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ORIGINAL ARTICLE

Identification of Quantitative Trait Loci for Resistance to RSIVD in Red Sea Bream (*Pagrus major*)

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Abstract Red sea bream iridoviral disease (RSIVD) is a major viral disease in red sea bream farming in Japan. Previously, we identified one candidate male individual of red sea bream that was significantly associated with convalescent individuals after RSIVD. The purpose of this study is to identify the quantitative trait loci (QTL) linked to the RSIVD-resistant trait for future marker-assisted selection (MAS). Two test families were developed using the candidate male in 2014 (Fam-2014) and 2015 (Fam-2015). These test families were challenged with RSIV, and phenotypes were evaluated. Then, de novo genome sequences of red sea bream were obtained through next-generation sequencing, and microsatellite markers were searched and selected for linkage map construction. One immune-related gene, *MHC class IIβ*, was also used for linkage map construction. Of the microsatellite markers searched, 148 and 197 were mapped on 23 and 27 linkage groups in the female and male linkage maps, respectively, covering approximately 65% of genomes in both sexes. One QTL linked to an RSIVD-resistant trait was found in linkage group 2 of the candidate male in Fam-2014, and the phenotypic variance of the QTL was 31.1%. The QTL was closely linked to *MHC class IIβ*. Moreover, the QTL observed in Fam-2014 was also significantly linked to an RSIVD-resistant trait in the candidate male of Fam-2015. Our results suggest that the RSIVD-resistant trait in the candidate male was controlled by one major QTL closely linked to the *MHC class IIβ* gene and could be useful for MAS of red sea bream.

Keywords Red sea bream · Red sea bream iridovirus · RSIVD · QTL · Microsatellite · *MHC class IIβ*

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Introduction

Red sea bream (*Pagrus major*) is an important aquaculture fish species in Japan because of its palatability and high economic value. Nowadays, around 80,000 tons of this species is cultured per year, approximately 5-fold that of production by catch (Miyashita and Seoka 2005). Red sea bream aquaculture is mainly conducted in the west side of Japan, such as in Ehime Prefecture, and most of the aquaculture farms are located in the southern district of this prefecture (e.g., Uwajima and Ainan) because of the warm water temperature during the year. Thousands to millions of the fish (depending on fish size) are usually cultured in square net-cages, set side by side (Miyashita and Seoka 2005). The density of the fish in the net-cages is high and the aquaculture area is crowded compared with a wild environment, and therefore, infectious diseases caused by viruses, bacteria, and parasites often occur in red sea bream aquaculture, and sometimes, outbreaks of

infectious diseases cause serious economic loss in the red sea bream aquaculture industry (Miyashita and Seoka 2005).

Red sea bream iridoviral disease (RSIVD) caused by the red sea bream iridovirus (RSIV) is a major cause of mortality in red sea bream farming in Japan (Nakajima and Kurita 2005). RSIVD was first reported in cultured red sea bream in an outbreak in Ehime Prefecture, Japan, in 1990 (Inouye et al. 1992), and since then, this viral disease has been observed around Japan (Nakajima and Kurita 2005). This viral disease has also been confirmed in 30 other cultured marine fish species (Kawakami and Nakajima 2002). After being infected with RSIV, fish become lethargic and suffer from severe anemia, petechial of the gill, and enlargement of the spleen (Inouye et al. 1992). The vaccine for RSIV was approved in 1999 for juvenile red sea bream, and vaccination significantly reduces mortality of this disease (Nakajima et al. 1999). However, vaccination of a large number of fish is costly and labor-intensive, and not all farmers use the vaccine. Injecting vaccine into millions of small seedlings (approximately 3 to 4 cm) is also impractical in hatcheries. Therefore, RSIVD is still prevalent in red sea bream aquaculture, and developing a prevention method for RSIVD without vaccination is required to improve production efficiency and to sustain the red sea bream aquaculture industry.

Previously we identified a novel male broodstock (based on pedigree tracing) significantly associated with convalescent individuals that survived an outbreak of RSIVD (Sawayama and Takagi 2017). The survival rate of offspring associated with the male individual was higher than other broodstock. Therefore, the male broodstock is suggested to have an RSIVD-resistant trait that could be useful for future breeding programs. Quantitative trait loci (QTL) linked to disease-resistant traits have been identified in many aquaculture fish species in the past decade (Fuji et al. 2006; Ozaki et al. 2007; Moen et al. 2009; Houston et al. 2010; Ozaki et al. 2010; Ozaki et al. 2013). DNA markers tightly linked to QTL have been used for marker-assisted selection (MAS). Several disease-resistant strains and seedlings constructed through MAS have now been successfully developed and have improved production efficiency in the aquaculture industry (Fuji et al. 2007; Moen et al. 2015; Moldal and Bornø 2015; Chavanne et al. 2016). If QTL linked to an RSIVD-resistant trait are identified in the candidate male, an RSIVD-resistant strain of red sea bream can be developed using MAS in a short time period.

