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RESEARCH ARTICLE

Sequence variability analysis on major histocompatibility complex class II DRB alleles in three felines

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Abstract The variation of the exon 2 of the major histocompatibility complex (MHC) class II gene DRB locus in three feline species were examined on clouded leopard (*Neofelis nebulosa*), leopard (*Panthera pardus*) and Amur tiger (*Panthera tigris altaica*). A pair of degenerated primers was used to amplify DRB locus covering almost the whole exon 2. Exon 2 encodes the β1 domain which is the most variable fragments of the MHC class II molecule. Single-strand conformational polymorphism (SSCP) analysis was applied to detect different MHC class II DRB haplotypes. Fifteen recombinant plasmids for each individual were screened out, isolated, purified and sequenced finally. Totally eight distinct haplotypes of exon 2 were obtained in four individuals. Within 237 bp nucleotide sequences from four samples, 30 variable positions were found, and 21 putative peptide-binding positions were disclosed in 79 amino acid residues. The ratio of nonsynonymous substitutions (d_N) was much higher than that of synonymous substitutions (d_S), which indicated that balancing selection probably maintain the variation of exon 2. MEGA neighbor joining (NJ) and PAUP maximum parsimony (MP) methods were used to reconstruct phylogenetic trees among species, respectively. Results displayed a more close relationship between leopard and tiger; however, clouded leopard has a comparatively distant relationship form the other two.

Keywords major histocompatibility complex (MHC), DRB locus, exon 2, feline, variability

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1 Introduction

Major histocompatibility complex (MHC) is a multigene family, which has a firm relationship with organism self-immune function in all vertebrates (Klein and Figueroa, 1986). In the previous studies, the MHC family includes two major sub-families: class I and class II (Bernatchez and Landry, 2003). In mammals and aves, the class I and class II genes are linked together in a single gene complex (Hughes and Yeager, 1998; Hess and Edwards, 2002; Kennedy et al., 2002). However, the class I and class II genes have some different aspects on self-construction, function and evolution (Abbas et al., 1991; Nei and Hughes, 1991). Because of high polymorphism in MHC class II genes exon 2, most emphases are put on the studies of it (Shia et al., 1995; Kennedy et al., 2002).

To date, as for the felines, most studies of their MHC genes concentrate on small felids, such research work included domestic cat (*Felis catus*) (Yuhki and O'Brien, 1997; Kuwahara et al., 2000; Kennedy et al., 2002), Ocelot (*Leopardus pardalis*) and Margay (*Leopardus wiedii*) (Yuhki et al., 2000). As for large feline animals, Drake et al. (2004) have been investigated the sequences polymorphism of MHC DRB allele's exon 2 in Cheetah (*Acinonyx jubatus*). Yet until now, few investigations have been taken on the other felines, especially large ones. Now 13 felids species distribute on China, in which Amur tiger (*Panthera tigris altaica*), leopard (*Panthera pardus*) and clouded leopard (*Neofelis nebulosa*) were listed in IUCN Red Data Book of threatened or endangered and ranked the first class national protected animals in China as well (Wang, 1998). However, the former research work on felid species was focusing on ecology, phylogenetic evolution and classification (Janczewski et al., 1995; Masuda et al., 1996; Nowell and Jackson, 1996; Johnson and O'Brien, 1997; Slattery and O'Brien, 1998; Zheng et al., 2005), but

relatively fewer studies were on genetic diversity. Thus, the investigations on the genetic polymorphism from the perspective of the MHC genes were rarely reported. Based on the reasons mentioned above, the variability of exon 2 in MHC class II DRB alleles on Amur tiger, leopard and clouded leopard were examined, hoping to throw light on some scientific foundation for the putting forward, drafting and carrying out of the policies in protecting species. In addition, the phylogenetic relationship among the three felines was analyzed on the basis of the MHC data in the present study.

2 Materials and methods

2.1 Materials

Four muscle samples (preserved at −80°C until DNA extraction) were used in this study, including two clouded leopard samples (from Ningguo, Anhui; code: MA99019, MA99023), one leopard sample (from Yi county, Anhui; code: MA049020) and one Amur tiger (provided by Ningguo specimen factory; code: MA99017).

