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Short communication

Identification of 48 full-length MHC-DAB functional alleles in miiuy croaker and evidence for positive selection

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A B S T R A C T

Major histocompatibility complex (MHC) molecules play a vital role in the immune response and are a highly polymorphic gene superfamily in vertebrates. As the molecular marker associated with polymorphism and disease susceptibility/resistance, the polymorphism of MHC genes has been investigated in many tetrapods and teleosts. Most studies were focused on the polymorphism of the second exon, which encodes the peptide-binding region (PBR) in the α1- or β1-domain, but few studies have examined the full-length coding region. To comprehensive investigate the polymorphism of MHC gene, we identified 48 full-length miiuy croaker (*Miichthys miiuy*) MHC class IIB (Mimi-DAB) functional alleles from 26 miiuy croaker individuals. All of the alleles encode 34 amino acid sequences, and a high level of polymorphism was detected in Mimi-DAB alleles. The rate of non-synonymous substitutions (d_N) occurred at a significantly higher frequency than that of synonymous substitutions (d_S) in the PBR, and this result suggests that balancing selection maintains polymorphisms at the Mimi-DAB locus. Phylogenetic analysis based on the full-length and exon 2 sequences of Mimi-DAB alleles both showed that the Mimi-DAB alleles were clustered into two major groups. A total of 19 positive selected sites were identified on the Mimi-DAB alleles after testing for positive selection, and 14 sites were predicted to be associated with antigen-binding sites, which suggests that most of selected sites are significant for disease resistance. The polymorphism of Mimi-DAB alleles provides an important resource for analyzing the association between the polymorphism of MHC gene and disease susceptibility/resistance, and for researching the molecular selective breeding of miiuy croaker with enhanced disease resistance.

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1. Introduction

The major histocompatibility complex (MHC) is a genomic region that plays an important role in innate and adaptive immune responses. Historically, MHC was conveniently divided into three major regions: class I, class II and class III region [1]. The MHC class I and II genes are involved in the adaptive immune response by presenting antigens to T cell receptors while discriminating self from non-self [2], and some genes in the MHC class III region participate in the innate immune response [3,4]. The MHC II molecules are heterodimers comprising of α and β chains, and present foreign peptides derived from extracellular pathogens to helper CD4$^+$ T cells through T-cell receptors [5]. MHC II genes contain IIA and IIB genes which encode the α and β chain, respectively. Vertebrates have many different MHC IIA and MHC IIB genes. In order to distinguish between these genes, three letters are assigned to the different genes [6]. Genes belonging to the MHC class II are represented by the letter D, the different MHC II genes are represented by the second letter "X" and the third letter is either A or B to distinguish between the alpha and beta chain.

A prominent characteristic of MHC genes is the high level of polymorphism, due to numerous alleles could be found at one given locus and each gene may identify multiple loci within populations [1]. This feature confers MHC in each individual a direct functional relevance for immune response and enables the organism to deal with a great range of pathogens [7,8]. There are several hypotheses that have been postulated to explain the evolution and maintenance of allelic polymorphism in MHC genes: heterozygous advantage, overdominant selection [9,10], frequency-dependent selection [11], and balancing selection [12]. Several lines of evidence showed that polymorphic MHC loci are subject to balancing selection [13]. The major component of balancing selection is the

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pressure exerted by pathogens and parasites [14]. Therefore, the main selective force of the polymorphism of MHC genes was hypothesized by pathogen-mediated selection. Moreover, mate choice and other behaviors such as kin recognition may also be important selective forces [15,16]. The excess rate of non-synonymous substitutions (d_N) over synonymous substitutions (d_S) are regarded as good indicators for an ongoing adaptive evolutionary process [12,17].

The high level of polymorphism of MHC genes attracted the attention of evolutionary geneticists and immunologists, as the polymorphism of MHC genes may strengthen the resilience of many species to various stressful and complex living environments. MHC genes have been researched in detail with respect to polymorphism, protein function and evolution among tetrapods [18–21], teleosts [22–25] and cartilaginous fishes [26,27]. However, most investigation of the polymorphism of MHC II genes was focused on exon 2 in teleost as this exon encodes the PBR in the α1- or β1-domain [28–31], and only few studies concentrate on the full-length coding region [32–34].

As an important species in the marine industry, miiuy croaker (*Miichthys miiuy*) is vulnerable to bacterial and parasitic infection. MHC genes participate in the adaptive immune response and are associated with disease resistance. To investigate the functional characteristics and evolutionary mechanism of MHC genes from miiuy croaker, we analyzed the polymorphism of full-length of MHC class IIB from miiuy croaker (Mimi-DAB). In the present study, 48 full-length Mimi-DAB functional alleles were identified from 26 miiuy croaker individuals, and the polymorphism of Mimi-DAB alleles was analyzed. Molecular evolutionary analysis was also conducted to explore the ongoing evolutionary mechanisms of DAB gene.

