Mitochondrial DNA analysis of two Southern African indigenous cattle breeds

S.C.Parfitt*
Animal Improvement Institute, Irene, Private Bag X2, Irene, 0062, South Africa

H.H. Huismans
University of Pretoria, Pretoria, 0002 South Africa

Received 31 August 1996; accepted 20 March 1998

Mitochondrial DNA cleavage patterns from representative animals of the Afrikaner and Nguni sanga cattle breeds, indigenous to Southern Africa, were compared to the mitochondrial DNA cleavage patterns of the Brahman (zebu) and the Jersey (taurine) cattle breeds. Identical restriction patterns were found with nine restriction enzymes in all four breeds. The restriction profiles obtained from these sanga breeds fall into the Afro-European lineage as described previously. High resolution restriction analysis was performed on representative Nguni and Afrikaner animals in order to obtain information on the mitochondrial DNA variation within these breeds. For this, the hypervariable displacement loop region and adjacent genes were amplified using the polymerase chain reaction. The amplified product was analysed with nine restriction enzymes. An additional restriction site was observed in two unrelated Nguni animals with the enzyme *Msp I*. This mutation is situated in the 12S rRNA gene. As in other Southern African animal species, the variation observed in the indigenous breeds is low and this can be attributed to the effect that bottlenecks have played in the evolution of the species. It is concluded that mitochondrial DNA studies may be less informative in these Southern African cattle breeds, than similar studies performed in the northern hemisphere.

Mitochondriale DNA verteringsensiempatrone van verteenwoordigende diere van die Afrikaner en Nguni sanga beesrasse, inheems in Suider-Afrika, is vergelyk met die mitochondriale DNA verteringsensiempatrone van die Brahman (zebu) en die Jersey (taurus) beesrasse. Identiese verteringsprofiele vir al vier die rasse is gekry met nege verskillende verteringsensieme. Die verteringsprofiele van die sanga rasse stem ooreeen met die profiele van die Afro-Europese lyn. 'n Hoë resolusie verteringsanalise is op verteenwoordigende Nguni- en Afrikaner-diere uitgevoer om die mitochondriale DNA variasie binne hierdie rasse te bestudeer. Hiervoor is die hiperveranderlike D-lus area en naasliggende gene met behulp van die polimerase-kettingreaksie geamplifiseer. Die geamplifiseerde produk is met behulp van nege verteringsensieme geanaliseer. 'n Addisionele snypunt met die ensiem *Msp I* is in twee onverwante Nguni-profiele gevind. Die mutasie lê in die 12S rRNA geen. Soos in ander Suider-Afrikaanse dierespesies is die variasie wat gevind is baie laag. Dit kan toegeskryf word aan die effek wat 'bottlenecks' op die ontwikkeling van die spesies gehad het. Die gevolgtrekking word gemaak dat mitochondriale DNA-studies minder inligting oplewer in hierdie Suider-Afrikaanse beesrasse, as soortgelyke studies wat op beesrasse in die noordelike halfrond uitgevoer is.

Keywords: mitochondrial DNA, restriction fragment length polymorphism, cattle breeds

*Author to whom correspondence should be addressed

Introduction

Domestic cattle are classified into two major types or subspecies, the zebu or humped cattle (*Bos taurus indicus*) and the taurine or humpless cattle (*B. taurus taurus*). It is believed that these subspecies have evolved from the same ancestor, the now extinct auroch (*B. primigenius*) (Epstein, 1971). African indigenous cattle breeds can be classified as zebu, taurine or sanga. The African sanga breeds are believed to be an admixture of taurine and indicine cattle.

The different cattle breeds indigenous to Southern Africa are individually adapted to particular sets of harsh environmental conditions (e.g. high or low temperature, drought or floods, high altitude and extreme temperature combinations, parasites, poor quality grazing, etc.) and perform well under these suboptimal conditions. It is therefore imperative to conserve these breeds. Genotypic characterisation as well as data on the geographic distribution of animals provide a foundation for the conservation and enhancement of threatened livestock resources. The investigation of genetic variation in these breeds may assist breeders in developing successful breeding strategies. Mitochondrial DNA (mtDNA) has been found to be extremely useful in assessing genetic relationships between closely related species as a result of the mode of inheritance, its rapid mutation rate and lack of recombination (Brown *et al.*, 1979).

