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Full Length Research Paper

Genetic diversity and bottleneck analysis of Yunnan mithun (*Bos frontalis*) using microsatellite loci

Kai-Xing Qu[1,2,3], Sang Ngoc Nguyen[2,4], Zhan-Xing He[3], Bi-Zhi Huang[3], Xi-Ping Yuan[3], Ya-Ping Zhang[2] and Lin-Sen Zan[1,5]*

[1]College of Animal Science and Technology, Northwest A & F University, Yangling, Shaanxi 712100 China.
[2]Yunnan Key Laboratory of Molecular Biology of Domestic Animal & Laboratory of Molecular Evolution and Genome Diversity, Kunming Institute of Zoology, the Chinese Academy of Sciences, Kunming 650223 China.
[3]Yunnan Academy of Grassland and Animal Science, Kunming 650212, China.
[4]Graduate University of Chinese Academy of Sciences, Beijing, 100094, China.
[5]National Beef Cattle Improvement Centre of China, Yangling, Shaanxi 712100, China.

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Mithun or gayal (*Bos frontalis*) is endemic to the Gaoligongshan Mountains and Drung River Basin in Yunnan, China; a rare and endangered *Bos* species. To evaluate the genetic diversity and bottleneck effect of Yunnan mithun population, we screened 16 bovine microsatellite loci of Yunnan mithun (N = 34) to provide genetic information for its conservation strategies and breeding programmes. A total of 126 microsatellite alleles and large allele size variance were detected. All loci displayed low genetic diversity with overall mean of Na = 7.88, polymorphism information content (PIC) = 0.60, and H_E = 0.63, which were the lowest among the beef cattle breeds. Despite the smaller population size, the normal L-shaped distribution of allelic frequencies without any mode-shift indicate the absence of genetic bottleneck in Yunnan mithun population, suggesting that Yunnan mithun population might moderately have underwent gene introgression from zebu and yellow cattle.

Key words: Yunnan mithun (*Bos frontalis*), genetic diversity, bottleneck effect, microsatellite loci.

INTRODUCTION

Mithun or gayal (*Bos frontalis*), a descendent of wild gaur, is found in Bhutan, Myanmar, Bangladesh, Malaysia, Yunnan Province of China and the Northeast Mountains of India (Simoons, 1984; He et al., 2009; Mondal et al., 2010; Rosli et al., 2011). In China, mithun, known as Dulong cattle, is endemic to the rainforests of Gaoligongshan Mountains and Drung River Basin, and feeds on bamboos and leaves of arbors and shrubs. It is a semi-wild rare and endangered bovid species, and mithun was listed in 130th notice of "National Preservation Record of Genetic Resource of Domestic Animal and Poultry" by the Chinese Minister of Agriculture in August, 2000. According to the census data, less than a population of 100 was estimated to have remained in Yunnan province in the 1980s and mithun was also recorded as an endangered species for meat-purpose breed in the *Records of Domestic Livestock and Fowl Breeds of Yunnan Province* (1987). However, the domestic form (*Bos frontalis*) of gaur (*Bos gaurus*) is excluded from the red-listing considerations by the International Union for the Conservation of Nature (IUCN). After some *in-* or *ex-situ* conservation strategies, the population recovered about 3,000 animals in 2006 (Qu et al., 2008). Most of the mithuns with white stockings are reared under the free-range system (Figure 1), and moved seasonally into extensive natural forests (He et al., 2007, 2009).

Microsatellite are useful markers to illustrate the genetic variability and relationship, population structure, bovine admixture and migration pattern in many cattle breeds (MacHugh et al., 1997; Maudet et al., 2002; Pandey et al., 2006; Egito et al., 2007; Wang et al., 2008). Strong evidence supported the distinct bifurcation

*Corresponding author. E-mail: zanls@yahoo.com.cn. Tel: +86-29-87091923.

