ORIGINAL ARTICLE

Comparative Genomics Identifies Candidate Genes for Infectious Salmon Anemia (ISA) Resistance in Atlantic Salmon (*Salmo salar*)

Jieying Li · Keith A. Boroevich · Ben F. Koop · William S. Davidson

Abstract Infectious salmon anemia (ISA) has been described as the hoof and mouth disease of salmon farming. ISA is caused by a lethal and highly communicable virus, which can have a major impact on salmon aquaculture, as demonstrated by an outbreak in Chile in 2007. A quantitative trait locus (QTL) for ISA resistance has been mapped to three microsatellite markers on linkage group (LG) 8 (Chr 15) on the Atlantic salmon genetic map. We identified bacterial artificial chromosome (BAC) clones and three fingerprint contigs from the Atlantic salmon physical map that contains these markers. We made use of the extensive BAC end sequence database to extend these contigs by chromosome walking and identified additional two markers in this region. The BAC end sequences were used to search for conserved synteny between this segment of LG8 and the fish genomes that have been sequenced. An examination of the genes in the syntenic segments of the tetraodon and medaka genomes identified candidates for association with ISA resistance in Atlantic salmon based on differential expression profiles from ISA challenges or on the putative biological functions of the proteins they encode. One gene in particular, HIV-EP2/MBP-2, caught our attention as it may influence the expression of several genes that have been implicated in the response to infection by infectious salmon anemia virus (ISAV). Therefore, we suggest that HIV-EP2/MBP-2 is a very strong candidate for the gene associated with the ISAV resistance QTL in Atlantic salmon and is worthy of further study.

Keywords Infectious salmon anemia · Atlantic salmon · Disease resistance · Comparative genomics

Introduction

Infectious salmon anemia (ISA) is a highly communicable viral disease that affects salmonids. ISA can be particularly devastating to marine-farmed Atlantic salmon with cumulative mortalities ranging up to 100% in some instances. ISA was first recognized as a new disease entity in Atlantic salmon in 1984 in Norway (Thorud and Djupvik 1988), and since then, outbreaks have occurred in Scotland, the Faroe Islands, Denmark, Maine, and eastern Canada (see Kibenge et al. 2004 for details). ISA has also recently had a major negative impact on salmon production in Chile (Godoy et al. 2008). Such is the threat to salmon aquaculture that ISA is on the European Union list of the most dangerous fish diseases.

The causative agent of ISA is infectious salmon anemia virus (ISAV), which is classified as a member of the *Orthomyxoviridae* and the only species in the genus *Isavirus* (Falk et al. 1997; Rimstad and Mjaaland 2002). The biochemical events involved in the infection and replication of ISAV are similar (Eliassen et al. 2000; Rimstad and Mjaaland 2002; Hellebo et al. 2004) but not identical (Falk et al. 1997; Krossoy et al. 2001) to what has been observed in their relatives, the influenza viruses. The genome of ISAV resembles that of influenza virus A as it is composed of eight linear single-stranded, negative-sense

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RNA segments with a total size of 14.3 kb, which encode at least ten proteins, nine of which are structural (Falk et al. 2004; Kibenge et al. 2004).

Although vaccination is used successfully in Atlantic salmon farming to control many bacterial diseases, effective vaccines against viral diseases have proved more difficult to produce. An alternative strategy is to identify quantitative trait loci (QTL) affecting resistance to the viruses and to make use of this information in a marker assisted selection breeding program. Gjoen et al. (1997) showed that there is a genetic component to ISA resistance in Atlantic salmon and estimated the narrow-sense heritability to be 0.19. A preliminary study using amplified fragment length polymorphisms and a multistage testing strategy identified several putative QTL (Moen et al. 2004a), and it was subsequently shown by the addition of microsatellite markers that a major QTL is located on linkage group (LG) 8 in the Atlantic salmon genetic map (Moen et al. 2004b). The recent integration of the linkage map and karyotype (Phillips et al. 2009) showed that LG8 corresponds to chromosome (Chr) 15, and this nomenclature will be used hereafter. Further investigation confirmed the presence of the QTL for ISA resistance on Chr15 in Atlantic salmon and showed that this QTL explains a significant fraction (32–47%) of the additive genetic variation of the trait (Moen et al. 2007). However, a transmission disequilibrium test and combined linkage disequilibrium/linkage analysis revealed that the QTL markers were not in a population-wide association with ISA resistance, highlighting the need for finer mapping and/ or the use of salmon genomic resources to identify the gene and the functional polymorphism itself.

