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ORIGINAL ARTICLE

A Genome Scan for Quantitative Trait Loci Associated with *Vibrio anguillarum* Infection Resistance in Japanese Flounder (*Paralichthys olivaceus*) by Bulked Segregant Analysis

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Abstract A recent genetic linkage map was employed to detect quantitative trait loci (QTLs) associated with *Vibrio anguillarum* resistance in Japanese flounder. An F1 family established and challenged with *V. anguillarum* in 2009 was used for QTL mapping. Of the 221 simple sequence repeat (SSR) markers used to detect polymorphisms in the parents of F1, 170 were confirmed to be polymorphic. The average distance between the markers was 10.6 cM. Equal amounts of genomic DNA from 15 fry that died early and from 15 survivors were pooled separately to constitute susceptible bulk and resistance bulk DNA. Bulked segregant analysis and QTL mapping were combined to detect candidate SSR markers and regions associated with the disease. A genome scan identified four polymorphic SSR markers, two of which were significantly different between susceptible and resistance bulk (P=0.008). These two markers were located in linkage group (LG) 7; therefore, all the SSR markers in LG7 were genotyped in all the challenged fry by single marker analysis. Using two different models, 11–17 SSR markers were detected with different levels of significance. To confirm the associations of these markers with the disease, composite interval mapping was employed to genotype all the challenged individuals. One and three QTLs, which explained more than 60 % of the phenotypic variance, were detected by the two models. Two of the QTLs were located at 48.6 cM. The common QTL may therefore be a major candidate region for disease resistance against *V. anguillarum* infection.

Keywords *Paralichthys olivaceus* · *Vibrio anguillarum* · Simple sequence repeat · Bulked segregant analysis · Linkage analysis

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Introduction

Japanese flounder (*Paralichthys olivaceus*) is an economically important fish in China, Japan, and Korea. In recent years, bacterial and viral diseases have seriously interfered with the development of Japanese flounder aquaculture. To prevent or at least diminish the devastating effects of these diseases, one useful method is to cultivate disease-resistant families or strains. Classical breeding programs focused on disease resistance traits can be highly problematic because the phenotypic determination of these traits is often complex and expensive (Rodriguez-Ramilo et al. 2011). Marker-assisted selection (MAS) is a rapid approach to culture strains of fish resistant to certain diseases (Fuji et al. 2007; Moen et al. 2009). For example, a single major genetic locus controlling the resistance to lymphocystis disease in Japanese flounder has been

identified (Fuji et al. 2006) and successfully used in MAS (Fuji et al. 2007).

Traditional association analysis is a time-consuming and expensive method. Bulked segregant analysis (BSA) is a rapid procedure for screening the main loci of quantitative traits or the loci linked with quality traits (Giovannoni et al. 1991; Michelmore et al. 1991; Wang and Paterson 1994; Venuprasad et al. 2012; Yang et al. 2013). With its high efficiency, low cost, and simplicity, BSA has come to be widely used in quantitative trait locus (QTL) mapping and association analysis (Mackay and Caligari 2000). In the past few years, a number of studies that have combined BSA with next-generation sequencing (Trick et al. 2012; Wang et al. 2013) have been reported. For example, Trick et al. (2012) demonstrated that RNA sequencing (RNA-seq) could be used to discover single nucleotide polymorphisms (SNPs) in polyploidy species and that BSA was an effective method to target SNPs in specific genetic intervals to fine map genes without the need for sequenced genomes. Wang et al. (2013) demonstrated that bulked segregant RNA-seq (BSR-seq) was useful for identifying genes associated with disease resistance against enteric septicemia of catfish (ESC) through expression profiling and mapping of significantly associated SNPs. They proposed that BSR-seq could be used in analyzing candidate genes associated with various traits without the vast investment needed to establish large genotyping platforms such as SNP arrays.

Several Japanese flounder genetic linkage maps have been reported (Kang et al. 2008; Castano-Sanchez et al. 2010; Song et al. 2012). The highest-density genetic linkage map currently available is based on genomic information and covers 96.7 % of the entire genome with an average interval of 1.22 cM (Song et al. 2012). This map can be used for genome-wide scanning to detect markers linked with a variety of traits.