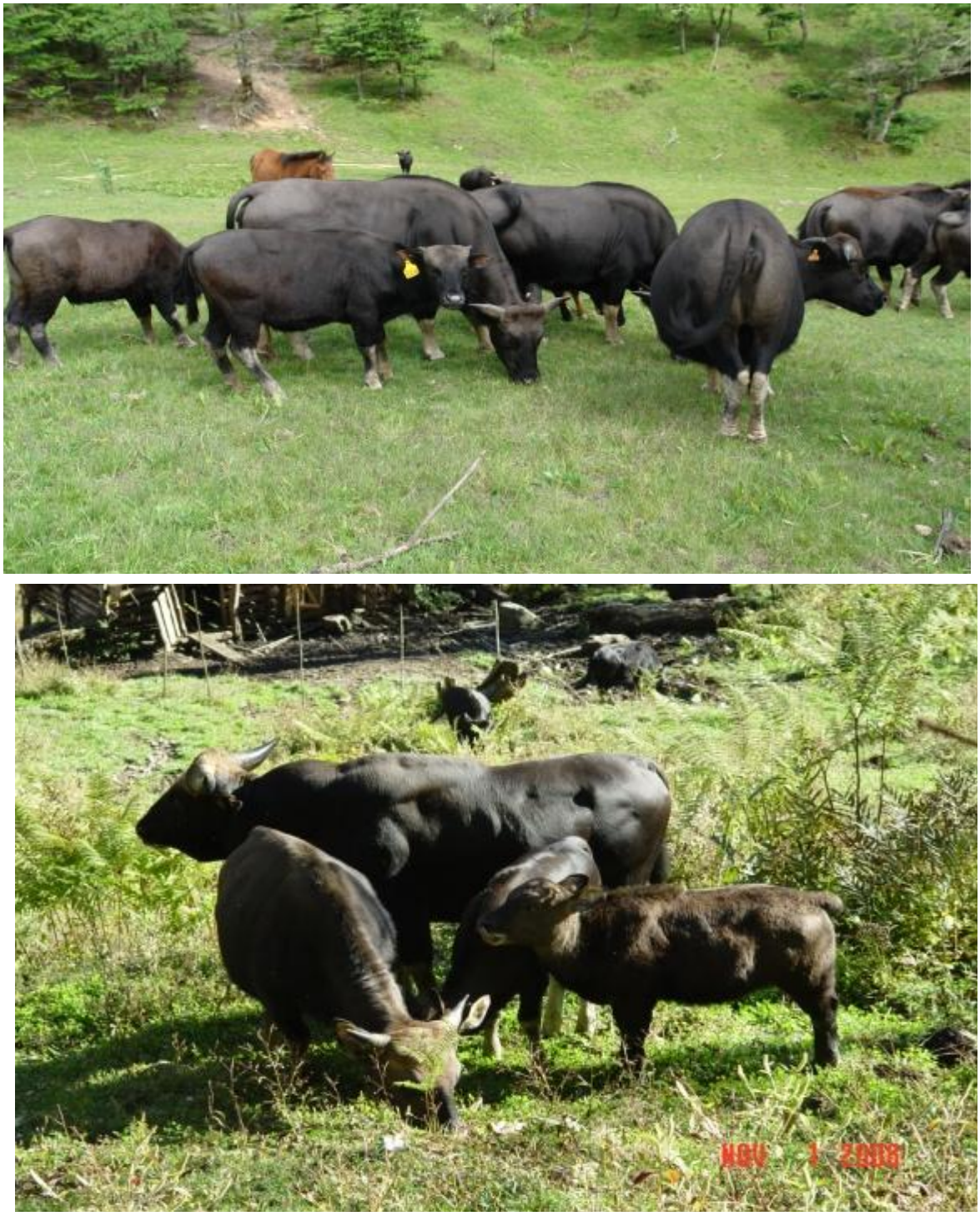

Figure 1. Grazing mithun population in Phoenix Mountains, Yunnan.

