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Hybridization of banteng (*Bos javanicus*) and zebu (*Bos indicus*) revealed by mitochondrial DNA, satellite DNA, AFLP and microsatellites

IJ Nijman[1,2], M Otsen[1], ELC Verkaar[1], C de Ruijter[1], E Hanekamp[1], JW Ochieng[3], S Shamshad[4], JEO Rege[5], O Hanotte[3], MW Barwegen[6], T Sulawati[7] and JA Lenstra[5]

[1]*Institute of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 1, 3584 CL Utrecht, The Netherlands;* [2]*Institute of Equine Sciences, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 1, 3584 CL Utrecht, The Netherlands;* [3]*International Livestock Research Institute, P.O. Box 30709, Nairobi, Kenya;* [4]*National Institute of Animal Biotechnology, Jerantut 27000, Pahang D.M., Malaysia;* [5]*International Livestock Research Institute, P.O. Box 5689, Addis Ababa, Ethiopia;* [6]*Royal Institute of Linguistics and Anthropology, Reuvensplaats 2, 2300 RA Leiden, The Netherlands;* [7]*Animal Reproduction Laboratory, Animal Husbandry Faculty, Brawijaya University, Malang, Indonesia*

Hybridization between wild and domestic bovine species occurs worldwide either spontaneously or by organized crossing. We have analysed hybridization of banteng *(Bos javanicus*) and zebu (*Bos indicus*) in south-east Asian cattle using mitochondrial DNA (PCR-RFLP and sequencing), AFLP, satellite fragment length polymorphisms (SFLP or PCR-RFLP of satellite DNA) and microsatellite genotyping. The Indonesian Madura zebu breed is reputed to be of hybrid zebu–banteng origin, but this has never been documented and Bali cattle are considered to be a domesticated form of banteng. The banteng mitochondrial type was found in all animals sampled on the isle of Bali, Indonesia, but only in 35% of the animals from a Malaysian Bali-cattle population. The Madura animals also carried mitochondrial DNA of either zebu and banteng origin. In both populations, zebu introgression was confirmed by AFLP and SFLP. Microsatellite analysis of the Malaysian Bali population revealed for 12 out of 15 loci screened, Bali-cattle-specific alleles, several of which were also found in wild banteng animals. The tools we have described are suitable for the detection of species in introgression studies, which are essential for the genetic description of local breeds and the preservation of their economic and cultural value.
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Introduction

Hybridization between species may occur if closely related species share an overlapping habitat or through human intervention during captive breeding. Hybrid offspring may combine desired properties of the parental species, but fertility is often restricted to the homogametic sex (Forsdyke, 2000). This can be overcome by repeated backcrossing to animals from one of the parent species until a viable hybrid population is established. Uncontrolled hybridization, as it occurs in nature, may have had a significant impact on the formation of domestic breeds, but can also affect the genetic integrity of domestic and wild species. Monitoring the species composition of these animals may become essential for the future preservation of genetic diversity.

Within the group of the *Bovini*, comprising cattle-like species, several hybrids have been described: yakows (Felius, 1995; Tumennasan *et al*, 1997), a cross of yak (*Bos grunniens*) and taurine cattle; the selembu, a cross of gayal (*Bos gaurus*) and zebu or the North-American beefalo, a cross of bison (*Bison bison*) and taurine cattle. In Africa, introgression of Indian zebu bulls (*Bos indicus*) in taurine herds occurs and has improved the tolerance of the cattle (*Bos taurus*) to hot and dry environments (Epstein, 1971; Bradley *et al*, 1994, 1996; Loftus *et al*, 1994b; Frisch *et al*, 1997; Hanotte *et al*, 2000). Bongso *et al* (1988) described a spontaneous hybridization of wild gaur and zebu.

