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MOLECULAR CHARACTERIZATION, POLYMORPHISM AND EXPRESSION ANALYSIS OF SWAMP EEL MAJOR HISTOCOMPATIBILITY COMPLEX CLASS II B GENE, AFTER INFECTION BY *AEROMONAS HYDROPHILIA*

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ABSTRACT

Major histocompatibility complex (MHC) class II genes play a crucial role in the immune system of vertebrates. Here we report the cloning of full-length cDNA from the swamp eel (*Monopterus albus*) MHC class II B (MHC-DAB) gene. We described its genomic structure, molecular polymorphism and expression profiles. The full-length cDNA (Gen Bank accession No.:JQ236680) is 1339 bp, encoding a 249 amino acid peptide. The genomic sequence was identified to be 3066 bp in length, which contained six exons and five introns. Sequence comparison showed that the proposed amino acid sequence shared between 26.4 and 74.7% identity with other species. Thirty seven distinct alleles were isolated from sixty individuals. There were between two and five alleles per individual. The presence of five alleles in an individual suggested that there were at least three MHC IIB loci in the genome. At the protein-binding region d_N was significantly greater than d_S, providing evidence of strong positive selection among swamp eel sequences. RT-PCR demonstrated high MHC-DAB expression in stomach, spleen, blood cell and liver, moderate expression in brain, skin and kidney, and low or negligible expression in heart, intestine and muscle. Great changes were observed in liver, spleen and intestine after challenged with pathogenic bacteria, *Aeromonas hydrophilia.*

Key words: Major histocompatibility complex (MHC); cDNA; genomic structure; polymorphism; swamp eel (*Monopterus albus*).

INTRODUCTION

The major histocompatibility complex (MHC) encodes the cellular glycoproteins responsible for presenting self or non-self antigens to T cell receptors (TCR), and thereby initiate immune responses in invertebrates (Rothbard *et al.*, 1991; Sommer, 2005). MHC molecules, in general, are divided into two main subgroups according to their chemical structure and molecular function (Piertney *et al.*, 2006; Srisapoome *et al.*, 2004). MHC class II proteins are heterodimers, composed of two peptides (alpha and beta chains). The alpha-1 and beta-1 domains of the two chains form the peptide-binding region (PBR), which facilitates the T lymphocyte-mediated immune recognition of pathogens (Carroll *et al.*, 2002). Major changes in the amino acid sequences of the PBRs alter the peptide binding capabilities of the MHC locus and also affect pathogen recognition (Brown *et al.*, 1993). All MHC molecules are characterized by an extremely high degree of polymorphism, mainly due to the large number of alleles that exist within populations, and the high sequence variation between alleles (Grimholt *et al.*, 2003).

Since the first fish MHC was cloned from carp in 1990 (Hashimoto *et al.*, 1990), a large number of MHC genes have been isolated from different teleosts (Ristow *et al.*, 1999; Xu *et al.*, 2009; Zhou *et al.*,2013; Pang *et al.*, 2013;Sültmann *et al.*, 1994). With the development of study, growing eidence showed that high degrees of MHC polymorphism are closely related to resistance or susceptibility to disease in mammals (Medina *et al.*, 1998; Paterson *et al.*, 1998;Hill *et al.*,1991;Tang *et al.*, 2012), poultry (Briles *et al.*, 1983; Nikolich-Zugich *et al.*, 2004; Banat *et al.*, 2012) and teleost fish (Zhang *et al.*, 2006b; Wynne *et al.*, 2007; Rakus *et al.*, 2009; Du *et al.*, 2011). Therefore, MHC genes are likely candidates as gene markers for disease resistance. As far as we are concerned, at present, there is no report on the swamp eel MHC molecules.

Swamp eels, *Monopterus albus*, are an important economical freshwater fish in China and other Asian countries (Zhou *et al.*, 2002). However, traditional breeding of swamp eels rely heavily on the natural resources (Cai *et al.*, 2008). The production of swamp eel has become seriously challenged not only because of the poor bacterial-resistance of farmed populations, but also because of decline in wild populations due to large scale application of pesticides and overfishing (Lei *et al.*, 2012). In order to conserve and sustainably exploit these resources, new resistant genes, urgently need to be

introduced into the marker-assisted selection (MAS) breeding program.

In this study, we firstly reported the cloning and structural analysis of the swamp eel MHC-DAB gene. We also described its molecular polymorphism and expression in tissues in response to infection with pathogenic bacteria. The objective of the study was to facilitate better understanding of vertebrate immunity and thereby help to formulate disease management strategies for farming swamp eels.

MATERIALS AND METHODS

Sampling and challenge of swamp eel with bacteria: Two hundred, healthy, swamp eels (weighing 60 to 70g) were purchased from the Taihu Fishery Farm in Jingzhou, China. The fish were raised in tanks at 20 ºC for 1 week before genetic analysis. Ten different tissues (heart, liver, spleen, stomach, kidney, blood cells, intestine, skin, muscle, brain) were removed and immediately immersed in liquid nitrogen at -80 ºC until RNA extraction.

Challenge of swamp eel with pathogenic bacteria was performed as reported previously (Zhou *et al.*, 2013) with minor modifications. Briefly, the pathogenic bacteria (*Aeromonas hydrophilia*) were cultured at 28ºC to mid-logarithmic growth in LB medium. The bacteria were then collected by centrifugation at 4500 rpm/min for 5 min, and resuspended at approximately 2.2×10^7 CFU/mL in phosphate-buffered saline (PBS). The challenge concentration was determined in pre-challenge experiments. The fish were anesthetized by immersion in 3-Aminobenzoic acid ethyl ester methanesulfonate (MS222, Sigma) and injected intraperitoneally with 20 µL of bacterial suspension or with the same volume of PBS (controls). Infected and control fish were sacrificed 4, 12, 24, 48 and 72 h after injection. Three tissues (liver, spleen and intestine) were collected and kept at -80ºC for RNA extraction.