between *Bos taurus* and *Bos indicus*, and *Bos taurus* domesticated in Near East while *Bos indicus* (zebu) originated from Indian subcontinent (MacHugh et al., 1997; Loftus et al., 1999; Troy et al., 2001). The huge influx of Indian zebu from Asian to East coast of Africa were inferred from zebu introgression into African cattle which presented zebu morphologies without indicine maternal lineages and inherited an resistance to rinderpest and trypanosomes in accordance with the historic event and extensive trading (MacHugh et al., 1997; Maudet et al., 2002; Freeman et al., 2005). Recently, facing population fragment, inhabitation reduc-

tion and genetic erosion, the genetic diversity and genetic structure of the local breeds are becoming a major concern. Mithun has a genetic complex and shows a monophyletic clade involving concurrent pattern of multi-origin which is strongly concerning gaur and zebu (indicine) or taurine cattle based on maternal lineage (Verkaar et al., 2004; Ma et al., 2007; Li et al., 2008; Dorji et al., 2010; Rosli et al., 2011; Tanaka et al., 2011). A few works have been documented for mithun (*Bos frontalis*) revealed by microsatellite markers (Liao et al., 2008). Herein, the aim this study was to provide new insights into the genetic diversity, bottleneck effect and future management of the extant mithun population in Yunnan Province by scanning bovine microsatellite loci.

MATERIALS AND METHODS

Sampling

A total of 34 mithun blood or ear tissue samples were collected from Phoenix Mountains of Lushui County and Jiumudang Conservation Farm of Gongshan County, Nujiang Prefecture, China. Blood samples (2 to 5 ml) were collected from jugular vein or tail artery and stored in vacutainer tubes containing heparin as anticoagulant.

Microsatellite genotyping

17 microsatellite loci were selected from the database of the Food and Agriculture Organization (FAO) and the International Society for Animal Genetics (ISAG) (Table 1). Forward primers were 5'-labeled with phosphoramidite dye. Polymerase chain reaction (PCR) products were diluted and electrophoresed on an ABI PRISM 3730 DNA analyzer. Allele scoring was performed using Genemapper software version 3.2 with LIZ 500 size standard.

DNA extraction and PCR conditions

The DNA was isolated according to earlier works (Wang and Shi, 1993; Huang et al., 1999) and laboratory manual, then 20 to 50 ng genomic DNA was used as a template for PCR amplification. PCR reactions were carried out in a 10 µl volume comprising 20 to 50 ng/µl total DNA, 1.0 pmol of each primer, 0.125 U Taq polymerase, 0.1 mmol/L of each dNTPs, 1.0 mmol/L $MgCl_2$ and 1.0 µl 10×PCR Buffer. PCR procedure involved initial denaturation at 94°C for 4 min followed by 25 cycles of 30 s at 94°C, 40 s at 52.1 to 60.0°C for annealing temperature, 30 s at 72°C, and a final extension at 72°C for 10 min. PCR products were diluted and mixed with LIZ 500 size standards according to the procedure about microsatellite markers and electrophoresed on an ABI PRISM 3730 DNA analyzer (ABI, USA). Allele scoring was performed using Genemapper software.

Statistical analysis

All loci were collected as an Excel file, and then converted into a computable file through Excel microsatellite Toolkit version 3.1. (http://animalgenomics.ucd.ie/sdepark/ms-toolkit/index.php).
Calculation of allele frequencies, the observed and expected heterozygosity (H_O and H_E), numbers of alleles per locus (Na), and exact test for Hardy-Weinberg equilibrium were performed using the Genepop software (Version 3.4, Raymond and Rousset, 1995). Polymorphism information content (PIC) of each locus was calculated according to Botstein et al. (1980). The alleles were classified into 10 frequency classes, which were used to test whether the distribution followed the normal L-shaped form by using the SMM model (stepwise mutation model) of bottleneck version 1.2.02 (Piry et al., 1999; http://www.ensam.inra.fr/URLB).

