Original Article

MARINE BIOTECHNOLOGY

Major Histocompatibility Complex Class IIB Allele Polymorphism and Its Association with Resistance/Susceptibility to *Vibrio anguillarum* in Japanese Flounder (*Paralichthys olivaceus*)

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Abstract

The full length of major histocompatibility complex (MHC) class IIB cDNA was cloned from a Chinese population of *Paralichthys olivaceus* by homology cloning and rapid amplification of cDNA ends-polymerase chain reaction (RACE-PCR). The MHC IIB genomic sequence is 1,864 bp long and consists of 34-bp 5′UTR, 741-bp open reading frame, 407-bp 3′UTR, 96-bp intron1, 392-bp intron2, 85-bp intron3, and 109-bp intron4. Phylogenetic analysis showed that the putative MHC class IIB amino acid of the Chinese *P. olivaceus* shared 28.3% to 85.4% identity with that of the reported MHC class IIB in other species. A significant association between MHC IIB polymorphism and disease resistance/susceptibility was found in Chinese *P. olivaceus*. Thirteen different MHC IIB alleles were identified among 411 clones from 84 individuals. Among the 280 (268) nucleotides, 32 (11.4%) nucleotide positions were variable. Most alleles such as alleles *a*, *b*, *c*, *d*, *e*, *f*, *j*, *k*, *i*, *m* were commonly found in both resistant and susceptible stock. Via χ^2 test, allele *d* was significantly more prevalent in individuals from susceptible stock than from resistant stock, and their percentages were 23.80% and 7.14%, respectively. In addition, allele *g* occurred in 9 and allele *h* in 4 of 42 resistant individuals that were not present in the susceptible stock; their percentages were 21.4% and 9.52%, respectively. Although allele *l* was found only in 8 individuals from the susceptible stock, its percentage is 19.05%.

Keywords: cDNA — disease resistance — disease susceptibility — Japanese flounder — major histocompatibility complex class IIB (MHC IIB) — *Paralichthys olivaceus* — polymorphism

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Introduction

Major histocompatibility complex (MHC) loci encode glycoproteins that bind foreign peptides and thus initiate immune responses through the interaction with T cells. There are two classes of glycoprotein which differ in structure, peptide binding specificity, and the subset of T cells they activate (Klein, 1986; Rothbard and Gefter, 1991). The MHC class II molecules are heterodimers consisting of α and β chains that are mainly expressed on antigen-presenting cells. The α_1 and β_1 domains form the peptide binding region (PBR), in which peptides are bound and then recognized by CD^{4+} helper T-cell receptors. MHC genes have been isolated and characterized in almost all major vertebrate taxi, including cartilaginous fish (Bartl and Weissman, 1994; Ohta et al., 2000), bony fish (Stet et al., 1998; Kruiswijk et al., 2002; Chen et al., 2004a), amphibians (Flajnik et al., 1991; Liu et al., 2002), reptilians (Grossberger and Parham, 1992; Wittzell et al., 1999), birds (Miller et al., 1994), and mammals (Trowsdale, 1995; Hughes, 2000).

MHC genes are characterized by their high levels of polymorphism in terms of both the large amount of alleles present in populations and the high sequence variation between alleles. The classic MHC genes represent the most polymorphic genes known to date, with multiple loci and considerable number of alleles at each given locus in mammal (Hoelzel et al., 1999) and teleost (Walker et al., 1994; Chen et al., 2006). This diversity results in each

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individual having direct functional relevance for the immune response. Variation in exon 2 is responsible for most of the polymorphism observed in the class II genes, and most of it is concentrated in the codons specifying the PBR. Several hypotheses have been postulated to explain the vast polymorphism of the MHC genes: heterozygous advantage, overdominant selection, and frequency-dependent or balancing selection (Parham and Ohta, 1996). Pathogen-driven selection favors genetic diversity of the MHC genes

