see above), 5 μl of the PCR product was digested by the addition of 5–10 U of *Bfa*I (New England Biolabs) and the recommended concentrated reaction buffer. The samples were digested for 1.5 h at 37°C and fractionated on a 2.5% agarose/0.5 × TBE gel. Sequencing was performed with 200 ng PCR product and a Cy5 Big Dye terminator kit (Applied Biosystems) on an ABI Prism 310 Sequencer.

Amplification of the *TSPY* multicopy genes was performed as described above with 50 ng of primers TSPY-L (5′-TCA TCA GCG AAG ACG TGT GGG, positions 2172–2192 of Genbank entry U75895, Vogel *et al*, 1997) and 50 ng of TSPY-R (5′-AAG TGG CGA GAG CGT GTT TTG G, positions 3188–3123). For differentiation of cattle and zebu versus bison, wisent and yak, 5 μl of the purified PCR product was digested by the addition of 2–5 U of *Rsa*I (Promega) and the recommended concentrated reaction buffer. The samples were digested for 1 h at 37°C and fractionated on a 1% agarose/ 0.5 × TBE gel.

Results

Differentiation of cattle and bison

Previously, it has been demonstrated that at least 6% of American bison carry mitochondria from taurine cattle (Polziehn *et al*, 1995; Ward *et al*, 1999; Minard, 2003). In a population of imported bison in Belgium, we found by *Xba*I and *Hin*fI PCR–RFLP analysis (Verkaar *et al*, 2002), mitochondria of taurine origin in two out of 72 bison (data not shown). In contrast, no taurine-specific alleles of the Y-chromosomal BYM1 microsatellite were found in American bison (Ward *et al*, 2001). However, the hybrid beefalo cattle are bred with bulls from both species.

We designed a convenient method for differentiation of the bison and taurine Y-chromosomes by PCR–RFLP analysis of a C→T mutation on position 1225 (numbering of AB039748), which has led to the loss of a *Btr*I site in the *SRY* gene from American bison and wisent, but not in *SRY* from taurine cattle (Figure 1). *Btr*I analysis of a beefalo bull available for analysis suggested that this animal descends from a taurine or zebu bull (Figure 1).

An alternative Y-chromosomal PCR–RFLP assay may be based on a G→A mutation on position 2916 (Genbank entry U75895, Vogel *et al*, 1997) of the multicopy *TSPY* gene. This mutation abolishes an *Rsa*I site and distinguishes American bison, wisent and yak (fragments of 36, 112 and 870 bp) from taurine cattle and zebu (fragments of 36, 112, 271 and 599 bp; Figure 1).

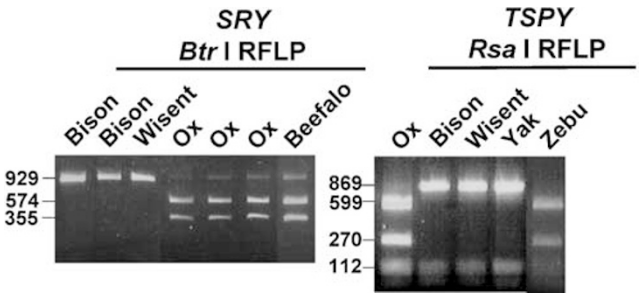

Figure 1 Differentiation of bison and bovine Y-chromosomes by PCR–RFLP analysis of *SRY* and *TSPY* from the indicated species.

Differentiation of American bison and wisent

The wisent was almost extinct in 1922 (Felius, 1985; Lenstra and Bradley, 1999). Essential in the revival program, which started with 22 bulls and 22 cows, was the protection against the introgression of American bison. The Y-chromosomes from bison and wisent can be distinguished by a G→T mutation at position 1130 of *SRY* (Genbank entry AB039748). Since this did not create or abolish a restriction site, we designed a competitive PCR in which the forward primer competes with a third, bison-specific primer (SRY-bison) with the diagnostic mutation at its 3′-end. By varying the primer concentration, we found that an unambiguous differentiation of bison and wisent was obtained by a 1:4:5 ratio of the concentrations of the forward primer, internal forward primer and reverse primer, respectively. This generates the full-length product (SRY-1 to SRY-3, 929 bp) with wisent and a predominant shorter product (SRY-3 to SRY-bison, 689 bp) with bison. Competitive PCR with several samples of taurine cattle (3), yak (4), wisent (3) and American bison (6) yielded the 689 bp fragment only with bison (results for American bison and wisent are shown in Figure 2).

