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Cloning and characterization of major histocompatibility complex class II genes in the stone flounder *Kareius bicoloratus* (Pleuronectidae)

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ABSTRACT. Major histocompatibility complex (MHC) class II genes play important recognition roles in the immune system in vertebrates. We cloned the MHC class II genes *A* and *B* in the stone flounder (*Kareius bicoloratus*). The full-length cDNA and DNA sequences of both genes were obtained, and their characteristic motifs were analyzed. The DNA sequence of stone flounder MHC class II *A* consists of four exons, while gene *B* contains six exons. The extra intron in gene *B* might be a common feature in most of its Acanthopterygii orthologs. Several conserved motifs were identified by multiple deduced amino acid sequence alignments of the two genes and their orthologs. The peptide sequences of α chain and β chain shared identity of 86.0-30.1% and 69.8-31.3% with their orthologs, respectively. Bayes phylogenetic trees showed that the stone flounder is closely related to the spotted halibut (*Verasper variegates*), and the half-smooth tongue sole (*Cynoglossus semilaevis*). Real-time quantitative PCR showed that in the stone flounder, both genes *A* and *B* are highly or moderately

expressed in several tissues, including the intestine, spleen and gills, and less expressed or undetectable in the liver, kidney, brain, heart, and gonads. These expression patterns differed slightly from those in other teleosts. This might be a unique phenomenon in the stone flounder. This first study of MHC genes in stone flounder could provide reference data for comparative studies.

Key words: Major histocompatibility complex; Stone flounder; Cloning; Expression

INTRODUCTION

The major histocompatibility complex (MHC) is called "the center of the immune universe" (Trowsdale, 1995) because of its great contribution to immune recognition of antigens. MHC molecules are members of the large immunoglobulin superfamily, and they mainly encompass two classes, namely I and II. Class I molecules are ubiquitously expressed in almost all kinds of cells, while the expression of class II molecules is mainly restricted to specialized antigen-presenting cells, including dendritic cells, macrophages, B cells, and thymic epithelial cells (Antoniou et al., 2012). These molecules display the peptides of viruses and other pathogens on the surface of immune cells, such as macrophages (Edwards and Hedrick, 1998). Class I molecules function in the immune system by presenting peptide fragments to $CD8^+$ T lymphocytes (Flajnik and Kasahara, 2001) and natural killer cells (Quillet et al., 1988). Class II molecules bind antigenic peptides that enter lymphocytes via phagocytosis (Edwards et al., 1998) and present these peptides to $CD4^+$ T cells (Cresswell, 1994). In addition, functions of MHC molecules in fish sexual selection (Reusch et al., 2001) also attract the attention of many researchers.

Each MHC class II molecule is a heterodimer of an α chain and β chain (Brown et al., 1993), which noncovalently associate with each other. Each chain comprises a membrane distal extracellular domain (α1 or β1), a membrane proximal extracellular domain (α2 or β2), a transmembrane region, and a short cytoplasmic anchor (Rothbard and Gefter, 1991). The α chain possesses one conserved disulfide bond in the α2 domain and the β chain possesses two conserved disulfide bonds in the β1 and β2 domains (Antoniou et al., 2012). The α chain and the β chain together make up an open-ended and shallow peptide-binding groove, so there is no stringent sequence requirement for binding peptides (Jardetzky et al., 1990). The two chains are encoded by genes *A* and *B*, respectively (Klein and Figueroa, 1986). The most polymorphic sites are found in the peptide-binding region (PBR), which interacts with antigenic peptides (Apanius et al., 1997).

Studies on teleost MHC genes are abundant. Ever since the first isolated MHC genes were studied in carp (Hashimoto et al., 1990), researchers have found and analyzed MHC genes in many teleosts, such as Atlantic salmon (Grimholt et al., 2003), zebrafish (Ono et al., 1992), half-smooth tongue sole (Li et al., 2010), and miiuy croaker (Xu et al., 2011). Studies have focused on their genome structure (Sato et al., 2000; Roney et al., 2004), polymorphism (Graser et al., 1996; Du et al., 2011), evolution (Hughes and Nei, 1989; Eizaguirre et al., 2012), mate choice (Landry et al., 2001), and other aspects. Expression studies showed that MHC class II genes are ubiquitously expressed, with high expression in immune tissues, such as spleen, kidney, gill, and intestine (Chen et al., 2006; Li et al., 2010; Xu et al., 2011).

