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ORIGINAL ARTICLE

Linkage Mapping of Toll-Like Receptors (TLRs) in Japanese Flounder, *Paralichthys olivaceus*

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Abstract Toll-like receptors (TLRs) are responsible for the recognition of specific pathogen-associated molecular patterns and consequently activate signal pathways leading to inflammatory and interferon responses. The region surrounding several TLRs was previously found to be associated with resistance to specific disease. Hence, we determined the location of 11 TLRs in Japanese flounder (*Paralichthys olivaceus*) using polymorphic microsatellite markers. TLR1 and TLR3 were located on linkage group (LG) 21 and 7, respectively. Membrane TLR5 and soluble TLR5 were mapped to LG22. TLR7 and TLR8 were mapped to LG3. TLR9 was found on LG1 and TLR14 and TLR21 were located on the same linkage group, LG10. TLR22 was found on LG8. Interestingly, TLR2 was mapped with the previously reported Poli9-8TUF microsatellite marker which is tightly associated with lymphocystis virus disease resistance. Therefore, TLR2 is a candidate gene for resistance to lymphocystis disease. These results imply that the location of a TLR associated with a particular disease may be valuable for the research on the relationship between host immune response and disease resistance.

Keywords Toll-like receptors (TLRs) · *Paralichthys olivaceus* · Linkage map · Microsatellite marker

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Introduction

Toll-like receptors (TLRs) recognize pathogen-associated molecular patterns (PAMPs) in the innate immune response resulting in the production of inflammatory and interferon responses via the activation of intracellular signal pathways. They consist of an extracellular leucine-rich repeat (LRR) domain for ligand recognition, a transmembrane domain, and an intracellular Toll/interleukin 1 receptor (TIR) signaling domain (Akira and Takeda 2004; Medzhitov 2001). LRRs recognize conserved microbial features called PAMPs (Bell et al. 2003; Medzhitov 2001), such as bacterial cell-surface peptidoglycans, lipoproteins, LPS, and bacterial flagellin, and single- and double-strand viral RNA and the unmethylated CpG islands of bacterial and viral DNA. After PAMPs recognition by LRR, the TIR domain triggers recruitment of adaptor proteins for signaling which lead to modulation of several aspects of innate immune responses (Takeda et al. 2003). Therefore, TLRs play important roles in the immune response to protect the host against invading pathogens.

To date, 13 TLRs have been identified in mammals (Akira and Takeda 2004; Medzhitov 2001). In several teleost fish, however, more than 15 TLRs have been identified and mapped (Palti et al. 2006, 2010a, b) including mammalian TLR homologues as well as novel fish TLRs that have not been reported in mammals such as TLR5 soluble form (5S), TLR14, TLR20, TLR21, and TLR22 (Hirono et al. 2004; Hwang et al. 2010a, b; Jault et al. 2004; Oshiumi et al. 2003; Rebl et al. 2007; Takano et al. 2010; Tsoi et al. 2006). The regions surrounding several TLRs are closely linked to susceptibility or resistance to specific disease infection (Leveque et al. 2003; Sebastiani et al. 2000). These loci might be useful for genetic selection to improve disease resistance (Hu et

al. 1997; Leveque et al. 2003; Sebastiani et al. 1998, 2000). Thus, mapping of TLR location will help to better understand the roles in the host response to pathogens.

Genetic linkage mapping using molecular markers is a powerful tool for understanding genome evolution, comparative genomics, positional cloning of functionally important gene, and identification of quantitative trait loci (QTL) (Jaari et al. 2009; Koshimizu et al. 2010). One type of molecular marker is microsatellites, which consists of tandem repeats of 1–6 bp that vary in length. These lengths are useful to determine a particular location with high polymorphisms in population (Coimbra et al. 2003; Jaari et al. 2009; Sakamoto et al. 2000). Linkage maps have been constructed for commercially important aquaculture species, including channel catfish (*Ictalurus punctatus*), Japanese flounder (*Paralichthys olivaceus*), Pacific abalone (*Haliotis discus hannai*), Pacific oyster (*Crassostrea gigas*), rainbow trout (*Oncorhynchus mykiss*), sea bass (*Dicentrarchus labrax* L.), tilapia (*Oreochromis niloticus*), and whiteleg shrimp (*Litopenaeus vannamei*) (Castaño-Sánchez et al. 2010; Chistiakov et al. 2005; Coimbra et al. 2003; Kocher et al. 1998; Lee et al. 2005; Li and Guo 2004; Liu et al. 2003, 2006; McConnell et al. 2000; Meehan et al. 2003; Sakamoto et al. 2000; Sekino et al. 2006; Waldbieser et al. 2001). These linkage maps allow for the identification of markers linked to the objective trait and the genetic mechanism of complex traits in aquatic animals (Koshimizu et al. 2010; Liao et al. 2009; Liu et al. 2010). Notably, linkage maps assist in locating candidate genes and QTL within a region associated with resistance to a specific disease and subsequently may improve the control of disease by selective breeding (Johnson et al. 2008; Lallias et al. 2009).