Over the past decade, many genomic resources have been developed for Atlantic salmon, much of them through the Norwegian Salmon Genome Project, the Genomic Research on Atlantic Salmon Project (GRASP), and their successor, Consortium for Genomic Research on All Salmonids Project (cGRASP). These include the construction of a bacterial artificial chromosome (BAC) library (CHORI-214; Thorsen et al. 2005), a physical map comprising ~4,300 contigs based on Hind III fingerprinting of 223,781 BACs in Ng et al. (2005), 207,869 BAC end sequences that cover ~3.5% of the genome sequence and linkage maps with ~1,600 markers (Danzmann et al. 2008; Moen et al. 2008; Lorenz et al. 2009). More than half of the genetic markers are microsatellites or SNPs derived from BAC end sequences, and these have recently been used to integrate the linkage map, the physical map, and the Atlantic salmon karyotype (Phillips et al. 2009). In addition, ~495,000 ESTs have been generated (Rise et al. 2004; Adzhubei et al. 2007; Koop et al. 2008) and used to construct microarrays for expression studies (Rise et al. 2004; von Schalburg et al. 2008; Koop et al. 2008). These

genomic resources have been amalgamated and are publicly accessible at www.asalbase.org.

In the absence of a sequence for the genome of the Atlantic salmon, we wondered if it would be feasible to link these salmon genomic resources associated with ISA to the sequenced genomes of other teleosts, in particular those of the pufferfish (*Tetraodon nigroviridis*), medaka (*Oryzias latipes*), zebrafish (*Danio rerio*), and three-spined stickleback (*Gasterosteus aculateus*), in the manner illustrated by Sarropoulou et al. (2008) for the commercially important fish species: *Sparus aurata*, *Dicentrarchus labrax*, and *Oreochromis* spp. This approach has been used successfully to identify candidate genes for QTL in the blind Mexican cavefish, *Astyanax mexicanus*, based on conservation of synteny with zebrafish (Gross et al. 2008). Here, we report the results of a search for candidate genes associated with the ISA resistance QTL in Atlantic salmon using comparative genomics.

Materials and Methods

Design of Oligonucleotide Hybridization Probes and Polymerase Chain Reaction Primers

Prior to probe and primer design, the sequences of the microsatellite markers and their flanking regions were first masked for repetitive DNA using the Repeat Masker program on the University of Victoria cGRASP website (http://lucy.ceh.uvic.ca/repeatmasker/cbr_repeatmasker.py). Then, the masked sequences were used as the input to design oligonucleotide probes (40 mers) and corresponding complementary primers (20 mers) using OLIGO 4.0 software (Rychlik and Rhoads 1989). The oligonucleotides were designed such that they had a GC content of 50% or higher, with a Tm of the probe at least 55°C. The probes and primers used in this study are listed in Table 1.

Identification of BACs Containing Specific Microsatellites and their Assignment to Fingerprint Contigs

We screened the CHORI-214 Atlantic salmon BAC library (Thorsen et al. 2005) with the 40-mer probes designed from the flanking sequences of the microsatellite markers (Table 1). The 40 mers were end-labeled with $^{32}P\gamma ATP$ using T4 polynucleotide kinase and hybridized to six BAC filters at a time as described by Johnstone et al. (2008). Briefly, prehybridization was carried out in 5× saline–sodium citrate buffer (SSC), 0.5% sodium dodecyl sulfate (SDS), and 5× Denhardt's solution at 65°C for 3 h, and then the ^{32}P-labeled probes were added and incubated overnight. The filters were washed three times, each for 1 h at 50°C in 1× SSC and 0.1% SDS. The filters were wrapped in Saran™ wrap and exposed to phosphor screens that were

Table 1 Oligonucleotide probes and primers used for screening the Atlantic salmon BAC library

Primer names	Nucleotide sequences
BHMS130F and probe	AAAACACTCTAATGGTTGTGTCAGTCAGAGACAACCCTCC
BHMS130R	TGTCAGTCTGCTAAACACTG
BHMS177F and probe	TTATCTGGGATCACTGATTAGAGCTGTTCATCTGGCTGTG
BHMS177R	CTTTCCATTTCCTCCCCCAG
BHMS553R and probe	GAGTGCTAACTTCAAGGCTTCTCCACTAATAGTCTGAAGG
BHMS553F	CTGTAAACATCACAGGCG
Ssa0130ECIGF and probe	CAGTTAGGTGAGGGGGTTAGGGGAAAATGTCAAGGCAAAG
Ssa0130ECIGR	GGACACACCTACTCATTCCA
Ssa0821BSFUF	TAACGAATGACAGCTTGCTA
Ssa0821BSFUR	AGCCTTTTTCAAAGAATGTG
S0076B01T7F and probe	GTAGTGGGACTCAGGTGGTATGCTATTTCCCTTCCCAATA
S0076B01T7R	GTACAGACTGTGGGTTGAGG
Ssa0578BSFUF	TTGAAACCCTTTCTGTGATT
Ssa0578BSFUR	CTGACTCTGGCATGTACAAA

