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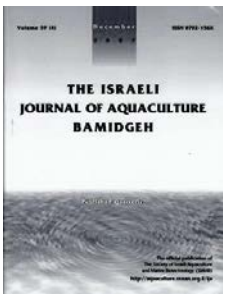

Molecular Cloning and Expression of Major Histocompatibility Complex Class Iα Gene of Swamp Eel (*Monopterus albus*)

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Keywords: swamp eel; MHC Iα; genomic structure; *Aeromonas hydrophila*; infection

Abstract

Major histocompatibility complex (MHC) plays an important role in the immune response of vertebrates. In this study, we isolated MHC class Iα gene from swamp eel (*Monopterus albus*) by rapid amplification of cDNA ends polymerase chain reaction (RACE-PCR). In order to study the function of MHC Iα in swamp eel, tissue distribution and immune response of the MHC Iα after bacterial challenge were analyzed. All the characteristic features of MHC Iα chain structure were identified by the deduced amino acid sequence, including the leader peptide, α1, α2, and α3 domains, connecting peptide, transmembrane and cytoplasmic regions, conserved cysteines, and N-glycosylation site. The deduced amino acid sequence of the MHC Iα shares 27.3% to 69.6% similarity with those of other vertebrates. Quantitative real-time PCR (qRT-PCR) demonstrated that MHC Iα gene was ubiquitously expressed in ten tissues, comparatively higher in immune related tissues, including the liver and intestine. Challenge of *M. albus* with the extracellular pathogen, *Aeromonas hydrophila*, resulted in a significant increase in the expression of MHC Iα within 72 h after infection and these high levels were maintained for 72 h in kidneys, spleen, and skin, whereas in the intestine and liver, it was found first in the form of up-regulated and then down-regulated gene. These results demonstrated that MHC Iα gene played an important role in response to bacterial infection in *M. albus*.

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Introduction

Major histocompatibility complex (MHC) class I genes encode a family of structurally related glycoproteins which can be subdivided into the class Iα and Iβ genes. The class Iα genes encode the heavy chains of the classical transplantation antigens, which are expressed on the surface of cells in nearly all tissues, are highly polymorphic, and function to present an antigen to the T-cell receptor of CD8+ T lymphocytes. The class Iβ genes are expressed in a more tissue-restricted manner and are relatively non-polymorphic (Stroynowski 1990). The class Iβ genes can functionally regulate the adaptive response to certain bacteria and viruses (Rodgers and Cook, 2005). Since MHC genes have been recognized as significant elements of the adaptive immunity in vertebrates (Barribeau et al., 2008; Luo et al., 2014), many MHC genes have been isolated and characterized in various fish species (Ristow et al., 1999; Srisapoome et al., 2004; Xu and Chen, 2011; Pinto et al., 2013).

Swamp eel, *Monopterus albus* is an economically important freshwater fish in China and other Asian countries. However, outbreaks of disease in cultured fish can cause heavy losses, limiting profitability and aquaculture development.

Aeromonas hydrophila is one of the most virulent bacteria for *M. albus* and can cause great losses in eel production (Xu et al. 2013). The use of antibiotics has partially solved the problem of bacterial diseases, but has raised concerns regarding antibiotic residue in fish, the environment, and antibiotic resistance. A better choice includes vaccines and breeding between disease resistant stock. Hence a potential approach is to culture strains of fish with enhanced resistance to bacterial disease via marker assisted selection (MAS) breeding. MHC genes are likely candidates as gene markers associated with disease resistance and can be used for future MAS research in swamp eel.

For this purpose, cloning and analysis of MHC genes of swamp eel are very important. Until now, the cloning of MHC IIA cDNA has been undertaken and its tissue expression in *M. albus* has been studied (Li et al. 2014). However, cDNA sequence and tissue expression of MHC Iα gene in *M. albus* have not been reported in previous studies.

In this study we cloned and exhibited the structural analysis of swamp eel MHC Iα gene. We also analyzed MHC Iα gene expression in different tissues when challenged with *A. hydrophila*. According to the gene expression in different tissues, potential information can be found for further study of the function of MHC Iα gene in *M. albus*. Activated MHC Iα gene plays an important role in protecting swamp eel from diseases.