The stone flounder (*Kareius bicoloratus* Basilewsky, 1855) is a kind of demersal flatfish. It lives on sandy and muddy bottoms in coastal areas at depths of up to 150 m. Its native habitat is the temperate waters of the northwest Pacific, from northern China to Japan, the Kuril Islands, the Korean Peninsula, and South China Sea. It is oceanodromous and breeds in autumn and winter. Adult individuals can grow up to 50 cm in length, and may reach 12 years of age. Because of its advantages in low-temperature resistance, disease resistance and aquaculture cost, stone flounder has become one of the main aquaculture species in northern China (Liu et al., 2009).

In the present study, both genes *A* and *B* of MHC class II in stone flounder were cloned and characterized. The genomic sequences of the two genes were obtained and multiple sequence analysis was conducted. Expression levels of these genes in various tissues were analyzed. This is the first study on MHC genes in stone flounder and will provide basic materials for further research on disease resistance, evolution analysis and other possible aspects.

MATERIAL AND METHODS

Fish and sampling

Healthy wild stone flounders were caught in the Yellow Sea, northern China. They were sexually mature and the gender could be distinguished by the morphology of their gonads. Besides, female individuals were much bigger than males. Tissues (muscle, brain, gill, heart, kidney, spleen, liver, intestine, and ovary/testis) were removed and preserved at -80°C until use.

DNA and RNA extraction and cDNA synthesis

Genomic DNA was extracted from muscle samples of each individual with the phenol-chloroform method. Total RNA was extracted from each of the eight tissues with Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer protocol. 3'-RACE-Ready cDNA was synthesized with BD SMART™ RACE cDNA Amplification kit (BD Biosciences Clontech, Palo Alto, CA, USA). First-strand cDNA in the spleen of 8 individuals (4 females and 4 males) was synthesized with the PrimeScript™ RT-PCR kit (TaKaRa, Dalian, China) following the manufacturer protocol.

Primer design and cloning

In a previous study, the whole length sequence of MHC class II *B* cDNA (GenBank Nos. GQ273943.1 and GQ273944.1) and the 5'-end sequence of MHC class II *A* cDNA in stone flounder were identified (Li et al., 2010). One specific primer (sf-MIIA-3'F, Table 1), together with the universal primer (NUP) were used to amplify the 3'-end cDNA sequence of gene *A*. Primers (sf-MIIADNA-Fw and sf-MIIADNA-Rv) were designed to amplify the whole length DNA sequence of gene *A*, and primers (stoneBDNA-Fw and stoneBDNA-Rv) were for gene *B* DNA amplification. The polymerase chain reaction (PCR) system was 25 µL with about 500 ng template DNA/cDNA. The reaction was catalyzed by Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen). PCR products were separated by agarose gel electro-

phoresis and purified with the Quick Gel Extraction kit (CWBIO, Beijing, China). Purified fragments were ligated to *pEASY*™-T1 Cloning vectors (TransGen Biotech, Beijing, China) and cloned to Trans5α Chemically Competent Cells (TransGen Biotech). Positive clones were screened by PCR with M13 primers and sequenced.

Table 1. Primers used in this study.

Primer	Sequence (5'-3')
sf-MIIA-3'F	GGGCTGCTCGGTGTCGCT
NUP	AAGCAGTGGTATCAACGCAGAGT
sf-MIIADNA-Fw	GGAGTGTGTGTCTGTGAGTGTC
sf-MIIADNA-Rv	AGGTGAGAAAAAGCAGGAAC
stoneBDNA-Fw	GAGTGTTTGTCAGTGAGCAGAGG
stoneBDNA-Rv	CAGTCCAGCAGATGGCAGC
SF-A-Fw	TCCGATGATCTACACCGAAG
SF-A-Rv	GTTGGGATAGGGAACATTGAG
SF-B-Fw	AGAGAAGATTTCCTGTGTGGTG
SF-B-Rv	CAGTCCTGAGGTTCCGATG
18S-70-Fw	GGTAACGGGGAATCAGGGT
18S-70-Rv	TGCCTTCCTTGGATGTGGT
M13-Fw	GCCAGGGTTTTCCCAGTCACGAC
M13-Rv	GCGGATAACAATTTCACACAGGA

Sequence alignments and data analysis

According to the deduced amino acid sequence, the secondary structure of MHC class II α and β chains was predicted by PREDICT PROTEIN (http://www.predictprotein.org/). The signal peptide was predicted by SignalP 4.0 Server (http://www.cbs.dtu.dk/services/SignalP/). The amino acid sequences of stone flounder MHC class II α and β chains were aligned with their orthologs by the Clustal W method (MEGA 5.1). Bayes phylogenetic trees were constructed by the MrBayes version 3.2 software.

