MHC polymorphism and disease resistance to *vibrio anguillarum* in 8 families of half-smooth tongue sole (*Cynoglossus semilaevis*)

Min Du1,2,3, Song-lin Chen1*, Yan-hong Liu3, Yang Liu1 and Jing-feng Yang1

**Abstract**

**Background:** Genes in the major histocompatibility complex (MHC) have a critical role in both the innate and adaptive immune responses because of their involvement in presenting foreign peptides to T cells. However, the nature has remained largely unknown.

**Results:** We examined the genetic variation in MHC class IIB in half-smooth tongue sole (*Cynoglossus semilaevis*) after challenge with *vibrio anguillarum*. Two thousand and four hundred fry from 12 half-smooth tongue sole families were challenged with *Vibrio anguillarum*. To determine any association between alleles and resistance or susceptibility to *V. anguillarum*, 160 individuals from four high-resistance (HR, < 40.55% mortality) families and four low-resistance (LR, > 73.27% mortality) families were selected for MHC IIB exon2 gene sequence analysis. The MHC IIB exon2 genes of tongue sole displayed a high level of polymorphism and were discovered at least four loci. Meanwhile, the dN/dS [the ratio of non-synonymous (dN) substitutions to synonymous (dS) substitutions] in the peptide-binding region (PBR) was higher than that in the non-peptide-binding region (non-PBR). Eighty-eight alleles were discovered among 160 individuals, and 13 out of 88 alleles were used to analyze the distribution pattern between the resistant and susceptible families. Certain alleles presented in HR and LR with a different frequency, while other alleles were discovered in only the HR or LR families, not both. Five alleles, *Cyse-DBB*6501, *Cyse-DBB*4002, *Cyse-DBB*6102, *Cyse-DBB*5601 and *Cyse-DBB*2801, were found to be associated with susceptibility to *V. anguillarum* with a frequency of 1.25%, 1.25%, 1.25%, 1.25% and 2.5% in the HR families, and 35%, 33.75%, 27.5%, 16.25%, 15% in the LR families (p < 0.01, 0.01, 0.01, 0.01, 0.01), respectively. Four alleles, *Cyse-DBB*3301, *Cyse-DBB*4701, *Cyse-DBB*6801 and *Cyse-DBB*5901, were found to be associated with resistance to *V. anguillarum*, with a frequency of 13.75%, 11.25%, 11.25%, 8.75% in the HR families and 1.25%, 1.25%, 1.25% and 1.25% in the LR families (p < 0.01, 0.05, 0.05 and p = 0.064), respectively.

**Conclusions:** Elucidation of the role of MHC II B genes in half-smooth tongue sole should prove to be helpful to the in-depth development of marker-assisted selective breeding in half-smooth tongue sole.

**Keywords:** *Cynoglossus semilaevis, Vibrio anguillarum*, polymorphism, MHC IIB, susceptibility, resistance

**Background**

Major histocompatibility complex (MHC) molecules play a critical role in both innate and adaptive immunity by presenting foreign peptides to T cells in vertebrate organisms, and have been considered candidate molecular markers of an association between polymorphisms and resistance/susceptibility to diseases [1]. A combination of balanced and directional selection is thought to be responsible for allelic variation of MHC genes in vertebrate populations, because pathogen pressure varies at different times and locations [2]. Two classes of MHC are found in fish, MHC class I and class II molecules. The genes encode glycoproteins which bind peptides for the presentation of self and non-self peptides to T-cell receptors (TCR) [3].
The MHC class II molecules are symmetrical heterodimers, consisting of one alpha chain and one beta chain, with non-covalent contacts in which the alpha1 and beta1 domains form a peptide-binding region (PBR). In mammals, MHC class II genes are constitutively expressed in antigen-presenting cells such as macrophages, B cells, monocytes and dendritic cells, and have direct functional relevance in the immune response. Class I antigens are expressed in all somatic cells [1,4,5]. In teleosts, class I and class II genes were found to reside on different linkage groups [6-8]. Many MHC genes have been isolated, characterized expressed and analyzed in at least 30 different fish species over the last twenty years [9-14]. Multiple loci and a considerable number of alleles at each given locus were found in the classical MHC genes. The peptide-binding region (PBR) contains the highest level of polymorphisms in the MHC genes [15-29]. Certain MHC alleles of the class II genes linked to viral and bacterial diseases have been reported in some species [30-37]. The link between disease susceptibility/resistance and MHC polymorphism is crucial for detecting MHC alleles related to resistance in marine aquaculture species for molecular marker-assisted selective breeding programs [38].

