Indian Journal of Animal Sciences **82** (9): 1012–1015, September 2012

Yak introgression into Tibetan Yellow cattle: evidences from mitochondrial DNA D-loop sequence analysis and ß-lactoglobulin genetic variants

ZHENGXIN LIU[1], BANGMIN LU[2], YONGJIANG MAO[3], YUPING LI[4], SUYU JIN[5] and YUCAI ZHENG[6]

Southwest University for Nationalities, Chengdu 610041 P R China

Received: 18 September 2011; Accepted: 13 January 2012

ABSTRACT

The objective of this study was to investigate the possible introgression of highland yak (*Bos grunniens*) into Tibetan Yellow cattle (*Bos taurus*) based on milk protein and mtDNA D-loop analysis. Tibetan Yellow cattle (43) were used to assay polymorphisms of ß-lactoglobulin (ß-Lg) and to extract genomic DNA for the PCR amplification of mtDNA D-loop. Native polyacrylamide gel electrophoresis (PAGE) of milk ß-Lg showed that 3 milk samples exhibited the yak-specific ß-Lg E variant among the 43 milk samples tested. mtDNA D-loop sequences were successfully amplified by PCR using genomic DNA extracted from milk. A total of 16 sequences of the mtDNA D-loop were obtained for analysis, which contained 133 variable sites (97 parsimony-informative sites and 36 single sites) and defined 15 different haplotypes. The 3 Tibetan Yellow cattle carrying ß-Lg E variant were exactly clustered with yak when mtDNA D-loop sequences were employed for cluster analysis, while other yaks assayed (13) clustered with cattle. This research demonstrated that there existed matrilineal introgression of yak during the evolution or natural breeding of Tibetan Yellow cattle population on Qinghai-Tibet plateau.

Key words: ß-lactoglobulin, Gene introgression, mtDNA D-loop, Tibetan Yellow cattle, Yak

Maternally inherited mitochondrial DNA (mtDNA) analysis is a valuable tool for investigating intraspeciûc variation, population structure, evolution, phylogeny and phylogeography in animals (Cozzi *et al*. 2004, Blancher *et al*. 2008). The mtDNA control region is useful for genetic profiling analysis. By sequencing the mtDNA D-loop, Jia *et al*. (2010) found that cattle from 6 Asian countries fell into 3 groups (*Bos taurus, Bos indicus,* and *Bos grunniens*). Studies were carried out using mtDNA D-loop analysis in genetic diversity and conservation (Ginja *et al*. 2010, Berthouly *et al*. 2010), and phylogeny and phylogeography (Kumar *et al*. 2007). Tibetan Yellow cattle are distributed in the highland region of yaks, and therefore crossbreeding may occur during evolution or natural breeding. Yu *et al*. (1999) reported the introgression of yak genes into local Yellow cattle population at Yunnan province in China, by PCR-RFLP analysis. Introgression between other cattle species was also reported (Halbert and Derr 2007, Berthouly *et al*. 2010). Jiulong Tibetan Yellow cattle is an excellent Yellow cattle breed adapted to the unique hypoxic environment of Qinghai-Tibet plateau, with the number of only about 50000. The aim of this study was to investigate the genetic diversity of mtDNA D-loop hypervariable region and polymorphisms of ß-lactoglobulin (ß-Lg) in Jiulong Tibetan Yellow cattle, in order to evaluate its potential genetic relationships to yak, which will be useful for the breeding and preservation of this breed.

Present address: [1, 2]Research Fellow (599141004@qq.com; lubangmin@126.com), [3]Associate Professor (maoyongjiang @googlemail.com), College of Animal Science and Technology, Yangzhou University, Yangzhou 225009 P R China.

[4]Research Fellow (lyp_gz@sina.cn), Agricultural and Animal Husbandry Bureau of Jiulong County, Jiulong County, 610000, P R China.

[5]Associate Professor (syjin65@163.com), [6]Professor (yucaizheng65@hotmail.com) College of Life Science and Technology.

