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MHC polymorphism and disease-resistance to *Edwardsiella tarda* in six turbot (*Scophthalmus maximus*) families

DU Min[1,2,3], CHEN SongLin[1*], LIU YanHong[2], NIU BaoZhen[2], YANG JingFeng[1] & ZHANG Bo[1]

[1] *Key Laboratory for Sustainable Utilization of Marine Fisheries Resources, Ministry of Agriculture, Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao 266071, China;*
[2] *College of Life Sicence and Technology, Honghe University, Mengzi 661100, China;*
[3] *College of Aqua-Life Science and Technology, Shanghai Ocean University, Shanghai 200090, China*

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This study examined genetic variation in the major histocompatibility complex (MHC) Class II B gene in turbot (*Scophthalmus maximus*) by virulent bacterial pathogen challenge. One hundred fry from each of six families were infected with *Edwardsiella tarda* by intraperitoneal injection. Family mortality ranged from 28.0% to 83.3%. Complete exon 2 and intron 1 sequences of MHC Class II B genes were amplified from five survivor and five non-survivor individuals per family using the clone-sequence method. Thirty-seven sequences from 60 individuals revealed 37 different alleles, 25 of which were unique to this study. The 25 unique alleles belonged to 16 major allele types. Nine alleles were used to examine the association between alleles and resistance/susceptibility to disease. Five alleles were present in an individual, suggesting a minimum of three loci or copies of the turbot MHC Class II B gene. The rate of non-synonymous substitution (d_N) was 2.30 and 1.58 times higher than synonymous substitution (d_S) in the peptide-binding regions (PBR) and non-PBR in whole families, respectively, which suggested balancing selection on exon 2 of the MHC Class II B gene in turbot. One allele, *Scma-DBB1*02*, was significantly more prevalent in survivor stock than in non-survivor stock (P=0.001). Therefore, this allele might be associated with resistance to bacteria. A second allele, *Scma-DBB1*10*, was significantly more prevalent in non-survivor stock (P=0.021), and is likely associated with susceptibility to bacteria.

turbot (*Scophthalmus maximus*), *Edwardsiella tarda*, major histocompatibility complex II B, polymorphism, resistance, susceptibility

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The major histocompatibility complex (MHC) is a multigene family that plays a central role in the adaptive immune system. The MHC family includes two major subfamilies, termed Class I and Class II genes. Classical Class I and Class II MHC genes are heterodimeric, highly polymorphic, and encode cell-surface proteins that present antigenic self and non-self peptides to the T-cell receptor (TCR), initiating a cascade of complex immune responses [1,2]. Classical MHC Class I molecules contain an α and a β_2-microglobulin chain and are expressed on the surface of all nucleated somatic cells, while classical MHC Class II molecules contain one α and one β chain, and are constitutively expressed on antigen-presenting cells of the immune system [3].

Following the identification of carp (*Cyprinus carpio* L.) [4] MHC gene fragments two decades ago, MHC Class I, Class II A and Class II B genes have been isolated and characterized in a large variety of fish species, including rainbow trout [5], Atlantic salmon [6], zebrafish [7], cichlid fish [8,9], carp [4,10], nurse-shark [11], cartilaginous fish [12], turbot [13], Japanese flounder [14,15], red sea bream

*Corresponding author (email: chensl@ysfri.ac.cn)

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[16], and half-smooth tongue sole [17]. Surprisingly, MHC classes I and II genes are found on the same linkage groups in all studied mammals, while in teleosts the MHC Class II genes were not linked with MHC Class I genes [18–24].

The presence of multiple loci and a considerable number of alleles at each given locus within populations has meant that classical MHC genes are the most polymorphic genes studied to date [25]. Extensive variability of MHC Class II B genes was discovered in the β1 domain (exon 2), which encodes the functional peptide binding region (PBR) [17,26]. Allelic diversity is critical for resistance to a variety of parasites [27]. Several selective mechanisms have been proposed to elucidate the high level of polymorphism of the MHC genes, including parasite resistance, maternal-fetal interaction, negative-assortative mating, overdominant selection, heterozygous advantage, and frequency-dependent balancing selection [28,29]. However, the significance of the generation of new variants is still unknown.