We used this assay in combination with a mitochondrial test on the maternal descendance (Verkaar *et al*, 2002) for an analysis of three bulls from a privately held bison herd in Halle, Belgium. As evident from Figure 2a, c and d, bison 1 descends paternally and maternally from bison, bison 2 has a maternal wisent and a paternal bison origin, respectively, and bison 3 descends both paternally and maternally from wisent. This suggests that, in contrast to the differential taurine introgression in bison (Ward *et al*, 2001), crossbreeding in mixed populations of American bison and wisents is symmetrical.

Differentiation of zebu and banteng

The predominant cattle species in Indonesia is the zebu (*Bos indicus*), which is reported to carry mitochondrial DNA of the Banteng type (Kikkawa *et al*, 2003). Bali cattle descend from the wild banteng (*Bos javanicus*), although zebu introgression has been suspected (Namikawa, 1981). Bali cattle bred outside their region of origin are clearly of hybrid origin, as shown by the analysis of mitochondrial DNA, AFLP, SFLP and microsatellite analysis of Malaysian Bali cattle (Nijman *et al*, 2003). Madura cattle has the typical zebu hump, but

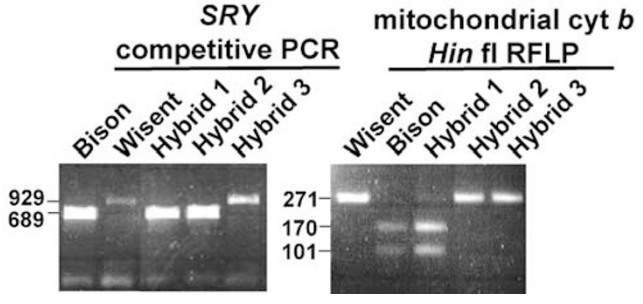

Figure 2 Differentiation of bison and wisent Y-chromosomes and mitochondrial DNA and analysis of three individuals (hybrids 1, 2 and 3, respectively) from a Belgian mixed wisent–bison population by competitive PCR of *SRY* and PCR–RFLP of the mitochondrial cytochrome *b* gene (Verkaar *et al* 2002; Nijman *et al*, 2003).

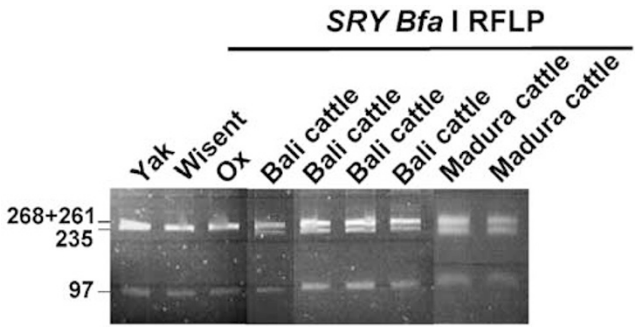

Figure 3 *Bfa*I PCR–RFLP of *SRY* from the indicated bovine species, Bali cattle (four animals) and Madura cattle (two animals), respectively. In separate experiments, it was checked that the nucleotide sequences as well as the corresponding *Bfa*I cleavage patterns of banteng and Bali cattle were identical and different from other bovine species.

is also supposed to result from zebu–banteng hybridization (Payne and Rollinson, 1976). The hybrid origin of the Madura nuclear genome has been verified by SFLP and AFLP analyses, while the mitochondrial DNA was either of zebu or banteng origin (Nijman *et al*, 2003).

However, no data are available yet about parental lineages in Bali and Madura cattle. The Y-chromosome from banteng can be detected via an A→G mutation in the *SRY* gene at position 2018 of database entry AB039748, which creates an extra *Bfa*I site. PCR–RFLP with *Bfa*I and bovini other than banteng yield fragments of 268, 261 and 97 bp, respectively, while in banteng the 261 bp fragment is cleaved in a 235 and a 26 bp fragment (representative results are shown in Figure 3).