In a number of studies, the mtDNA of cattle has been investigated. These studies focused on regional breed groups (Watanabe *et al.*, 1985; Watanabe *et al.*, 1989; Bhat *et al.*, 1990; Suzuki *et al.*, 1993; Kikkawa *et al.*, 1995) or on breeds at a global level (Loftus *et al.*, 1994a; Loftus *et al.*, 1994b). The sanga breeds of Africa have not been included in any of these studies.

The aim of this study was to assist in the clarification of the mitochondrial relationship between the sanga, taurine and zebu groups of cattle. Data is presented from an investigation of the mtDNA restriction fragment length polymorphisms (RFLP) observed in two sanga breeds, a taurine and a zebu breed, as well as the mtDNA variation within these sanga breeds. The sanga breeds chosen for the study are the Nguni, which is adapted to the hot, humid conditions of the eastern parts of Southern Africa and the Afrikaner, which is best suited to the dry, hot western regions of the country. Both these breeds show the typical taurine submetacentric Y-chromosome morphology, as opposed to the acrocentric zebu Y-chromosome (Meyer *et al.*, 1984). Their morphology, however, shows zebu characteristics.

Materials and Methods

DNA samples

Whole blood samples (20–40 ml) were collected in EDTA (1% 0.5 M) from representative animals from four different breeds of cattle. Eleven unrelated Afrikaners, twelve Nguni's, a registered Brahman (*B. taurus indicus*) selected to be representative of the breed and a registered Jersey (*B. taurus taurus*), selected to be representative of the breed, were sampled. The Afrikaner and Nguni samples were collected from the Afrikaner and Nguni experimental herds of the ARC. These herds consist of different breeding lines and have been compiled to represent the gene pool of the breeds in Southern Africa.

mtDNA extraction

Each sample of whole blood was separated into layers by centrifugation (180 × g). The platelet-rich plasma and the leucocyte layers were collected. The plasma was washed with 30 ml T-Wash (0.9% NaCl, 10 mM EDTA) and centrifuged at 145 × g for 15 min at room temperature to remove any leucocytes or erythrocytes. The platelets were then collected from the supernatant by centrifugation at

10 000 × g for 15 min and resuspended in 500 ml lysis buffer (10 mM NaCl, 10 mM EDTA, 50 mM Tris/HCl, pH 8). To this 12.5 ml SDS (10%) was added and the lysate was incubated at 37°C for 5 min, whereafter the mtDNA was extracted with phenol/chloroform (Ausubel *et al.*, 1988). The mtDNA from the leucocytes was extracted separately using the method described by Wrischnik *et al.* (1987). Agarose gel electrophoresis (1% v/v) indicated that the mtDNA extracted from the leucocytes had nuclear DNA contamination, whereas the mtDNA from the platelets was pure.

Restriction enzyme analysis

The enzymes *Bam HI, Bgl II, Eco RI, Hind III, Pst I, Pvu II, Sac I, Sal I,* and *Xho I* (Boehringer Mannheim) were used for the RFLP analysis. These enzymes have been used successfully in previous studies (Watanabe *et al.*, 1985; Watanabe *et al.*, 1989; Bhat *et al.*, 1990). They all specify six base pair (bp) recognition sequences. DNA samples were separated on 1% (v/v) agarose gels and visualised either by EtBr staining under ultra-violet light, or by hybridization with a mtDNA probe or end-labelling of the digested fragments.

Polymerase chain reaction (PCR) amplification

For a higher resolution restriction analysis, the displacement loop (D-loop) and adjacent genes of the Nguni and Afrikaner mtDNA preparations were amplified. The primers were obtained from W. Mann from the Technical University of Munich. Their sequences are as follows:

mtP1: 5' GAA GAA ACT GCA GTC TCA CC 3'
mtP2: 5' GGC TCG TTA GGC ATG TCA CC 3'.