Turbot (*Scophthalmus maximus*) is one of the most widely cultured marine fish species [13,30], and *Edwardsiella tarda* is one of the most significant pathogens affecting turbot farming [31]. The use of antibiotics in farmed fish is strictly controlled, but antibiotic residues may be harmful to human health and also have adverse effects on the environment. Selective breeding, especially gene-assisted selection of turbot for disease-resistant lines/families, is a promising solution [30,32]. Family members with a known genetic background were used for selective breeding because of their clear background information. In recent years, much research has gone into the molecular cloning, expression, and polymorphism analysis of turbot MHC II A and B genes [13]. Xu et al. [30] identified two alleles associated with susceptibility to *E. tarda,* with another allele associated with resistance in turbot populations.

This study aimed to estimate the number of MHC Class II loci in turbot, ascertain the extent of MHC molecular polymorphism, test for balancing selection in PBR, and identify any MHC alleles associated with variation in resistance or susceptibility to *E. tarda* in turbot families.

1 Materials and methods

1.1 Fish families and rearing

The six full-sibling families of turbot assessed in this study were established from six sires and six dams, as described previously [32,33]. These parents were reared and mated at the Aqua-Breeding Station (LaiZhou MinBo aquatic CO., Ltd.) in Laizhou, located in the north of Shandong Province, China. The parentage of the sires and dams was unknown, although it was assumed that they were unrelated. Following mating, fertilized eggs from each family were incubated, hatched in a separate tank from August 1–8 of 2009, and reared at the same breeding station in Laizhou. Fry were fed *Brachionus plicatilis*, brine shrimp, and a commercial compound feed using a routine feeding formula.

1.2 Challenge experiments

Approximately 600 fish were used in this study. Random samples of 100 fish from each family were divided into two replicate batches of approximately 50 individuals. Fry were allocated a separate tank with fresh seawater supply at (21±0.5)°C under flow-through status. Prior to challenge, fish were fed a routine daily diet, while post challenge they were fed according to appetite. A pre-challenge experiment to estimate the median lethal bacterial concentration was performed using similarly sized fish (mean weight 18.2 g, mean body length 10.2 cm). For the challenge test in turbot, 50 fish from each family were inoculated by intraperitoneal (i.p.) injection of 0.2 mL *E. tarda* bacterial suspension (~1.4×10^7 CFU/individual) according to the method of Zhang et al. [13]. As a control, 14 fish were injected with 0.2 mL 0.9% saline solution [15,25]. One individual per control group died during the test period. The challenge trial began on November 6, 2009 and lasted for 32 d. Dead fish were collected from each tank daily and the number was recorded during the challenge period. Surviving fish were counted at the termination of the challenge trial and fin tissues from all trial fish were preserved individually in 100% ethanol.

1.3 Sampling and DNA isolation

Groups of dead and live fish from each family were selected for further analysis following the bacterial challenge (Table 1). Genomic DNA was extracted from the fin samples of both the first five fish to die and the last five survivors of each family, using a modified phenol-chloroform method, as described previously [15,34].

1.4 Primer design and polymerase chain reaction (PCR) amplification

A pair of gene specific primers, TPMHCIIBN1 (5′-CGCCCTCCTCTTCATCACTGT-3′) and TPMHCIIBC1 (5′-TCA-

Table 1 Numbers of dead and surviving individuals selected from six families of turbot

Family	Individuals per family		Total
	Dead	Surviving	
Family 1	5	5	10
Family 2	5	5	10
Family 3	5	5	10
Family 5	5	5	10
Family 6	5	5	10
Family 7	5	5	10
Total	30	30	60

