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Insight into gene evolution within Cervidae and Bovidae through genetic variation in MHC-DQA in the black muntjac (*Muntiacus crinifrons*)

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ABSTRACT. The critical role that the major histocompatibility complex plays in the immune recognition of parasites and pathogens makes its evolutionary dynamics exceptionally relevant to ecology, population biology, and conservation studies. The black muntjac is a rare deer endemic to a small mountainous region in eastern China. We found that this species has two *DQA* loci through cDNA expression and sequence variation analysis. The level of variation at both *DQA* loci was found to be extremely low (three alleles for *DQA1* and four alleles for *DQA2*), possibly because of past bottlenecks and the species' relatively solitary behavior pattern. The ratio of d_N/d_S in the putative peptide binding region of the *DQA2* locus (13.36, P = 0.012) was significantly larger than one but not that of *DQA1* (0.94, P = 0.95), suggesting strong positive selection at the *DQA2* but not at the *DQA1* locus. This difference might reflect different sets of evolutionary selection pressures acting on the two loci. The phylogenetic tree showed that *DQA1* alleles from two species of Cervidae and two of Bovidae grouped together, as did the *DQA2* alleles. However, different genes from the four species were located in separate branches. These results lead us to suggest that these *DQA* alleles are derived from primordial *DQA* genes

from a common ancestor and are maintained in Cervidae and Bovidae since their divergence around 25.5-27.8 million years ago.

Key words: Black muntjac; MHC; DQA; Trans-species evolution; Positive selection

INTRODUCTION

The major histocompatibility complex (MHC) consists of a group of closely linked genes that form one of the most important genetic systems in vertebrates. These genes encode MHC class I and II molecules that bind antigens derived from pathogens and parasites and present them to T-lymphocytes, which trigger appropriate immune responses (Hughes and Yeager, 1998). The critical role that the MHC plays in the immune recognition of parasites and pathogens renders it evolutionarily relevant in a dynamic fashion to ecology, population biology, and conservation (Piertney and Oliver, 2006). Genes within the MHC that are involved in antigen presentation are usually highly polymorphic, and those polymorphic sites are mainly confined to the codons forming the peptide-binding region (PBR) (Piertney and Oliver, 2006). It has been documented that MHC gene diversity is usually affected by many ecological factors, such as population bottlenecks, demographics, environment, and social structure (Piertney and Oliver, 2006; Cutrera and Lacey, 2006).

The MHC class II genes encode polymorphic cell-surface glycoproteins comprising non-covalently linked α and β subunits. These play a pivotal role in the initiation of the immune response to pathogen-derived peptide antigens (Hughes and Yeager, 1998). An increasing number of studies have examined diversity at MHC class II loci in different species so as to investigate the ecological repercussions of this diversity on resistance to parasites (Bernatchez and Landry, 2003). The second exon has been shown to be highly polymorphic and under positive selection, and the class II DQA gene has recently attracted more attention (Bryja et al., 2006; Cutrera and Lacey, 2006; Amills et al., 2008; Goüy et al., 2009). In artiodactyls, research on the DQ gene structure and polymorphisms of economically important animals such as cattle and sheep is remarkably extensive (Ballingall et al., 1997; Hickford et al., 2000, 2004; Zhou and Hickford, 2004). In contrast, studies on MHC class II genes in deer is largely restricted to the DRB locus (Van Den Bussche et al., 1999), only rarely considering the DQ gene (Wan et al., 2011). Furthermore, all these studies have focused on deer of the genera Cervinae and Odocoileinae, leaving polymorphism patterns and evolutionary mechanisms of MHC genes in the Muntiacinae unresolved.

The black muntjac (*Muntiacus*, Muntiacinae, Cervidae) is a rare deer endemic to eastern China. Because of its unique karyotype (2n = 8♀/9♂) and the phylogenetic position that it holds with other muntjac species, it is considered a valuable model for studying karyotypic and genomic evolution (Yang et al., 1997; Wang and Lan, 2000). Unfortunately, due to habitat fragmentation, illegal poaching, etc., the species is currently distributed in a confined mountainous region of southern Anhui and western Zhejiang (Sheng, 1987). Based on a survey of fur harvesting in the early 1980s, the population was estimated to be only 7000-8500 individuals in the wild (Sheng, 1987). For conservation purposes, a captive population of the species was established in the Hefei Wild Animal Park (Hefei, Anhui Province) in the late 1980s (Wu and Fang, 2005). Neutral genetic marker analysis suggests that the genetic diversity of the captive population was much lower than that of wild stocks, and that the founders had caused a large difference in the genetic balance of later generations (Wu and Fang, 2005; Ni et al., 2009). In recent years, the population has been af-

