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Identification of MHC class IIβ resistance/susceptibility alleles to *Aeromonas salmonicida* in brook charr (*Salvelinus fontinalis*)

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ABSTRACT

Pathogen-driven selection is believed to be important in the evolution and maintenance of the polymorphism of the major histocompatibility complex (MHC) genes but have been tested for very few vertebrates. In this study, we first investigate by SSCP (single strand conformational polymorphism) the diversity found at the MHC class IIβ gene in a population of brook charr (*Salvelinus fontinalis*) from the Rupert River (Québec, Canada). Secondly, to explore the survival performances conferred by specific alleles and genotypes, individuals from 23 half- and full-sibling families were infected with *Aeromonas salmonicida*, the causative agent of furonculosis. From the initial brook charr population, a total of six MHC class IIβ alleles were identified; four complete and two partial coding sequences that include the complete polymorphic β1 domain. One allele, *Safo-DAB*0101*, was significantly associated with resistance against *A. salmonicida*. In addition to homozygotes for this allele, its resistance effect was also detected in heterozygotes for two specific genotypes. Other allelic combinations, namely heterozygous genotypes *Safo-DAB*0201/*0301* and *Safo-DAB*0301/*0401* were significantly associated with increased susceptibility to furonculosis. Given that its frequency was relatively low (0.0873), the negative frequency-dependent selection hypothesis could explain the advantage associated with the allele *Safo-DAB*0101* over the other alleles and highlight the importance of this mechanism to sustain variation at the MHC in brook charr.

1. Introduction

The major histocompatibility complex (MHC) class I and class II genes encode cell-surface proteins specialised in the presentation of self- and non-self-antigen peptides to T-lymphocytes in the adaptive immune system. These represent the most polymorphic genes known to date, with multiple loci and high allelic diversity at each of these loci (Trowsdale and Parham, 2004). Unlike the situation in other vertebrates, the two classical MHC regions, class I and class II, are not found in a complex in bony fishes (Bingulac-Popovic et al., 1997; Sato et al., 2000). For this reason, the expression of "major histocompatibility" (MH) genes is more appropriate in teleosts (Dixon and Stet, 2001). Studies have shown that rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) express a single "minimal, essential" gene of the MH class I (Aoyagi et al., 2002; Grimholt et al., 2002) and class II (Glamann, 1995; Langefors et al., 2000). Also, MH class IIα and class IIβ loci are genetically linked in Atlantic salmon (Stet et al., 2002).

The evolution and maintenance of the polymorphism of the MHC genes can be attributed to two major types of mechanisms: the pathogen-driven and reproductive mechanisms (Aguilar and Garza, 2007; Bernatchez and Landry, 2003). Two main hypotheses have been proposed to explain the pathogen-driven selection: the overdominance and the negative frequency-dependent selection hypothesis. In the overdominance model (or heterozygous advantage), the heterozygous individuals are assumed to present a broader range of pathogen-derived antigens due to a larger number of different MHC molecules and have increased fitness relative to homozygous (Hughes and Nei, 1989). The second model of negative frequency-dependent selection (or rare-allele advantage) argues that individuals bearing low-frequency alleles have an advantage because of the limited co-evolution of the pathogens facing these MHC alleles (Takahata and Nei, 1990; Slade and McCallum, 1992). A third mechanism implicates selection that fluctuates in time and/or space (Hedrick, 2002).

The implications of different MHC class I and class II alleles in disease resistance or susceptibility have been documented previously (e.g. Zekarias et al., 2002; Gebe et al., 2002; Singer et al., 1997; Carrington and O'Brien, 2003; Shiina et al., 2004). In fish, the functional implications of MH polymorphism on the resistance against or susceptibility to infectious diseases have essentially

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all been investigated in Atlantic salmon (*S. salar*). Langefors et al. (2001) were the first to establish a correlation between survival probability and three different MH class IIβ alleles following *Aeromonas salmonicida* exposure. Their observations were confirmed by Lohm et al. (2002) in an experiment where the effect of non-MH background genes could be controlled. Another study identified significant associations between resistances towards infectious diseases caused by both bacterial or viral pathogens and MH class I and class II alleles (Grimholt et al., 2003). It is noteworthy that different alleles were identified as associated with resistance to each pathogen. More recently, a combination of particular MH class I and class II alleles has been associated with resistance or susceptibility to infectious salmon anaemia virus (ISAV) (Kjoglum et al., 2006). Finally, results that could be interpreted as the manifestation of a disease-mediated natural selection have been observed for the MH class II locus (de Eyto et al., 2007).

A. salmonicida, a Gram-negative, facultatively anaerobic, rod-like bacterium, is an invasive pathogen capable of surviving and replicating within intraperitoneal macrophages and non-phagocytic cells (Daly et al., 1996; Garduno et al., 2000). This economically important pathogen is the causative agent of furonculosis that has a strong impact on the survival of salmonids. Previous studies have demonstrated an important genetic component associated with resistance to this pathogen (Perry et al., 2004; Gjedrem, 2000). Brook charr (*Salvelinus fontinalis*) is an important endemic salmonid fish from eastern North America, but there has been no research regarding the MH genes and their performance against *A. salmonicida*, although a heritable genetic basis for resistance to furonculosis has been evidenced in this species (Perry et al., 2004). In this study, our aims were (i) to document the diversity of alleles at the MH class IIβ gene of brook charr and (ii) for the first time, to document survival conferred by specific alleles and/or genotypes following exposure to furonculosis in a fish other than Atlantic salmon.