GAGCAGCTTCATGGTCATTTC-3′), was designed based on known turbot MHC II B cDNA sequences [13]. The primers were used to amplify a fragment of the turbot MHC II B gene containing part of exon 1 and all of intron 1 and exon 2. Reactions contained approximately 100 ng of template DNA, 1× *Taq* polymerase buffer (TransGen Biotech, Beijing, China), 1.5 mmol L^{-1} $MgCl_2$, 0.2 mmol L^{-1} deoxynucleotide triphosphate, 0.2 μmol L^{-1} of the forward and reverse primers, and 1 U of *Taq* polymerase (TransGen Biotech). Cycling conditions were as follows: 5 min at 94°C, followed by 30 cycles of 94°C for 40 s, 53°C for 40 s, and 72°C for 50 s, and a final extension of 10 min at 72°C. PCR reactions were performed on a Peltier Thermal Cycler (PTC-200). PCR products were visualized by electrophoresis on a 1% agarose gel and imaging on a Molecular Imager Gel Doc XR system (BioRad, Hercules, CA, USA).

1.5 Cloning and sequencing

PCR products were electrophoresed on 1.5% agarose gels and the expected amplified fragments were excised before being purified with a QIAEX II gel extraction kit (Qiagen, Hilden, Germany). PCR amplicons were cloned into the PBS-T vector (Takara Bio, Otsu, Japan) and propagated in TOP 10 *Escherichia coli* competent cells (TransGen Biotech) following manufacturer's instructions. Vector specific sequencing primers M13 (5′-CGCCAGGGTTTTCCCAGTCACGAC-3′) and RV-M (5′-AGCGGATAACAATTTCACACAGG-3′), were used to confirm positive clones by PCR. Five positive clones from each individual were sequenced using an ABI 3730 automated sequencer (Applied Biosystems, Foster City, CA, USA).

1.6 Amino acid sequence analysis and statistical tests

All sequences were aligned using ClustalW [35]. The rate of non-synonymous substitution (d_N) and synonymous substitution (d_S) in the putative PBR and non-PBR was determined using MEGA version 4.0 [36], employing the Nei and Gojobori method with *p*-distance [37]. Statistical analyses were carried out using SPSS 13.0 (IBM, Armonk, NY, USA) and polymorphic value analysis was performed using DnaSP 4.0 [38].

Novel alleles, as determined by the deduced amino acid sequences, were designated *Scma-DAB*0101-Scma-DAB*1601* according to the rules proposed by Davies et al. [39]. The abbreviation Scma refers to *Scophthalmus maximus*, D to Class II, A to uncharacterized family designation, and B to the β chain-encoding genes. In the four digits following an asterisk, the first two digits refer to the major type (alleles that differ by at least five amino acid substitutions), and the next two digits to the subtype (alleles that differ by less than five amino acid substitutions within a single major type) [15,40].

2 Results

2.1 Disease resistance/susceptibility comparison of six turbot families

All individuals from six full-sibling families were i.p. injected with a bacterial suspension of approximately 2.8×10^6 CFU of *E. tarda*. The average mortality rate was 72.5%, with the within-family mortality rate ranging from 66.1%–83.3%. The mortality rate for individual families was 66.2%, 83.3%, 76.5%, 66.1%, 71.6%, and 71.1% for families 1, 2, 3, 5, 6, and 7, respectively. Resistance to disease was assessed as a binary trait (died or survived until termination of the challenge trials) [24].

2.2 Sequence polymorphism analysis of exon 2 of the MHC II B gene

Three hundred and two sequences from 30 resistant turbot (survivor individuals) and 30 susceptible fish (dead individuals) were analyzed. The length of the amplified fragment was either 364, 383 or 402 bp. Based on sequence alignment with the turbot MHC II B cDNA complete sequence [13], and the intron-exon boundary GT-AG paradigm, the expected fragment contained part of exon 1 (32 bp) and the complete intron 1 region (68, 87 or 106 bp, containing zero, one or two copies of a 19 bp repeat loci, gatcaatacactgagcact). The complete exon 2 region was contained on a 294 bp fragment, which was used for further analysis. Analysis of the exon 2 fragment revealed 37 different amino acid sequences, which represented 37 alleles. Of these 37 alleles, 25 were novel and were deposited in GenBank (Table 2) (GenBank accession No. HQ698599–HQ698623).

