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Full Length Research Paper

Genetic diversity analysis of DRB3.2 in domestic yak (*Bos grunniens*) in Qinghai-Tibetan Plateau

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DRB3 gene has been extensively evaluated as a candidate marker for association with many bovine disease and immunological traits. A hemi-nested polymerase chain reaction-sequencing method was used to investigate the polymorphisms of DRB3.2 gene from 209 individuals in three different domestic yak (*Bos grunniens*) populations (62 Tianzhu white yaks, 78 Gannan yaks and 69 Datong yaks) from the Qinghai-Tibetan Plateau. Sixty-three polymorphic sites and 143 haplotypes were detected. The percentage of polymorphic sites in Gannan Yak (GNY), Tianzhu white Yak (TWY) and Datong Yak (DTY) were 21.80, 29.95 and 12.95%, while the haplotype diversity were 0.9987, 0.9984 and 0.9855, respectively. At the amino acid level, Glu had the highest content; the percentage was 12.326%, followed by Arg (10.315%), Phe (10.804%), Val (8.346%), Gly (8.315%), Leu (6.606%) and Ala (5.851%), whereas Met and Ile were below than 1%. Only 19 amino acids were found in DTY, Met was lost. Among the synonymous codons, whose third base was G and/or C had a higher usage frequency. Most variability were found in amino acid residues 11, 13, 26, 28, 30, 32, 37, 56, 57, 59, 60, 61, 67, 70, 71, 72, 73 and 74. In GNY, the residues at positions 71, 11 and 72 were highly polymorphic with 8, 7 and 7, at 50, 58, 70, 74 and 78, the residues were selectively polymorphic than other yak populations; the other polymorphic sites were common in the populations. The results of this study indicated that the Chinese domestic yak populations in the Qinghai-Tibetan Plateau have abundant polymorphism in DRB3.2, and the GNY was the highest, followed by TWY and DTY.

Key words: Domestic Yak, Hemi-nested PCR, BoLA-DRB3.2, polymorphism.

INTRODUCTION

The bovine lymphocyte antigen (BoLA) system is the major histocompatibility complex (MHC) of cattle. The genes located in the MHC class II region encode glycoproteins that are composed of α- and β-chains that bind exogenous peptides within the cell and present them to CD4-positive T helper cells (Banchereau and Steinman, 1998). The immunological importance of the MHC genes and their possible role in disease resistance have been a major impetus for research on the MHC system in cattle denoted BoLA. The MHC class II genes in cattle have been shown to be similar to those of humans in structure (Bensaid et al., 1991). So far, a single DRA locus and three DRB loci, two DQA and DQB loci, and single DOB, DNA, DYA, DYB and DIB loci have been characterized (Andersson et al., 1986a, b, 1988a; Andersson and Rask, 1988b; Stone and Muggli-Cockett, 1990). DR and DQ have been identified as the two-principal class II molecule in ruminants. In DR sub-region of cattle, at least three different DRB loci have been described along with pseudogene and gene fragments (Ellis and Ballingall, 1999). However, DRA and DRB3

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have been found as major expressed gene pair (Lewin et al., 1999). Furthermore, DRB3 has been found to be highly polymorphic and it is responsible for the difference in the susceptibility to infectious disease. Polymorphism of BoLA-DRB3 is confined mainly to the second exon that encodes for β1 domain, responsible for peptide-binding sites (Sachinandan De et al., 2011).

