Genetic Variation and Trans-species Polymorphism of MHC Class II B Genes in Reptiles

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Abstract.- Trans-species polymorphism has been extensively documented for the major histocompatibility complex (MHC) in mammals, fishes and birds, but not for non-avian reptiles. Our study has addressed this by focusing on three species of the reptiles: *Chinemys reevesii*, *Plestiodon chinensis* and *Alligator sinensis*. Using polymerase chain reaction (PCR) and nucleotide sequence analyses, we examined a total of twenty-five sequences of exon 2 of the MHC class II B genes in these species. High allelic variability was observed among sequences within each of these species, indicating extensive MHC polymorphism. Nonsynonymous substitution rates (d_N) exceeded synonymous substitution rates (d_S) greatly within the antigen-binding sites (ABS), suggesting the effect of balancing selection. Phylogenetic analysis of these reptile sequences clearly supports the hypothesis of trans-species polymorphism. We therefore confidently conclude that trans-species polymorphism in the MHC is now known for reptiles, as well as mammals, fishes and birds. This suggests that the main function of the MHC (presentation of peptides to T lymphocytes) has remained largely unchanged despite of long periods of evolution.

Keywords.- Major Histocompatibility Complex (MHC), reptiles, trans-species polymorphism.

Introduction

The genes of the major histocompatibility complex (MHC) code for polymorphic membrane glycoproteins that play a key role in the T-cell mediated immune response (Klein, 1986). There are two distinct classes of MHC molecules, class I and class II, which are encoded by separate but tightly linked loci. The diverse, but always specific, antigen-binding properties of the MHC class I and II molecules determine which foreign peptides can be identified to trigger an immune response. Such MHC-dependent recognition of certain antigens has been considered as an important contributing factor in susceptibility to disease (Klein, 1986). In many vertebrate species, the MHC class I and class II loci exhibit an extraordinarily high degree of polymorphism, particularly in exon 2 of the beta genes. This variation is probably maintained through some kind of balancing selection related to interactions between the immune system and pathogens (Parham and Ohta, 1996), although it has not been resolved as to whether the selection is overdominant (heterozygote advantage hypothesis), frequency dependent (rare-allele advantage hypothesis) or a combination of these factors (Hughes and Hughes, 1995; Hughes and Yeager, 1998; Hill et al., 1992; Hughes, 2000; Thurz et al., 1997).

A characteristic feature of the MHC genes is trans-species polymorphism, i.e. the existence of allelic lineages shared by related species, supporting the theory that the divergence of MHC allelic lineages predate speciation (Graser et al., 1996; Klein, 1987; Ottova' et al., 2005). For MHC genes, this kind of polymorphism has been well-documented in mammals, but for other vertebrate classes, the data on trans-species polymorphism are either fragmentary or unavailable. Only in fish (Klein et al., 1998; Ottová et al., 2005) and birds (Hess and Edwards, 2002; Richardson and Westerdahl, 2003) is there clear evidence for the interspecific sharing of MHC alleles, but these species are of recent origin and do not provide information about long-term persistence of allelic lineages. In an attempt to obtain such information, we decided to compare the polymorphism in three reptiles (*Alligator sinensis*, *Chinemys reevesii* and *Plestiodon chinensis*) with that in two other closely-related reptiles (*Alligator mississippiensis* and *Caiman crocodilus*).

In this study, we investigate genetic variation at exon 2 of the MHC class II B genes, including part of the putative antigen-binding sites, in three reptiles. We choose this particular exon because it is known to be highly polymorphic in primates and a variety of other terrestrial species. Our purposes in this study are: first, to analyze the variability of MHC class II B genes among the species listed above; second, to test for the influence of selection on amino-acid polymorphism, i.e. a positive (balancing) selection in exon 2; and finally, to document whether the trans-species polymorphism in MHC class II B genes also exits in reptiles.

Table 1. The genetic parameters of the sequences within each analysed species.

Species	N	L	*S*	π	*p*
C. reevesii	8	166	84	0.22203	35.80%
P. chinensis	7	166	12	0.0218	5.20%
A. sinensis	10	166	38	0.09236	18.40%

Note: N: number of sequences; L: sequence length (pb); S: variable sites; π: nucleotide diversity; p: amino acid diversity.

Materials and Methods

Isolation of genomic DNA.- Total genomic DNA was isolated from 20–50 µL of blood using standard phenol-chloroform extraction methods (Sambrook and Russell, 2001). The sampled individuals (without existing sequence data on GenBank) included a total of four *Chinemys reevesii* (terrapin) and two *Plestiodon chinensis* (saurian) from natural populations.