DNA isolation and cDNA synthesis: Genomic DNA was isolated from the liver of swamp eels using a DNA isolation kit (Omega). Total RNA was extracted from the tissues using Trizol reagent (Invitrogen) according to the manufacturer's instructions. cDNA synthesis was carried out with PrimeScript® RT reagent kit (Takara) according to manufacturer's instructions. The synthesized cDNA was kept at -80ºC until further use.

Primer design, amplification of MHC-DAB fragment and RACE-PCR amplification: A pair of degenerate primers, seMHC-N1 and seMHC-C1 (Table 1), were designed according to the conserved sequences of MHC II B genes from other species, such as large yellow croaker (ABV48908), striped sea bass (AAA49379), spotted halibut (GU253881), rainbow trout (AF115529) and cichlid (AAB27553). 5' RACE and 3' RACE PCR amplification of the MHC-DAB gene were performed using a Smart Race cDNA amplification kit (Clonetech) according to the manufacturer's instructions. Nested PCR was carried out using the first-PCR products as templates. Touchdown polymerase chain reaction was used for RACE PCR. The sequence was performed as described in Xu *et al.* (2009): 94ºC for 3 min, 94 ºC for 1 min, 70 ºC for 45 s, 72 ºC for 2 min, for 5 cycles. This was followed by 94ºC for 1 min, 65 ºC for 50 s, 72 ºC for 1 min, for 30 cycles; and 72ºC for 10 min for elongation. The PCR products were resolved and purified by QIAEX gel extraction kit (Qiagen).The purified fragments were ligated into pMD-18T (Takara) and cloned to Top10 cells. Positvie clones were screened via PCR with M13+/- primers. At least three clones were sequenced per fragement using ABI PRISM 3730 DNA sequencer with M13 primer.

Genomic sequences of swamp eel MHC-DAB gene: Based on the full-length cDNA sequence of MHC-DAB, gene-specific primers were synthesized to amplify the introns in order to further characterize swamp eel MHC class II B (Table 1). The conditions of the PCR were as follows: pre-denaturalization at 94ºC for 4 min, followed by 35 cycles of 94ºC for 30 s, 50-60ºC for 35 s and 72ºC for 1 min 30 s, followed by one extension cycle at 72ºC for 10 min. Exon-intron junctions were deduced according to the known MHC IIB genes.

Molecular polymorphism analysis of the swamp eel MHC-DAB: According to the full-length cDNA sequence of MHC-DAB, two gene-specific primers (DAB-alle-F and DAB-alle-R), were designed to amplify the complete exon 2 in order to analyze molecular polymorphism of the swamp eel MHC-DAB gene (Table 1). Sixty healthy eels were randomly selected for these experiments. The alleles were named and determined according to the principle published by Klein *et al*. (1990) and Kennedy *et al*. (2002). Peptide-binding region (PBR) sites were determined according to Brown *et al.* (1993). The average rates of synonymous (d_S) and nonsynonymous (d_N) substitutions per site were calculated using the method described by Nei and Gojobori (Nei *et al.*, 1986) with the Jukes and Cantor correction as implemented in the MEGA 4.0 software. P values and standard error was determined by 1000 bootstrap replications and the rates were compared with the Z-test of selection (Tamura *et al.*, 2007).

Sequence alignment and phylogenetic tree construction: The alignment of deduced amino acid sequences of MHC class II B was performed using the Clustal W procedure in MEGA version 4.0 (Tamura *et al.*, 2007). A phylogentic tree was constructed using deduced amino acid sequences from MHC class II B genes using the neighbor-joining method (Saitou and Nei,

1987). Bootstrap tests were replicated 1000 times to derive the confidence values for the phylogeny analysis.

Expression analysis of swamp eel MHC-DAB: Real-time quantitative (RT)-PCR was performed to determine the expression profiles of the MHC-DAB mRNA in normal tissues and to investigate the regulation of the MHC-DAB following bacterial challenge. Total RNA was extracted from ten normal tissues and three infected fish tissues. Based on the full-length cDNA sequence of MHC-DAB, primer DAB-rt-F1 and DAB-rt-R1 (Table 1) were designed by primer premier 5.0 to amplify the gene fragments. Expression of beta-actin was used as internal control.

RT-PCR was performed on a 7500 Real-time PCR system (Applied Biosystems, USA). The 20 µL reaction system contained 1 µL cDNA template, 10 µL SYBR Premix Ex Taq^{TM} (Takara), 0.4 µL ROX reference dye II, 0.4 µL of each primer and 7.8 µL of sterile H_2O. Reactions carried out without the template were used as a blank control. PCR was performed in triplicate wells, three samples per treatment, using the following sequence: 30 s at 95ºC, followed by 40 cycles consisting of 5 s at 95ºC, 25 s at 54ºC and 1 min at 72ºC. Dissociation curve analysis was performed after each assay to determine target specificity.

RESULTS AND DISCUSSION

Sequences analysis of swamp eel MHC-DAB: Full-length cDNA of swamp eel MHC-DAB that was designated as moal-DAB*0101 is 1339 bp, including 10 bp 5' UTR, 750 bp encoding region and 579 bp 3' UTR (GenBank accession No.JQ236680) (Figure 1). The swamp eel MHC-DAB genomic DNA was 3066 bp in length. Six exons and five introns were identified, which is similar to that of the Atlantic salmon (Kjøglum *et al.*, 2006), spotted halibut (Li *et al.*, 2011), Mi-iuy croaker (Xu *et al.*, 2011) and Nile tilapia (Zhou *et al.*, 2013; Pang *et al.*, 2013). However, the sequence was markedly different from the general five-exon-four-intron structure of other teleosts, such as the turbot (Zhang and Chen, 2006a) and half-smooth tongue sole (Xu *et al.*, 2009) (Figure 2). Interestingly, a 15-bp repeat sequence (ACTGTCTACACAGCA) was found both in exon 1 and intron 1, which suggested that alternative splicing was involved in the prematuration process of MHC-DAB mRNA.

