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Major histocompatibility complex loci are associated with susceptibility of Atlantic salmon to infectious hematopoietic necrosis virus

Kristina M. Miller[a], James R. Winton[b], Angela D. Schulze[a], Maureen K. Purcell[b] & Tobi J. Ming[a]
[a]*Department of Fisheries and Oceans Canada, Pacific Biological Station, Nanaimo, BC, V9T 6N7, Canada (e-mail: millerk@pac.dfo-mpo.gc.ca)*
[b]*Western Fisheries Research Center, 6505 NE 65th St., Seattle, WA 98115, U.S.A.*

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Synopsis

Infectious hematopoietic necrosis virus (IHNV) is one of the most significant viral pathogens of salmonids and is a leading cause of death among cultured juvenile fish. Although several vaccine strategies have been developed, some of which are highly protective, the delivery systems are still too costly for general use by the aquaculture industry. More cost effective methods could come from the identification of genes associated with IHNV resistance for use in selective breeding. Further, identification of susceptibility genes may lead to an improved understanding of viral pathogenesis and may therefore aid in the development of preventive and therapeutic measures. Genes of the major histocompatibility complex (MHC), involved in the primary recognition of foreign pathogens in the acquired immune response, are associated with resistance to a variety of diseases in vertebrate organisms. We conducted a preliminary analysis of MHC disease association in which an aquaculture strain of Atlantic salmon was challenged with IHNV at three different doses and individual fish were genotyped at three MHC loci using denaturing gradient gel electrophoresis (PCR-DGGE), followed by sequencing of all differentiated alleles. Nine to fourteen alleles per exon-locus were resolved, and alleles potentially associated with resistance or susceptibility were identified. One allele (*Sasa*-B-04) from a potentially non-classical class I locus was highly associated with resistance to infectious hematopoietic necrosis ($p < 0.01$). This information can be used to design crosses of specific haplotypes for family analysis of disease associations.

Introduction

Genes of the major histocompatibility complex (MHC) are crucial elements of adaptive immunity in vertebrate organisms and have been linked to resistance to numerous pathogenic diseases (reviews in Apanius et al. 1997, Lamont 1998). MHC genes encode polypeptides which recognise and bind both self and foreign peptides and present them to T-cells. MHC-bound foreign peptides are recognised by the T-cells which signal the induction of a cellular or humoral immune response. For the most part, foreign peptides produced by the degradation of intracellular pathogens (e.g. viruses) are bound by MHC class I molecules and presented to cytotoxic T-cells, eliciting a cellular immune response and killing of the cell. Alternately, foreign peptides produced extracellularly (e.g. most bacteria) are bound by MHC class II molecules and presented to helper T-cells which secrete cytokine mediators required for humoral (antibody), cytotoxic and inflammatory immune responses (Brodsky et al. 1999). Because the peptide binding ability varies by the composition of the peptide binding residues (PBR) of the MHC molecules, some pathogens may escape recognition by certain MHC molecules; this can lead to increased susceptibility of individuals homozygous for those alleles (Potts & Slev 1995). Alternatively, resistance to pathogens may be derived through high affinity binding

of certain peptides by specific MHC alleles (Sidney et al. 1996). However, the strong potential for MHC-based disease associations is significantly reduced in most vertebrate species by the presence of multiple polymorphic MHC loci, thereby reducing the effects of individual allele binding repertoires and creating redundancy in the binding capabilities of the MHC as a whole (Potts & Slev 1995, Kaufman et al. 1995).

The recognition and binding of foreign peptides is the primary function of classical MHC genes. Classical MHC genes can be defined by key conserved N- and C-terminal residues in their peptide binding site as well as a high rate of non-synonymous mutation in other PBRs, their ubiquitous expression, high levels of polymorphism, and the retention of old allelic lineages achieved through balancing selection (Nei & Hughes 1991). The number of classical MHC classes I and II genes varies both within and among species. Within species, haplotype variation creates different numbers and compositions of MHC molecules contained within individuals. Among species, different levels of MHC gene duplication exist as MHC genes are repeatedly gained and lost through expansion and contraction of the MHC (Parham 1994, Miller et al. 2002). Although theoretically the peptide binding repertoire should increase with the number of MHC variants carried by an individual, there appears to be a limit to the number of MHC variants tolerated. This limit may stem from restriction of the T-cell repertoire through negative selection by MHC genes (Takahata 1995). In general, most species contain two to three classical MHC classes I and II loci. Species with higher numbers of MHC class I and/or class II loci use haplotype diversity to reduce the number of MHC variants per individual. However, seven or more expressed loci have been observed in some non-mammalian species (Murray et al. 2000, Sammut et al. 1999, Malaga-Trillo et al. 1998, Timon et al. 1998), and in one teleost species, Atlantic cod, as many as 42 expressed MHC class I loci have been isolated from a single individual (Miller et al. 2002).