Yak species (*Bos grunniens*) is the most important grazing livestock for beef and milk productions on the Qinghai-Tibetan Plateau, as it represents a unique bovine species adapted to the Tibetan Plateau of China at altitudes of 3 000 m above sea level, where oxygen content is only 33% of that at sea level and the intensity of ultraviolet radiation is 3 to 4 times that in lowland areas (Storz et al., 2010). Consequently, due to natural selection adapted to such environment, yak likely have special physiological mechanisms of resistance to infectious diseases because they are usually not artificially immunized. Recent studies have demonstrated that the MHC allele diversity is associated with the ability to recognize a large number of antigens, resulting in a more efficient immune response (Behl et al., 2007; Fernández et al., 2008). Analysis of the BoLA-DRB3 gene is of special interest at least for two reasons: a high functional importance of the gene (one of the key genes control bacterial infections) and a high level of polymorphism (Ali and Abbas, 2011). So it may be interesting to assess the level of allele diversity in the BoLA-DRB3 gene in a population that is under great pressure for survival under tough conditions, such as the domesticated yak populations in the Qinghai-Tibetan Plateau. In addition, this polymorphism can be used to study the genetic relationships between populations and to assess their levels of genetic differentiation.

Presently, extensive information is available on the levels of genetic diversity of exon 2 of the DRB3 gene in different populations of cattle obtained by amplification of this segment by PCR, and subsequent digestion with endonucleases (Gilliespie et al., 1999; Mota et al., 2004; Behl et al., 2007). However, information on DRB3.2 gene and its polymorphisms in domestic yak still remains very scarce. The objective of this study was to investigate the level of genetic diversity present in the BoLA-DRB3.2 locus in three domestic yak populations from Qinghai-Tibetan Plateau in Northwest China.

MATERIALS AND METHODS

Sample collection and genomic DNA isolation

Blood samples were obtained from 209 yaks belonging to three Chinese domestic yak populations: Tianzhu White Yak (TWY, n = 62), Gannan Yak (GNY, n = 78) and Datong Yak (DTY, n = 69). Approximately 10 ml of blood was collected from each animal via the jugular vein. The whole blood was preserved in acid citrate dextrose solution and stored at -70°C. Genomic DNA was isolation from the blood by the Relax Gene Blood DNA System (TIANGEN Biotech, China).

Amplification of DRB3.2

A hemi-nested PCR method was used to amplify the DRB3.2 gene by using the primers published by Van Eijk et al. (1992). The oligonucleotide primers HL030 (5′-ATCCTCTCTCTGCA-GCACATTTCC-3′), HL031 (5′-TTTAAATTCGCGCTCACC-TCGCCGCT-3′) and HL032 (5′-TCGCCGCTGCACAGTGAAAC-TCTC-3′) were used in the polymerase chain reaction (PCR). The first round PCR was carried out in a final volume of 25 µL containing: 12.5 µL 2×Hotstart Taq PCR Master Mix, 0.5 mM of each HL030 and HL031 primers, 100 ng of DNA, and ddH_2O up to 25 µL. The cycling conditions were as follows: an initial denaturation step of 4 min at 95°C followed by 10 cycles of 1 min at 94°C, 2 min at 60°C, and 1 min at 72°C. The last polymerization step was extended for 10 min at 72°C.

Briefly, 3 µL of first-round reaction product was transferred to a new tube with 50 µL of PCR buffer containing primers HL030 and HL032. Primer HL032 is internal to the sequence of the amplified product of the first-round PCR and has eight bases that overlap with primer HL031 (underlined in the text above) and 25 µL of 2×Hotstart Taq PCR Master Mix at the same concentrations as described above. The cycling conditions for the second round of PCR were as follows: 30 cycles of 45 s at 94°C and 90 s at 65°C as the annealing extension step, followed by a final extension step of 5 min at 72°C and conserved at 4°C.

Sequence preparation and genetic analysis

All of the DNA samples were purified by using a TIANgel Midi Purification Kit (TIANGEN Biotech, China), then tested and entrusted to Sangon Biotech Co., Ltd. (Shanghai, China) for sequencing. The results were corrected by comparing with the sequencing peak map; all the sequences were blasted with the DRB3.2 gene of yak sequence, and the inaccurate and non-coding fragments of the gene were deleted. The BioEdit program v7 (Hall TA, 1999.) was used to ClustalW multiple alignment. The DnaSP program v5 (Librado and Rozas, 2009) was used to analyze DNA polymorphism and haplotype. The MEGA program v5 (Kumar et al., 1993) was used to calculate the relative frequencies of non-synonymous (dy) and synonymous substitutions (ds) according to Nei and Gojobori (1986), Jukes and Cantor's (1969) correction was applied for multiple hits. The peptide binding groove was according to the model of Brown et al. (1993).

