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MHC mediated resistance to *Piscirickettsia salmonis* in salmonids farmed in Chile

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ABSTRACT

Piscirickettsia salmonis is the most persistent and threatening pathogen for Chilean aquaculture. In our search for alternatives to control the disease, we analyzed class II polymorphic genes of the Major Histocompatibility Complex (MHC) in juvenile fish trying to find alleles that may confer resistance or susceptibility to the agent in the three major salmonid species cultured in Chile: *Salmo salar*, *Oncorhynchus mykiss* and *Oncorhynchus kisutch*. DNA from farmed fish naturally exposed to the pathogen in different aquaculture centers in Chile were analyzed via PCR for the MHC class II alpha and beta loci (*DAA* and *DAB*), and characterized by denaturant gradient gel electrophoresis (DGGE) followed by DNA sequencing. The comparison between alleles present in either healthy or diseased individuals clearly demonstrated allelic differences between the two populations, specifically for the *DAB* locus in the three species and only for the *DAA* locus in *S. salar*. We conclude that a thorough analysis of the allelic differences identified between fish naturally susceptible/resistant to *P. salmonis* could potentiate MHC to become a powerful genetic marker for both prophylactic and breeding purposes.

1. Introduction

Salmonid Rickettsial Septisemia (SRS) is a bacterial disease caused by the facultative pathogen *Piscirickettsia salmonis*, a gram negative bacteria isolated for the first time in Southern Chile in 1989 (Fryer and Hedrick, 2003). Although the disease has also been described in the Northern hemisphere (Brocklebank et al., 1993; Olsen et al., 1997; Rodger and Drinan, 1993), its impact has largely been associated with the Chilean farmed salmon industry, where it is responsible for causing severe mortalities that average 10% yearly and that have reached 30% to 90% in some affected aquaculture centers (Lannan and Fryer, 1993; Reid et al., 2004). Chile is the second largest producer of farmed salmon worldwide and the effect of SRS has by far surpassed the impact of any other infectious diseases, with annual losses of US$ 150 million (Wilhelm et al., 2006). Despite the positive effects of newly developed good biosecurity practices, SRS continues causing high mortalities every year. Several vaccines have been recently available in the market (Sommerset et al., 2005), but they have not yielded the expected protection and in fact, the disease has maintained a negative impact on the salmon industry (Wilhelm et al., 2006). As a consequence, the main countermeasure prevailing in Chile has been the massive use of antibiotics, which surpass the 100 metric tons per year (Cabello, 2006).

Selecting breeding for SRS resistance appears as the most attractive alternative to the use of antibiotics and vaccines; but given salmonids' average generation time (5 years (Hindar et al., 2004)), it would require decades before achieving commercially significant results. Alternatively, marker-assisted selection (MAS) could potentially speed up the process of genetic enhancement in breeding schemes (Meuwissen and Sonesson, 2004). MAS is still rarely applied to aquaculture (but see Ferguson and Danzmann, 1999) despite its potential for selective breeding in relation to aquaculture-related traits particularly difficult to measure such as disease resistance. Some of the most promising candidate genes for this type of studies are the genes of the Major Histocompatibility Complex (MHC) that are commonly associated with disease resistance in vertebrates (Grimholt et al., 2003; Kjøglum et al., 2006; Rakus et al., 2009; Xu et al., 2008). MHC genes encode membrane proteins involved in the distinction between self and non-self peptides, and as such represent the first line of defense of the adaptive immune response (Steinmetz and Hood, 1983). Since the adaptive response depends on capture and presentation of peptides by the MHC receptor, pathogens have evolved to produce proteins that are harder to bind. Correspondingly, the part of the MHC genes that encode the peptide binding residues (PBR) have co-evolved into the most polymorphic coding region known to date in vertebrates, in order to increase the possibility to survive disease outbreaks (Grimholt et al., 2003; Reche and Reinherz, 2003). This high polymorphism pattern is

Abbreviations: *Sasa*, *Salmo salar*; *Onmy*, *Oncorhynchus mykiss*; *Onki*, *Oncorhynchus kisutch*.

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settled in the species as a whole, with each individual carrying a unique set of MHC alleles responsible for conferring either resistance or susceptibility to a particular pathogen.

Here we compared the MHC class II alpha and beta genotypes of individuals susceptible and resistant to *P. salmonis* outbreaks in three of the most commonly farmed salmonid species: *Salmo salar, Oncorhynchus mykiss and Oncorhynchus kisutch*. We focused our study on the peptide binding region (PBR) of the MHC class II receptors, known to be involved in the specific immune responses against bacteria.

We found significant differences in the MHC class II allele distribution of susceptible and resistant salmonids. Also, we found specific alleles for MHC class II alpha and beta that were significantly correlated with resistance or susceptibility to *P. salmonis* in all species studied.

2. Materials and methods

2.1. Origin of the samples, fish infection and DNA extraction

Juveniles from three salmonid species were randomly sampled from different hatcheries exposed to *P. salmonis* bacteria in Southern Chile. A total of 57 *S. salar* individuals from 5 hatcheries, 44 *O. mykiss* individuals from 3 hatcheries, and 88 *O. kisutch* individuals from 9 hatcheries were analyzed. The origin of the eggs is unknown, but despite the fact that the three species are not native to Chile, it is known that 82% of the eggs currently used in the national salmon industry are domestically produced (http://www.fao.org/fishery/countrysector/naso_chile/es).