Non-classical MHC genes are generally derived from classical genes but carry mutations in key residues that limit or eliminate their ability to bind peptides (Parham 1994). Non-classical MHC genes thus evolve new immune system functions, often playing a supportive role for classical genes or binding only a very specific set of peptides. Highly degenerate non-classical genes can even evolve functions that are not related to immunity at all. Non-classical MHC genes are generally not polymorphic, and their expression is limited to certain tissues and/or specific stages of development. However, the distinction between classical and non-classical MHC genes can be obscure at times, as non-classical genes are continuously evolving from classical genes, and many intermediates exist.

Most MHC-based disease associations documented in mammals are relatively weak and are greatly influenced by background genes (Apanius et al. 1997). Conversely, in chickens, which contain a 'minimal essential MHC' consisting of single highly expressed classes I and II loci, a number of strong MHC-based disease associations exist (Kaufman & Salomonsen 1997). These observations led Kaufman and Salomonsen to speculate that the lack of redundancy in the chicken MHC results in stronger levels of pathogen-driven selection, a situation that ultimately may make chickens more susceptible to disease than most mammals. Interestingly, for the most widely recognised MHC-associated disease (Marek's disease of chickens), resistance is not due to a higher affinity binding of a pathogenic peptide to a specific MHC molecule. Instead, resistance derives from the reduced expression of the MHC resistance alleles, resulting in a stronger innate immune response for individuals carrying these alleles (Kaufman et al. 1995). However, other diseases of chickens, such as Rous sarcoma virus, have more classical associations with MHC molecules, whereby the differential binding capabilities of the MHC alleles favours resistance or susceptibility to the disease (Kaufman et al. 1995).

The number of expressed MHC genes varies greatly among the teleost fishes. Most early teleost species, or euteleosts, contain 1–3 classical MHC classes I and II genes, while extensive gene duplication in the neoteleost species has greatly expanded the numbers of MHC loci (Miller & Withler 1998, Miller et al. 2002). Salmon, a somewhat intermediate euteleost, contain only a single MHC class II gene (DAB), and thus far, only one confirmed classical class I gene (UBA) has been found (UBA, Shum et al. 2001, Aoyagi et al. 2002, Miller & Withler 1996). In addition, two potentially non-classical class I genes have been discovered. The 'B' locus shares homology with the classical UBA locus and its status as a classical or non-classical gene is yet to be conclusively established (termed B by Miller & Withler 1998 or U71 by Shum et al. 2002). Alternately, the 'UAA' locus is considered a non-classical locus as it is ancient and highly differentiated from all classical loci (Shum et al. 1999).

Finally, there exists at least one psuedogene that has likely lost its function altogether (UFA – Aoyagi et al. 2002; U41 – Shum et al. 2002). Our experience in the aquaculture of salmon and trout suggests that salmonid species are quite susceptible to disease, and variation in degrees of susceptibility exist among stocks (Withler & Evelyn 1990). Given the limited numbers of classical MHC genes found in salmon, there may exist a higher potential for strong associations of MHC alleles with resistance or susceptibility to disease, as observed in chickens. Thus, in the move towards mapping QTL markers in salmon for use in selective breeding programs, targetting the MHC genes in particular may yield useful QTL markers for disease resistance.

The fish rhabdovirus, infectious hematopoietic necrosis virus (IHNV), is responsible for extensive losses among several species of salmon and trout in many areas of the world (Bootland & Leong 1999, Wolf 1988). Typical of most rhabdoviruses, IHNV has a relatively broad host range and is among the oldest and best-studied viral pathogens of fish. Historically, IHNV was endemic among many populations of wild Pacific salmon in the western portion of North America. During the past several decades, the virus has spread to stocks of cultured rainbow trout, *Oncorhynchus mykiss*, in other areas of the United States, Asia and Europe, probably as a result of the movement of infected fish or eggs (Winton 1991). More recently, IHNV has been recovered from both salmonid and non-salmonid hosts in the Pacific Ocean (Kent et al. 1998, Traxler et al. 1997) suggesting that the virus is endemic among certain marine fish species as well.