Material and methods

Fish and sampling

One hundred and fifty healthy *M. albus* (weighing 80-100 g) were purchased from Haining Fishery Farm (Anqing, China). The fish were acclimatized in tanks at 20°C before genetic analysis. Ten different tissue samples (liver, spleen, stomach, intestine, kidney, skin, heart, blood cells, muscle, and brain) were dissected, collected and immediately immersed in 1 ml Trizol (Promega) at -80°C until RNA extraction.

Cloning of MHC Iα cDNA and gene sequence in swamp eel

Based on the conserved sequence of MHC Iα homologues in other teleost, a pair of degenerate primers MHC-Iα-F/MHC-Iα-R (Table 1) was designed to amplify the initial region of swamp eel MHC Iα. To obtain the full-length MHC Iα cDNA sequence, primers were designed from the initial fragment obtained (Table 1). 5'- and 3'-RACE PCR were performed using the Clontech SMART cDNA synthesis kit following the manufacturer's protocol. Purified PCR products were ligated into pMD18-T (Takara) for cloning and sequencing.

Table 1 Primers used in this study

Name	*Primers sequences (5'-3')*	*Amplification target*
MHC Iα-F	TBMCARAYTTYCCMGAGTWTG	cDNA fragment of MHC Iα
MHC Iα-R	ACCAKARARCTGRAACACACA	
MHC Iα-GSP5'	CCTGGTGTTGCTGTCATAGT	RACE-PCR of MHC Iα
MHC Iα-GSP3'	CATCAGTGAAACCTGAAGACTGGAGC	
MHC Iα-ex-F	GCCAGTCTTCAAAGCCAACA	Expression of MHC Iα
MHC Iα-ex-R	CATCATCCCATTCACAGCCG	
HS actin-F	CCCCATTGAGCACGGTATTG	Expression of HS actin
HS actin-R	GTTGGCTTTGGGGTTCAGAG	

***Notes*:** Degeneracy bases, B = C or G or T; K = G or T; M = A or C; R = A or G; Y = C or T; W = A or T

Sequence analysis.

BLAST was used to identify homologous sequences within the NCBI database. Protein prediction was performed using software provided by ExPASy Molecular Biology Server. Signal peptide and potential glycosylation sites were predicted using SignalP 4.0 and NetNGlyc 1.0 Server, respectively.

Real-time PCR quantification of MHC Iα in organs and tissues of the swamp eel.

Quantitative real-time PCR (qRT-PCR) was performed to determine the expression profile of the MHC Iα mRNA in liver, spleen, stomach, intestine, kidney, skin, heart, blood cells, muscle, and brain. MHC Iα response in liver, kidney, spleen, intestine, and skin was also investigated when challenged with bacterial antigen. Total RNA was extracted using Trizol reagent (Invitrogen) from each tissue sample and treated with DNase I (Fermentas), before being transcribed into cDNA using the Revert-Aid™ First Strand cDNA Synthesis Kit (Fermentas), following the manufacturer's instructions. qRT-PCR was performed using the primers MHC Iα-ex-F and MHC Iα-ex-R (Table 1), in 20 ul PCR reaction mixture containing 10 ul 2 × iQ^{TM} SYBR Green Supermix (Bio-Rad), 1 ul of each primer (0.4 uM), 1 ul template and 7 ul H_2O on the Bio-Rad CFX96™ Real-Time System (Bio-Rad), with the initial denaturation cycle of 95 °C for 3 min, then 45 cycles of 95°C for 15 s, 55°C for 20 s, 72°C for 20 s. Each sample was run in triplicate on 96-well qPCR plate (Bio-Rad). The relative gene expression level was calculated using the $2^{-\triangle\triangle CT}$ method (Livak and Schmittgen, 2001).

Sequence alignment and phylogenetic tree construction.

The alignment of deduced amino acid sequences of MHC Iα was performed using the Clustal-X procedure in the MEGA software version 4.0 (Tamura et al. 2007). A phylogenetic tree was constructed using deduced amino acid sequences from MHC Iα genes using the neighbor joining method (Saitou & Nei 1987). Bootstrap tests were replicated 10,000 times to derive the confidence values for the phylogeny analysis.

Results

cDNA and genomic sequences of swamp eel MHC Iα

The full-length cDNA of MHC Iα gene (Gen-Bank: KP690079) that was designated as Swam-UAA is 1511bp, including252 bp 5' terminal UTR, 1071 bp encoding region, 188 bp 3' UTR with a 26 bp poly A tail. The 1071 bp encoding region was found to code a protein with a 356 amino acid residue (Fig. 1).