Individuals were initially classified as healthy or diseased based upon clinical symptoms. The presence of *P. salmonis* bacteria was then confirmed in liver, kidney and brain smears by IFAT test using a specific anti-*P. salmonis* monoclonal antibody and a secondary anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC) (Bios Chile I.G.S.A., Chile) (Ferguson and Danzmann, 1999; Grimholt et al., 2003).

Liver tissues were collected from all the juveniles and kept in 95% ethanol at −20 °C for DNA extraction. DNA was extracted using Chelex-100 resin (Bio-Rad Laboratories, CA, US) according to the manufacturer's instructions.

2.2. Amplification and identification of MHC class II genes

The second exon of the MHC class II α (*DAA* locus) and β (*DAB* locus) genes was amplified by PCR using the same primers and conditions as in Gómez et al. (2010).

To identify putative different alleles of the MHC class II α and β present in our population, individual PCR products were resolved by Denaturing Gradient Gel Electrophoresis (DGGE) using the Dcode™ Universal Mutation Detection System (Bio-Rad Laboratories, CA, US) as previously described (Gómez et al., 2010). Individual bands corresponding to a single allele were purified, re-amplified and submitted for DNA sequencing at Macrogen Corporation (Seoul, Korea).

Sequences from both MHC class II loci and for each species were manually edited and aligned using the ClustalW sub-routine of Bioedit (Hall, 1999). The final length of the sequences considered for this study were 240 bp for the *DAA* locus of *S. salar*, 171 bp for the *DAA* locus of *O. mykiss* and of *O. kisutch*, 261 bp for the *DAB* locus of *S. salar*, and 219 bp for the *DAB* locus of *O. mykiss* and of *O. kisutch*.

Only nucleotide changes found in more than one individual were taken into account and alleles were defined on the basis of deduced amino acid sequence. Novel alleles sequences reported in this study have been uploaded into the NCBI GenBank database under the accession numbers: HQ839642–HQ839659.

2.3. Genetic differentiation test

To determine the level of heterozygosis in individuals at each farming center, Hardy–Weinberg probability test was performed based on a Markov-chain method using the GENEPOP software (Raymond and Rousset, 1995a). The parameters were set to 1000 dememorizations, 100 batches and 1000 iterations per batch and statistical differences were calculated by the Fisher's method with a significance level of 0.05.

In order to define tendencies, we pooled the data for each species considering two large populations “healthy” and “diseased” in every case, and all further analyses were done using this criterion.

Pairwise FST comparisons and exact tests for genetic differentiation between healthy and diseased fish were performed using Arlequin 3.01 (Excoffier et al., 2005). The parameters were set to 5000 permutations for the FST test and 10,000 Markov steps in the exact test. p-Values<0.05 were considered significant for this study and are indicative of differences between both populations.

DnaSP software (Librado and Rozas, 2009) was used to calculate the individual MHC dissimilarity as an estimate of the non-synonymous substitutions rate (*Ka*) between the two alleles within individuals at each locus and specie (Consuegra and Garcia de Leaniz, 2008). Differences in mean *Ka* between healthy and diseased individuals were tested for statistical significance using a *t*-test implemented in Excel (Microsoft Corp., Washington, US).

2.4. Association analyses

Allele and genotype frequencies for each locus, species and health condition were calculated using Genalex (Peakall and Smouse, 2006).

We used the L_D statistic (Hindar et al., 2004) to evaluate the association between MHC alleles from both loci with individual health status. This statistic considers a null hypothesis in which the allele distribution is homogeneous between two populations and an alternative hypothesis in which one or more alleles have the highest representation in a particular population. The statistic makes a multiple comparison similar to Bonferroni's test based on contingency tables. After every comparison the highest L_D value obtained is contrasted with the corresponding chi-squared value and a significance level of 0.0001 (Araneda et al., 2009).

We also calculated the contribution of every allele to the separation of the healthy and diseased populations performing a SIMPER analysis (Clarke, 1993) using the Primer 5 software (Plymouth Marine Laboratory, UK).

3. Results and discussion

To our knowledge, this is the first study correlating resistance or susceptibility of salmonids fish to the pathogenic bacteria *P. salmonis* with the variability at immunorelated genes. By comparing the MHC class II allele diversity of farmed salmonids that either survive or died during *P. salmonis* outbreaks, we were able to identify differences in allelic frequencies between healthy and diseased fish, strongly suggesting that particular alleles could reflect the ability of a fish to respond to the infection of this bacterial agent.

We analyzed a region covering most of the second exon of the MHC class II alpha (*DAA* locus) and beta (*DAB* locus) which contains the nucleotides encoding for the peptide binding region or PBR (Steinmetz and Hood, 1983). In the three salmonids species analyzed we found a high level of polymorphism at both loci. For the *DAA* locus, we found 12 different alleles in *S. salar*, 11 in *O. mykiss* and 5 in *O. kisutch*; while for the *DAB* locus, the number of sequences for each species was 13, 13 and 14, respectively. Some of these sequences have been already used to perform an evolutionary analysis of MHC class II in salmonids (Gómez et al., 2010), but 18 sequences are novel.