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MHC class IIα gene polymorphism and its association with resistance/susceptibility to *Vibrio anguillarum* in Japanese flounder (*Paralichthys olivaceus*)

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ABSTRACT

Association between polymorphism of the major histocompatibility complex (MHC) genes and disease resistance has been documented for few teleosts. In this study, we first investigate the genetic variation at the MHC IIα gene in Japanese flounder (*Paralichthys olivaceus*) for survival after challenge with bacterial infection. To explore the specific allele associated with disease resistance, about 6000 individuals from 60 families challenged with *Vibrio anguillarum*, which causes significantly different mortality in flounder families. 15–20 individuals from each of six high-resistance (HR) and six low-resistance (LR) families were screened for their MHC class IIα genotypes using sequence analysis. High polymorphism of MHC IIα gene and at least two loci were discovered in Japanese flounder and the rate of d_N occurred at a significantly higher frequency than that of d_S in PBR and non-PBR, the balancing selection hypothesis could explain the high polymorphism of MHC IIα gene in flounder. From the 212 individuals, a total of 55 MHC class IIα alleles were identified, and 9 alleles were used to study association between alleles and resistance to disease. Three alleles, *Paol-DAA*1301*, *Paol-DAA*1401* and *Paol-DAA*2201* were significantly associated with resistance against *V. anguillarum*, and *Paol-DAA*1001* and Paol-*DAA*1501* alleles were significantly associated with increased susceptibility to *V. anguillarum*. This study confirmed the association between alleles of MHC class IIα gene and disease resistance or susceptibility to bacterial infection in flounder, and the disease resistance-related MHC markers could be used for molecular marker-assisted selective breeding in the flounder.

1. Introduction

The major histocompatibility complex (MHC) is one of the most studied regions of the genome in mammalian, non-mammalian known to date, which has been thought candidate for molecular markers association between polymorphism and resistance/susceptibility to diseases. There are two classes MHC in vertebrates, MHC class I and class II genes encode cell-surface glycoproteins responsible for binding foreign peptides for the presentation of self- and non-self peptide to T-cells receptor (TCR), and triggering a specific immune response towards the pathogen from which the peptides are derived in the adaptive immune system (Croisetiere et al., 2008). The MHC class I molecule consisting of one alpha chain and β2-microglobuin, present foreign peptide product by the degradation of intracellular pathogens to cytotoxic $CD8^+$ T cells (Srisapoome et al., 2004); The MHC class II molecule, consisting of one alpha chain and one beta chain, present foreign peptides derived from extra cellular pathogens and present to helper $CD4^+$ T cells (Kjøglum et al., 2006). In mammals, class I antigens are expressed in all somatic cells, and class II antigens are expressed on antigen presenting cells. Surprisingly, unlike mammals and other vertebrates, class I gene and class II genes were found to reside on different linkage groups in teleosts (Graser et al., 1996; Hansen et al., 1999; Sato et al., 2000). Thus, some scholars believed that the major histocompatibility (MH) genes are more appropriate designation in teleosts (Stet et al., 2002).

High polymorphism is one of the most characteristic features of the MHC genes, which results in the presence of multiple loci and numerous alleles at each given locus within populations (Grimholt et al., 2003). Generally, class Iα and class II genes are highly polymorphic and the highest level of polymorphism is concentrated observed in the exon 2 that encode the peptide-binding region (PBR). Three major type of mechanisms can be explained the evolution and maintenance of the polymorphism and allelic variation of the MHC genes within vertebrate populations: the pathogen-driven, reproductive mechanisms and selection of

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pathogen pressure varies in space and/or time (Edwards and Hedrick, 1998; Hedrick, 2002). Several hypotheses have been proposed to explain the vast polymorphism of the MHC genes: heterozygous advantage selection, negative frequency dependent selection and balancing selection, and the pathogen-driven selection favors genetic variation through heterozygous advantage and frequency dependent (Parham and Ohta, 1996).

Different allelic MHC molecules bind and present unique specificity peptides, the response of an organism towards certain pathogens can be influenced by the MHC alleles present in different haplotypes (Rakus et al., 2008). It is very important to detect resistant allele of MHC in economic species for molecular marker-assisted selective breeding program. The associations between disease susceptibility/resistance and MHC polymorphism have been established in some species (Briles et al., 1983; Hill et al., 1991; Medina and North, 1998; Paterson et al., 1998). For instance, one MHC haplotype was found significantly with resistance to Marek's disease in chicken (Bacon, 1987), the MHC is also found to associate with resistance toward other pathogens (Lamont, 1998). In teleosts, association between MHC polymorphism and the resistance against or susceptibility to some viral and bacteria diseases have been documented in salmonid fish species by comparing individual alleles of MHC genes, specific alleles of MHC have been documented to correlate with some viral and bacterial disease (Grimholt et al., 1994, 2003; Gjedrem et al., 1991; Glover et al., 2007; Lohm et al., 2002; Wynne et al., 2007).