IHNV causes a disease characterised by acute viremia with resulting haemorrhage and widespread necrosis of major organ systems (Wolf 1988). Depending upon the species and size of fish, strain of virus, and environmental conditions such as temperature, an outbreak of infectious hematopoietic necrosis (IHN) may result in explosive and untreatable losses that can approach 100% (Winton 1991, Wolf 1988). Currently, the only method for control of IHN is avoidance of exposure to the causative virus through the use of virus-free stocks of fish, pathogen-free water supplies, and strict sanitation procedures (Winton 1991, Wolf, 1988). Vaccines to control IHN are in the experimental stage (Winton 1997, 1998) as are genetic approaches based on the selection of disease resistant stocks (Parsons et al. 1986, Bootland & Leong 1999, Kono et al. 2000). The purpose of this study was to determine if alleles within loci of the MHC of Atlantic salmon were associated with differences in susceptibility to IHNV.

Methods

Fish

Approximately 400 juvenile (4–5 g) Atlantic salmon were obtained from Cyprus Island Inc. in Rochester, Washington, a commercial facility with no history of IHNV. The fish originated from an outbred population of broodstock maintained at the site, and both broodstock and juveniles were reared in pathogen-free freshwater. Fish were transported to the Western Fisheries Research Center in Seattle, WA and reared in 15°C, pathogen-free freshwater until used.

Virus

IHNV isolate 4814 was provided by R. Busch (Seattle, Washington). This isolate was recovered from Atlantic salmon reared in marine netpens in British Columbia and associated with mortality in this species. The virus was propagated using monolayer cultures of the epithelioma papillosum cyprini (EPC) cell line to produce a stock containing 6.32×10^6 plaque-forming units (pfu) ml^{-1} of tissue culture fluid.

IHNV Challenge

Fish were assigned randomly to provide eight replicate groups of 50 fish for the challenge experiment. Each group of 50 fish was placed in 2 l of pathogen-free, 15°C freshwater in a 5 l aquarium provided with constant aeration. For the trial, 30 ml of minimum essential medium (MEM) was added to each of two aquaria containing groups of fish that served as negative controls, while the remaining six groups of fish were challenged by addition of 30 ml of MEM adjusted to provide IHNV concentrations in each of two aquaria of 1.05×10^2 pfu ml^{-1} (low dose), 3.16×10^3 pfu ml^{-1} (medium dose) or 9.48×10^4 pfu ml^{-1} (high dose). After 20 min in the static bath challenge, water flow was resumed at $\sim$1 l min^{-1}. The fish were observed daily for mortality. Fish were not fed during the 28-day challenge period, and once each day mortalities were collected and the dorsal or caudal fins excised and placed into 70% ethanol until assayed.

MHC genotyping

MHC class II molecules are dimers consisting of an α chain and a β chain. As these chains are encoded separately on the chromosome and both contain exons with PBR ($\alpha 1$ and $\beta 1$), both exons of the single MHC class II gene (DA) were analysed (DAA and DAB). MHC class I genes are dimers of an α chain and a $\beta 2$-macroglobulin molecule. Because the two PBR-encoding exons are linked on the α-chain ($\alpha 1$ and $\alpha 2$), we limited our survey to the $\alpha 1$ exon of the class I B locus. Due to the presence of numerous highly divergent allelic lineages in the two peptide encoding exons of UBA, and the inherent difficulties in genotyping highly differentiated alleles (seven to eleven primersets per exon are utilised in our laboratory), we utilised a microsatellite from the 3' untranslated region of the UBA locus for genotyping (Grimholt et al. 2002). The three exons and microsatellite from UBA were amplified separately using the primers and PCR conditions outlined in Tables 1 and 2. Alleles derived from the amplification of exons were differentiated by denaturing gradient gel electrophoresis (DGGE) with conditions outlined in Miller et al. (1999) and summarised in Table 1. Exons were amplified using GC-clamped primer sets. Polyacrylamide gels with parallel gradients (see Table 1 for locus-specific gradients) were poured using a Gradient Maker™ (Model 475 Gradient Delivery System; Bio-Rad, Hercules, Calif). Three standard sets containing all known alleles for each exon were loaded into four positions on each gel and used to directly identify individual alleles. Two microliters of PCR product were loaded into each well, and the gels were run using the conditions outlined in Table 1 with the Dcode™ electrophoresis system (Bio-Rad). Gels were stained with ethidium bromide and alleles were scored directly from the allelic ladders. Allele identifications were confirmed for a subset of individuals by direct sequencing from bands excised from the DGGE gels, with at least three confirmations