The oligonucleotide probe also acted as one of the PCR primer pairs for checking the hybridization-positive BACs

subsequently scanned using the Typhoon Imaging System and visualized using ImageQuant software, giving an image of the ^{32}P-labeled hybridization-positive BACs containing the microsatellite markers. The hybridization-positive BAC clones were picked from the library, cultured in 5 mL LB media containing chloramphenicol (50 μg/mL) overnight at 37°C shaking at 250 rpm, and made into glycerol stocks for subsequent polymerase chain reaction (PCR) verification that they did indeed contain the appropriate microsatellite marker. The PCR reaction mixture contained 1 μL of 10× PCR buffer containing $MgCl_2$ (QIAGEN), 1 μL of 2 mM dNTPs, 0.5 μL of 10 μM 40-mer probe, 0.5 μL of 10 μM complementary primer, 0.15 μL of Taq DNA polymerase (QIAGEN), and 6.8 μL of dH_2O. A small amount of BAC clone glycerol stock was added into the PCR reaction mix as template. The PCR conditions comprised 95°C for 5 min followed by 35 cycles of 95°C for 45 s, 65°C for 45 s and 72°C for 2 min, and then 72°C for 10 min. PCR products were separated by electrophoresis through a 1.3% agarose gel with 1× TAE, stained with ethidium bromide, and visualized using a UV trans-illuminator (Alpha Innotech). Hybridization and PCR-positive BACs for the microsatellite markers were matched to fingerprint contigs within the Atlantic salmon physical map (Ng et al. 2005 as shown on www.asalbase.org).

Construction of Minimum Tiling Paths for BAC Contigs and Chromosome Walking

The construction of minimum tiling paths for fingerprint contigs followed the procedure outlined in Quinn et al. (2008). The approach to probe and primer design for chromosome walking was as described above except that the program used was Primer3 Input (version 0.4.0; http://frodo.wi.mit.edu/primer3/) instead of OLIGO 4.0. The

probes and corresponding complementary primers were designed to produce a PCR product of approximately 200 bp. BAC library screening and PCR testing for confirmation of true positives followed the procedure described above. Two fingerprint contigs were considered joined if a BAC from one contig was confirmed to be a true positive of a probe from another contig.

Linkage Mapping

Linkage analysis was carried out using the two Atlantic salmon SALMAP mapping families as previously described (Danzmann et al. 2008; Phillips et al. 2009). The most recent version of the merged female Atlantic salmon genetic map can be seen on www.asalbase.org.

Comparative Genome Analysis

A freely accessible Web-based database, ASalBase (www.asalbase.org), has been built to display the Atlantic salmon linkage map, physical map, and associated information such as BAC end sequences (Boroevich et al. 2008). This website provides a rich resource for comparative genomic analysis using the Atlantic salmon BAC end sequences. BAC end sequences (207,869) with an average length of 666 bp (~3.5% of the genome) are shown on this site. After masking the BAC end sequences using the Repeat Masker program on the University of Victoria cGRASP website (http://lucy.ceh.uvic.ca/repeatmasker/cbr_repeatmasker.py), they were subjected to a BLASTx sequence similarity search against the tetraodon, medaka, stickleback, and zebrafish (Ensembl release 53 protein databases). These comparisons enabled us to identify putative regions of the genomes of these four fish that are syntenic with the Atlantic salmon ISA QTL region.

Results and Discussion

Identification of BACs Containing the Genetic Markers Associated with the ISA Resistance QTL

The results from QTL mapping of ISA resistance in Atlantic salmon identified three microsatellite markers, BHMS177, BHMS553, and BHMS130, most closely associated with this trait (Moen et al. 2007). These microsatellites are tightly linked spanning, approximately 4.3 cM from 30.4 to 34.7 cM on Chr15 of the integrated female map (Phillips et al. 2009; www.asalbase.org). We noted that the marker Ssa0130ECIG also mapped to this region. We designed oligonucleotide probes from the flanking regions of these four microsatellite markers (Table 1) and used them to screen the CHORI-214 Atlantic salmon BAC library (Thorsen et al. 2005). The hybridization-positive BACs were examined by PCR using the primers that amplify the four microsatellite markers (Table 1). BACs which gave amplification products of the expected size are listed in Table 2 along with the four

Table 2 BACs, and the contigs to which they have been assigned (Ng et al. 2005), that were positive by hybridization and PCR confirmation for genetic markers in the Atlantic salmon ISA QTL region (Moen et al. 2007)

Markers	Positive BACs	Contig
BHMS130	S0019O02	1180
	S0026I01	1180
	S0123H01	1180
	S0006L24	Singleton
BHMS553	S0036K05	386
	S0045L14	386
BHMS177	S0353K21	1907
Ssa130ECIG	S0819O15	620
	S0835A07	620
	S0848I03	620
	S0881C19	620
	S0927L15	620
	S0933B05	620
	S0941O10	620
	S0958C23	Not fingerprinted
Ssa0821BSFU	S0092G24	620
S0076B01T7	S0076B01	620
	S0143G23	1907
	S0228D13	1907
	S0824B12	1907
	S0928O23	1907
	S0939C19	Not fingerprinted
	S0960I24	Not fingerprinted
Ssa0578BSFU	S0038H22	227

fingerprint contigs of the physical map (Ng et al. 2005; www.asalbase.org) to which they belong, namely, contigs 1180, 386, 1907, and 620. Contig 620 contains a microsatellite marker, Ssa0821BSFU, derived from a BAC end sequence. Ssa0812BSFU was mapped in the Atlantic salmon SALMAP mapping families (Danzmann et al. 2008) to Atlantic salmon Chr15 at position 30.4 cM of the integrated female map, which is consistent with the mapping of the other markers listed above.