A

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1                                                                ACGCGGGGAGTGTTCGTCAAGTGAACAGAGGAAC
35      ATGGCTTCATTCATCCTCAGCTTCTCCCTCTTCTTCATCACGGTCTGCACAGCAGgtaggatcagaacactgatcactgatcaatacact
    1    M  A  S  F  I  L  S  F  S  L  F  F  I  T  V  C  T   A
125     gatcaatacactgatcaatacatggatcattctctgactcgttttcttgtctgctcttcagATGGATTTCTGCATTATACGGTGACAGAC
    18                                                                D  G  F  L  H  Y  T  V  T  D
215     TGTGAGTTTAACTCCTCGAAGCTGAACGACATCGAGTACACACAGTCTTACTATTACAACAAACTGGAGATCGTCAGGTTCAGCAGCAGT
    29   C  E  F  N  S  S  K  L  N  D  I  E  Y  T  Q  S  Y  Y  Y  N  K  L  E  I  V  R  F  S  S  S
305     GTGGGGAAGTACGTTGGATACACTGAGTTTGGAATCAAGAACGCTGAGAGGTGGAACAATGGTCCAGAGGTGATCTCTAGAAGAGGTGAG
    59   V  G  K  Y  V  G  Y  T  E  F  G  I  K  N  A  E  R  W  N  N  G  P  E  V  I  S  R  R  G  E
395     AAGGAGAGCTACTGCTTTCACAACGTTGGAATCTTCACAGAATCTGCTCTGACGA AGTCAGgtgagtgtgtgtgttgtatgtgtgtga
    89   K  E  S  Y  C  F  H  N  V  G  I  F  T  E  S  A  L  T  K  S
485      gtgtgtgttgtatgtgtgtgtgtgtgtgtgtgtgtgtttggattgtatgtgtgagtgtgtgttgtatgtgttgtatgtgtgtgtgt
575     tgtgtgtgttcttcatgtt ccagatgaaattcataaagaatctgatgagtcaaactttttatttattgtctttgtttcctggaaatagact
665     cagctcggtgtcaccacctgctggtcacacgctgtaactacagccgctgacactgaagcttctctttgcttctttatgaaataaaataaa
755     ggatgaaacaatcctctgatgttttcagttgatcaatcgaaccacagactaagatccagttcacacctgacgtgacc aacatctgtcttc
845     cagCCAAACCCTACGTCAGGCTTAACTCTGTGGCGCCCCCAGCTGGCAAACATGCCATGTTGGTCTGCAGCGTCTTTGACTTCTACCCCA
    109    A  K  P  Y  V  R  L  N  S  V  A  P  P  A  G  K  H  A  M  L  V  C  S  V  F  D  F  Y  P
935     AACGGATCAAAGTGAGCTGGCAAAGAGACGGACAGGAAGT CACCTCTGATGTCACTTCCACTGATGAGCTGGCAGACGGTGATTGGTACT
    138 K  R  I  K  V  S  W  Q  R  D  G  Q  E  V  T  S  D  V  T  S  T  D  E  L  A  D  G  D  W  Y
1025    ACCAGATCCACTCCCACCTGGAGTACATGCCCAAGTCTGGAGAGAAGATTTCCTGTGTGGTGGAACATGCCAGCCTGAGTAAACCTCTGA
    168 Y  Q  I  H  S  H  L  E  Y  M  P  K  S  G  E  K  I  S  C  V  V  E  H  A  S  L  S  K  P  L
1115     TCACTGACTGGGAgtaaatacctgtctgtcacttgtctgtctgccccctcgtctgtgtgctcacctgctcagctgtctttctccctttct
    198I  T  D  W  D
1205    gtgctcagTCCATCCATGCCTGAGTCAGAAAGAAACAAGATCGCTATTGGGACCTCAGGACTGATCCTGGGTCTGACCTTATCTCTGGCT
    203          P  S  M  P  E  S  E  R  N  K  I  A  I  G  T  S  G  L  I  L  G  L  T  L  S  L  A
1295    GGATTCATCTACTACAAGAGGAAGGCCCAAGgt cagagccagaaacccatttcacaccagtttatacagaccaaatccagtttagaccag
    230 G  F  I  Y  Y  K  R  K  A  Q
1385    ttttaatggtgtgtatttgttttgacccttgtctcttctctttaaaccagGACGGGATCCTGGTTCCCACTAACTGAGTCTTGTCCTGGA
    240                                                      G  R  D  P  G  S  H  *
1475    CCTGGACTTGGTCCTGGATCTATTTCTGTTTTCCTGATGGAAGTAAAATTAGCTGCTGCTTCAACCTGCTTTCTGTTTTCAGTCCAGCAG
1565    ACTCAGCGCTGCCACCTGCTGGACTTGCTGAACACCAAATTCTCTGATGGTCTGTGCTG GTCTGAACCTGATCTAGAACCAGTCCTGTTC
1655     TCACTGTGGATCCAGACTCTGGTACATCAGCTGGATCACAAACACCTGATTTGTTGCTTCACGGTGTGAAATCTATATTTTTTTTCTCAT
1745    CCTCCTGTTGTTAAACAGTTTTAAATCCTCTTTGATTCAGATCTCAGGTGTCTATACTTTTACTGTTTTCTAATCAA AATAAACTTTGAA
1835    CCCAAAAAAAAAAAAAAA AAAAAAAAAAAA
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B

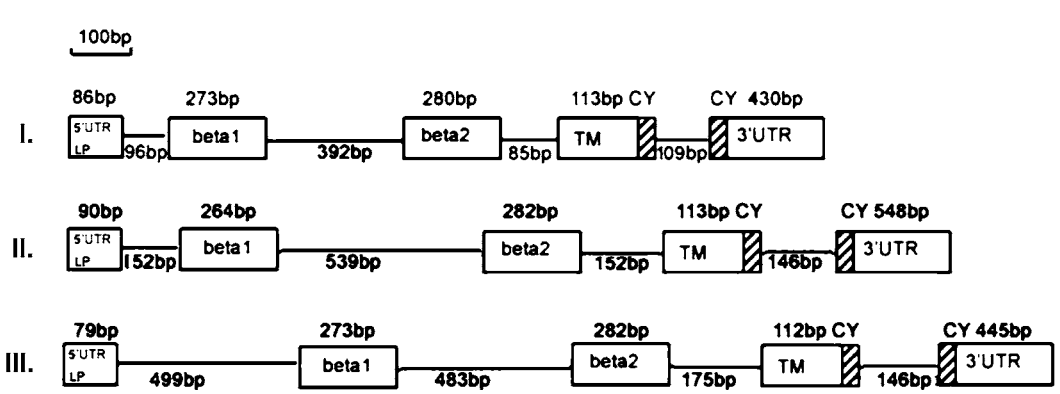
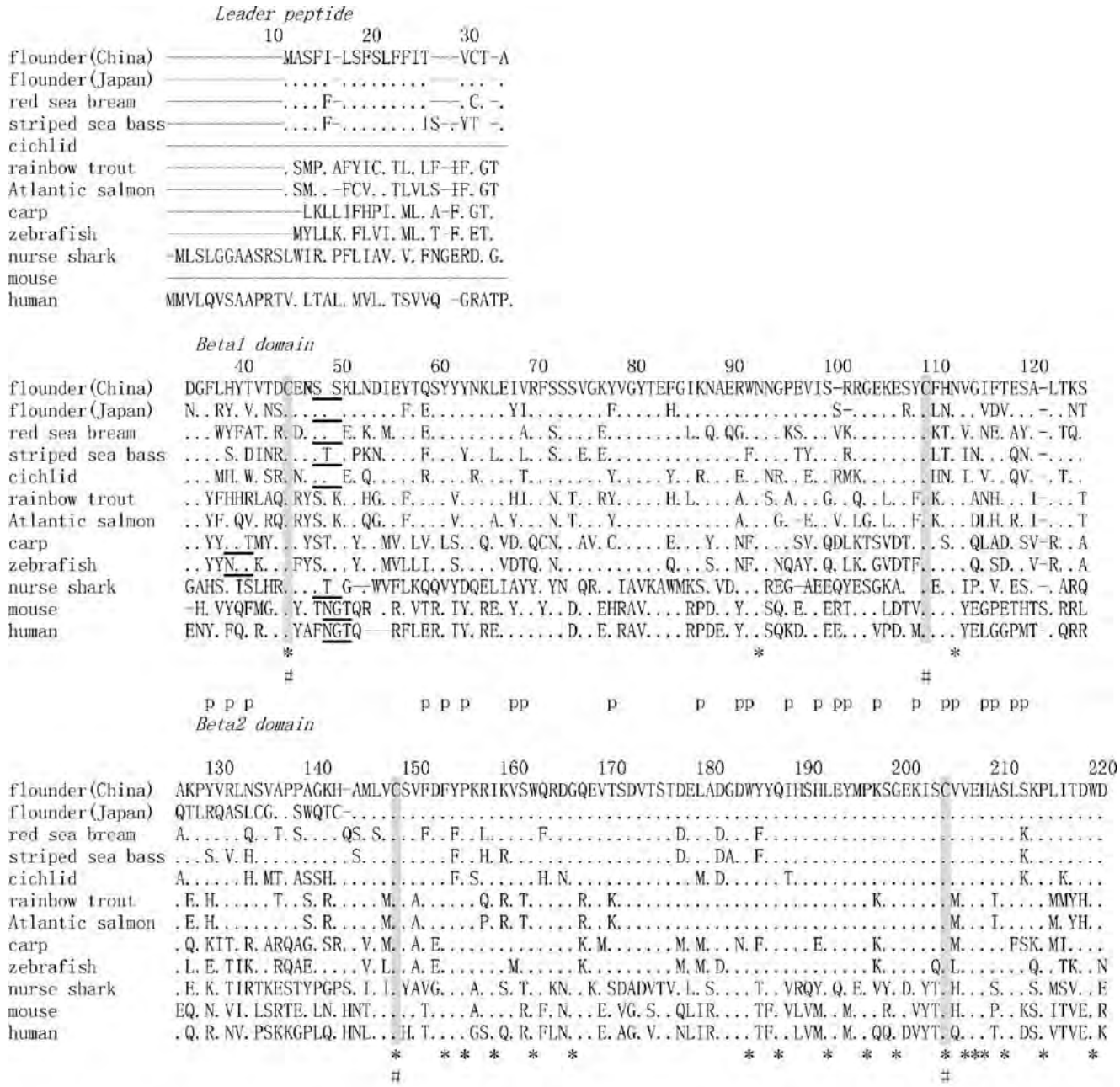