RESULTS AND DISCUSSION

Genetic diversity of Yunnan mithun

Of 17 microsatellite loci, TGLA 227 failed to amplify, and 16 polymorphic microsatellite loci were identified in Yunnan mithun. Among the 16 microsatellite polymorphic loci, high allelic size variations and a total of 126 alleles were identified (Table 2). The observed numbers of alleles per locus ranged from 2 (BoLA DR2B) to 12 (BM2113), with an average of 7.88 (Table 3). All loci were highly polymorphic except for BoLA DR2B and ETH10, of which PIC was less than 0.25 (0.16 and 0.11, respectively). The PIC per locus ranged from 0.11 (ETH10) to 0.85 (HAUT24) (Table 3). The overall mean value of H_O (0.53), H_E (0.63), and PIC (0.60) across all the loci was low, indicating that genetic diversity of Yunnan mithun was low (Table 3). Yunnan mithun displayed the lowest heterozygosity among the cattle breeds [Brazilian Holstein (0.74), Jersey (0.71), Nellore (0.72), Gyr (0.72) (Egito et al., 2007), Indian Kherigarh cattle (0.72) (Pandey et al., 2006), French Charolais (0.66), Limousin (0.68), Holstein (0.69) (Maudet et al., 2002), and Chinese Yellow cattle (0.66 to 0.80) (Wang et al., 2008)].

Hardy-Weinberg test

Of the 16 microsatellite loci, four (ETH3, BMS2533 ETH10 and HEL5) deviated from Hardy-Weinberg equilibrium ($P < 0.05$) due to heterozygote deficiency. HEL5 highly deviated from Hardy-Weinberg equilibrium ($P < 0.0001$) because of the presence of null alleles. The deficiency of heterozygotes in Yunnan mithun population should be attributed to several factors involving population substructure, null alleles or assortative mating (individual relatedness) (Nei, 1987). Inbreeding coefficient (F_{IS}) was low (6.26%) for all loci according to Weir and Cockerham (1984) (Table 3). The overall positive value of F_{IS} indicate departures from random mating, which was consistent with the potential intercrossing between mithun and other bovid species such as zebu (*Bos indicus*) and yellow cattle (*Bos taurus*).

Bottleneck effect

A normal L-shaped distribution of allelic frequencies without any mode-shift showed the absence of genetic bottleneck in Yunnan mithun population by the qualitative graphical test (Figure 2), which was similar to the scenarios of Kherigarh cattle, Banni and Nagpuriin buffalo

Table 1. Primer sequences and size range of the 17 microsatellite loci.

Locus	5’ to 3’	Annealing temperature (°C)	Size range (bp)	Chromosome
ETH225 (D9S1)	F: GATCACCTTGCCACTATTTCCTA R: CATGACAGCCAGCTGCTACT	60	134 – 158	9
BMS2533	F: TGAAGTAAGTAAGCACACAAGCA R: TTGATCATCTTTAGGTCCATCC	56	122 – 156	15
ETH10 (D5S3)	F: GTTCAGGACTGGCCCTGCTAACA R: CCTCCAGCCCACTTTCTCTTCTC	58	206 – 216	5
ETH3 (D19S2)	F: GAACCTGCCTCTCCTGCATTGG R: ACTCTGCCTGTGGCCAAGTAGG	60	103 – 127	19
BM2113 (D2S26)	F: GCTGCCTTCTACCAAATACCC R: CTTCCTGAGAGAAGCAACACC	54	121 – 157	2
BM1824 (D1S34)	F: GAGCAAGGTGTTTTTCCAATC R: CATTCTCCAACTGCTTCCTTG	58	180 – 188	1
TGLA126 (D20S1)	F: CTAATTTAGAATGAGAGAGGCTTCT R: TTGGTCTCTATTCTCTGAATATTCC	58	117 – 127	20
TGLA122 (D21S6)	F: CCCTCCTCCAGGTAAATCAGC R: AATCACATGGCAAATAAGTACATAC	54	135 – 165	21
SPS115 (D15)	F: AAAGTGACACAACAGCTTCTCCAG R: AACGAGTGTCCTAGTTTGGCTGTG	58	240 – 256	15
POTCHA	F: GTAAACACAGTTCCCTGGAGAG R: ATGCCAACTTTTCCCATCAC	59	135 – 151	15
ILSTS006 (D7S8)	F: TGTCTGTATTTCTGCTGTGG R: ACACGGAAGCGATCTAAACG	56.5	274 – 300	7
HEL5 (D21S15)	F: GCAGGATCACTTGTTAGGGA R: AGACGTTAGTGTACATTAAC	55	138 – 164	21
HAUT24 (D22S26)	F: CTCTCTGCCTTTGTCCCTGT R: AATACACTTTAGGAGAAAAATA	52.1	106 – 132	22
INRA023 (D3S10)	F: GAGTAGAGCTACAAGATAAACTTC R: TAACTACAGGGTGTTAGATGAACTC	58	196 – 214	3
BoLA DR2B	F: AGGCAGCGCCGAGGTGAGCGA R: TCCAACACTCACCTGGACGTAGC	60	144 – 152	23
BoLA DRBPl	F: ATGGTGCAGCAGCAAGGTGAGCA R: GGGACTCAGTCTCTCTATCTCTTT	55	110 – 132	23