Construction of Minimum Tiling Paths and Chromosome Walking in the ISA Resistance QTL Genomic Region

We made use of the extensive BAC end sequence database and designed PCR primers from BACs in contigs 1180, 386, 1907, and 620. This allowed us to construct minimum tiling paths for these contigs. We then attempted to carry out chromosome walking by screening the BAC library with oligonucleotide probes designed from BAC end sequences of BACs that were at the ends of the contigs. This proved very difficult, primarily because of the repetitive nature of the Atlantic salmon genome (de Boer et al. 2007); however, the probe derived from the T7 end sequence of BAC S0076B01, which is in contig 620, hybridized to several BACs from contig 1907 (Table 2), indicating that contigs 620 and 1907 overlap one another. When we examined the putative syntenic regions of these four contigs on the medaka genome using www.asalbase. org, we noted that contig 227 was adjacent to contig 1907. Contig 227 contains the BAC end sequence-derived genetic marker Ssa0578BSFU. Using the Atlantic salmon SALMAP mapping families, we mapped Ssa0578BSFU to 34.7 cM on Chr15, which is at one end of the ISA resistance QTL region.

Comparative Genomic Analyses

We used the BAC end sequences from contigs 1180, 386, 1907, 620, and 227 to carry out similarity searches against the genomic sequences of tetraodon (*T. nigroviridis*), medaka (*O. latipes*), stickleback (*Gasterosteus aculeatus*), and zebrafish (*D. rerio*) (Ensembl release 53). Table 3 provides a summary of the results. It appears that this region of Chr15 in Atlantic salmon shares extensive synteny with LG14 of tetraodon, LG24 of medaka, LGXVIII of stickleback, and LG20 of zebrafish. It should be noted that in the comparisons of Atlantic salmon with tetraodon, medaka, and stickleback, there appears to be a discontinuity between contigs 227 and 1180, although the linkage group is conserved in all cases. This is illustrated for medaka in Fig. 1. Similarly, the comparison of the Atlantic salmon and zebrafish suggests that some rearrangements have occurred in these genomes, but their nature is

Table 3 Regions of fish genomes that are syntenic to the ISA QTL in Atlantic salmon based on similarity searches using the BAC end sequences from contigs that contain genetic markers associated with ISA resistance (Moen et al. 2007)

ISA QTL markers	Map position	Contig	Tetraodon LG14	Medaka LG24	Stickleback LGXVIII	Zebrafish LG20
BHMS553	30.4	386	9,450k–9,550k	15,600k–16,400k	13,200k–13,300k	39,500k–40,500k
BHMS177	30.4	1907	9,550k–10,000k	15,600k–16,400k	15,800k–15,900k	29,700k–30,000k
Ssa0130ECIG	30.4	620	9,970k–10,000k	23,300k–23,900k	12,700k–12,800k	29,500k–30,000k
Ssa0821BSFU	30.4	620	9,970k–10,000k	23,300k–23,900k	12,700k–12,800k	29,500k–30,000k
Ssa0578BSFU	34.7	227	10,200k–10,250k	23,700k–24,400k	16,000k–16,100k	26,200k–26,300k
BHMS130	34.7	1180	8,350k–8,400k	2,700k–3,200k	2,500k–2,530k	56,400k–56,600k

not immediately obvious. Another possibility is that the resolution of the linkage map is imprecise because of the nature of the SALMAP mapping families used for its construction (see Danzmann et al. 2008 for details). However, it was both reassuring and satisfying to discover that there is an apparent conservation of synteny among all of these phylogenetically diverse fish genomes corresponding to this segment of Chr15 in Atlantic salmon. Tetraodon has one of the most compact vertebrate genomes (Jaillon et al. 2004), and therefore, we decided to examine the region from 8,350k to 10,250k in tetraodon LG14 in more detail. We also examined the region from 15,600k to 23,900k in medaka LG24. We developed gene lists from these regions of the tetraodon and medaka genomes using the BioMart tool from Ensembl (Electronic Supplementary Material Tables 1 and 2). We expected that these lists would contain one or more orthologs of candidate genes that could explain the ISA resistance in Atlantic salmon.