Fig. 1. (**A**) Full length of Japanese flounder (China) MHC class IIB genome. The polyadenylation signal sequence (AAT AAA) is underlined. The introns sequences are shown in lowercase letters. (**B**) Comparison of MHC class IIB intron-exon organization of Japanese flounder with that of turbot and red sea bream. (A) indicates the sketch map of MHC class IIB of Japanese flounder; (B) indicates that of turbot; (**C**) indicates that of red sea bream. CY, Cytoplasmatic tail; LP, leader peptide; TM, transmembrane region; 3′UT, 3′-untranslated sequence; the numbers above the boxes and the lines indicate the length of the introns and exons.


Leader peptide
Beta1 domain
Beta2 domain
Connecting Peptide
Transmembrane region
Cytoplasm tail
flounder(China)
flounder(Japan)
red sea bream
striped sea bass
cichlid
rainbow trout
Atlantic salmon
carp
zebrafish
nurse shark
mouse
human

◀ **Fig. 2.** Alignment of MHC class IIB amino acid sequences of Japanese flounder (Chinese), Atlantic salmon (X70167), carp (X95431), cichlid (S63770), human (AB062112), mouse (M36939), nurse shark (L20275), rainbow trout (AF115529), red sea bream (AY190711), striped sea bass (L33966), and zebrafish (AL672158). Identity is indicated by dots (.....), and gaps used to maximize the alignment are shown by dashes (– –). Asterisks under the sequences denote identical residues; "p" indicates the correlative amino acid that combines the antigen; shading denates the cysteine resides; # and gray boxes that indicate four cysteine residues were observed in the β1 as well as in the β2 domains; the *N*-linked glycosylation signal is underlined.

through both heterozygote advantage (overdominance) and frequency-dependent selection.

Genes of the MHC are obvious candidates as they have an important role in both the innate and adaptive immune response. Moreover, specific MHC alleles have been well documented to correlate with disease resistance in chicken (Briles et al., 1983), mouse (Medina and North, 1998), sheep (Paterson et al., 1998), and human (Hill et al., 1991; Pedro et al., 2003) and in salmonid species (Gjedrem et al., 1991; Grimholt et al., 1994; Lohm et al., 2002; Wynne et al., 2004).

Japanese flounder (*Paralichthys olivaceus*) is a widely cultured marine fish species in Asian countries and is highly valued because of its good taste. However, diseases of the cultured fish have frequently occurred and losses resulting from infectious diseases limit profitability and development of aquaculture. The use of antibiotics has partially solved the problem, but has raised concerns regarding antibiotic residues in fish, environmental pollution, and antibiotic resistance development. There is extensive interest in enhancing resistance of the cultured fish to diseases. Therefore, it is necessary to assist selective breeding of a resistant strain via molecular techniques.

A report was published on the cloning and sequence of MHC class IIB cDNA in a Japanese population of *Paralichthys olivaceus* (Srisapoome et al., 2004), but no data on the genomic structure, gene polymorphism of class IIB, and its association with disease resistance was available for this fish species. In this article, we report the genomic structure of the MHC class IIB gene from a Chinese population of Japanese flounder (*P. olivaceus*) as a base, and we examine the polymorphism of MHC class IIB and the relationship between the polymorphism and disease resistance/susceptibility.