Constructing a linkage map with a large number of polymorphic DNA markers is essential to identify QTL. Data were used to construct linkage maps for several important aquaculture fish species, and these maps are used to identify QTL linked to economic traits such as growth and morphometric- and disease-resistant traits (Yue 2013). The linkage map of red sea bream has already been constructed by Inami et al. (2005) using polymorphic microsatellite markers, but map and marker information are not available. Therefore, developing a

genetic infrastructure, and a large number of microsatellite DNA markers, and constructing the new linkage map are needed to identify the RSIVD-resistant trait of the candidate male and also for future breeding programs in this fish species. In addition, using not only microsatellite DNA markers located on anonymous regions of genomes but also DNA markers located on immune-related genes for linkage map construction should provide useful information to predict quantitative trait genes (QTG) and quantitative trait nucleotides (QTN) for the RSIVD-resistant trait of the candidate male.

In the present study, we conducted QTL analysis for the RSIVD-resistant trait of the candidate male for future breeding programs. Subsequently, large numbers of microsatellite DNA markers of red sea bream were designed from de novo-assembled genomic sequences constructed using next-generation sequencing (HiSeq 2000). These newly developed microsatellite markers and also one immune-related gene (*MHC class IIβ*) were used to construct genetic linkage maps of an F_1 test-cross, and QTL for the RSIVD-resistant trait in two different F_1 populations associated with the candidate male were identified.

Materials and Methods

Fish

Milt was collected from the previously identified RSIVD-resistant male (Sawayama and Takagi 2017) for artificial fertilization. Two F_1 families were produced by artificial fertilization using the milt and egg from the female normally used for production at a seed production company (Marua Suisan Co., Ltd., Ehime, Japan) in 2014 (Fam-2014) and 2015 (Fam-2015). Rearing procedures followed the hatchery manual. Fish from Fam-2014 ($n = 120$; average body weight ± SD: $28.1 ± 7.4$ g) were used for the first challenge test for linkage map construction and screening QTL, and fish from Fam-2015 ($n = 400$; average body weight ± SD: $15.6 ± 4.7$ g) were used for the second challenge test to re-confirm the QTL identified from Fam-2014. All experiments described in this study were carried out in accordance with the Guide for the Care and Use of Laboratory Animals from Ehime University.

Challenge Experiments

Red sea bream specimens diagnosed with RSIVD were collected from two different farms in 2014 (Ainan, Ehime, Japan) and 2015 (Kamijima, Ehime, Japan), and spleen tissues of these fish from each year were sampled. The spleen tissues collected each year were used for two challenge experiments conducted in 2014 and 2015. The collected spleen tissues were pooled and minced using scalpels and stored at − 80 °C until the experiment. These tissues were homogenized in a 30-fold volume of minimum

essential media (MEM) and centrifuged at 3000g for 20 min at 4 °C. The supernatant was filtered through a 0.45-μm membrane filter, and the filtrate was diluted to 300-fold volume using MEM and used for the challenge experiments. The approximate viral titer was measured using PCR as described by Kim et al. (2005) because RSIV failed to grow in common cell lines. The viruses collected in 2014 and 2015 were detectable until the homogenates were diluted by a factor of 10^3 and 10^4, respectively. The injection volume of the medium per fish was set at 0.1 mL. After injecting the RSIV-containing medium, fish were kept in 200-L polycarbonate tanks, and water temperature was maintained at 25–30 °C. All challenge experiments were conducted at the rearing facility of Ehime University (Ainan, Ehime, Japan). Dead fish were collected every day during the challenge experiment, and all the surviving fish were sampled at 14 days post-challenge. The phenotypes were scored as dead (1) or survived (0) for further analysis. These specimens were stored in 99.9% ethanol or at − 20 °C until DNA extraction.

