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A novel C-type lectin gene is a strong candidate gene for Benedenia disease resistance in Japanese yellowtail, *Seriola quinqueradiata*

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ABSTRACT

Little is known about mechanisms of resistance to parasitic diseases in marine finfish. Benedenia disease is caused by infection by the monogenean parasite *Benedenia seriolae*. Previous quantitative trait locus (QTL) analyses have identified a major QTL associated with resistance to Benedenia disease in linkage group Squ2 of the Japanese yellowtail/amberjack *Seriola quinqueradiata*. To uncover the bioregulatory mechanism of Benedenia disease resistance, complete Illumina sequencing of BAC clones carrying genomic DNA for the QTL region in linkage group Squ2 was performed to reveal a novel C-type lectin in this region. Expression of the mRNA of this C-type lectin was detected in skin tissue parasitized by *B. seriolae*. Scanning for single nucleotide polymorphisms (SNPs) uncovered a SNP in the C-type lectin/C-type lectin-like domain that was significantly associated with *B. seriolae* infection levels. These results strongly suggest that the novel C-type lectin gene controls resistance to Benedenia disease in Japanese yellowtails.

1. Introduction

Marine fish of the genus *Seriola*, commonly known as yellowtails or amberjacks, are globally distributed and aquaculturally important in countries such as Japan, China, South Korea, the United States, New Zealand and Australia (Sicuro and Luzzana, 2016). In yellowtail aquaculture production, Benedenia disease is a serious parasitic disease caused by infection by the monogenean parasite *Benedenia seriolae*. Eggs of *B. seriolae* drift freely in water and attach to fish cages in aquaculture farms, where their free-swimming larvae parasitize the skin surface of yellowtails. Yellowtails infected by *B. seriolae* show reduced growth and also scrape their bodies against the cages to remove parasites, leading to secondary infection due to viral or bacterial disease.

Previous quantitative trait locus (QTL) analyses of Benedenia disease resistance using wild F_1 strains of the Japanese yellowtail/amberjack *Seriola quinqueradiata* identified two major QTLs: *BDR-1* and *BDR-2* (Ozaki et al., 2013). The highest detected LOD (logarithm of odds) score was for *BDR-1*, which was located within a 5.5 cM interval (at the 95% confidence level) of the proximal region of linkage group Squ2. *BDR-1* was found to explain 20.1%–21.4% of the phenotypic variance. The location of *BDR-2* was narrowed to an 11-cM interval (95% confidence level) at the edge of linkage group Squ20. *BDR-2* explained 12.8%–14.1% of the phenotypic variance. Although yellowtails use the same genetically regulated immune mechanisms to reject and/or protect against *B. seriolae* infection, the bioregulatory mechanisms underlying Benedenia disease resistance are unclear. *In vitro* experiments on the parasitism of *B. seriolae* have indicated that extracts of yellowtail skin epithelia induce the attachment and deciliation of *B. seriolae*, while some sugar-related compounds such as lectin suppress the attachment capacity (Yoshinaga et al., 2002). The results of these experiments suggest that sugar-related compounds are also involved in host specificity. In addition, parasite–host fish interactions via skin mucus chemicals have been reported in various fishes (Buchmann, 1999; Buchmann and Lindenstrøm, 2002; Alvarez-Pellitero, 2008). These results suggest that the chemical environment of yellowtail skin is critical for protecting these fish against parasites. Specific

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chemical components may thus be present in the skin mucus of yellowtail individuals exhibiting Benedenia disease resistance.

Many parasitic diseases exist in vertebrates; these include the especially well-known parasitic diseases of humans caused by malaria and hookworm, where these protozoa parasitize the skin and ultimately the intestines through the bloodstream (Hotez et al., 2004; Sinnis and Zavala, 2012). Another example, in an aquaculture species, is whirling disease caused by infection by the myxosporean parasite *Myxobolus cerebralis*. A QTL for resistance against this disease has been identified in rainbow trout, *Oncorhynchus mykiss* (Baerwald et al., 2011). Relatively few researchers have focused on parasitic diseases compared with viral and bacterial infections. Consequently, the bioregulatory mechanisms underlying resistance against parasitic diseases are still unclear.

Resistance to Benedenia disease in yellowtails is a good model for studying parasitic diseases in vertebrates. Because bioregulatory mechanisms against parasitic diseases may be evolutionally conserved among vertebrates, information about bioregulation against Benedenia disease may aid the elucidation of the mechanism(s) of bioregulation against other parasitic diseases.

The aim of the present study was to reveal bioregulatory mechanisms against parasitic diseases caused by monogenean parasites. To achieve this goal, in this study we performed a detailed analysis of the QTL region of linkage group Squ2 in Japanese yellowtail and identified a strong candidate gene for Benedenia disease resistance. Complete sequencing of the QTL region of linkage group Squ2 uncovered a novel C-type lectin. The expression of this C-type lectin mRNA was detected in skin tissue parasitized by *B. seriolae*. A search for single nucleotide polymorphisms (SNPs) in the open reading frame of this C-type lectin revealed that one SNP located in the C-type lectin/C-type lectin-like domain was associated with infection levels of *B. seriolae*. These results strongly suggest that the novel C-type lectin gene is responsible for Benedenia disease resistance in yellowtails. This is the first report of a gene controlling resistance to parasitic diseases in teleost fish and provides the first genetic evidence that the protein conformation of C-type lectin in skin is important for resistance and protection against *B. seriolae*.