2. Materials and methods

2.1. Fish and challenge test

Wild brook charr (*S. fontinalis*) originating from the Rupert River and associated waterways were maintained at the LARSA (LAboratoire Régional des Sciences Aquatiques; Université Laval). This strain is of particular interest for aquaculture because it demonstrates rapid growth and large adult size that may exceed 5 kg (Sutton et al., 2002). Fish kept at LARSA were used to generate 23 half- and full-sibling families as previously described (Perry et al., 2004). This allowed us to take into account the effect of genetic background in interpreting the putative role of MH alleles in resistance to furonculosis. To identify individuals of interest for breeding, adult brook charr were first genotyped at the MH class IIβ locus by SSCP (single strand conformational polymorphism). Fish were exposed to a virulent strain of *A. salmonicida* by a bath immersion method (Dautremepuits et al., 2006; Perry et al., 2004; Lutwyche et al., 1995) at 1×10^6 bacteria/ml previously determined to be the LD_{50} at 72 h. Individual survival was recorded every 4 h for the following 90-h period. No mortality was observed in control fish. Dead and surviving individuals were collected and SSCP genotyped at the MH class IIβ locus. The final experimental dataset contained the surviving time of 860 genotyped individuals for statistical analyses.

2.2. MH class IIβ genotyping of brook charr

Genomic DNA from 63 adult brook charrs was extracted from fin adipose for genetic analysis. The different MH class IIβ alleles were first identified using SSCP. A 315–318 bp fragment containing the complete MH class IIβ exon 2, coding for the polymorphic β1 domain of the protein, was amplified by PCR (polymerase chain reaction) using intronic sense primer SP4501 (5′-CCTGTATTTATGTTCTCCTTTC-3′) and antisense primer SP4502 (5′-TAAGTGTTGCTACGGAGCC-3′) in a thermocycler (Biometra). PCR was carried out in a total volume of 50 μl containing 20–40 ng of genomic DNA, 0.2 mM dNTPs, 8 μl of 10× PCR buffer (200 mM Tris–HCl pH 8.4, 500 mM KCl), 2 mM $MgCl_2$, 5 pmol each of SP4501 and SP4502 primers, 1 unit of *Taq* DNA polymerase. The following PCR conditions were used: denaturation for 3 min at 94 °C followed by 35 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 47 °C and extension for 1 min at 72 °C. The final extension was for 10 min at 72 °C. Three microliters of formamide were added to 9 μl of the PCR products which were then loaded onto a non-denaturing acrylamide gel (10% 49:1 acrylamide:bis-acrylamide, 5% glycerol and 1× TBE) for a 2.5 h and 200 V migration in a Mini-protean III tank (Bio-Rad) in an ice-cold water bath. The procedure resolves alleles into discrete bands that were finally revealed on the gels by silver staining (Budowle et al., 1991).

2.3. RNA isolation from peripheral blood leukocytes (PBL) of brook charr

RNA from PBL of brook charr was extracted to assure the identification of expressed MH class IIβ alleles. Fish were anesthetised with 0.1% of tricaine methanesulfonate (MS-222) (Sigma). The blood was collected from the caudal vein using a heparinised 3 ml syringe with a 21G1 needle (Becton Dickson) and added to 1 volume of ice-cold PBS containing 10 U/ml heparin sodium salt (Sigma). The cellular suspension (1 ml) was then spun at 500 × *g* for 5 min at 4 °C and the buffy layer and plasma transferred to a new Eppendorf. The PBL were then pelleted and suspended in Trizol (Invitrogen) for total RNA extraction according to the manufacturer's protocol.

2.4. Reverse transcriptase (RT)-PCR from PBL

Total RNA (500 ng) from fish of different genotypes was reverse transcribed using SuperScript II reverse transcriptase (Invitrogen), oligo$(dT)_{12–18}$ primer (Invitrogen) and RNAseOUT (Invitrogen) according to the manufacturer's protocol. The complete open reading frame of MH class IIβ was obtained by PCR using primers in the 5′-UTR (P472; 5′-CAGCAGAGGAACATGTCGATG-3′) and the 3′-UTR (P473; 5′-TTTCTGCTGCAGATTCAGCA-3′) designed from sequences at positions conserved between Atlantic salmon and rainbow trout (Syed et al., 2003). The reactions were carried out on homozygous individuals for product homogeneity. PCR was carried out in a total volume of 50 μl containing 0.25 μl of cDNA from a 1/20 dilution of the RT reaction, 0.2 mM dNTPs, 5 μl of 10× Expand High Fidelity buffer (Roche), 2 mM $MgCl_2$, 5 pmol each of P472 and P473 primers, 2.6 units of Expand High Fidelity enzyme mix (Roche). The following PCR conditions were used: denaturation for 3 min at 94 °C followed by 40 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 45 °C and extension for 1 min at 72 °C. The final extension was for 10 min at 72 °C. Sequencing of the PCR products was realized on a 3730 DNA analyser (Applied Biosystems).

2.5. Data analysis

Alignment of the deduced amino acid sequence of MH class IIβ peptide was performed using ClustalX (Thompson et al., 1994). Sequences used for comparison and their GenBank™ accession numbers were as follows: *O. mykiss*: U20943 (Glamann, 1995); *S. salar*: X70166 (Hordvik et al., 1993); *Salmo trutta*: AF296398 (Shum et al., 2001); *Cyprinus carpio*: Z49064 (Ono et al., 1993); *Brachydanio*

rerio: L04805 (Ono et al., 1992); human: P04229; UniProt (Bell et al., 1985).

Nucleotide sequences were analysed with the Syn-SCAN program (Gonzales et al., 2002) to calculate synonymous and nonsynonymous nucleotide substitution rates according to the method of Nei and Gojobori (1986) using a Jukes–Cantor correction.

Phylogenetic tree was created using the Neighbor-Joining method (Saitou and Nei, 1987). The percentages of replicate trees in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007).

We calculated the proportion of mortality for each genotype with 95% exact confidence intervals obtained by using the F distribution method given in Collett (1991) and also described by Leemis and Trivedi (1996). To compare survival times between the genotypes for MH class II protein, we conducted a log-rank test with the procedure LIFETEST. Then the Cox proportional hazards model (Cox, 1972) was fitted by using the procedure TPHREG to compare survival times between genotypes with adjustment for covariates (weight and infection tank) selected by the stepwise method. In the Cox model, each individual has its own hazard function expressed as

$$\lambda_i(t) = \lambda(t; Z_i) = \lambda_0(t)\exp(\mathbf{Z}_i'\beta)$$

where $\lambda_i(t)$ is the risk function for the individual i at time t, $\mathbf{Z}_i$ is the vector of covariates for individual i, $\lambda_0(t)$ is the common part of the model and $\exp(\mathbf{Z}_i'\beta)$ is the individual specific part of the model. Results are presented as a hazards ratio (HR). To compare allele and genotype effect on survival time, we computed contrasts on HR with 95% confidence intervals.