MATERIALS AND METHODS

Experimental cattle and sampling: Milk samples (43) were collected from Jiulong Tibetan Yellow cattle at mid-lactation. The experimental cattle were reared at Jiulong County of Sichuan Province in China. The habitat of the experimental Tibetan Yellow cattle is located at the main distribution region of Jiulong yaks (a yak breed in China), with an altitude of approximate 3500 m. All milk samples were stored at –80°C until analysis. Five milk samples were collected from Jiulong yaks for electrophoresis comparison. The experiment was conducted according to the guidelines of Chinese government for the use of experimental animals and EC Directive 86/609/EEC for animal experiments.

Polymorphism analysis of milk ß-lactoglobulin: Skim milk was prepared by the centrifugation of whole milk at 3000g at 4°C for 20 min. Whey preparation and native polyacrylamide gel electrophoresis (PAGE) for detecting the polymorphism of ß-Lg was conducted as reported (Medrano and Sharrow 1989). The relative percentages of A and B variants of heterozygous ß-Lg were estimated according to band intensity on the gels measured by software of gel imager.

Western blotting of milk ß-lactoglobulin genetic variants: Whey proteins were separated on a 14.2% native PAGE (Medrano and Sharrow 1989) and transferred onto NC membrane by semi-dry transfer apparatus. The membrane was blocked in PBS containing 0.1% tween-20 and 5% bovine serum albumin overnight at 4°C. After 3 washes, the membrane was incubated with the primary antibody (properly diluted rabbit against bovine ß-Lg serum) for 1 h at RT. After 3 washes, the membrane was incubated for 1 h at RT with the HRP-conjugated secondary antibody (1:2000). A kit was used to detect signals on membrane.

DNA isolation from milk: Genomic DNA was isolated from the whole milk (Amills *et al*. 1997) and stored at –20°C for PCR analysis.

Amplification of mtDNA D-loop sequence: The D-loop region of mtDNA of Tibetan Yellow cattle (16) was amplified by PCR using 2 primers: L15737: 5'-CTGCAGTCTCACCATCAACC-3' and Yc-R: 5' -GGGGTGTAGATGCTTGC -3' (Loftus *et al*. 1994, Wu *et al*. 2000). The 16 Tibetan Yellow cattle tested included all the 5 individuals suspected carrier of ß-Lg E variant among the 43 Tibetan Yellow cattle. The PCR condition was as follows: one cycle of denaturation at 95°C for 3 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 60 s and extension at 72°C for 60 s; a final extension at 72°C for 10 min. PCR products were gel purified and cloned into pMD 18-T Vector (TaKaRa) for transfection and sequencing. For each sample at least 3 positive clones were sequenced from both strands.

Phylogenetic analysis: The D-loop sequences of 16 Tibetan Yellow cattle were edited and aligned with reference to the D-loop sequences of the domestic cow (*Bos taurus*) (Accession No. EU281540) using DNASTAR package and were checked manually. Three D-loop sequences each for cattle (*Bos taurus*), zebu (*Bos indicus*) and yak (*Bos grunniens*) were obtained from GenBank. Nucleotide composition was analyzed using the computer program MEGA 3.1 (Kumar *et al*. 2004). The haplotype diversity (Hd) and nucleotide diversity (Pi) were calculated by the software DNAsp 4.1 (Rozas *et al*. 2003). The Neighbor-joining (NJ) phylogenetic tree was reconstructed on the basis of a Kimura two-parameter model using the computer program MEGA 3.1 (Kumar *et al*. 2004). Levels of resolution at internal nodes of the phylogenetic tree were evaluated by bootstrap resampling with 1000 iterations (Felsenstein 1985).

RESULTS AND DISCUSSION

Polymorphisms of milk ß-lactoglobulin: Native PAGE analysis showed that only 3 milk samples exhibited yak origin ß-Lg E variant among the 43 milk samples assayed (Fig.1a). Western blot demonstrated the identity of ß-Lg bands and that the weakly stained band with similar mobility to ß-Lg E variant in sample No. 4 and 34 were not ß-Lg (Fig.1b). The genotype frequencies of AB and BB accounted for the most, and the variant A was the dominant allele (Table 1).