The disease caused by *Vibrio anguillarum* is one of the most serious diseases in Japanese flounder because it can cause significant mortality to Japanese flounder at any point in the entire growth phase. The major histocompatibility complex (MHC) gene family plays an important role in both innate and adaptive immunity (Sheng et al. 2011), and previous studies have reported the relationship between MHC polymorphisms and resistance/susceptibility to *V. anguillarum* in Japanese flounder (Zhang et al. 2006; Xu et al. 2008, 2010; Du et al. 2011). Some MHC alleles have been detected as candidate genes associated with disease resistance. However, these genes may not be used to assist breeding because their selection may increase susceptibility of the selected individuals to other parasites and pathogens because of the loss of MHC variation (Johnson et al. 2008). It is therefore important to identify QTLs linked with this disease. QTL mapping is the first step for MAS. In China, the family selection of Japanese flounder with resistance to *V. anguillarum* has been conducted since 2007. In 2007, 59 of 63 Japanese flounder families were infected with *V. anguillarum* by intraperitoneal injection and families with a high resistance and with a low resistance to the disease were selected. In 2009, several families (F1 families) were established by crossing a high-resistance family with the lowest-resistance family. In this study, a genome scan was performed to detect simple sequence repeat (SSR) markers associated with resistance to *V. anguillarum*. To screen disease-resistant markers effectively, BSA and QTL mapping procedures were combined to detect loci linked with

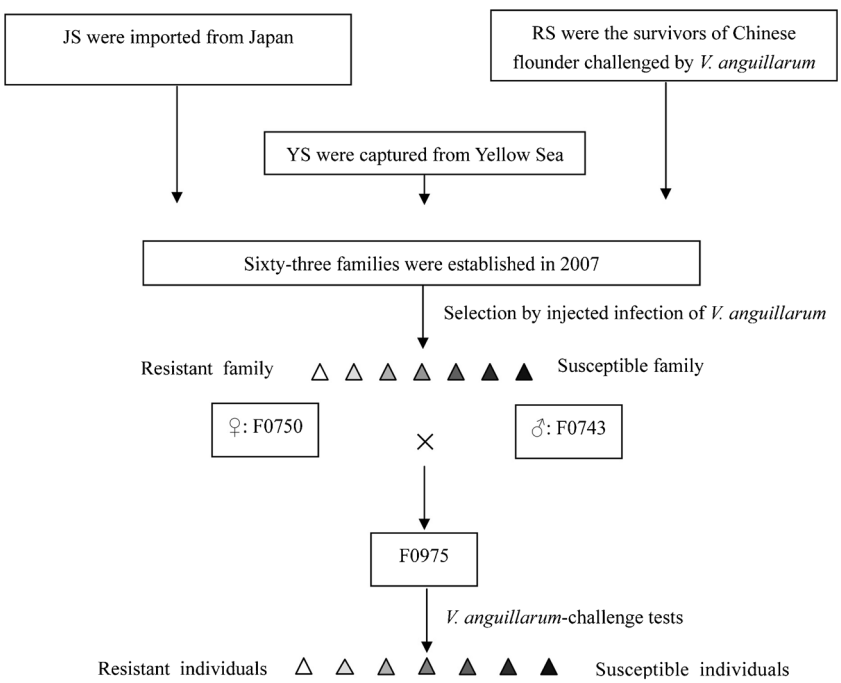

Fig. 1 *V. anguillarum* challenge and disease classification for QTL. *JS* Japanese stock, *RS* resistance stock, *YS* Yellow Sea wild stock

resistance to *V. anguillarum* in an F1 family challenged with *V. anguillarum*.

Materials and Methods

Japanese Flounder Families

Three basic populations of Japanese flounder were established in 2007, namely, Japanese stock, Chinese resistance stock (selected by spontaneous challenge and intraperitoneal injection of *V. anguillarum*), and Yellow Sea wild stock. Sixty-three families were established in 2007 and 59 of them were challenged with *V. anguillarum*. Of the 59 challenged families, F0750 (naming rule: F + year + family number) had the second highest survival rate (64.05 %) and F0743 had the lowest survival rate (7.27 %) (Chen et al. 2008). F0975, which we selected for QTL mapping, is an F1 family that was obtained from crossing of F0750 and F0743. A total of 81 fry derived from F0975 were challenged by intraperitoneal injection (Fig. 1). In addition, a brother–sister inbred family (F0905) was established and challenged in the same year as the control family. All the families mentioned above were raised and challenged in Haiyang Yellow Sea Aquatic Product Company (Yantai, China).