The domestication of the banteng (*Bos banteng*) is likely to have occurred either in Java or on mainland southeast Asia, and later the domesticated variety was spread and bred (Rollinson, 1984). Bali cattle are a domesticated form of banteng and are kept throughout Indonesia and Malaysia. This breed is of considerable economic importance since it is adapted to poor-quality fodder, yields good-quality meat and provides draft power. Their small posture makes them particularly suitable for training and work in small- and irregular-shaped fields (Rollinson, 1984). Although the presence of zebu cattle on the island of Bali has been forbidden since 1913 (Felius, 1995), biochemical polymorphisms suggested that zebu and possibly taurine introgression might have occurred (Namikawa, 1981). Furthermore, Bali cattle may have been crossbred to zebu animals outside their region

Correspondence: IJ Nijman, Faculty of Veterinary Medicine, Utrecht University Yalelaan 1, 3584 CL Utrecht, The Netherlands.
E-mail: I.Nijman@vet.uu.nl
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of origin. The Madura breed, which on the island of Madura is used for the traditional bull racing competition (*kerapan sapi*, Felius, 1995), is believed to be a cross between wild banteng or Bali cattle and zebu, originating approximately 1500 years ago (Payne and Rollinson, 1976), but this has never been documented. Hybridization of banteng and zebu is supposed to have occurred in other Indonesian breeds, either spontaneously or via breeding (Epstein, 1971; Felius, 1995). Zebu introgression is considered as a threat of the genetic purity of the endangered wild banteng (Epstein, 1971).

However, the supposed zebu–banteng hybridization has never been verified by genome analysis. Several DNA markers are now available to analyse the genetic background of hybrid *Bovini*. Mitochondrial DNA (mtDNA) markers reveal introgression via the maternal lineage (Loftus *et al*, 1994a; Ward *et al*, 1999; Verkaar *et al*, 2003). The species origin of the nuclear genome can be inferred from species-specific microsatellite alleles (Frisch *et al*, 1997; MacHugh *et al*, 1997), AFLP patterns (Vos *et al*, 1995; Savelkoul *et al*, 1999; Buntjer *et al*, 2002) or mutations in satellite DNA. Previously, we showed that satellite fragment length polymorphism (SFLP), as well as amplified fragment length polymorphisms (AFLP) correlated with introgression of zebu (*Bos indicus*) in African cattle populations (Nijman *et al*, 1999). In this paper, we demonstrate the use of mitochondrial D-loop and cytochrome-*b* typing, AFLP, SFLP and microsatellite genotyping in order to detect zebu–banteng hybridization in Madura and Malaysian Bali cattle.

Materials and methods

DNA samples

Tissue (skin) and six blood samples from Madura cattle were collected at local slaughterhouses on Madura, Indonesia and preserved in preservation buffer (4.5 M NaCl, 25% DMSO) until DNA purification with SDS, proteinase K and phenol. A mixed blood sample of three Madura bulls was obtained at the scene of the bull racing with permission of the owners. Blood samples of 17 Bali cattle were taken from animals in the south and east of the Malaysian peninsula and from 11 individual Bali cattle of the Indonesian isle of Bali. DNA was isolated from the whole blood using standard SDS/proteinase K methods and phenol/chloroform extractions (Sambrook *et al*, 1989). Dr DG Bradley (Dublin, Ireland) kindly donated two zebu DNA samples (Indian Sahiwal breed, pure *Bos indicus*). DNA from four bantengs was isolated from liver tissue obtained from Blijdorp zoo (Rotterdam, The Netherlands) as described above. Microsatellite genotypes were compared with data from three Indian zebu breeds (Nelore, Ongole and Sahiwal, $n = 97$) and three European taurine breeds (Jersey, Friesian and Charolais, $n = 103$).

Analysis of mtDNA

A fragment of the mitochondrial D-loop was amplified from 50 ng template DNA with the primers D-loop-L (5′-AAAAATCCCAATAACTAACACAG-3′; positions 15 848–15 870) and D-loop-R (5′-TACAATAGATGC-TCCGGGTCAG-3′; 126–105), both derived from bovine mtDNA (Genbank J01394) in a standard PCR reaction (50ng primers, 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 1.25 U *Taq* polymerase (Promega) with the following programme: predenaturation for 2 min at 92°C, 30 cycles of 15 s at 92°C, 45 s at 52°C and 45 s at 72°C). Products were separated in 1% agarose gels in 1 × TAE, pH 8. The excised fragments were purified with the QIAquick system (Qiagen, USA) and inserted in the pGem-T Easy vector (Promega, USA) by following the protocols of the manufacturers. Clones were purified with the Qiagen Midipep kit and sequenced using the Cy5-labelled T7 and SP6 primers and an ALF automatic sequencer (Amersham-Pharmacia Biotech). For each species, sequences were based on the analysis of two independent clones and aligned by the program Bioedit (Hall, 1999). For comparison, a D-loop sequence from zebu (Genbank AF13114610) and one from taurine cattle (AF3114579) were retrieved from the database. The banteng, Bali and Madura sequences have been submitted to Genbank (accession codes AF162486-AF162490). Amplification of the mitochondrial cytochrome *b* gene and diagnostic digestions with *Hin*f I (present in zebu, absent in banteng) and *Taq* I (absent in zebu, present in banteng) has been performed as described previously (Meyer *et al*, 1995; Verkaar *et al*, 2002).