Polymorphism of ß-Lg has been extensively studied and at least 7 genetic variants were discovered, with A and B variants dominate in Holstein (Tsiaras *et al*. 2005). Our result on the gene frequency of ß-Lg B in Tibetan Yellow cattle is similar to that of Holstein cows. The discovery of the unique ß-Lg E variant of yak in Tibetan Yellow cattle population (Fig.1a) clearly demonstrated the existence of gene introgression from yak.

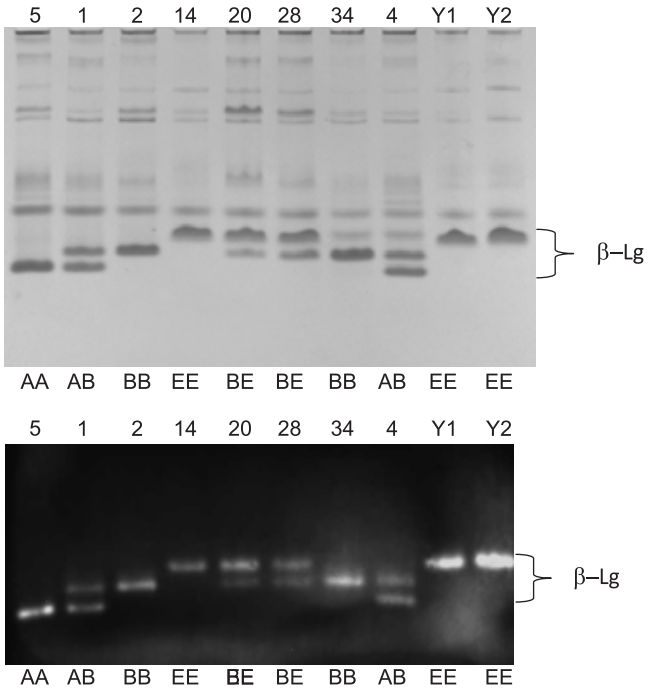

Fig. 1. Native PAGE and western blot of ß-Lg of Tibetan Yellow cattle. Sample numbers are indicated on the top of the figures. Y1 and Y2 are yaks, others are Tibetan Yellow cattle. The letters beneath the figures are genotypes of ß-Lg. **(a)** 14.2% PAGE of whey proteins from Tibetan Yellow cattle and yak; **(b)** Western blot of ß-Lg.

Table 1. Genotype and gene frequency of ß-Lg of Tibetan Yellow cattle (n=43)

Genotype	AA	AB	BB	BE	EE
Frequency	0.116	0.395	0.419	0.047	0.023
Gene	A	B	E		
Frequency	0.314	0.640	0.046		

Band intensity analysis showed that the relative percentage of ß-Lg A and ß-Lg B were similar (50.1±7.6% ß-Lg A *vs* 49.9±7.6% ß-Lg B) among the Tibetan Yellow cattle carrying heterozygous ß-Lg (AB genotype, n=17). The level of ß-Lg B variant is lower than A variant in bovine milk (Lum *et al*. 1997), which might be related to mutation in promoter of ß-Lg B gene (Kuss *et al*. 2003). However, the expression of ß-Lg A and B variants was similar in Tibetan Yellow cattle carrying heterozygous ß-Lg. This is different from Holstein cows, and the reason is unknown at present. We guess that the cloning and sequencing of the promoter of ß-Lg A and B of Tibetan Yellow cattle may help to elucidate the mechanism of their expression difference.

Sequence analysis of mtDNA D-loop: mtDNA D-loop sequences were successfully amplified by PCR using genomic DNA extracted from milk (figure not shown). A total of 16 sequences of the mtDNA D-loop of Tibetan Yellow cattle were obtained for analysis, which comprised 914 bp. The average base composition was 28.7% T, 24.9% C, 32.6% A and 13.8% G, respectively. The obtained D-loop sequence contained 133 variable sites, of which 97 parsimony-informative sites, and 36 single sites. A total of 15 different haplotypes were defined. The haplotype diversity (Hd) and nucleotide diversity (Pi) calculated 0.992 and 0.03957, respectively.