Development of Microsatellite Markers

DNA extracted from the milt of a single male individual was used for the microsatellite DNA isolation. Genomic DNA was extracted using a High Pure PCR Template Preparation Kit (Roche Diagnosis, Tokyo, Japan). Approximately 1 μg of genomic DNA was used for DNA library preparation with an insert size of 500 and 100 bp pair-end sequencing of HiSeq 2000 (Illumina, San Diego, CA, USA) following the manufacturer's instructions. After pair-end sequencing, the raw data was treated as follows: deleted adapter sequence, removed the low-quality reads (quality value ≤ 5 (E) was ≥ 50%), and deleted duplicated reads. The filtered reads were then assembled using SOAP de novo (Li et al. 2010). SSRIT was used to scan the assembled sequences for perfect di-, tri-, tetra-, penta-, and hexa-nucleotide tandem repeats that had at least four repeat units (Temnykh et al. 2001). Primer 3 (Rozen and Skaletsky 2000) was used to design primer pairs in the flanking region of the microsatellite loci with the following specifications: melting temperatures (Tm) 60–65 °C with a maximum of 1 °C difference between paired primers; amplicon length range 80–300 bp; primer size 20–28 bp. Finally, we aligned the designed primer sequences to the assembled genome sequences again using SOAP2 (Li et al. 2009), and only unique primer sequences were selected. Tri- to hexa-nucleotides of the newly developed markers were selected for genotyping because tri- and tetra-nucleotide microsatellites have less stuttering and lower genotyping errors than di-nucleotide microsatellites (Butler 2005). In addition, size differences between the tri- and tetra-nucleotide microsatellites can be distinguished using the newly developed capillary electrophoresis system (Advanced Analytical, Iowa, USA), which is more cost effective and less time consuming than capillary sequencers and gel electrophoresis. Newly developed microsatellite markers were named

with the prefix *Pma* (*Pagrus major*) with a repeat motif number and serial number of markers separated by an underscore (e.g., *Pma4_001* for tetra-nucleotide repeat microsatellite marker no. 001).

Genotyping

Genomic DNA was extracted using a QuickGene Tissue kit (Kurabo, Osaka, Japan) from portions of caudal fin of preserved specimens according to the manufacturer's protocol. PCR for the newly developed microsatellite DNA markers was conducted with 25 ng of extracted DNA, 0.5 μM primer, 1 mM dNTPs, 0.1 unit KOD Fx polymerase (Toyobo, Osaka, Japan) with 2× buffers in a total volume of 5 μL. The PCR reactions were done under a touch-down thermal profile: 2-min hold at 94 °C, followed by five cycles of pre-PCR consisting of 10 s at 98 °C for denaturing, 30 s at 65 °C for annealing, and 30 s at 68 °C for primer extension reaction. PCR amplification was then continued for 30 more cycles at a constant 61 °C annealing temperature, and the rest of the pre-PCR parameters was kept unchanged. After the PCR procedures were completed, the samples were stored at 4 °C. The PCR products were separated using a Fragment Analyzer (Advanced Analytical, Iowa, USA) at 8 kV for 80 min using a DNF-900 dsDNA Reagent Kit. Band sizes of PCR fragments were compared with a 25-bp DNA step ladder (Promega, WI, USA) and calculated using *PRO Size™* software (Advanced Analytical).

We also genotyped eight di-nucleotide microsatellite markers (*Pma-2*, *-3*, *-4*, and *-5* (Takagi et al. 1997); *Pma22-9* and *Pma4-32* (Hatanaka et al. 2006); and *Kpm2* and *Kpm22* (Blanco-Gonzales et al. 2012)) that were used for population genetics and parentage analysis of red sea bream in our previous studies (Sawayama and Takagi 2011, 2015, 2016). PCR amplification and allele detection followed the method of previous studies (Sawayama and Takagi 2015, 2016).

All the primers used for genotyping are shown in supplemental Table 1.

Candidate Gene Analysis

To assess association between the immune-related gene and the RSIVD-resistant trait, we also developed a DNA marker using the major histocompatibility complex class IIβ gene (*MHC class IIβ*) of red sea bream following the sequence cloned by Chen et al. (2006). A primer pair amplifying a 14 bp of minisatellite located in intron I was designed based on the deposited sequence in GenBank (AY848956) and named as *pmaMHC2b*.

The PCR protocol of *pmaMHC2b* was 25 ng of extracted DNA, 0.3 μM primer, 0.4 mM dNTPs, 0.2 unit KOD Fx polymerase with 2× buffers in a total volume of 10 μL. The PCR reactions were done as follows: one initial denaturing step for

2 min at 94 °C, followed by 30 cycles at 98 °C for 10 s, 63 °C for 30 s, and 68 °C for 60 s. The PCR products were separated with the Fragment Analyzer at 8 kV for 85 min using a DNF-910 dsDNA Reagent Kit. Band sizes were calculated using *PRO Size™*.

Linkage Mapping and Genome Size Estimation

Linkage analysis was performed using LINKMFEX version 2.3 (Danzmann 2006): this application can separate alleles originating from a dam and sire and check for the accuracy of genotypes in their progeny. Genotype data were converted to a backcross format even though the grandparent genotype was unknown. Pairwise analyses were performed, and markers were sorted in linkage groups at a minimum LOD score of 3.0. Double recombination events were checked with Map ManagerQTX version 2.0 (Manly et al. 2001), and a final marker order was determined. Graphic map files were generated using MapChart version 2.2 (Voorrips 2002).