PCR-RFLP on satellite DNA

Fragments from bovine satellite IV DNA were amplified as described previously (Jobse *et al*, 1995; Nijman and Lenstra, 2001). One-fifth of the PCR product was digested twice with 5–10 U of *Ban* II (at 37°C), *Mse* I (at 37°C) or the *Mse* I isoschizomer *Tru*9 (at 65°C) for 3 h and fractionated on a 2% agarose gel. As a second digestion did not alter the restriction patterns, we considered the digestion to be complete. DNA was transferred onto a nylon membrane (Amersham) by standard Southern blotting protocols and fixed with UV radiation. After prehybridization for 1 h, filters were hybridized to ^{32}P-labelled PCR product of *Bos indicus* and washed twice for 10 min in 1 × SSPE and 0.1% SDS, all steps being carried out at 55°C. Hybridization was detected by autoradiography on a Molecular Dynamics 400 phosphorimager set at 176 μm and 680 V. Quantitative hybridization signals were measured with the Imagequant software (Molecular Dynamics, version 3.22) and results calculated relative to the total signal (amount of DNA) in the gel lane.

AFLP, scoring and interpretation

AFLP analyses were basically carried out as described (Vos *et al*, 1995). Briefly, 250 ng DNA of two banteng, two zebu, three Bali and one Madura animal was digested with *Taq* I and *EcoR* I (Promega) successively in the buffer supplied by the manufacturer. *Taq* I and *EcoR* I adapters (Ajmone-Marsan *et al*, 1997) were ligated to the fragment ends and after preamplification with E01L and T01, separate AFLP reactions were performed using the primer combinations: E31/T39, E34/T45 and E31/T42 (Table 1). The primers E31 and E34 were end-labelled by using T4 polynucleotide kinase and [γ-^{33}P-ATP] and AFLP products were separated on 5% polyacrylamide gels. Bands were scored as dominant markers using the program Cross Checker (Buntjer and Otsen, 2000) to generate 1(presence)/0(absence) matrices. These data sets were analysed by the program NTSYS (Rohlf, 1993). Jaccard similarity values (number of shared bands divided by the total number of bands in either species)

Table 1 Nucleotide sequences of AFLP primers

Name	*Sequence (5′–3′)*
E01L	AGACTGCGTACCAATTCA
T01	GATGAGTCCTGACCGAA
E32	GACTGCGTACCAATTCAAC
E35	GACTGCGTACCAATTCACA
T32	GATGAGTCCTCACCGAAAC

were calculated and visualized by principal coordinate analysis (PCOORD, Jackson, 1991).

Microsatellite analysis

Fifteen autosomal microsatellites were genotyped including nine microsatellites (*ILSTS005*, *ILSTS006*, *ILSTS008*, *ILSTS023*, *ILSTS028*, *ILSTS033*, *ILSTS036*, *ILSTS50* and *ILSTS103*) that were originally isolated from N′Dama taurine cattle (Kemp *et al*, 1995), and six microsatellites that are commercially available (*TGLA122*, *TGLA126*, *TGLA227*, *MGTG4B*, *AGLA293* and *TGLA48* Applied Biosystems StockMark Kit 1). Analyses were carried out as described (Hanotte *et al*, 2002).