Challenge Experiments

The challenge experiment was conducted when the whole length of the fry was approximately 10 cm. Eighty-one individuals were selected randomly and cultured in a tank of 0.28 m^3 under flow-through conditions with a fresh water supply at 23±0.5 °C. *V. anguillarum* at a concentration of 3.58×10^5 colony-forming units (CFU)/ml were injected intraperitoneally. The fries were observed once every 3 h after infection and the number, whole length, and quantity of the dead fries were recorded each time. After 7 days, no further dead fry were observed and the surviving fry showed no disease symptoms. The number, whole length, and quantity of the surviving fry were recorded. At the time of recording, the tail fins of the fry were sampled and preserved in absolute alcohol.

Table 1 *V. anguillarum* challenge and disease classification for QTL mapping

Time after infection/h	Dead number	Model 1	Model 2
38	16		5
38–47	9		4
47–53	6		3
53–65	8		2
65–113	5	1	1
Survival number	37	0	0

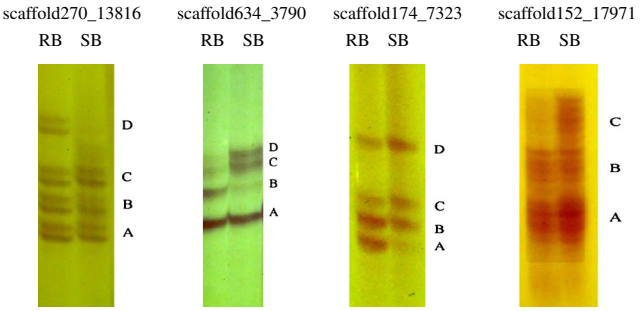

Fig. 2 Polymorphic SSR markers between RB and SB

SSR and BSA

A total of 221 SSR markers selected from a recent genetic linkage map (Song et al. 2012) were used to detect polymorphisms in the parents (F0750 and F0743) of F0975. Equal amounts of DNA from 15 fry that had died early and from 15 survivors were pooled separately to constitute the susceptible bulk (SB) and the resistance bulk (RB) DNA, respectively.

PCR Amplification and Denaturing Polyacrylamide Gel Electrophoresis (PAGE)

The polymerase chain reaction (PCR) system of 15 μL included 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.27 mM dNTP mixture, 0.4 mM primers, 0.5 unit of Tap DNA polymerase, and 50 ng of genomic DNA. Primer information is given in Online Resource 1. PCR reactions were performed on a Veriti 96-well thermal cycler (Applied Biosystems, Inc) under the following conditions: 5 min at 95 °C, followed by 30 s at 94 °C, 30 s at 57.5 °C (all with an annealing temperature of 57.5 °C) (Song et al. 2012), 40 s at 72 °C for 30 cycles, and 5 min at 72 °C for the final extension. The denatured PCR products were genotyped with 6 % denaturing PAGE.

Table 2 Fisher's exact chi-square test of polymorphic microsatellite SSR markers in BSA

Locus	Accession no.	15R[a]:15S[b]	LG	*P* value
Scaffold270_13816-D	JN900906	7:3	1	0.469
Scaffold634_3790-B	JN901018	10:2	7	0.008
Scaffold634_3790-D	JN901018	5:13	7	0.008
Scaffold174_7323-A	JN901035	10:2	7	0.008
Scaffold174_7323-D	JN901035	5:13	7	0.008
Scaffold152_17971-C	JN901408	10:5	15	0.066