Half-smooth tongue sole (Cynoglossus semilaevis) is widely cultured throughout the coastal areas of North China [39]. However, viral and bacterial diseases frequently occur in this cultured fish, and losses due to infectious disease limit the profitability and the extent of the development of the aquaculture [40,41]. One pathogen which is a significant threat to half-smooth tongue sole is Vibrio anguillarum [42]. Antibiotics have partially solved this problem, but antibiotic residues in fish, environmental pollution and antibiotic resistance are questions about which grave concerns remain [43]. Therefore, the selective breeding of tongue sole with disease resistance, based on molecular techniques which can enhance the resistance to specific pathogens, may be a good approach to solving these problems.

The half-smooth tongue sole MHC class IIB cDNA sequence and cDNA polymorphisms have been reported [40]. However, the polymorphisms at the DNA level and the link between specific alleles and resistance to *V. anguillarum* have not been elucidated yet. In the present study, we investigated the single nucleotide polymorphism (SNP) sites and polymorphisms in MHC II B exon2, and the association between certain alleles and disease resistance or susceptibility to *Vibrio anguillarum*, across 8 families of half-smooth tongue sole.

**Methods**

**Fish and rearing**

Eighteen full-sib families were established as reported [44], using a method for producing strains with a high growth rate and disease resistance. Male parents came from wild populations while female parents came from farming populations. Fertilized ova were hatched and reared at the breeding station at Minbo aquatic Co., Ltd. Located in Laizhou city, Shandong province, China. Each family was kept in a separate tank. The fry were fed a commercial diet using a standard feeding regimen [45].

**Challenge test**

For the challenge test, 200 individuals of each family (12 out of 18 families were large enough to be included), ten months old, were intraperitoneally injected with a 0.2 ml bacterial suspension of approximately 10,000,000 cells of *V. anguillarum*, while 16 individuals were injected with 0.9% saline as control [15]. Each fry weighed approximately 12-15 grams. The fry of each family were kept in a 1 m³ single tank with a fresh seawater supply at 23°C. This challenge experiment was performed twice and lasted for approximately two weeks. Mortality was recorded every day and the fin clips of all the fish were collected and preserved in absolute ethanol until use. The gross signs of fish mortality were based on a previous reporting method [42].

**Sampling and DNA isolation**

To identify whether MHC IIB exon2 alleles are associated with resistance or susceptibility to *V. anguillarum*, fin samples from each family of half-smooth tongue sole were collected and recorded from the first 20 to die and the last survivors at the time the bacterial challenge was terminated and preserved in absolute ethanol until use. High-resistance families (HR) with a survival rate (SR) > 59.45% and susceptible families or low-resistance families (LR) with a SR < 26.73% were selected from the challenge trials. The numbers fish which died or survived after the infection recorded for each family (Additional file 1).

Genomic DNA was isolated from the dorsal or caudal fin samples of 20 individuals per family (from the 4LR and 4HR families) using the phenol-chloroform method as described by Chen et al. [46]. The quality and concentration of DNA were assessed by agarose gel electrophoresis and then measured with a GENEQUANT Pro (Pharmacia Biotech Ltd.) RNA/DNA spectrophotometer. Finally, DNA was adjusted to 100 ng/μl and stored at -20°C.

** Primer design and Polymerase Chain Reaction (PCR)**

A pair of gene-specific primers was used for the PCR amplification of the MHC II B gene: hMPN12 (5’- CTCTCTTCTTTTCTCTTACC-3’) and hMPC12 (5’- ACA CTCACTGTATTTAGCCA-3’). They were designed according to reported half-smooth tongue sole MHC II B cDNA sequences [40]. The primer pair was
used to amplify part of exon1, and all of intron1 and exon2 from half-smooth tongue sole using a Polymerase Chain Reaction technique. A 25 μl PCR reaction mixture contained 1 μl of template DNA, 2.5 μl of 10×Taq polymerase buffer (TransGen Biotech), 1.5 mM MgCl$_2$, 0.2 mM dNTP mix, 0.2 μM of the forward and reverse primers, and 1 unit of Taq polymerase (TransGen Biotech). The amplifications were performed on a Peltier Thermal Cycler (PTC-200). A Molecular Imager Gel Doc XR system (Bio-rad) was used to determine the PCR products by electrophoresis on a 1% agarose gel.