The secondary structure of the deduced amino acid was analyzed. All the characteristic domains present in the MHC Iα protein of other fish species could be found in the swamp eel MHC Iα sequence, including a leader peptide, three extracellular domains (α1, α2 and α3), a connecting peptide, a transmembrane region, and a cytoplamic domain (Fig. 2). In addition, two N-linked glycosylation site (N-X-S/T), three protein kinase C phosphorylation sites (S/T-X-R/K), one tyrosine kinase phosphorylation site (KPEDWSRY), one N-myristoylation site (GLHTST), and three casein kinase II phosphorylation sites (S/T-X-X-D/E). In addition, five conserved cysteine residues were found in α1, α2 and α3 domain, which could form disulfide bonds of immunoglobulin domain.

Fig. 1. Schematic illustration of cDNA sequence. Encoding region are in uppercase, 5' UTR, and 3' UTR are in lowercase. The stop codon is indicated by an asterisk.

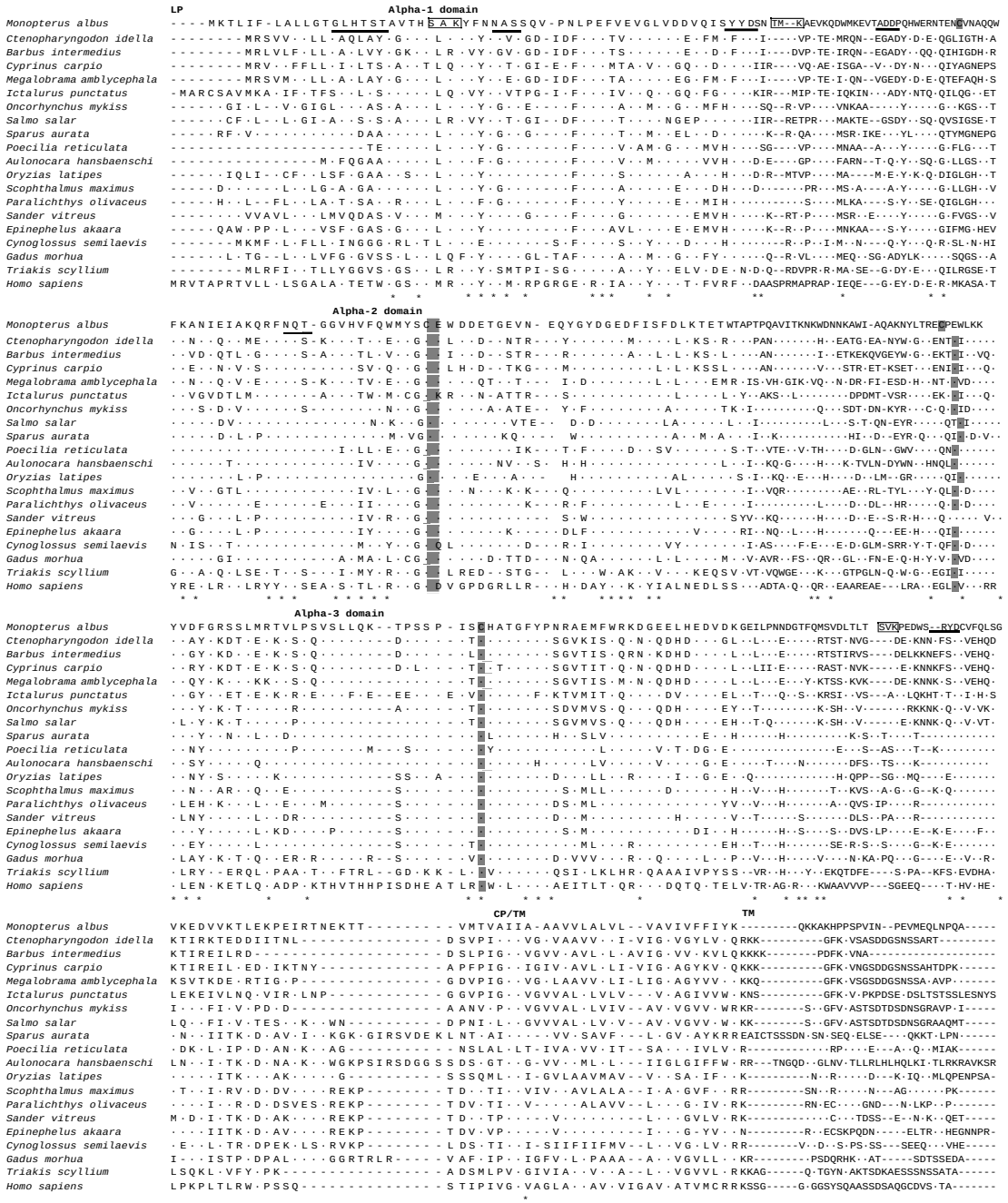

Fig. 2 Alignment of deduced amino acid sequences of the *Monopterus albus* MHC class Iα with those of other vertebrates. Identity is indicated by *dots*, and gaps used to maximize the alignment are shown by *dashes*, asterisks under the sequences denote mostly identical residue, gray boxes denote the cysteine residues in teleost, protein kinase C phosphorylation sites are in the normal frames, N-linked glycosylation and casein kinase II phosphorylation sites, and N-myristoylation site are underlined.