Only the biggest different allele of every SSR marker was displayed

[a] Fifteen individuals from resistance bulk

[b] Fifteen individuals from susceptible bulk

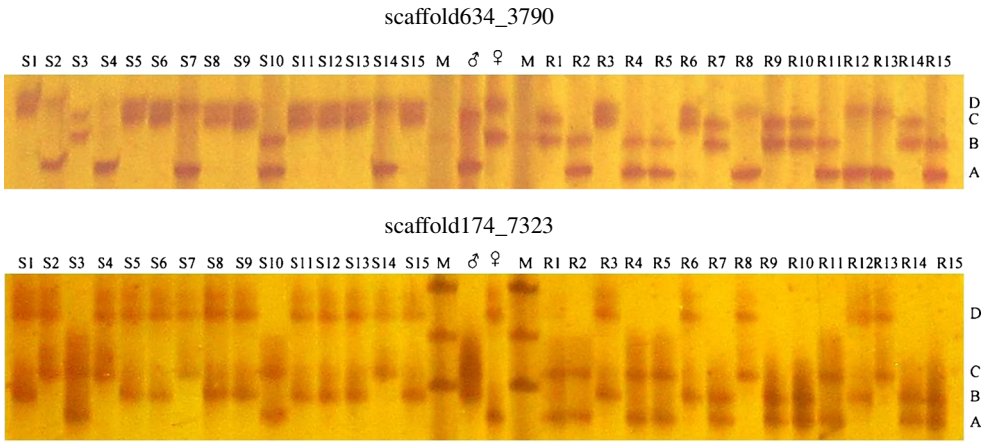

Fig. 3 Amplification of scaffold634_3790 and scaffold174_7323 in 30 individuals of SB and RB

Linkage Analysis

The polymorphic markers that were detected by BSA were confirmed in the RB and SB DNA from the 30 individuals. Fisher's exact chi-square test of the different alleles between the RB and SB was performed with cross tabulation using the SPSS 17.0 software. Linkage was considered to be significant for $P<0.05$ (the significant threshold of association with marker to trait was set at $P<0.05$). We selected the SSR markers from the linkage groups (LG) which had significantly different genotypes in the RB and SB groups to genotype all the infected fry. The JoinMap 4.0(Van Ooijen. 2006) and WinQTLCart 2.5 software (Wang et al. 2007) were used to map the genotyped markers and analyze the QTLs associated with disease resistance, respectively.

Results

V. anguillarum Challenge Experiment and Phenotype Analysis

After the challenge, the survival rates of F0905 and F0975 were 70.8 and 45.7 %, respectively. The F0975 family was deemed suitable for QTL mapping. Two QTL mapping models were used (Table 1). In model 1, the fry were classified as dead (1) or survived (0) (Houston et al. 2008; Ozaki et al. 2010). In model 2, the dead fry were classified at different levels as 5, 4, 3, 2, and 1 based on the time of death; the surviving fry were identified as "0" (Shoba et al. 2012).

SSR Polymorphisms and BSA

After testing, 170 of the 221 SSR markers were found to be polymorphic in the parents with a polymorphic rate of 76.9 %. The average distance between the markers was 10.6 cM. The polymorphic SSRs were derived from 24 linkage maps and 4–9 SSR markers were found per linkage group.

It is reasonable to assume that the markers that display uniform bands between the SB and RB were not related with the disease, while markers that display different bands or bands of different concentration between the two bulks may be associated with the disease. When the 170 polymorphic SSR markers were compared between the RB with SB, four polymorphic SSR markers, scaffold270_13816 in LG1, scaffold152_17971 in LG15, and scaffold634_3790 and scaffold174_7323 in LG7, were detected (Fig. 2). This result suggested that these four markers may be associated with disease resistance. All the SSR alleles were named A, B, C, or D according to their length from small to large. For example, the smallest allele of scaffold634_3790 was named scaffold634_3790-A.