Cloning and sequencing
The PCR products were resolved by electrophoresis on 1.5% agarose gels. The fragments of interest were excised and purified with the QIAEX II gel extraction kit (Qiagen). The purified fragments were cloned into a PBS-T vector (Takara) according to the standard PBS-T vector protocol (Takara) and then transformed into TOP 10 Escherichia coli competent cells (TransGen Biotech). Forward and reverse M13 primers were used to screen for positive clones via PCR. Ten positive clones from the upper purified fragments were sequenced with an ABI 3730 automated sequencer using the M13+/- primer.

Genotyping, sequence analysis and statistical tests analysis
Sequence data were analyzed using DNASTAR 5.0 and DNAMAN software. The alignment was performed with MEGA4.0 [47]. The rate of synonymous substitution (dS) and non-synonymous substitution (dN) was calculated according to an earlier report [47] using MEGA4.0 software. DAMBE and DnaSP5.0 software packages were used to analyze the polymorphisms [48]. Statistical analysis was carried out with SPSS13.0. Differences in the allelic frequency were verified using Fisher’s exact test and the significance level [49] was determined for every individual (n = 160) and each family (n = 8).

The new alleles were designated Cyse-DBB*0101 to Cyse-DBB*6601 on the basis of the rules reported by Davies et al. [50]. Cyse refers to Cynoglossus semilaevis, D to class II, the first B to an uncharacterized family and the second B to β chain-encoding genes. In the first four digits after the asterisk, the first two digits refer to the major type (alleles that differ by at least five amino acid substitutions), while the last two digits refer to the subtype (alleles that differ by less than five amino acid substitutions within a single major type) [51,52].

Results
To analyze disease resistance among 12 half-smooth tongue sole families
The first specific mortality appeared after 16 h due to an ip injection of *V. anguillarum*, and the challenge test lasted two weeks, at which time the overall accumulated mortality reached 42.24%. The survival rate among the 12 test families ranged from 15% to 79.25%, which was determined on the basis of each family. Here, we selected four high-resistance and four low-resistance families to ascertain whether MHC IIB exon2 alleles were associated with resistance to *V. anguillarum* among the 12 families of half-smooth tongue sole. The mean prevalence of survival of the four high-resistance families was 59.45%, while that of the four low-resistance families was considerably less at 26.73%.

To elucidate sequence polymorphism within exon2 of MHC IIB gene in 8 half-smooth tongue sole families
Eighty individuals from the four high-resistance families and eighty individuals from the four low-resistance families were used in the present study (Additional file 1). Nine to twelve positive clones per individual were sequenced and 1618 sequences were obtained. A fragment of 397 bp was obtained in reference to the complete half-smooth tongue sole MHC IIB cDNA sequence [40] and intron-exon boundary GT-AG rule. This fragment of 397 bp contains a part of exon1 (35 bp), the entire intron1 (84 bp, containing a 12 bp CA repeat sequence) and the entire exon2 of MHC IIB. A fragment of 270 bp containing the complete exon2 which encodes the β1 domain of the MHC IIB gene was also analyzed. The results indicated 88 different sequences, in which 88 novel alleles were designated (Table 1) belonging to 57 major allele types, following established allele nomenclature method [49,50].

Gaps were not found in the full alignment of the 270 bp exon2 of the MHC IIB gene. A putative 90 amino acid peptide was based on a sequence alignment with the half-smooth tongue sole MHC II B cDNA sequence [40]. Among the 270 nucleotides, 72 regions and 121 (44.8%) nucleotide positions were variable. The numbers of two-nucleotide mutation, three-nucleotide mutation and four-nucleotide mutation were 24, 11 and 1, respectively (Table 2). At the SNP sites, there were two kinds of nucleotide substitutions, i.e. transition (Table 2, Serial No. 1, 7, 11, 13, 18, 23, 28, 29, 32, 33, 35, 42, 43, 44, 46, 49, 52, 53, 54, 60 and 69) and transversion (Table 2, Serial No. 20, 21, 25, 59). Three kinds of mutation per site (Table 2, Serial No. 2, 4, 6, 9, 14, 15, 16, 22, 26, 30, 31, 36, 37, 41, 51, 56, 58, 61, 62, 63, 65, 67, 68 and 71) which revealed the mutation hotspots. 36 out of 72 mutation regions were multi-nucleotide co-mutations, ranging from two to five nucleotides per region. The SNP sites were located in a tight region from position 9 to 29 (Table 2), so this were most of the mutation hotspots of MHC exon2 herein must be located. The frequency ratio ranged from 0.989:0.011 (Table 2, Serial No.1, 23, 32, 49, 59 and 60) to
null
Table 2 Distribution of SNP sites within exon2 of MHC IIB allelic sequences of half-smooth tongue sole