Table 1. PCR and DGGE conditions for MHC loci surveyed. PCR was carried out in a total volume of 50 µl containing 0.2–0.5 µg of DNA, 15 pmol of each primer, 200 µM of each dNTP and 2.5 U UltraTherm™ DNA polymerase. A GC-clamp, denoted^{CL}, with the sequence 5′-GCCCGCCCCCGCGCCCTGCCCGCGCCCCGCGCCGCCCGCCC-3′, was added to 5′ end of the Sense primer(s) for the B locus and the antisense (as) primer for the DAB and DAA loci.

Locus	Primer	Primers used	PCR conditions	DGGE conditions	Standard alleles
UBA		UBA-3′UTs × UBA-3′UTas	94 deg/45″ 54 deg/45″ 70 deg/45″ 30 cycles 72 deg/10′	ABI 377	12 alleles
	UBA-3′UTs	5′-GGAGAGCTGCCCAGATGACTT-3′			
	UBA-3′UTas	5′-GTTTCATTACCACAAGCCCGCTC-3′			
B		A1B-s2^{CL} × A1B-I2as1	94 deg/1′ 50 deg/2′ 68 deg/2′ 35 cycles 72 deg/10′	45%–60% denat. 7.5% acryl. 120V/6hrs @54 deg & 45%–60% denat. 7.0% acryl. 120V/6hrs @55 deg	9 alleles
	A1B-s2^{CL}	5′-GCCCTGAAGTATTTCTACACTGCG-3′			
	A1B-I2as1	5′-TGAGGTAAAGATGAAGGAC-3′			
DAB		B1s × B1as^{CL}	94 deg/1′ 50 deg/2′ 68 deg/2′ 34 cycles 72 deg/10′	45%–60% denat. 7.5% acryl. 60V/15hrs @56 deg & 60V/15hrs @54 deg	13 alleles
	B1s	5′-CCGATACTCCTCAAAGGACCTGCA-3′			
	B1as^{CL}	5′-GGTCTTGACTTG[AC]TCAGTCA-3′			
DAA		CIIA1s × CIIA1Ias^{CL}	94 deg/1′ 50 deg/2′ 71 deg/2′ 35 cycles 71 deg/10′	45%–60% 7.5% acryl. 120V/6hrs @54 deg & 120V/6hrs @56 deg	9 alleles
	CIIA1s	5′-CTCTTCTGGGTTCTTGTAAGC-3′			
	CIIA1Ias^{CL}	5′-GGTTTCTTTTCTCAGTTCTGCAT			

Abbreviations are as follows: denat. – denaturant, acryl. – acrylamide.

Table 2. Fisher's exact test for each challenge group and exact tests for stratified RXC tests.