Materials and Methods

Fish and Sampling. Resistant and susceptible stocks were prepared as follows: Pathogenic bacteria, *Vibrio anguillarum*, were cultured at 28°C to mid-logarithmic growth on Difco medium 2216E, and then resuspended after centrifugation to approximately 4.6×10^9 colony-forming units (cfu) ml^{-1} in saline. About 350 individuals weighing 10 to 50 g of a Chinese population of Japanese flounder were anesthetized by immersion in MS 222 and injected intraperitoneally with 0.5 ml of bacterial suspension. After 20 h, the fish began to die, with the highest rate at 24 h; the number of dead fishes was relatively constant after 30 h. About 200 individuals died after challenge, and 150 in-dividuals survived. Fifty dying individuals with early symptoms of infection were used as susceptible individuals for collecting blood, and surviving Japanese flounder were classified as resistant individuals.

Forty-two resistant individuals and 42 susceptible individuals were used in the present study. Blood samples were collected from these fish and stored frozen (−80°C) until use.

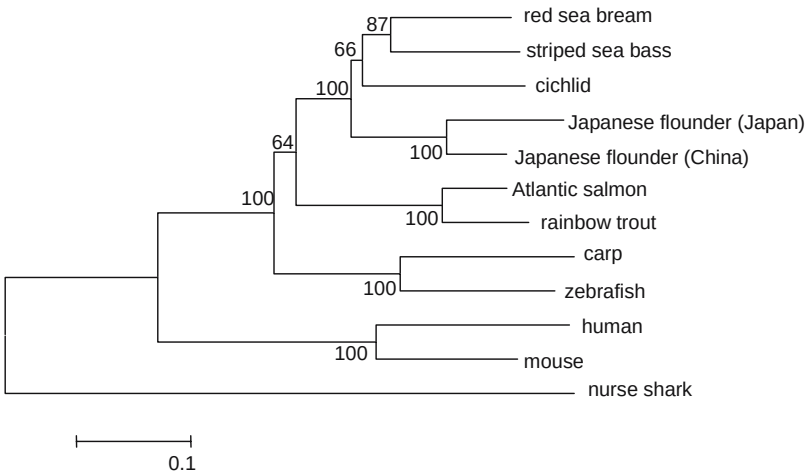
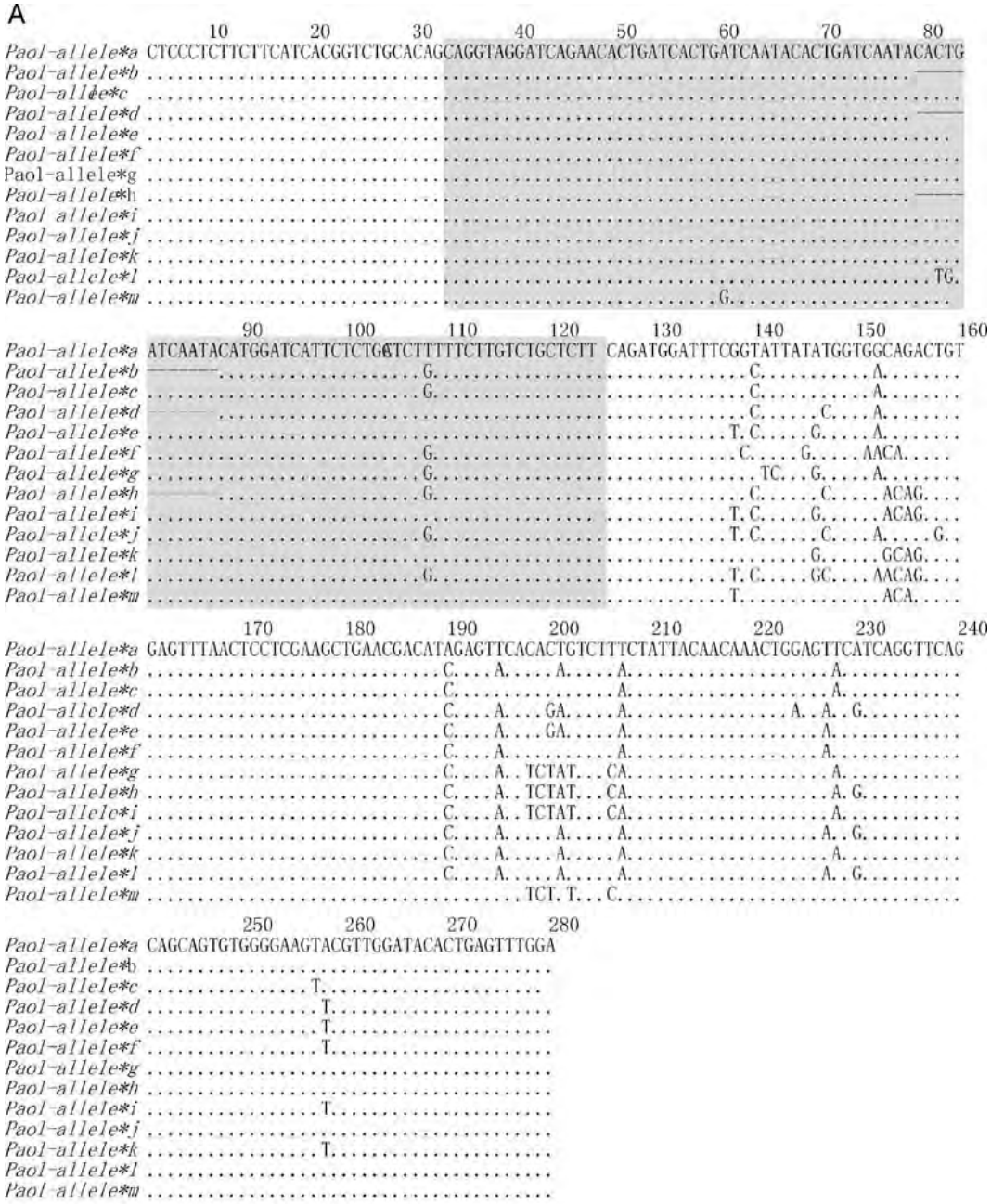


Fig. 3. Phylogenetic tree constructed via a neighbor-joining method based on alignment of full deduced class IIB amino acids of a Chinese population of *Paralichthys olivaceus* with that of other vertebrates. Genetic distance was calculated based on nucleotide difference (*p*-distance) with complete deletion of gaps. The number at each node indicates the percentage of bootstrapping of a 1000 replications. The phylogenetic tree was constructed using the nurse shark MHC class IIB sequence as an outgroup. Scale bar = 0.1.