Genome length was estimated in sex-specific maps using two methods. G_{e1} was calculated according to Fishman et al. (2001): chromosome ends of two times the average framework marker spacing were added to the length of each linkage group, where the framework marker was denoted as the marker providing statistical support for marker order and spacing in the genetic linkage map. G_{e2} was calculated according to Chakravarti et al. (1991): the length of each genetic linkage group was multiplied by $(m + 1)/(m - 1)$, where m is the number of framework markers on the linkage groups. The estimated genome length (Ge) for each sex was used as an average of the two estimated values.

QTL Analysis

Genome- and chromosome-wide QTL analysis was performed using J/qtl (Smith et al. 2009), which is the graphical interface of R/qtl (Broman et al. 2003), by implementing the binary model to test 10,000 permutations of the genotypic vector generated for each parent using EM likelihood. This approach allowed us to obtain estimates for significance levels of segregating markers tested within individuals (significant ($P < 0.10$) and highly significant ($P < 0.05$) QTLs). Single QTL locations were identified (scanone) across all linkage groups. Percent phenotypic variance (%) attributable to a locus was also estimated by modeling the binary trait as continuous using J/qtl.

Results

RSIV Challenge Test and Trait Measurement

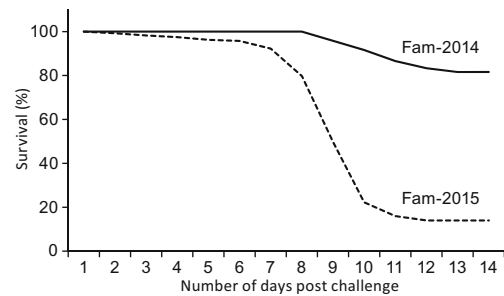The survival rate (%) of the Fam-2014 and Fam-2015 families in the RSIV-challenge tests are shown in Fig. 1. The

Fig. 1 Survival rates of Fam-2014 and Fam-2015 in injection medium containing RSIV-infected spleens of red sea bream. Cumulative mortalities of Fam-2014 and Fam-2015 were 18.4 and 86.0%, respectively

cumulative mortalities of Fam-2014 and Fam-2015 were 18.4 and 86.0%, respectively. All the dead fish ($n = 21$) and 23 surviving fish were used for genotyping in Fam-2014 (total 44 individuals). For Fam-2015, 44 each of the surviving and dead fish (total 88 individuals) were used for genotyping. Highly susceptible individuals were selected and used for genotyping in Fam-2015 because of high mortality observed in the challenge test in Fam-2015.

Isolation of Microsatellite DNA Markers

We obtained a total of 13.02 Gb of clean data on red sea bream generated from HiSeq 2000 sequencing. These data were assembled, and 843.4 Mb of sequence data was obtained from 886,260 scaffolds with a length range from 100 to 454,737 bp. After screening of microsatellite motifs and primer designing, a total of 172,172 primer pairs were designed within 79,776 scaffolds. The most abundant type of repeat motif was a di-nucleotide ($n = 93,709$; 54.4%), followed by a tri-nucleotide ($n = 56,517$; 32.8%), tetra-nucleotide ($n = 16,534$; 9.6%), penta-nucleotide ($n = 4264$; 2.5%), and hexa-nucleotide ($n = 1148$; 0.7%). A total of 449 newly developed microsatellite markers (64 of tri-, 360 of tetra-, 16 of penta-, and 9 of hexa-nucleotide repeats) were selected and used for linkage mapping (supplemental Table 1). Sequencing information and data were registered in BioProject PRJDB5593. Scaffolds and microsatellite sequences were also deposited in GenBank (BDUH01000001–BDUH01886260 and LC323197–LC323645, respectively).