Results

Mitochondrial DNA analysis

Figure 1 shows an alignment of the D-loop sequences obtained from a Madura cow, one Malaysian Bali individual and one banteng with taurine and zebu sequences for reference. The banteng sequence is clearly distinct from the zebu and taurine sequences with several point mutations, deletions, insertions and an expanded repeat-like structure (510–550 bp, see Figure 1). The Bali sequence is nearly identical (99.5%) to the zebu sequence, while the Madura sequence is nearly identical (99.7%) to banteng. However, PCR-RFLP analysis of the other samples revealed in both breeds the mitochondrial type of either banteng or zebu. From the Madura breed, four animals carried the banteng mtDNA type and three the zebu variant while in a mixed blood sample from three Madura bulls, both zebu and banteng cytochrome *b* variants were detected (not shown). Analysis of Malaysian Bali samples revealed that 11 out of 17 samples were from maternal zebu origin (Figure 4b and c), while no zebu patterns were found in the 11 animals sampled on the isle of Bali (not shown).

Satellite DNA polymorphisms

Sequence analysis of satellite IV DNA (Nijman and Lenstra, 2001) revealed a heterogeneity (TTMA; M = C or A) that creates an *Mse* I site (TTAA) in part of the repeat units of banteng, while the zebu sequence (TTCA) on the same position is almost homogeneous. Conversely, a GAGCTC *Ban* II site in zebu is replaced by GAGCTM in banteng. This allows an estimation of the species composition by SFLP or RFLP analysis of satellite DNA (Nijman *et al*, 1999; Nijman and Lenstra, 2001). The SFLP patterns of both Madura samples are clearly intermediate between the zebu and banteng patterns: *Mse* I digests (Figure 2a) reveal the presence of banteng-like repeat units, while *Ban* II digests (Figure 2c) show that the zebu-like units are also present. The patterns of two Bali cattle are different, but again both restriction enzymes generate intermediate patterns, suggesting the presence of both zebu and banteng satellite DNA in Madura and Bali cattle. To quantify the presence of the satellite units of zebu and banteng, band intensities were measured by transferring the DNA to nylon membrane and hybridizing to a satellite IV probe. The intensities of the smaller restriction fragments, indicative of the specific restriction site, were measured relative to the total amount of DNA (Figure 2b and d). Both the *Mse* I and *Ban* II digestions indicate that Madura is mostly like zebu, but apparently contains a banteng component, while Bali cattle has a smaller zebu component. Analysis of more Malaysian Bali samples by SFLP with *Mse* I (Figure 4a) and *Ban* II (not shown) confirmed a variable degree of zebu introgression, which, however, did not correlate strictly with the origin of the mitochondrial DNA (Figure 4b,c).

AFLP

AFLP similarities were based on 120 variable markers and were condensed in a PCO plot (Figure 3). Although only one Madura sample was suitable for AFLP analysis, Madura, as well as Bali cattle, have PCO coordinates intermediate between zebu and banteng with Bali cattle closer to the banteng position (Figure 3). The difference between the Bali individuals, as observed by SFLP, is also apparent from the AFLP data.

Microsatellite analysis

Malaysian Bali individuals were also analysed with 15 microsatellites and the allele frequencies were compared with the frequencies in a group of European taurine, banteng and zebu cattle (data available on request). The microsatellites were highly polymorphic in all breeds (number of detected alleles ranging from 5 -*TGLA48*- to 21 -*TGLA122*), with a few exceptions: *ILSTS008* and *MGTB4b* were monomorphic in the banteng samples only, while *TGLA227* was monomorphic in banteng but showed low polymorphism in zebu. Although the banteng sample size was probably insufficient to detect the full range of alleles, Malaysian Bali specific alleles were obtained for nine loci (*AGLA293*, *ILSTS005*, *ILSTS023*, *ILSTS028*, *ILSTS050*, *ILSTS103*, *MGTG4b*, *TGLA048* and *TGLA227*). Predominant Bali alleles are mostly shorter (*AGLA293*, *ILSTS005*, *ILSTS008*, *ILSTS023*, *MGTG4b*, *TGLA126* and *TGLA227*), while some are longer (*TGLA48*, *ILSTS103*) than the taurine and indicine alleles. The Bali alleles of *ILSTS050* and *ILSTS036* differed one base pair in length from the zebu or taurine alleles. Several banteng animals share Bali-cattle-specific alleles, including the alleles of *ILSTS036* with the 1-bp difference. In fact, in four loci (*MGTG4b*, *ILSTS008*, *TGLA126* and *TGLA227*), the banteng alleles are only found in Bali cattle.