These primers amplified a region between positions 15 731 and 1 800 of the mitochondrial genome, passing through the origin, and yield a PCR product of 2 407 bp. This includes the whole D-loop and the 12S rRNA, $tRNA^{Phe}$, $tRNA^{Val}$ and part of the $tRNA^{Pro}$ and 16S rRNA genes (Figure 1).

Approximately 150 ng of mtDNA was subjected to a PCR primed by 100 ng each of mtP1 and mtP2 primers in a 50 µl reaction mix. The PCR mix contained 400 µM dNTPs, 2.5 mM $MgCl_2$, 2 units Taq polymerase in 1 × PCR buffer (Boehringer Mannheim), and was covered by a layer of mineral oil. The temperature cycling program was as follows:
One cycle of 90 s at 94°C; 45 s at 60°C and 90 s at 72°C, followed by 30 cycles of 45 s at 94°C; 45 s at 60°C and 90 s at 72°C, with a final extension at 72°C for 4 min. All PCR amplifications included a negative control reaction which lacked template DNA. No product was observed in any negative control reaction.

The enzymes *Ava II, Asp HI, Bam HI, Hae III, Msp I, Pst I, Sca I, Taq I* and *Xba I* (Boehringer

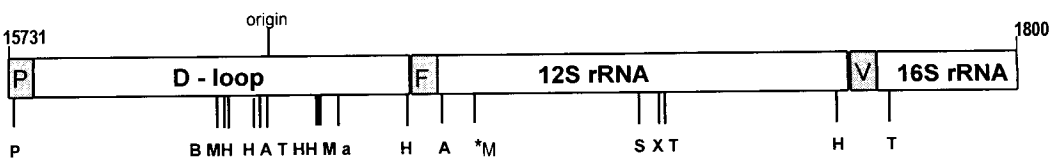

Figure 1 A diagrammatic representation of the restriction sites in the amplified region. This includes the D-loop, the 12S rRNA gene and a section of the 16S rRNA gene as well as the $tRNA^{Val}$ (V), $tRNA^{Phe}$ (F) and part of the $tRNA^{Pro}$ (P) gene. *Ava II* – A, *Asp HI* – a, *Bam HI* – B, *Hae III* – H, *Msp I* – M, *Pst I* – P, *Sca I* – S, *Taq I* – T, *Xba I* – X.

Mannheim) were used to digest the amplified products. Double digests were performed using a buffer suitable for both enzymes. The digested products were separated on 16 cm vertical slabs of 4% or 6% polyacrylamide (29% acrylamide, 1% bis acrylamide) and the DNA visualized under ultra-violet light by EtBr staining.

Results

The number of cleavage sites and fragment lengths for each of the enzymes used to compare the four cattle breeds is summarized in Table 1. Identical restriction patterns were observed for all the animals. The complete sequence for bovine mtDNA has been determined by Anderson *et al.* (1982) and the patterns generated in this study corresponded exactly to the predicted cleavage sites.

Higher resolution restriction analysis was performed on the 2 407 bp PCR-product of the mtDNA D-loop and the adjacent genes from twelve Ngunis and eleven Afrikaners (Figure 1). The cleavage sites obtained with the nine enzymes are summarized in Table 2. A polymorphic restriction pattern

Table 1 Number of cleavage sites and molecular lengths of restriction fragments in the mitochondrial genome

Restriction endonuclease	Number of sites	Approximate molecular length of fragments (kb)
Bam HI	3	11.2 3.2 1.9
Bgl II	2	9.7 6.6
Eco RI	3	7.4 4.8 4.3
Hind III	3	10.2 4.5 1.7
Pst I	2	9.4 7.0
Pvu II	3	13.3 2.5 0.6
Sac I	1	16.4
Sal I	1	16.4
Xho I	1	16.4