Comparison of QTL Gene Lists with Microarray Gene Expression Lists from ISA-Infected and Uninfected Atlantic Salmon

Several studies have examined the changes in gene expression in Atlantic salmon tissues and cultured salmon cells that result from exposure to ISA (Jørgensen et al. 2007, 2008; Kiling et al. 2007; McBeath et al. 2007; Schiøtz et al. 2008; Workenhe et al. 2008, 2009). Although we considered the results from all of these publications, we concentrated on the recent work of Workenhe et al. (2009). Our rationale was that this study used the most comprehensive microarray (i.e., the cGRASP 16,000 cDNA array described by von Schalburg et al. 2008) to examine the changes that occur in the macrophage/dendritic-like cell line TO infected with a either a low pathogenic or a highly pathogenic strain of ISA at 24 and 72 h post infection. We first compared the complete catalog of differentially

Fig.1 Schematic representation of the genetic map of Atlantic salmon Chr15 (LG8) showing the ISA QTL region identified by Moen et al. (2004a, 2007) and the corresponding syntenic regions of medaka LG24
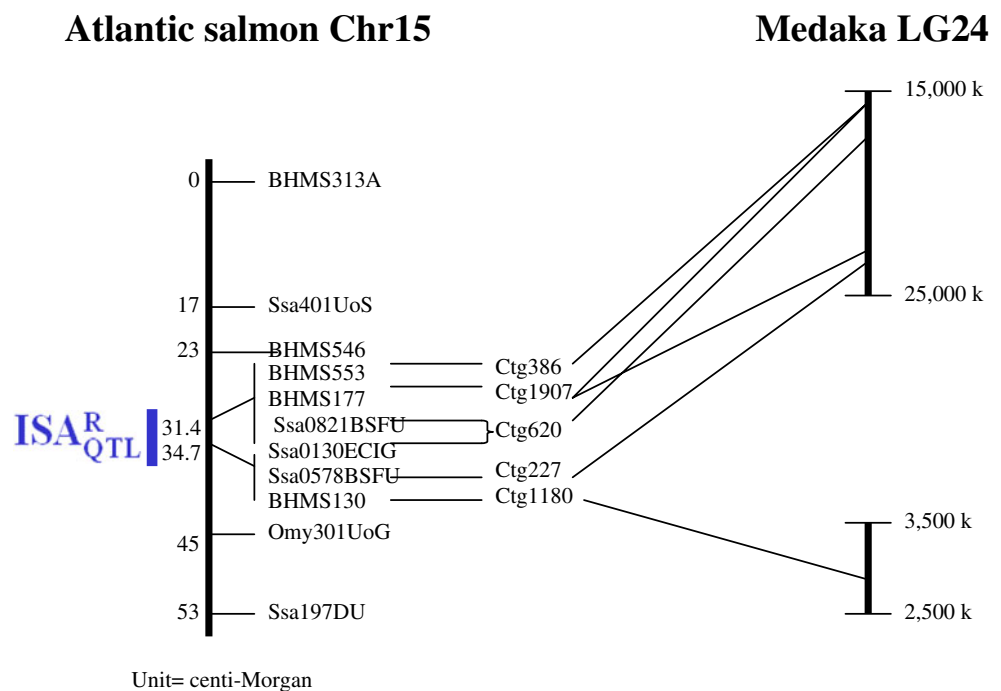
regulated genes (Workenhe et al. 2009) with the list of genes in the ISA resistance QTL putative syntenic region of the tetraodon genome (LG14 position 8,350k–10,250 k) as well as the corresponding region in the medaka genome from 15,600k–24,400 k on LG24. The annotation of fish genomes as well as the cDNAs on the cGRASP microarray is an imprecise science as it is based on sequence similarities primarily with mammalian genes. This is further complicated by the relatively recent genome duplication in the common ancestor of extant salmonids (Allendorf and Thorgaard 1984). We therefore took a very liberal approach when making our comparisons. For example, we considered galectin-3 and galectin-8 as being potential orthologs of galectin-9 and included proteasome subunits irrespective of them being classified as alpha or beta. The results of this comparison are shown in Table 4. The tetraodon comparisons revealed 14 annotated genes that had a putative ortholog identified as being differentially expressed from the microarray experiments. These included four genes classified as E3 ubiquitin-protein ligases (RNF8, RNF19B, RNF217, and UBR7), three N-acetyl transferases (ESCO2, NAT12, and NAT5), two tripartite motif-containing genes (TRIM54 and TRIM67), two galectins (LGALS3 and LGALS8), a single copy of a tetratricopeptide repeat protein (TTC13), and two members of the proteasome (26S protease regulatory subunit 4, PSMC1, and proteasome subunit alpha type-6, PROS-27). We note that galectin-9 was one of the four genes, along with 5-lipoxygenase activating protein, cytochrome P450 2K4-I,

and annexin AI, which were sufficient for correct assignment of individual salmon to early mortality, late mortality, and uninfected groups (Jørgensen et al. 2008). Only three of the 14 genes from the tetraodon selected gene list were annotated in the corresponding region of the medaka genome: TTC13, RNF217, and NAT5. The transcript corresponding to TTC13 was increased 26.88-fold and 30.24 fold in TO cells infected with ISA at the 24 and 72-h post-infection time points, respectively. In contrast, there was no significant increase in the abundance of RNF217 transcripts in the TO cells after 24 h of infection, but it rose 16.61-fold over that found in the control after 72 h of infection. The change in expression of NAT5 was only observed in TO cells 72 h post infection with the highly pathogenic ISAV isolate. We suggest that the genes listed in Table 4, particularly TTC13, RNF217, and NAT5, are worthy of further consideration as potential candidates that could contain alleles that are causal for the ISA resistance QTL. Furthermore, we suggest that they be screened for polymorphisms that alter their expression pattern as well as cause changes in their protein products.