A


10 20 30 40 50 60 70 80
Paol-allele*a CTCCCTCTTCTTCATCACGGTCTGCACAGCAGGTAGGATCAGAACACTGATCACTGATCAATACACTGATCAATACACTG
Paol-allele*b
Paol-allele*c
Paol-allele*d
Paol-allele*e
Paol-allele*f
Paol-allele*g
Paol-allele*h
Paol-allele*i
Paol-allele*j
Paol-allele*k
Paol-allele*l
Paol-allele*m
90 100 110 120 130 140 150 160
Paol-allele*a ATCAATACATGGATCATTCTCTGGTCTTTTTCTTGTCTGCTCTT CAGATGGATTTCGGTATTATATGGTGGCAGACTGT
Paol-allele*b
Paol-allele*c
Paol-allele*d
Paol-allele*e
Paol-allele*f
Paol-allele*g
Paol-allele*h
Paol-allele*i
Paol-allele*j
Paol-allele*k
Paol-allele*l
Paol-allele*m
170 180 190 200 210 220 230 240
Paol-allele*a GAGTTTAACTCCTCGAAGCTGAACGACATAGAGTTCACACTGTCTTTCTATTACAACAAACTGGAGTTCATCAGGTTCAG
Paol-allele*b
Paol-allele*c
Paol-allele*d
Paol-allele*e
Paol-allele*f
Paol-allele*g
Paol-allele*h
Paol-allele*i
Paol-allele*j
Paol-allele*k
Paol-allele*l
Paol-allele*m
250 260 270 280
Paol-allele*a CAGCAGTGTGGGGAAGTACGTTGGATACACTGAGTTTGGA
Paol-allele*b
Paol-allele*c
Paol-allele*d
Paol-allele*e
Paol-allele*f
Paol-allele*g
Paol-allele*h
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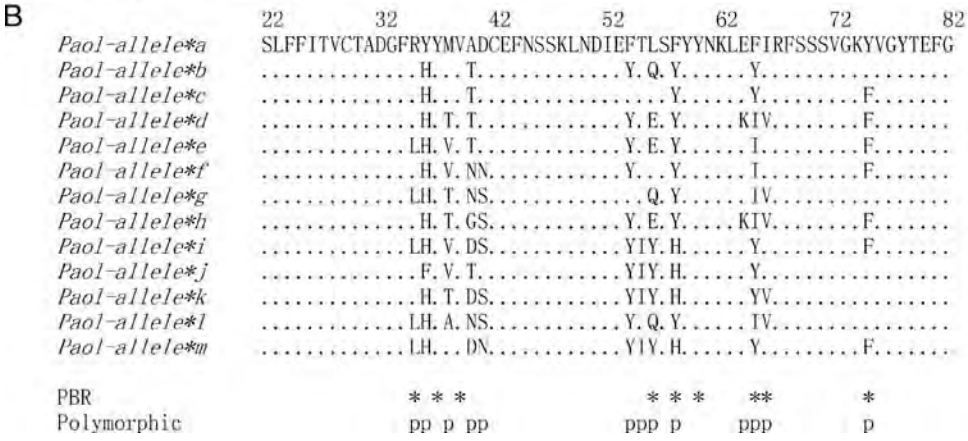
Paol-allele*l
Paol-allele*m

B


22 32 42 52 62 72 82
Paol-allele*a SLFFITVCTADGFRYYMVADCEFNSSKLNDIEFTLSFYYNKLEFIRFSSSVGKYVGYTEFG
Paol-allele*b
Paol-allele*c
Paol-allele*d
Paol-allele*e
Paol-allele*f
Paol-allele*g
Paol-allele*h
Paol-allele*i
Paol-allele*j
Paol-allele*k
Paol-allele*l
Paol-allele*m
PBR * * * * * * ** *
Polymorphic pp p pp ppp p ppp p

◀**Fig. 4.** (**A**) Thirteen different MHC class IIB genotypes of Japanese flounder MHC class IIB nucleotide sequences including partial exon1, complete intron1, and most of exon2; the intron1 sequence is shaded. (**B**) Sequence comparison of putative amino acids from different MHC class IIB genotypes based on Clustal W (..), Identity with the consensus sequence; *indicates putative PBR positions in human genes; p denotes polymorphic residues in flounder genes.

Primer Design. A pair of degenerate primers, dfMHCF (5′-GGTWTGTSGGWTACACTGA-3′) and dfMHCR (5′-TCAGTGGYYGTSACATCAGA-3′) (W = A/T; S = G/C; Y = C/T), were designed according to conserved sequences in other known vertebrates (Klein et al., 1993; Hordvik et al., 1994; Sültmann et al., 1994; Walker et al., 1994; Van Erp et al., 1996; Chen et al., 2004a) and used to amplify a class IIB cDNA fragment of about 300 bp from flounder liver cDNA. To isolate full-length class IIB cDNA, rapid amplification of the cDNA ends (5′RACE and 3′RACE) was performed. Two specific primers (GSP5′ and GSP3′) for Japanese flounder were designed according to the above amplified partial class IIB cDNA sequence. GSP5′ primer (5′-GTGACTTCCTGTCCGTCTCTTTGCCA-3′) was used for amplification of the 5′end, and GSP3′ primer (5′-GGAATCAAGAACGCTGAGAGGTGG AA-3′) was used for the 3′ end of the class IIB cDNA. The universal primer used for 5′-RACE and 3′-RACE were long primer (5′-CTAATACGACTCACTA TAGGGCAAGCAGTGGTATCAACGCAGAGT-3′) and short primer (5′-CTAATACGACTCACTA TAGGG-3′). Intron1 and intron2 were amplified with a primer pair fMHC2bIn12N (5′-CTCCCTC TTCTT CATCACGGT-3′) and fMHC2bIn12C (5′-GTAGAAGTCAAAGACGCTGC-3′). Intron2 and intron3 were amplified with a primer pair fMH C2bIn23N (5′-AGAATCTGCTCTGACGAA GT-3′) and fMHC2bIn23C (5′-CTTCTGACTCAGGCATG GATG-3′). Intron3 and intron4 were amplified with a primer pair fMHC2bIn34N (5′-GTCTGGAGA GAAGATTTCCTGTGT-3′) and fMHC2bIn34C (5′-AAGCAGGTTGAAGCAGCAGC-3′). A primer pair, fMHC2bIn12N (5′-CTCCCTCTTCTTCAT CACGGT-3′) and fMHC2bC1 (5′-TCCAAACT CAGTGTATCCAACG-3′), was used for amplification the sequence including partial exon1, complete intron1, and most of exon2 to determine the polymorphism of MHC class IIB in the Chinese population of *P. olivaceus*.