Table 1. Continues.

TGLA227* (D18S1)	F: CGAATTCCAAATCTGTTAATTTGCT R: ACAGACAGAAACTCAATGAAAGAC	56	77 – 105	18

* TGLA227 failed to amplify.

Table 2. The allelic frequencies of the 16 microsatellite loci in Yunnan mithun population.

Loci	Allelic frequency		Loci	Allelic frequency		Loci	Allelic frequency	
	134	2.86		117	5.71		106	7.14
	136	4.29		119	72.86		112	4.29
	138	1.43	TGLA126	123	1.43		114	1.43
	144	4.29		125	18.57		116	21.43
	146	1.43		127	1.43		118	17.14
ETH225	150	5.71				HAUT24	120	17.14
	152	7.14					124	7.14
	154	60.00					126	8.57
	156	10.00					128	8.57
	158	2.86					130	1.43
							132	5.71
	122	15.71		135	1.43		196	4.29
	124	35.71		141	1.43		198	5.71
	126	4.29		143	4.29		200	14.29
	130	4.29		147	1.43		202	35.71
	132	7.14		151	7.14		204	2.86
BMS2533	138	7.14	TGLA122	153	1.43	INRA023	206	2.86
	140	2.86		157	5.71		208	21.43
	156	22.86		159	18.57		210	1.43
				161	10.00		212	10.00
				163	27.14		214	1.43
				165	21.43			
	206	94.29		240	1.43		110	1.43
	208	1.43		242	1.43		118	7.14
	210	1.43		244	7.14		120	44.29
	216	2.86		246	1.43	BoLA	124	4.29
ETH10			SPS115	250	54.29	DRBP1	126	11.43
				252	1.43		128	20.00
				254	17.14		130	4.29
				256	15.71		132	7.14
	103	1.43		135	37.14		180	7.35
	105	2.86		137	1.43		182	14.71
	111	4.29	POTCHA	139	2.86	BM1824	186	73.53
	113	5.71		141	55.71		188	4.41
ETH3	115	31.43		151	2.86			
	117	11.43						
	123	5.71						
	125	10.00						
	127	27.14						

Table 2. Continues.

	121	58.57		274	5.71		138	3.13
	123	1.43		276	37.14		144	1.56
	125	4.29		278	5.71		150	31.25
	127	7.14		280	2.86		152	26.56
	129	5.71		284	11.43		154	15.63
BM2113	131	1.43	ILSTS006	286	7.14	HEL5	156	12.50
	133	1.43		290	4.29		162	7.81
	135	10.00		292	5.71		164	1.56
	137	2.86		296	5.71			
	139	2.86		298	2.86			
	141	2.86		300	11.43			
	157	1.43						
BoLA DR2B	144	10.00						
	152	90.00						

Table 3. Genetic characteristics of 16 microsatellite loci in Yunnan mithun population.