fected by gastrointestinal disease caused by parasites and has gone into decline. MHC diversity is adaptively significant in disease resistance, and high diversity has been shown to allow response to a wider range of parasites and pathogens than low diversity. The association between resistance to endoparasites and MHC II locus has been demonstrated by previous studies (Spurgin and Richardson, 2010). Consequently, MHC data are particularly pertinent to black muntjac conservation. Prior to the present study, little was known about the MHC of black muntjac.

Recently, we successfully amplified the *DQA2* exon 2 of the black muntjac according to the method described by Ballingall et al. (1997) through a two-step procedure, and obtained four distinct *DQA2* alleles from 40 individuals (Zhu et al., 2011). However, the primer sets failed to amplify the *DQA1* locus. In the present study, we initially isolated and characterized MHC class II *DQA1* exon 2 sequence variations in the black muntjac using a primer pair specifically designed for the American bison (Traul et al., 2005). We then evaluated the total DQA loci variation based on the sequences isolated here along with the four *DQA2* alleles previously isolated. Our aims were 1) to determine the expression of the two *DQA* loci at the cDNA level; 2) to characterize the pattern of polymorphism at the *DQA* loci by comparison with those of other mammal species; 3) to examine the possible mechanisms of maintenance of *DQA* allele diversity in this species; and 4) to provide insight into *DQA* gene evolution within the Cervidae and Bovidae.

MATERIAL AND METHODS

Sampling

A total of 42 individuals sampled from three sources during 1998-2003 were used for *DQA* variation analysis. In detail, 24 skin and seven muscle samples were collected from the wild, and 11 blood samples were obtained from Hefei Wild Animal Park. Additionally, one fresh blood sample obtained from the Xiuning Wild Animal Rescue Center in 2010 was used for cDNA expression analysis. DNA was extracted from blood, tissue, and skin samples using procedures described by Ni et al. (2009).

Primer design and PCR

Initially, a primer pair (BoDQA1FP-E2A/BoDQA-RP-E3A) specific to the *DQA1* locus of the American bison (Traul et al., 2005) was used to amplify three genomic DNA samples of the black muntjac. PCR mixtures of 40 µL contained 50-100 ng genomic DNA, 1X ExTaq buffer (Takara, China), 200 µM dNTP, 2.0 mM $MgCl_2$, 0.3 µM of each primer, and 1.5 U ExTaq polymerase (Takara). Amplification was carried out in an iCycler (Bio-Rad, USA) and consisted of an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 30 s, with a final extension at 72°C for 5 min. PCR products were separated on a 1.5% agarose gel, recovered using the Agarose Gel Extraction kit (AxyGene), and then ligated into a pMD18-T vector (Takara) according to manufacturer instructions. A 2- to 4-µL aliquot of the ligation mixture was used to transform competent *Escherichia coli* cells (Sangon) following the protocol recommended by the manufacturer. The positive clones were verified by PCR using the primers BoDQA1FP-E2A and BoDQA-RP-E3A and then sequenced in both directions by GenScript Inc. (Nanjing, China). The amplified *DQA* fragment products were about 727 bp in length, including all but 13 nucleotides of exon 2, all of intron 2, and 47 nucleotides from the 5'-end of exon 3. Subsequently, based on the conserved regions across the three 727-

bp sequences, we designed the primer pair MucrDQA1-F (5'-CTC CGA CTC AGC TGA CCA CAT TGG-3') and MucrDQA1-R (5'-CAC TCT GCT TCT CTT TAT TAA-3') to amplify a 294-bp DNA fragment including 219 bp of the exon 2 sequence for analysis of *DQA1* variation. To confirm transcription of the *DQA* loci under investigation, two exon 2 primer pairs were designed to amplify *DQA1* (primers DQA1cup: 5'-ACG TGG ACC TGG AAA AGA-3' and DQA1cdn: 5'-AGC AGC GGT AGA GTT GGA-3') and *DQA2* (primers DQA2cup: 5'-TAT GTG GAC CTG GGG AAG A-3' and DQA2cdn: 5'-GGG GTA AAG TTG GAG CGT-3') from cDNA.