No insertion/deletion, stop, or frame-shift mutations were found in the 294 bp sequences. Among the 294 nucleotides, 132 (44.9%) sites were polymorphic, and 54 (18.4%) parsimony informative sites were examined. Alignments showed a high degree of similarity between the 37 sequences (93.9%), with a maximum of five different amino acids at each variable codon. Sixty-three (64.3%) out of the 98 amino acids were variable. The average number of nucleotide differences (*k*) was 24.7, and the nucleotide diversity (Pi) and the theta value per site (Theta-W) were 0.084 and 0.107, respectively. Figure 1 shows the spatial distribution of nucleotide variability. The lowest Pi value is presented in the middle, while two peaks were found in the upstream and downstream regions of exon 2.

The putative PBR and non-PBR amino acids in turbot were identified based on the human Class II molecule described by Brown et al. [41]. The values of nonsynonymous (d_N) and synonymous (d_S) substitution rates in the PBR were higher than those in the non-PBR (Table 2). These values were assessed using the 8, 9, 7, 9, 6, 10, and 38 sequences for families 1, 2, 3, 5, 6, 7 and whole families, respectively.

Table 2 Novel alleles and GenBank accession number

Allele	GenBank accession No.	Allele	GenBank accession No.	Allele	GenBank accession No.
*Scma-DAB*0101*	HQ698599	*Scma-DAB*0801*	HQ698606	*Scma-DAB*1203*	HQ698616
*Scma-DAB*0102*	HQ698614	*Scma-DAB*0901*	HQ698607	*Scma-DAB*1204*	HQ698623
*Scma-DAB*0103*	HQ698619	*Scma-DAB*1001*	HQ698608	*Scma-DAB*1205*	HQ698622
*Scma-DAB*0201*	HQ698600	*Scma-DAB*1101*	HQ698609	*Scma-DAB*1301*	HQ698612
*SCMA-DAB*0301*	HQ698601	*Scma-DAB*1102*	HQ698615	*Scma-DAB*1401*	HQ698613
*Scma-DAB*0401*	HQ698602	*Scma-DAB*1103*	HQ698618	*Scma-DAB*1402*	HQ698620
*Scma-DAB*0501*	HQ698603	*Scma-DAB*1201*	HQ698610	*Scma-DAB*1501*	HQ698617
*Scma-DAB*0601*	HQ698604	*Scma-DAB*1202*	HQ698611	*Scma-DAB*1601*	HQ698621
*Scma-DAB*0701*	HQ698605				

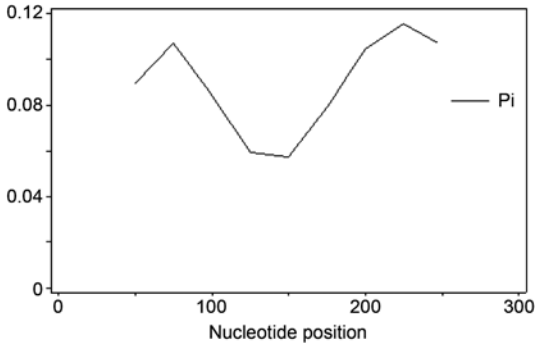

Figure 1 Nucleotide variability within exon 2 of the turbot MHC II B gene in the 37 alleles denoted by Pi (p). Sliding window length: 100; step size: 10.

The values of d_N in PBR were significantly higher than those of d_N in non-PBR, while values of d_S in PBR were marginally higher than those of d_S in non-PBR. Rates of d_N/d_S in PBR were higher than those of d_N/d_S in non-PBR in families 1, 2, 3, 5 and whole families, but the d_N/d_S rates in PBR were lower than those of d_N/d_S in non-PBR in families 6 and 7 (Table 3).