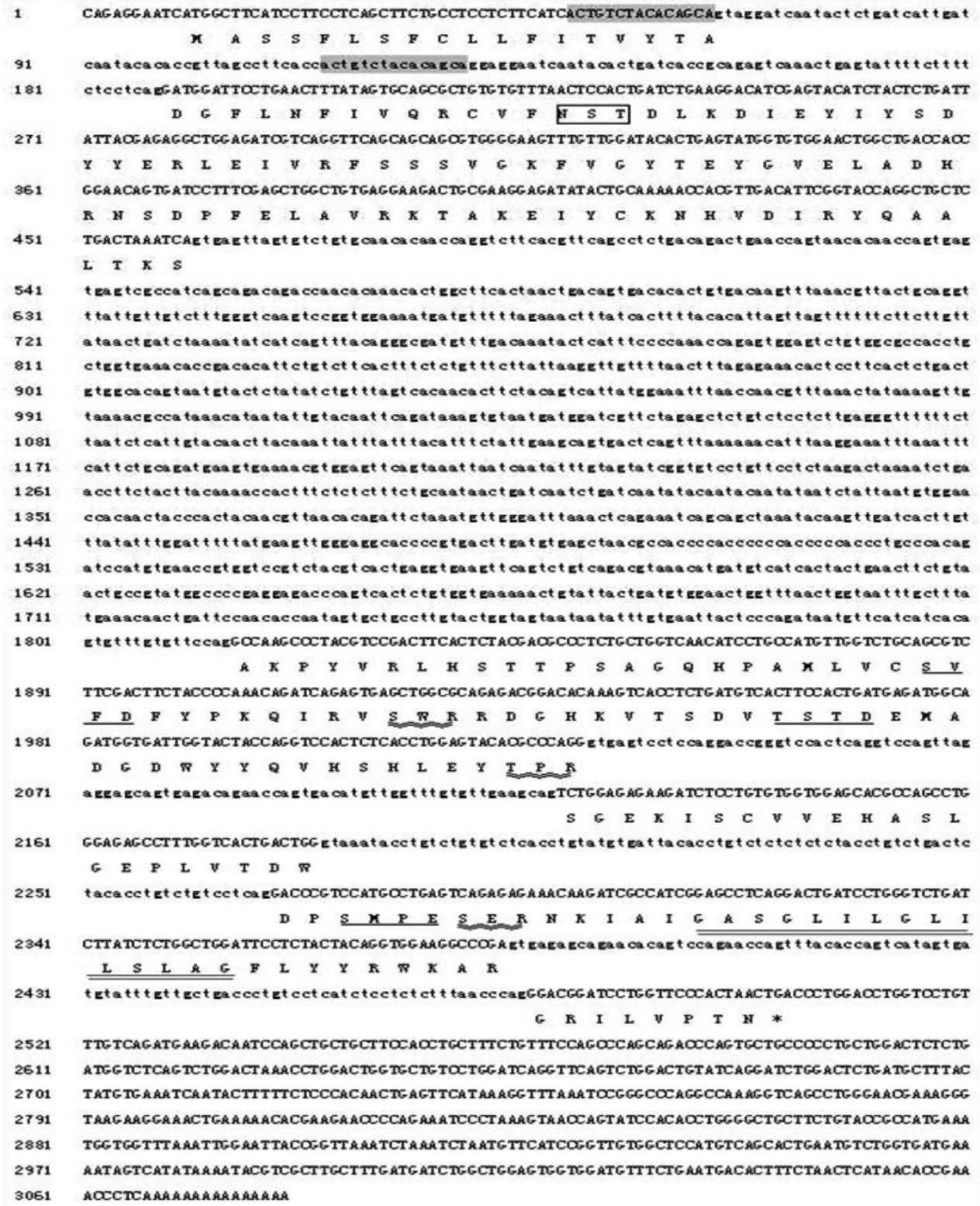
Compared with that of other teleosts, the putative amino acid sequence of swamp eel MHC IIB consisted of two extracellular domains, one connecting peptide, one transmembrane region and one cytoplasmic domain. Moreover, a single N-glycosylation site (N-X-S/T-X) was found in the beta-1 domain. Three protein kinase C phosphorylation sites (S/T-X-R/K), three kinase II phosphorylation sites (S/T-X-X-D/E) and a GXXGXXXGXXXXXXG motif were observed in the ORF (Figure 3). Four conserved cysteine residues were found in the beta-1 and beta-2 domains, which are conserved among other fishes (Figure 3). These structure features of swamp eel were very similar to those in mammalian and teleosts (Brown *et al.*, 1993; Xu *et al.*, 2009; Li *et al.*, 2011; Zhou *et al.*, 2013; Pang *et al.*, 2013).

Molecular polymorphism of MHC-DAB: Sixty randomly selected healthy eels were used to study the allelic polymorphism. Primer DAB-alle-F and DAB-alle-R were used to amplify the complete exon 2 fragment of MHC-DAB from every individual. All the PCR products were purified and ligased to pMD-18T. Positvie clones were screened via PCR with M13+/- primers. Five or six positive clones from each eel were randomly sequenced.Thirty-seven alleles belonging to 30 allele major types, were isolated (Figure 4). The proportion of variable sites for nucleotide and amino acid sequences across the 37 alleles were 40.3% (110/273) and 50.6% (46/91), respectively. There were between two and five alleles per individual, with 56 (93.3%) of the 60 eels displaying at least three alleles; one of 56 eels displayed 5 alleles, and 9 of the 56 displayed 4 alleles. More than four alleles of MHC IIB have also been identified in a single individual from other fish species, suggesting the existence of at least three loci of class IIB (Li *et al.*, 2011;Zhou *et al.*, 2013; Pang *et al.*, 2013;Zhang and Chen, 2006a; Xu *et al.*, 2009). However, only one MHC class II B locus was identified in Chinese long snout catfish (Shen *et al.*, 2011).