We analyzed five Bali bulls from breeding farms with the *Bfa*I PCR–RFLP Y-chromosomal test. As shown in Figure 3 for four of the animals, the Bali cattle bulls appeared to descend from banteng bulls. In addition, a mitochondrial PCR–RFLP test (Verkaar *et al*, 2002, Nijman *et al*, 2003) also showed that the maternal lineage is also of banteng origin. Interestingly, the two Madura bulls that were available for analysis also carried banteng Y-chromosomes (Figure 1).

These results suggest that in combination with typing of nuclear and mitochondrial DNA (Verkaar *et al*, 2002; Nijman *et al*, 2003), Y-chromosomal assays are a useful tool for the conservation of the original Indonesian cattle breeds.

Discussion

In this paper, we have used sequence variation in the Y-chromosomal *SRY* gene for the design of convenient tests of paternal lineages in bovine populations (Table 1). *SRY* is the most obvious target for these assays, since it is the only single-copy gene without an X-chromosomal homologue. As shown in Figure 1, the concerted evolution of the multicopy *TSPY* gene allows an alternative assay for the origin of paternal lineages (Tosi *et al*, 2000). In addition to the tests described in this paper, similar tests can be designed for the detection of taurine or yak Y-chromosomes (Table 1). The differentiation of taurine and indicine Y-chromosomes by a *Tru*91 digestion would provide a convenient way of tracing the paternal origin of cattle

Table 1 Diagnostic restriction sites in the bovine Y-chromosomal *SRY* gene

Position	*Restriction enzyme*	*Bovine species identified via PCR–RFLP*	
		(in the presence of site)	*(in the absence of site)*
1009	*Mae*I	Yak	
1225	*Btr*I		Bison, wisent
1708	*Tru*91		Ox
1843	*Mae*I	Yak	
2016	*Bfa*I	Banteng	

The numbering refers to Genbank entry AB039748 of the taurine *SRY* (Kako *et al*, 1995). The numbers in bold indicate sites used in this study.

populations (Bradley *et al*, 1994; Hanotte *et al*, 2000; Kikkawa *et al*, 2003).

Previously described tests were based on Y-chromosomal microsatellites. However, this requires radiochemical or fluorescent labels as well as sequencing equipment. Furthermore, microsatellites are only informative for the species origin if species-specific alleles have been fixed (Edwards *et al*, 2000; Vila *et al*, 2003). It may be worth noting that wisent did not have the short bison-like alleles of the BYM1 marker, illustrating the dynamic evolution of microsatellites (results not shown). In contrast, the homogeneity within bovine species and the phylogeny of bovine Y-chromosomal variants (this work and unpublished results) indicate that the sequence variants of *SRY* are more stable and that the segregation of species-specific Y-chromosomal variants has been complete. However, the mutation in the *SRY* gene of yak on position 1292 observed by Kikkawa *et al* (2003) was not observed in the yak bulls sampled by us (unpublished results). If more single-copy Y-chromosomal sequences are becoming available (Hellborg and Ellegren, 2003), assignments can be based on more than one mutation as a safeguard against intraspecies polymorphism.

Several methods are available for the detection of the *SRY* point mutations. PCR–RFLP and competitive PCR require modest equipment and are suitable for low-throughput implementations in zoos and wildlife-management institutes. With appropriate controls it can be checked if the enzymatic digestion has been successful.

Uniparentally inherited markers may detect the origin of a population even if many generations of breeding has obscured the original species composition (Bradley *et al*, 1994; Ward *et al*, 2001). Since the female calves normally stay in the herd where they were born, the mitochondrial DNA sequence reveals the species origin of the herd. In mammalian species, females are often philopatric, while the males cover a broader geographic range (Lyrholm *et al*, 1999; Wang and Schreiber, 2001). So, Y-chromosomal markers may be expected to have another geographical distribution than the mitochondrial markers. In addition, paternally inherited markers may reveal male introgression via contacts of the herd with species from the neighboring habitats. If introgression has been systematic, for instance upgrading of a breed, Y-chromosomal markers will correlate with the phenotypic variation and autosomal markers.

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