3. Results

3.1. Sequences of brook charr MH class IIβ gene

Six MH class IIβ alleles were identified from the 63 brook charr individuals by means of SSCP. The cDNA coding region (741–738 bp) of the four most frequent alleles were obtained from PBL (Peripheral Blood Leukocytes) total RNA by using 5′-UTR and 3′-UTR primers. According to accepted nomenclature rules (Klein et al., 1990), these alleles were designated *Safo-DAB*0101* to *Safo-DAB*0401*. Partial coding sequences of two alleles, denoted *Safo-DAB*0501* and *Safo-DAB*0601*, were obtained from amplified exon 2 by PCR on genomic DNA using primers in intron 1 and intron 2 because of the impossibility to proceed blood extraction. The sequences of these two far less frequent alleles cover the complete polymorphic β1 domain. The nucleotide alignment of the six coding sequences of brook charr MH class IIβ alleles is shown in Fig. 1 (GenBank accession nos. EU478851, EU478852, EU478853, EU478854, EU478855 and EU478856). The frequency of each allele in the initial population was: *DAB*0101* – 8.73%, *DAB*0201* – 39.7%, *DAB*0301* – 17.5%, *DAB*0401* – 30.2%, *DAB*0501* – 3.17% and *DAB*0601* – 0.79%. The open reading frame of *Safo-DAB* alleles encodes a 245–246 residues long polypeptide chain. Alignment of the sequences with teleosts and mammal representative sequences revealed a characteristic structure typical of the MHC class IIβ chain (Fig. 2).

3.2. Polymorphism of MH class IIβ

The pairwise nucleotide diversity of the sequences obtained from the four brook charr complete cDNA clones ranged from 3.8% to 5.4% (28–40 nucleotides) with a mean of 4.6% (34 nucleotides). Derived amino acid sequences divergence ranged from 7.3% to 11% (18–26 residues) with a mean of 8.6% (21 residues) (Table 1). The level of diversity was higher in the β1 domain coding sequences compared to the non-β1 domain.

Within the polymorphic β1 domain of the six reported sequences from brook charr, the mean pairwise nucleotide divergence was 9.5% (range: 3.0–12%), while it was 18% (7.6–22%) for the amino acids. Among the teleost β1 domain amino acid sequences, 25 positions were conserved whereas a deletion of one residue was shared by the *Safo-DAB* alleles (Fig. 2). A deletion of two residues was shared in the alleles *DAB*0401* and *DAB*0601* that also showed the greatest similarity between the alleles with only 7.6% divergence at amino acid variation. Sequence comparison showed that 30 of the 91 residues were polymorphic, each involving replacement of two to five different amino acids. The polymorphic positions found in *Safo-DAB* alleles corresponded well with the PBR (peptide-binding region) residues identified in HLA-DRB (Brown et al., 1993), particularly in the N-terminal region of the domain.

Non-β1 domain coding sequences were much less variable, with pairwise divergence values ranging from 0.85% to 2.8% (mean of 1.7%) for nucleotides and from 1.3% to 3.9% (mean of 2.37%) for amino acid positions. The 20 residues long signal peptide of *DAB*0101* allele differed from the three other alleles at four positions, with the exception of *DAB*0301*. The β2 domain, connecting peptide, transmembrane region, and the cytoplasmic tail were all highly conserved (Fig. 2).

The average rates of synonymous (d_S) and nonsynonymous (d_N) nucleotide substitutions and their ratio (d_N/d_S) for the brook charr MH class IIβ sequences are presented in Table 1. For the complete cDNA comparisons, d_S and d_N were similar and the resulting ratio d_N/d_S was 1.1 (±0.13). In contrast, for the 91 codons of the polymorphic β1 domain, the rate of synonymous nucleotide substitutions was 2.2 (±1.1) times higher than the nonsynonymous nucleotide substitution rates, providing evidence for the role of positive selection in maintaining diversity in this domain, as generally reported. On the other hand, the corresponding ratio for the non-β1 domain was 0.31 (±0.082), being more indicative of purifying selection.

A phylogenetic tree constructed with the exon 2 from the six MH class IIβ alleles of brook charr indicated that the sequences clustered together and separately from those observed in Atlantic salmon (Hordvik et al., 1993) and Pacific salmon (*Oncorhynchus*) species (Miller and Withler, 1996). The two sequences characterized by an additional deletion, *DAB*0401* and *DAB*0601*, formed a separate outgroup in the brook charr cluster (Fig. 3).

3.3. Single expressed locus

We sequenced the intron 2 of the six MH class IIβ alleles identified in brook charr. We identified in all the alleles a minisatellite composed of a 32 nucleotide motif: ACATTACAGTATGACTAGTCAGCTATGTAGTG, which has already been shown to be present in *S. salar* (Stet et al., 2002) and as a single motif in Pacific salmon (*Oncorhynchus*) species (Miller and Withler, 1996). With the exception of the minisatellite varying in size, the identity of intronic sequences ranged from 84% to 99% (mean of 91%). From each fish, only one or two different PCR products covering intron 2 was obtained, for which a particular length always corresponded to the same allele. In addition to SSCP and sequencing analysis that indicated no more than two alleles per fish, these results strongly suggest that the identified alleles corresponded to a single locus. As for other salmonids (Glamann, 1995; Langefors et al., 2000), it thus appears that a single locus expresses the MH class IIβ gene in the brook charr.