RNA extraction and cDNA synthesis

Total RNA was isolated from peripheral blood with TRIzol (Invitrogen) and cleaned up using an RNeasy kit (Qiagen) according to manufacturer instructions. The RNA sample was treated with DNase I, and first-strand cDNA was reverse-transcribed using a MMLV First-strand cDNA Synthesis kit (Sangon) following manufacturer instructions. Regular PCR was then carried out using the exon-located primer pairs DQA1cup/DQA1cdn and DQA2cup/DQA2cdn to detect expression of *DQA1* and *DQA2*, respectively. The PCR products were subject to cloning and sequencing.

PCR-SSCP analysis, sequencing, and nomenclature

All samples were amplified by PCR using primer pair MucrDQA1-F and MucrDQA1-R. PCR mixtures of 15 μL contained 30-50 ng genomic DNA, 1X ExTaq buffer (Takara), 200 μM dNTP, 1.5 mM $MgCl_2$, 0.2 μM of each primer and 1 U ExTaq polymerase (Takara). Amplification was carried out in an iCycler (Bio-Rad, USA) and consisted of an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 30 s, and extension at 72°C for 30 s, with a final extension at 72°C for 5 min. To identify allelic diversity of *DQA1* exon 2, all individuals were subjected to single-stranded conformation polymorphism (SSCP) analysis. An aliquot of 5 μL per amplicon was mixed with an equal volume of 2X gel loading dye. After denaturation at 95°C for 3 min, samples were rapidly cooled on ice and then loaded on 16 x 18 cm, 8% non-denatured polyacrylamide gels (acrylamide:bis-acrylamide = 49:1) containing 10% glycerol (Bio-Rad) gels. Electrophoresis was performed using the Bio-Rad Dcode™ Universal Mutation Detection System at 300 V for 10-15 h at room temperature in 0.5X TBE buffer. The SSCP bands were visualized by silver staining.

Allele bands were recovered from the gel and cloned using the pMD18-T cloning system (Takara) as described above. For each cloned allele, SSCP analysis was performed on the PCR products of several recombinant clones using the reaction conditions and thermal profiles described above. PCR amplicons from cloned inserts were run alongside amplified products from the individual, and clones with SSCP patterns identical to the genomic band profile were chosen for sequencing.

In the present study, all alleles were confirmed either by sequencing the same allele from more than one individual or by checking SSCP banding patterns between cloned PCR products and PCR products of the individual from which the allele was isolated. The nomenclature of black muntjac *DQA1* alleles follows that of Klein et al. (1990).

Sequence analysis

Nucleotide and amino acid sequences were aligned with the ClustalX program

(Thompson et al. 1997) and MEGA version 4.0 (Tamura et al., 2007). Molecular evolutionary analyses of relative frequencies of synonymous (d_S) and non-synonymous (d_N) substitution were calculated by MEGA version 4.0 (Tamura et al., 2007) using the method developed by Nei and Gojobori (1986) and the Jukes and Cantor (1969) correction for multiple substitutions. The significance of the differences between these rates was tested using a Z-test with selection at the 5% level whereby the P values designated the probability of rejecting the null hypothesis of neutrality ($d_N = d_S$; Nei and Kumar, 2000). Amino acid positions involved in putative PBRs were determined according to *HLA-DQA* (Reche and Reinherz, 2003). To evaluate the total variation of *DQA* loci in the black muntjac, we combined the four *Mucr-DQA2* alleles recently isolated by our research group (Zhu et al., 2011).

Allelic frequencies, observed heterozygosity (H_O), expected heterozygosity (H_E), and deviation from Hardy-Weinberg equilibrium were computed for both loci using a Markov chain with 100,000 steps, as implemented with GENEPOP4.0 (Rousset, 2008).