| Serial number | Position (n = 88) | Base type | Allele no. | Frequency | Position (n = 88) | Base type | Allele no. | Frequency |
|---------------|------------------|-----------|------------|-----------|------------------|-----------|------------|-----------|
| 1             | 6                | T         | 87         | 0.989     | 39               | ATC       | 51         | 0.580     |
|               |                  | C         | 1          | 0.011     |                  | ATT       | 1          | 0.011     |
| 2             | 9-11             | CTA       | 52         | 0.591     |                  | ATG       | 1          | 0.011     |
|               |                  | GTA       | 1          | 0.011     |                  | CAG       | 35         | 0.398     |
|               |                  | GAG       | 35         | 0.398     | 40               | TCG       | 84         | 0.955     |
| 3             | 12               | C         | 17         | 0.193     |                  | TCA       | 2          | 0.023     |
|               |                  | T         | 34         | 0.386     |                  | CCG       | 1          | 0.011     |
|               |                  | A         | 7          | 0.080     |                  | TTG       | 1          | 0.011     |
|               |                  | G         | 30         | 0.341     | 41               | GGA       | 49         | 0.557     |
| 4             | 13               | A         | 82         | 0.932     |                  | AGA       | 12         | 0.136     |
|               |                  | T         | 5          | 0.057     |                  | GAG       | 27         | 0.307     |
|               |                  | G         | 1          | 0.011     | 42               | A         | 56         | 0.636     |
| 5             | 14-15            | AT        | 30         | 0.341     |                  | T         | 32         | 0.364     |
|               |                  | AC        | 1          | 0.011     | 43               | C         | 2          | 0.023     |
|               |                  | TT        | 55         | 0.625     |                  | T         | 86         | 0.977     |
|               |                  | CT        | 2          | 0.023     | 44               | AT         | 49         | 0.557     |
| 6             | 16               | C         | 32         | 0.364     |                  | TA         | 8          | 0.091     |
|               |                  | T         | 20         | 0.227     |                  | TT         | 31         | 0.352     |
|               |                  | A         | 36         | 0.409     | 45               | CC         | 1          | 0.011     |
| 7             | 18               | G         | 39         | 0.443     |                  | TA         | 24         | 0.273     |
|               |                  | A         | 49         | 0.557     |                  | TC         | 63         | 0.716     |
| 8             | 19               | T         | 33         | 0.375     | 46               | C          | 87         | 0.989     |
|               |                  | G         | 20         | 0.227     |                  | T          | 1          | 0.011     |
|               |                  | C         | 30         | 0.341     | 47               | AG         | 71         | 0.807     |
|               |                  | A         | 5          | 0.057     |                  | GC         | 17         | 0.193     |
| 9             | 20               | G         | 46         | 0.523     | 48               | ATG        | 75         | 0.852     |
|               |                  | A         | 34         | 0.386     |                  | ATT        | 9          | 0.102     |
|               |                  | C         | 8          | 0.091     |                  | TTG        | 3          | 0.034     |
| 10            | 21-23            | ACA       | 53         | 0.602     |                  | ACT        | 1          | 0.011     |
|               |                  | GTG       | 35         | 0.398     | 49               | G          | 1          | 0.011     |
| 11            | 24               | G         | 74         | 0.841     |                  | A          | 87         | 0.989     |
|               |                  | A         | 14         | 0.159     | 50               | GA         | 86         | 0.977     |
| 12            | 25               | A         | 35         | 0.398     |                  | AA         | 1          | 0.011     |
|               |                  | G         | 31         | 0.352     |                  | GG         | 1          | 0.011     |
|               |                  | T         | 19         | 0.216     | 51               | GTG        | 80         | 0.909     |
|               |                  | C         | 3          | 0.034     |                  | ATC        | 7          | 0.080     |
| 13            | 26               | T         | 5          | 0.057     |                  | GAA        | 1          | 0.011     |
|               |                  | C         | 83         | 0.943     | 52               | C          | 24         | 0.273     |
| 14            | 28-29            | CC        | 52         | 0.591     |                  | T          | 64         | 0.727     |
|               |                  | CA        | 1          | 0.011     | 53               | A          | 62         | 0.705     |
|               |                  | GA        | 35         | 0.398     |                  | G          | 26         | 0.295     |
| 15            | 32-33            | CG        | 35         | 0.398     | 54               | A          | 69         | 0.784     |
|               |                  | TG        | 1          | 0.011     |                  | G          | 19         | 0.216     |
|               |                  | TC        | 52         | 0.591     | 55               | GG         | 40         | 0.455     |
| 16            | 38-39            | CA        | 52         | 0.591     |                  | GT         | 18         | 0.205     |
|               |                  | CG        | 1          | 0.011     |                  | TG         | 8          | 0.091     |
|               |                  | TA        | 35         | 0.398     |                  | CG         | 22         | 0.25      |
| 17            | 40-41            | AC        | 51         | 0.580     | 56               | A          | 67         | 0.761     |
|               |                  | GC        | 1          | 0.011     |                  | C          | 20         | 0.227     |
|               |                  | AT        | 35         | 0.398     |                  | G          | 1          | 0.011     |
|               |                  | CT        | 1          | 0.011     | 57               | GA         | 62         | 0.705     |
| 18            | 44               | C         | 36         | 0.409     |                  | AC         | 3          | 0.034     |