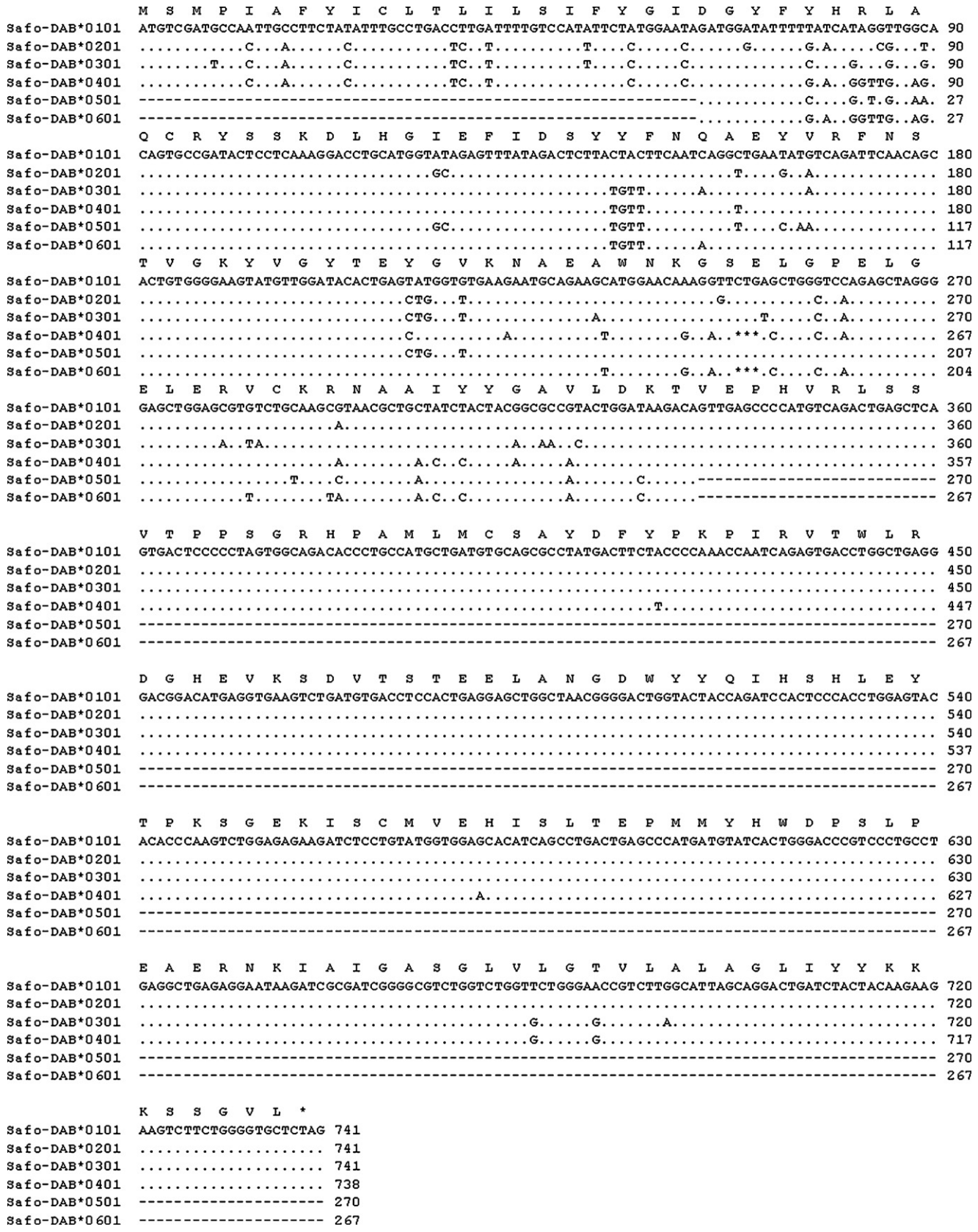

Fig. 1. Nucleotide sequences of the MH class IIβ cDNA and partial gDNA of six different alleles from brook charr. On top is the amino acid sequence translated from *Safo-DAB*0101*.

