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Phylogeny of bovine species based on AFLP fingerprinting

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The *Bovini* species comprise both domestic and wild cattle species. Published phylogenies of this tribe based on mitochondrial DNA contain anomalies, while nuclear sequences show only low variation. We have used amplified fragment length polymorphism (AFLP) fingerprinting in order to detect variation in loci distributed over the nuclear genome. Computer-assisted scoring of electrophoretic fingerprinting patterns yielded 361 markers, which provided sufficient redundancy to suppress stochastic effects of intraspecies polymorphisms and length homoplasies (comigration of non-homologous fragments). Tree reconstructions reveal three clusters: African buffalo with water buffalo, ox with zebu, and bison with wisent. Similarity values suggest a clustering of gaur and banteng, but bifurcating clustering algorithms did not assign consistent positions to these species and yak. We propose that because of shared polymorphisms and reticulations, tree topologies are only partially adequate to represent the phylogeny of the *Bovini*. Principal-coordinate analysis positions zebu between a gaur/banteng cluster and taurine cattle. This correlates with the region of origin of these species and suggests that genomic distances between the cattle species have been influenced by genetic exchange between neighbouring ancestral populations.
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Introduction

The tribe Bovini comprises cattle and buffalo species, several of which have been domesticated. Taurine cattle (ox) and zebu have only survived as domestic animals, while yak and water buffalo still exist in the wild. Domestic gayal (or mithan) descends from gaur and Bali cattle from banteng. Bison, wisent and African buffalo have not been subjected to systematic domestication.

The reconstruction of the Bovini phylogeny has so far resisted traditional approaches (Lenstra and Bradley, 1999). The only consistent outcome of comparisons of morphological (Bohlken, 1961; Groves, 1981; Geraads, 1992) or molecular (Miyamoto *et al*, 1989; Schreiber *et al*, 1990; Wall *et al*, 1992; Janecek *et al*, 1996; Hassanin and Douzery, 1999a, b; Schreiber *et al*, 1999; Ritz *et al*, 2000) characters is the early branching of buffalo-like species (genera *Syncerus* and *Bubalus*). Mitochondrial phylogenies are complicated by intraspecies hybridization (Janecek *et al*, 1996; Bradley *et al*, 1996; Schreiber *et al*, 1999), while variation of nuclear gene sequences is not informative at this level of relatedness (Chikuni *et al*, 1995). Generally, the evolution of a single locus may be influenced by selection or drift and does not necessarily reflect the species phylogeny (Pamilo and Nei, 1988).

We have used amplified fragment length polymorphism (AFLP) (Vos *et al*, 1995) to generate nuclear DNA fingerprints that display variation of loci dispersed over the nuclear genome. AFLP is based on the selective amplification of restriction fragments by primers that bind to adapters and extend at their 3′ ends one to three extra nucleotides beyond the restriction sites. It has been developed as genetic mapping tool for plants, but is now used for genomic typing of several prokaryotic and eukaryotic organisms (Ajmone-Marsan *et al*, 1997; Heun *et al*, 1997; Mueller and Wolfenbarger, 1999; Savelkoul *et al*, 1999). In this study we show that with appropriate precautions AFLP fingerprints are informative for phylogeny and indicate recent speciation events of cattle species better than mitochondrial DNA. However, it is proposed that the phylogeny of the cattle species can only partially be represented by a hierarchical topology. A three-dimensional representation by principal-coordinate analysis suggests that the genetic distances of cattle species correlate with geographical origin.

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Materials and methods

DNA samples

DNA was isolated from blood or tissue samples from ox (*Bos taurus*; one Holstein-Friesian, one Meuse-Rhine-Yssel, one Galloway and two West-African N′Dama); zebu (*Bos indicus*, one Hariana and one Sahiwal); banteng (*Bos banteng*, Blijdorp Zoo, Rotterdam); gaur (*Bos gaurus*, obtained via Dr. DG Bradley, Dr P Arctander and Dr J Womack, respectively); yak (*Bos grunniens*, Ouwehand Zoo, Rhenen); bison (*Bison bison*, Artis Zoo, Amsterdam); wisent (*Bison bonasus*, Artis Zoo); African buffalo

(*Syncerus caffer*, Zimbabwe); water buffalo (*Bubalus bubalis*, Italian population); bongo (*Taurotragus eurycerus*, Antwerp Zoo), respectively, as described previously (Ciulla *et al*, 1988; Sambrook *et al*, 1989).