In mammals, MHC polymorphism is maintained over long times by positive (balancing) selection at the nonsynonymous sites specifying the PBR of the MHC molecule (Graser *et al.*, 1996). The rate of nonsynonymous substitutions significantly exceeds that of synonymous substitutions in MHC genes, the dN/dS ratio is greater than 1, as would be expected if the locus were evolving under balancing selection (Hughes and Nei, 1989). In our study, the rate of non-synonymous substitution (d_N) was significantly higher than the rate of synonymous substitution (d_S) in the peptide-bridging regions (PBR) (Z-test, d_N/d_S=3.018, P=0.005), suggesting that the polymorphism of MHC-DAB alleles may be maintained by positive selection (Table 2).

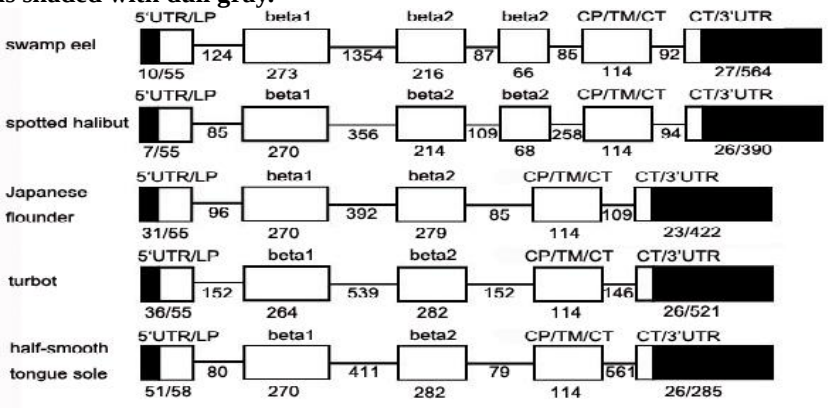
Sequence alignment and phylogentic analysis: Phylogenetic analysis demonstrated that the deduced amino acid sequence of swamp eel MHC-DAB had 29.6%, 27.1%, 29.0%, 29.6%, 39.7%, 49.8%, 51.1%, 51.6%, 54.9%, 67.1%, 69.6%, 70.7%, 71.9%, 73.5%, 74.2% and 74.7%identity with those of nurse shark, chicken, human, mouse, guppy, zbrafish, common carp, rainbow trout, Atlantic salmon, spotted halibut, tongue sole, turbot, cichlid, red sea bream, stripped sea bass, and large yellow croaker, respectively. The phylogenetic trees

exhibited a close relationship with other teleosts. The swamp eel MHC-DAB was clustered with those from the cichlid and other teleosts (Figure 5). All these results