	Low		Mid		High		Exact estimates		
	p	OR	p	OR	p	OR	p	$p_{corrected}$	OR
UBA									
1	NA	NA	NA	NA	1.0000	∞	1.0000	1.0000	∞
2	0.7769	0.812	0.4618	2.965	1.0000	1.400	0.7500	1.0000	1.237
3	1.0000	∞	0.0077	0.000	1.0000	∞	0.1622	0.8804	0.188
4	0.2413	1.573	0.0459	0.435	0.8296	0.889	0.7547	1.0000	0.912
5	0.7873	0.777	0.7665	1.406	0.7907	0.807	0.9018	1.0000	0.920
6	0.3855	0.678	0.0494	6.922	0.0454	∞	0.1722	0.8965	1.646
7	1.0000	1.250	1.0000	1.016	0.3806	0.463	0.7792	1.0000	0.773
8	0.2616	0.3526	1.0000	0.756	1.0000	1.282	0.5457	0.9999	0.691
9	0.5246	3.173	0.495	0.500	0.1734	0.000	0.9750	1.0000	0.680
10	0.3052	0.475	0.6877	2.102	0.6287	0.613	0.6706	1.0000	0.753
11	0.6204	1.395	0.6877	2.102	0.5215	0.705	0.8117	1.0000	1.177
12	1.0000	∞	1.0000	∞	1.0000	∞	0.4411	0.9991	∞
B									
1	0.2879	∞	1	∞	0.5813	∞	0.1439	0.7885	∞
2	1	1.048	0.8032	1.324	1	1.060	0.7743	1	1.131
3	0.8803	1.091	0.4537	0.739	0.7052	1.187	1	1	0.998
4	**0.1630**	**0.522**	**0.0484**	**0.371**	**0.0071**	**0.279**	**0.0008**	**0.0080**	**0.379**
5	1	1.123	0.6903	2.473	0.3472	∞	0.3168	0.9778	1.758
6	1	1.246	0.6735	0.784	0.4934	0.630	0.7536	1	0.799
7	0.8357	1.134	0.0303	7.077	0.2554	2.571	0.0595	0.4585	1.899
8	1	1.878	1	0.788	0.5057	0.518	1	1	0.995
9	1	0.615	NA	NA	NA		1	1	0.617
10	0.1448	0	1	1.333	1	∞	1	1	0.837
DAB									
1	1	1.164	0.3401	∞	0.5828	∞	0.3297	0.9945	2.213
2	**1**	**0.953**	**0.1882**	**0.596**	**0.0392**	**0.444**	**0.0550**	**0.5207**	**0.650**
3	0.4702	0.765	0.6472	1.377	0.0918	2.383	0.4894	0.9998	1.219
4	1	1.044	0.2666	0.543	0.0873	0.348	0.1368	0.8523	0.594
5	0.2418	1.751	1	1.028	0.1907	0.523	0.9084	1	1.067
6	1	∞	1	∞	NA	NA	0.8982	1	∞
7	1	0.691	1	∞	1	∞	1	1	1.757
8	0.4472	0.561	0.5948	0.461	0.1077	0.123	0.1111	0.7837	0.370
9	**0.5303**	**1.660**	**0.0728**	∞	**0.0736**	∞	**0.0084**	**0.1039**	**4.4620**
10	0.5303	1.660	0.2212	0.371	1	1.574	1	1	0.940
11	0.7183	0.682	0.3401	∞	0.0775	∞	0.1877	0.9330	2.283
12	0.3217	0.442	0.3401	∞	1	1.850	1	1	0.986
13	NA	NA	NA	NA	1	∞	1	1	∞
DAA									
1	1	1.109	0.2920	3.814	1	1.021	0.4979	0.9980	1.489
2	1	0.909	0.0729	0.371	0.086	5.976	1	1	1.002
3	0.3020	0.691	0.2714	0.657	0.1024	0.480	0.0354	0.2770	0.620
4	0.7233	0.723	0.5743	∞	0.239	0.423	0.6701	1	0.745
5	0.7031	0.842	1	0.998	0.2622	1.899	0.8017	1	1.098
6	0.0159	3.436	1	1.048	0.1376	0.429	0.5188	0.9986	1.246
7	0.6119	1.391	0.0216	∞	0.3752	2.321	0.0367	0.2857	2.369
8	0.6513	0.479	0.6088	0.528	1	1.138	0.5669	0.9995	0.639
9	0.6513	0.479	1	∞	1	1.375	1	1	0.876

Each table shows the statistical results for one of the four loci examined: MHC class I UBA, B and MHC class II DAB and DAA. Alleles with significant p-values less than 0.01 (although not necessarily significant under $p_{corrected}$) for the exact estimates are shown in bold, with frequencies in Figure 2.

per allele-type to check whether all alleles were, in fact, differentiated.

Statistical analysis

Tests for genic homogeneity and fit to Hardy–Weinberg expectations were performed using GENEPOP (V.3.1) (Raymond & Rousset 1995). Survival curves were estimated by Kaplan–Meier and compared by the log-rank test using SYSTAT V.8 (SPSS Inc., Chicago, IL). Association of MHC alleles to mortality or survival for each challenge group was tested by Fisher's exact tests (Svejgaard et al. 1974). To analyse allele association across the three challenge groups combined, we first determined whether the odds ratios (OR) for each allele across all three challenges were homogeneous. If homogeneity was not rejected, the exact confidence interval for the common OR was calculated and tested against the null hypothesis of unity. All exact tests were done using STATXACT V.4 (Cytel Software Inc.). When appropriate, p-values were corrected for multiple comparisons as $p_c = 1-(1-p)^n$, where n = number of alleles analyzed at a locus (Svejgaard and Ryder 1994).