Table 2 Cleavage sites of the 2407 bp PCR product

Enzyme	Number of sites	Position of sites	Fragment sizes (bp)
Ava II	2	16302, 440	1360, 571, 476
Asp HI	1	151	1649, 758
Bam HI	1	16201	1937, 470
Hae III	6	16222, 16285, 92, 102, 351, 1248	897, 552, 491, 249, 145, 63, 10
Msp I A	2	16218, 110	1690, 487, 230
B	3	16218, 110, 497	1303, 487, 387, 230
Pst I	1	15742	2396, 11
Sca I	1	917	1524, 883
Taq I	3	16332, 991, 1479	997, 601, 488, 321
Xba I	1	969	1576, 831

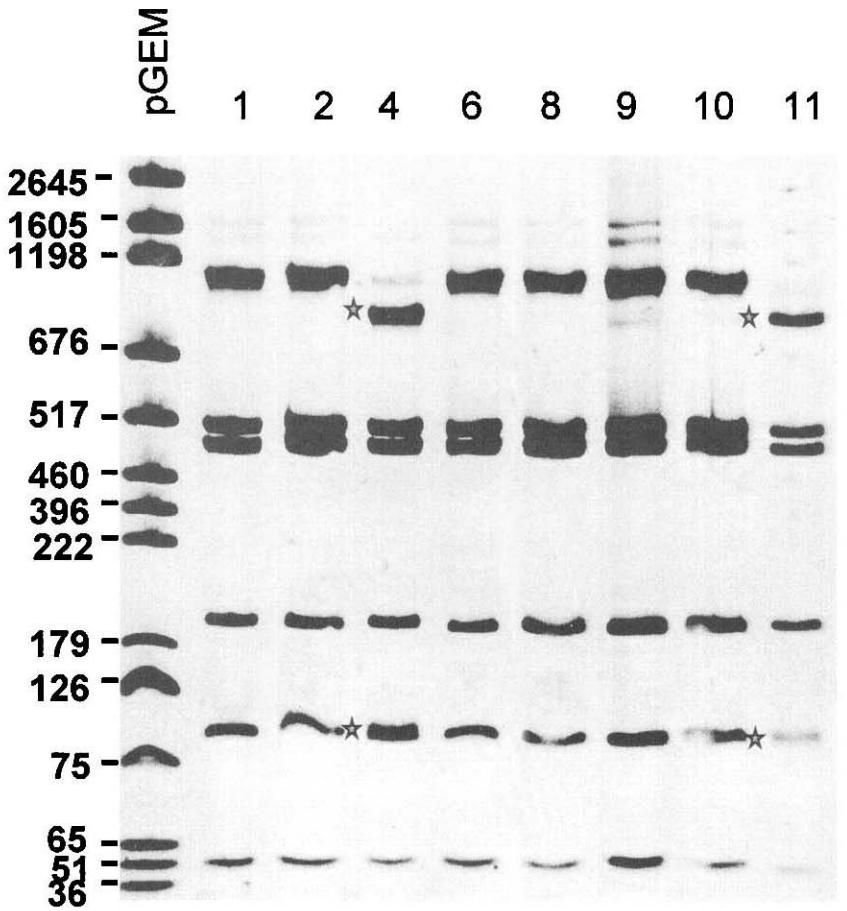

Figure 2 A polyacrylamide gel of the amplified products of eight of the Nguni mtDNA preparations (1, 2, 4, 6, 8, 9, 10 and 11) digested with *Msp I*. The molecular weight marker pGEM (Promega), of which the sizes are indicated in bp on the figure, was used as a size standard. The polymorphic bands are indicated by ★ in lanes 4 and 11.

was observed with the enzyme *Msp I* in two of the Nguni mtDNA preparations (Figure 2). The additional *Msp I* site was determined from double digests with the enzymes *Taq I, Ava II* and *Hae III* and by comparison with the complete sequence of mtDNA in cattle (Anderson *et al.*, 1982). It was established that the mutation lies in position 497, and is the result of an A to G transition.

No polymorphisms were observed in the Afrikaner animals tested. The fragment sizes that were obtained were in agreement with those predicted from the sequence published by Anderson *et al.* (1982).