Discussion

We have shown that mtDNA, SFLP, AFLP, as well as microsatellites, are informative for the detection of zebu–banteng hybridization. Both Madura and Malaysian Bali cattle individuals have a mixed banteng–zebu origin, while no zebu introgression has been found in the individuals from the isle of Bali.

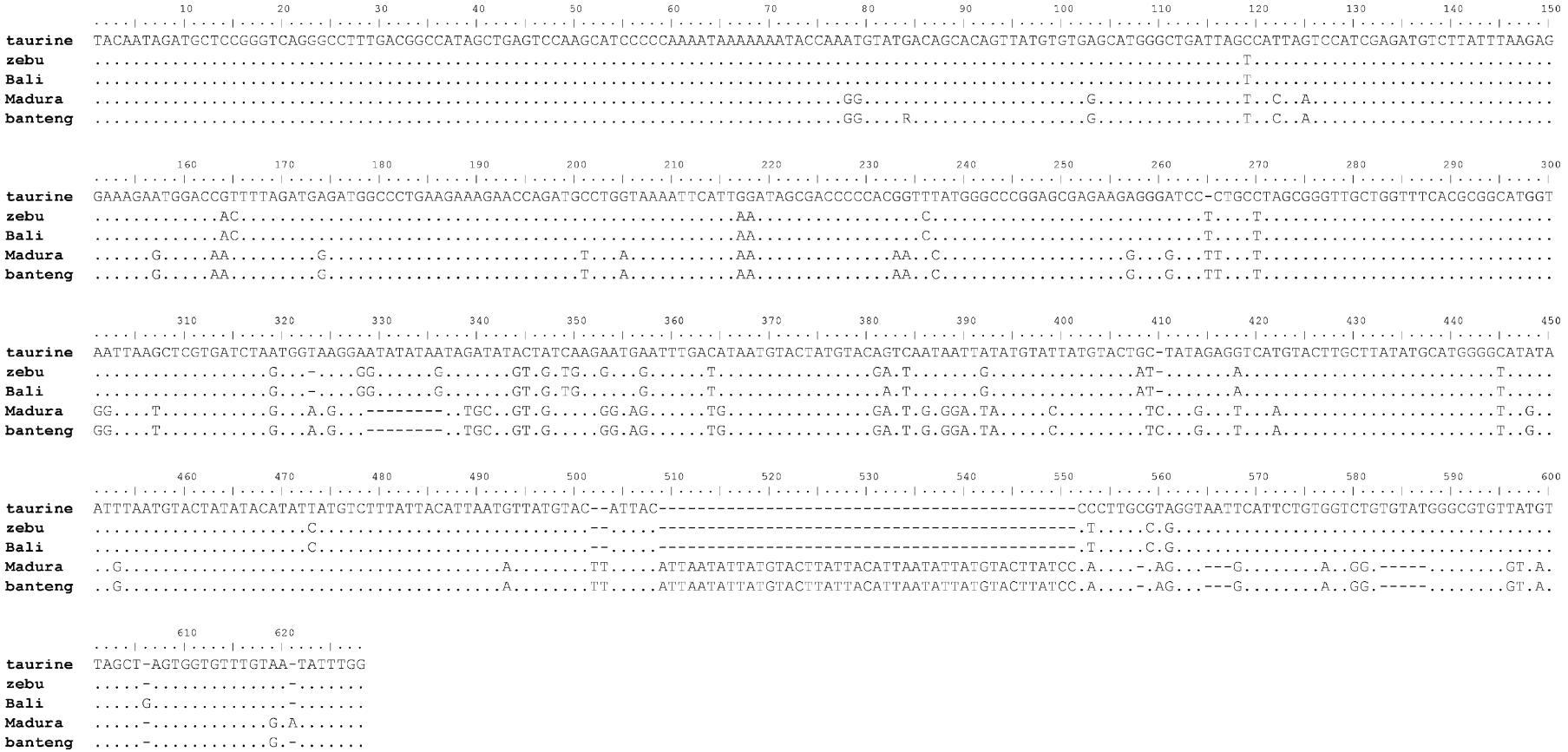

Figure 1 Alignment of mitochondrial D-loop sequences of zebu (AF1314610), taurine (AF3114579) banteng, Bali and Madura cattle individuals. Dots indicate identical bases and dashes indicate insertions/deletions to optimize the alignment.