RESULTS

The result of polymerase chain reaction

Using the primers HL030, HL031and HL032 to amplify yak DRB3.2, we got a 284 bp segment (Figure 1), blast with the DRB3.2 gene of yak (download from the GenBank) found that it is the DRB3.2 gene of yak.

The nucleotide diversity of DRB3.2 in yak

Polymorphic loci variation

In the 209 animals tested, 63 polymorphic sites in BoLA-DBR3.2 were indentified (Table 1). The percentage of polymorphic sites is 31.50%, singleton variable sites are 17 (26.98%), and parsimony informative sites are 46

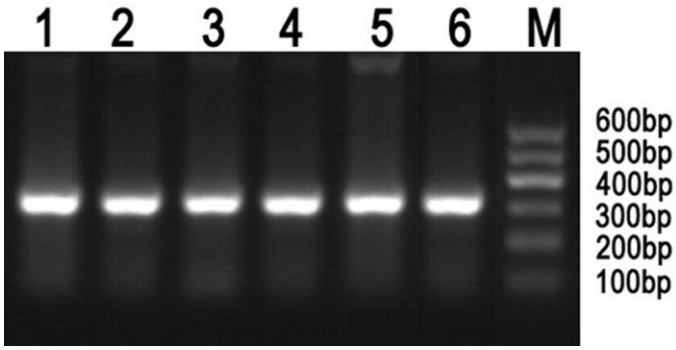

Figure 1. The result of polymerase chain reaction. Lanes 1 to 6, PCR product; lane M, 100 bp DNA ladder marker.

Table 1. Polymorphic information of DRB3.2 in yak.

Populations	N	P (%)	SP	PIP
TWY	62	29.95	21	41
DTY	29	12.95	14	15
GNY	46	21.80	2	44
Yak	63	31.50	17	46

N, Number of polymorphic sites; P, percentage of polymorphic sites; SP, single polymorphic sits; PIP, parsimony informative polymorphic sites.

Table 2. Transition and transversion of DRB3.2 in three yak populations.

Population		A	T	C	G	N	C*	N/C*
DTY	A	-	*5.94*	*7.37*	**7.69**	38.85	61.14	0.64
	T	*6.7*	-	**14.55**	*10.56*			
	C	*6.7*	**11.73**	-	*10.56*			
	G	**4.88**	*5.94*	*7.37*	-			
GNY	A	-	*4.42*	*6.02*	**11.81**	52.81	47.19	1.12
	T	*5.12*	-	**19.32**	*8.04*			
	C	*5.12*	**14.16**	-	*8.04*			
	G	**7.52**	*4.42*	*6.02*	-			
TWY	A	-	*5.18*	*7.05*	**9.38**	43.24	56.76	0.76
	T	*6.45*	-	**15.92**	*9.7*			
	C	*6.45*	**11.71**	-	*9.7*			
	G	**6.23**	*5.18*	*7.05*	-			

Each entry shows the probability of substitution (r) from one base (row) to another base (column). For simplicity, the sum of r values is made equal to 100. Rates of different transitional substitutions are shown in bold and those of transversionsal substitutions are shown in italics. The transition/transversion rate ratios are k1 = 0.906 (purines) and k2 = 2.175 (pyrimidines). The overall transition/transversion bias is R = 0.689, where R = [A*G*k1 + T*C*k2]/ [(A+G)*(T+C)]. N is transition and C* is transversion.

(73.02%). The transition and transversion of DRB3.2 in the three yak populations are shown in Table 2. In DTY and TWY, transversion ratio was higher than transition, in GNY this ratio is contrary, and T/C variance is the major variation in transition.

Haplotype distribution

For the 209 sequences, 143 haplotypes were detected (Table 3), and GNY had the highest haplotype diversity, while DTY had the lowest.