Discussion

Nine enzymes, some of which have been reported in the literature to show polymorphisms in the mitochondrial genome of cattle (Anderson *et al.*, 1982; Watanabe *et al.*, 1989; Bhat *et al.*, 1990; Loftus *et al.*, 1994b; Kikkawa *et al.*, 1995), were used for the RFLP comparison of the four breeds.

Twenty-eight restriction sites were covered. No polymorphisms were detected at these sites and therefore no breed specific restriction sites could be identified. This result corresponds with Watanabe *et al.* (1985), Watanabe *et al.* (1989), Bhat *et al.* (1990), Suzuki *et al.* (1993) and Loftus *et al.* (1994b) who, although they found polymorphisms, found none that were breed specific. The restriction profiles observed in this study corresponded to the profiles predicted from the reference sequence by Anderson *et al.* (1982).

In a study of 13 breeds of cattle from Europe, Africa and Asia, Loftus *et al.* (1994b) distinguished consistently between Afro-European (African and European) and Asian cattle breeds with the enzymes *Bam HI, Bgl II* and *Hind III.* The predominant Afro-European haplotype was also found with the enzymes *Bam HI, Bgl II* and *Hind III* for the animals in this study. No profiles typical of Asian breeds, which show a different profile for these restriction enzymes, (Loftus *et al.*, 1994b) were observed in the Afrikaner or Nguni, or in the Brahman which is a zebu breed. Unfortunately the Brahman was not a good choice to represent the indicine type. The breed was developed through the amalgamation of four strains of *B. taurus indicus* cattle, but it may contain a considerable amount of *B. taurus taurus* blood (Mason, 1988). The presence of the taurine influence was supported by the mtDNA profiles of the animal investigated in this study.

The mtDNA of the Nguni and Afrikaner or any of the other indigenous Southern African cattle breeds have not been studied previously. This study revealed that the restriction patterns observed for the indigenous breeds were similar to the European type. This has also been reported by Loftus *et al.* (1994b) for central African cattle breeds. The mitochondrial type as well as the Y-chromosome conformation of sanga cattle correspond to the taurine breeds, while their body conformation (e.g. the hump) shows zebu characteristics.

The D-loop is the area with the highest mutation rate in the mitochondrial genome. This region was therefore chosen to study the variation within the indigenous breeds. In order to study intra-breed variation a comparison was made of the restriction enzyme sites in the variable D-loop and adjacent genes of 11 unrelated Nguni's and 12 unrelated Afrikaners. Eleven of the 19 restriction sites surveyed with the nine enzymes listed in Table 2, lie in the D-loop. These cover 50 bp or 5.5% of the 910 bp D-loop. An *Msp I* polymorphism was observed in the 12S rRNA gene. The position of the *Msp I* site was determined by double digestions. When the approximate position was compared to the sequence of Anderson *et al.* (1982), the site of the polymorphism was mapped to position 497.

A very low level of variation was observed between the different cattle used in this study. A higher level of variation was expected. Polymorphisms in the D-loop region have, for example, been used successfully by Olivo *et al.* (1983) to distinguish between maternal lineages in Holstein cattle. A possible reason for the lack of variation has been provided by Grant & Leslie (1993), who found that, in the Southern Hemisphere, the mtDNA variation was much lower than in the northern hemisphere. They ascribe this to bottlenecks, which have occurred much more frequently in the Southern than in the northern hemisphere. mtDNA variation is much more sensitive to bottlenecks than nuclear DNA, because of its maternal inheritance and the lack of recombination. Therefore when studying indigenous cattle in Southern Africa it is suggested that techniques employing nuclear DNA, e.g. microsatellites, should be of greater value in the process of determining genetic variability.

This investigation was aimed at determining variability within and between indigenous Southern African breeds, but results indicated that mtDNA variation in the two breeds studied is either non-existent or very low. However it is clear that the taurine mitotype is dominant in the mitochondrial genome of these animals. The role of bottlenecks in the evolution of the cattle breeds indigenous to the Southern Hemisphere in general, and Southern Africa in particular, needs further investigation.