Analysis of phylogenetic tree: The results showed that 3 sequences among the 16 sequences were clustered with yak, while all others clustered with cattle (Fig. 2). These 3 sequences exactly corresponded to the 3 Tibetan Yellow cattle carrying ß-Lg E variant (Sample no. 12, 20, and 28 in Fig. 1).

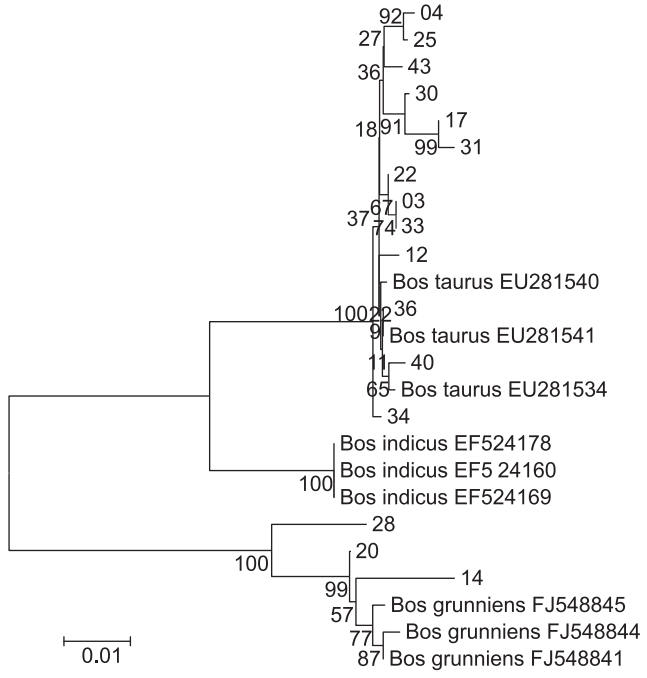

Fig. 2. N-J phylogenetic tree constructed based on mtDNA D-loop sequences. Numbers at nodes represent bootstrap values (%) with 1000 replicates.

Gene introgression between species has been reported in cattle (Halbert and Derr 2007, Berthouly *et al*. 2010). Based on mtDNA D-loop sequence comparisons, our experiment found that Tibetan Yellow cattle have rich genetic diversity (Hd=0.992), and most Tibetan Yellow cattle clustered with cattle except the 3 Tibetan Yellow cattle carrying ß-Lg E variant. This further proved that the gene introgression from yak was matrilineal. In theory, the crossbreeding between male yak and female Tibetan Yellow cattle (called cattle-yak, which looks like yak) may also occur, and in this case some of their hybrids can carry ß-Lg E variant. However, we did not observe such individuals (ß-Lg E variant carrier but clustered with cattle based on mtDNA D-loop analysis). We suppose this is perhaps due to the relatively small numbers of mtDNA D-loop being sequenced for the Tibetan Yellow cattle.

In conclusion, the analysis of mtDNA D-loop sequence can distinguish between Tibetan Yellow cattle carrying yak matrilineal mtDNA genes and pure Tibetan Yellow cattle, although they share similar appearance. This will provide useful information for the preservation of Tibetan Yellow cattle.

ACKNOWLEDGMENTS

This work was supported by Animal Science Discipline Program of Southwest University for Nationalities (No. 2011XWD-S0905).

REFERENCES

Amills M, Francino O, Jansa M and Sanchez A. 1997. Isolation of genomic DNA from milk samples by using Chelex resin. *Journal of Dairy Research* **64**: 231–38.

Berthouly C, Maillard J C, Doan L P, Van T N, Bed'Hom B, Leroy G, Thanh H H, Laloë D, Bruneau N, Chi C V, Dang V N, Verrier E and Rognon X. 2010. Revealing fine scale subpopulation structure in the Vietnamese H'mong cattle breed for conservation purposes. *BMC Genetics* **11**: 45, doi: 10.1186/1471–2156–11–45.