Gene expression

Eight tissues (intestine, spleen, liver, kidney, brain, heart, gill, and testis/ovary) of 2 male individuals and 1 female individual were prepared for cDNA template in real-time quantitative PCR. Primers SF-A-Fw/SF-A-Rv and primers SF-B-Fw/SF-B-Rv were designed for real-time quantitative PCR of genes *A* and *B*, respectively. 18S (18S-70-Fw and 18S-70-Rv) was chosen to be the internal control for both genes. The PCR system was 20 μL with 20 ng template cDNA. The experiments were conducted on a 7500 Real-time PCR system (Applied Biosystems, Foster City, CA, USA) following the manufacturer protocol. The PCR results were standardized by standard plasmids of the two genes and 18S fragment. Data were analyzed with the SPSS Statistics 19 (IBM) software.

RESULTS

Structure and genomic sequence of MHC class II *A*

The full-length cDNA of gene *A* (GenBank accession No. JX647844) was obtained.

It is 974 nt in length, including a 40-nt 5'-untranslated region (UTR), a 226-nt 3'-UTR, and a 708-nt open reading frame (ORF), which is predicted to encode 235-amino acid residues. An AATAAA motif and poly (A) tail were found in the 3'-UTR.

In the putative α peptide (Figure 1), the first sixteen amino acids form the signal peptide. The transmembrane region is from amino acid 209 to 226, followed by a cytoplasmic tail. There is a motif GxxxGxxGxxxG (x stands for any hydrophobic residue other than Gly) in the transmembrane region. One N-glycosylation site (N-V-T-E), three protein kinase C phosphorylation sites (S-Y-R, S-F-R, and T-A-R) and four casein kinase II phosphorylation sites (S-D-S-D, S-F-I-D, S-Y-R-E, and T-K-N-E) were identified. One immunoglobulin and MHC protein signature (Y-S-C-T-V-S-H) exist in the α2 domain. Six conserved cysteine residues were found in the mature peptide, which can participate in the formation of disulfide bonds.

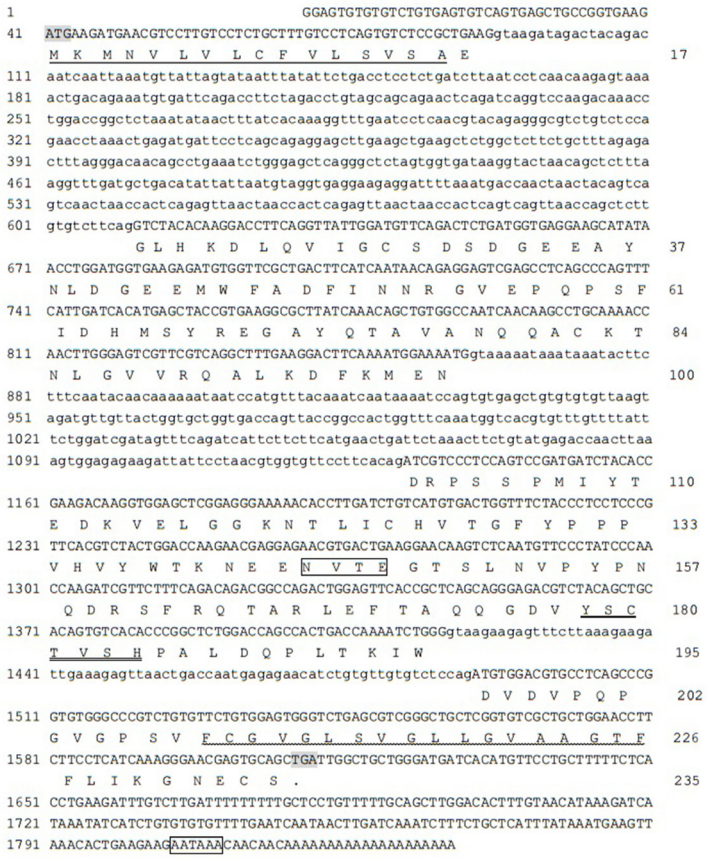

Figure 1. DNA sequence and putative amino acid sequence of gene *A*. The initiation codon and the termination codon are in shade. The signal peptide consisting of sixteen amino acid residues is underlined. One N-glycosylation site and the signal of polyadenylation tail are in frame. The transmembrane region is marked by a wavy line. One immunoglobulin and MHC proteins signature are double underlined.