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|---|---|---|---|---|---|
| 19 | 47-49 | AAA | 52 | 0.591 | GG | 8 | 0.091 |
|   |   | AAG | 1 | 0.011 | 58 | 210-211 | AA | 86 | 0.977 |
|   |   | TAA | 19 | 0.216 |   |   | GA | 1 | 0.011 |
|   |   | TGA | 16 | 0.182 |   |   | AT | 1 | 0.011 |
| 20 | 51 | G | 53 | 0.602 | 59 | 218 | G | 87 | 0.989 |
|   |   | C | 35 | 0.398 |   |   | T | 1 | 0.011 |
| 21 | 53 | G | 36 | 0.409 | 60 | 220 | A | 87 | 0.989 |
|   |   | C | 52 | 0.591 |   |   | G | 1 | 0.011 |
| 22 | 55-56 | AC | 69 | 0.784 | 61 | 226-227 | TG | 47 | 0.534 |
|   |   | AT | 18 | 0.205 |   |   | TA | 40 | 0.455 |
|   |   | GC | 1 | 0.011 |   |   | CG | 1 | 0.011 |
| 23 | 58 | A | 87 | 0.989 | 62 | 228 | A | 50 | 0.568 |
|   |   | G | 1 | 0.011 |   |   | C | 29 | 0.330 |
| 24 | 63-64 | GC | 51 | 0.580 |   |   | T | 9 | 0.102 |
|   |   | GT | 1 | 0.011 | 63 | 229 | A | 82 | 0.932 |
|   |   | GA | 1 | 0.011 |   | T | 2 | 0.023 |
|   |   | CA | 35 | 0.398 |   |   | G | 4 | 0.045 |
| 25 | 67 | A | 68 | 0.773 | 64 | 231-234 | AAC | 61 | 0.693 |
|   |   | T | 20 | 0.227 |   |   | ACT | 2 | 0.022 |
| 26 | 72 | C | 13 | 0.148 |   |   | CAC | 20 | 0.227 |
|   |   | G | 40 | 0.455 |   |   | AGC | 5 | 0.057 |
|   |   | T | 35 | 0.398 | 65 | 237-238 | GG | 79 | 0.898 |
| 27 | 74 | C | 40 | 0.455 |   |   | GA | 5 | 0.057 |
|   |   | G | 48 | 0.545 |   |   | AG | 4 | 0.045 |
| 28 | 78 | C | 38 | 0.432 | 66 | 240-241 | AA | 10 | 0.114 |
|   |   | T | 50 | 0.568 |   |   | AT | 54 | 0.614 |
| 29 | 80 | C | 35 | 0.398 |   |   | CT | 23 | 0.261 |
|   |   | T | 53 | 0.602 |   |   | GT | 1 | 0.011 |
| 30 | 82-83 | AC | 52 | 0.591 | 67 | 242-243 | TG | 46 | 0.523 |
|   |   | AT | 35 | 0.398 |   |   | TT | 19 | 0.216 |
|   |   | GT | 1 | 0.011 |   |   | GG | 23 | 0.261 |
| 31 | 84-85 | TT | 30 | 0.341 | 68 | 245-246 | CT | 68 | 0.773 |
|   |   | CT | 23 | 0.261 |   |   | CA | 19 | 0.216 |
|   |   | TA | 35 | 0.398 |   |   | AT | 1 | 0.011 |
| 32 | 87 | A | 87 | 0.989 | 69 | 248 | T | 2 | 0.023 |
|   |   | G | 1 | 0.011 |   |   | C | 86 | 0.977 |
| 33 | 90 | A | 86 | 0.977 | 70 | 250-253 | ACCA | 10 | 0.114 |
|   |   | G | 2 | 0.023 |   |   | ACCG | 64 | 0.727 |
| 34 | 92-94 | ACT | 52 | 0.591 |   |   | AGCC | 1 | 0.011 |
|   |   | GAG | 36 | 0.409 |   |   | GGAC | 12 | 0.136 |
| 35 | 96 | G | 35 | 0.398 |   |   | AGCG | 1 | 0.011 |
|   |   | A | 53 | 0.602 | 71 | 254-256 | TGC | 70 | 0.796 |
| 36 | 98-99 | GA | 42 | 0.477 |   |   | TGG | 12 | 0.136 |
|   |   | GT | 11 | 0.125 |   |   | GTT | 6 | 0.068 |
|   |   | AT | 35 | 0.398 | 72 | 256-258 | TT | 12 | 0.136 |
| 37 | 100 | C | 1 | 0.011 |   |   | TC | 63 | 0.716 |
|   |   | A | 38 | 0.432 |   |   | CC | 2 | 0.023 |
|   |   | T | 49 | 0.557 |   |   | TG | 11 | 0.125 |
| 38 | 101-102 | CA | 34 | 0.386 |   |   | |   |
|   |   | CG | 16 | 0.182 |   |   | |   |
|   |   | GA | 3 | 0.034 |   |   | |   |
|   |   | TG | 31 | 0.352 |   |   | |   |
|   |   | TA | 4 | 0.046 |   |   | |   |
individuals. The distribution of the alleles was unequal. Certain alleles had a low frequency and were excluded from allele distribution analysis between the HR and LR families. Thirteen alleles were used for distribution analysis (Figure 2). The alleles Cyse-DBB*3301, Cyse-DBB*4701, Cyse-DBB*6801 and Cyse-DBB*5901 were more prevalent in individuals from the HR families \((P = 0.005, 0.018, 0.018 \text{ and } 0.064, \text{respectively } n = 160 \text{ individuals})\), while Cyse-DBB*6501, Cyse-DBB*4002, Cyse-DBB*6102, Cyse-DBB*5601 and Cyse-DBB*2801 were more prevalent in individuals from low-resistance families, as shown by chi-square test \((P < 0.01, 0.01, 0.01, 0.01, 0.01 \text{ respectively } n = 160 \text{ individuals})\). Some alleles were not significantly different in the HR and LR families, such as Cyse-DBB*0101 \((P = 0.247)\), Cyse-DBB*4602 \((P = 0.117)\) and Cyse-DBB*5003 alleles \((P = 0.159)\). Here we (deduced) show that Cyse-DBB*3301, Cyse-DBB*4701, Cyse-DBB*6801 and Cyse-DBB*5901 were associated with resistance, while Cyse-DBB*6501, Cyse-DBB*4002, Cyse-DBB*6102, Cyse-DBB*5601 and Cyse-DBB*2801 were associated with susceptibility to \(V.\ anguillarum\) in half-smooth tongue sole. Alignment of the 13 deduced MHC IIB amino acid sequences (Figure 3) indicated that no specific single amino acid substitution was evidently involved in the resistance or susceptibility, as there was no specific amino acid substitution difference between the HR families and LR families.