AFLP

AFLP reactions were basically performed as described (Vos *et al*, 1995; Ajmone-Marsan *et al*, 1997). *Eco*RI and *Taq*I digested DNA was ligated to adapters 5′-CTCGTAG ACTGCGTACC/3′-CATCTGACGCATGGTTTAA and 5′-GACGATGAGTCCTGAC/3′-TACTCAGGACTGGA. For multiplex PCR, primers consisted of adapter sequences (5′-GACTGCGTACCAATTC and 5′-GATGAGTCCTGAC CGA) and 3′ selective extensions. Pre-amplification was done with primers with single adenosine residue as extensions of both primers. Final amplifications were done with the following primer combinations: *Eco*RI primer -AAA, *Taq*I primer -AGA; *Eco*RI primer -AAA, *Taq*I primer -AGT; *Eco*RI primer -AAA, *Taq*I primer ATG. The -AAA *Eco*RI primer was labelled with the fluorescent dye FAM and the -AAT *Eco*RI primer with the fluorescent dye JOE. AFLP products were electrophoresed on an ABI Prism377 (Perkin-Elmer) and analyzed by proprietary image analysis programs (Keygene N.V.).

Phylogenetic analysis

Electrophoretic patterns were converted into binary matrices (1 for presence, 0 for absence of a band) and used for calculation of the Jaccard index for each pair-wise comparison (number of shared bands/number of bands either lane). The program PHYLTOOLS (http://www.spg.wau.nl/pv/pub/pt/) has been developed to generate input files for the Phylip program package (Felsenstein, 1995) and included options for: (i) calculation of Jaccard values while ignoring in each pairwise comparison band positions with missing values; (ii) application of the bootstrapping procedure on the binary matrix generating of multiple matrices of Jaccard indices; (iii) generation of multiple data files with for each species one randomly chosen individual. This last option allowed, analogous to bootstrap values, for each node the calculation of a genetic sampling value, which indicates the percentage of trees with one individual per species that give the same clustering of species.

Neighbour-joining trees were calculated with the Phylip program NEIGHBOR. Maximum parsimonious trees were constructed with PAUP (Swofford, 1985) and Phylip programs MIX and DOLLOP. Bootstrap and genetic sampling values were calculated by the Phylip programs SEQBOOT and CONSENSE. Phylogenetic consistency indices were calculated by PAUP. Trees were rooted by the branches towards the bongo, a *Bovinae* species outside the Bovini tribe.

Split-decomposition has been carried out with the program SPLITSTREE (Huson, 1998). Three-dimensional principal coordinate analysis of the Jaccard similarities was done with the NTSYS-pc modules DCENTER, EIGEN and MOD3D (Rohlf, 1993).

Results

A representative part of AFLP fingerprints of the *Bovini* species is shown in Figure 1. One to five individuals per species have been analysed. Three *Eco*RI-*Taq*I adapter-primer combinations yielded 361 markers, 120 of which were polymorphic within one or more species. Table 1 (tinted area) shows the similarity of species, expressed as the Jaccard values averaged over pairwise comparisons of individuals. Intraspecific variation of AFLP was clearly lower than the difference between species. The similarity of species that can produce completely fertile hybrid offspring is clearly higher than of other species. The same trends are apparent from similarity values based on the subset of 241 markers without intraspecies polymorphism (Table 1, untinted area).

