(Original paper)

Mitochondrial DNA variation and genetic construction of indigenous cattle population in Bhutan

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ABSTRACT

To determine genetic diversity and relationship of Bhutanese indigenous cattle, we analyzed mtDNA D-loop sequence of cattle from three populations of Bhutanese native cattle (*Thrabum*) and two Bhutanese populations each of Tibetan (*Goleng*) and Indian (*Jaba*), and together with a Nepalese cattle population. Although Bhutanese native cattle morphologically reveal them as *B.indicus* with the prominent hump, the three *Thrabum* populations included both *B.indicus* and *B.taurus* types of mtDNA haplotypes. Almost all of Bhutanese indigenous populations included both haplotypes assigned to either I1 or I2 clade of *B.indicus* with adequate frequencies, while *B.taurus* type of haplogroups of T1, T3 and/or T4 clades were observed in all the five Bhutanese indigenous populations. The result indicated that indigenous cattle of Bhutan are admixture of *B.taurus* and *B.indicus*, and that ancient taurine had contributed in the formation of Bhutanese native cattle.

Key words: Bhutan, cattle, diversity, mitochondrial DNA

Introduction

Bhutan is a small country located in the Himalayas and the agriculture is the main occupation of the people. In Bhutan, cattle provide important source of draught power, manure and dairy products. Cattle in Bhutan include; i) native Zebu (locally called as *Nublang* for male and *Thrabum* for female), ii) *Goleng* (dwarf Tibetan humpless cattle found in high altitude areas), iii) *Jaba* (non-descript Indian Zebu in the southern foothills of Bhutan) and iv) introduced European breeds (Jersey and Brown Swiss). Mithun (*Bos frontalis*) as well as yak (*Bos grunniens*) are also used for hybridization with indigenous cattle in Bhutan (Dorji *et al.* 2010a; b).

The native *Nublang* and *Thrabum* cattle reveal them morphologically as *B.indicus* type with prominent hump. They inhabit under wide range of environment from 200 m to 3000 m altitude above mean sea level (AMSL) in Bhutan and are adapted to the traditional transhumance rearing systems that migrate to subtropical broadleaf forest in winter and return to temperate areas in summer. Payne & Hodges (1997) described the native cattle of Bhutan as an

intermediate type with influence of *B.taurus* from the Tibetan plateau and *B.indicus* from the Indian plains. However, genetic information of the native cattle populations of Bhutan has been limited.

Mitochondrial DNA (mtDNA) is one of powerful genetic markers investigating domestic livestock origins. The mtDNA polymorphism, especially in the displacement loop (D-loop) region, has been widely used for studying origin, diversity and genetic relationships in cattle (Loftus *et al.* 1994; Bradley *et al.* 1996; Mannen *et al.* 1998; Troy *et al.* 2001; Sasazaki *et al.* 2006). The *B.indicus* mtDNA types were reported in the humpless Nepalese Lulu cattle (Fugise *et al.* 2003), Mongolian cattle (Mannen *et al.* 2004) and Chinese cattle (Lai *et al.* 2006). In addition, comprehensive *B.indicus* mtDNA analyses were carried out to investigate

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the origins and the diversity (Chen *et al.* 2010). Although the mtDNA is almost exclusively maternally inherited and the investigation is limited on maternal genetics only, the mtDNA analysis still has strong advantage to trace the origin of any modern domestic lineage. Since mtDNA is exempt from genetic recombination, it is possible to identify ancestral and immigrated mtDNA lineages recruited into the domestic pool.

The objective of this study was to investigate the origin, the genetic diversity and the genetic structure of indigenous Bhutanese cattle by analyzing mtDNA D-loop variations.

MATERIALS AND METHODS

Animals

Tail hair samples of 107 individuals were collected from six populations. The Bhutanese *Thrabum* cattle samples included three different populations. 1) Sangbey *Thrabum* from Haa Sangbeykha valley in western Bhutan. The place is considered to be the original habitat of purebred Nublang and *Thrabum*. There is no known history of introducing other foreign breeds in the valley. 2) Farm *Thrabum* from the National Nublang Breeding Farm (Tashiyangphu Farm). The farm is dedicated to the conservation of this native breed. It is located in Tashigang in east Bhutan. 3) Village *Thrabum*, sampled randomly from village populations from central and eastern Bhutan (Bumthang, Lhuntse, Tashiyangtse, Tashigang Proper, Trongsa, Zhemgang, Sarpang, Dagana, Chukha, Samtse). 4) The *Goleng* were sampled from 3000 m AMSL near Bhutan-Tibet border in Gasa and Tashigang Sakten. 5) The *Jaba* was sampled from the India-Bhutan borders of Samdrupjonkhar. 6) In addition, the Nepalese cattle samples were also collected from Ilam district in east Nepal. The numbers of Bhutanese indigenous animals sampled from different locations are shown in Figure 1.

Genomic DNA was extracted from five hair follicles per animal using ISOHAIR kit (Nippon Gene Co., LTD, Tokyo, Japan) according to the manufacturer instruction.