Blancher A, Bonhomme M, Crouau-Roy B, Terao K, Kitano T and Saitou N. 2008. Mitochondrial DNA sequence phylogeny of 4 populations of the widely distributed cynomolgus macaque (*Macaca fascicularis fascicularis*). *Journal of Heredity* **99**: 254–64.

Cozzi M C, Strillacci M G, Valiati P, Bighignoli B, Cancedda M and Zanotti M. 2004. Mitochondrial D-loop sequence variation among Italian horse breeds. *Genetics, Selection, Evolution* **36**: 663–72.

Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**: 783–91.

Ginja C, Penedo M C T, Sobral M F, Matos J, Borges C, Neves D, Rangel-Figueiredo T and Cravador A. 2010. Molecular genetic analysis of a cattle population to reconstitute the extinct *Algarvia* breed. *Genetics, Selection, Evolution* **42**: 18, doi: 10.1186/1297–9686–42–18.

Grossi S F, Lui J F, Garcia J E and Meirelles F V. 2006. Genetic diversity in wild (*Sus scrofa scrofa*) and domestic (*Sus scrofa domestica*) pigs and their hybrids based on polymorphism of a fragment of the D-loop region in the mitochondrial DNA. *Genetics and Molecular Research* **5**: 564–68.

Halbert N D and Derr J N. 2007. A comprehensive evaluation of cattle introgression into US federal Bison herds. *Journal of Heredity* **98**: 1–12.

Jia S, Zhou Y, Lei C, Yao R, Zhang Z, Fang X and Chen H. 2010. A new insight into cattle's maternal origin in six Asian countries. *Journal of Genetics and Genomics* **37**:173–80.

Kumar S, Nagarajan M, Sandhu J S, Kumar N and Behl V. 2007. Phylogeography and domestication of Indian river buffalo. *BMC Evolutionary Biology* **7**: 186 doi:10.1186/1471–2148–7–186.

Kumar S, Tamura K and Nei M. 2004. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics* **5**: 150–63.

Kuss A W, Gogol J and Geidermann H. 2003. Associations of polymorphic AP–2 binding site in the 52 -flanking region of the bovine beta-lactoglobulin gene with milk proteins. *Journal of Dairy Science* **86**: 2213–18.

Loftus R T, MacHugh D E, Bradley D G, Sharp P M and Cunningham P. 1994. Evidence for two independent domestications in cattle. *Proceedings of the National Academy of Sciences of the United States of America* **91**: 2757–61.

Lum L S, Dovc P and Medrano J F. 1997. Polymorphisms of bovine beta-lactoglobulin promoter and differences in the binding affinity of activator protein-2 transcription factor. *Journal of Dairy Science* **80**: 1389–97.

Medrano J F and Sharrow L. 1989. Milk protein typing of bovine mammary gland tissue used to generate a complementary deoxyribonucleic acid library. *Journal of Dairy Science* **72**: 3190–96.

Mirol P M, Giovambattista G, Lirón JP and Dulout F N. 2003. African and European mitochondrial haplotypes in South American Creole cattle. *Heredity* **91**: 248–54.

Rozas J, Sαnchez-DelBarrio J C, Messeguer X and Rozas R. 2003. DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics* **19**: 2496–97.

Tsiaras A M, Bargouli G G, Banos G and Boscos C M. 2005. Effect of kappa-casein and beta-lactoglobulin loci on milk production traits and reproductive performance of Holstein cows. *Journal of Dairy Science* **88**: 327–34.

Wu J M, Smith R K, Freeman A E, Beitz D C, McDaniel B T and Lindberg G L. 2000. Sequence heteroplasmy of D-loop and rRNA coding regions in mitochondrial DNA from Holstein cows of independent maternal lineages. *Biochemical Genetics* **38**: 323–35.

Yu Y, Nie L, He Z Q, Wen J K, Jian C S and Zhang Y P. 1999. Mitochondrial DNA variation in cattle of South China: origin and introgression. *Animal Genetics* **30**: 245–50.