Prediction of Candidate Genes for the ISA Resistance QTL Based on Putative Functions of Their Protein Products

It is widely recognized that interferons and the products of the genes they induce play a critical part in the innate immunity system against viruses. Although more than 100 interferon-stimulated genes have been identified, to date,

Table 4 Genes, previously identified as being differentially expressed in ISA-infected salmon by microarray analysis from Workenhe et al. (2009), that are annotated in the ISA QTL syntenic regions of the medaka and tetraodon genomes (Electronic Supplementary Material Tables 1 and 2, respectively)

Common name	Tetraodon gene	Medaka gene	Fold increase at 24h post infection	Fold increase at 72h post infection
N-acetyltransferase (ESCO2)[a]	ESCO2	Not annotated	Not significant	3.47[f]
Tripartite motif-containing protein 67 (TRIM9-like protein)[b]	TRIM67	Not annotated	3.67–4.83	5.40–14.4.1
Tetratricopeptide repeat protein 13	TTC13	TTC13	26.88	30.24
E3 ubiquitin-protein ligase (RING finger protein 8)[c]	RNF8	Not annotated	Not significant	16.61
E3 ubiquitin-protein ligase (RING finger protein 19B)[c]	RNF19B	Not annotated	Not significant	16.61
E3 ubiquitin-protein ligase (RING finger protein 217)[c]	RNF217	RNF217	Not significant	16.61
N-acetyltransferase 12[a]	NAT12	Not annotated	Not significant	3.47[f]
Galectin-3 (GALBP)[d]	LGALS3	Not annotated	2.76–6.01[d]	37.09–167.22
Galectin-8 (Gal-8; PCTA-1)[d]	LGALS8	Not annotated	2.76–6.01[d]	37.09–167.22
26S protease regulatory subunit 4 (P26s4 ATPase 1)[e]	PSMC1	Not annotated	Not significant	25.41
Proteasome subunit alpha type-6 (PROS-27)[e]	PSMA6[b]	Not annotated	Not significant	3.42–4.52
Tripartite motif-containing protein 54 (MURF-3)[b]	TRIM54	Not annotated	3.67–4.83	5.40–14.4.1
N-acetyltransferase 5[a]	NAT5	NAT5	Not significant	3.47[f]
E3 ubiquitin-protein ligase UBR7 (N-recognin-7)[c]	UBR7	Not annotated	Not significant	16.61

The genes are listed in the order in which they occur in the tetraodon genome. Genes encoding proteins with the same function are identified by the same superscript. We took a very liberal approach with annotation comparisons (e.g., we took galectin-3 and galectin-8 as being a potential homolog of galectin-9 and proteasome subunits irrespective of them being alpha or beta). The fold increase results are with infection with a low pathogenic ISAV isolate (RPC/NB-04-085-1) except for [f] N-acetyltransferase at 72 h post infection with a highly pathogenic ISAV isolate (NBISA01)

Mx is the only one that has been shown to possess antiviral properties (Haller et al. 2007; Robertsen 2008). It has been shown that ISA is a powerful inducer of key genes of the type I interferon system in Atlantic salmon, including Mx, but the virus is not inhibited by the interferon-dependent pathway or the presence of Mx prior to infection (Kiling et al. 2007). Therefore, we considered what is known about resistance to viruses in other systems and if this information could be applied to identifying candidate genes for ISA resistance in Atlantic salmon. Several genes have been identified with allelic variation that influences HIV infection and disease progression (Heeney et al. 2006). Among these are the tripartite motif-containing protein TRIM5α; cytokines such as IL-10; coreceptor/ligands such as CCR5, CCL2, CCL7, CCL11, and DC-SIGN; and components of the adaptive immune response, namely, subtypes of HLA-A, HLA-B, and HLA-C. Similarly, it has recently been shown that the host genetic background strongly influences the response of influenza A virus infections in mice, although the molecular basis for this phenomenon has yet to be identified (Srivastava et al. 2009). As indicated in the review article by Beutler et al. (2007), it is quite feasible to consider mutations arising in the host that confer virus resistance by affecting some of the key steps in viral entry into a cell, its replication, and ultimately its assembly and spread to other cells. In the case of ISA resistance in Atlantic salmon, it is possible that the allele(s) associated with the QTL is in a gene(s) that is not directly involved in the interferon response pathways. Moreover, a significant change in the expression of the gene may not be required for the effect to be realized. Therefore, we searched the BioMart gene lists from the ISA resistance QTL putative syntenic regions of the tetraodon and medaka genomes and identified some potentially interesting genes whose products have been discussed in the context of naturally occurring mutations associated with viral resistance (Heeney et al. 2006; Beutler et al. 2007). The list of seven potential ISA

resistance candidate genes we wish to consider is given in Table 5.