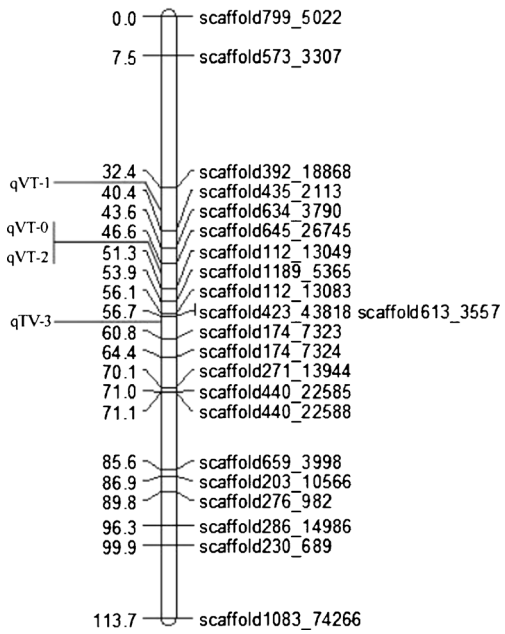

Fig. 4 A small linkage map and QTLs location

Confirmation of the Polymorphic SSR Markers

To confirm the allele difference between RB and SB groups, the individuals of the two bulks were genotyped. Different alleles were tested through the cross tabulation. The alleles of each of the SSR markers showed the biggest differences between the two bulks are displayed in Table 2. Chi-square tests indicated that scaffold270_13816 (P=0.469) and scaffold152_17971 (P=0.066) were not significantly different (P>0.05) in the RB and SB groups, while scaffold634_3790 (P=0.008) and scaffold174_7323 (P=0.008) were significantly different (P<0.05) between the two populations. Scaffold634_3790-B and scaffold174_7323-A had the same frequency in the RB (66.7 %) and SB (13.3 %) groups; their frequency in RB was significantly higher than their frequency in SB (P=0.008, <0.05). In contrast, scaffold634_3790-D and scaffold174_7323-D had the same frequency in the RB (33.3 %) and SB (86.7 %) groups; their frequency in RB was significantly lower than their frequency in SB (P=0.008, <0.05). Therefore, we concluded that these two markers may be associated with resistance to *V. anguillarum* infection (Fig. 3).

Single Marker Analysis

Because the two candidate markers (scaffold634_3790 and scaffold174_7323) were located in LG7, all the LG7 SSR markers were genotyped in the 30 challenged fry (15 in the SB and 15 in the RB groups). Twenty-two of the 44 SSR markers were used to construct a new linkage map (Fig. 4) and single marker analysis was used to locate QTLs. The total length of the small linkage map was 113.7 cM. In the first model, there were five and six significant markers at the 5 and 1 % levels, respectively. The phenotypic variance (R^2) and the additive effect of these 11 markers were estimated to range from 5.04 to 12.31 % and from −0.353 to 0.328, respectively. In model 2, there were seven, six, and four significant markers at the 5, 1, and 0.1 % levels, respectively. The R^2 and additive effect of the 17 markers were estimated to range from 4.85 to 14.26 % and from −1.558 to 1.494, respectively. In both models, the highest R^2 and additive effect were found for scaffold435_2113 (Table 3). Details about the SSR markers derived from LG7 are provided in Online Resource 2.

Table 3 Single marker analysis in the two models

Locus	Position/cM	Model 1			Model 2		
		Significance[a]	R^2/%	Add.	Sig.	R^2/%	Add.
Scaffold799_5022	0		0.03	−0.037		0.01	−0.208
Scaffold573_3307	7.5	–*	7.5	−0.277	–*	8.16	−1.148
Scaffold392_18868	32.4	–**	9.05	0.302	–**	10.03	1.307
Scaffold435_2113	40.4	–**	12.31	−0.353	–***	14.26	−1.558
Scaffold634_3790	43.6	–**	10.69	−0.328	–***	13.2	−1.494
Scaffold645_26745	46.6	–*	7.68	−0.278	–**	12.33	−1.444
Scaffold112_13049	51.3		5.81	0.22	–*	7.64	0.974
Scaffold1189_5365	53.9	–*	6.62	−0.256	–**	8.16	−1.168
Scaffold112_13083	56.1	–**	11.63	−0.353	–**	11.29	−1.458
Scaffold423_43818	56.7	–**	10.69	−0.328	–***	13.2	−1.494
Scaffold613_3557	56.7	–**	10.69	0.328	–***	13.2	1.494
Scaffold174_7323	60.8	–*	7.21	−0.269	–**	12.29	−1.444
Scaffold174_7324	64.4	–*	5.04	−0.227	–**	9.41	−1.27
Scaffold271_13944	70.1		4.02	0.158	–*	7.67	1.02
Scaffold440_22585	71		4.16	−0.204	–*	6.99	−1.085
Scaffold440_22588	71.1		3.68	−0.204	–*	6.48	−1.085
Scaffold659_3998	85.6		1.49	−0.122	–*	4.85	−0.906
Scaffold203_10566	86.9		0.89	0.095		3.86	0.806
Scaffold276_982	89.8		0.92	0.095		3.96	0.806
Scaffold286_14986	96.3		1.49	0.122	–*	5.4	0.956
Scaffold230_689	99.9		0.04	0.05		0.95	0.518
Scaffold1083_74266	113.7		0.02	−0.021		0.19	0.119