Neighbour-joining (NJ) trees of the Jaccard (Figure 2a) or Nei (not shown) indices of band sharing were in good agreement. Both trees support with high bootstrapping and genetic sampling values the clustering of water buffalo with African buffalo, of ox with zebu and of bison with wisent. Other clusterings have lower bootstrapping values and also depended on the organization of the data. For instance, a tree of all 28 individuals had the same branching order of species, but in a tree based on average Jaccard values of species (Table 1, tinted) gaur is tied to the zebu-ox cluster and separated from banteng, while yak is separated from the other *Bos* and *Bison* species. In addition, genetic sampling values (see Materials and methods) indicate that the positions of yak, gaur and banteng in species trees based on all 361 markers depended on the choice of the individual.

A maximum parsimony (MP) tree was calculated directly on the basis of presence or absence of the markers. Figure 2b shows a tree without intraspecies variation reconstructed with the Wagner parsimony algorithm. The same species topology was obtained by including intraspecies variation. Both the Wagner (Figure 2b) and the Dollo parsimony algorithms support the same robust clusters as found by neighbour-joining. However, the positions of gaur, banteng and yak were sensitive to the algorithm and, as indicated by the genetic sampling values, on the choice of the individual.

Split decomposition (Bandelt and Dress, 1992; Huson, 1998) reveals if a given dataset supports a unique tree rather than a tree-like network. Figure 2c shows a split-decomposition graph based on the 241 markers without intraspecies variation, which again supports the bison-wisent and ox-zebu clusters, but suggests that the phylogeny of the other species is not completely tree-like. Split decomposition of average similarity values similarity value (Table 1, tinted) was more complete and also clustered bison with wisent and ox with zebu.

Principal coordinate analysis (PCO; Jackson, 1991) allows a visualisation of genetic distance data without assuming a hierarchical topology. Figure 3 shows a PCO that displays 75% of the variation of Table 1 (untinted) in three dimensions. In this plot gaur and banteng group together and share the values of the two major components. Zebu is between the gaur/banteng cluster and ox, while yak and the cluster of bison-wisent have separate positions. Essentially the same pattern was generated by PCO on the basis of average Jaccard values based on 361 markers (Table 1, tinted) and containing 68% of the variation. A PCO of 18 individual animals, accounting for 82% of the total variation clearly clustered animals from the same species and again yielded the same species pattern but with banteng closer to zebu and ox than gaur.