The DNA sequence of gene *A* (GenBank accession No. JX647845) is 1837 bp in length. Exon 1 of 52 bp encodes the leader peptide. Exon 2 is 249 bp, encoding the α1 domain. Exon 3 of 285 bp encodes the α2 domain. The connecting peptide, transmembrane region and cytoplasmic tail are encoded by exon 4 of 122 bp.

Structure and genomic sequence of MHC class II *B*

The full-length cDNA of gene *B* (GenBank accession No. JX645176) was obtained in a previous experiment. It is 1210 nt in length, including a 26-nt 5'-UTR, a 747-nt ORF, and a 437-nt 3'UTR with an AATAAA motif and a poly (A) tail. The ORF is predicted to encode 248-amino acid residues.

The first eighteen amino acids of β peptide form the signal peptide (Figure 2). Four conserved cysteine residues were found in the mature peptide, which can participate in the formation of disulfide bonds. The transmembrane region is from amino acid 217 to amino acid 234. The motif GxxGxxxGxxxxxxG (x stands for any hydrophobic residue other than Gly) was found in this region. Besides, one N-glycosylation site (N-S-T-E), four protein kinase C phosphorylation sites (T-F-R, T-E-R, T-P-R, and S-E-R) and four casein kinase II phosphorylation sites (T-S-C-E, T-S-T-D, S-H-L-E, and S-M-P-E) exist in β1 and β2 domains.

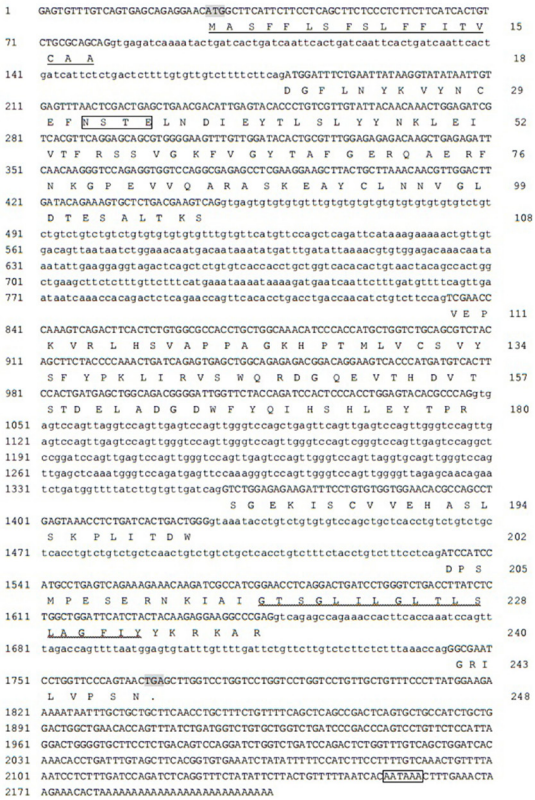

Figure 2. DNA sequence and putative amino acid sequence of gene *B*. The initiation codon and the termination codon are in shade. The signal peptide consisting of eighteen amino acid residues is underlined. One N-glycosylation site and the signal of polyadenylation tail are in frame. The transmembrane region is marked by a wavy line.

The DNA sequence of gene *B* (GenBank accession No. JX647846) is 2206 bp. It consists of six exons and five introns. The 55-bp exon 1 encodes the leader peptide and the 270-bp exon 2 encodes the β1 domain. Exon 3 of 214 bp, together with exon 4 of 68 bp, encodes the β2 domain. The 114-bp exon 5 encodes the connecting peptide, transmembrane region and partial cytoplasmic tail. The rest part of the cytoplasmic tail is encoded by exon 6.

Multiple sequence alignment and phylogenetic tree

Alignment of multiple peptide sequences of stone flounder MHC class II α chain (Figure 3) and β chain (Figure 4) with other species showed that the motifs GxxxGxxGxxxG and GxxGxxxGxxxxxxG are shared by most vertebrates. Besides, the conserved cysteine residues exist in most teleosts.