PCR amplification and sequencing

We amplified the D-loop region of mtDNA using the forward primer (b-15706F: 5' -ACAACTAACCTCCCTAAG ACTC-3') and reverse primer (b-971R: 5' GATTATAGAA CAGGCTCCTCTA-3'). These primers and an internal

Fig. 1. Sampling locations and sampling numbers for Thrabum, Jaba, Goleng in Bhutan. Numbers in parenthesis indicate the sampling number of animal used in this study. Numbers in right side single parenthesis are consistent with numbers in "Animals" paragraph of Materials and Methods and Table 1.

primer (b-1709F: 5'-ACTAACCTCCCTAAGACTCAAG-3') were also used for sequencing. Amplification and purification was done according to the method of Mannen *et al.* (2004). Standard double-strand DNA sequencing was performed using 20 ng of the amplified product with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied BioSystems, CA, USA) and ABI PRISM 3100 Genetic Analyzer (Applied BioSystems).

Sequence Analysis

In this study, we included the representative D-loop sequences of *B.indicus* type (FJ492194 (I1 type) and AB268559 (I2)) and *B.taurus* (L27714 (T1), L27720 (T1), AB117082 (T2), AB117084 (T2), L27717 (T3), L27725 (T3) and U87637 (T4)) defined by previous studies (Troy *et al.* 2001; Mannen *et al.* 2004; Lai *et al.* 2006). Alignment of sequence was achieved using the CLUSTALW package (Thompson *et al.* 1994). Site representing a gap in any of the aligned sequences were excluded from the analysis, and distances between D-loop sequences were estimated using the substitution model of Tamura & Nei (1993). Unrooted neighbor joining phylogenies were constructed using these distances. The distance computation and phylogenetic tree construction were incorporated in the MEGA package Ver 4.1 (Tamura *et al.* 2007).

RESULTS

We have analyzed D-loop sequences of 710 bp (15869 bp to 240 bp according to gene accession number of V00654) for 96 Bhutanese and 11 Nepalese cattle. All

sequences were deposited in the database (AB570113 - AB570175). Of the 96 Bhutanese samples, 17 animals could be assigned to *B. taurus* type and 79 to *B. indicus* type. Of the 11 Nepalese samples, three were to *B. taurus* type and eight to *B. indicus* type. The *B. taurus* types were observed in all populations including Sangbey *Thrabum* (genetic frequency = 0.333), Farm *Thrabum* (0.143), Village *Thrabum* (0.143), *Goleng* (0.667) and *Jaba* (0.067) (Table 1).

Table 1 Frequency of Bhutanese and Nepalese mtDNA haplotypes assigned by the *B. taurus* and *B. indicus* haplogroups

Populations	<i>B. taurus</i> clade				<i>B. indicus</i> clade		
	T1	T3	T4	Total	I1	I2	Total
1) Sangbey <i>Thrabum</i> (n=15)	3	1	1	5	6	4	10
2) Farm <i>Thrabum</i> (n=28)	1	3	0	4	19	5	24
3) Village <i>Thrabum</i> (n=35)	0	4	1	5	14	16	30
4) <i>Goleng</i> (n=3)	0	2	0	2	1	0	1
5) <i>Jaba</i> (n=15)	0	1	0	1	9	5	14
Total in Bhutanese cattle	4	11	2	17	49	30	79
Nepalese cattle (n=11)	1	0	2	3	3	5	8

Numbers in right side single parenthesis are consistent with numbers in “Animals” paragraph of Materials and Methods and Figure 1.

In the 79 Bhutanese *B. indicus* sequences, 43 haplotypes were found based on polymorphism at 70 sites. Of the 70 variable positions, one represented insertion-deletion (indel) event of a single base pair and the remaining 69 were nucleotide substitutions including six transversions. In 17 Bhutanese *B. taurus* sequences, 9 haplotypes were found based on polymorphism at 20 sites. Of the 20 variable positions, one represented indel event of a single base pair and the remaining 19 were nucleotide substitutions including one transversion.

Unrooted neighbor joining phylogenetic trees were constructed using individual sequences in order to investigate the genetic relationships among haplotypes. Figure 2 shows the phylogenetic tree using *B. indicus* haplotypes with two published representative sequences assigned to I1 and I2, and the major feature of this tree was existence of the two major clades (I1 and I2) as described by Lai *et al.* (2006). The I1 haplogroup illustrated a clear starburst phylogenetic pattern. However, the I2 starburst pattern was seemingly ambiguous and did not show numerous sequences at the center of this radiation. The I1 haplogroup contained 49 Bhutanese haplotypes that occurred in 62.0 % (49/79), while the I2 contained 30 haplotypes occurred in 38.0 % (30/79), of the total *B. indicus*

sequences in the Bhutanese samples. In Nepalese samples, three were included in I1 haplogroup and five were in I2 haplogroup (Table 1).

Fig. 2. Unrooted neighbor-joining tree constructed by mtDNA sequences of Bhutanese and Nepalese cattle with representative *B. indicus* haplogroups, I1 and I2. The bar scale indicates genetic distance by using the substitution model of Tamura and Nei (1993).