LP, leader peptide; CP, connecting peptide; TM, transmembrane region; CT, cytoplasmic domain.

Sequence alignment and phylogenetic analysis

Alignment of deduced amino acid sequence in swamp eel MHC Iα with that of other vertebrates revealed 27.3-69.6% similarity with other species (Table 2). Phylogenetic analysis demonstrated that the deduced amino acid sequence of swamp eel MHC Iα had a close relationship with *Sander vitrius*, *Epinephelus akaara*, *Sparus aurata* (Fig. 3).

Table 2. MHC I amino acid identity determined by Cluster W.

Species	GenBank Acc. No.	Identity (%)
Sander vitreus	AAL11412	69.6
Paralichthys olivaceus	BAD13368	67.5
Scophthalmus maximus	ABJ98693	66.6
Oryzias latipes	BAB83849	64.4
Epinephelus akaara	ABX80521	64.3
Sparus aurata	ABB04087	62.5
Poecilia reticulata	CAA90791	62.4
Cynoglossus semilaevis	FJ372720	59.0
Aulonocara hansbaenschi	AAD37812	55.6
Oncorhynchus mykiss	AAG25199	55.6
Salmo salar	AAN75116	53.9
Gadus morhua	AAL14532	52.3
Megalobrama amblycephala	AEE87248	46.8
Ctenopharyngodon idellus	AAS76087	45.0
Ictalurus punctatus	AAG29241	43.0
Cyprinus carpio	CAA62497	42.8
Barbus intermedius	CAD44955	41.5
Triakis scyllium	AAB97322	35.3
Homo sapiens	CAC15502	27.3

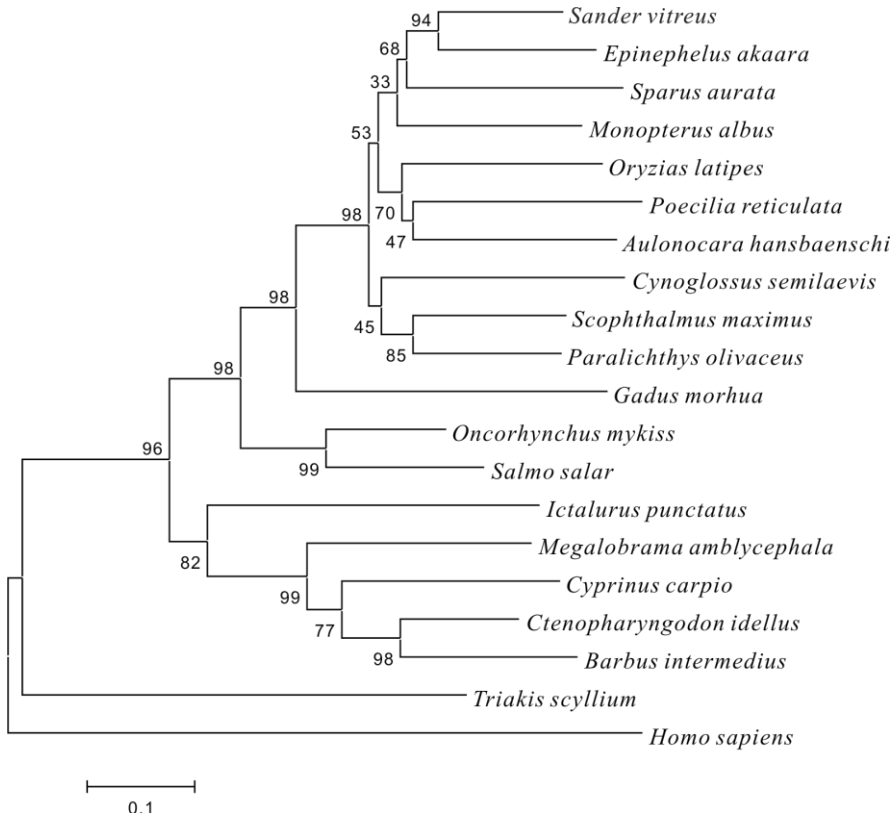

Fig. 3. Phylogenetic tree of MHC Iα gene from swamp eel and other vertebrates.