Japanese flounder (*Paralichthys olivaceus*) is one of the most important marine aquaculture species and widely cultured throughout the coastal areas of North China due to its good taste and abundant nutrition. Because of the frequently occurred viral and bacterial diseases of cultured flounder, the production of the fish has not increased in the last decade in spite of extensive fishery management efforts. This situation resulted in limit profitability of fisher folk and embarrassed the development of marine aquaculture (Liu et al., 2005a). Many bacterial and viral diseases especially occurred in step of juvenile flounder, of which *Vibrio anguillarum* is of particular concern due to its ubiquitous presence and frequent occurrence, it brings on a significant threat to commercial production in marine aquaculture (Liu et al., 2005b). The use of antibiotics has partially solved the problem of bacterial diseases, but has raised concerns regarding antibiotic residues in body, environmental pollution, and antibiotic resistance development. Hence, an important approach to disease prevention is to culture strains of fish with enhanced resistance to some major diseases using molecular marker-assisted selective breeding. There has been no research regarding association between MHC IIα gene polymorphism and their resistance to *V. anguillarum* known to date. In the present study, we decided to analyze the polymorphism of exon 2 of the MHC IIα gene, because exon 2 encodes for the α1 domain and is the most polymorphic region of the MHC IIα gene. The aim of the present study were to document the allelic diversity at the MHC IIα gene of Japanese flounder and to detect specific allele which has high resistance to *V. anguillarum* across selected families of Japanese flounder.

2. Materials and methods

2.1. Fish, challenge experiment and sampling

Base populations of Japanese flounder derived from the yellow sea wild stocks, Japan sea wild stock and resistance stock which was selected according to the resistance against *V. anguillarum* as described by Zhang et al. (2006). Sixty half-sibling and full-sibling families of flounder were established as previously described (Chen et al., 2008). Fertilized eggs were incubated, hatched and reared at Aqua breeding station in Haiyang and were kept in separate tanks. The fry were fed a commercial diet according to standard feeding scheme.

Table 1
Number of individuals from high-resistance (HR) and low-resistance (LR) families.

	No. of family	No. of individuals per family		Total individuals
		Dead	Surviving	
HR	6		15–20	108
LR	6	15–20		104
Total	12	104	108	212

For the *V. anguillarum* challenge experiment in Japanese flounder, all the test fishes were inoculated by intraperitoneally injection. In order to determine the median lethal concentration, different concentration of the *V. anguillarum* had been tested in a pre-challenge experiment on fish of the same size as the test fish prior to challenge trial. Each family was reared in a separate tank with a fresh water supply at 20 °C. About 100 individuals per family were inoculated intraperitoneally with 9.8×10^5 colony forming units (CFU) of *V. anguillarum*. The test lasted for approximately 6 days, mortality was recorded every 6 h and fin of all dead and surviving individuals were stored in 95% ethanol. No mortality was observed in control fish.

To document whether MHC class IIα gene alleles are associated with resistance/susceptibility to *V. anguillarum*, samples of resistance and susceptible stocks of each family were collected from the first 30 to die and the last survivors of the bacterial challenge. Six high-resistance families (HR, survivor rate (SR) >50%) and six low-resistance families (susceptible families, LR, SR <20%) were selected from the challenge tests. Both individuals that had died and those that had survived during infection were sampled from HR and LR families (Table 1).

2.2. DNA and RNA isolation and cDNA synthesis

Genomic DNA was extracted from fin samples of 15–20 individuals per HR and LR flounder families with the method of phenol–chloroform as descried (Xu et al., 2008). The quality and concentration of DNA were assessed by agarose gel electrophoresis and measured with a GeneQuant Pro (Pharmacia Biotech Ltd.) RNA/DNA spectrophotometer. Finally, DNA was adjusted to 100 ng/μl and was stored at 4 °C for future use. Total RNA was extracted from liver tissue of adult individuals using Trizol reagent (Qiagen) according to the manufacture'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 manufacture's instructions.

2.3. Primer design

To isolate full-length cDNA of MHC class IIα gene, two specific primers GSP5′ (5′-GACCGACGCTCAGACCCACTCCACA-3′) and GSP3′ (5′-TGTGGAGTGGGTCTGAGCGTCGGTC-3′) were designed according to the published partial class IIα cDNA sequence (GenBank accession No.: AU091246). GSP5′ primer was used for amplication of the 5′end, and GSP3′ primer was used for the 3′end of the class IIα cDNA. The universal primer used for 5′-RACE and 3′-RACE were long primer (5′-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3′ and short primer (5′-CTAATACGACTCACTATAGGG-3′).

To identify of MHC class IIα genomic organization for subsequently future research, three pairs primer were designed to amplify three introns using genomic DNA across putative