2.2 Experimental methods

2.2.1 Total DNA extraction

Genomic DNA was extracted from each muscle tissue sample. Standard SDS/Proteinase, Phenol/Chloroform method was adopted (Sambrook and Russell, 2001). Extracted DNA was examined by 1% agarose gels electrophoresis.

2.2.2 Polymerase chain reaction (PCR) amplification

Exon 2 of the MHC class II DRB genes was amplified by PCR using a pair of degenerate primers. These primers sequences for subsequent PCR amplification were FDF: 5′-CCACACAGCACGTTTC(C/T) TG-3′; FDR: 5′-CCGCTG-CACTGTGAAGCT-3′, on the basis of the previous studies of Yuhki and O'Brien (1997). The PCRs were carried out in volume of 30 μL containing 10 mM of Tris-HCl (pH 8.3), 1.5 mM of $MgCl_2$, 50 mM of KCl, 150 μM of dATP, dCTP, dGTP and dTTP, 10 μM of primer (forward and reverse respectively), 1 unit of Taq DNA polymerase and 20-100 of total DNA, the deficient part is supplemented by ddH_2O. The procedure of the experiment is denaturation for 50 s at 94°C, annealing for 50 s at 54°C, and extension for 50 s at 72°C, altogether 34 cycles. A 5-min pre-denaturation at 94°C is carried before the first cycle, and a 10-min post-extension at 72°C follows the last cycling. To verify successful amplification, 3 μL PCR products of each sample were checked with 1% agarose gels electrophoresis.

2.2.3 Cloning

The PCR products of each sample were purified by 2% agarose gels, and the targeted band corresponding to the amplified product was isolated with the DNA Gel Extraction Kit (V-gene). The recovered DNA was re-suspended in 15 μL of double distilled water.

All purified PCR products were cloned into the pMD18-T vector with a TA cloning kit (TaKaRa), and then transformed in *Escherichia coli* DH5*α*-competent cells was performed according to Sambrook and Russell (2001). For each individual, 15 positive clones were picked after the blue/white selection method as described in the TaKaRa protocol, and grew overnight in separate tubes with 3 mL of LB-medium, which is followed by a another PCR, were used to examine positive clones, using M13 universal primers, and then the products with the expected band will be used for single-strand conformation polymorphism (SSCP) analysis.

2.2.4 SSCP analysis

The SSCP analyses were used to discriminate the different haplotype. The 5 μL of PCR product was mixed with 6 μL of loading buffer (70% formamide, 0.1% bromophenol blue, 0.1% xylene cyanole FF), and denatured at 95°C for 10 min, and then the samples were immediately cooled on ice for 5 min after denaturizing, and loaded onto 1.5 mm thick, 6% polyacrylamide gels. Then the mixtures transferred through electrophoresis at 350 V for 5–6 h. Finally, the gel was stained with 0.02 M silver nitrate, and the PCR products sharing different bands were selected and the corresponding cloned cells were sequenced.

2.2.5 Sequencing and data analysis

All different bands were selected by SSCP and the corresponding cloned cells were sequenced. After confirming the sequences were MHC class II DRB exon 2 of feline using a BLAST analysis with GenBank. To determine the variable nucleotide sites and unique alleles, and then the resultant sequences were aligned with the program Clustal X (Jeanmougin et al., 1998), and corrected by manual too. Statistical analyses of nucleotide and amino acid sequences were computed in MEGA Version 2.1 (Kumar et al, 2001) and DnaSP4.0 (Rozas et al., 2003) software.

Genetic distance (with Standard Error, SD) and rate (number) of transition or transversion in the nucleotide sequences are calculated by MEGA, on the basis of the Kimura two-parameters (Gamma) model. Relative proportion of synonymous (d_S) and non-synonymous (d_N) substitution in the overall sequences were calculated, using the model of modified Nei and Gojobori (1986), applying the correction of Jukes and Cantor (1969) for multiple hits. The significance of the difference between these rates was tested with a *Z*-test of selection at 5% level, whereby the *P*-values are the probability of rejecting the null hypothesis of neutrality ($d_N = d_S$). The selection of peptide binding sites is based on the 24 codons of peptide-binding region (PBR) in human class II (Brown et al., 1993). Heteozygosity per site of amino acid was calculated according to the method of Hedrick et al. (1991).