2.3 Association between MHC II B alleles and disease resistance/susceptibility to *E. tarda*

Table 4 shows the number of alleles in each fish and its corresponding individual number. All individuals in this

Table 3 Synonymous (d_S) and non-synonymous (d_N) substitution rates in the putative peptide binding region (PBR) and non-peptide binding region (non-PBR) in turbot families

Family	Region	No. of codons	d_N(SE)	d_S(SE)	d_N/d_S
Family 1	PBR	23	0.128±0.038	0.031±0.026	4.13
	Non-PBR	75	0.056±0.014	0.030±0.015	1.87
	Total	98	0.073±0.014	0.030±0.013	2.43
Family 2	PBR	23	0.110±0.037	0.022±0.016	5.00
	Non-PBR	75	0.051±0.016	0.022±0.013	2.32
	Total	98	0.065±0.014	0.022±0.010	2.96
Family 3	PBR	23	0.188±0.036	0.096±0.044	1.96
	Non-PBR	75	0.091±0.015	0.053±0.014	1.72
	Total	98	0.114±0.015	0.064±0.016	1.78
Family 5	PBR	23	0.155±0.047	0.037±0.031	4.19
	Non-PBR	75	0.054±0.016	0.025±0.013	2.16
	Total	98	0.078±0.017	0.028±0.013	2.79
Family 6	PBR	23	0.098±0.035	0.031±0.030	3.16
	Non-PBR	75	0.046±0.014	0.010±0.011	4.60
	Total	98	0.058±0.014	0.016±0.011	3.63
Family 7	PBR	23	0.107±0.033	0.047±0.026	2.28
	Non-PBR	75	0.051±0.011	0.022±0.010	2.32
	Total	98	0.064±0.011	0.028±0.010	2.29
Whole	PBR	23	0.154±0.038	0.067±0.032	2.30
	Non-PBR	75	0.079±0.016	0.050±0.013	1.58
	Total	98	0.095±0.015	0.054±0.013	1.76

Table 4 The number of Scma-DAB alleles in each fish per family and its corresponding individual number

Family	1 allele	2 alleles	3 alleles	4 alleles	5 alleles
Family1	6	1	2		1
Family 2	3	4	3		
Family 3	2	7	1		
Family 5	5	3	1	1	
Family 6	4	3	3		
Family 7		4	4	1	1
Total	20	22	14	2	2

study had between one and five alleles. Approximately five clones from each individual were sequenced, which suggests that turbot have at least three MHC II B loci or copies. All families included both homozygous and heterozygous individuals at exon 2 of the MHC Class II gene, except family 7 which contained only heterozygotes.

Thirty-seven MHC II exon 2 alleles were identified in the 60 fish samples. Several alleles were present in low frequencies and were excluded from allele association analysis between survivor and non-survivor individuals in each family and in whole families. Nine alleles were selected for association analysis (Table 5). Varied distribution frequencies of alleles in survivors and non-survivors from each family were observed. For example, the *Scma-DBB1*02* allele was significantly more frequent in survivors than in non-survivors from whole families ($P<0.01$), while the frequency of the same allele was marginally greater in survivors than in non-survivors from family 3. This allele was present only in survivors from family 2. The *Scma-DBB1*10* allele was significantly more frequent in non-survivors than in survivors from whole families ($P=0.02$). The frequencies of some alleles were not significantly different between survivors and non-survivors from whole families or from each family, including the *Scma-DBB1*16* allele ($P=0.999$, 0.0001, 0.24, 0.09) in families 1, 5, 7, and pool respectively, and the *Scma-DBB1*09* allele ($P=0.0001$, 0.999, 0.12) in families 2, 6 and pool. We therefore deduced that the *Scma-DBB1*02* allele was highly associated with resistance to *E. tarda* in turbot, while the *Scma-DBB1*10* allele was associated with susceptibility to *E. tarda*.

3 Discussion

Most of the previous research into MHC genes in fish species has been focused on the extent of polymorphism within loci [13,17,42–48], as well as association between alleles and resistance/susceptibility to pathogens [15,30,49–52]. In this paper, polymorphisms within the turbot MHC Class II B exon 2 gene in six families, and associations between MHC alleles and resistance/susceptibility to *E. tarda*, were examined.