R^2 (%) proportion of the explained phenotypic variance, *Add.* the additive effects

[a] Significance at the 5, 1, and 0.1 % levels are indicated by *, **, and ***, respectively

Composite Interval Mapping

To confirm the association between the SSR markers and disease resistance, composite interval mapping was employed to reanalyze the genotyped markers. The logarithm of odds (LOD) threshold was set at 2.5 to identify the association of QTLs with disease resistance. In model 1, a single QTL (qVT-0) peaked at 48.6 cM with a LOD score of 2.8. This QTL explained 65 % of the total R^2 with an additive effect of −0.83751. The SSR markers in scaffold645_26745 and scaffold112_13049 that were located to the left and right of the QTL explained 6.54 and 0.04 % of R^2, respectively. In model 2, three QTLs (qVT-1, qVT-2, and qVT-3) were identified. These QTLs peaked at 37.5, 48.6, and 57.8 cM with LOD scores of 9.7, 5.0, and 7.9, respectively (Fig. 5).

QTLs qVT-1, qVT-2, and qVT-3 explained 63, 66, and 66 % of R^2 with additive effects of −3.605, −3.464, and 3.496, respectively (Table 4). The SSR markers located to the left and right of the three QTLs explained from 0.74 to 9.73 % of R^2. The recombination frequency between the QTLs and their closest markers ranged from 1.2 to 5.1 %. The SSR marker in scaffold645_26745 explained the highest amount of R^2 (Table 5). The qVT-0 and qVT-2 QTLs were located at the same position of 48.6 cM in the two models.

Discussion

In QTL mapping studies, either an F2 population or a backcross population is commonly used. Because the traits of the two populations segregate clearly, F1 populations can also be used in QTL mapping (Wang et al. 2006; Ozakil et al. 2007; Guo et al. 2012; Song et al. 2012), but the F1 parents should have a rich genetic background and enough polymorphisms to segregate DNA markers and phenotypic values in the offspring (Yue 2013). Japanese flounder take 3 years to reach sexual maturity, making it difficult to establish a pure line of Japanese flounder. In this study, the parents of F0975 came from the mating of Japanese stock and Chinese resistance stock. The two populations have a high polymorphism rate and great differences in genetic background (Shao et al. 2009). Therefore, F0975 was deemed suitable for the QTL mapping reported in this paper.

BSA was used originally to detect major effect loci (Giovannoni et al. 1991; Michelmore et al. 1991; Wang and