DNA and RNA Isolation and cDNA Synthesis. Genomic DNA was extracted with phenol-chloroform from blood as described (Liu

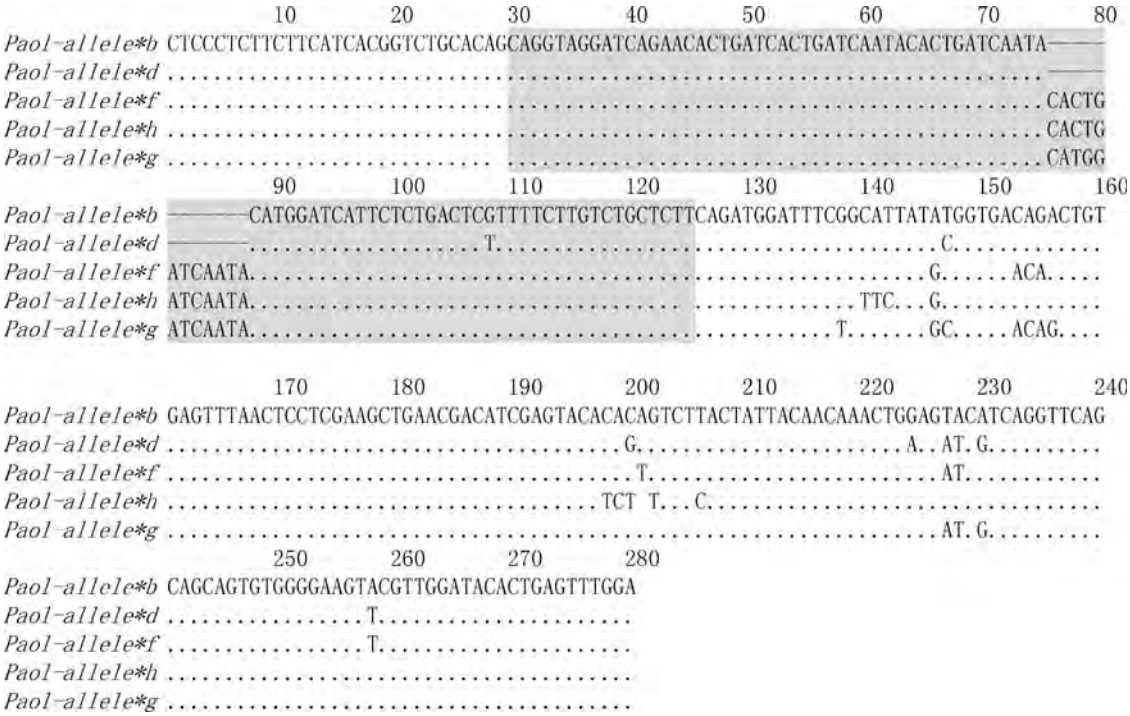
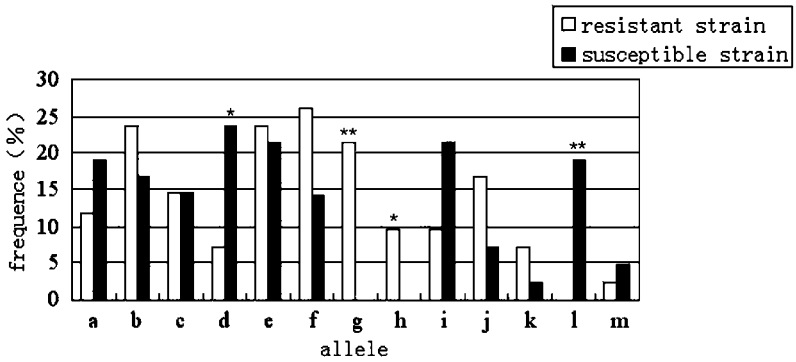


Fig. 5. Five different sequences including partial exon1, complete intron1, and most of exon2 of the MHC class IIB alleles from a single individual of a Chinese population of *Paralichthys olivaceus* from resistant stock; intron1 sequences are shaded.

A



B

Paol-allele	a	b	d	e	f	g	h	i	j	k	l	m
Resistance stock	11.90	23.80	7.14	23.80	26.19	21.4	9.52	9.52	16.67	7.14	0.0	2.38
Susceptibility stock	19.05	16.67	23.80	21.43	14.29	0.0	0.0	21.43	7.14	2.38	19.05	4.76
Chi-square (p)	0.3655	0.4152	0.0347*	0.7942	0.1745	0.0015**	0.0404*	0.1315	0.1778	0.3055	0.0029**	0.3795

Fig. 6. (**A**) Distribution of MHC class IIB alleles in resistant (white bars) and susceptible stock (black bars) of Japanese flounder (China population). $*P < 0.05$; $**P < 0.01$. (**B**) Percentages of MHC class IIB alleles in resistant and susceptible stocks of Japanese flounder (Chinese population) and χ^2 test result (*P*). $*P < 0.05$; $**P < 0.01$.

et al., 2005). The total RNA was extracted via Trizol reagent (Qiagen) according to the man-ufacturer's instructions. Poly $(A)^+$ RNAs were isolated from the total RNA using Oligotex™ spin-column kit (Qiagen). cDNA was synthesized using BD Smart™ RACE cDNA Amplification Kit (Clontech) according to the manufacturer's instructions.