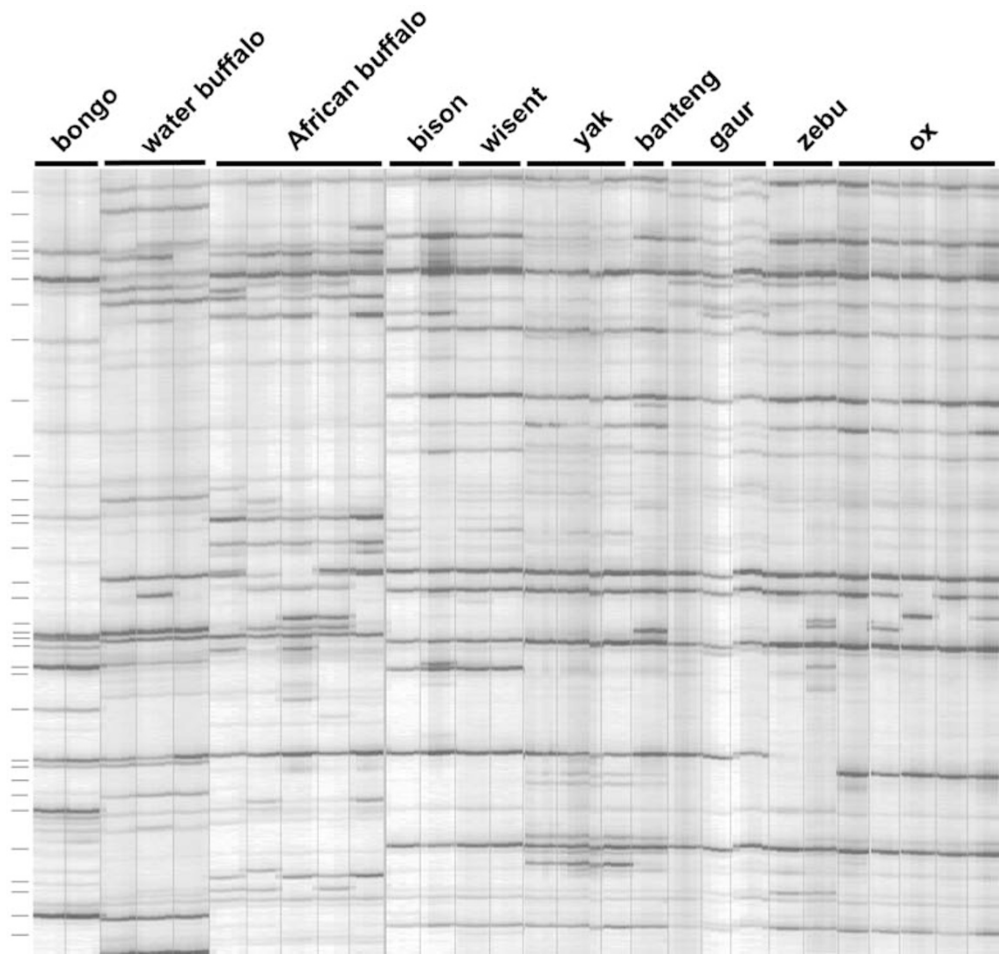

Figure 1 Representative part of an ABI Prism377 AFLP virtual gel pattern generated by an *Eco*RI primer with an AAT-3′ extension and a *Taq*I primer with an ATG-3′ extension. Individuals from different *Bovini* species have been analysed. Lanes have been rearranged electronically. Markers that have been scored are indicated at the left.

Table 1 Similarity of AFLP patterns. Jaccard values of band-sharing were averaged over pairwise comparisons (tinted area) or based on markers without intraspecies variation (untinted)

	Bongo	*Water buffalo*	*African buffalo*	*Bison*	*Wisent*	*Yak*	*Banteng*	*Gaur*	*Zebu*	*Ox*
bongo (2)	**0.983**	0.277	0.266	0.248	0.261	0.270	0.285	0.273	0.271	0.262
water buffalo (3)	0.267	**0.941**	0.442	0.330	0.324	0.385	0.357	0.357	0.375	0.361
African buffalo (5)	0.281	0.480	**0.882**	0.327	0.326	0.336	0.336	0.320	0.349	0.320
bison (2)	0.276	0.335	0.344	**0.957**	***0.848***	*0.679*	*0.686*	*0.701*	*0.667*	*0.619*
wisent (2)	0.282	0.333	0.343	***0.903***	**0.984**	*0.668*	*0.668*	*0.705*	*0.655*	*0.633*
yak (3)	0.271	0.400	0.328	*0.729*	*0.736*	**0.961**	*0.700*	*0.721*	*0.653*	*0.621*
banteng (1)	0.307	0.352	0.347	*0.725*	*0.719*	*0.715*	—	*0.770*	*0.738*	*0.708*
gaur (3)	0.298	0.369	0.342	*0.745*	*0.752*	*0.761*	0.805	**0.914**	*0.753*	*0.725*
zebu (2)	0.306	0.377	0.342	*0.694*	*0.701*	*0.673*	0.788	0.825	**0.888**	***0.808***
ox (3)	0.288	0.375	0.333	*0.667*	*0.685*	*0.658*	0.769	0.819	***0.885***	**0.928**

Figures between parentheses indicate the number of individuals per species. **Bold** indicate intraspecific comparisons, ***bold italic*** comparisons of species with fertile hybrid offspring, and *italic* comparisons of species with fertile female and sterile male hybrid offspring. Standard deviations (not shown) were in the range 0.005–0.015.