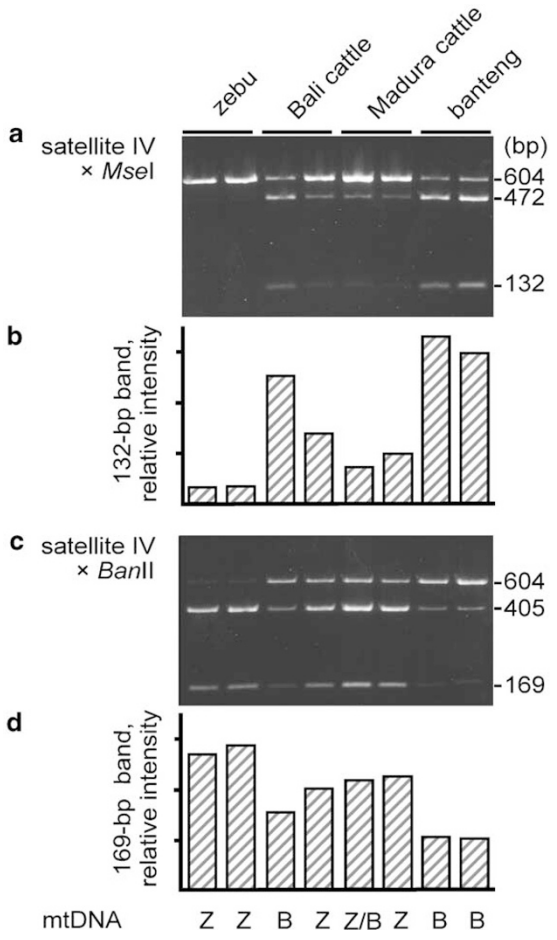

Figure 2 (a,c) Cleavage patterns of satellite IV PCR fragments. Mitochondrial haplotypes have been determined by restriction analysis of cytochrome *b* and are indicated with Z (zebu) and B (banteng). (b,d) Relative intensities of the smallest restriction fragments were measured by phosphor imaging of a Southern blot, hybridized to a satellite IV radio-labelled probe and have been recorrected for the total amount of DNA in a lane.

mtDNA is a marker of the maternal lineage, which in cattle corresponds to the history of the herd. So cows of both banteng and zebu origin were used for the breeding of Madura cattle and the Malaysian Bali-cattle population. By SFLP, AFLP and microsatellite typing we analysed the species composition of the nuclear genome, which is also influenced by male-mediated introgression and may also reveal introgression in earlier generations. This may be the result either from intentional upgrading or from natural contact with bulls of another species.

Centromeric satellite DNA (stDNA) is a sensitive marker of species origin (Bachmann *et al*, 1993; Buntjer *et al*, 1995, 1998; Jobse *et al*, 1995; Grenier *et al*, 1997). As a result of concerted evolution, repeat units of satellite DNA are more similar within than between species (Dod *et al*, 1989; Waye and Willard, 1989; Elder and Turner, 1995). Related species that share a repeat family can be distinguished by SFLP analysis of repeat variants (Nijman *et al*, 1999; Nijman and Lenstra, 2001). In the present study, the relative amounts of restriction fragments were quantified by blotting, hybridization and phosphoimaging. In a preliminary experiment, SFLP quantification was successfully achieved by capillary electrophoresis

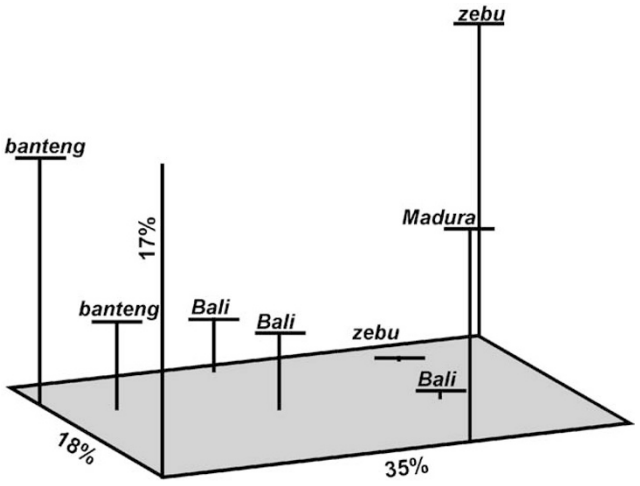

Figure 3 PCO plot of Jaccard similarity values (% band sharing) as calculated from AFLP data based on 120 polymorphic markers. Percentages indicate amount of variation displayed per axis.