Table 3. Haplotype diversity of DRB3.2 in yak.

Population	N	Np	Pi (%)	K	HD
DTY	69	54	2.155	4.806	0.9855
GNY	78	74	5.530	11.667	0.9987
TWY	62	59	4.117	8.481	0.9984
Yak	209	143	3.594	7.188	0.9843

N, Sample size; Np, no. of haplotype; Pi, nucleotide diversity; K, average number of nucleotide differences; HD, haplotype diversity.

Table 4. The net genetic distance (Da) and nucleotide divergence (Dxy) among three yak populations.

Population	DTY	GNY	TWY
DTY	-	0.0057	0.0029
GNY	0.0406	-	0.0013
TWY	0.0302	0.0446	-

Above diagonal is the net genetic distance (Da) and below diagonal is the nucleotide divergence (Dxy).

Nucleotide divergence and net genetic distance

Among the three yak populations, GNY and DTY have the biggest net genetic distance, whereas GNY and TWY is the smallest (Table 4). The biggest nucleotide divergence was between TWY and GNY, while the smallest was between TWY and DTY.

The amino acid polymorphism of DRB3.2 in yak

Amino acid constitute

The amino acid constituents in the three yak populations are very different. There are 20 amino acids in GNY and TWY, but DTY only have 19 ones (Met is lost). Glu has the highest percentage (12.326%), followed by Arg, Phe, Val, Gly, Leu and Ala, with percentage of 10.3151, 10.804, 8.3463, 8.3151, 6.606 and 5.8512%, respectively. Met and Ile were below 1%.

Amino acid variation

The frequencies of codon usage in DRB3.2 among the three yak populations are different. For synonymous codons, the ones whose third base is G and/or C have a higher usage frequency (Table 5). The sharing of DRB3.2 polymorphism at the amino acid level found in yak populations is presented in Table 6. Most variability were found in amino acid residues at sites 11, 13, 26, 28, 30, 32, 37, 56, 57, 59, 60, 61, 67, 70, 71, 72, 73 and 74. In GNY, amino acid residues at positions11, 71 and 72 were highly polymorphic with 7, 8 and 7 amino acids. However, residues at sites 50, 58, 70, 74, 78 showed more selectively polymorphic than other yak populations. The amino acids for other polymorphic sites were common in the experimental populations. The level of polymorphism was the highest in GNY, followed by TWY and DTY.

DISCUSSION

Information about bovine MHC polymorphisms is important in the beef and dairy industry since MHC contributes substantially to fitness and resistance/ susceptibility to disease (Ripoli et al., 2004). Therefore, we used a hemi-nested PCR-sequencing method to detect polymorphisms in Chinese domestic yak populations from Qinghai-Tibetan Plateau. PCR products were represented by a 284 bp fragment that was expected on the basis of the nucleotide sequence of the gene (Figure 1).

Polymorphic loci variations analyses demonstrated that the yak DRB3.2 locus had a high degree of polymorphism. Li et al. (2005) indicated that the polymorphism of yak DRB3 exon 2 in 24 Chinese domestic yaks was rich, and they also observed 115 polymorphic sites in sequence of 234 bp segment and the percentage of polymorphism loci was 49.15%. The results were similar to our findings. Moreover, comparing to other bovine breeds, the DRB3.2 gene of yak was almost similar in the Holstein herds studied by other researchers (Sofia and Leif, 1995; Gelhaus et al., 1995).

The haplotype analysis of the three Chinese yak populations showed that GNY has the highest haplotype diversity, nucleotide divergence and net genetic distance, followed by TWY and DTY. The reason for this result may be that as GNY is an original breed and artificial selection is focus on the older, obvious physical defects and frail individuals, so the selective pressure is very small. The DTY is a bred variety, and in the process of breeding formation, artificial selection plays a leading role, not only concerned about body appearance, but also on high adaptability, disease resistance and production performance. Hence, they faced a greater pressure in artificial selection.

Table 5. The codon usage frequency of yak.