Polymerase chain reaction (PCR).- A 166 bp fragment of exon 2, from the class II B genes coding for part of the peptide-binding region, was amplified by PCR using the following degenerate primers reported by Shi et al. (2004): the forward (sense) primer MHC-UP 5'-AAGG(T/G/C)C(C/G)AGTG(T/C)TACT(T/A)(C/T)A(T/G/C)(T/G/C)AACGG-3'; the reverse (anti-sense) primer MHC-DP 5'-TAGTTGTG(C/G)C(G/T)GCAG(A/T)A(C/G)GTGTC-3'. PCR reaction were performed in 30 µL of reaction mixture containing 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 150 µM dNTP, 1 µM of each primer, 20–100 ng of isolated genomic DNA and 1 unit of *Taq* DNA Polymerase (Promega). Thermocycler conditions were as follows: an initial denaturation for 5 min at 94°C, followed by 35 cycles, each consisting of 30 s at 94°C, 40 s at 52°C, and 40 s at 72°C. The final extension at 72°C was for 10 min. PCR products were separated in a 2% agarose gel containing ethidium bromide (0.5 µg mL^{-1}). Separated PCR products were visualized under UV light and photographed to examine the banding patterns.

Cloning and sequencing.- Following agarose gel electrophoresis, PCR products of appropriate size were recovered, purified and concentrated using the DNA Gel Extraction Kit (V-gene Biotechnology Limited). The purified PCR products was ligated into the pGEM®-T Vector using the TA cloning kit (Promega); Competent *Escherichia coli* DH5α cells were transformed in a ligation reaction, and positive clones were identified by blue/white selection, as described in the manufacturer's protocol. Twenty to thirty positive clones were selected for each individual. Insert size was verified by PCR using M13 universal forward and reverse primer. Different inserts were screened by single-strand conformation polymorphism (SSCP) analysis and sequenced using the dideoxy nucleotide chain termination method (Sanger et al., 1977) on an Applied Biosystems 377 automated sequencer.

Data analysis.- Nucleotide and inferred protein sequences were aligned using the CLUSTAL X software (Jeanmougin et al., 1998). MHC sequences from closely-related species were acquired using the GenBank BLAST program (Altschul et al., 1990). Genetic distances were measured using the two-parameter method (Kimura, 1980). The computer package MEGA 2.1 (Kumar et al., 2001) was used to estimate the rate of nonsynonymous (d_N) and synonymous (d_S) substitutions according to Nei and Gojobori (1986), applying the Jukes and Cantor (1969) correction for multiple hits. The differences between these rates was evaluated with a *t*-test with infinite degrees of freedom according to the test statistic $t = d/s(d)$; $s(d)$ is the standard error of d and

Table 2. Numbers (mean±standard error) and relative rate (d_N/d_S) of nonsynonymous (d_N) and synonymous (d_S) substitutions per nucleotide in exon 2 sequences given for all sites and for pABS and non-pABS for comparison of three species.

Species	Sites	No. of codons	d_N (S.E.)	d_S (S.E.)	d_N /ds
C. reevesii	pABS	14	0.669±0.171	0.429±0.149	1.60*
	non-pABS	41	0.230±0.046	0.149±0.047	1.54*
	Total	55	0.311±0.049	0.204±0.047	1.52**
P. chinensis	pABS	14	0.043±0.027	0.032±0.036	1.34
	non-pABS	41	0.016±0.007	0.018±0.013	0.89
	Total	55	0.023±0.008	0.020±0.012	1.15
A. sinensis	pABS	14	0.245±0.087	0.045±0.034	5.44**
	non-pABS	41	0.083±0.025	0.058±0.032	1.43**
	Total	55	0.120±0.028	0.055±0.024	2.18**

Asterisks indicated the significance of two-tailed t-test in the order: * $p < 0.01$, ** $p < 0.001$.

Figure 1. Amino acid sequences translated from nucleotide sequences of exon 2 from MHC class II B genes of *C. reevesii, P. chinensis* and *A. sinensis*. The *Alsi* sequences are from our laboratory work (Shi et al. 2004). Asterisks (*) indicate the putative antigen-binding sites correspond with those for human class II sequences; dots (.) indicate identity with the consensus sequence at the top; dashes (–) gaps introduces to achieve optimal sequence alignment.