(lab-on-a-chip, Agilent Technologies, Palo Alto, CA, USA). This experiment confirmed the variable species composition of Madura animals, while no zebu introgression was detected in the original Bali samples (unpublished results). The banteng genome contains relatively low amounts of satellite IV (Nijman and Lenstra, 2001), so quantitative analysis of restriction sites may have overestimated the zebu component in our study.

However, the finding of zebu DNA in Madura and Malaysian Bali cattle was also confirmed by AFLP and microsatellite typing. Differences in AFLP patterns correspond to point mutations in or near restriction sites or to insertions and deletions. Depending on the degree of relatedness, AFLP patterns generated by a single primer combination are as informative as the typing of 10–30 SNPs or other bi-allelic markers with negligible effect of co-migration of nonhomologous fragments (Buntjer *et al*, 2002). AFLP markers have a less dynamic mode of evolution than microsatellites, but still clearly reveal genetic variation. Genetic distances based on band-sharing parameters can be condensed by coordination analysis (Jackson, 1991) to visualize the relative effect of intra- and interspecies variation. A three-dimensional principal-coordinate analysis (PCO; Figure 3) shows appreciable differences between animals of the same breed, but suggests that the first dimension (representing about one-third of the total variation) is most informative for detection of the banteng–zebu hybridization.

In addition, we found at most microsatellite loci, characteristic alleles for Bali cattle and banteng. If additional genotyping of Bali and banteng supports the specificity of these alleles, these are potentially valuable for assaying the purity of Bali and other banteng breeds. Most of these alleles are shorter than the zebu–taurine alleles, which is in agreement with the general idea that microsatellite alles are shorter in other species than in the species from which they were originally cloned (Ellegren *et al*, 1995). Allele frequencies in Bali cattle are intermediate between the frequencies in taurine and indicine cattle and the frequencies in banteng.

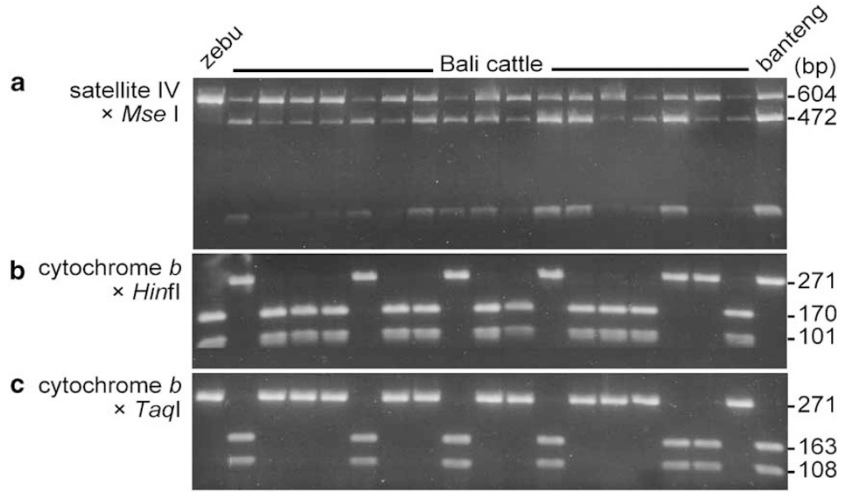

Figure 4 Typing of Malaysian Bali cattle by SFLP and PCR-RFLP of the mitochondrial cytochrome *b* gene.

Our data, although based on a limited sample size, consistently indicate a hybrid origin of the Madura breed and the Malaysian Bali-cattle population. The molecular tools described in this paper may be applied to a larger panel of samples. This would result in a more comprehensive survey of the unique species composition of Indonesian cattle and contribute to a preservation of their economic and cultural value.

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