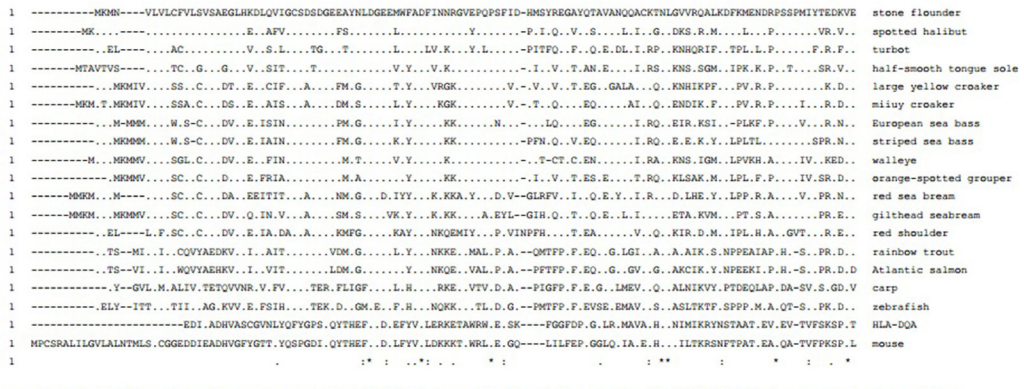

Figure 3. Multiple sequence alignment of stone flounder MHC class II *A* and its orthologs. Asterisks indicate positions that have a single, fully conserved residue. Colons indicate conservation between groups of strongly similar properties - scoring >0.5 in the Gonnet PAM 250 matrix. Periods indicate conservation between groups of weakly similar properties - scoring ≤0.5 in the Gonnet PAM 250 matrix. The motif GxxxGxxGxxxG is in shadow.

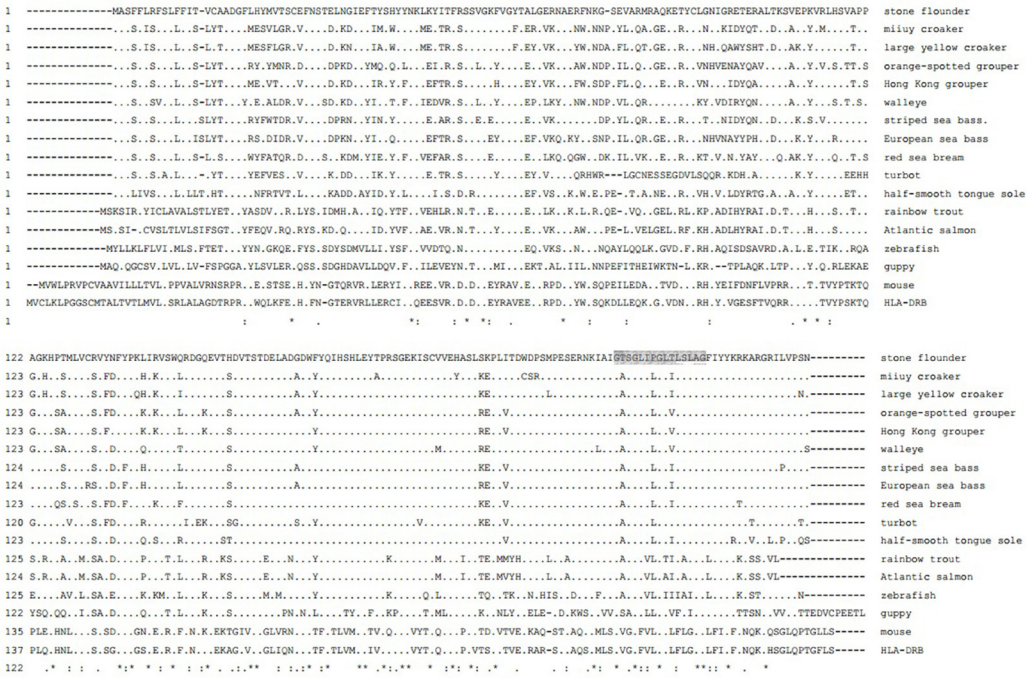

Figure 4. Multiple sequences alignment of stone flounder MHC class II *B* and its orthologs. Asterisks indicate positions that have a single, fully conserved residue. Colons indicate conservation between groups of strongly similar properties - scoring >0.5 in the Gonnet PAM 250 matrix. Periods indicate conservation between groups of weakly similar properties - scoring ≤0.5 in the Gonnet PAM 250 matrix. The motif GxxGxxxGxxxxxxG is in shadow.

Phylogenetic analysis showed that the deduced amino acid sequence of stone flounder MHC class II α chain shares 86.0, 72.3, 70.2, 70.2, 70.2, 69.8, 69.7, 68.8, 68.1, 65.8, 62.4, 60.7, 54.1, 52.4, 49.8, 48.9, 30.3, and 30.1% identity with that of spotted halibut, miiuy croaker, large yellow croaker, turbot, orange-spotted grouper, European sea bass, striped sea bass, walleye, half-smooth tongue sole, gilthead sea bream, red sea bream, red shoulder, Atlantic salmon, rainbow trout, zebrafish, carp, human, and mouse, respectively. A Bayes phylogenetic tree (Figure 5) further confirmed that stone flounder clusters with spotted halibut and that they both belong to the same family Pleuronectidae.