Discussion

AFLP as phylogenetic tool

AFLP generates complex patterns of fragments with the same distribution over the genome as the restriction sites used for cleavage. The use of fingerprinting methods for inferring phylogeny has been criticised (Seberg and Petersen, 1998). However, unlike random amplified polymorphic DNA (RAPD) patterns, AFLP patterns are reproducible between laboratories (Ajmone-Marsan *et al*, 1997; Jones *et al*, 1997), while their rather uniform band inten-

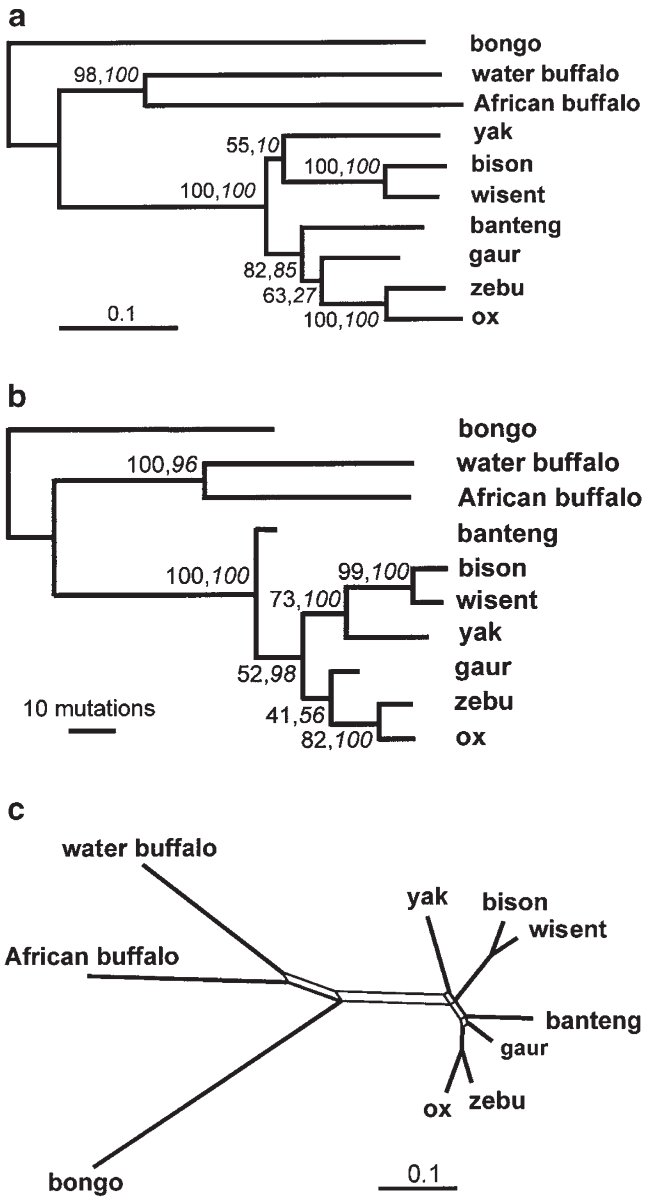

Figure 2 (**a**) Neighbour-joining tree based of the Jaccard similarity of band sharing, calculated on the basis of 241 markers without intraspecies variation. (**b**) Wagner parsimony tree base on the same markers with a consistency index of 0.66 and a length of 334 mutations. Bootstrap percentages from 500 iterations are indicated. Genetic sampling values in italics are based on 500 selections and indicate to which degree the species topology, based on 361 markers with or without intraspecies polymorphism, is independent of the choice of the individual animal (see Materials and methods). (**c**) Tree generated by the split decomposition algorithm with the Jaccard value based on 241 markers without intraspecies variation.

sities allow an unambiguous scoring. In our panel the differences between species clearly exceed the intraspecies polymorphism of up to 10% (this study; Ajmone-Marsan *et al*, 1997).