In a previous study, a high-density sex-specific linkage map of Japanese flounder has been constructed. This linkage map covers 79% of the female genome and 82% of the male genome. In females and males, the high rate of recombination was observed in the centromeric and teleomeric regions, respectively (Castaño-Sánchez et al. 2010). Here, we investigated the location of 11 TLR genes in the linkage map of Japanese flounder using microsatellites and characterized the potential role of TLR in conferring resistance to specific disease. We envision these results to later serve as a guide in breeding genetically improved strains of Japanese flounder resistant to disease.

Materials and Methods

Isolation of Japanese Flounder TLRs from BACs

We have already sequenced 11 TLRs of Japanese flounder, including TLR1, TLR2 (Hirono et al. 2004), TLR3, TLR5 membrane (5M) (Hwang et al. 2010a), TLR5S (Hwang et al. 2010a), TLR7, TLR8, TLR9 (Takano et al. 2007), TLR14 (Hwang et al. 2010b), TLR21, and TLR22 (Hirono et al. 2004). The TLR genes were isolated from a bacterial artificial chromosome (BAC) library of Japanese flounder (Katagiri et al. 2000). Specific probes for each TLR gene were designed (200–500 bp) and PCR-amplified probes were labeled with α-^{32}P [CTP] using a random primer kit (Takara). The BAC membranes were hybridized with the probes for 2 h at 65°C and membranes were washed three times at 65°C using saline sodium citrate containing sodium dodecyl sulfate. The membranes were visualized using a FLA 9000 image scanner (Fuji Film). The positive BAC clones of TLR genes were sequenced using an ABI 3130xl Genetic analyzer (Applied Biosystems).

Identification of Microsatellite Marker

BAC clones that were positive for each TLR gene were used in constructing the libraries. Plasmid DNA of the positive BAC clones was digested with *Sau*3AI and the fragments (around 2 kb) were eluted from the gel. The DNA was ligated in pBluescripts SK plasmid vector which was digested with *Bam*HI. Transformation was performed using JM 109 *Escherichia coli* competent cells. The library was screened for microsatellite repeat sequences by colony hybridization with a γ-^{33}P [ATP] end-labeled $(CA)_{10}$ probe. The positive microsatellite clones were sequenced on an ABI 3130xl Genetic analyzer (Applied Biosystems). Primers were designed based on the regions flanking the microsatellite repeat sequences.

PCR and Linkage Analysis

PCR was carried out with genomic DNA from two parents and 45 progenies that were previously used to construct a second generation genetic linkage map for Japanese flounder (Castaño-Sánchez et al. 2010). PCR was performed in a 12-μl solution containing 0.7 pmol of forward primer and 0.32 pmol of reverse primer end labeled with 0.02 MBq of γ^{33}-P [ATP], 0.25 U of *Taq* polymerase, 0.2 mM of each dNTP, 1% BSA, and 50 ng of genomic DNA. PCR was carried out under the following conditions: initial denaturation at 95°C for 2 min, 35 cycles of denaturation at 95°C for 30 s, annealing at corresponding annealing temperature for 1 min and extension at 72°C for 1 min and final extension at 72°C for 3 min. Amplified PCR products were electrophoresed on 6% polyacrylamide (acrylamide/bisacrylamide ratio, 19:1)–8 M urea gels. After electrophoresis, gels were dried on a standard gel drier for 30 min. Imaging plates were scanned with the FLA-9000 image scanner (Fuji Film). Microsatellites were scored as dominant markers with the genotype indicated by the presence or absence of a band. Linkage mapping analysis

was carried out with Map Manager QT (Manly and Olson 1999). Microsatellites of the $\log_{10}$ of odds >3.0 were assigned to the same linkage group. The linkage map was visualized using MapChart version 2.0 (Voorrips 2002).