Neighbor-joining tree for the sequences of MHC class II DRB exon 2 were reconstructed on the model of Nucleotide: Jukes-Cantor in MEGA Version 2.1 and 1000 bootstrap replications were performed to determine the reliability of the branching order (Kumar et al., 2001). The maximum parsimony (MP) was also reconstructed by heuristic search with PAUP4.0 (Phylogenetic Analysis Using Parsimony) (Swofford, 1998) software. To construct the phylogenetic trees, the following homologous sequences were used, including Cheetah (*Acju-DRB*, AY312962), Ocelot (*Lepa-DRB*, AF057836), Domestic cat (*Feca-DR*, U51983) and Domestic dog (*Cafa-DRB*, X93572).

3 Results and analysis

3.1 Sequences polymorphism analysis

In total, eight distinct nucleotide sequences from four individuals were acquired, and the full length of these sequences was 237 bp without any gaps. However, only one sequence was obtained from Amur tiger. To examine the reliability of the results, the PCR product was directly sequenced again. Different sequenced method showed the identical results. Three and four sequences were investigated from leopard and clouded leopard, respectively. These allelic sequences have been deposited in GenBank (Accession number: DQ189257-DQ189264). Based on the recommended regulations of the MHC Nomenclature Committee, the sequences acquired from clouded leopard, Amur tiger and leopard are named as *Nene-DRB**1, *Nene-DRB**2, *Nene-DRB**3 and *Nene-DRB**4; *Pati-DRB**1; *Papa-DRB**1, *Papa-DRB**2 and *Papa-DRB**3, respectively.

The average proportions of T, C, A and G in leopard nucleotide sequences were 16.3%, 22.8%, 22.1% and 38.8%. Additionally, one transversion was found, but no transition was examined. In Amur tiger nucleotide sequence, average proportions of T, C, A and G was 18.1%, 21.1%, 22.4% and 38.4%. The average proportions of T, C, A and G are 19.5%, 21.6%, 23.0% and 35.9% in clouded leopard sequences. Eight parsimony informative sites, eight transitions, ten transversions and one site were also found experienced both a transition and transversion. In total eight unique sequences, it was screened out that the proportions of T, C, A and G were 18.1%, 22.0%, 22.6% and 37.3% respectively, and also 35 parsimony informative sites were found. Low degree of differences were shown in base pairs between the species, but clouded leopard nucleotide sequence contributed higher variable frequencies at the transition and transversion than the other animals in this study. The reason may lies in the high variation among clouded leopard sequences.

After the full alignment of the eight nucleotide sequences, 59 variable sites were examined in the entire length. Of these 59 variable sites determined above, eight variants were screened at the first, eight at the second and nine at the third base position of the amino acid codons, and the percentage was 32.0%, 32.0% and 36.0%, respectively. The variable sites included ten transitions and 15 transversions, but no site experienced both transition and transversion. By the Kimura two-parameter methods, the percentage variations of genetic distance among eight sequences ranged from 0.4% to 19.1%, with an average of 11.7% (Table 1). The maximum percentage variation of genetic distance within specie was 0.109, shown between *Nena-DRB**1 and *Nena-DRB**3, however, the minimum value was only 0.004, shown between *Papa-DRB**2 and *Papa-DRB**3. Compared with the figures mentioned above, the between-species genetic divergences were much larger, and the maximum variable value was 0.191, shown between *Nena-DRB**3 and *Papa-DRB**3, while the minimum variable values were 0.085, shown between *Pati-DRB**1 and *Papa-DRB**2, *Papa-DRB**3. Analysis of genetic distance indicated that the variation of clouded leopard sequences were much higher than the sequences variation of leopard and Amur tiger, and the comparatively closed relationship of genetic distance was showed between leopard and Amur tiger.