We used the CODEML program from the PAML version 4 package (Yang, 2007) to test for the presence of codon sites affected by positive selection and to identify those sites. Positive selection is indicated by $\omega = d_N / d_S > 1$. Following the method of Schaschl et al. (2006) used in *DRB* of chamois, only model M7 (beta) and M8 (beta and ω) (Yang et al., 2000) were considered in the present study. Under the model M7 (beta) the ω ratio varies according to the β-distribution and does not allow for positive selected sites ($0 < \omega < 1$). In this way, it serves as a null model by comparison with model M8 (β and ω). Model M8 adds an additional site class to the β model to account for sites under positive selection ($\omega > 1$). Models M7 and M8 can be compared in pairs using the likelihood-ratio test (LRT) (Nielsen and Yang, 1998). The LRT statistic calculates twice the log-likelihood difference compared to a χ^2 distribution, with degrees of freedom equal to the difference in the number of parameters between the two compared models. A Bayesian approach, implemented in CODEML, was used to identify residues under positive selection in the MHC class II *DQA* sequences.

To test for evidence of trans-species polymorphism, a neighbor-joining tree was constructed based on black munjtac *DQA* alleles (Figure 1) along with sequence data from three other species, including the sika deer (*DQA1*: AY679433-AY679444; *DQA2*: AY679445-AY679460), cattle (*DQA1*: Z48190, Z48191, Z48193-Z48196, D50454; *DQA2*: U80866, U80862, U80861, Y07820, Z79515, Z79516, Z79518), and sheep (*DQA1*: Z28418, AY230210, M33304, AY230209, AF317617, AY230208; *DQA2*: AY312375-AY312379, AY312397). Phylogenetic trees were constructed based on Kimura's two-parameter distances using MEGA version 4.0 and rooted with the *DQA* sequence of the cotton rat (AF155914). The bootstrap confidence interval was obtained using 1000 replicates.

RESULTS

Genomic DNA from 41 of 42 black munjtac individuals were well amplified by the primers MucrDQA1-F/MucrDQA1-R, with only one individual sampled from wild showing no PCR amplification. No more than two sequences were detected in each animal. None of the sequences showed deletions, insertions, or stop codons, implying that the identified *DQA* sequences represented functional molecules. More importantly, RT-PCR for detecting the expression of blood cDNAs using the exon-located primer pairs DQA1cup/DQA1cdn and DQA2cup/*DQA2* produced a single discrete band of expected size for each locus. These sequencing results showed that the cDNA products did indeed represent the *DQA1* and *DQA2*

loci. These findings demonstrated that the black muntjac *DQA1* and *DQA2* were both expressed.

DNA sequence and SSCP analysis revealed only three unique *Mucr-DQA1* alleles among 41 black muntjac individuals (accession No. GU984228-GU984230). Of the three alleles, *Mucr-DQA1**01 and *Mucr-DQA1**02 had similar frequencies of 43.75 and 47.5%, respectively. This was in sharp contrast to that of *Mucr-DQA1**03 (8.75%). For the *DQA2* locus, *Mucr-DQA2**01 was the dominant allele, with a frequency of 56.25% followed by *Mucr-DQA2**02 (36.25%). Both *Mucr-DQA2**03 and *Mucr-DQA2**04 were rare alleles with frequencies of 5 and 2.5%, respectively. In addition to similar distribution patterns of allele frequency, significant deviation from Hardy-Weinberg equilibrium due to heterozygosity deficiency was detected at both loci ($P < 0.001$).

Alignments of nucleotide and amino acid sequences revealed only 5% of nucleotide substitutions (11/219) and 11% of amino acid substitutions (8/73) among the three *Mucr-DQA1* alleles (Figure 1). Compared to the *DQA1* gene, the *DQA2* gene of black muntjac manifested a relatively high level of polymorphism. Of the 219 nucleotide sites, 21 (9.6 %) translated to 15 polymorphic amino acids. These constituted 20.5% of the 73 residues that were found to be polymorphic across the four *Mucr-DQA2* alleles (Figure1).

The rates of non-synonymous (d_N) and synonymous (d_S) substitutions were calculated for the putative PBR and non-PBR amino acid positions (Table 1). For the putative PBR of *DQA1*, the d_N/d_S ratio was 0.942 ($P = 0.953$), indicating that there was no significant excess of non-synonymous over synonymous substitutions. However, for *DQA2*, the d_N of the putative PBR was significantly higher than the corresponding d_S ($d_N/d_S = 13.357$, $P = 0.012$), thus rejecting the null hypothesis of neutrality.