Expression analysis of swamp eel MHC Iα gene

Quantitative real-time PCR showed that swamp eel MHC Iα gene was ubiquitously expressed in ten tissues, but the expression level was distinctly, significantly and noticeably different (Fig. 4). The high levels of MHC Iα transcripts were detected in blood, brain, intestine and liver; moderate expression in kidney, muscle, skin and spleen; low expression in heart and stomach (Fig. 4).

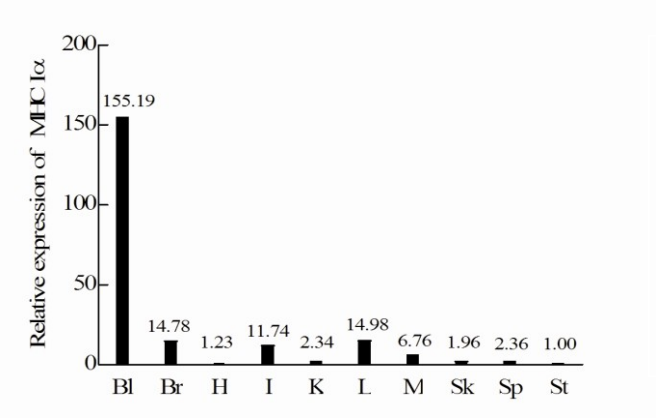

Fig. 4. Expression analysis of MHC Iα mRNA in various tissues (blood (Bl), brain (Br), heart (H), intestine (I), kidney (K), liver (L), muscle (M), skin (Sk), spleen (Sp) and stomach (St)) of swamp eel (uninfected). MHC Iα mRNA was expressed as a ratio relative to HS actin levels in the same samples after real-time PCR.

Challenge of swamp eel with pathogenic bacteria, *A. hydrophila*, resulted in marked changes in the pattern of expression levels of MHC Iα. In the liver, expression level of MHC Iα gene sustained a normal level from the start to 4 hours post infection then increased from 12 to 24 h. There was a marked decrease between 24 to 72h and the lowest expression level was detected after 72 h. In the intestine, expression level of MHC Iα gene sustained a normal level from infection starting time to 24 h then significantly increased at 48 h, followed by a recovery to normal level at 72 h. In the skin, the expression level of MHC Iα gene sustained a normal level from infection starting time to 12 h, then increased significantly at 24 h; this high level was maintained to 72 h post infection. In spleen and kidney, expression levels of MHC Iα gene were found up-regulated from infection starting time to 72 h, and the highest levels were checked at 72 h (Fig. 5).