The genetic parameter of three species on nucleotide and amino acid polymorphism sites were concluded by DnaSP4.0 software. Table 2 listed polymorphism position, mutant positions, nucleotide differences (K) and nucleotide diversity (Pi). From Table 2, it can be seen that high variation was shown among four clouded leopard sequences. However, only two diagnostic sites could be found among three leopard sequences. Fifty-nine nucleotide polymorphism sites were examined among the eight sequences, with a proportion of 33.47%, and 35 parsimony informative sites were also found, with a proportion of 14.5%. Thirty amino acid polymorphism sites were identified among 79 amino acid residues, with a proportion

Table 1 Genetic distances and their standard errors among eight nucleotide sequences

	1	2	3	4	5	6	7	8
1. *Papa-DRB**1		0.004	0.006	0.020	0.029	0.029	0.031	0.023
2. *Papa-DRB**2	0.004		0.004	0.020	0.029	0.028	0.031	0.022
3. *Papa-DRB**3	0.009	0.004		0.020	0.029	0.029	0.031	0.023
4. *Pati-DRB**1	0.090	0.085	0.085		0.029	0.029	0.030	0.025
5. *Nene-DRB**1	0.170	0.165	0.170	0.170		0.004	0.023	0.019
6. *Nene-DRB**2	0.164	0.159	0.164	0.164	0.004		0.022	0.019
7. *Nene-DRB**3	0.191	0.186	0.191	0.181	0.109	0.104		0.022
8. *Nene-DRB**4	0.109	0.104	0.109	0.129	0.090	0.075	0.099	

Note: numbers below the diagonal are genetic distances and numbers above the diagonal are standard errors.

of 38%. The identical variable sites were not screened out between leopard and clouded leopard amino acid residues. The average value of nucleotide differences and nucleotide diversity were 24.286 and 0.102 5 between leopard and clouded leopard, respectively. Nucleotide replacements number per each site (Da) was 0.141 0 between species, whereas the average value of nucleotide replacement (Dxy) was 0.101 3.

Table 2 Genetic parameter of three species

	Panthera pardus	*Neofelis nebulosa*	*Panthera tigris altaica*	Total sequences
Polymorphic positions	2	31	0	59
Mutant positions	2	33	0	65
Nucleotide differences (K)	1.333 3	17.500 0	0.000 0	25.178 6
Nucleotide diversity (Pi)	0.005 6	0.073 8	0.000 0	0.106 2

At present, the exact PBR had not been located at the locus in the feline genes. Thus, the PBR was selected on the basis of the 24 codons of PBR in human class II (Brown et al., 1993). In this study, it was named as putative peptide-binding region. Table 3 summarized the measures of the rate of non-synonymous (d_N) and synonymous (d_S) substitutions for the putative PBR and non-PBR and their ratio among different three species (because only one Amur tiger sequence has been acquired, the values mentioned above could not be obtained). It can be inferred from Table 3 that the rate of non-synonymous was very higher than synonymous substitutions at the putative PBR in clouded leopard. Its ratio, d_N/d_S, was 2.595. However, the relative frequency of non-synonymous substitution was significantly higher than the frequency of synonymous substitution ($P = 0.019$).

Figure 1 illustrated the heterozygosity of amino acid sequences. As the figure shows, 30 variable positions from eight amino acid sequences have been identified. Also, 14 variant putative PBR sites (indicated by asterisk,*) were screened out from these residues. The maximum and minimum heteozygosity were disclosed at position 20th and 52nd, where the highest and lowest values were 0.91 and 0.051 respectively. These two positions sited justly at putative PBR, with a distance of 0.859. The average value of heterozygote for the whole positions was 0.442. The results of statistics indicated that the variability of amino acid in PBR much higher than that of in non-PBR.

3.2 Phylogenetic analysis

Figure 2 provided neighbor-joining tree of the eight nucleotide sequences. In it, sequences were divided into two clades. *Pati-DRB**1, *Papa-DRB**1, *Papa-DRB**2 and *Papa-DRB**3

Table 3 Comparison of the rate of non-synonymous (d_N) and synonymous (d_S) substitutions for the putative PBR and non-PBR and their ratio among different three species