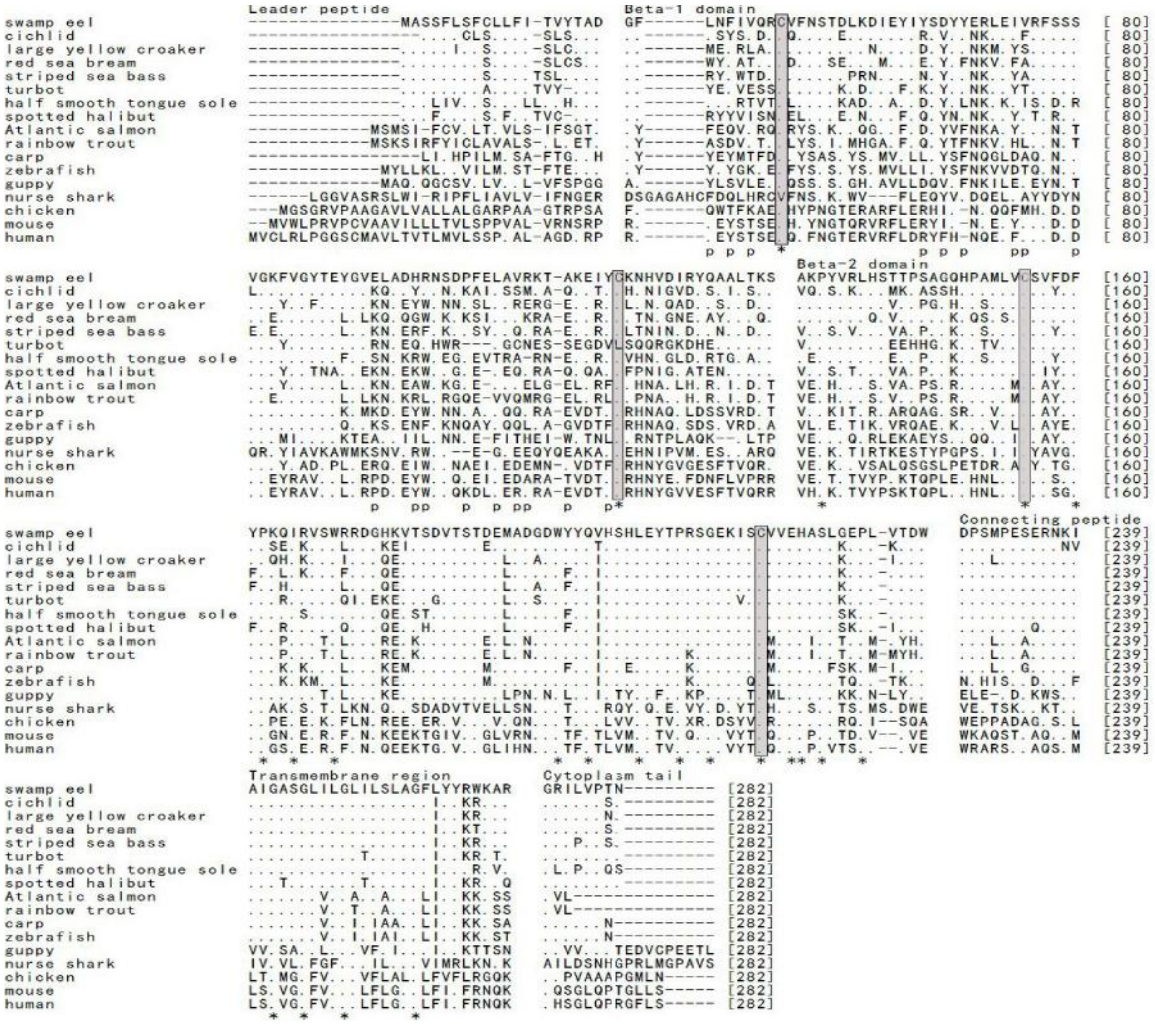


Li *et al*., Figure 1 Genomic sequence of swamp eel MHC class II B gene. Exons are in uppercase and introns are in lowercase. The stop codon is indicated by an asterisk. N-linked glycosylation site is represented with a box; protein kinase C phosphorlation sites are underlined with ⎯; casein kinase II phosphorylation sites

are underlined with ——; the GXXGXXXGXXXXXXG motif was underlined with ═.The 15-bp repeat sequence was shaded with dull gray.



Li *et al.*, Figure 2 Schematic illustration of the swamp eel MHC-DAB gene. Boxes represent exons, and the lines represent introns. LP: leader peptide; CP:connecting peptide; TM:transmembrane region; and CT: cytoplasmatic tail. Numbers indicated the length of the introns and exons.



Li *et al.*, Figure 3 Alignment of swamp eel MHC-DAB amino acid sequences with those of other species. The GenBank accession numbers of them are listed in Figure 5. "p" indicates the correlative amino acid that combines the antigen; the N-linked glycosylation signal is underlined with ——; asterisks show the identical residues; the conserved four cysteine residues were boxed.

strongly suggest that swamp eel MHC class II B gene was a novel member of the MHC superfamily.

Expression analysis of swamp eel MHC-DAB: Quantitative real-time RT-PCR showed that MHC-DAB transcripts were ubiquitously expressed in ten tissues, but expression levels were distinctly different (Figure 6). There was relatively high expression of MHC-DAB in stomach, spleen, blood cell and liver; moderate expression in brain, skin and kidney, and low or negligible expression in heart, intestine and muscle (Figure 6). The swamp eel MHC-DAB gene expression pattern is consistent with what had been found in other fish and mammalian (Brown *et al.*, 1993; Xu *et al.*, 2009; Li *et al.*, 2011; Zhou *et al.*, 2013; Pang *et al.*, 2013). Relatively low or negligible expression of MHC class II B in muscle seen in swamp eels has also been found in carp (Rodrigues *et al.*, 1995), spotted halibut(Li *et al.*, 2011), red sea bream (Chen *et al.*, 2006), half-smooth tongue sole(Xu *et al.*, 2009) and Nile tilapia(Zhou *et al.*, 2013; Pang *et al.*, 2013). In contrast, the high expression in spleen, kidney, liver and intestine suggested that these organs might involve in the adaptive immune system (Chen *et al.*, 2006; Li *et al.*, 2011; Xu *et al.*, 2009).

Challenge of swamp eel with the pathogenic bacteria, *Aeromonas hydrophilia*, resulted in marked changes in the pattern of expression levels of MHC-DAB. The expression level of MHC-DAB transcripts was initially up-regulated in the intestine from 0 h to 4 h. It then decreased acutely between 4 and 48 h, followed by a marked increase at 72 h. Expression of MHC-DAB transcripts showed evidence of a sustained decrease in liver tissue up to 24 h post infection, followed by a slight increase from 48 h to 72 h. Expression of MHC-DAB transcripts in the spleen was slowly down-regulated from 0 to 72 h with minor fluctuations (Figure 7). Previous studies have shown that expression levels of MHC class II B mRNA are markedly affected when challenged with pathogenic bacteria or lipopolysaccharide (LPS) (Koppang *et al.*, 1999; Chen *et al.*, 2006; Li *et al.*, 2011; Xu *et al.*, 2009; Zhou *et al.*, 2013; Pang *et al.*, 2013). Different pathogenic bacteria, produce different changes in MHC class II B gene expression (Zhou *et al.*, 2013;Pang *et al.*, 2013). In the same way, challenge with same pathogen always resulted in different expression profiles in different fishes (Li *et al.*, 2011; Xu *et al.*, 2009;Chen *et al.*, 2006). Therefore, it is reasonable to conclude that high diversity of MHC class II B gene results in individual vertebrate species being able to bind and present a variety of peptide ligands that trigger different immune responses (Srisapoome *et al.*, 2004; Croistetiere *et al.*, 2008). Further studies will be needed for elucidating the precise role and mechanism of MHC genes in defense response in swamp eels.