Codon	Count	RSCU	Codon	Count	RSCU	Codon	Count	RSCU	Codon	Count	RSCU
UUU(F)	0	0.01	UCU(S)	0.1	0.23	UAU(Y)	2	0.96	UGU(C)	1.5	1.19
UUC(F)	8.4	1.99	UCC(S)	0.1	0.23	UAC(Y)	2.1	1.04	UGC(C)	1	0.81
UUA(L)	0	0	UCA(S)	0	0	UAA(*)	0	0	UGA(*)	0	0
UUG(L)	0.1	0.18	UCG(S)	0	0.03	UAG(*)	0	3	UGG(W)	2	1
CUU(L)	0	0	CCU(P)	0	0	CAU(H)	2.3	1.18	CGU(R)	0	0.03
CUC(L)	0	0.02	CCC(P)	0	0	CAC(H)	1.6	0.82	CGC(R)	1	0.67
CUA(L)	0.1	0.15	CCA(P)	0	0	CAA(Q)	0	0.02	CGA(R)	0	0
CUG(L)	3.9	5.65	CCG(P)	0.9	4	CAG(Q)	1.6	1.98	CGG(R)	5.6	3.83
AUU(I)	0	0	ACU(T)	0	0	AAU(N)	1.3	0.67	AGU(S)	0	0
AUC(I)	0.3	3	ACC(T)	2	2.71	AAC(N)	2.6	1.33	AGC(S)	2.9	5.52
AUA(I)	0	0	ACA(T)	0	0	AAA(K)	0	0.01	AGA(R)	2	1.38
AUG(M)	0	0	ACG(T)	1	1.29	AAG(K)	3.9	1.99	AGG(R)	0.1	0.09
GUU(V)	0	0	GCU(A)	0	0.04	GAU(D)	0	0	GGU(G)	0	0
GUC(V)	0.7	0.57	GCC(A)	2.1	2.7	GAC(D)	5.1	2	GGC(G)	1	1
GUA(V)	0	0	GCA(A)	0	0	GAA(E)	1	0.21	GGA(G)	1	0.99
GUG(V)	4	3.43	GCG(A)	1	1.26	GAG(E)	8.3	1.79	GGG(G)	2	2.01

All frequencies are averages over all taxa. The relative synonymous codon usage (RSCU) is given in parentheses following the codon frequency; the numbers in the box means the usage frequency of this synonymous codon is high than others.

Mammalian genomes are highly heterogeneous in base composition (Laurent et al., 2002). A strong synonymous codon usage biased towards the codons ending at C or G was observed in DRB3.2 genes (Table 5). Codon analysis of a variety of biology confirmed that the composition limits (Bulmer, 1991), transcription selection (Sharp et al., 1988), tRNA abundance (Pan and Fu, 2001), mutation pressure (Pan and Fu, 2001), gene function (Ma et al., 2002; Comeron, 2004), protein secondary structure (Gu et al, 2004; Kahali et al., 2007), gene length (Marais and Duret, 2001; Miyasaka, 2002) and CpG islands (Scaiewicz et al., 2006; Woo et al., 2007) are factors that form codon bias. In our study, high GC3s content was mainly due to an important factor in keeping the structure and function of gene under selection constraint.

Extensive polymorphism was also revealed in the peptide-binding amino acid region in yak populations. Out of all peptide-binding sites, in position 71, eight different amino acids were encountered followed by seven amino acids in the position 11. In the non peptide-binding region position 72, 74 and 57 were found to be highly variable, containing seven, six and six amino acid substitutions. Sachianandan et al. (2011) analyzed the variation of allelic forms of MHC-DRB3.2 of cattle and buffalo and compared the variation with sheep, goats and other ruminant species. They found that in peptide-binding region (PBR), positions 37 and 11 encountered seven and six amino acids. Furthermore, in the non peptide-binding region, positions 57 and 67 were found containing four amino acid substitutions. In this study, we found a high ratio of non-synonymous substitution to synonymous substitution in PBR, and this high ratio indicates that non-synonymous sites evolved faster than synonymous sites and implies balancing selection favored new variants and increased allelic polymorphism (Bergstrom and Gyllensten, 1995). The pattern and level of DRB3.2 polymorphism revealed in our study could be a consequence of adaption to the cold and oxygen-deficient climate of Qinghai-Tibetan Plateau, and lack or abundance of the forage that relatively changes with the seasons.