Linkage Mapping and Genome Coverage Estimation

A total of 458 markers were used for genotyping. Of the 458 markers, 250 were polymorphic (54.6%) composed of 5 di-nucleotide (62.5%), 27 tri-nucleotide (42.2%), 165 tetra-nucleotide (45.8%), 7 penta-nucleotide (43.8%), 4 hexa-nucleotide (44.4%), and one gene-associated marker (*pmaMHC2b*) in Fam-2014. A total of 148 markers were successfully mapped on the female-specific maps distributed in 23 linkage groups (Fig. 2). The total genome size of the female map was estimated as 769.3 cM. The number of markers per
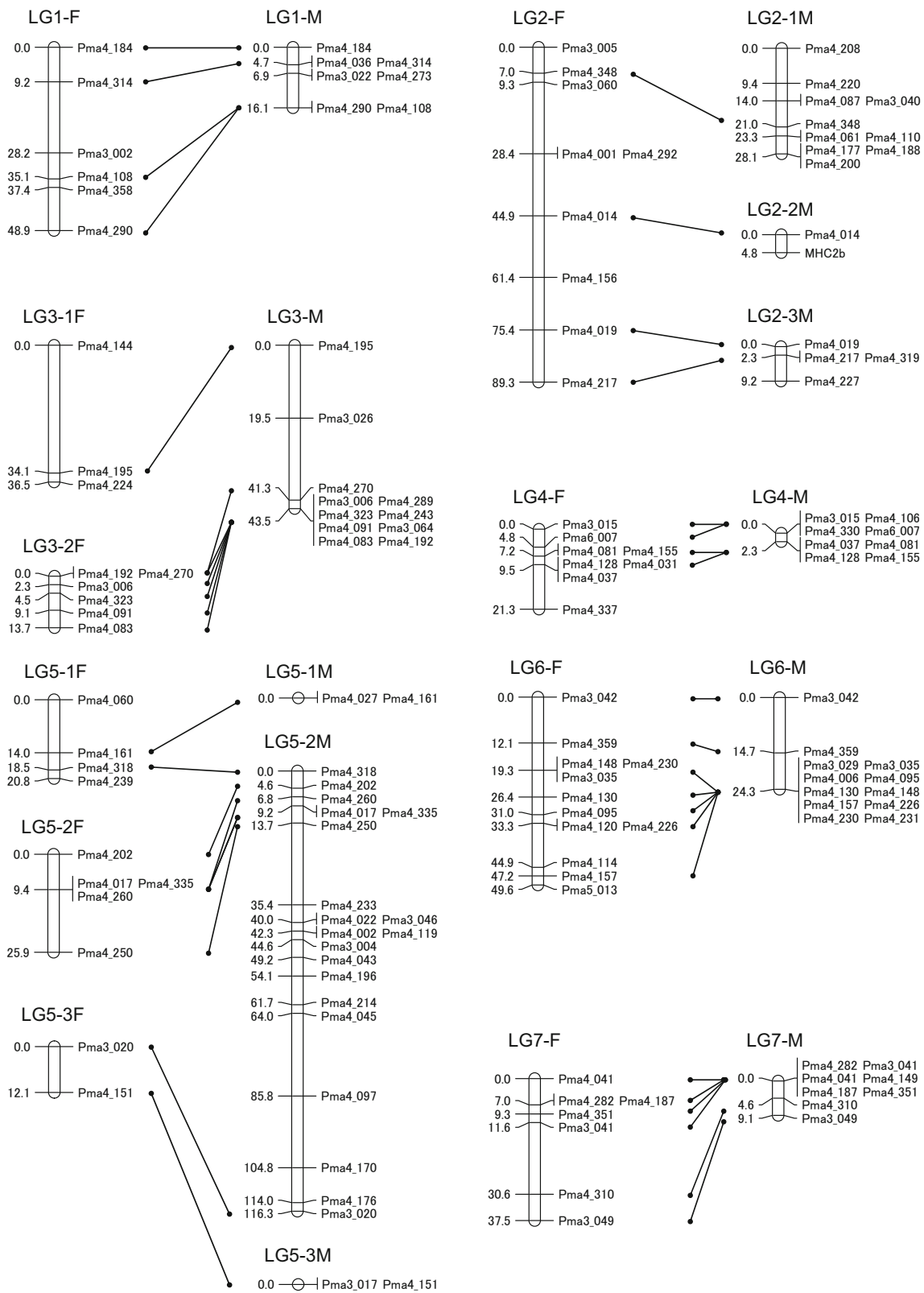
Fig. 2 Red sea bream female (left) and male (right) maps. Co-segregation microsatellite loci were connected between the female and male maps. Map distances calculated between markers are expressed in Kosambi function (cM)

linkage group varied from 2 to 17, with an average of 6; the longest linkage group of the female map extended to 89.3 cM