### 4. Discussion

It is well known that MHC genes are vital components of both the innate and adaptive immune system. They present foreign peptides to T cells. Cloning and cDNA polymorphism of the MHC II B gene has been discussed [40]. In the present study, partial sequences of the MHC class IIB gene in different families of half-smooth tongue sole were isolated, then molecular polymorphisms as well as the link between alleles and resistance/susceptibility to \(V.\ anguillarum\) were analyzed.

Among the 72 mutated regions in the complete sequence of MHC IIB exon2, 36 regions were multinucleotide co-mutations, which indicate inter-allelic recombination took place in these regions. Moreover, no deletion, insertion or stop codon was observed, indicating that all of these alleles were functional genes. The frequency ratio of substituted nucleotides per mutation region was not equally distributed, which suggests that different regions might have different impact.

The rate of non-synonymous substitutions to synonymous substitutions \((d_{SN}/d_S)\) in the PBR and non-PBR of

| Region     | No. of codons | \(d_{SN}(SE)\) | \(d_S(SE)\) | \(d_{SN}/d_S\) |
|------------|---------------|----------------|-------------|----------------|
| PBR        | 23            | 0.261 ± 0.033  | 0.153 ± 0.052 | 1.70           |
| Non-PBR    | 67            | 0.087 ± 0.016  | 0.159 ± 0.034 | 0.547          |
| Total      | 90            | 0.132 ± 0.017  | 0.157 ± 0.027 | 0.841          |

Table 3 Synonymous \((d_S)\) and nonsynonymous \((d_{SN})\) substitution rate in the putative peptides binding region (PBR) and non-peptides binding region (non-PBR) among half-smooth tongue sole alleles

![Figure 1](http://www.biomedcentral.com/1471-2156/12/78)
MHC IIB exon2 of half-smooth tongue sole was studied (Table 3). The \( \frac{d_N}{d_S} \) ratio was higher in the PBR than non-PBR, which corresponds with the results reported in other species [43,54-56]. The \( \frac{d_N}{d_S} \) ratio in exon2 was higher than 1. The location of the PBR sites in the MHC genes of fish was not yet defined, therefore PBR sites were identified using the model of Brown et al. [53] to define HLA-DRB, It was also in accordance with a previous application by Xu et al. [38] for half-smooth tongue sole. The 23 positions were used as PBR sites for in-depth study: 3, 5, 7, 25, 27, 34, 35, 44, 53, 57, 58, 62, 65, 67, 71, 74, 77, 78, 82, 83, 85 and 86 (Figure 3).

It is possible that the PBR sites in fish do not exactly correspond to those in humans [57]. In mammals, MHC polymorphisms are maintained over long periods of time by balanced selection or positive selection at the non-synonymous sites specifying the PBR of the MHC [7]. The ratio between non-synonymous and synonymous substitutions in PBR sites of MHC IIB exon2 genes is greater than 1 (Table 3), as would be expected if the locus were evolving under a condition of balanced selection [58]. The number of alleles per individual ranged from 1 to 5, which showed that at least three loci existed per individual, a result is in accordance with previous studies [22,28,40]. Polymorphism of the 88 alleles in the 160 individuals was higher in half-smooth tongue sole than in Atlantic salmon [57,59] and cyprinid fish [54], and each family had 25-38 alleles. A few hypotheses have been put forward to interpret the abundant polymorphism of the MHC genes, including