	10	20	30	40	50	55
Chre-1	T E R V R Y L Y R D	I Y N G R Q D L H F	D S D V G V H V A D	T E L G Q P D A E Y	W N S Q P E I L A D	R R A A V
Chre-2	. . . . .	. . . . .	. . . . R	. . . . .	. . . . .	. . . . .
Chre-3	. . . A	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
Chre-5	. Q . . . . . H	V W . Q E . . A . .	. . . . . Y . . .	M . . . E . . . K .	. . . . K . V M E L	. . G E .
Chre-4	. . . . . H . Y	. . D R . . I A . .	. . . L . . Y . . .	. . . . R L	. . . . . T . Q Y	Y . . V .
Chre-6	. . . I . L . V . W	. . . . Q . Y . . .	. . E L . R Y . . .	. . P . R . S . . .	. . K D K A E . D H	. . G E A
Chre-7	. . . . . L . V S W	. . . . Q . Y A . .	. . E L . R Y . . .	. . P . R . S . . F	. . K D K A V . D H	. . G E A
Chre-8	. . . . . F . F . Y	T . . R Q P L A . .	. . . . . F E . .	. . . . R A Q . . I	. . K D K A L . . Q	A . G E .
Plch-1	. Q . . . . . H	V W . Q E . . A . .	. . . . . Y . . .	M . . . G . . . K .	. . . . K . V M E L	. . G E .
Plch-2	. Q . . . . . H	V W . Q E . . A . .	. . . . . Y . . .	M . . . E . . . K .	. . . . K . V M E L	. . G E .
Plch-3	. Q . . . . . H	V W . Q E . . A . .	. . . . . Y . . .	M . . . E . . . K .	. . . . K . V M E L	. . G E .
Plch-4	. Q . . . . . Y	V W . Q E . . A . .	. . . . . Y . . .	M . . . E . . . K .	. . . . K . V M E L	. . G E .
Plch-5	. Q . . . . . H	V W . Q E . . A . .	. . . . . Y . . .	M D . . E . . . K .	. . . . K . V M E H	. . G E .
Plch-6	. Q . . . . . H	V W . Q E . . A . .	. . . M . . Y . . .	M . . . E . . . K .	. . . . K . V M E H	. . G E .
Plch-7	. Q . . . . P P . H	V W . Q E . . A . .	. . . . . Y . . .	M . . E E . . . K .	. . . . K . V M E L	. . G E .
Alsi-1	. Q . . . . . H	V W . Q E . . A . .	. . . . . Y . . .	M . . . E . . . K .	. . . . K . V M E L	. . G E .
Alsi-2	. Q . . . . . H	V W . Q E . . A . .	. . . . . Y . . .	. . . . E . . . K .	. . . . K . V M E L	. . G E .
Alsi-3	. Q . . . . . H	V W . Q E . . A . .	. . . . . Y . . .	. D . . E . . . K .	. . . . K . D M E Y	. . I G P
Alsi-4	. Q . . . . V S . .	. W D R . . . V . .	. . . . . Y . . .	. . . . E . . . K .	. . . . K . E . E .	D W . . .
Alsi-5	. Q . . . . . H	V W . Q E . . A . .	. . . . . Y . . .	. . . . E . . . K .	. . . . K . V . E Y	. . G S .
Alsi-6	. Q . . . . P . . H	V W . Q E . . A . .	. . . . . Y . . .	M . . . E . . . K .	. . . . K . V . E Y	. . G S .
Alsi-7	. Q . . . . V . . .	V W D R . . . V . .	. . . . . Y . . .	. . . . E . . . K .	. . . . R . E . E .	D W . . .
Alsi-8	. Q . . . . . V S	. W D R . . . V . .	. . . . . W . . .	. . . . E . . . K .	. . . – – – V E . E L	. . G V .
Alsi-9	. Q . . . . V . . .	V W D R . . . V . .	. . . . . Y . . .	. . . . E . . . K .	. . . . K . V . D Y	. . G . .
Alsi-10	. Q . . . . V . . .	V W D R . . N V . .	. . . . . Y . . .	. . . . E . . . K .	. . . . K . V M E Y	. . G S .
	* * *	* * *	*	* * * *	* * * *	* *