Another potential source of artefacts is the comigration of fragments originating from different loci, which would increase the apparent similarity ($J_{observed}$) of two patterns. Assuming random distribution of fragments over the gel, the effect of comigration is approximated by

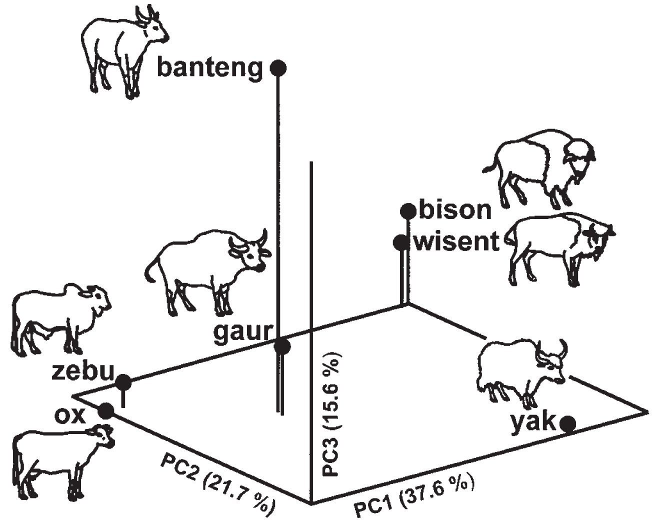

Figure 3 First three dimensions of a principal coordinate analysis of the values of Table 1, tinted area. The percentages of the total variation represented by the principal coordinates PC1, PC2 and PC3, respectively, are indicated.

$$J_{observed} = J + \frac{O(1-J)^2}{4(1-OJ)}$$

(unpublished), where O is the fraction of gel positions that are occupied by a band. So under our conditions ($J \approx 0.70$ and $O \approx 0.12$), this effect is negligible ($J_{observed} \approx 0.703$), but at higher O and/or lower J comigration has the same effect as parallel and back mutations.

The proposed clusterings of species did not critically depend on program parameters, the choice of which depends on assumptions on the molecular basis of the variation in band patterns. Appearance or disappearance of bands can be caused by point mutations in the restriction sites, point mutations in the adjacent 3 bp or by insertions/deletions (Vos *et al*, 1995). In plants and animals about 90% of the AFLP polymorphisms reflect point mutations and most AFLP fragments are independent loci (unpublished results). Comparative sequencing confirmed that also within *Bovini* species point mutations are more frequent than length variation (not shown). We also checked with a simulated dataset that interdependence of characters by length variation of at the expected level of 10% of the markers did not change the topology.

The Wagner algorithm was used for parsimony analysis, since this weights equally the appearance and disappearance of a band. The Wagner parsimonious tree (Figure 2b) had generally higher bootstrapping and genetic sampling values than the Dollo tree of the same dataset.

We conclude that with appropriate precautions AFLP is informative for phylogeny. It generates a large set of basically equivalent and neutral molecular markers that are dispersed over the genome. As indicated by this study, this is particularly informative for the comparison of closely related species if different regions of the genome have different histories.

Phylogeny of the Bovini

The phylogeny of the Bovini has been studied by morphological and molecular methods, but this has not led to a consistent phylogeny (Lenstra and Bradley, 1999; Ritz *et* *al*, 2000). A few clusters that are obvious from morphological data (Bohlken, 1961; Groves, 1981; Geraads, 1992) have not been supported by all molecular studies.

The clustering of the buffalo species (genera *Bubalus* and *Syncerus*; Groves, 1981; Wall *et al*, 1992; Janecek *et al*, 1996) is confirmed by both the AFLP data, which also support the monophyly of the other bovine (*Bos* and *Bison*) species. A comparison of nuclear ribosomal genes (Wall *et al*, 1992) and mitochondrial DNA D-loop sequences (Bradley *et al*, 1996) confirmed a close relationship of taurine cattle (*Bos taurus*) and zebu (*Bos indicus*). This is also apparent from the complete fertility of their hybrid offspring, but this was not supported by a deviating mitochondrial cytochrome *b* sequence from zebu (Schreiber *et al*, 1999). Likewise, there is no reproductive barrier between American bison (*Bison bison*) and wisent (*Bison bonasus*, European bison). However, independent comparisons of mitochondrial DNA (Janecek *et al*, 1996; Schreiber *et al*, 1999; Verkaar *et al*, unpublished results) did not cluster these species, suggesting an anomaly in one of the maternal lineages. AFLP reproduces both the zebu-ox and the bison-wisent clusters and also confirmed the intermediate position of African hybrid breeds between the Indian zebu and taurine cattle (Nijman *et al*, 1999).