(LG2). Meanwhile, 197 markers were successfully mapped on the male-specific maps containing 27 LGs (Fig. 2). The total
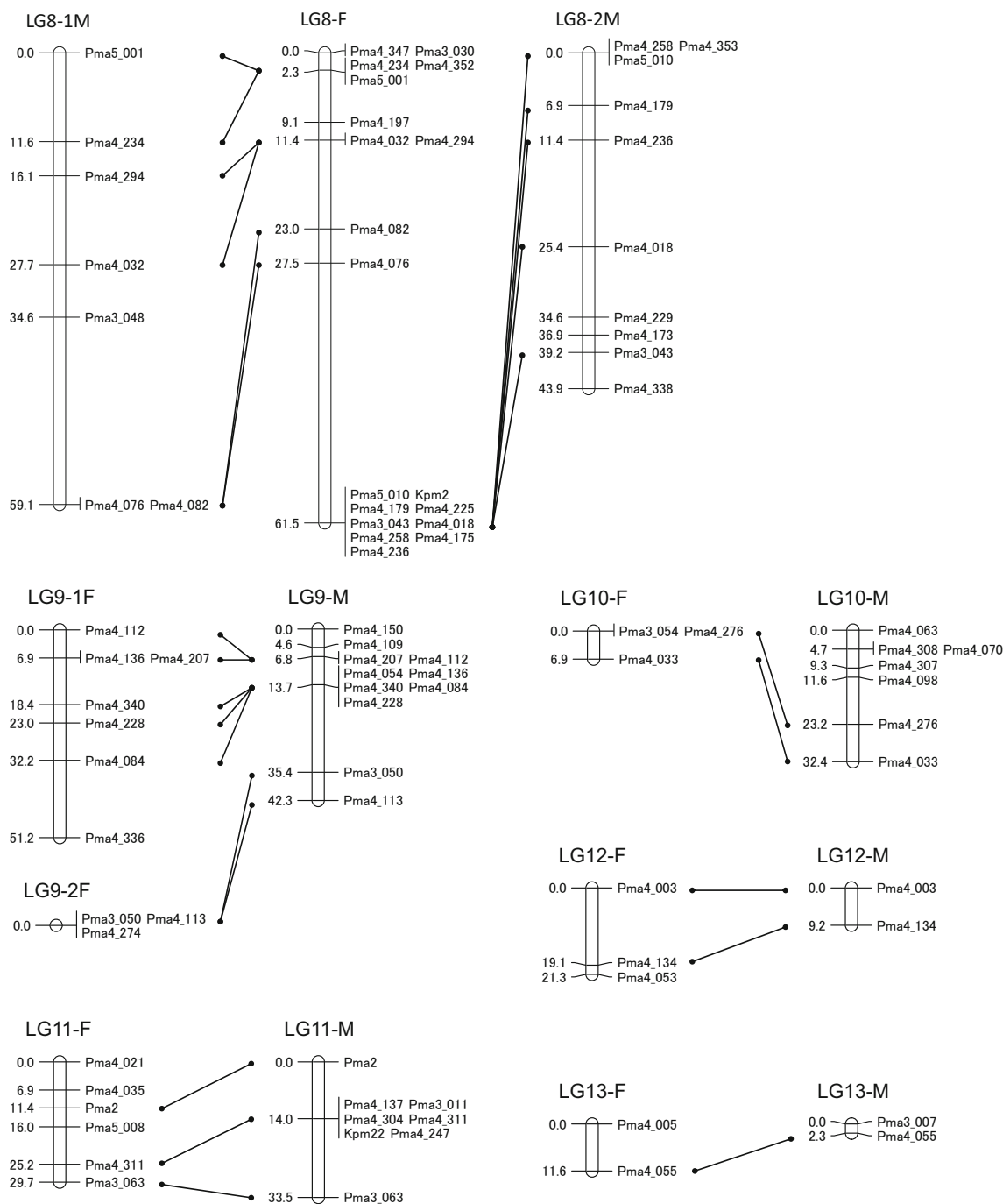
Fig. 2 (continued)

genome was estimated at 626.0 cM. The longest linkage group of the male map extended to 116.3 cM (LG5), and the average number of markers per linkage group was 7 and varied from 2 to 22. There were 21 linkage groups shared with both sexes. A newly developed gene-related marker (*pmaMHC2b*) was mapped onto the male map of LG2M. Four of the dinucleotide microsatellite DNA markers used in previous population genetic research and parentage analysis were mapped on three different LGs: *Kpm2* on LG8F, *Pma2* on LG11F and M,

Kpm22 on LG11M, and *Pma4–32* on LG24M. One polymorphic marker, *Pma4*, was not mapped on any linkage groups constructed in this study.