Table 1. Relative frequencies of non-synonymous (d_N) and synonymous (d_S) substitutions in the peptide binding region (PBR) and non-PBR for the *DQA1* and *DQA2* loci of black muntjac.

Locus	PBR	$d_N \pm SE$	$d_S \pm SE$	d_N/d_S	P[a]
DQA1	PBR	0.065 ± 0.033	0.069 ± 0.073	0.942	0.953
	non-PBR	0.019 ± 0.011	0.041 ± 0.029	0.463	0.513
DQA2	PBR	0.187 ± 0.069	0.014 ± 0.015	13.357	**0.012**
	non-PBR	0.035 ± 0.014	0.028 ± 0.02	1.25	0.396

[a]P values are the probability that d_N and d_S are different using a Z-test. Standard errors (SE) were estimated by 1000 bootstrap replicates.

The LRT statistic comparing the two models indicated that M8 fit the data of the *DQA2* locus significantly ($P < 0.01$) better than did M7 (Table 2). Four residues of *DQA2* sequences were found to be under strong positive selection ($\omega = 11.11$) by Bayes empirical Bayes infer-

Table 2. Log-likelihood values and parameter estimates for the major histocompatibility complex class II *DQA* loci of black muntjac.

Locus	Model	LnL[a]	Estimates of parameters	$2\Delta L$[b] (M7 *vs* M8)	Positively selective sites[d]
DQA1	M7 (beta)	-346.928	$p = 3.013743$, $q = 0.0054$	0.182[c]	Not allowed
	M8 (beta and ω)	-346.837	$p_0 = 0.00001$, ($p_1 = 0.99999$)	($P > 0.1$)	
			$p = 0.6054$, $q = 1.402$, $\omega = 1.342$		
DQA2	M7 (beta)	-408.411	$p = 27.154$, $q = 0.005$	9.434[c]	Not allowed
	M8 (beta and ω)	-403.694	$p_0 = 0.628$, ($p_1 = 0.372$),	($P < 0.01$)	14E* 61G**
			$p = 0.005$, $q = 1.717$, $\omega = 11.109$		65A* 69A*

[a]LnL = log-likelihood value, ω = selection parameter, and p_n = proportion of sites that fall into the ω_n site class. [b]$2\Delta L$ = computed as $2(L_b\text{-}L_a)$, where L_a and L_b are the log-likelihood values for models M7 and M8, respectively. [c]$df = 2$. [d]Sites inferred to be under positive selection are given at the 95% (*) and 99% (**) confidence intervals.

ence. All four codons shown to be under positive selection belonged to the putative PBR (Figure 1). In contrast, for the *DQA1* locus, the selection parameter (ω) from M8 was only 1.34 and the LRT revealed no significant difference (P > 0.1) between M8 and M7 (Table 2).

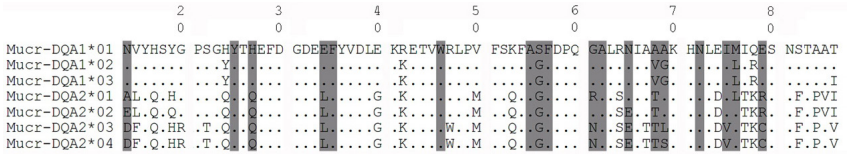

Figure 1. Predicted amino acid sequences of the second exons of black muntjac *DQA* alleles. The *DQA1* alleles were isolated in the present study, and the *DQA2* alleles were isolated by Zhu et al. (2011). The numbers above the aligned sequences refer to the human DQα chain (Reche and Reinherz, 2003). Each dot indicates the same amino acid as in *Mucr-DQA1**01, and the putative peptide binding regions as proposed for the human DQα molecules are shaded.

Phylogenetic analysis of the *DQA* exon 2 alleles from black muntjac, sika deer, cattle, and sheep revealed monophyletic relationships for the two *DQA* loci. As shown in Figure 2, the 52 *DQA* alleles formed two large clades. One clade comprised all *DQA1* alleles (clade