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01  moal-DAB*0101 DGFLNFIVQR CVFNSTDLKD IEYIYSDYYE RLEIVRFSSS VGKFVGYTEY GVELADHRNS DPFELAVRKT AKEIYCKNHV DIRYQAALTK S [91]
02  moal-DAB*0102 .......... .......... .......... .......... .......... .......... .......... .......... ..E....... . [91]
03  moal-DAB*0103 .......... .......... .......... .......... .......... .......... ...M...... .......... ..E....... . [91]
04  moal-DAB*0104 ......T... .......... .......... .......... .......... ...Q...... ...V...... .......... ..E....... . [91]
05  moal-DAB*0105 .......... .......... .......... ..K....... .......... ...Q...... ...V...... .......... ..E....... . [91]
06  moal-DAB*0201 .......... .......... .......... .........R .......... ...Q...... ...V...... .......YNI ..D....... . [91]
07  moal-DAB*0301 .......... .......... .......... K......... .......... ...Q...... .........N Q..R...HNI ..D....... . [91]
08  moal-DAB*0401 .......... .......... .......... .........R .........F ..KQ..NW.. ...V.....N Q..RF..HNI ..D....... . [91]
09  moal-DAB*0402 .......... .......R.. .......... .........R .........F ..KQ..NW.. ...V.....N Q..RF..HNI ..D....... . [91]
10  moal-DAB*0403 .......... .......R.. .......... .........R .........F ..KQ..NW.. ...V.....N Q..RF..HNI NNE....... . [91]
11  moal-DAB*0501 ....E.F.D. .......... ......T..N K..FA..... .......... ..KQ..YW.K ..SV...... L..S...HNI ..E....... . [91]
12  moal-DAB*0601 ....E.V.N. .......... .......H.N KV.LI..... .......... ..KQ..YW.. ..SV..L..N E..R...HNI ..D....... . [91]
13  moal-DAB*0701 ....RYD.D. .......... ......K..N K...I..... ...Y.....L ..KN.ERW.. G.-....... ...R....NI NNE....... . [91]
14  moal-DAB*0801 .....YD.V. .......... ..F...A..D K..FA..... .......... ..KQ...W.K G.E-.VQM.N Q..R....NI ..D....... . [91]
15  moal-DAB*0901 ......A.D. .......... ......M..D K..YA..... ...L.....F ..KQ..NW.N ..SQ..L..N E..R...PTI ..D....... . [91]
16  moal-DAB*1001 ....RYS.D. .......... ......T..N K..FA..... .......... ..KQ..YW.. ..SQ..L..N E..R...PNI .SD....... . [91]
17  moal-DAB*1101 .....YR.G. ......E... ....Q.YV.D K......... .........F ..KQ..NW.. G.E-.VQM.N E..R...HNI .......... . [91]
18  moal-DAB*1201 .....YD.V. .......... ..F...T..D K..FA..... .......... ..K...YW.K G.E-.VQA.N D..R...HNI ..D....... . [91]
19  moal-DAB*1301 ....KY..N. ......E... ......T..N K..LA..... .......... ..KN.ERW.N ..S...R..N E..R...HNI .VD....... . [91]
20  moal-DAB*1401 ....RYD.D. .......... ......K..N K...A..... ...Y.....L ..KN.ERW.K ..S...R..N E..R...PNI .SD....... . [91]
21  moal-DAB*1501 ......D.D. ......E... ......I..N K...T..... .........L ..KN.ERW.. .AGY.VEM.N E..R...HNI ..D....... . [91]