This study identified 37 turbot MHC Class II B exon 2 alleles in 60 individuals, of which 25 were novel, while the remaining 12 alleles (*Scma-DBB1*08*, *Scma-DBB1*13*, *Scma-DBB1*16*, *Scma-DBB1*04*, *Scma-DBB1*14*, *Scma-DBB1*02*, *Scma-DBB1*09*, *Scma-DBB3*02*, *Scma-DBB1*22*, *Scma-DBB1*10*, *Scma-DBB1*07*, *Scma-DBB3*05*. GenBank accession Nos. EF555735, EF555740, EF555743, EF555731, EF555741, EF555729, EF555736, EF555770, EF555749, EF555737, EF555734, EF555773) had been identified in previous studies [13,30]. The six turbot families (1–6) examined in this study contained 8, 9, 7, 9, 6, and 10 alleles respectively. The alleles were also unique to each family. A previous study in flounder identified 76 alleles in 60 individuals [15], while another study in water vole found five alleles in 100 individuals [46]. Therefore, the allelic diversity of turbot demonstrated in this study is consistent with other species.

One of the experimental aims was to identify MHC Class II B genes in turbot that were associated with resistance to *E. tarda*, which has a devastating economic impact on turbot farming. Using survival rates based on a preliminary bacterial infection test, we screened for any correlation between MHC allele and resistance to *E. tarda* in turbot. We determined that allele frequency differed between survivor and non-survivor individuals from different families. Many alleles were present at a very low frequency and therefore had no value for distribution analyses; only nine alleles were used for association analyses (Table 5). The distribution frequency of the *Scma-DBB1*02* allele was 27.5% in survivors in family 2, but the allele was not present at all in non-survivors ($P=0.0001$). The distribution frequency of the same allele was 13.7% and 3.9% in survivors and non-survivors respectively from family 3 ($P=0.081$), while the distribution frequency of *Scma-DBB1*02* was 6.2% and 0.6% in survivors and non-survivors respectively in whole families ($P=0.0001$). It is therefore likely that the *Scma-DBB1*02* allele is associated with resistance to *E. tarda* in turbot. The frequency of the *Scma-DBB1*10* allele was 15.7% and 0% in non-survivors and survivors from family 2, respectively, while this allele was present at a frequency of 20% and 16% in non-survivors and survivors respectively from family 6. The frequency of the same *Scma-DBB1*10* allele was 5.9% and 2.4% in non-survivors and survivors in whole families, respectively. We inferred that the *Scma-DBB1*10* allele might associate with susceptibility to *E. tarda* in turbot (Table 5). In a previous study, Xu et al. [30] discovered that the *Scma-DBB1*10* and *Scma-DBB1*04* alleles were associated with susceptibility to *E. tarda* in turbot, while *Scma-DBB2*01* was associated with resistance to *E. tarda.* As two studies have now associated the *Scma-DBB1*10* allele with *E. tarda* susceptibility, the MHC Class II B exon 2 has been confirmed as a useful gene marker. In this study, we could not find an association between the *Scma-DBB2*01* allele and resistance to *E. tarda* in turbot. This may be a result of using different populations with different genetic backgrounds.

Table 5 The allele frequency (>13.7%) in survivors and non-survivors in six families[a)]