Results

Challenge study

The challenge study used three doses of IHNV to insure a range of responses. Both mortality and mean-day-to-death during the 28-day challenge period showed a strong dose-response (Figure 1). Fish dying during the experiment showed typical signs of IHN. At the end of the experiment, 39% of the fish survived the low dose, 27% survived the mid dose, and 20% survived the high dose.

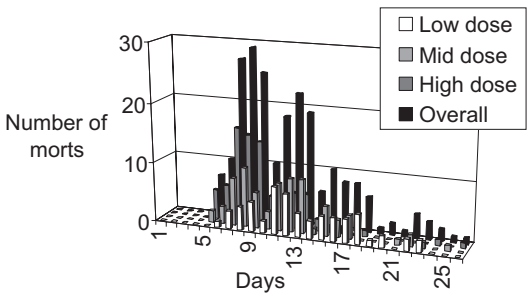

Figure 1. Challenge-dosage curve, depicting time, in days, to mortality after challenged with low, medium and high doses of IHNV. All mortality ceased after 25 days, and the experiment ended, and surviving fish sacrificed on Day 28.

Prior to the experiment, fish were randomly assigned to replicate and treatment groups. Genetic homogeneity was confirmed across all replicates and treatments (all loci combined p = 0.10). Each challenge dose (low, medium, and high titre) had two replicates. Comparisons of survival curves between the two replicates within each challenge dose detected no significant differences in survival rate (high dose, p = 0.45; medium dose, p = 0.337; low dose, p = 0.058); consequently, replicates were pooled for all further analyses.

MHC analysis

Expected heterozygosities were high for all three loci, with values of 0.81 for class I UBA, 0.76 for class II DAA, 0.79 for class I B, and 0.85 for class II DAB. Tests for Hardy–Weinberg equilibrium revealed a significant heterozygote deficiency at the class I UBA locus (p = 0.00; Ho 0.70), possibly indicative of the presence of null alleles or haplotype diversity at this locus. There was no statistically significant relationship with heterozygosity at any of the loci and susceptibility to IHNV.

Twelve class I UBA microsatellite alleles were detected, while DGGE typing of MHC exons detected, 13 alleles for class II DAB, 10 alleles for class I B, and 9 alleles for class II DAA. Each allele was tested for association to challenge outcome (survival or mortality) using Fisher's exact tests. Analysing each challenge dose separately, several loci were significant ($p < 0.05$) but none were highly significant and capable of withstanding p-value correction. Next, we evaluated each allele across all three challenges (strata) using a stratified RXC test. None of the alleles rejected homogeneity of OR across strata so the common odds ratio and the exact p-value were calculated for each allele. Using this approach, the class I *Sasa*-B-04 allele, which was three times more common in survivors than in the fish that died (morts) in the high dose group, was highly significant (p = 0.0008) and remained significant even after correction (p = 0.0080; Table 2 and Figure 2a). The OR for this allele was 0.38 indicating that the allele is protective. Sixty-one percent of the fish surviving the high dose of IHNV carried allele *Sasa*-B-04, whereas only 21% of fish that died in the high dose challenge group carried the allele. The class II *Sasa*-DAB-09 allele, which was only found in fish dying in the mid- and high-dose challenges, was also significant before correction (p = 0.0084) but was not significant after correction (p = 0.1039); the OR was 4.462 indicating that the allele is associated