Locus	Na	H_O	H_E	PIC	H-W	F_{IS}
BoLA DR2B	2	0.20	0.18	0.16	0.77	-0.0549
BM1824	4	0.35	0.44	0.40	0.92	-0.1289
SPS115	8	0.54	0.65	0.61	0.70	-0.1287
TGLA126	5	0.28	0.44	0.39	0.06	0.2512
BoLA DRBP1	8	0.77	0.75	0.71	0.60	0.0121
ILSTS006	11	0.71	0.83	0.80	0.74	-0.0560
HAUT24	11	0.68	0.88	0.85	0.10	0.0649
ETH3	9	0.60	0.81	0.77	0.02*	0.1869
TGLA122	11	0.60	0.84	0.80	0.81	-0.0841
ETH225	10	0.71	0.62	0.60	0.80	-0.0474
BMS2533	8	0.54	0.79	0.75	0.01*	0.1180
ETH10	4	0.08	0.11	0.11	0.02*	0.2732
INRA023	10	0.66	0.80	0.76	0.21	0.0053
POTCHA	5	0.51	0.56	0.46	0.96	-0.1071
HEL5	8	0.66	0.80	0.75	0.00**	0.6471
BM2113	12	0.60	0.64	0.62	0.29	0.0490
Mean	7.88	0.53	0.63	0.60	-	0.0626

Na, numbers of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; PIC, polymorphism information content; H-W, significance of deviation from Hardy-Weinberg equilibrium; * significant at the level of 5%; ** significant at the level of 1‰; F_{IS}, value evaluated according to Weir and Cockerham (1984).

in India (Pandey et al., 2006; Mishra et al., 2009; Kataria et al., 2009). Though Yunnan mithun population had relatively lower heterozygosity and smaller population size, there was no signal of severe population bottleneck in the recent past. The reasons could be the common potentials of intercrossing between mithun and other bovid species (Winter et al., 1988; Phanchung and Roden, 1996; He et al., 2008; Qu et al., 2008; Gou et al., 2010; Tanaka et al., 2011; Baig et al., personal communication). The results suggest that Yunnan mithun population might have underwent moderate gene introgression from local zebu and yellow cattle.

These results support the usefulness of bovine microsatellite markers in evaluating genetic diversity and genetic structure of *Bos* species. The genetic information of mithun in this study would be quite helpful to illuminate

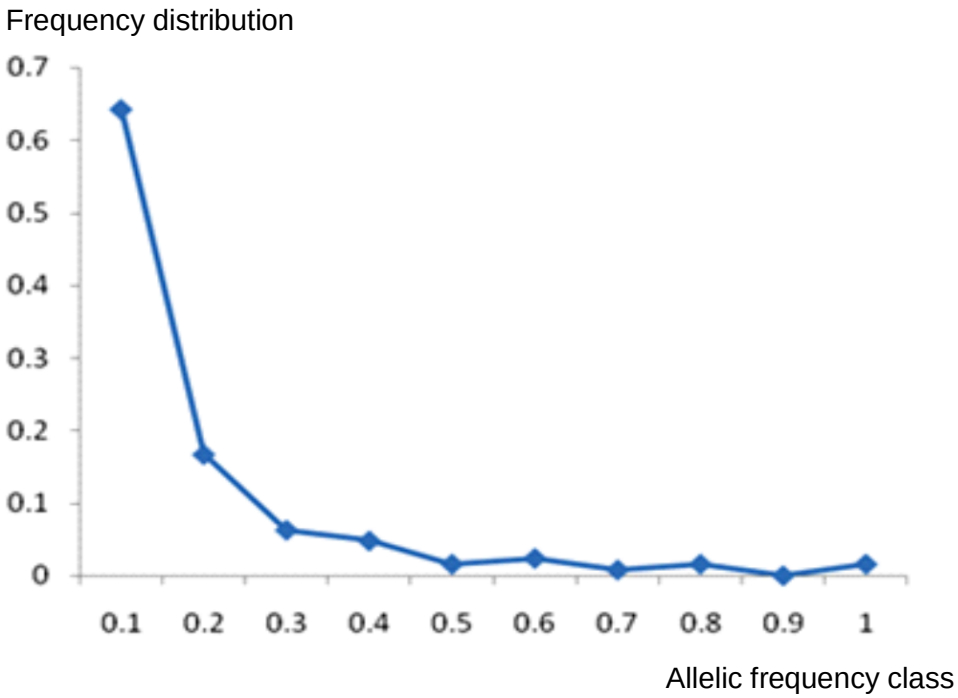

Figure 2. Mode-shift test for bottleneck analysis in Yunnan mithun population.

its phylogeny, and to plan the conservation strategies and breeding programmes in Yunnan Province.

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