HDDC2 (NS5A-TP2) is an HD domain-containing protein that is induced by the non-structural 5A (NS5A) protein of hepatitis C virus (Yang et al. 2004). The hepatitis C virus NS5A plays multiple roles in virus infection including viral replication and cellular signaling pathways (see MacDonald and Harris 2004 for review). It has been suggested that hepatitis C virus resistance to interferon could be explained by the ability of NS5A to bind to and inhibit the dsRNA-activated, interferon-induced kinase PKR (Gale et al. 1997). We speculate that variation in proteins that are induced by NS5A, or its equivalent in other single-stranded RNA viruses such as ISA, may be associated with differential responses to viral infection, and thus, HDDC2 (NS5A-TP2) becomes a candidate for the Atlantic salmon ISA resistance QTL.

Toll/interleukin-1 receptor-like protein 5 (TLR5) is a member of the Toll-like receptor (TLR) family (Akira et al. 2006). Recognition of specific ligands by TLRs induces MyD88-dependent signaling via the Toll–IL-1 receptor pathways, which activate dendritic cells and help induce their differentiation into antigen presenting cells (Takeda et al. 2003). Single-stranded RNA serves as a physiological ligand for TLR7 and TLR8 (Heil et al. 2004; Diebold et al. 2004). TLR5 is generally thought to be involved in flagellated bacterial pathogen detection (Hayashi et al. 2001), and a novel transcript encoding a soluble form of TLR5 was observed in the liver of Atlantic salmon during *Aeromonas salmonicida* infection (Tsoi et al. 2007). We speculate that a variant of TLR5 in Atlantic salmon may have become a ligand for ISAV and that this could explain the ISA resistance QTL that occurs in Atlantic salmon.

HIV-EP2 is a member of the metal-finger protein family that binds to the human immunodeficiency virus type 1 enhancer (Nomura et al. 1991). HIV-EP2 is expressed at high levels in T cells, and its expression is induced by

Table 5 Candidate genes for ISA resistance in the QTL syntenic regions of the tetraodon and medaka genomes based on the predicted functions of gene products

| Common gene name	Tetraodon gene	Medaka gene
HD domain-containing protein 2 (hepatitis C virus NS5A-transactivated protein 2)	HDDC2	HDDC2
Toll-like receptor 5 Precursor (Toll/interleukin-1 receptor-like protein 3)	TLR5	TLR5
Transcription factor HIVEP2 (HIV-EP2; MHC-binding protein 2)(MBP-2))	HIVEP2	HIVEP2
Interleukin-1 receptor-associated kinase 1-binding protein 1	IRAK1BP1	Not annotated in syntenic region
NF-kappa-B inhibitor alpha (MHC complex enhancer-binding protein MAD3)	NFKBIA	Not annotated in syntenic region
Feline leukemia virus subgroup C receptor-related protein 1	FLVCR1	Not annotated in syntenic region
Interleukin-20 receptor alpha chain Precursor (cytokine receptor class-II member 8)	Not annotated in syntenic region	IL20RA

mitogen and phorbol ester treatment, suggesting that it is involved in the regulation of T cell growth. The transcription factor HIV-EP2 also binds to the enhancer of the major histocompatibility complex (MHC) class I genes, and thus, it is also known as MHC-binding protein 2 (MBP-2; van't Veer et al. 1992). Related enhancer motifs to those of MHC and HIV occur in the regulatory regions of immunoglobulin genes, the β_2 microglobulin gene, the interferon β gene, and the interleukin 2 receptor gene. Therefore, changes in the structure of HIV-EP2/MBP-2 may influence the expression of several genes that have been implicated in the response to infection by ISAV. It is noteworthy that three of the microsatellite markers associated with the ISA resistance QTL (BHMS177, Ssa0821BSFU, and Ssa0130-CIG) map to the syntenic region in the medaka genome where HIV-EP2/MBP-2 occurs (Fig. 2). We suggest that HIV-EP2/MBP-2 is a very strong candidate for the gene associated with ISAV resistance in Atlantic salmon and that it is definitely worthy of further study.

Complex signaling pathways link the recognition of pathogens by TLRs and the activation of the transcription factor NF-$\kappa\beta$ which regulates the expression of genes essential for host defense and inflammation (Akira et al. 2006). We identified two genes in the Atlantic salmon ISA resistance QTL syntenic region of the tetraodon genome that are part of the NF-$\kappa\beta$ regulatory pathway, namely, interleukin receptor-associated kinase 1 binding protein 1 (IRAK1BP1) and NF-$\kappa\beta$ inhibitor alpha (NFKBIA; Li and

Verma 2002). We suggest that allelic variation in one of these genes that are involved in modulating the NF-$\kappa\beta$ signaling pathway could be responsible for variation in ISA resistance in Atlantic salmon. Thus, they are worthy of further investigation.