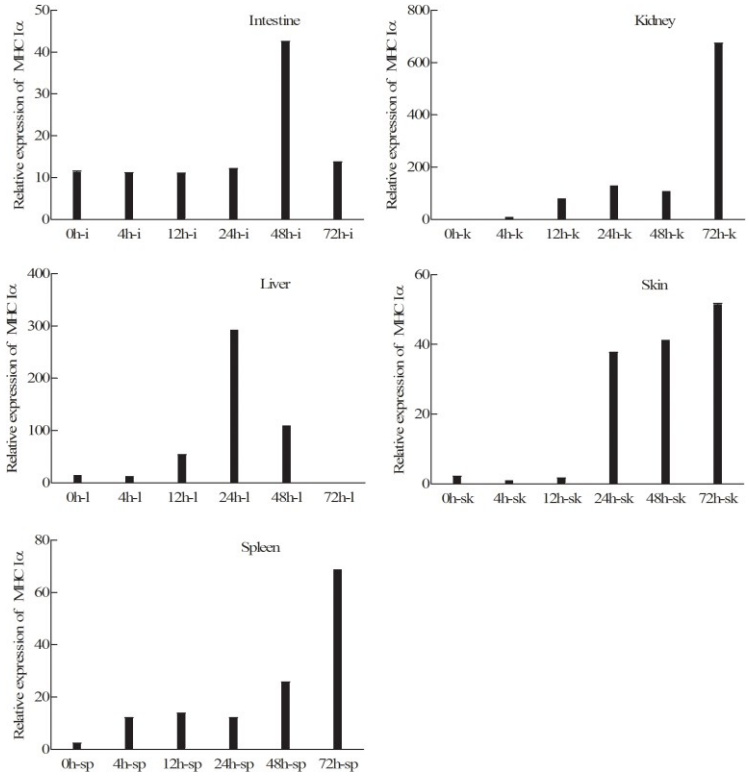

Fig. 5. Expression of swamp eel MHC Iα gene in five tissues after challenge with pathogenic bacteria *A. hydrophila*, and sampled at 0, 4, 12, 24, 48, and 72 h after challenge. Abbreviations: i, intestine; k, kidney; l, liver; sk, skin; sp, spleen. MHC Iα gene mRNA levels were expressed at a ratio relative to HS actin levels in the same samples after real-time PCR.

Discussion

MHC is a large genomic region and plays a vital role in the immune response of vertebrates (Croisetiere et al. 2008). Identification and characterization of MHC genes provide solid foundation for future studies that show how these genes function by elucidating innate immune response against pathogen invasion. In this study, the full-

length cDNA sequences and the complete genome sequence of MHC Iα gene from *M. albus* were cloned. Furthermore, the genomic structure, expression levels in different tissues, and immune response were also investigated.

The deduced amino acid sequence of swamp eel MHC Iα shared 27.3-69.6% similarity with vertebrates. Phylogenetic analysis revealed that swamp eel clustered with *Sander vitrius*, *Epinephelus akaara*, and *Sparus aurata*. Alignment analysis showed that the MHC Iα consists of three extracellular domains, a connecting peptide, a transmembrane region, and a cytoplasmic domain. Five conserved cysteine residues were found in α1, α2, and α3, domain and four of the five domains were found in α2 and α3 domain in all the fish. Two N-linked glycosylation site were found in α1 and α2, these structures of swamp eel MHC Iα are similar to those of half-smooth tongue sole (Xu and Chen 2011) and Japanese flounder (Srisappome et al. 2004).

The swamp eel MHC Iα gene transcripts were detected in all the ten tissues, but the expression levels were distinctly different. Similar results of MHC Iα gene have been reported in other fish (Xu and Chen, 2011; Srisapoome et al, 2004). The presence of MHC Iα in all tissues may be a result of expression in all nucleated cells (Antao et al. 2001). The most abundant expression of MHC Iα was observed in blood and liver, which was similar to that in half-smooth tongue (Xu and Chen, 2011) and flounder (Srisapoome et al, 2004). This phenomenon may imply that these tissues are important immune organs in fish and the immune response could be initiated when pathogenic antigens were introduced.

Expression levels of MHC Iα mRNA may be significantly affected in fish when challenged with pathogenic bacteria (Luo et al. 2014; Xu and Chen, 2011; Srisapoome et al, 2004). Challenge with *Vibrio anguillarum* resulted in a significant increase of MHC Iα expression in various tissues of half-smooth tongue sole after infection (Xu and Chen, 2011). Challenge of Blunt snout bream with *A. hydrophila*, resulted in a significant increase in the expression of MHC I mRNA within 72 h after infection, followed by a recovery to normal level after 120 h (Luo et al. 2014). In the present study, expression levels of MHC Iα mRNA were significantly elevated when challenged with *A. hydrophila* in swamp eel. These results showed that MHC Iα is an important immune related gene in fish. Significant increases were found in liver, spleen, kidney, intestine, and skin, which may imply that these organs are important in defending against pathogenic antigens. However, further studies are required to explain the precise role and mechanism of MHC Iα in defense response in fish.

In summary, we characterized the genome structures of MHC Iα from individual swam eel. Furthermore, the swamp eel MHC Iα gene transcripts were detected in different tissues and the expression levels of MHC Iα mRNA were also investigated when challenged with pathogenic bacteria. Results from this study may complement investigation and further research of innate immune systems in fish. MHC genes are likely candidates as gene markers associated with disease resistance and could be used for future expansion of MAS research in swamp eel since diseases of cultured swamp eel occur frequently. In addition, losses due to infectious diseases limit profitability and development of aquaculture. Hence, an important approach to disease prevention is the use of MAS breeding to culture of strains of swamp eel with enhanced resistance to some freshwater diseases.

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