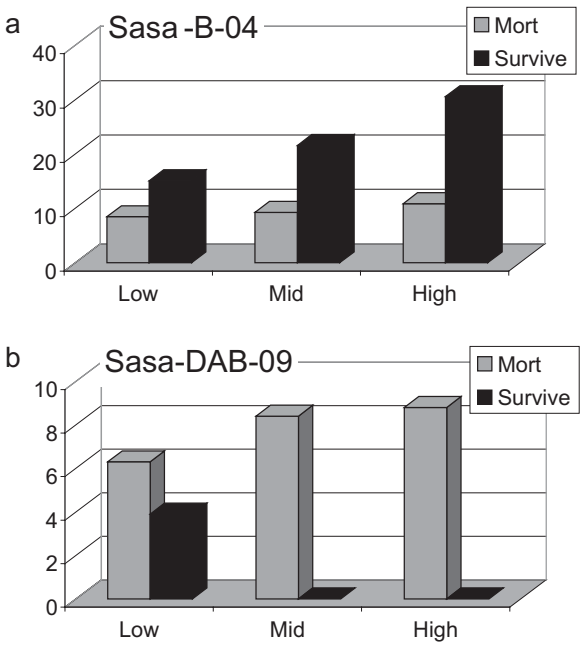

Figure 2. Frequency histograms for alleles with significant associations to IHN. Histograms show the percentage of survivors *versus* mortalities under each challenge dose, with the two replicates under each dose combined. (a) *Sasa*-B-04, highly significant association with resistance, (b) *Sasa*-DAB-09, suggestive allele not significant after p-value correction for multiple tests.

with increased susceptibility (Figure 2b). Additionally, the *Sasa*-DAB-02 allele, which was nearly significant before correction ($p = 0.055$, $p = 0.52$ after correction), was almost twice as common in the survivors as compared to the morts of the high dose (0.4 vs. 0.22) (not shown). None of the class I UBA alleles were significantly associated with susceptibility or resistance to IHN.

Discussion

Establishing associations of genetic loci with disease resistance can be daunting, given the vast number of potential gene candidates. With the sequencing of the human genome and the development of widely dispersed single nucleotide polymorphisms (SNPs), genome-wide scans can now be used to identify resistance markers to human pathogens through linkage disequilibrium mapping (Kruglyak 1999). However, this approach is not only very expensive, but requires detailed linkage maps containing numerous polymorphic markers spaced evenly throughout the genome, something that is not yet available for salmon. Another approach is to select potential candidate genes that are either mechanistically established to play a role in the disease etiology of a specific pathogen, or to simply choose genes that are associated with a number of other related diseases. We chose the latter approach, and examined three genes of the MHC in salmon. This approach seemed plausible, given the vast number of diseases at least weakly associated with MHC genes in a variety of species. The similarity of the simple MHC of salmon with that of chickens, for which MHC associations are strong, provided an even greater impetus to test this complex for associations in salmon.

The criteria to determine significance in MHC association studies is still under debate. Even with a scaled down number of candidate genes, the need for p-value corrections for multiple tests can require very large sample sizes to establish statistically significant associations (Svejgaard & Ryder 1994, Apanius et al. 1997). One approach around this is to conduct a study twice, the first time to establish a hypothesis for loci/alleles with associations, and the second to test the hypothesis (Hill 1991). In following this two-pronged approach, we present the data from the first experiments, conducted to establish a hypothesis, herein. Experience from the human HLA field suggests that associations with p-values between 0.01 and 0.05 are often not reconfirmed in subsequent studies (Svejgaard & Ryder, 1994). In this study, the *Sasa*-B-04 allele was associated with resistance to IHN and was observed at a frequency of 0.09 to 0.13 in the mid and high dose morts and 0.21 to 0.33 in the survivors. We used a conservative correction procedure but still detected a significant association for the *Sasa*-B-04 allele with a p-value less than 0.01.

A weak association of the MHC class II gene and IHNV resistance was documented in a recent study conducted on rainbow trout/cut-throat trout backcrosses ($p = 0.024$; Palti et al. 2001). Their study was based on the class II intron sequence between $\beta 1$ and $\beta 2$, as opposed to our analysis of the coding $\beta 1$ sequence, so unfortunately, no specific comparison of alleles is possible. Previous to their study, cutthroat trout were identified as being highly resistant to IHNV, while rainbow trout were susceptible, hence, they theorised that any allele conferring resistance should come from cutthroat trout. Contrary to their expectations, the allele associated with resistance was from the rainbow trout. As the p-values were

relatively low, this study requires confirmation using a greater number of families.

In most genetic associations, the MHC genes themselves do not cause the disease, they merely influence the susceptibility or resistance to the disease. Additional genes may also be involved in susceptibility to a particular disease, and may or may not interact with the MHC genes. Hence, associations of MHC alleles may depend upon the genetic background of individuals, and different strains or populations may carry different allelic associations (reviewed in Apanius et al. 1997). If MHC-based associations are to be used to enhance disease resistance for the aquaculture industry, it would be judicious not only to re-test preliminary associations on the populations or strains from which they were originally found, but also to test associations on other aquaculture strains before using them for selective breeding or vaccine development. The Atlantic salmon utilised herein have been derived over the past 30 years from multiple strains of Atlantic salmon, including two from Norway, three from the East Coast of Canada, and a single strain from Scotland. Although the strains have been extensively interbred, microsatellite analysis suggests that they most closely resemble the fish from one of the Norway strains which was added to the broodstock in the early 1990s (Withler & Miller, unpubl. data).

