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Identification of a novel allele of peroxisome proliferator-activated receptor gamma (PPARG) and its association with resistance to *Aeromonas salmonicida* in Atlantic salmon (*Salmo salar*)

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ABSTRACT

Bacterial and viral diseases are major problems in Atlantic salmon aquaculture, but may be challenged through selection of brood stock with enhanced survival to diseases. Today's selection strategy is based on controlled challenge tests using siblings of the breeding candidates, and is thus indirect. Direct trait records on breeding candidates can potentially be provided through identification of genetic variation linked to the susceptibility to the disease. Peroxisome proliferator-activated receptor gamma (PPARG) is a lipid-sensing transcription factor primarily known for inducing fat-accumulation in adipocytes, but also in lipid-accumulating macrophages, in mammalian species. Here we report a novel allele of PPARG, *pparg-2*, in Atlantic salmon. *pparg-2* has an insertion of sixty nucleotides that encodes two additional copies of the almost perfect decapeptide motif, (F/C/Y)NHSPDR(S/N)HS, compared to the previously described *pparg-1*. *pparg-1* contains six copies of this repeat unit whereas eight copies are present in the novel *pparg-2* allele. *pparg-2* mRNA was detectable in kidney and spleen of random Atlantic salmon samples. Here, we studied the effect of *pparg-1* and *pparg-2* on survival upon challenge to a highly virulent bacterium, *Aeromonas salmonicida*, causing furunculosis, and the virus causing infectious salmon anaemia (ISA), respectively, in a Norwegian aquaculture population of Atlantic salmon. *ppar* alleles were found to be significantly associated with survival upon challenge to *A. salmonicida*, but not to ISA. *pparg-2* was the better allele in terms of survival in the challenge test for furunculosis, survival rates being 0.32, 0.40 and 0.42 for animals with the *pparg-1,-1*, *pparg-1, -2* and *pparg-2, -2* genotypes, respectively. We conclude that *pparg-2* is in linkage disequilibrium (LD) with, or identical to, a locus contributing to different susceptibility to furunculosis in Atlantic salmon. PPARG was mapped to linkage group eight (LG8) but could only be positioned on the male linkage map since all the informative parents in the mapping families were males. This is the first report showing an association between *pparg* alleles and an enhanced immune response in fish.

1. Introduction

The immune response to pathogens in teleost fish have many features in common with that of other higher vertebrates, but the ability of the fish to respond depends on the environmental conditions [1]. Innate, non-adaptive, protective mechanisms are the first line of defence against disease, and under intensive aquaculture conditions some of the innate defences are easily compromised, particularly the mucus and epidermal barriers. Furunculosis is caused by *Aeromonas Salmonicida* ($A_{sal.}$), and has affected wild and farmed salmonids throughout the world. The heritability of mortality due to furuncunlosis in Atlantic salmon (*Salmo salar*) is high ($h^2 = 0.48$), and selection towards increased resistance to the disease has been part of several breeding objectives in Norway since the mid-1990s [2,3]. Though the prevalence of furunculosis in salmon aquaculture has declined much due to the introduction of an effective vaccine in 1991 [4], not only it is still part of the breeding goal of some breeding companies, partly as a representative

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of bacterial diseases in general, but also because vaccination is considered a suboptimal solution. Vaccination may cause adverse side-effects like adhesions, granuloma formation and pigmentations sometimes leading to reduced growth, particularly prominent with the use of oil-based adjuvants [4]. An important aspect of furunculosis is the carrier-state, i.e.; in some cases, non-immunised fish appear to co-exist with highly virulent pathogens in a carrier state, without showing any signs of morbidity [5], so presumably the innate mechanisms of defence provide some degree of protection. Infectious salmon anaemia (ISA), is a virally induced disease that was first identified in Norwegian farmed fish in 1984 [6], and has later been identified in Canadian and Scottish farmed salmon [7,8]. ISA has been reported to cause 15–100% mortality during a disease outbreak [9]. Vaccination has not been allowed in Europe, while two commercial vaccines are available in Canada/USA [10–12].