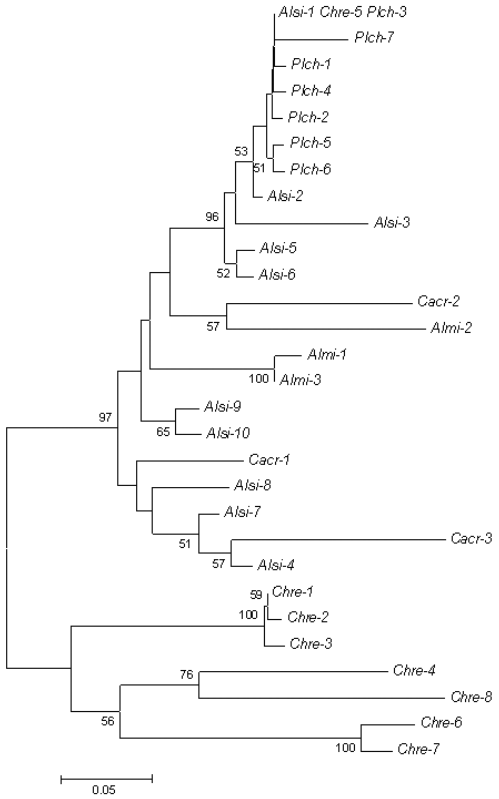

Figure 2. Phylogenetic tree of MHC class II B genes nucleotide sequences in different reptiles constructed by neighbor-joining method in MEGA. Bootstrap values from 1000 replications were indicated above the branches, values less than 50% are not shown.

is given by $s(d) = [\mathrm{Var}(d_S) + \mathrm{Var}(d_N)]^{1/2}$ (Kumar et al., 1993). These analyses were performed on the 24 codons comprising the ABS sites (as defined in the crystal structure of a human class II molecule, DRB1 (Brown et al., 1993)) and on all remaining non-ABS codons in the amplified segment. The phylogenetic tree was constructed using MEGA 2.1 program for distance-based methods, applying the neighbour-joining (NJ) algorithm. Bootstrap analysis (1000 replications) was performed to determine the reliability of the branching pattern in the phylogenetic tree. To further quantify polymorphism, nucleotide diversity (π) was also calculated using the computer application DnaSP (Rozas et al., 1999).

Results

Amount and extent of variation.- In total, fifteen sequences were obtained from *Chinemys reevesii* and *Plestiodon chinensis* (see Table 1); sequence identity was confirmed by sequencing multiple clones in both directions. These sequences are designated *Chre* for *Chinemys reevesii* and *Plch* for *Plestiodon chinensis*, in accordance with the proposed nomenclature (Klein et al., 1990), and have been deposited in GenBank under the accession numbers AY937200~AY937207 (for *Chre*-1~*Chre*-8), AY772946~AY772951 and AY764032 (for *Plch*-1~*Plch*-7). Ten sequences (*Alsi*-1~*Alsi*-10; GenBank accession numbers AY491421~AY491430) of exon 2 of the class II B genes from three *Alligator sinensis*, used for the subsequent data analysis, were also submitted. All sequences except for one from *A. sinensis* (*Alsi*-8), which had six nucleotides deletions, were 166bp in length. Published sequences were aligned with those derived here, illustrating numerous variable sites in *Chinemys reevesii* (84 [=50.6%]), *P. chinensis* (12 [=7.2%]) and *A. sinensis* (38 [=22.9%]); these numbers are consistent with those seen for this fragment in other species. Nucleotide diversity was also calculated within species, with all three species exhibiting a mean pairwise nucleotide diversity of 0.22203, 0.02180 and 0.09236, respectively. Furthermore, *Chre*-5 was also found to be identical to *Plch*-3 in *P. chinensis*, and *Alsi*-1 in *A. sinensis*. Sharing of these same alleles in different species was also reported in Hedrick et al. (2002).

Amino acid variation within *Chinemys reevesii*, *Plestiodon chinensis* and *Alligator sinensis* was 35.8%, 5.2% and 18.4%, respectively (Table 1). Aligned amino acid sequences are presented in Figure 1. The putative antigen-binding sites (pABS), corresponding to those in the human class II sequences (Brown et al., 1993), are indicated by an asterisk. The term "putative" has used here because the actual antigen-binding sites for reptiles have not yet been verified. Among the *Chinemys reevesii* sequences, 92.9% (13 out of 14 codons) of pABS are variable and 63.4% (26 out of 41 codons) of the nonbinding sites (non-pABS) are variable. Within *A. sinensis*, 71.4% of pABS and 31.7% of the non-pABS are variable, and in *P. chinensis*, 21.4% of pABS (3 of 14) are variable, while 12.2% (5 of 41 positions) of non-pABS are variable. The numbers of synonymous substitutions (d_S) and nonsynonymous substitutions (d_N) per nucleotide in exon 2 sequences are given in Table 2. The ratio of d_N to d_S tended to be greater than 1.0, particularly for pABS, which has a ratio consistent with that seen in other MHCs, suggesting that there is selection for amino acid replacements in the antigen-binding region. Meanwhile, d_N/d_S for pABS and non-pABS in *Chinemys reevesii* and *A. sinensis* were all larger than 1.0.