Associations of MHC with susceptibility to specific pathogens can also be derived through linkage disequilibrium with a susceptibility locus, and not the MHC gene itself (Bengtsson & Thomson 1981). In situations where there is not a causal relationship with disease, it can be difficult to discern whether associations are due to linkage or the associated gene. The statistical procedures used herein derives association values that are directly correlated with linkage disequilibrium values, such that the locus with the highest values should be in the closest linkage disequilibrium with the susceptibility locus, or may actually be the susceptibility locus (Svejgaard & Ryder 1994). Unlike the mammalian MHC, whereby class I and II genes reside on a single chromosome, the class II DAB/DAA, class I UBA and class I B genes of salmon each reside on separated chromosomes (Phillips et al. 2003). The *Sasa*-B locus contained the highest linkage disequilibrium values. One may surmise from this that the B locus is a susceptibility locus; however, as many other class Ib loci reside on the same chromosome and are closely linked to the B locus (ibid; Miller unpublished data), confirmation of this requires association analyses of loci linked to B and establishment of the role of the class I B locus in the IHN disease etiology.

One cannot discount the possibility that the associations found herein are an artefact of strain or family differences in survival. Strain/population differences in susceptibility to a variety of pathogens have been documented in coho and chinook salmon (Withler & Evelyn 1990, Beacham & Evelyn 1992a,b, Balfry et al. 2001). A preliminary analysis of about half of the fish utilised herein suggested that the fish were derived from at least 30 families. This finding, and the fact that the *Sasa*-B locus was in Hardy–Weinberg equilibrium (hence no inbreeding effects), suggest that family effects may be minimal. In addition, no effort has been made by this particular hatchery to keep the strains of Atlantic salmon separate, hence, they are likely highly introgressed. However, in our next study, we will analyse the MHC composition of the three strains of Atlantic salmon used as broodstock and we will design crosses to examine both strain differences and allele-specific associations with susceptibility to IHN.

The MHC class I *Sasa*-B locus was originally isolated in gDNA (Grimholt et al. 1994, Miller and Withler 1998). There are no published cDNA sequences for the MHC class I *Sasa*-B locus, and it has not been shown to exist on northern blots, although it was not probed specifically (Aoyagi et al. 2002). For this reason, the B locus has not previously been considered a coding locus. However, recent work in three laboratories suggests that it is, in fact, present in cDNA but is expressed at lower levels than the UBA gene (Shum et al. 2002, K.M. Miller, unpubl. data, John Hansen, pers. commun., Center of Marine Biotechnology, Baltimore, Maryland). The question remains, is the B locus a classical MHC gene, or is it non-classical? Other than a potential relatively low level of expression, the B locus contains some features of a classical locus. It is highly polymorphic, with 10 alleles and a heterozygosity of 0.79 ($n = 300$) in Atlantic salmon $\alpha 1$, and the ratio of dN to dS in the PBR is greater than one for the $\alpha 1$ exon of Atlantic salmon (1.77 herein; unpubl. data), suggesting that selection maintains alleles with differential binding repertoires. However, a previous analysis of the B locus $\alpha 2$ exon in Pacific salmon did not yield a dN to dS ratio greater than one, and analysis of the key conserved N- and C-terminal peptide binding sites for classical loci suggests a lack of conformance of some residues (Shum et al. 2002). Hence, as the B locus has properties of both classical and non-classical loci, it is likely in the process of degenerating to a non-classical

gene, but may still be capable of binding a reduced set of peptides, or at least still play a role in immune system function.

The information gained from this study can be used to conduct a second challenge experiment, this time using families derived from parents with known MHC genotypes, to verify linkage of the *Sasa*-B-04 allele with increased survival from IHNV challenge. In addition, we will specifically target alleles with nearly significant associations, such as *Sasa*-DAB-09 and -02. This second study will confirm the statistical relevance of the associations found herein for the mixed strain of Atlantic salmon used in this study. If associations are confirmed, further analysis of additional aquaculture strains may be in order.

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