overdominant selection or heterozygous advantage [60], negative frequency-dependent selection [61,62] and balanced selection [24]. Pathogen-driven selection [26,60] is reported to be contributing to MHC gene diversity through both frequency-dependent selection and heterozygote advantage (over-dominance) [15]. In the present study, the high rate of \( \frac{d_N}{d_S} \) score and high levels of polymorphism which occurred in half-smooth tongue sole revealed that balanced selection is responsible for presence in the PBR domain of the MHC class IIB exon2 gene. This results in the high polymorphism levels in MHC IIB genes in half-smooth tongue sole.

**Figure 2** Sequence polymorphism analysis within exon 2 of MHC IIB gene. (Asterisks indicate the correlative amino acid that combines the antigen).

**Figure 3** Distribution of MHC class IIB alleles in high-resistance families individuals (white bars) and low-resistance families individuals (black bars) of half-smooth tongue sole. *Asterisks denote P < 0.05. ** denote P < 0.01.
Due to the polymorphic nature of MHC genes, certain alleles/haplotypes may be associated with increased disease resistance. In the present study, the distinct distribution pattern of the alleles exhibited a relationship between MHC class IIB alleles and resistance/susceptibility to *V. anguillarum* in half-smooth tongue sole.

The *Cyse-DBB*\(^3\)*301, *Cyse-DBB*\(^4\)*4701 and *Cyse-DBB*\(^6\)*6801 alleles which was found in three families, and the *Cyse-DBB*\(^5\)*5901 allele in two families, were markedly more frequent in HR families (13.75%, 11.25%, 11.25%, 8.75% respectively) than in LR families (1.25%, 1.25%, 1.25%, respectively). This suggests an association of the *V. anguillarum* disease resistance alleles in half-smooth tongue sole. The *Cyse-DBB*\(^6\)*501, *Cyse-DBB*\(^4\)*002 and *Cyse-DBB*\(^5\)*601 alleles were found in two LR families (35%, 33.75% and 16.25% respectively) and one HR family (1.25%, 1.25% and 1.25%, respectively), while the *Cyse-DBB*\(^6\)*102 allele was found in three LR families (27.5%) and one HR family (1.25%), *Cyse-DBB*\(^2\)*801 was found in two LR families (15%) and two HR families (2.5%), which might be associated with susceptibility to *V. anguillarum* in half-smooth tongue sole. In the present study, statistical analysis was used to reveal the associations between the alleles and resistance or susceptibility to *V. anguillarum* in half-smooth tongue sole. The observed link between alleles *Cyse-DBB*\(^3\)*301, *Cyse-DBB*\(^4\)*4701, *Cyse-DBB*\(^6\)*6801, *Cyse-DBB*\(^5\)*5901, *Cyse-DBB*\(^6\)*501, *Cyse-DBB*\(^4\)*002, *Cyse-DBB*\(^6\)*102, *Cyse-DBB*\(^5\)*601 and *Cyse-DBB*\(^2\)*801 and resistance/susceptibility to *V. anguillarum* supported the hypothesis that frequency-dependent selection is crucial for the maintenance of MHC variation [63]. This experimental result was in accord with reports in Atlantic salmon [64] and flounder [38]. However, it was not possible to identify a single allele which appeared in all HR families or all LR families. This might indicate the importance of multiple polymorphisms. One MHC haplotype has been reported to be significantly associated with resistance to Marek’s disease in chickens [65], and MHC polymorphism was significantly associated with both juvenile survival and resistance to nematode parasites was also reported in Soay sheep [31].