2. Materials and methods

2.1. Yellowtail source and measurements

Japanese yellowtail/amberjack (*S. quinqueradiata*) individuals from a wild population were provided by the Goto Laboratory of the Seikai National Fisheries Research Institute (Nagasaki, Japan). Individual yellowtails were labeled with PIT (Passive Integrated Transponder) tags. To count infection numbers of *B. seriolae* acquired under natural aquaculture conditions at the fish farm, fish were individually dipped in a freshwater tank. Parasites released from the fish bodies were collected and counted. These parasite measurements were performed on December 27 in 2007 and February 5, March 18, June 4 and July 9 in 2008. The sum of the five measurements was used as the number of parasites. Parasite numbers recorded for 40 yellowtail individuals are given in Supplemental Table 1. After the final measurement, the caudal fin of each yellowtail was fixed in 100% ethanol. All animal experiments were carried out with the approval of the Institutional Animal Care and Use Committee of the Tokyo University of Marine Science and Technology.

2.2. Screening for BAC clones

A BAC (Bacterial Artificial Chromosome) library was constructed from genomic DNA of sperm cells taken from one male yellowtail (Fuji et al., 2014). BAC library three-dimensional pools were generated according to a PCR-based protocol (Bruno et al., 1995; Bouzidi et al., 2006). The PCR amplifications were carried out using Takara Ex *Taq* (Takara Bio, Shiga, Japan) under the following cycling conditions: initial denaturation for 2 min at 95 °C, followed by 30 cycles of 30 s at 95 °C, 30 s at 56 °C and 30 s at 72 °C, with a final extension of 3 min at 72 °C. To determine BAC end sequences, BAC DNA extracted using a PureLink HiPure Plasmid Miniprep kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions was sequenced from both SP6 and T7 ends on an Applied Biosystems 3730xl DNA Analyzer (Life Technologies, Carlsbad, CA, USA). Primer sets for BAC end markers are shown in Supplemental Table 2. Novel simple sequence repeat (SSR) markers in linkage group Squ2 were developed from the BAC clones using the microsatellite hybrid capture technique according to a previous protocol (Prochazkal, 1996). Microsatellite genotyping was performed as previously described using the same mapping family as in that study (Ozaki et al., 2013). QTL analysis based on simple interval mapping was conducted using R/qtl software (Broman et al., 2003).

2.3. Sequencing and gene detection in BDR-1

Sequencing libraries with insert sizes of approximately 350 bp were individually constructed and subjected to 101-bp paired-end sequencing on a HiSeq2000 system (Illumina, San Diego, CA, USA) by InfoBio (Tokyo, Japan). The generated sequencing data were submitted to the DDBJ Sequence Read Archive (DRA) under accession number DRA005631. After filtering low-quality reads from the data, we performed *de novo* assembly on each clone separately using two programs: Velvet version 1.2.03 and Newbler version 2.9 (Roche Diagnostics, Mannheim, Germany), which were based respectively on *de Bruijn* graph (Zerbino and Birney, 2008) and overlap consensus algorithms. The sequence read assembly in Velvet was performed using *k*-mer sizes of 93 for #066_e22, 95 for #013_p20 and #101_o14, and 97 for #102_l17, #090_d18, #013_a02 and #070_j15, while the *de novo* assembler Newbler was used with default parameters. For finishing, gaps within contigs and ambiguous nucleotides were sequenced by Sanger sequencing using the primers shown in Supplemental Table 3. Names and GenBank accession numbers of the generated BAC clone sequences are as follows: #102_l17 (AP017998), #013_p20 (AP017999), #066_e22 (AP018000), #090_d18 (AP018001), #013_a02 (AP018002), #070_j15 (AP018003) and #101_o14 (AP018004). For *ab initio* gene detection, repetitive elements were identified from the genomic sequence and masked by RepeatMasker (Smit et al., 2013). To predict genes in the QTL region of linkage group Squ2, we used GENSCAN (Burge and Karlin, 1997; Burge, 1998; GENSCAN Web server at MIT http://genes.mit.edu/GENSCAN.html), Augustus version 3.1.0 (Stanke et al., 2008) and GrimmerHMM version 3.0.3 (Majoros et al., 2004) with default parameters. Augustus was trained using stickleback genomes (Ensembl Release 81; Yates et al., 2016). GrimmerHMM was used with a zebrafish training model. Amino acid sequences of predicted genes were searched and annotated against the NCBI protein database using Blastp with an *E*-value cutoff of 1×10^{-5}.

2.4. cDNA cloning

Yellowtail skins were fixed in RNAlater solution (Thermo Fisher Scientific). Total RNA was then extracted from the fixed skin using an RNeasy Mini kit (Qiagen, Hilden, Germany) and used for first-strand cDNA synthesis with an Ominiscript RT kit (Qiagen) and oligo-dT primers. The cDNA fragments of the yellowtail C-type lectin were amplified by RT-PCR using Takara Ex *Taq* (Takara Bio)