High polymorphism of DRB3 gene promotes its use as a highly informative marker in molecular genetics and phylogenetic studies. The functional roles of DRB3 gene are different. There were many works have been done in other mammals, such as the relationship between BoLA-DRB3 gene and resistance/susceptibility to persistent lymphocytosis caused by bovine leukemia virus (Xu et al., 1993; Udina et al., 2003), mastitis caused by *Staphylococcus* sp. (Rupp et al., 2007; Zahra et al., 2003), and other diseases (Maillard et al., 1996; Lewin et al., 1999). Previous studies showed that many diseases observed in cattle were also reported in yak, and it appears that the incidences of some diseases may be high and this is attributed to lack of economic incentive for prevention and treatment in many cases (Gerald et al., 2003). But in our previous study, we found that the prevalence of yak is obviously smaller than cattle (unpublished data).

Up to now, the relationship between the polymorphism of MHC and the disease resistance in yak is still unknown. Therefore, the understanding of MHC diversity could be very useful, and our future work is to discover

Table 6. Comparison of polymorphic amino acid substitutions for DRB3.2 molecules in yak populations.

Code position	Amino acid of TWY	Amino acid of GNY	Amino acid of DTY	Code position	Amino acid of TWY	Amino acid of GNY	Amino acid of DTY
6	H	H	H	*28	DHE	DHE	DH
7	F	F	F	29	RS	R	R
8	LF	L	L	*30	YCHSQ	YCSHP	Y
*9	EQ	EQ	E	31	FY	FY	F
10	Y	Y	Y	*32	YHN	YHN	YHN
*11	SYCHR	SYCHRFA	SYCHR	33	N	N	N
12	KT	KT	KT	34	G	G	G
*13	SRGK	SRGK	SR	35	E	E	E
14	E	E	E	36	E	E	E
15	C	C	C	*37	FYVT	FYSNTL	F
16	H	H	H	*38	V	V	V
17	F	F	F	39	R	R	R
18	F	F	F	40	F	F	F
19	N	N	N	41	D	D	D
20	G	G	G	42	S	S	S
21	T	T	T	43	D	D	D
22	E	E	E	44	W	W	W
23	R	R	R	45	GD	GD	G
24	VL	VL	V	46	E	E	E
25	R	R	R	*47	FY	FY	F
26	FLY	FLY	FY	48	R	R	R
27	L	L	L	49	AP	A	A
50	V	VL	V	67	FIL	FILTS	FILT
51	T	T	T	68	L	L	L
52	E	E	E	69	E	E	E
53	LE	L	L	*70	REQ	REQG	REQ
54	G	G	G	*71	EGRK	EGRKANQT	ERK
55	RQ	RQ	R	72	ARGES	ARGESDT	ARS
*56	PRQ	PRQ	PRQ	73	ARG	ARG	AG
57	ADV	ADVSFP	ADVF	74	ENK	ENKATS	ENK
58	A	APT	A	75	V	V	V
59	EK	EKSVD	EK	76	D	D	D
*60	HYSQ	HYSQTA	HYSQ	77	TR	TR	T
*61	WCLR	WCL	W	78	Y	YV	Y
62	N	N	N	79	C	C	C
63	S	S	S	80	R	R	R
64	Q	Q	Q	81	H	H	H
65	K	K	K	82	KN	KN	KN
66	DE	DE	D				

the relationship between them.

Conclusion

The results obtained from the current study suggest that the Chinese domestic yak populations also have abundant polymorphism in DRB3.2, and GNY was the highest, followed by TWY and DTY, thus suggesting an unfavorable state of the DTY population that is probably caused by inbreeding depression due to a long-term isolation and a small population size.

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