As mentioned above, it is possible that a mutation in Atlantic salmon could confer ISA resistance by affecting a key step in viral entry into a cell. It was interesting, therefore, to find feline leukemia virus subgroup C receptor-related protein 1 (FLVCR1) in the ISA resistance QTL syntenic region of the tetraodon genome. The preferred receptor for the feline leukemia virus subgroup C, a γ-retrovirus, is a transporter involved in the export of heme (Tailor et al. 2003). We speculate that mutations in the Atlantic salmon FLVCR1 ortholog may be associated with ISA resistance.

The final gene that we identified as a possible candidate associated with the ISA resistance QTL in Atlantic salmon is interleukin-20 receptor alpha chain (IL20RA), which was observed in the ISA resistance QTL syntenic region of the medaka genome but not in the corresponding region of the tetraodon genome. IL20RA encodes one of the heterodimers of the IL-20R complex through which Signal Transducer and Activator of Transcription activation occurs when bound to IL-19, IL-20, or mda-7 (Dumoutier et al. 2001). We speculate that signaling through the IL-20R complex may provide an alternative mechanism for activating a host defense against ISA in Atlantic salmon.
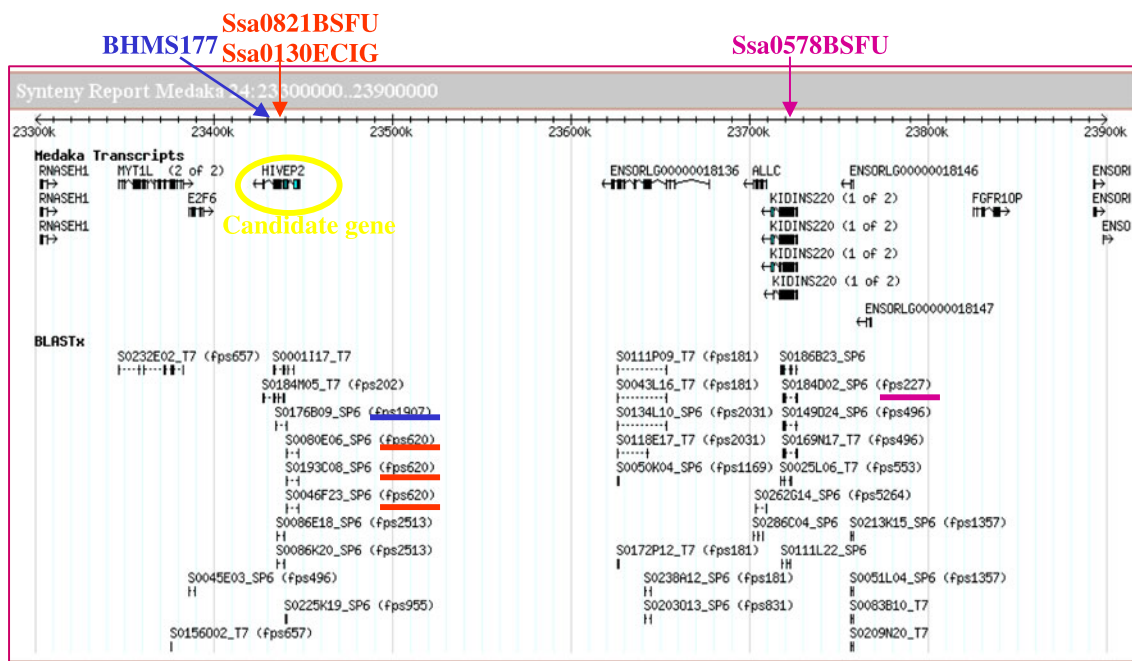
Fig. 2 A screenshot from www.asalbase.org that shows the annotated genes and transcripts from 23,300k–23,900k of medaka LG24 as well as the Atlantic salmon BAC end sequences that align with them. This region also contains one of the microsatellites, BHMS177, associated with the ISA resistance QTL (Moen et al. 2007) and three additional markers, Ssa0821BSFU, Ssa0130ECIG, and Ssa0578BSFU, that are tightly linked to BHMS177 (Danzmann et al. 2008; Phillips et al. 2009)

Conclusions

In the absence of a sequence for the Atlantic salmon genome, we have taken a comparative genomics approach to identify candidate genes that may be associated with the QTL for ISA that has been mapped to Chr 15 (Moen et al. 2004a, 2007). We recognize that we have indulged in broad speculation in developing the list of candidate genes given in Tables 4 and 5 but wish to point out that even when a well-annotated genomic sequence becomes available for Atlantic salmon, it will still be necessary to identify candidate genes based on their putative physiological functions and to test them in ISA-resistant and susceptible families. Of course, having a sequence of the Atlantic salmon genome should provide additional genetic markers, which would allow finer mapping of the QTL.

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