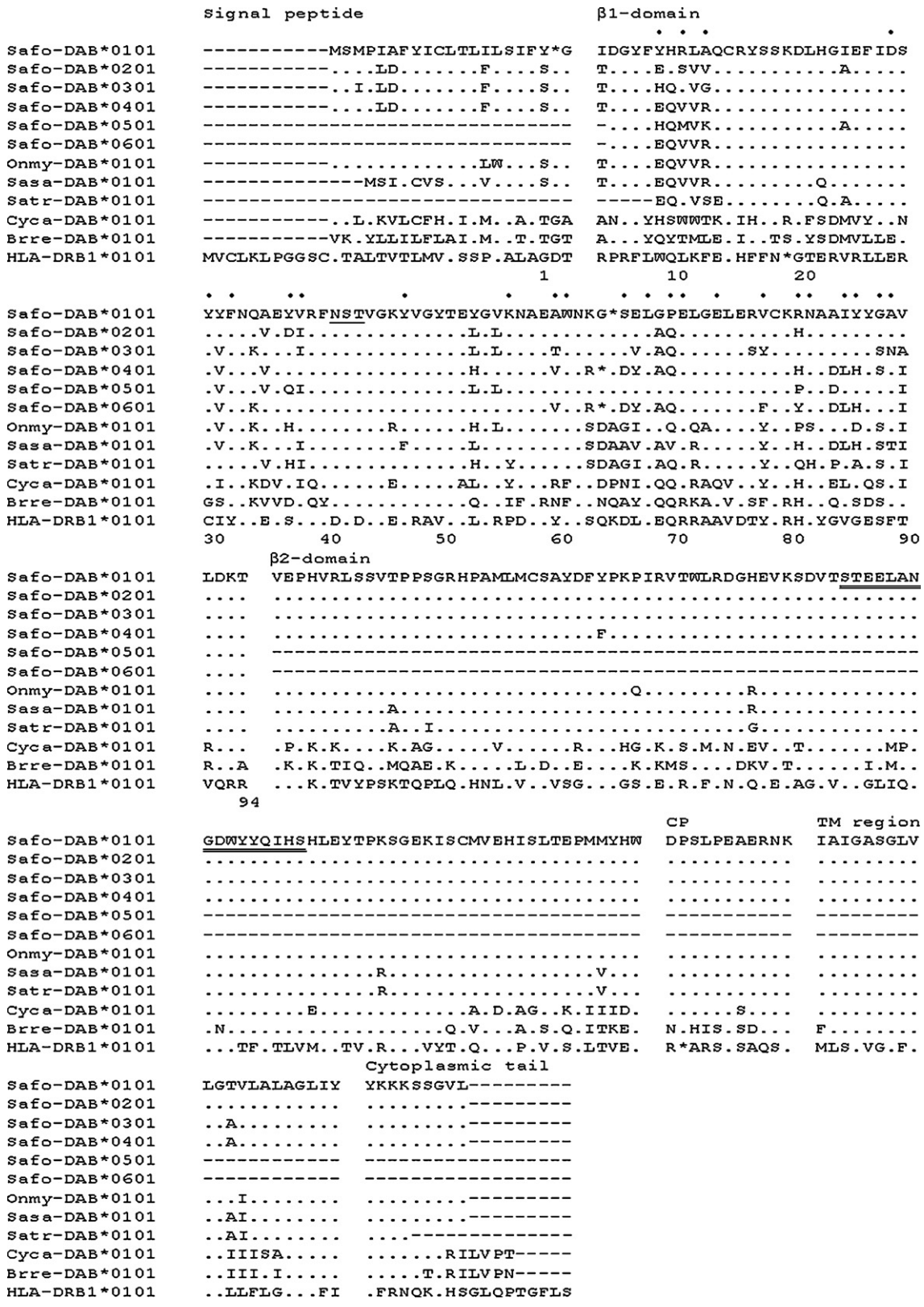

Fig. 2. Alignment of brook charr MH class IIβ amino acid sequences with MH class IIβ sequences from other vertebrates. Protein domains are indicated. Dots indicate identity with *Safo-DAB*0101*; asterisks, gaps introduced to maximize the alignment; dashes, unavailability of sequence information; cysteine residues are marked with light gray boxes; putative N-linked glycosylation sites are underlined; conserved CD4-binding residues are double underlined. Numbering below and residues involved in antigen binding denoted by dots above the β1-domain sequence refer to HLA-DRB*0101 (Brown et al., 1993). CP: connecting peptide; TM: transmembrane. In addition to brook charr (*Salvelinus fontinalis:* Safo) sequences, *Oncorhynchus mykiss* (Onmy), *Salmo salar* (Sasa), *Salmo trutta* (Satr), *Cyprinus carpio* (Cyca), *Brachydanio rerio* (Brre) and human (HLA) sequences are aligned.

Table 1
Mean diversity in the nucleotides (nt) and amino acid (AA) sequences, mean proportion of observed synonymous (d_S) and nonsynonymous substitutions (d_N) and the ratio between d_N and d_S of brook charr MM class IIβ gene

MH class IIβ sequence	*N*	Diversity (%)		d_S	d_N	d_S/d_N
		nt	AA			
Complete cDNA	4	4.6 [3.8–5.4]	8.6 [7.3–11]	0.046 (0.007)	0.048 (0.007)	1.1 (0.13)
β1 domain	6	9.5 [3.0–12]	18 [7.6–22]	0.061 (0.027)	0.11 (0.029)	2.2 (1.1)
Non-β1 domain	4	1.7 [0.85–2.8]	2.4 [1.3–3.9]	0.036 (0.014)	0.012 (0.007)	0.31 (0.082)

N gives the number of alleles included in the analysis. Standard errors are in parentheses and ranges are in brackets.

3.4. Analysis of MH class IIβ in resistance to furonculosis

The survival time of 860 *A. salmonicida* infected brook charrs was recorded at 4 h intervals for a total of 90 h following infection (Fig. 4). The mortality started 52 h after the challenge, peaked at 68 h and reached a cumulative mortality of 94.4% at the end of the experiment.

Fish harbouring genotypes *DAB*0101/*0101* or *DAB*0101/*0201* showed retarded mortality and superior survival at the end of the experiment compared to the other individuals. In single allele analysis, individuals possessing the MH class IIβ *DAB*0101* or *DAB*0301* alleles were the most resistant, while those with *DAB*0201* or *DAB*0401* were the most susceptible to furonculosis (Table 2). However, fish carrying the *DAB*0101* allele were the only individuals to show a statistically significant increase in resistance to *A. salmonicida*. Thus, observed mortality for fish carrying the *DAB*0101* allele was 84.03%, which was approximately 10% lower than that observed for fish not possessing this allele.

In the combined (genotype) analysis, two allelic combinations returned statistically significant results. Namely, the genotypes *DAB*0201/*0301* and *DAB*0301/*0401* were associated with increased susceptibility to furonculosis (Table 3). The allele *DAB*0101* was mostly associated with genotypes showing increased resistance, and homozygote genotype *DAB*0101/*0101* was nearly significant ($p = 0.0657$). Thus, survival of individuals with the *DAB*0101/*0101* and *DAB*0101/*0201* genotypes was clearly higher than the other combination of alleles, their respective mortality being 84.62% and 79.71%, or 8.5–13.4% lower than for the other allelic combinations on average. Finally, fish with *DAB*0201/*0201* genotypes showed a tendency towards susceptibility, albeit not significant.

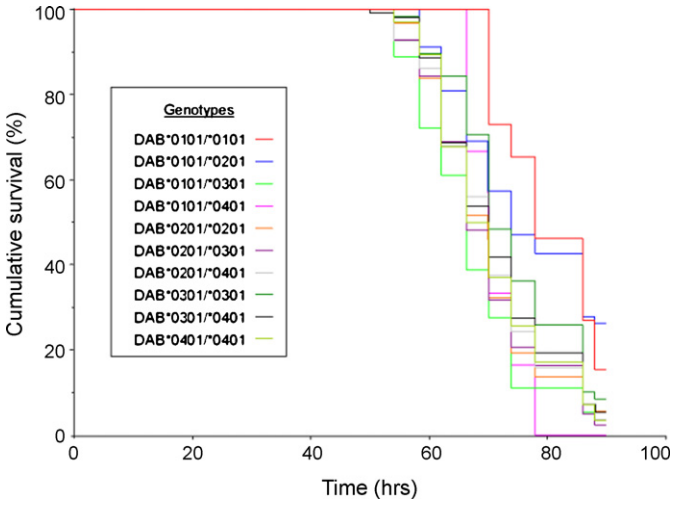

Fig. 4. Kaplan–Meier plot showing the survival of different MH class IIβ genotypes in brook charr exposed to the challenge experiment with *Aeromonas salmonicida*. Cumulative survival of fish harbouring one of the ten genotypes found in this study was calculated following harvest every four hours during the infection experiment. Survival was reflected by remaining individuals at 90 h.