Results and Discussion

TLRs play important roles in the immune response against invading pathogens. Eleven TLRs have been identified in Japanese flounder. In this study, we identified microsatellites from BACs containing each TLR and carried out linkage analysis of the TLRs using a previously reported sex-specific linkage map of Japanese flounder (Castaño-Sánchez et al. 2010). The 11 TLRs were located on different linkage group.

Japanese flounder TLR1 was located on linkage group (LG) 21. The female parent was heterozygous for TLR1, while the male parent was homozygous at that locus and we were unable to determine it (Fig. 1).

Mammalian TLR2 recognizes a broad range of ligands, including bacterial cell wall components and unidentified DNA viral surface components, and subsequently induces an inflammatory response (Morrison 2004; Aravalli et al. 2005; Sørensen et al. 2008). Japanese flounder TLR2 mapped with the Poli9-8TUF microsatellite marker on LG15 (Fig. 1), which is tightly associated with the lymphocystis-virus-disease-resistant locus (Fuji et al. 2006). Marker-assisted breeding using Poli9-8TUF microsatellite led to a strain in which there was no incidence of lymphocystis virus disease (Fuji et al. 2007). Thus, Japanese flounder TLR2 is a candidate gene for resistance to lymphocystis virus disease. The host genetic factor of TLR genes and other immune-related genes in pathogen-resistant locus has strongly affected the ability of the host to respond to invasion of pathogens (Hu et al. 1997; Leveque