Figure 3 shows a phylogenetic reconstruction using *B. taurus* haplotypes in conjunction with representative published sequences (T1, T2, T3 and T4) defined by previous studies (Troy *et al.* 2001; Mannen *et al.* 2004). Three *B. taurus* haplogroups of T1, T3 and T4 were observed in Bhutanese cattle. These observed numbers of *B. taurus* and *B. indicus* types of mtDNA haplotypes in Bhutanese and Nepalese cattle studied in this study were summarized in Table 1.

Fig. 3. Unrooted neighbor-joining tree constructed from *B. taurus* haplotypes of Bhutanese, Nepalese and representative D-loop sequences. Open circles by dashed line indicate branching point of primary *B. taurus* haplotypes defined by previous study (Troy *et al.* 2001; Mannen *et al.* 2004). The bar scale indicates genetic distance by using the substitution model of Tamura and Nei (1993).

DISCUSSION

We have analyzed D-loop sequence for three indigenous Bhutanese *Thrabum* populations and also determined the sequence of the Tibetan *Goleng*, Indian *Jaba* and Nepalese lowland cattle. Our result showed that Bhutanese native cattle have two genetic sources, *B. taurus* and *B. indicus*. The Bhutanese native cattle morphologically resemble *B. indicus* with prominent hump. High altitude environment in Bhutan mountain area and the cooler climate do not offer a selective advantage for the heat-tolerant zebu cattle. However, they also show high altitude adaptability for even 2500 to 3500 m AMSL. In this study, the high proportion of *B. indicus* mtDNA found in the native cattle is consistent with its morphological features (Table 1).

Recently, intimate analyses on the basis of mtDNA were carried out in order to examine origins and diversity of *B. indicus* cattle (Chen *et al.* 2010). The examination of *B. indicus* mtDNA revealed two moderately divergent predominant haplogroups of the clades, I1 and I2 (Lai *et al.* 2006), which were defined separately as Z2 and Z1 described in other articles (Magee *et al.* 2002; Baig *et al.* 2005). The two haplogroups indicated starlike topology strikingly analogous to that seen in *B. taurus* cattle. I1 haplogroup was predominant in East, Southeast and Central Asia, while I2 haplogroup was observed in South Asia with high frequency (Chen *et al.* 2010). In indigenous Bhutanese cattle, the haplotype frequency in the I1 and I2 haplogroups were 0.62 and 0.38, respectively. In addition, almost all of Bhutanese populations had both haplotypes in the I1 and I2 haplogroups with adequate frequencies (Table 1). This result is consistent with that of previous study (Chen *et al.* 2010) and supports that the origin of I2 haplogroup would be traced into Indian subcontinent as described by Chen *et al.* (2010).

Troy *et al.* (2001) identified four *B. taurus* haplogroups (T, T1, T2, T3) in European, African and Near East cattle. The haplogroup distribution showed clear geographic differentiation. The T1 haplogroup was exclusively found in African cattle, while T, T2 and T3 haplogroups were observed in the cattle from Near East and Western Europe. The unique African mtDNA haplotype, which was rare in the Near East, supported separate domestication of *B. taurus* in Africa (Bradley *et al.* 1996; Troy *et al.* 2001). Mannen *et al.* (2004) showed that cattle from North East Asia (Mongolia, Korea and Japan) have T,

T2-T4 *B. taurus* mtDNA haplotype, while *B. indicus* haplotypes were also found in the Mongolian cattle. Since T4 haplotype was not observed in European, African and Near Eastern cattle populations, Mannen *et al.* (2004) suggested the incorporation of East Asian wild ox gene source into the bovine pool, either as a separate domestication or via adoption of female animals into existing herds.

In Bhutanese cattle, three *B. taurus* haplogroups of T1, T3 or T4 were detected in several populations. T3 haplogroup is predominantly distributed in European cattle breeds (Loftus *et al.* 1994; Troy *et al.* 2001). The T3 detected in Bhutanese village *Thrabum* populations may be the recent introgression from introduced Jersey and Brown Swiss breeds of cattle for crossbreeding purpose in Bhutan (Lin *et al.* 2007). However, interestingly, Bhutanese cattle also have T1 or T4 haplotypes which were not included in European breeds. This fact suggests that Bhutanese *B. taurus* mtDNA does not originate from only the recent introgression from European breeds, and indicates that indigenous cattle of Bhutan are admixture of *B. taurus* and *B. indicus* as described in previous studies (Payne & Hodges 1997). In particular, detection of *B. taurus* mtDNA in the purely native populations of Sangbey *Thrabum* and Farm *Thrabum*, which had no contact with recent European breeds, may indicate ancient taurine contribution in the formation of Bhutanese native cattle.

In conclusion, this study indicated that the Bhutanese cattle have measurable genetic frequency of *B. taurus* mtDNA which were not originated from recent genetic introgression of *B. taurus* mtDNA from European breeds. Further studies of Bhutanese cattle using additional individuals and genetic markers would be important to elucidate the formation and genetic construction of native cattle raised in Himalayan Mountain with several environmental conditions.

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