Allele	Mode	Number	Frequency	Family	Allele	Mode	Number	Frequency	Family
*Scma-DBB1*07*	S	14	0.28	Family 1	*Scma-DBB1*16*	S	10	0.2	Family 1
	NS	8	0.16			NS	10	0.2	
	Total	22	0.44			Total	20	0.4	
*Scma-DBB1*02*	S	14	0.275**	Family 2	*Scma-DBB1*09*	S	6	0.118	Family 2
	NS					NS	5	0.098	
	Total	14	0.275			Total	11	0.217	
*Scma-DBB3*10*	S	8	0.157**	Family 2	*Scma-DBB1*10*	S			Family 2
	NS					NS	8	0.157**	
	Total	8	0.157			Total	8	0.157	
*Scma-DBB1*16*	S	12	0.235	Family 3	*Scma-DBB1*02*	S	7	0.137	Family 3
	NS	7	0.137			NS	2	0.039	
	Total	19	0.373			Total	9	0.176	
*Scma-DBB1*04*	S	10	0.2	Family 5	*Scma-DBB1*16*	S	10	0.2	Family 5
	NS	17	0.34			NS			
	Total	27	0.54			Total	10	0.2	
*Scma-DBB3*10*	S	10	0.2	Family 6	*Scma-DBB1*10*	S	8	0.16	Family 6
	NS	10	0.2			NS	10	0.2	
	Total	20	0.4			Total	18	0.36	
*Scma-DBB1*14*	S	6	0.12**	Family 6	*Scma-DBB1*09*	S	7	0.137	Family 7
	NS	1	0.02			NS	9	0.177	
	Total	7	0.14			Total	16	0.314	
*Scma-DBB1*07*	S	9	0.176	Family 7	*Scma-DBB1*16*	S	2	0.039	Family 7
	NS	5	0.098			NS	5	0.098	
	Total	14	0..275			Total	7	0.137	
*Scma-DBB1*07*	S	23	0.068	Whole	*Scma-DBB1*10*	S	8	0.024	Whole
	NS	13	0.039			NS	20	0.059*	
	Total	36				Total	28		
*Scma-DBB1*16*	S	35	0.104	Whole	*Scma-DBB1*09*	S	32	0.095	Whole
	NS	23	0.068			NS	13	0.039	
	Total	58				Total	45		
*Scma-DBB1*04*	S	15	0.045	Whole	*Scma-DBB3*10*	S	16	0.047	Whole
	NS	25	0.074			NS	22	0.065	
	Total	40				Total	38		
*Scma-DBB1*14*	S	6	0.018	Whole	*Scma-DBB1*08*	S	4	0.012	Whole
	NS	6	0.018			NS	9	0.027	
	Total	12				Total	13		
*Scma-DBB1*02*	S	21	0.062**	Whole					
	NS	2	0.006						
	Total	22							

a) S denotes survivor individual and NS denotes non-survivor individual during the challenge tests. * denotes significant difference at 0.05 (P<0.05), ** denotes significant difference at 0.01 (P<0.01).

Other studies have shown associations between MHC alleles and disease resistance in salmonids [25,43,53–55], turbot [13,30], Japanese flounder [14,15], and whitefish [56]. It remains difficult to screen a single allele which is present in survivors or non-survivors from each family, possibly because of environmental factors or because of other genes that are in linkage disequilibrium and that are associated with resistance or susceptibility to *E. tarda* in turbot [30,55].

The rate of non-synonymous (d_N) to synonymous (d_S) substitutions is often used for the assessment of balancing selection [57]. Some studies have reported higher non-synonymous mutations in the PBR of MHC genes [3,7,13, 58–60]. In this study, the rate of d_N/d_S in PBR was higher than that of d_N/d_S in non-PBR in families 1, 2, 3, 5 and whole families, which is consistent with results in other fish species [14,53,60,61]. This finding suggests that positive selection is acting on the PBR of exon 2 of turbot MHC II B genes. However, there were two exceptions in this study, in families 6 and 7, where d_N/d_S in the PBR was lower than that of in the non-PBR, which has also been observed in other fish species [14,53]. It is possible that the PBRs in fish do not exactly correspond to those in humans [14,49], or that the structure of the PBRs is different between MHC Class II B exon 2 alleles at the same locus [62]. Further studies are needed to understand the actual functions of MHC genes.

In conclusion, exon 2 of the MHC Class II B gene in turbot is polymorphic, and the *Scma-DBB1*02* allele is associated with resistance to *E. tarda*, while *Scma-DBB1*10* is associated with susceptibility to *E. tarda* in turbot. These markers might be helpful in a selective breeding program for improved bacterial survivability.

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