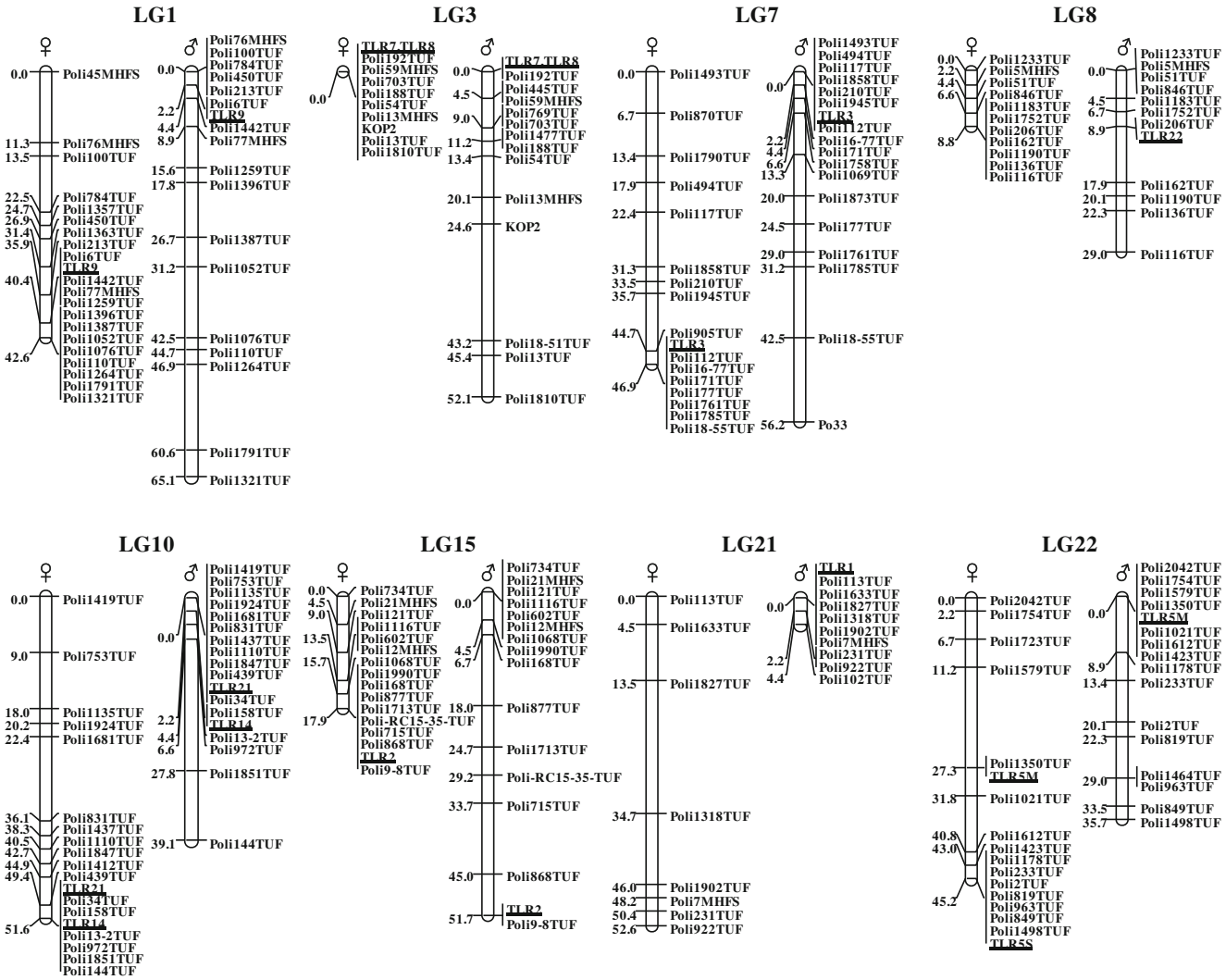

Fig. 1 Linkage maps of the TLRs in female (*female sign*) and male (*male sign*) Japanese flounder. Japanese flounder TLRs are indicated by *underline*. Marker distances are shown in centimorgans

Table 1 Characterization of microsatellite markers used for making TLRs map in Japanese flounder

TLR gene	Repeat motif	Primer sequence	Ta (°C)	Female	Male	LG
TLR1	$(CA)_6GA(CA)_5GA(CA)_5$	ACGGTTCGAACTGATTGTCTCC AAGGCTGTCTCTGTTCCTGGAC	55	Homo	Hetero	21
TLR2	$(CA)_{23}$	GGAACTCTCCCCGAACACTTTA CGTGGTAACTGAAAGGACGTTG	55	Hetero	Hetero	15
TLR3	$(CA)_{45}$	GCCTGTAAAGCATATTGATCCTG TCATGCCCAAAGTGTAGGTGAG	55	Hetero	Hetero	7
TLR5M	$(CA)_2$ $CT(CA)_8$	GGCTGCTTTTTAAGATGCTTGTCTCG GTACCGAATATCCCTGATGGCTGT	62	Hetero	Hetero	22
TLR5S	$(CA)_{10}$	CCAATTAGAGGAGCCTGCACA CCGGTTTGGAGCGTAATAAGAC	58	Hetero	Homo	22
TLR7 and 8	$(TG)_{15}(CA)_1(TG)_6$	AATGGTGGTGGGAGATGAGTGT CTCAACCCTTCCCTGCTTTCT	55	Hetero (Same hetero pattern in both sexes)	Hetero	3
TLR9	$(CA)_9TA(CA)_{11}TA(CA)_3$	CCCCTCTGAACTAAAATGAGAAGACC AGAGCGTGGTGGGTATGTGG	55	Hetero	Hetero	1
TLR14	$(CA)_{11}$	TGACCGTAACACACCGTTCCAA GTGGTTCCATAGAGTCGGGCA	62	Hetero	Hetero	10
TLR21	$(CT)_{14}$	CATGCAGCAAGCAGCTAAATCT TCTTAACTTGCTCTGTGCGTGT	60	Hetero	Hetero	10
TLR22	$(GT)_{12}$	TCGTGTCCCTGATCCTGTATTG CCAGTGGCTAAAACACTGCTCT	58	Homo	Hetero	8

Ta is optimal annealing temperature, *LG* is linkage group

et al. 2003; Sebastiani et al. 1998, 2000). Single nucleotide polymorphisms, especially in the coding region of LRRs, reduce the ability to recognize PAMPs and interfere with the innate immune response (Leveque et al. 2003). Therefore, further studies on host genetic variation of TLR2 between resistant and susceptible lines may improve genetic selection for resistance to lymphocystis virus disease.

TLR3 was mapped to LG7 (Fig. 1). TLR3 was clustered with Poli112TUF, Poli16-77TUF, and Poli171TUF in both sexes. Comparing the location of Japanese flounder TLR3 with zebrafish, TLR2 and TLR3 of Japanese flounder were mapped to the different linkage groups while TLR3 of zebrafish was located with TLR2 on the same chromosome (Table 2). The difference appears to be related to genome rearrangements during teleost evolution.