A Bayes phylogenetic tree (Figure 6) constructed based on stone flounder MHC class II β chain and its orthologs confirmed that stone flounder clusters with half-smooth tongue sole and that they both belong to the same order Pleuronectiformes. The deduced amino acid sequence of stone flounder MHC class II β chain shares 69.8, 69.8, 69.6, 69.4, 69.0, 68.1, 67.9, 55.4, 54.1, 49.8, 41.0, 31.3, and 31.3% identity with that of walleye, red sea bream, half-smooth tongue sole, large yellow croaker, miiuy croaker, orange-spotted grouper, turbot, Atlantic salmon, rainbow trout, zebrafish, guppy, human, and mouse, respectively.

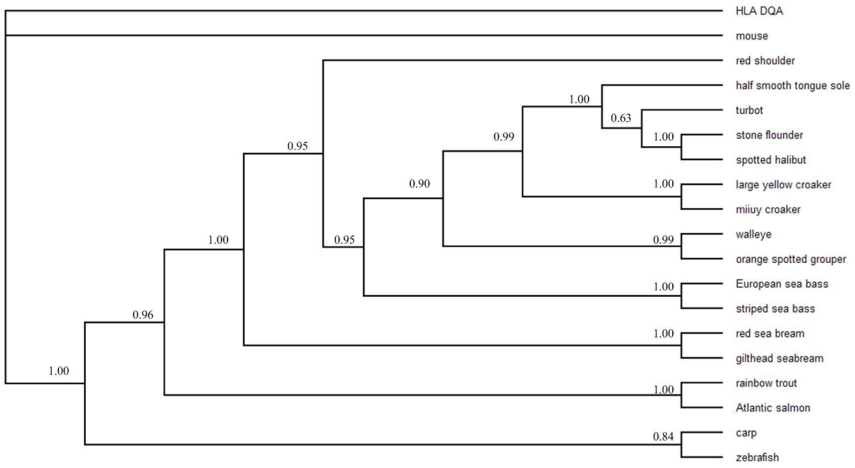

Figure 5. Bayes phylogenetic tree of MHC class II *A*. The sequences analyzed are: spotted halibut (GU253882), miiuy croaker (GU936787), large yellow croaker (EF681861), turbot (DQ094170), orange-spotted grouper (FJ598317), European sea bass (DQ821106), striped sea bass (AAB67867), walleye (AY158872), half-smooth tongue sole (FJ372721), gilthead sea bream (DQ019411), red sea bream (AAW21980), red shoulder (AF212850), Atlantic salmon (AAL40122), rainbow trout (CAB96452), zebrafish (CAD60677), carp (CAA64707), human (*HLA-DQA*, AAC41950), and mouse (BAE42123).

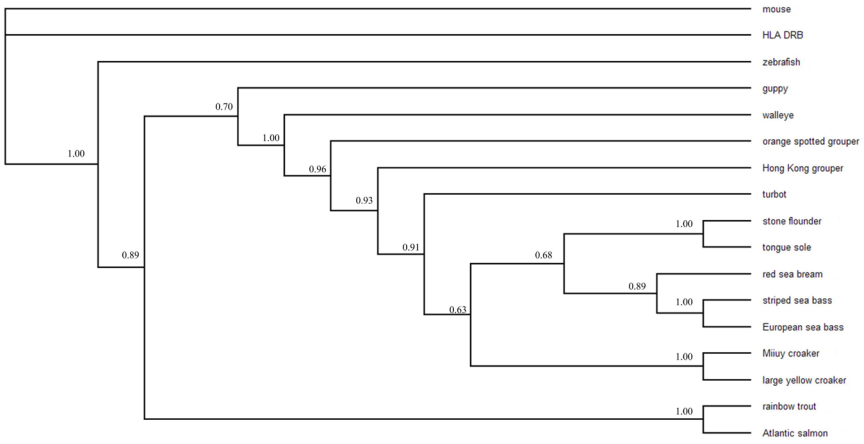

Figure 6. Bayes phylogenetic tree of MHC class II *B*. The sequences analyzed are: walleye (AY158837), red sea bream (AY190711), half-smooth tongue sole (FJ372722), large yellow croaker (EF681865), miiuy croaker (HM236158), orange-spotted grouper (FJ598318), turbot (DQ001730), Atlantic salmon (CAA49726), rainbow trout (AF115529), zebrafish (CAD87794), guppy (AF080585), human (*HLA-DRB*, M11161), and mouse (P18469).