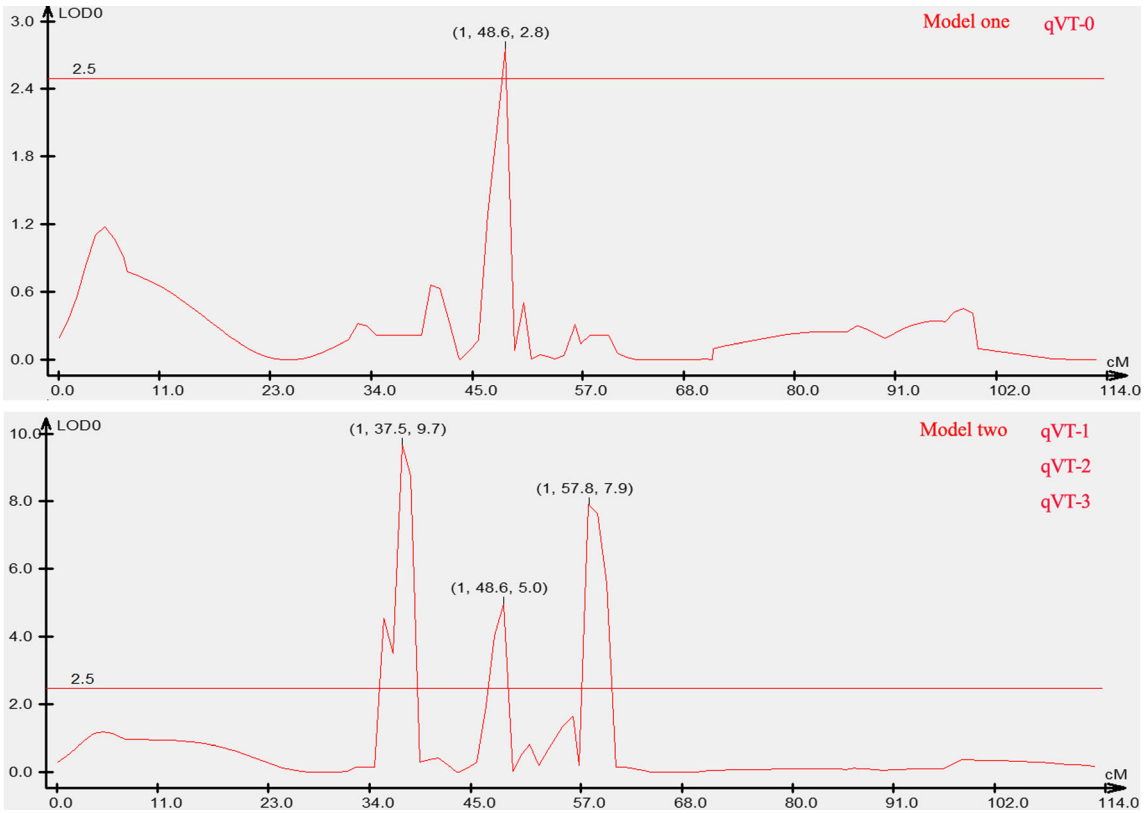

Fig. 5 LOD curve graphs of four QTLs in two models. *Abscissa* indicates the relative position on the linkage groups; *vertical coordinates* indicate the value of LOD; the *red line* whose value of LOD is 2.5 represents significant threshold of QTLs; and *the figure in parentheses* represents group, position, and value of LOD. Because only one genetic linkage group was constructed in this paper, the group was named as LG1. In fact, it was LG7 in the recent genetic linkage maps (Song et al. 2012)

Table 4 Parameters of the QTLs and estimation of the genetic effects

Model	QTL	Peak of cM	Peak of LOD	QTL internal	R^2 (%)	Add
Model 1	qVT-0	48.6	2.8	48.3–48.6	65	−0.83751
Model 2	qVT-1	37.5	9.7	35–39.2	63	−3.605
	qVT-2	48.6	5	46.9–49.1	66	−3.464
	qVT-3	57.8	7.9	57.1–60.3	66	3.496

Paterson 1994); now, because this method has high efficiency and low cost, it has also been used to analyze complex traits. In fish research, BSA has been used mainly to identify sex-related markers (Ezaz et al. 2004; Lee et al. 2004; Keyvanshokooh et al. 2007; Wang et al. 2009a; Wang et al. 2009b). However, the false-positive rate in BSA is reportedly high. Indeed, in our study, we found that two of the four polymorphic markers were false positives. Nevertheless, when BSA is used critically, it can provide a rapid and economical approach to help detect markers associated with traits. How many individuals should be selected to constitute the RB and SB depend on the following reasons. First, the amount of challenged number is an important factor because RB and SB require extreme individuals. In this paper, the survivors and early died fry were demanded to constitute the RB and SR. The intermediate fry were not used to constitute bulk. Second, many researchers generally believed that 10 to 20 individuals have adequate representation to screen candidate markers (Giovannoni et al. 1991; Livaja et al. 2013; Randhawa et al. 2013).

Here, we used two models to analyze the phenotype. Model 1 has always been used to analyze qualitative traits that were controlled by a few major genes, while model 2 has usually been used to analyze traits that were controlled by multiple minor genes. While model 1 has been used frequently to detect disease resistance markers in fish (Fuji et al. 2006; Houston et al. 2008; Ozaki et al. 2010), model 2 has generally been applied to screen markers associated with diseases in plants (Shoba et al. 2012). Although model 2 has not been reported previously in fish research, we used it here in an effort to obtain novel findings. We identified only one QTL using model 1 and three QTLs using model 2. A QTL at position 48.6 cM was detected by both models, suggesting that this QTL may be the QTL most usefully associated with *V. anguillarum* disease resistance.