Species	Positions	*N*	Non-synonymous (d_N)	Synonymous (d_S)	d_N/d_S	*P*
Neofelis nebulosa	PBR	21	0.109 (0.041)	0.042 (0.035)	2.595	0.198 (ns)
	Non-PBR	58	0.044 (0.016)	0.152 (0.034)	0.289	0.019
	All	79	0.061 (0.016)	0.122 (0.034)	0.5	0.091 (ns)
Panthera pardus	PBR	21	0.172 (0.070)	0.178 (0.107)	0.966	0.322 (ns)
	Non-PBR	58	0.091 (0.035)	0.035 (0.019)	2.6	0.325 (ns)
	All	79	0.113 (0.031)	0.066 (0.024)	1.712	0.147 (ns)
Panthera tigris altaica	PBR	21	–	–	–	–
	Non-PBR	58	–	–	–	–
	All	79	–	–	–	–
Total sequences	PBR	21	0.250 (0.069)	0.113 (0.066)	2.212	0.085 (ns)
	Non-PBR	58	0.072 (0.020)	0.113 (0.032)	0.637	0.266 (ns)
	All	79	0.116 (0.022)	0.112 (0.026)	1.036	0.909 (ns)

Note: standard errors are given in parenthesis; N is the number of codons and P-values are the probability of rejecting the null hypothesis of neutrality ($d_N = d_S$).

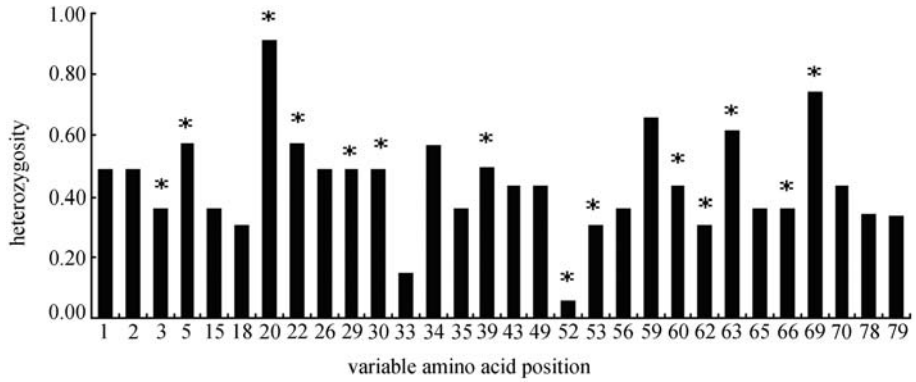

Fig. 1 Average h heterozygosity of the variable amino acid positions among eight different sequences

constituted the clade A. It may reflect that leopard has a relatively closed relationship with Amur tiger. *Nene-DRB** 1–4 constructed clade B.

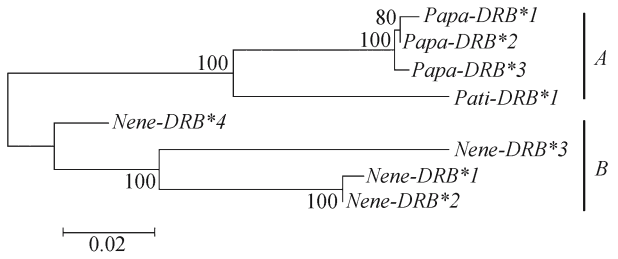

Fig. 2 NJ tree based on the eight sequences of MHC DRB gene exon 2 with confident values of Bootstrap 1000 indicated above each branch

The homologous sequences were downloaded from the GenBank to reconstructed NJ tree (Fig. 3) and MP tree (Fig. 4), with Domestic dog as outgroup. Figure 3 reveals that a consistent clustering of the *Pati-DRB**1, *Papa-DRB**1, *Papa-DRB**2 and *Papa-DRB**3 construct into single clade II, while the rest feline sequences clustered each other as the clade I. Clade II in Fig. 4 also presents a firmly phylogenetic relationship among *Pati-DRB**1, *Papa-DRB**1, *Papa-DRB**2 and *Papa-DRB**3. However, Clade I has more complex in MP

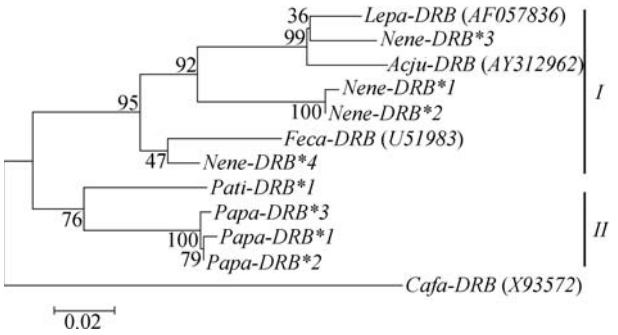

Fig. 3 NJ tree based on the eight sequences of MHC DRB gene exon 2 with confident values of Bootstrap 1,000 indicated above each branch

tree. *Nene-DRB**1 and *Nene-DRB**2 form one sub-clade, while *Nene-DRB**3, *Acju-DRB* and *Lepa-DRB* construct another sub-clade. The two sub-clades plus *Nene-DRB**4 and *Feca-DRB* construct a large clade I.