Rapid Amplification of cDNA Ends. Both 5′-RACE and 3′-RACE were performed using a Smart RACE cDNA amplification kit (Clontech) according to the manufacturer's instructions. Touchdown polymerase chain reaction (PCR) was used for RACE amplification: 94°C for 2 min, 94°C for 1 min, 70°C for 50 s, 72°C for 1 min, for 5 cycles; 94°C for 1 min, 65°C for 50 s, 72°C for 1 min, for 30 cycles; and 72°C for 10 min for elongation. The objective fragments were cloned and then sequenced.

Cloning and Sequencing of Class IIB Genomic Sequence. PCR was performed using genomic DNA to amplify the flounder class IIB intron sequences. Amplifications were performed on a Peltier Thermal Cycler-200 (PTC-200). The conditions for PCR amplification were as described (Chen et al., 2004b): in brief, 1 cycle of 94°C for 5 min, then 35 cycles of 94°C for 1 min, 50 to 60°C for 90 s and 72°C for 90 s, followed by 1 cycle of 72°C for 10 min and then holding at 4°C. The PCR products were resolved by electrophoresis on 1% agarose gels and the fragments of interest were excised and then purified via the QIAEX II Gel Extraction Kit (Qiagen). The purified fragments were cloned into pMD18-T vectors (Takara) and propagated in DH5α *Escherichia coli* competent cells. The insert size was then checked by double digestion with *Eco*RI and *Hin*dIII. The positive clones were sequenced.

Sequence Analysis. Sequence data were analyzed using DNASTAR 5.0 software. The alignment of the putative class IIB amino acid sequence of Japanese flounder and other known vertebrates was performed using CLUSTAL W program (Thompson et al., 1994). The GenBank accession numbers of sequences used for comparison were as follows: Japan population of Japanese flounder (AB126915; Srisapoome et al., 2004), Atlantic salmon (CAA49726; Hordvik et al., 1994), carp (CAA64706; Van Erp et al., 1996), cichlid (AAB27553; Klein et al., 1993), human (AB062112), rainbow trout (AF115529), red sea bream (AY190711; Chen et al., 2004a), striped sea bass (AAA49379; Walker et al., 1994), zebrafish (CAD87794; Sültmann et al., 1994), mouse (P18469; Acha-Orbea and Scarpellino, 1991), and nurse shark (L20275; Bartl and Weissman, 1994). The phylogenetic tree was constructed using the neighbor-joining methods in MEGA 2.0 (Kumar et al., 2001). Transition and transversion were analyzed using DnaSP 4.0 analysis software. χ^2 was used to test the significance of the MHC IIB allele frequency in resistant and in susceptible stock.

Results

Isolation and Analysis of MHC Class IIB Gene. MHC class IIB cDNA was isolated from Japanese flounder via PCR and RACE-PCR. A fragment of 295 bp was first amplified using degenerate primers dfMHCF and dfMHCR. Based on the sequence of the 295-bp fragment, two specific primers, GSP5′ and GSP3′, were designed and used for 5′-RACE and 3′-RACE, respectively. A 497-bp 5′-RACE fragment and a 945-bp 3′-RACE fragment, respectively, were amplified. After the two fragments were assembled, a full length of MHC IIB cDNA fragment of 1,182 bp was obtained (GenBank Accession No. AY848955). Based on the full length of flounder MHC IIB cDNA sequence, intron sequences were amplified by PCR. As in other teleosts, such as turbot (Zhang and Chen, 2006) and red sea bream (Chen et al., 2006), five exons and four introns were identified in Japanese flounder MHC class IIB gene (Figure 1A). Exon 1 included a 34-bp 5′-UTR and encodes the leader peptide (signal peptide). Exon 2 encodes the β1 domain. Exon 3 encodes the β2 domain. Exon 4 encodes the transmembrane region and partial cytoplasmic region, while exon 5 encodes a partial cytoplasmic region and also included a 407-bp 3′-UTR (Figure 1B).

Comparison of the deduced amino acid sequences of class IIB with other teleost and human class IIB showed that four cysteine residues were observed in the β1 as well as in the β2 domains of all fish and mammalian sequences. A putative *N*-linked glycosylation site was observed in the β1 domain. Alignment of flounder deduced MHC IIB amino acid sequences and other known MHC IIB amino acid sequences indicated that tyrosine at positions 62, 64, 79, and 110; lysine at position 88; isoleucine at 100; and glutamate at 106 of 24 correlative amino acids were essentially conserved among teleosts, and asparagine at position 114 was present in piscine, mouse, and human (Figure 2).

Phylogenetic Analysis. A phylogenetic tree was constructed via the neighbor-joining method and the MHC IIB sequence of nurse shark served as an outgroup (Figure 3). The deduced MHC IIB amino acid sequence of Japanese flounder (Chinese population) had 85.4%, 57.6%, 55.7%, 66.4%, 34.4%, 34.0%, 28.3%, 55.3%, 72.5%, 72.1%, and 51.0% identity with that of the Japanese population of *P. olivaceus* (AB126915), Atlantic salmon (CAA49726), carp (CAA64706), cichlid (AAB27553), human (AB062112), mouse (P18469), rainbow trout (AF115529), red sea bream (AY190711), striped sea bass (AAA49379), and zebrafish (CAD87794), respectively.

MHC Class II B Polymorphism. Genomic DNA from 42 susceptible and 42 resistant flounder individuals was amplified via PCR using the primer pair fMHC2bIn12N and fMHC2bC1. Four to seven positive clones per individual were sequenced. For the flounder class IIB alleles, a 268/280-bp PCR product was obtained. Among the 280 (268) nucleotides, 32 (11.4%) nucleotide positions were variable, and 22 (7.86%) nucleotide positions were parsimonious. Thirteen different MHC class IIB alleles that encoded 13 different amino acid sequences were identified from 411 clones from the 84 individuals (Figure 4A, B). When compared with the human genes, 13 (21.3%) of the 61 amino acids were polymorphic and 12(19.7%) amino acid sites were parsimonious; 6 (66.67%) of 13 sites were variable within the PBR (Figure 4B). In addition to the polymorphism in the protein encoding region, five different intron1 sequences were identified. Polymorphism in the first intron included a 12-bp deletion and various base substitutions (Figure 4A). In addition, 59 (70.24%) individuals from a total of 84 individuals displayed at least two different MHC class IIB sequences; 5 of 59 individuals displayed at least three different MHC class IIB sequences, and 1 of these 5 individuals displayed five different MHC class IIB sequences (Figure 5).