References

ANDERSON, S., DE BRUIJN, M.H., COULSON, A.R., EPERON, I.C., SANGER, F. & YOUNG, I.G., 1982. Complete sequence of bovine mitochondrial DNA, conserved features of the mammalian mitochondrial genome. *J. Mol. Biol.* 156, 683.

AUSUBEL, F.M., BRENT, R., KINGSTON, R.E., MOORE, D.D., SEIDMAN, J.G., SMITH, J.A. & STRUHL, K., 1988. Current Protocols in Molecular Biology. John Wiley & Sons.

BHAT, P.P., MISHRA, B.P. & BHAT, P.N., 1990. Polymorphism of mitochondrial DNA (mtDNA) in cattle and buffaloes. *Biochem. Genet.* 28, 311.

BROWN, W.M., GEORGE, M. JR & WILSON, A.C., 1979. Rapid evolution of animal mitochondrial DNA. *Proc. Natl. Acad. Sci. USA* 76, 1967.

EPSTEIN, H., 1971. The origin of the domestic animals of Africa. Vol 1. Africana Publishing Corporation, New York.

GRANT, W.S. & LESLIE, R.W., 1993. Effect of metapopulation structure on nuclear and organellar DNA variability in semi-arid environments of Southern Africa. *S. Afr. J. Sci.* 89, 287.

KIKKAWA, Y., AMANO, T. & SUZUKI, H., 1995. Analysis of genetic diversity of domestic cattle in east and southeast Asia in terms of variations in restriction sites and sequences of mitochondrial DNA. *Biochem. Genet.* 33, 51.

LOFTUS, R.T., MACHUGH, D.E., BRADLEY, D.G., SHARP, P.M. & CUNNINGHAM, P., 1994a. Evidence for Two Independent Domestications of Cattle. *Proc. Natl. Acad. Sci. USA* 91, 2757-2761.

LOFTUS, R.T., MACHUGH, D.E., NGERE, L.O., BALAIN, D.S., BADI, A.M., BRADLEY, D.G. & CUNNINGHAM, E.P., 1994b. Mitochondrial genetic variation in European, African and Indian cattle populations. *Anim. Genet.* 25, 265.

MASON, I.L., 1988. A World Dictionary of Livestock Breeds, Type and Varieties, 3rd ed., C A B International, Wallingford, Oxon, UK.

MEYER, E.H.H., HARRIS, E.J. & NEL, N.D., 1984. Preliminary results of a cytogenetic study on Malawi Zebu cattle. *6th European Colloquiom on Cytogenetics of Domestic Animals.* Zürich, Switzerland 16–20 July 1984.

OLIVO, P.D., VAN DE WALLE, M.J., LAIPIS, P.J. & HAUSWIRTH, W.W., 1983. Nucleotide sequence evidence for rapid genomic shifts in the bovine mitochondrial DNA D-loop. *Nature* 306, 400.

SUZUKI, R., KEMP, S.J. & TEALE, A.J., 1993. Polymerase chain reaction analysis of mitochondrial DNA polymorphism in N'Dama and Zebu Cattle. *Anim. Genet.* 24, 339.

WATANABE, T., HAYASHI, Y., SEMBA, R. & OGASAWARA, N., 1985. Bovine mitochondrial DNA polymorphism in restriction endonuclease cleavage patterns and the location of the polymorphic sites. *Biochem. Genet.* 23, 947.

WATANABE, T., MASANGKAY, J.S., WAKANA, S., SAITOU, N. & TOMITA, T., 1989. Mitochondrial DNA polymorphism in native Phillipine cattle based on restriction endonuclease cleavage patterns. *Biochem. Genet.* 27, 431.

WRISCHNIK, L.A., HIGUCHI, R.G., STONEKING, M., EHRLICH, H.A., ARNHEIM, N. & WILSON, A.C., 1987. Length mutations in human mitochondrial DNA: direct sequencing of enzymatically amplified DNA. *Nucleic Acids Res.* 15, 529.