The recombination rate between the sex-specific genetic linkages was estimated using co-segregation markers. At least two microsatellite markers shared loci in the female and male maps and could be used to calculate the recombination rate among adjacently paired markers (Kessuwan et al. 2016). The total lengths of genetic distance obtained from 20 linkage
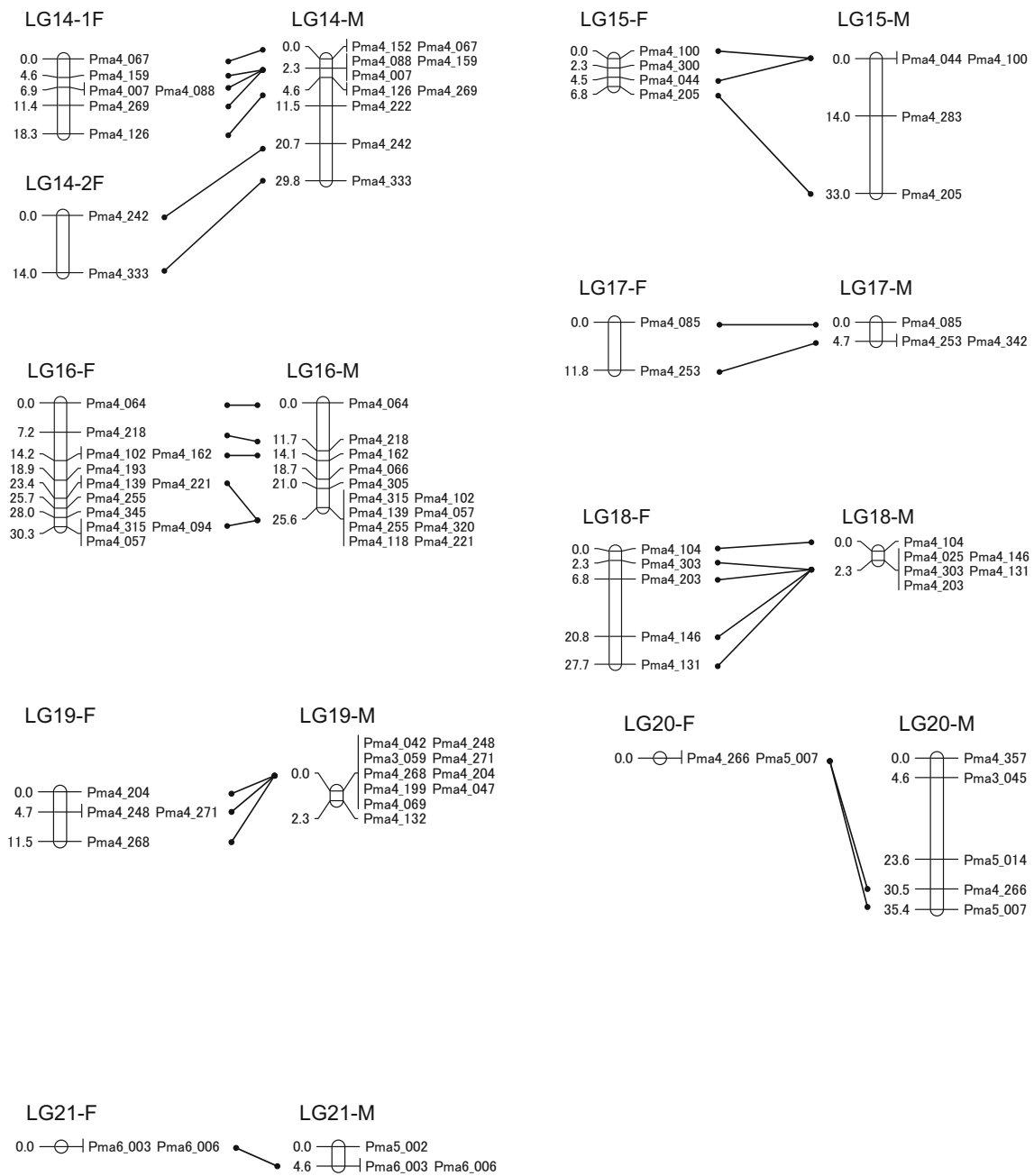
Fig. 2 (continued)

groups were 450.9 and 281.7 cM, respectively. LG13 was omitted from the calculation because it did not share at least two microsatellite markers. The relative recombination ratio between the female and male in these pairs was 1.6:1. Five linkage groups in the male map (LG8, 10, 11, 15, 16, and 20) showed higher recombination rates than in the female map.

The average genome length was estimated as 996.9 and 896.5 cM in the female and male maps, respectively. The female map was 1.11 times longer than the male map. The genome coverage of the female and male maps was estimated at 63.4 and 64.3%, respectively. The summary of the genetic

linkage map of red sea bream constructed in this study is shown in Table 1.