Single marker analysis is a simple and easy-to-use method that can be employed to identify the preliminary association between markers and traits. Composite interval mapping can be used to eliminate false positives and phantom QTLs so that QTLs can be mapped accurately. Therefore, when used together, these two methods can not only analyze a single marker but can also position QTLs precisely.

The four QTLs that we identified explained more than 60 % of the R^2 results; however, the markers around the QTLs explained, at most, only 9.73 % of the R^2. Further, there was only a 2 % recombination frequency between the markers and the QTLs; therefore, the R^2 of the QTLs may have been overestimated to some extent. One possible reason is that some of the minor genes were missed in the pre-filtering by BSA. Another possible reason is that there was an interaction effect between the three QTLs identified by model 2 because their locations were close together. This possibility warrants future investigation. The third reason may be that the classification method used in model 2 was not very accurate. The method of classifying the disease levels is of particular importance. For quantitative traits, selection based on just one particular allele or marker is difficult. In this study, the highest R^2 explained by the detected markers was 9.73 %, which is not sufficient for the selection of a strain with bacterial resistance. Further, the relationship between the MHC alleles, which have been detected previously as candidate genes associated with disease resistance (Zhang et al. 2006; Xu et al. 2008, 2010; Du et al. 2011), and the QTLs that were detected in the present study, is still unresolved. The MHC is encoded by an important family of immune-related genes with many genes and copies. Polymorphisms in MHC genes are closely related with environmental adaptability (Hughes et al. 1998; Cohen et al. 2006). Therefore, if individuals with candidate resistance MHC alleles are selected for breeding, careful monitoring is required.

Recently, some BSA studies combined with RNA-seq have been reported. Trick et al. (2012) used RNA-seq and BSA to fine map genes in polyploidy wheat in target genetic intervals when a sequenced genome was not available. The advantage

Table 5 Recombination frequencies and phenotypic variances of markers around the QTLs

QTL	The left marker	RF/%	R^2	The right marker	RF/%	R^2
qVT-0	Scaffold645_26745	2.0	6.54	Scaffold112_13049	2.7	0.04
qVT-1	Scaffold392_18868	5.1	0.74	Scaffold435_2113	2.9	1.69
qVT-2	Scaffold645_26745	2	9.73	Scaffold112_13049	2.7	4.21
qVT-3	Scaffold613_3557	1.2	1.04	Scaffold174_7323	2.9	0.81

RF recombination frequency

of this method is that it can be applied effectively in species for which no sequenced genome is available. A disadvantage is that it can be applied only to some specific genetic intervals. Wang et al. (2013) combined BSA with RNA-seq together with the sequenced catfish genome to detect expression and positional candidate genes and allele-specific expression for disease resistance against ESC. This method proved to be a rapid and efficient way to screen genes or SNPs associated with disease resistance against ESC. It is also an economical way to map significant SNPs without the need for a large genotyping platform. A shortcoming was that the method detected the different genes and SNPs associated with the trait only in one tissue and not in the whole individual. In our study, the foundation of our research was the construction of high-density genetic linkage maps based on genome sequencing of Japanese flounder. Therefore, we mapped QTLs associated with *V. anguillarum* infection resistance in Japanese flounder by BSA, which is a rapid and effective method to map major QTLs at the genome level. However, our study cannot discover differences in gene expression at the transcriptome level. In the mean time, SNPs are distributed more widely than SSRs, and SNPs are usually linked more tightly with genes than SSRs. A method to rapidly analyze the transcriptomes in whole individuals will be an important contribution to future researches.

In summary, the candidate regions associated with resistance to *V. anguillarum* infection in Japanese flounder were detected. One of our following interests is to research the relationship between the QTLs detected in this study and the MHC alleles. Our another interest is to establish a high-density genetic linkage map using single nucleotide polymorphism to find some markers linked with *V. anguillarum* resistance more closely and look for candidate genes associated with the major *V. anguillarum* resistance QTL in Japanese flounder.

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