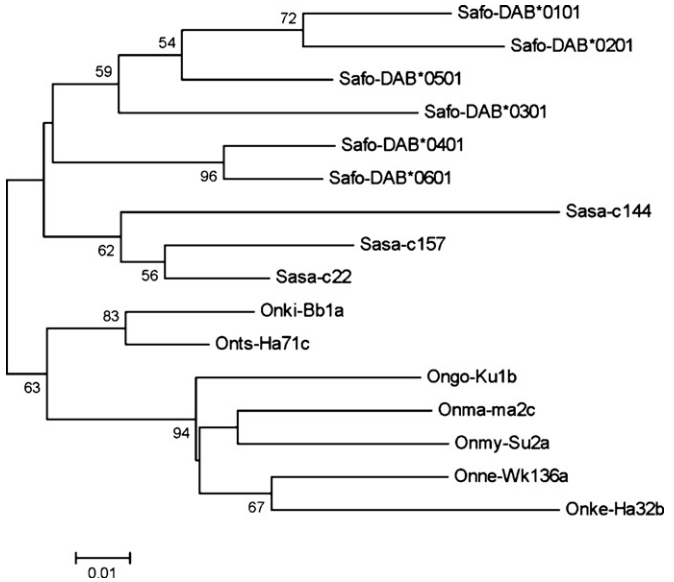

Fig. 3. Neighbor-joining tree of the second exon of the MH class IIβ gene in brook charr and other salmonids. Six sequences from brook charr (*S. fontinalis*: *Safo-DAB*0101* to *Safo-DAB*0601*; this study), three from Atlantic salmon (*Sasa-c22*, *Sasa-c144*, *Sasa-c157*; Hordvik et al., 1993) and seven from Pacific salmon (*Onts-Ha71c*, *Onki-Bb1a*, *Ongo-Ku1b*, *Onma-ma2c*, *Onmy-Su2a*, *Onke-Ha32b*, *Onne-Wk136a*; Miller and Withler, 1996) are included. Tree was constructed using the method of Saitou and Nei, based on Maximum Composite Likelihood method in Mega software. Numbers on node indicate bootstrap confidence levels of 1000 bootstrap replications. Bootstrap values higher than 50% are shown.

4. Discussion

4.1. Identification of brook charr MH class IIβ alleles

The goals of this study were (i) to document the diversity of alleles at the MH class IIβ gene of brook charr and (ii) for the first time, to document survival conferred by specific alleles and/or genotypes following exposure to furonculosis in a fish other than Atlantic salmon. Overall, we observed a relatively low level of allelic diver-

Table 2
Hazard ratio in the single allele groups with at least one of the given class IIβ (DAB) alleles present

Allele	Hazard ratio	Confidence ($Pr > \chi^2$)	No. of fish
*DAB*0101*	**0.7187** (**0.1201**)	0.0480	119
*DAB*0301*	0.9896 (0.1454)	0.9434	388
*DAB*0401*	1.1133 (0.1493)	0.4235	412
*DAB*0201*	1.2255 (0.1918)	0.1938	453

Standard errors are in parentheses. Significant Hazard ratio ($p < 0.05$) is indicated in bold.

Table 3
Hazard ratio and mortality in groups with class IIβ (DAB) genotypes

Genotype	Hazard ratio (S.E.)	Confidence (Pr > χ^2)	Mortality (%) (CI)	No. of fish
*DAB*0101/*0101*	*0.6283* (*0.1586*)	0.0657	84.62 (65.13–95.64)	26
*DAB*0301/*0301*	0.7790 (0 1382)	0.1593	93.10 (83.27–98.09)	58
*DAB*0101/*0201*	0.7944 (0.1427)	0.2002	79.71 (68.31–88.44)	69
*DAB*0401/*0401*	0.8662 (01131)	0.2715	95.71 (90.91–98.41)	140
*DAB*0101/*0301*	1.0090 (0.3028)	0.9763	94.44 (72.71.99.86)	18
*DAB*0201/*0401*	1.0256 (0.1417)	0.8547	97.20 (92.02–99.42)	107
*DAB*0201/*0201*	*1.2886* (*0.1695*)	0.0538	96.77 (91.95–99.11)	124
*DAB*0301/*0401*	**1.2942** (**0.1605**)	0.0376	95.60 (91.14–98.21)	159
*DAB*0101/*0401*	1.3027 (0.6178)	0.5771	100.0 (54.07–100.0)	6
*DAB*0201/*0301*	**1.3207** (**0.1642**)	0.0253	96.73 (92.54–98.93)	153

Standard errors (S.E.) and confidence intervals (CI) are in parentheses. Significant Hazard ratios ($p < 0.05$) are presented in bold whereas tendencies ($0.05 < p < 0.075$) are shown in italic.

sity in the studied population with a total of six alleles of the MH class IIβ gene identified in a group of 63 adult brook charr from the Rupert River. The high ratio of d_N/d_S observed for the β1 domain compared to the remaining of the protein indicates that a positive selection pressure is effective to select polymorphic mutations in that region. Furthermore, their identification from PBL mRNA indicates that alleles *Safo-DAB*0101* to *Safo-DAB*0401* are expressed and ultimately represent the surface molecule in charge of antigen presentation. We also found that one allele, *Safo-DAB*0101*, was significantly associated with resistance against *A. salmonicida*. In addition to homozygotes for this allele, its resistance effect was also detected in heterozygote individuals for two specific genotypes. In contrast, other allelic combinations, namely heterozygous genotypes *Safo-DAB*0201/*0301* and *Safo-DAB*0301/*0401* were significantly associated with increased susceptibility to furonculosis.