4 Discussion

4.1 Variation of the MHC class II DRB gene in clouded leopard, leopard and Amur tiger

The results of cloned sequencing and directed sequencing of PCR products revealed only one kind of MHC class II DRB sequence in Amur tiger. The previous research on Amur tiger of the Russian Far East suggested that the extremely low levels of haplotypes diversity of mitochondrial DNA control region was reached because of the bottleneck in the 1940s (Russello et al., 2004). In addition, researchers also found similar results in Cheetah and lion (*Panthera leo*) population (O'Brien et al., 1983; Packer et al., 1991).The varied levels of MHC genes on population were regard as the important factors of immune response to various pathogens and disease. Thus it may play a significant role in population resistance (O'Brien and Evermann, 1988). In the present study, we found several distinct alleles in leopard and clouded leopard, on the basis of relatively smaller samples. It indicated the highly varied levels in two species, and this high variability could have a positive influence on specie survival and reproduction. Of course, different researches could provide distinct results on different vertebrates. For example, small population of San Nicolas Island Fox (*Urocyon littoralis dickeyi*) has a high level of MHC gene variation, and yet low MHC class II diversity were found in European and North American Moose (*Alces alces*) population (Mikko and Anddersson, 1995; Aguilar et al., 2004). For the sake of concentrating on different results, more research is still required to illustrate MHC gene diversity in leopard and clouded leopard population.

All eight amino acid sequences have Cysteine residues at sites 7 and 71, which are necessary for the correct folding of

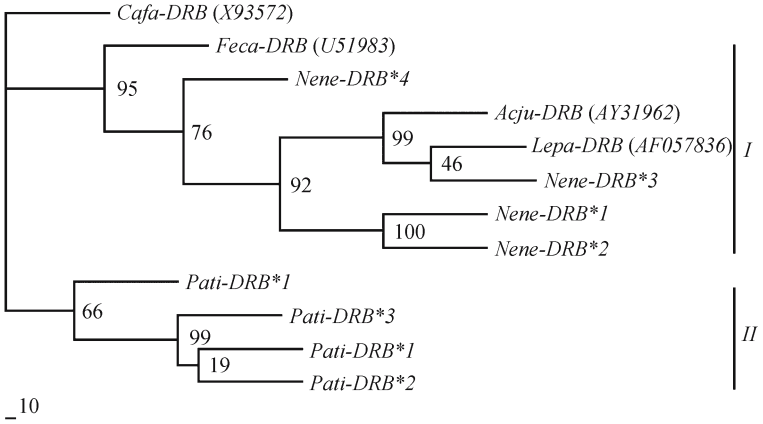

Fig. 4 MP tree based on the sequences of MHC DRB gene exon 2 with confident values of Bootstrap 1000 indicated above each branch

the DR molecules and none of the sequences show deletions, insertions, or stop codons, indicating that all sequences found could form functional region, and therefore could act some physiochemical function *in vivo* (Schaschl et al., 2004). One individual had more than two alleles suggests that these alleles are not deprived from the same locus, and thus could be regard as evidence of gene duplication. Similar examples had been found in the reports of Nino-Vasquez et al. (2000) and Elizabeth et al. (2000). Furthermore, the duplication of gene locus and gene polymorphism at locus increases the diversity of MHC gene products, and therefore could trigger response by inner and outer antigen in organisms.

Twenty-one putative PBR sites were found from 79 amino acid residues, in which 14 putative PBR sites varied in putative PBR sites, with a proportion of 66.7%. Yet, only 16 putative non-PBR sites varied in the rest 58 putative non-PBR sites, with a proportion of 27.6%. Significantly, evidences of amino acid substitutions in PBR were much higher than that in non-PBR. In all eight sequences, the relative rate of non-synonymous substitutions was significantly higher than that of synonymous substitutions in the PBR. In addition, all values of d_N/d_S in putative PBR are more than or near to 1 for all eight sequences. Clearly, amino acid substitutions tend to cluster around the PBR.