A link between MHC polymorphism and resistance/susceptibility to disease in fish has been reported. Kjøglum *et al.* [5] demonstrated that fish with the genotypes *UBA*\(^0\)*0201/*UBA*\(^0\)*030 and *DAA*\(^0\)*0201/*0201 were the most resistant to infectious anaemia in Atlantic salmon, while fish with the genotypes *UBA*\(^0\)*0601/*080, *DAA*\(^0\)*0501/*0501 and *UBA*\(^0\)*0201/*030, *DAA*\(^0\)*0301/*0501 were the most susceptible, based on an analysis of the combined MHC class I and class II A genotypes. It is reported [15] that the allele combinations *DAA*\(^0\)*0201-*0201 and *DAA*\(^0\)*0301-*0301 displayed a significantly lower prevalence of death in homozygous fish than in Atlantic salmon containing one copy or no copy of the allele in *Aeromonas salmonicida*-challenged Atlantic salmon.

The *Sasa-DAA-3*\(^U\)TR 239 allele [36] was shown to be significantly associated with a decrease in the severity of amoebic gill disease in Atlantic salmon. It was also reported [66] that *Sasa-B-04*, at the non-classical class I locus, was highly associated with resistance to infectious hematopoietic necrosis in Atlantic salmon. The alleles *Paol-DAB*\(^4\)*301 and *Paol-DAB*\(^1\)*601 were shown to be associated with resistance and susceptibility to *V. anguillarum* in flounder [38].

In this study in half-smooth tongue sole, the alleles *Cyse-DBB*\(^3\)*301, *Cyse-DBB*\(^4\)*4701, *Cyse-DBB*\(^6\)*6801 and *Cyse-DBB*\(^5\)*5901 were found to be associated with resistance while the *Cyse-DBB*\(^6\)*501, *Cyse-DBB*\(^4\)*002, *Cyse-DBB*\(^6\)*102, *Cyse-DBB*\(^5\)*601 and *Cyse-DBB*\(^2\)*801 alleles were associated with susceptibility to *V. anguillarum*. Associations of MHC with resistance or susceptibility to specific pathogens can also be derived through linkage disequilibrium with a resistance or susceptibility locus or gene, and may not be due to the MHC gene itself [55,67-69].

**Conclusions**

It can not ruled out that another linked gene, individual genetic background and different strains or populations may to some extent have caused the observed link, but here the *Cyse-DBB*\(^3\)*301, *Cyse-DBB*\(^4\)*4701, *Cyse-DBB*\(^6\)*6801 and *Cyse-DBB*\(^5\)*5901 alleles were associated with resistance to *V. anguillarum*, while the *Cyse-DBB*\(^6\)*501, *Cyse-DBB*\(^4\)*002, *Cyse-DBB*\(^6\)*102, *Cyse-DBB*\(^5\)*601 and *Cyse-DBB*\(^2\)*801 alleles were associated with susceptibility to *V. anguillarum* in half-smooth tongue sole. Further studies are needed to confirm the association between MHC class IIB exon2 gene with resistance to *V. anguillarum* in half-smooth tongue sole.

**Additional material**

**Additional file 1:** Results of the infection with bacterial. Results of the infection with bacterial is presented. Numbers of high-resistance (HR, SR > 59.45%) and low-resistance (LR, SR < 26.73%) families of *Vibrio anguillarum* were associated with resistance to *V. anguillarum*.

**Additional file 2:** The individual ID and corresponding number of allele. We presented the number of alleles per individual of half-smooth tongue sole and its corresponding individual number.

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