4.2. Variability of brook charr MH class IIβ alleles

The variability of certain amino acids of the peptide-binding region of the MHC class II alleles directly influences the binding properties for antigenic peptides as well as susceptibility to diseases (Jones et al., 2006). Is has been shown that a single residue mutated in the β1 domain N-terminal region could be correlated with increased resistance (Langefors et al., 2001). We compared the sequences of the β1 domain between the *Safo-DAB*0101* allele and the other three alleles present in the challenge test to identify exclusive residues, possibly responsible of a functional effect. Based on *HLA-DRB*0101* numbering, we identified six exclusive residues: β9Y, β12L, β13A, β53Y, β69G and β70P, with clustering of three of these residues between positions 9 and 13. The β9 position is particularly interesting for two reasons. First, observed substitution changes the chemical characteristics of the residue at this position since it is changed from a positively (β9H) or negatively (β9E) charged one to an aromatic polar uncharged one (β9Y). Second, this position is just beside the β10H residue that has been identified to be exclusively responsible for the emergence of resistance to furonculosis in European populations of Atlantic salmon (Langefors et al., 2001). It is interesting to note that β10H is also found in *Safo-DAB*0101*. Based on human MHC class II three-dimensional structure (Brown et al., 1993), this specific single changed residue identified by Langefors et al. (2001) corresponds to the β10Q position and is situated in the pocket 6 (P6) of the molecule (Jones et al., 2006). The presence of highly polymorphic charged residues is frequently observed in salmonids at corresponding positions between β9W and β13F of the *HLA-DRB1*0101* (Fig. 2). This sequence of residues is located in the bottom of the groove between pocket 6 (P6) and pocket 4 (P4), and the presence of particular residues could be critical for the efficient presentation of *A. salmonicida* antigens. From these studies, it appears that the polymorphic residues of the β1 domain present in these pockets could be important for the binding of antigenic peptide(s) from *A. salmonicida*. Namely, residue β53Y is located in the middle of an interface of dimerisation between two MH class II molecules and could possibly affect the stability or the structure of the dimer (Brown et al., 1993). Residues β69G and β70P are both located at corresponding positions, in *HLA-DR1* and *I-Ak*, that have been implicated in the binding to the TCR (Hennecke et al., 2000). The presence of a proline instead of the usually conserved glutamine at the position β70 of MH class II could have an important impact on immune system stimulation since this residue has the highest average number of contacts with the TCR (Rudolph et al., 2006).

4.3. Polymorphism of MH class IIβ leader sequences

Comparison of the signal sequence of the *DAB*0101* allele with the three other available sequences indicates two exclusive mutations. The replacement of the aspartic acid by an alanine (D6A) modifies the charged residue in amino-terminal of the signal sequence, while the phenylalanine by an isoleucine mutation (F14I) modestly alters the hydrophobicity. Compared to the other alleles, the differences in the signal sequence of the *DAB*0101* allele could affect both SRP binding and membrane integration leading to differences in cell surface expression and presentation (Peterson et al., 2003). Experiments are currently in progress to evaluate the surface expression levels of these different alleles.

4.4. Minisatellite in MH class IIβ intron 2

It is noteworthy that the minisatellite (motif: ACATTACAGTATGACTAGTCAGCTATGTAGTG) present in brook charr intron 2 also occurred in Atlantic salmon (Stet et al., 2002), but only as a single motif in Pacific salmon (*Oncorhynchus*) species (Miller and Withler, 1996). This result correlates and reinforces the phylogenetic tree constructed from β1 exonic sequences which establish a closer relationship between brook charr and Atlantic salmon alleles relative to Pacific salmon (Fig. 3). However, the identity of the intron 2 nucleotide sequences between the three species is highly conserved, suggesting a single and same active locus for the MH class IIβ genes. The single minisatellite motif found in Pacific salmon (*Oncorhynchus*) species is located between a partial *Hpa* 1 SINE (Short Interspersed Nucleotide Elements) and a complete *Hpa* 1 SINE (Miller and Withler, 1996), both absent from brook charr and Atlantic salmon. Minisatellites and SINEs are known to be implicated in recombination and gene conversion events (for reviews see: Jeffreys et al., 2004; Kazazian, 2004), raising the hypothesis that different molecular mechanisms may have been selected as a means to maintain high levels of MH class IIβ gene polymorphism among salmonid species.

4.5. Association of MH class IIβ alleles with resistance or susceptibility

In order to identify MH class II alleles associated with resistance or susceptibility to *A. salmonicida*, we performed a challenge experiment on 23 half- and full-sibling families. The observed resistance or susceptibility effect can falsely be attributed to a particular MH allele if no precautions are undertaken to control the genetic background effect. Here we could not integrate the genetic background effect in our statistical model because of insufficient degrees of freedom. However, the correlation of the allele *DAB*0101* with resistance to furonculosis resulted from the analysis of individuals issued from seven families for which two different sirs possessing the allele, and/or three different dams, have been utilized in the mating procedure. Thus, we believe that the use of families in this experiment minimized the effect of an eventual variable co-segregating gene while permitting variation at other unlinked genes.

From the four alleles that have been tested in the challenge experiment against *A. salmonicida*, the *DAB*0101* allele was significantly correlated with disease resistance in single analysis (Table 2). This result is strengthened by the combined analysis where fish homozygous for this allele, *DAB*0101/*0101*, also tended to be resistant to furonculosis (Table 3), which translated into a lower mortality in fish carrying allele *DAB*0101* in general and those homozygote at this allele in particular. Conversely, the results of the allele *DAB*0201* in single analysis and genotype *DAB*0201/*0201* in combined analysis are both suggestive of disease susceptibility (Tables 2 and 3). Interestingly, the relatively high resistance observed for the *DAB*0101/*0201* genotype also suggested that the dominant effect on disease resistance associated with *DAB*0101* was stronger than the dominant susceptibility conferred by *DAB*0201*. This type of effect has been demonstrated previously in Atlantic salmon (Lohm et al., 2002) where a co-dominant pattern of disease resistance/susceptibility was found, indicative of qualitative difference in the immune response between individuals carrying the high- and low-resistance alleles. Overall, the resistance or susceptibility to furonculosis infection seemed to be associated more to specific MH class IIβ alleles rather than MH heterozygosity.