Selection of breeding candidates with enhanced survival to disease is based on controlled challenge tests on siblings of the breeding candidates. Thus, the trait measure of the breeding candidates is indirect. The selection strategy could be made more efficient through identification of genetic variation linked to the trait, either the gene itself or genetic markers, which in turn could provide direct trait records on breeding candidates. So far, association between certain major histocompatibility complex class II alleles and susceptibility to furunculosis in Atlantic salmon has been reported [13,14]. The three peroxisome proliferator-activated receptor (PPAR) subtypes, PPARA, PPARB, and PPARG, belong to the superfamily of nuclear receptors. The DNA-binding domain of PPAR is highly conserved across species and contains two zinc finger motifs that promote binding of the receptor to specific response elements in the target gene promoter. Upon ligand-binding a conformational change of the receptor/ligand complex is induced that co-ordinately leads to transcriptional activation. All of the PPAR-subtypes can be activated by fatty acids (FA) and modulate the transcription of an array of genes that govern various pathways of FA metabolism [15]. The variable N-terminal A/B-domain of PPAR posses a ligand-independent transactivation function and in Atlantic salmon PPARG this domain contains 64 additional amino acids (aa) and displays only 28% aa sequence identity to rat PPARG2. Two PPARG isoforms have been reported in most mammalian species, of which PPARG2 encodes a 30 amino-acid extension, resulting from alternative splicing of exon B, compared to PPARG1. Whereas mammalian PPARG1 is relatively ubiquitously expressed [16–18], PPARG2 is highly expressed in adipose tissue and has a key regulatory role in the induction and maintenance of the adipocyte phenotype [18,19]. In fish species the existence of a single PPARG gene homologous to mammalian PPARG has been reported [20,22]. PPARG mRNA appears to be widely expressed in Atlantic salmon [20,21], similar to the expression profile in plaice and sea-bream [22]. However, we have recently identified an alternatively spliced form of PPARG that is induced during adipocyte differentiation, indicating that this isoform plays a role in lipid accumulation in adipocytes in Atlantic salmon [23]. Since piscine PPARG seems not be to be activated by mammalian specific ligands, and the activation profile by FA differ from that in mammalian PPARG, potential differences in function of PPARG in fish compared with mammals have been suggested [22].

Mammalian PPARG is also highly expressed in macrophages where it has anti-inflammatory activity [24]. In accordance, natural and synthetic PPARG ligands inhibit inflammatory response genes in macrophages and other cell types [24,25]. Based on its anti-inflammatory role in mammalian systems, the aim of this study was to reveal whether pparg alleles influence the susceptibility to disease in Atlantic salmon. Towards this goal, we performed an association study using pparg genotypes of both the parental and offspring generation of several families with survival data from offspring that had been exposed to infectious agents.

2. Materials and methods

2.1. Fish

The genetic material for testing gene-trait associations came from the 1999 year class population of a Norwegian breeding company (AquaGen AS, Trondheim, Norway). The population was founded in the early 1970, on the basis of salmon sampled from a number of different Norwegian rivers [2]. At the time the challenge tests were performed, the population had been selected for furunculosis and ISA two times.

For positioning PPARγ on the genetic linkage map, full- and half-sib groups from the 2005 year class of the same breeding company, and the parents of these groups, were used. The parents of these sib groups came from the 1999 year class as well as from three other year classes.

2.2. Disease challenge experiments

The challenge test for resistance to *A. salmonicida* and ISA were performed at VESO Vikan (Namsos, Norway). In both tests, more families were tested than those used in the present study. The challenge-tested animals originate from the 1999 year class of Aqua Gen AS, a Norwegian breeding company and egg producer. The parents of the challenge-tested animals arose from the 1995 year class. Furunculosis: Atlantic salmon presmolts, with average weight 27.0 g, were transported from Aqua Gen (Kyrksæterøra, Norway) to VESO Vikan (Namsos, Norway), and acclimated for 9 days. Thirty fish from each of 300 families were incubated with 297 furunculosis-infected cohabitants in a single tank (water temperature, 12 °C, tank volume 7 m^3). The fish were fed *ad libitum* during the challenge test. Deceased fish were collected every day during the test period, which was terminated at day 19 post challenge (at which point the overall mortality had reached 70%). The *A. salmonicida* strain was Jnr. 3175/88, and originated from Møre and Romsdal, Norway, October, 18, 1988. Bacterial dose was 5×10^4 *A. salmonicida* per cohabitant. The challenge test for ISA was performed similarly to the furunculosis test, except that 40 fish from each family were tested, the fish were infected by intraperitoneal injection, and the test was terminated at day 27 (53.6% overall mortality). The ISA virus was cultured at the Institute of Veterinary Science (Oslo, Norway), and diluted 1/3000 in 0.85% PBS at VESO Vikan. A dosage of 0.2 ml fish was injected. The virus was of strain Glaesvaer/2/90.

2.3. DNA extraction

Genomic DNA was isolated from about 20 mg frozen white muscle of Atlantic salmon, using either MagAttract DNA M48 Tissue kit on the Bio-Robot M48 (Qiagen, Hilden, Germany) or DNeasy 96 protocol (Qiagen, Hilden, Germany). Prior to isolation, muscle samples were lysed in Proteinase K at 56 °C over night according to the manufacturer's protocol. Insoluble materials after lysis were removed by centrifugation (300 × *g*, 1 min). DNA was extracted according to manufacturer's instruction with the inclusion of *RNaseA* (0.1 mg/sample RNase A R5503 (Sigma)), for 30 min at room temperature.

2.4. PCR

Genomic DNA was amplified using the forward oligonucleotide, f1:5′CAGATCCATATC-AGCGTGAA-3`and reverse, r1:5′ACAATCCCTT