22  moal-DAB*1601 ....YYA.D. .......... ....E.M..D K..FI..... .......... ..KN.ERW.. G.E-.VQM.N E..R...HN. ..D....... . [91]
23  moal-DAB*1701 ......A.S. .......... ..L.L.M... K..FA..... .........H ..KN.ERW.. G.E-.VQW.N E..R...HNI ..W....... . [91]
24  moal-DAB*1801 ......D.D. ......E... ......I..N K...T..... .........L ..KN.ERW.. .AGY.VEM.N E..R...HNI ..D....... . [91]
25  moal-DAB*1901 ....RYT.D. .......... ......I..V K..YIK.... .........H ..KN.ERW.K G.-...L... E..R...YNI ..D....... . [91]
26  moal-DAB*2001 ....RYD.D. .......... ......K..N K...A..... .........L ..KN.ERW.K G.-..VR..N E..R...PNI .SD....... . [91]
27  moal-DAB*2101 ....E.S.D. .......... ......M..D K..YI..... .........H ..KN.EFW.. .AGY.VEM.N E..R...HNI ..D....... . [91]
28  moal-DAB*2201 ....YYA.D. .......... ....E.H..D K..FI..... .........H ..KN.ERW.. G.E-.VQM.. E..R....NI .NE....... . [91]
29  moal-DAB*2301 ....RYT.D. .......... ......I..D K..YI..... .........H ..KN.ERW.. .AGY.VEM.N E..R...HNI ..D....... . [91]
30  moal-DAB*2401 ....HA..S. .......... ....R.YI.D K...I..... .........L ..KN.ERF.. G.E-.VGM.N E..RF..HNI ..W....... . [91]
31  moal-DAB*2501 ....E.F.S. .......... ....Q.YI.D K..YI..... ...Y.....F ..KN.KSW.. G.E-.VQV.N ...R...HNI ..W....... . [91]
32  moal-DAB*2601 ....RYT.D. .......... ......I..D K..YI..... .........H ..KN.ERW.. .AGY.VEM.N E..R...HNI ..D....... . [91]
33  moal-DAB*2701 ....E.Q.E. .......... ..L.H.L..D K..YA..... .........F ..KN.ERW.. G.E-.VQM.N E..RF..HNI ..D....... . [91]
34  moal-DAB*2801 ...Q.YQ.T. ......E... ....E.YV.D K..WA..... .........F ..KN.ERW.. ..SV..R..N E..RV..HNI ..D....... . [91]
35  moal-DAB*2802 ...Q.YQ.T. ......E... ....E.YV.D K..WA..... .........F ..KN.ERW.. G.E-.VR..N E..R...HNI ..D....... . [91]
36  moal-DAB*2901 ....HA..S. ......EM.. ....E.YI.N K......D.. ...Y.....F ..KN.ERF.K G.E-.VQA.N E..R...HNI .......... . [91]
37  moal-DAB*3001 ....E.A.V. .......... ..F.R.YI.D K...I..... ...Y....KF ..KN.ERF.. GQE-.VA..N K..S...HNI ..D....... . [91]
    Putative PBR     p p p              p p p     pp    p              p   pp    p  p pp   p   p  pp   pp pp
    Polymorphic      **** *   *    **     * * ** * *****  * *    *    **   ** *** * ******** * *  **  *** ***
    Sites
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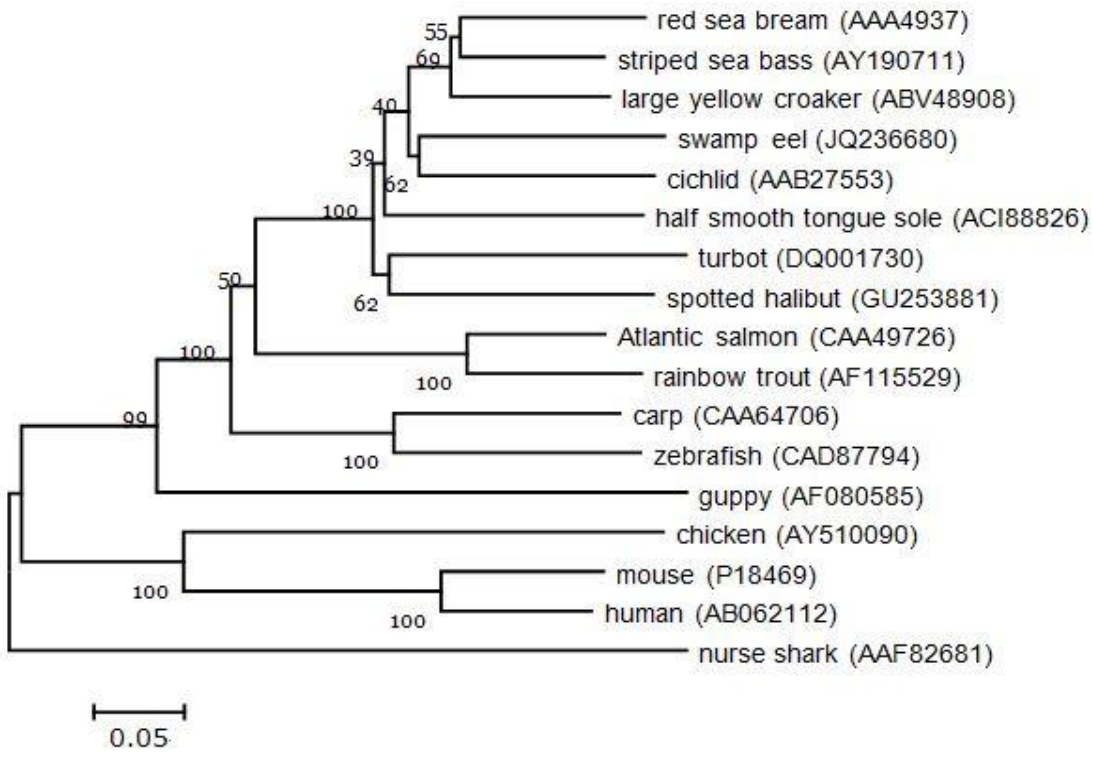
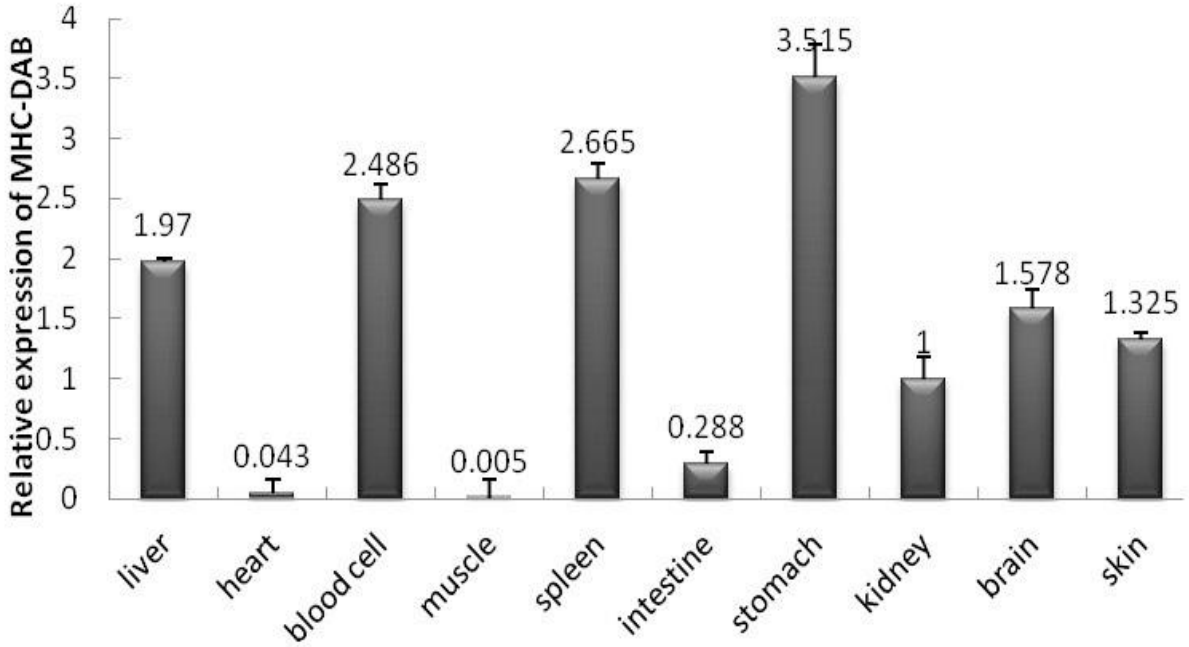
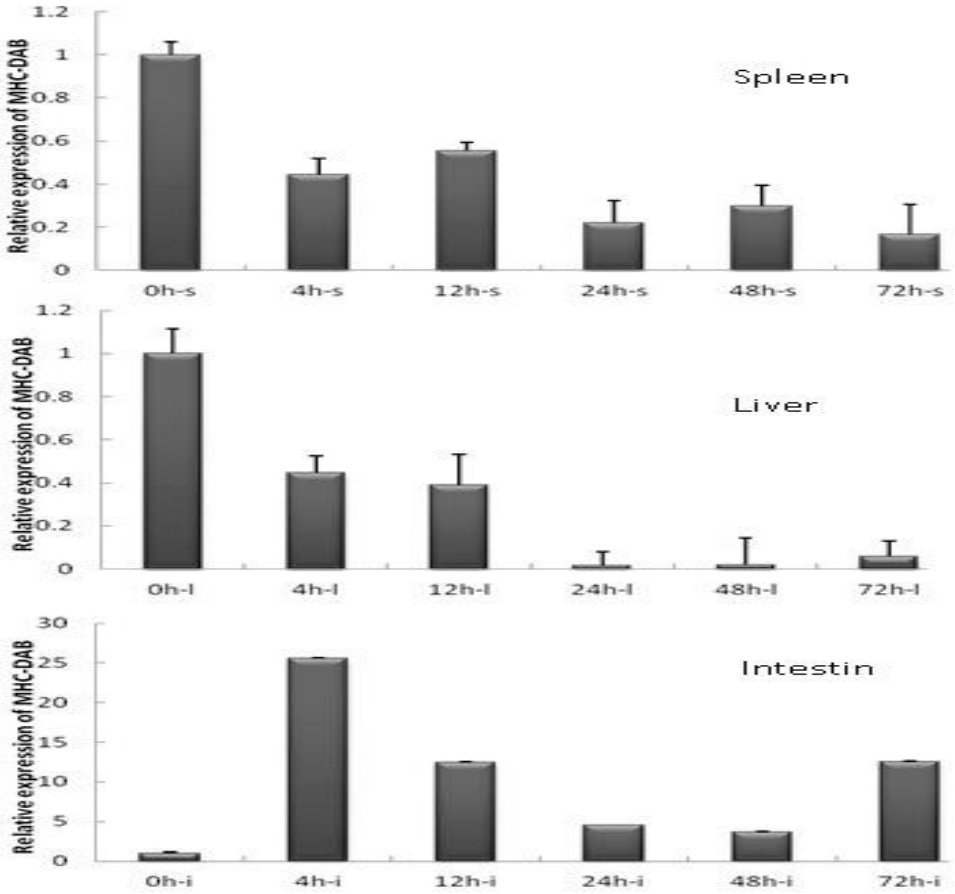
Li *et al.*, Figure 4 Sequence comparison of putative amino acids from different MHC-DAB alleles based on Clustal W. "p" showed the putative PBR positions in alleles; "*" indicated polymorphic sites in all the alleles.