The result obtained for fish harbouring genotype *DAB*0301/*0301*, although not statistically significant, was suggestive of increased resistance, whereas the high ratio values obtained for *DAB*0201/*0301* and *DAB*0301/*0401* were more suggestive of increased susceptibility. On the other hand, when looking at the single analysis for the *DAB*0301* allele, the intermediate hazard ratio was indicative of a null or confounded effect. Thus, it is possible that the apparent increased susceptibility in heterozygotes carrying this allele could have been caused by the alternate alleles to which it was associated in given heterozygous genotype such as allele *DAB*0201* and *DAB*0401* which gave high hazard ratios, albeit without statistically significant effect. Overall, these results are suggestive of a dominant effect of *DAB*0201* and *DAB*0401* alleles over *DAB*0301*.

4.6. Mechanism for pathogen-driven balancing selection

The negative frequency-dependent selection hypothesis may potentially explain the advantage of the allele *DAB*0101* over the other more common alleles, and underlines the importance of this mechanism in sustaining variation at the MH in brook charr. Thus, the *DAB*0101* allele was present in only 10 of the 63 brook charr (16%) originally used to generate the families, whereas the allele *DAB*0201* (76%) that was widely distributed in this population tended to be associated with disease susceptibility. With no evidence of bacterial outbreak in the Rupert River population, it is then reasonable to hypothesize that these allele distributions within the wild brook charr population from that system could reflect the adaptation of these fish to the dominant pathogen present in that river (Hedrick, 2002). Since we know that bacterial diversity represents an important factor to explain the MHC amino acid variability in wild salmonid populations (Dionne et al., 2007), it should be very useful for future studies of brook charr to combine the fish sampling with an analysis of the bacterial communities present in the water as well.

In population infection studies, an important factor influencing which MHC polymorphism mechanism of evolution will be observed is the type of infection the individual is facing. Single-pathogen infection studies failed to support the heterozygote advantage (Ilmonen et al., 2007), while others, in support to the frequency-dependent selection, demonstrated a selective benefit for individuals expressing rare alleles (Langefors et al., 2001; Lohm et al., 2002; Trachtenberg et al., 2003). Regarding the heterozygous advantage hypothesis, a recent study examined multiple pathogen infections and found a pattern of MHC superiority and reduced pathogen load in heterozygotes over homozygotes (McClelland et al., 2003). Penn et al. (2002) also showed that MHC heterozygotes were more resistant to infection and had higher fitness than homozygotes in multiple-strain infections.

Overall, the results of this study did not support a heterozygous advantage against a single pathogen. For example, the majority of the heterozygous individuals have hazard ratios indicative of susceptibility to furonculosis whereas three out of four homozygous genotypes of the combined analysis presented significant or a tendency towards resistance hazard ratios (Table 3). Homozygosity at the MH class II locus has already been suggested to be an advantage against furonculosis in Atlantic salmon (Grimholt et al., 2003). However, as for the brook charr in the present study, these fish were facing a single pathogen and the infection with multiple pathogens could have potentially revealed heterozygous advantage, assuming they are able to present a wider array of antigen peptides to T-cells to initiate the specific immune response (Hughes and Nei, 1992).

The selected infection mode by bath immersion (Lutwyche et al., 1995) represents a valuable standardized technique, where all fish used in this experiment were exposed to almost the same bacterial charge at the same time. The synchronicity of the infection could explain the period of high mortality rate that we observed (around 40 h) compared to published results of 6, 10 or 12 days with co-habitation infection method (Lohm et al., 2002; Grimholt et al., 2003; Kjoglum et al., 2006). Questions were previously raised regarding the rapid mortality occurring in these types of 'artificial' infections, especially pertaining to the sole contribution of the adaptive immunity (Grimholt et al., 2003). Because of the correlation of MH class IIβ genotype with survival in this model with a high mortality rate (<4 days), another avenue of investigation could be the effect of MH genotypes in the initiation of the immune response. Future studies could address the evaluation of early immune functions following infection in function of MH class II genotype. Those studies could include assay for the activation and proliferation of lymphocytes, as well as phagocytic activity and cytokine quantification (Kollner et al., 2002).

To conclude, this study brings further support to the hypothesis that specific MH class IIβ alleles can be associated to disease resistance or susceptibility in the brook charr, as reported previously for Atlantic salmon (Langefors et al., 2001; Lohm et al., 2002; Grimholt et al., 2003; Kjoglum et al., 2006). The results also demonstrated that the level of resistance or susceptibility is affected by specific alleles' combinations, which suggest different levels of dominance between them. However, the results presented in this study were obtained from a single pathogen infection, and it is likely that the fitness of different alleles would differ for different pathogens and

shifts over time (Penn and Potts, 1999). Consequently, future studies on the brook charr should compare different pathogens and combined infections. Even if precautions have been taken to control it, we cannot totally exclude a genetic background effect of co-varying genes closely linked to the MH class IIβ genes in the observed results. Finally, it should be noted that the resistance or susceptibility effect could also be imputed to particular residues on the alpha chain of the MH class II. Knowing that they are co-segregated (Stet et al., 2002), it should be interesting to sequence both chains of the MH class II and eventually modelize the different proteins. In combination, the identification of the major antigenic peptides derived from *A. salmonicida* could also be of importance for our understanding of the virulence of this pathogen and to refine the presentation complex model. The identification of a theoretical consensus resistance allele from sequences of different salmonids could also be relevant for the prediction of performance of new alleles to be discovered.

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