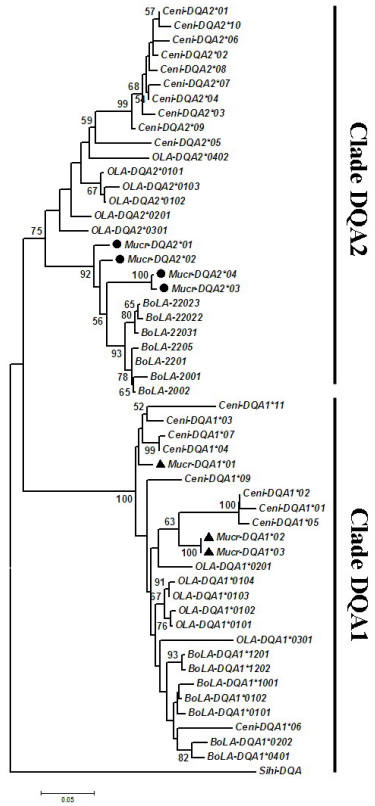

Figure 2. The neighbor-joining tree of major histocompatibility complex class II *DQA1* and *DQA2* exon 2 from black muntjac, sika deer, cattle, and sheep. This tree was constructed based on Kimura's two-parameter distances. The homologous sequence of the cotton rat was used as an outgroup. Bootstrap values at the forks come from 500 replicates and only the values larger than 50% are shown.

DQA1) and the other clade consisted of all *DQA2* alleles (clade *DQA2*). In clade *DQA1*, all alleles from Cervidae were clustered into two sister groups with one group comprising *Mucr-DQA1**01 and four *ceni-DQA1*, and the other group consisted of the remaining deer *DQA1* alleles and all the Bovidae *DQA1*. However, in clade *DQA2*, the four *Mucr-DQA2* alleles grouped with *BolA-DQA2* rather than *ceni-DQA2* with a bootstrap value of 92%.

DISCUSSION

In the present study, using primers designed to be specific to the black muntjac *DQA1*, MucrDQA1-F and MucrDQA1-R, 41 out of 42 samples were successfully amplified by PCR, with only one sample failing to be amplified. In our previous study, a similar method was used to amplify the black muntjac *DQA2* gene, and the black muntjac *DQA2* specific primers performed well in 40 out of 42 samples, with only two samples (one from southern Anhui Province and another from a captive population) failing to be amplified (Zhu et al., 2011). Notably, in the present study, the single sample that failed to be amplified by *DQA1* primers performed well for *DQA2* primers, and the two samples that failed to be amplified by *DQA2* primers in the previous study could be amplified by *DQA1* primers. These results indicate that the failure of these three samples to be amplified by PCR was not attributed to the quality of the DNA templates. We could not determine whether our inability to produce a PCR amplicon from these three black muntjac samples was due to the primers showing null alleles or to the fact that the number of *DQA* genes varies with haplotype (Ballingall et al., 1997; Snibson et al.,1998; Hickford et al., 2000; Bryja et al., 2006). This issue merits further analysis with additional PCR primers.

It has been shown that different mammalian species contain different numbers of expressed *DQA* genes. For example, most rodents have one *DQA* locus, but voles have two *DQA* copies (Bryja et al., 2006). Two or more functional and highly polymorphic *DQA* genes have been described in cattle and sheep (Ballingall et al., 1997; Snibson et al., 1998; Hickford et al., 2004). In deer, to date, the *DQA* gene has only been documented in Pére David's deer, and two expressed *DQA* loci were found to be monomorphic at their antigen-binding regions (Wan et al., 2011). As described above, analysis of cDNA expression and sequence variation in exon 2 suggested that black muntjac expressed two, most likely functional, MHC class II DQA genes. A phylogenetic analysis of the DQA exon 2 sequences supports this hypothesis.

A total of seven *DQA* alleles were identified in black muntjac, three for *DQA1* and four for *DQA2*. As has been found in sheep and goats (Snibson et al., 1998; Hickford et al., 2004; Zhou and Hickford, 2004; Amills et al., 2005), the black muntjac *DQA2* gene showed a relatively high level of polymorphism compared to *DQA1*. However, regardless of the number of alleles, the degree of sequence divergence between those alleles at both *DQA* loci was low in the black muntjac compared to that found in domestic ruminants (Ballingall et al., 1997; Snibson et al., 1998; Zhou and Hickford, 2004; Hickford et al., 2004; Amills et al., 2005). Considering that the samples of domestic ruminants used in analysis are usually larger than those used in the present study, and considering the increase in the number of black muntjac individuals, it was possible that the number of alleles would increase to some extent but that the difference between the sequences of the *DQA* alleles of the different species would not necessarily increase. Furthermore, the total level of *DQA* variation (including two loci) of the black muntjac was still lower (some of which contained only one *DQA*) when compared to that of other mammalian species and even some endangered animals. For example, researchers have