Phylogenetic analysis.- BLAST searches in genome sequence databases have revealed that a number of alleles from other reptiles exhibit a high degree of similarity to the sequences derived here. Of these, three *Caiman crocodilus* alleles (*Cacr*-1~*Cacr*-3; Accession Numbers AF256651, AF256652 and AF277661) and three *Alligator mississippiensis* alleles (*Almi*-1~*Almi*-3; Accession Numbers U24402~U24404) were chosen for

phylogenetic analysis. A neighbor-joining tree showing the relationships among these nucleotide sequences is presented in Figure 2. The sequences of *Chinemys reevesii* and *Plestiodon chinensis* tend to cluster together. Interestingly, *Caiman crocodilus* sequences were widely dispersed in the tree (supported by high bootstrap values), being more similar to the crocodile sequences. A cluster of four *Alsi* sequences (*Alsi*-2, *Alsi*-3, *Alsi*-5, *Alsi*-6) showed a higher degree of similarity to *Plch* sequences than to other lineages. The phylogenetic tree supports the hypothesis of trans-species polymorphism, as indicated by the clustering of lineages from different species and the presence of sequences from different species in the same allelic lineage. This trans-species allelic similarity is not unusual for MHC genes, as it has been proposed that MHC allelic lineages are maintained by selection and are often older than the species themselves.

Discussion

In the present study, we investigated exon 2 (of MHC class II B genes) sequences from three reptiles and examined within species polymorphism. The results revealed relatively high amounts of variability in both nucleotide and amino acid sequences (Table 1), as well as a pattern of evolution consistent with those seen in a variety of mammalian species, including humans. However, the level of genetic variation within each of these species differed, possibly reflecting different patterns of evolution and population genetic structure. Levels of polymorphism are higher in *Chinemys reevesii* and *Alligator sinensis* compared to *Plestiodon chinensis*, which may prove to be of value in future studies on population genetics and conservation biology.

As an important genetic component of the vertebrate immune system, variation in the MHC is significant to consider selective pressure due to parasitic or pathogen resistance. It has been suggested that species or populations with low MHC diversity might be particularly susceptible to infectious disease and parasites (Hedrick and Kim, 2000; O'Brien and Evermann, 1988). Furthermore, in this and other studies (Hedrick et al., 2002; Ottová et al., 2005), identical MHC alleles have been found amongst different species; the sharing of these likely homologous sequences may be due to exposure to similar (or the same) antigens present throughout the evolution of each species.

Balancing selection appears to play a determinant role in MHC evolution (Bernatchez and Landry, 2003), evidence of which is the presence of more nonsynonymous (d_N) than synonymous (d_S) substitutions in antigen-binding sites (Binz et al., 2001; David and Helena, 2003; Hedrick et al., 2002). In the present study, the observed excess of nonsynonymous substitutions, particularly at putative antigen-binding sites, indicates that nonsynonymous sites evolve faster than synonymous sites. This implies the presence of balancing selection (or positive Darwinian selection), which favors new variants and increases MHC diversity, which has been observed in a number of species (Hughes and Nei, 1989). In the case of *Chinemys reevesii* and *Alligator sinensis*, however, a significant excess of nonsynonymous substitutions was also found in non-pABS sites, possibly suggesting that reptile ABS sites may not exactly correspond to those in humans as originally defined by Brown et al. (1993). Similar findings were reported for the Pacific salmon (Miller and Withler, 1996) and Sonoran topminnow (Hedrick et al., 2001).

The phylogenetic tree suggests that some *Alsi* sequences are more similar to *Plch* sequences than to other *Alsi* sequences, with species sequences intermingling to form several significantly-supported clusters (Fig. 2). This intermixing suggests a trans-species persistence of MHC class II exon 2 sequences, with some allelic lineages predating species cladogenesis. The prolonged maintenance of MHC alleles is contrary to what would be expected from neutral loci, supporting the idea that long-term balancing selection on the MHC alleles has occurred (Figueroa et al., 1988). Our results are consistent with the theory of trans-species evolution in MHC alleles (Klein, 1987), which has been previously supported by studies on mammals and fish (reviewed in Hedrick, 2001), suggesting that MHC polymorphism is widespread in the Vertebrata.

In conclusion, we are presenting strong evidence for trans-species polymorphism at exon 2 of class II gene in reptiles. The polymorphism is putatively maintained by balancing selection and is restricted to what are apparently functional loci on (primarily) the ABS sites. These observations suggest that the MHC carries out the same function in reptiles as it does in mammals, but additional research needs to be conducted, particularly with regards to trans-species polymorphism and specific binding loci across taxa.

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