In our investigation, the value of d_N/d_S was 2.595 in putative PBR for clouded leopard, exceed 1 largely. If the division of PBR and non-PBR was disregarded in Leopard, the value of d_N/d_S was 1.712. The values was much higher than 1. In all eight alleles, the value of d_N/d_S was 1.036, also higher than 1. These evidences may indicate that non-synonymous sites have higher evolution frequencies than synonymous sites. Therefore, it may be inferred that MHC genes prepare a precondition for new gene variants and perpetually increased polymorphisms, and created conditions that MHC genes adapt continually variable antigens.

For leopard and clouded leopard sequences, not only the variability of nucleotide and amino acid sites, but also the ratio of non-synonymous substitutions to synonymous substitutions showed that the two species have high level of MHC class II genes DRB exon 2. To the present day, most researchers have considered that the level of MHC genes variability had firmly relationship with selection pressure organism burdened. However, the selection pressure for maintaining polymorphism in MHC genes is likely to vary in different organisms' survival environment and antigens. It has been reported that marine mammals have lower levels of MHC variability than terrestrial mammals (Murray et al., 1995; Hoelzel et al., 1999). The marine environment has been suggested to have a low level of infectious diseases and various pathogens, and therefore the selection pressure is weak. Parasites in tropical regions may impose stronger selection pressure on their hosts than that of in non-tropical regions. This could increase the selection pressure on pathogens, and hence the tropical animals usually maintaine a high level of MHC variability (Westerdahl et al., 2000).

4.2 Phylogenetic analysis on MHC genes

Hedrick et al. (2000; 2002) analyzed the phylogenetic relationship between Red wolf (*Canis rufus*), Coyotes (*Canis latrans*) and Grey wolf (*Canis lupus*). In the present study, effort has been made to describe the phylogenetic between three felines on the basis of MHC genes. However, the clustering results of Figs. 2–4 did not showed the similar scenarios to the results of Slattery et al. (1998) and Zheng et al. (2005). Several reasons may annotate the diplomas of this study. First, the reason may be that MHC genes have polymorphism and relative conservation, simultaneously. The study of Crespi and Fulton (2004) reflected similar phenomena. Second, to date, there were still a small quantity of studies on MHC genes of felid, so only few data could be used to compare and reconstruct feline phylogeny. Lastly, MHC was a multigene family, which may led that some sequences obtained from the present study were not orthologous genes, and therefore gene tree did not reflects completely specie tree. Additional, random errors and certain natural selection may influence the self-evolution on genes. Therefore, multiple genes must be used to evaluate gene tree or species tree (Nei and Kumar, 2002). Accordingly, it was presumed that single MHC gene maybe did not solve phylogenetic issues between species.

4.3 Trans-species polymorphism in MHC class II DRB genes of clouded leopard

The phylogenetic analysis indicated that MHC DRB sequences of clouded leopard did not clustered each other stably; in contrast, these sequences were mixed with other feline sequences, and then clustered as a large clade. In other words, DBR exon 2 sequences did not separate according to species. Trans-species polymorphism was thought to make contribution to the phenomena mentioned above. Trans-species polymorphism was the different species that have identical or similar alleles. It was well believed that the theory and trans-species evolution could elucidate the phenomena of trans-species polymorphism. The theory was that MHC allelic lineage exists a lone time, even across speciation events, and then these alleles were shared among species, within or between genera by speciation events. Some alleles even had been maintained for a long period. The concept of trans-species evolution of the MHC polymorphism was first formulated to explain the occurrence of serologically in distinguishable allelic products in geographically isolated populations and subspecies of the house mouse (*Mus musculus domesticus*) (Klein, 1980). Trans-species polymorphism has previously been discovered in many animal, e.g. fishes (Graser et al., 1996), aves (Hess and Edwards, 2002) and mammals (Klein et al., 1993). In the present study, the reason why the Trans-species polymorphism was observed in clouded leopard may be that the feline divergence is dated within 10 Myr (Wayne et al., 1991). Because of short time of speciation, some similar alleles were conserved among some closed relationship species.

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