2. Materials and methods

2.1. Sampling, total RNA isolation and cDNA synthesis

A total of 26 specimens of healthy miiuy croakers were collected from the East China Sea. Total RNA extraction was performed using Trizol reagent (Qiagen) from the spleen tissues of all specimens. The extracted RNA was stored at −80 °C until cDNA synthesis. cDNA was synthesized utilizing a QuantScript RT kit (TIANGEN) according to the manufacturer's instructions, and then stored at −20 °C.

2.2. Primer design, cloning and sequencing

According to the previous report by Xu et al. [35], specific primers were designed to obtain the full-length of MHC class IIB gene in miiuy croaker. Specific primers (MHC-IIB-F/R: 5′-GTTTGTGAAGTGAACAGACG-3′/5′-AGTTTAGACCAGCAGAGGAT-3′) were located on the 5′ and 3′ untranslated regions (UTRs) and different sequences were acquired to analyze the polymorphism of Mimi-DAB gene. Each 50 μL PCR system consisted of 5.0 μL of 10 × *Taq* Plus polymerase buffer, 0.2 mM dNTPS, 0.2 μM of the forward and reverse primers, 2.5 units of high-fidelity *Taq* Plus polymerase (TIANGEN), and 1 μL of cDNA template. Cycling conditions were as follows: 10 min at 95 °C for pre-denaturalization, followed by 35 cycles of denaturation at 95 °C for 40 s, annealing at 55 °C for 40 s and extension at 72 °C for 2 min, and a final elongation of 10 min at 72 °C.

The bands of the expected size were cut out from the gel and purified using a QIAEX II Gel Extraction Kit (Qiagen). The purified fragments were ligated into PMD18-T vectors (Takara) and transformed into DH5α competent cells in accordance with the standard protocol. Positive clones were identified via PCR screening with M13 primers. From each individual an average of 18 positive clones were selected and separately sequenced from both forward and reverse directions with M13 primers. The number of clones was sufficient to determine the genotype of each individual and to avoid reading errors or generating recombinant sequences during PCR amplifications [36].

2.3. Sequence analysis and phylogenetic tree construction

The nucleotide and amino acid sequences were aligned using Clustal W multiple alignment in MEGA 5.0. New alleles were identified and named in accordance with the accepted nomenclature rules [37]. In brief, alleles that differ by less than five amino acid substitutions in the analyzed domain of MHC molecule are considered as subtypes within a single major type. According to the method of Nei and Gojobori [38], the rate of d_S and d_N was calculated by MEGA software with the default setting. Genetic distances from the alignments of the complete CDS and the exon 2 nucleotide sequences of Mimi-DAB alleles were estimated using Kimura's two-parameter evolutionary model and then used as a basis to construct the neighbor-joining tree of all identified variants. The constructed tree was then used to identify the number of major evolutionary lineages and/or subgroups that Mimi-DAB alleles may represent in miiuy croaker.

2.4. Positive selection analysis

To estimate the effect of selective pressures on Mimi-DAB, positive selection analysis was performed using likelihood ratio model in the program CODEML of PAML v4 [39]. M0, M1a, M2a, M3, M7, and M8 models were used to test the possible positive selection sites among Mimi-DAB alleles. The likelihood ratio tests were

Table 1
The number of DAB alleles have been investigated in teleost.

Species	Full/Partial	Number of individuals	Number of alleles	References
Cynoglossus semilaevis	Exon 2	33	23	Xu et al. (2010)
Paralichthys olivaceus	Exon 2	60	72	Xu et al. (2008)
Pseudotropheus zebra	Exon 2	6	18	Ono et al. (1993)
Melanochromis auratus	Exon 2	12	28	Ono et al. (1993)
Gadopsis marmoratus	Exon 2	39	28	Bracamonte et al. (2015)
Macquaria australasica	Exon 2	44	41	Bracamonte et al. (2015)
Nannoperca australis	Exon 2	22	20	Bracamonte et al. (2015)
Nannoperca obscura	Exon 2	24	23	Bracamonte et al. (2015)
Salmo salar	3′UTRs	64	7	Grimholt et al. (2003)
Oreochromis niloticus	Full length	6	10	Pang et al. (2013)
Chrysophrys major	Full length	7	7	Chen et al. (2006)
Verasper variegates	Full length	5	12	Li et al. (2011)
Miichthys miiuy	Full length	26	48	In this study

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