characterized 50*DQA* alleles in European brown hare, 18 in rabbit (Surridge et al., 2008; Goüy et al., 2009), six in African elephants, four in Asian elephants (Archie et al., 2010), and 11 in the panda (7 *DQA1* and 4 *DQA2*) (Chen et al., 2010). The relatively low level of MHC polymorphism is usually attributed to either a consequence of reduced selection pressure or bottleneck effects (Goüy et al., 2009). In addition, many studies have demonstrated that MHC variation is usually subject to the influences of demographic and ecological factors (Kundu and Faulkes, 2004). Therefore, the reduced *DQA* diversity found in the black muntjac may be explained by the population bottlenecks that the species experienced during the last glacial period, the animal's solitary habits, and its mountainous habitat (Wu and Fang, 2005).

It is generally accepted that the polymorphism of MHC genes is most likely maintained by positive selection and that such selection usually results in more non-synonymous than synonymous changes in the codons of the PBR (Piertney and Olivier, 2006). In the present study, a significant selection signature was detected at the *DQA2* locus in that the d_N of the putative PBR was significantly higher than the corresponding d_S(Table 1), indicating that the locus was subject to positive selection. This was also shown by evidence from the likelihood ratio test. In this test, model M8 fit the data of the *DQA2* locus significantly better than did model M7, and estimates of M8 demonstrated that about 37% of the codons followed strong positive selection (Table 2). Four codons of the *DQA2* sequence were identified with highly significant posterior probability of being under positive selection by Bayes empirical Bayes inference, and all the sites belonged to the putative PBR. In contrast, non-synonymous substitutions occurred at about the same rate as synonymous substitutions in the putative PBR of *DQA1* (Table 1), and the LRT revealed no significant difference between M8 and M7 (Table 2). Hence, M8 did not fit the data of *DQA1* better than did M7, implying no distinct positive selection signature at the *DQA1* locus. Similar phenomena were also found at some primate *DQA* loci (Bergstrom and Gyllensten, 1995) and at sheep *DQA1* (Zhou et al., 2004). The difference in d_N/d_S ratios between black muntjac *DQA1* and *DQA2* loci may reflect different evolutionary selection pressures.

It has been suggested that positive selection can also act to maintain certain allele lineages across species over long evolutionary time frames (Klein, 1987). Similar *DQA1* sequences shared between black muntjac and sika deer and a common lineage of *DQA2* alleles observed between black muntjac and cattle (Figure 2) provide strong evidence of trans-species evolution existing at both the *DQA* loci of black muntjac. It has been documented that the *DQA1* and *DQA2* genes of sheep and cattle originated from gene duplication of an ancestral *DQA* gene and that the duplication event would have occurred before the divergence of the ovine and bovine lineages (Snibson et al., 1998). In the present study, evolutionary relationships of *DQA* alleles from four species (including two species of Cervidae, black muntjac and sika deer; and two species of Bovidae, cattle and sheep) revealed phylogenetic patterns similar to those previously described for cattle and sheep (Ballingall et al., 1997; Snibson et al., 1998; Hickford et al., 2004). In other words, the *DQA1* alleles of different species grouped together, as did the *DQA2* alleles, but different genes of any of the species were located in separate branches of the phylogenetic tree (Figure 2). These results suggest that an ancestral *DQA* gene may exist between Cervidae and Bovidae and that a gene duplication event occurring before the divergence of Cervidae and Bovidae gave rise to *DQA1* and *DQA2*. Because divergence between Bovidae and Cervidae can be traced to the late Oligocene (Hassanin and Douzer, 2003), the two *DQA* alleles lineages across the two genera must have been maintained for at least 25.5-27.8 million years. A similar evolutionary time frame has also been observed in primates,

wherein trans-species allelic lineages have been conserved for over 30 million years (Geluk et al., 1993). These results provide evidence that trans-species allelism has operated on both *DQA* loci across Bovidae and Cervidae. As more information on other MHC class II loci of deer species becomes available, it will be interesting to determine whether trans-species evolution, a common mechanism, operates on all MHC class II genes across Bovidae and Cervidae.

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