Li *et al.*, Figure 5 Phylogenetic tree constructed via neighbor-joining method based on alignment of full deduced amino acids of MHC class II B genes. Genetic distance was calculated based on nucleotide difference (p-distance) with complete deletetion of gaps. The number at each node indicates the percentage of bootstrapping of a 1000 replications. Nurse shark MHC class II B sequence was used as outgroup.



Li *et al.*, Figure 6 Expression analysis of swamp eel MHC-DAB gene in various tissues. MHC mRNA transcripts levels were determined as a ratio relative to beta-actin levels in the same samples after RT-PCR.



Li *et al.*, Figure 7 Expression analysis of swamp eel MHC-DAB gene in three tissues after challenge with pathogenic bacteria *A. hydrophila*. MHC mRNA transcripts levels were determined as a ratio relative to beta-actin levels in the same samples after RT-PCR.

Li *et al.*, Table1. Primers used in this study. Primers were designed by primer premier 5.0.

Primer name	Primers sequences (5'-3')	Utility in this study
Beta-actin1	GCTGTGCTGTCCCTGTA	Expression analysis of β-actin
Beta-actin2	GAGTAGCCACGCTCTGTC	
seMHC-N1	GGRAAGTWTGTKGGRTACACTGAG	cDNA fragment amplification
seMHC-C1	TTCYTCTTGTAGTAGATGARTCCWGC	
NUP	CTAATACGACTCACTATAGGG	RACE amplification
NestG5	CACAGCCAGCTCGAAAGGATC	5' RACE nested PCR
NestG3	GATCTCCTGTGTGGTGGAGCA	3' RACE nested PCR
seMHC-GSP5	CAGGCTGGCGTGCTCCACCACACAGGAG	5' RACE amplification
seMHC-GSP3	ACGTCCGACTTCACTCTACGACGCCCTC	3' RACE amplification
DAB-in1-F	CCTCTTCATCACTGTCTAC	Intron 1 amplification
DAB-in1-R	GCACTATAAAGTTCAGGAAT	
DAB-in2-F	GACATTCGGTACCAGGCTGCTCTG	Intron 2 amplification
DAB-in2-R	AAGACGCTGCAGACCAACATG	
DAB-in3-F	GGTGATTGGTACTACCAGGT	Intron 3 amplification
DAB-in3-R	TGGTTTGATGAGGAAGAAGGT	
DAB-in4-F	GAAGATCTCCTGTGTGGTGGA	Inrtron 4 and 5 amplification
DAB-in4-R	GGTCAGTTAGTGGGAACCAGGAT	
DAB-alle-F	CTCCTCTTCATCACTGTCTAC	Allele amplification
DAB-alle-R	TGATTTAGTCAGAGCAGCCT	
DAB-rt-F	GAGATGGCAGATGGTGATTGG	RT-PCR analysis of MHC II B
DAB-rt-R	AATCCAGCCAGAGATAAGATCAG	

Li *et al.*, Table2. Nonsynonymous substitution ratio (d_N) and synonymous substitution ratio (d_S) as well as the resulting ratio d_N/d_S for the sequenced section, the putative peptide binding region and non-PBR among swamp eel alleles.

Sites	No. of codons	d_N (S.E)	d_S (S.E)	d_N/d_S	p(Z-test)
All	91	0.160(±0.027)	0.063(±0.015)	2.539	0.000
Putative PBR	24	0.332(±0.074)	0.110(±0.041)	3.018	0.005
Non-PBR	67	0.110(±0.026)	0.048(±0.016)	2.292	0.021

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