Morphological studies (Groves, 1981; Geraads, 1992) and comparison of mitochondrial (Miyamoto *et al*, 1989; Schreiber *et al*, 1999; Hassanin and Douzery, 1999a) or nuclear (Pitra *et al*, 1997) sequences suggest a clustering of yak with the bison. An anomalous grouping of yak with *Bos taurus* indicated by the mitochondrial cytochrome oxidase genes (Janecek, 1996) is explained by maternal introgression in the lineage of the yak individual (Ward *et al*, 1999; Verkaar *et al*, unpublished). Yak clustered also with bison and wisent in most of the AFLP parsimonious trees, but with the neighbour-joining method this depended on the dataset.

In some classifications gaur and banteng are both designated as *Bibos* species. This clustering is in agreement with restriction-enzyme sites in the rDNA genes (Wall *et al*, 1992) and mitochondrial sequences (Janecek *et al*, 1996; Schreiber *et al*, 1999). These species indeed have a relatively high degree of AFLP band sharing (Table 1). However, the positions of both species in any of the trees were not stable.

The divergence times of the *Bos* and *Bison* species (ie, *Bovini* without the buffalo species) are estimated at a few Myr or less (Groves, 1981; Janecek *et al*, 1996; Ritz *et al*, 2000). Three phenomena are expected to disturb hierarchical clustering if speciation has been relatively recent: random allele sorting, shared polymorphisms and reticulation. The degree of allele sorting within the *Bovini* tribe is not clear, but this would be indicated if the clustering depends on the locus (Rogers, 1993; Moore, 1995; Harris and Disotell, 1998).

By shared polymorphisms (Avise and Wollenberg, 1997; Albertson *et al*, 1999) intraspecific and interspecific differences overlap. Indeed, although only a few individuals per species were analysed, 77 markers showed intraspecies polymorphism within the *Bos* and *Bison* species, 13 of which were polymorphic in more than one species. Consequently, the topology of AFLP trees with one individual per species partially depended on the individual, as indicated by the genetic sampling values in Figure 2a and 2b.

Reticulation occurs if a new species has emerged by interspecific hybridization and again leads to the situation that extant species are recombinants of ancestral haplotypes (Moore, 1995). Recent interspecific hybridizations have been well documented for the *Bos* and *Bison* species (Lenstra and Bradley, 1999; Nijman, 1999; Ward *et al*, 1999): zebu and ox in several tropical regions; zebu and banteng in Indonesia; taurine cattle and yak in China, Mongolia and Siberia, etc. Ox-zebu hybrids are completely fertile, while male progeny of other hybridizations are sterile. Earlier introgression events may be indicated by the anomalies in the mitochondrial phylogeny (see above) that are incongruent with trees of nuclear genes, AFLP fingerprints (these studies) and Y-chromosomal sequence variation (unpublished results).

One consequence of reticulation is that a tree topology is not adequate for representing the phylogeny. This is also indicated by the graph generated by the split composition algorithm (Figure 2c). An alternative way to visualize phylogenetic relations and the divergence of the species at the genomic level is coordination analysis, which does not impose a hierarchical topology. So in a principal-coordinate plot (Figure 3), gaur and banteng share the values of first two coordinates. Zebu is between ox and gaur/banteng and bison, while wisent and yak are positioned further away.

Ox and zebu descends from aurochs in the Middle East and on the Indian subcontinent, respectively (Bradley *et al*, 1996). Gaur are distributed from India to the Malaysian peninsula and banteng in South-East Asia and Indonesia. The bison species originate from Central Asia, while the yak is adapted to the high altitudes in and around Tibet. So the pattern of Figure 3 suggests a correlation of genetic distance and geographical origin of the species, at least for ox, zebu, gaur and banteng. Since exchange of genetic material depends on the geographical overlap of the regions inhabited by the species and their ancestors, this is consistent with the hypothesis that reticulation influenced the phylogeny of the *Bovini*.

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