Unlike mammalian TLR5, teleost fish have two types of TLR5, such as TLR5M and TLR5S. TLR5M, like other TLRs, consists of an LRR domain, a transmembrane domain and a TIR domain (Hwang et al. 2010a; Tsujita et al. 2004). On the other hand, TLR5S appeared through the duplication of the LRR domain of TLR5M and it lacked both a transmembrane domain and an intracellular TIR domain (Roach et al. 2005). TLR5M was mapped to LG22 in both sexes, while TLR5S was mapped to LG22 only in the female because the male was homozygous (Fig. 1). The

Table 2 Comparison of the locations of TLR genes in Japanese flounder, tetraodon, rainbow trout, zebrafish, and human

TLR gene	Japanese flounder	Tetraodon	Rainbow trout	Zebrafish	Human
TLR1	LG 21	Chr. 1	Chr. 14	Chr. 14	Chr. 4
TLR2	LG 15	Scaf. 7488	N/A	Chr. 1	Chr. 4
TLR3	LG 7	Chr. 18	Chr. 10	Chr. 1	Chr. 4
TLR5M	LG 22	Scaf. 13541	N/A	Chr. 20	Chr. 1
TLR5S	LG 22	Chr. 14	N/A	–	–
TLR7 and 8	LG 3	Chr. 2	Chr. 3	Chr. 21	Chr. X
TLR9	LG 1	Chr. 9	N/A	Chr. 8	Chr. 3
TLR14 or 18	LG 10	N/A	N/A	Chr. 16	–
TLR21	LG 10	N/A	Chr. 2	Chr. 16	–
TLR22	LG 8	Chr. 6	Chr. 11	Chr. 21	–

LG linkage group, *Chr* chromosome, *Scaf* scaffold, *N/A* not applicable, – not yet reported

distance between TLR5M and TLR5S was found to be 17.9 cM on the female map of LG22. Although both TLR5M and TLR5S in female flounder were located on the same linkage group, we could not rule out their syntenic relationships between teleost fish due to shortage of data available (Table 2).

In human, zebrafish, rainbow trout, and tetraodon, TLR7 and TLR8 are located adjacent to each other on the same chromosome by tandem duplication (Du et al. 2000; Palti et al. 2010a) (Table 2). A similar genomic organization was also observed in Japanese flounder, in which TLR7 and TLR8 are closely located to each other on the same BAC clone. This confirms that synteny between these TLR genes reveals high conservation from mammal to fish. The microsatellite of TLR7 and TLR8 in Japanese flounder was identified from a BAC clone containing both TLRs (Table 1). However, PCR results revealed that both parents were detected as the same heterozygous genotype pattern and their progeny have three genotypes, such as two types homozygous and one type heterozygous. Thus, we determined the location of TLR7 and TLR8 by analyzing the homozygous genotype pattern in the progeny and these genes were mapped to LG3 (Fig. 1).

TLR9 was located on LG1 (Fig. 1). TLR9 was commonly clustered with Poli6TUF microsatellite marker in both sexes. TLR14 and TLR21, which have not been identified from mammals, were mapped to LG10 (Fig. 1). Since TLR18 (corresponding to other fish TLR14) and TLR21 of zebrafish are located on the same chromosome (http://www.ensembl.org/index.html), the locations of the fish-specific TLR14 and TLR21 revealed that syntenic relationship between two genes are highly conserved in teleost line. In the male map, TLR14 and TLR21 are separated by 2.2 cM, while in the female map, they are located in the same cluster. These events are demonstrated by sex-specific difference in recombination of the microsatellites used for mapping of TLR14 and TLR21.

TLR22 was mapped to LG8 in the male and clustered with the Poli206 TUF microsatellite marker (Fig. 1). TLR22 and TLR9 of zebrafish were observed in the same chromosome, but these genes in Japanese flounder and tetraodon were mapped in different locations as TLR2 and TLR3 of Japanese flounder (Table 2).

In this study, we determined the location of 11 Japanese flounder TLRs in linkage group. TLR2 is especially interesting because it was found to be tightly linked to lymphocystis virus disease resistance locus, and is thus a candidate gene for disease resistance. Therefore, TLRs mapping might provide valuable information for future studies on the relationship between immune response and specific disease resistance. Furthermore, it can also serve as a guide for future genetic improvements to counter disease infection.

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