Expression analysis in various tissues

Expression levels in eight tissues (intestine, spleen, liver, kidney, brain, heart, gill, and gonads) of stone flounder MHC class II gene *A* (Figure 7) showed that gene *A* is highly

expressed in several immune tissues, such as spleen and gill. A moderate expression level was detected in intestine. In kidney, brain and heart, gene *A* was less expressed, and the expression levels in liver and gonads could hardly be detected.

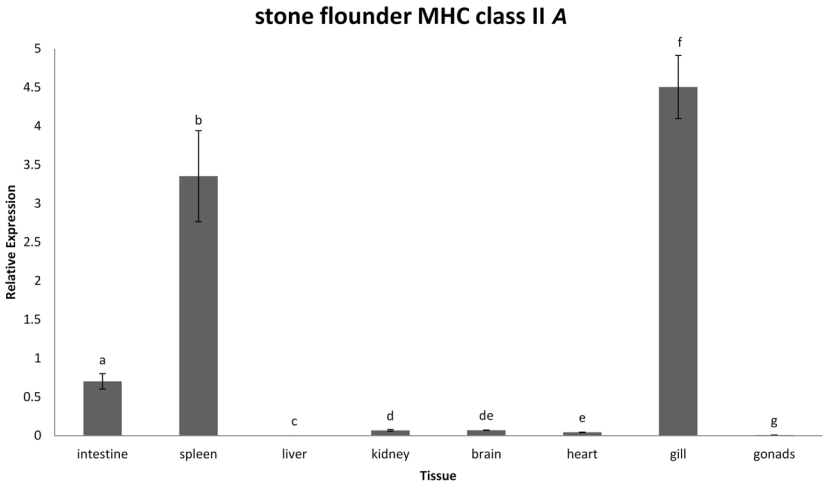

Figure 7. Expression levels of stone flounder MHC class II *A* in eight different tissues. Significance levels of different groups are marked by different letters according to data analyzed by Games-Howell method. 95% confidence intervals are marked by line segments.

The expression levels of stone flounder MHC class II B (Figure 8) displayed a similar pattern compared to gene *A*. It was highly expressed in intestine, spleen and gill, and showed lower expression levels in kidney, brain, heart, and gonads. Its expression could hardly be detected in liver.

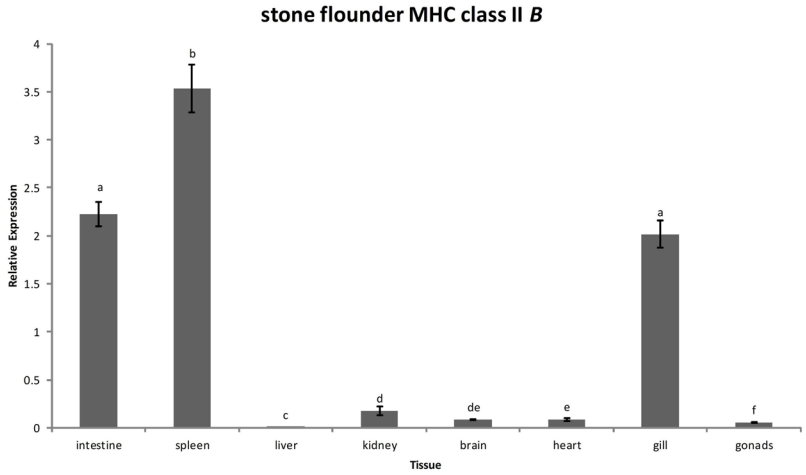

Figure 8. Expression levels of stone flounder MHC class II *B* in eight different tissues. Significance levels of different groups are marked by different letters according to data analyzed by Games-Howell method. 95% confidence intervals are marked by line segments.

DISCUSSION

The genome structure of stone flounder gene *A* was similar to that of other fishes, miiuy croaker (Xu et al., 2011) and half-smooth tongue (Xu et al., 2009), for instance. They all consist of four exons and three introns (Figure 9A). However, variant genome structures of gene *B* have been found in different fishes. The genome structure of gene *B* in stone flounder and miiuy croaker (Xu et al., 2011) both consist of six exons, while there are only five exons in most other orthologs, such as zebrafish (Sültmann et al., 1994) (Figure 9B). Several researchers have found that in fishes of the superorder Acanthopterygii, there is an extra intron in the immunoglobulin-like domain of this gene, which divides the original one exon into exons 3 and 4. However, this extra intron is absent in non-Acanthopterygii fishes (Figueroa et al., 1995), and it may serve as a phylogenetic marker of Acanthopterygii. In this study, the extra intron found in stone flounder can certify the common feature shared by Acanthopterygii fishes. However, there may be exceptions in Japanese flounder (*Paralichthys olivaceus*) and turbot (*Scophthalmus maximus*), which are kinds of Acanthopterygii fish without the extra intron in their gene *B* (Zhang and Chen, 2006; Zhang et al., 2006). Given the complicacy and variation of MHC structures, more surprising results will emerge through numerous further studies.