Association Between MHC IIB Alleles Polymorphism and Disease Resistance/Susceptibility. Most alleles were found in both resistant and susceptible stock, such as alleles *a, b, c, d, e, f, j, k, i,* and *m,* but the distribution of MHC class IIB alleles was significantly different between resistant and susceptible stock (Figure 6). After χ^2 test, allele *d* was significantly more prevalent in individuals from the susceptible stock than in individuals from the resistant stock: 23.80% and 7.14%, respectively. In addition, alleles *g* and *h* were observed only in the resistant individuals (21.4% and 9.52%, respectively), while allele *l* was found only in susceptible individuals (19.05%).

Discussion

MHC genes are known to be involved in the vertebrate immune system and encode antigen recognition proteins used in the adaptive immune response (Apanius et al., 1997; Edwards and Hedrick, 1998; Hedrick and Kim, 1999). To assess the effects of MHC class IIB genotypes on resistance to *V. anguillarum* in a Chinese population *P.*

olivaceus, we report here molecular cloning of the MHC class IIB cDNA, analysis of the gene structure, and polymorphism in relation to disease resistance and susceptibility. Sequence analysis indicated that the putative amino acid identity of MHC class IIB between the Japanese (Srisapoome et al., 2004) and Chinese population of *P. olivaceus* was 85.4%, suggesting a rapid divergence rate. Of course, the high polymorphism of the MHC class IIB gene may be one important reason for this. The cysteine residues involved in forming disulfide bridges are present in the β1 as well as the β2 domain of the flounder sequence, and one conserved amino acid on the mammalian β chain (N at position 114) was also conserved. An *N*-linked glycosylation site is present at amino acid positions 49, 50, and 51 (Figure 2). Similar *N*-linked glycosylation signals were observed in red sea bream (Chen et al., 2006), striped sea bass (Walker et al., 1994), cichlid (Klein et al., 1993), and channel catfish (Godwin et al., 1997).

The MHC class IIB gene has been described for some teleost species including Atlantic salmon (*Salmo salar*; Hordvik et al., 1994; Langefors et al., 2001a,2001b), rainbow trout (*Oncorhynchus mykiss*; Glamann, 1995; Ristow et al., 1999), large barbus (*Barbus intermedius*; Dixon et al., 1996), red sea bream (Chen et al., 2006), channel catfish (*Ictalurus punctatus*; Godwin et al., 1997), striped bass (*Morone saxatilis*; Walker et al., 1994), zebrafish (*Danio rerio*; Ono et al., 1992), cichlids (*Cichlidae*; Stet et al., 1998), and common carp (*Cyprinus carpio*; Figueroa et al., 2000). In the present study, 13 different IIB alleles were found among 411 clones from 84 individuals of flounder. Fifty-nine of 84 individuals displayed at least two different MHC class IIB sequences, indicating either a high degree of heterozygosity or the presence of multiple loci; 5 of 59 individuals displayed at least three different IIB sequences. One of 5 individuals displayed five different IIB sequences, and there are three different intron1 sequences, which suggests that there were at least three MHC class IIB loci. Similar phenomena were observed in zebrafish (Ono et al., 1992). The observations for the class II loci were in accordance with the results of previous work showing the presence of multiple loci in salmonids (Grimholt et al., 1994) and in lake whitefish (Binz et al., 2001).

In addition, 13 alleles are just two different types based on the length of intron1, of which three alleles are 84 bp, and 10 alleles are 96 bp in length. The sequences of intron1 ranged from 84 bp to 96 bp in length, and insertion/absence, transition, and transversion appeared in intron1. Comparison of the sequences at the exon-intron boundaries with the consensus sequences for these regions (Padgett et al., 1986) revealed the presence of potentially functional splice signals in most of the genes. Similar results were observed in zebrafish Sültmann et al., (1994) and rainbow trout (Palti et al., 2001).

Grimholt et al. (2003) investigated Atlantic salmon infection with anemia virus (ISAV) causing infectious salmon anemia and the *Aeromonas salmonicida* bacteria causing furunculosis. They found highly significant associations between resistance toward infectious diseases caused by both pathogens and MHC class I and class II polymorphism in Atlantic salmon. Although there was a report on class IIB cDNA structure from Japanese flounder (Srisapoome et al., 2004), no data on class IIB polymorphism and its association with disease resistance/susceptibility are available. To our knowledge, the present article reports the first study on class IIB polymorphism and association with resistance/susceptibility to a bacterial pathogen, *V. anguillarum,* in Japanese flounder, *P. olivaceus.* In the present study, we identified two alleles, *g* and *h*, which were observed only in individuals from resistant stock, and allele *l,* which was observed only in susceptible stock. These might provide evidences of actual association between certain MHC alleles and resistance to *V. anguillarum* in Japanese flounder. The observed association between alleles *g*, *h*, and *l* and resistance/susceptibility to *V. anguillarum* supported the hypothesis that frequency-dependent selection is important for the maintenance of MHC variation. This experimental result was in accordance with Asa Langefors's conclusion in Atlantic salmon (2001a). However, whether the gene itself is the main reason for the association between class IIB gene and disease resistance/susceptibility or whether the lopsided linkage with another interrelated gene is responsible for this relationship requires further study.

In summary, we have isolated and characterized MHC class IIB gene and established significant associations between class IIB allele polymorphism and disease resistance/susceptibility in a Chinese population of Japanese flounder. These class IIB genotypes might be potentially applied as gene markers in selecting Japanese flounder with enhanced resistance to disease caused by pathogenic bacteria.

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