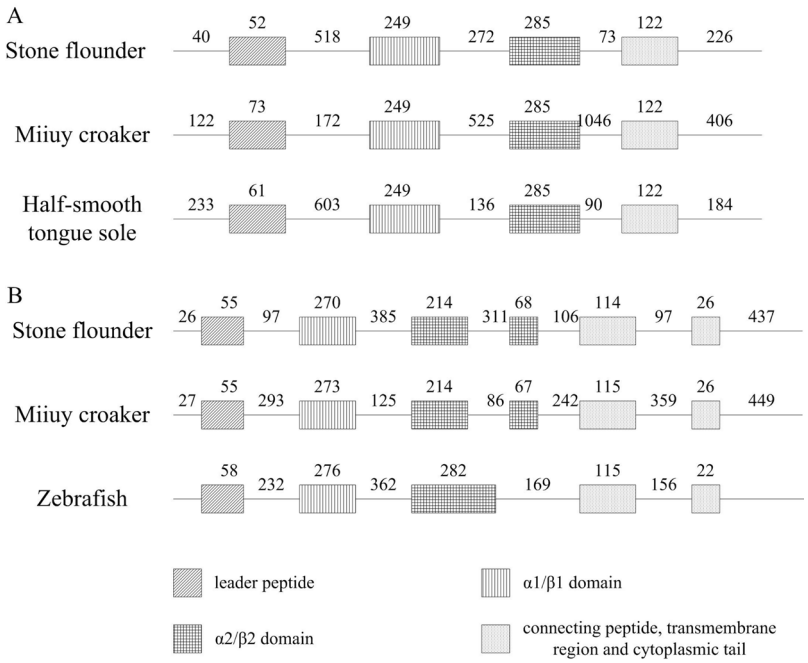

Figure 9. Genome structures of stone flounder MHC class II *A* (**A**) and *B* (**B**) and their orthologs. The boxes indicate exon regions. The lines indicate introns and untranslated regions.

Besides the N-glycosylation site and other conserved sites found in stone flounder putative α and β peptides, two Gly-rich motifs were also found in the two peptides. Motifs GxxxGxxGxxxG and GxxGxxxGxxxxxxG were both shown to be located in the transmembrane region of α and β peptides, respectively. They are supposed to be related to the correct

interaction of the two peptides. Mutations in the transmembrane regions of either peptide may result in dysfunctional proteins (Cosson and Bonifacino, 1992).

In teleost fishes, immune structures include gills, thymus, head kidney, mucous skin, liver, spleen, and gut-associated lymphoid tissue (Tort et al., 2003). In gilthead seabream, it is found that gene *A* is highly expressed in gill, head kidney, spleen, thymus, and peritoneal exudate leukocytes (Cuesta et al., 2006). In zebrafish, the gene is expressed in tissues with a high content of lymphoid/myeloid cells, such as spleen, pronephros, hepatopancreas, and intestine, and it is not detected in heart and ovaries (Sültmann et al., 1993). Here in stone flounder, the gene showed higher expression levels in spleen, gill, and intestine. This implies that these tissues are the main functioning parts of gene *A*. However, the gene did not show a high expression level in other immune tissues, such as liver and kidney. This may be a particular phenomenon in stone flounder, compared to other teleosts. In red sea bream, gene *B* is ubiquitously expressed, with high levels in head kidney, kidney, intestine, gill, stomach, heart, and spleen, low expression in muscle and blood (Chen et al., 2006). In Atlantic salmon, the gene shows low or negligible expression in brain and skeletal muscle, an intermediate level of expression in the heart, liver and foregut, and a high level of expression in the head kidney, spleen, hindgut, and gills (Koppang et al., 1998). As to the immune tissues in stone flounder, both genes *A* and *B* showed high or moderate expression levels in intestine, spleen, and gill, and low or undetectable expression levels in liver and kidney. Further studies need to be conducted to interpret this phenomenon.

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