QTL Mapping

The result of interval mapping for RSIVD resistance in Fam-2014 for all linkage groups is shown in Fig. 3. One marker (*Pma4_014* on LG 2, accession no. LC323274) for RSIV resistance was detected using permutation tests for single-locus association at the 5% genome-wide significance threshold in Fam-2014. The LOD score of this marker was 3.19. The
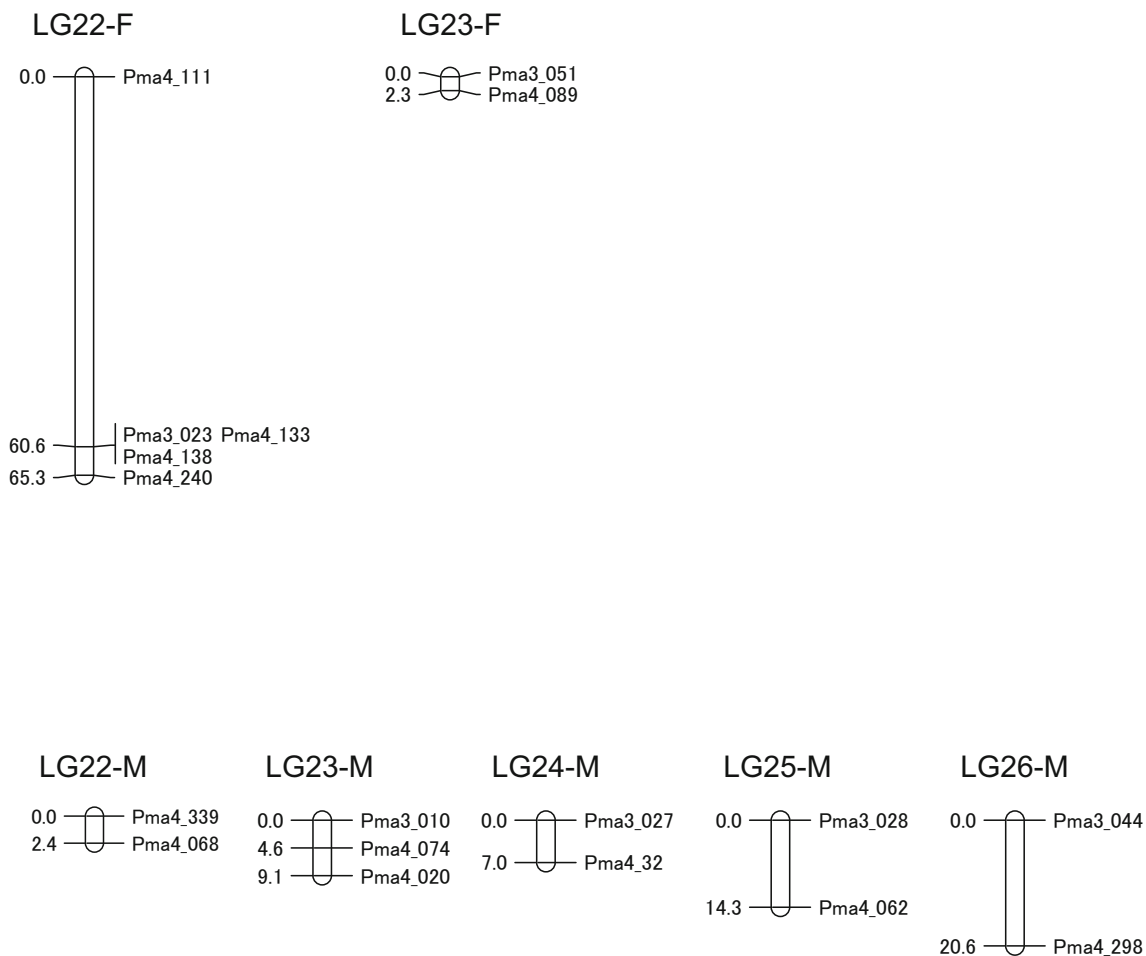
Fig. 2 (continued)

Table 1 Summary of the genetic linkage map of red sea bream

	Female	Male
Segregating markers	155	209
Mapped markers	148	197
Number of linkage groups	23	27
Number of triplets	1	0
Number of doublets	2	1
Maximum marker number per group	17	22
Minimum marker number per group	2	2
Average number of markers per group	6	7
Maximum length of linkage group (cM)	89.3	116.3
Minimum length of linkage group (cM)	0	0
Average marker spacing (cM)	9.3	7.7
Observed genome length (cM)		
Framework map	631.6	576.3
All markers	769.3	626.0
Estimated genome length (cM)		
Ge1	920.3	816.4
Ge2	1073.5	976.7
Ge	996.9	896.5
Genome coverage (%)	63.4	64.3
Recombination rate	1.6	1

Map distances are shown in centimorgans (cM). The estimated genome length (*Ge*) is estimated using two different methods (see "Materials and Methods")

phenotypic variance explained (PVE) attributed to this QTL, as a percentage, was 31.1%.

We also re-confirmed the QTL using the other family produced in 2015 (Fam-2015) with the marker set located on LG2, and at this time, the gene-related marker, *pmaMHC2b*, showed the chromosome-wide significant threshold ($P < 0.05$) with a LOD score of 2.43 (Fig. 4). The PVE of this QTL in Fam-2015 was 12.4%. *Pma4_014* was also significant in Fam-2015. In Fam-2015, another QTL (*Pma4_227*) located on LG2-3M was identified and modestly affected the phenotype (Fig. 4).

The summary of QTLs for resistance to RSIVD in Fam-2014 and -2015 is shown in Table 2.

Discussion

Isolation of Microsatellite DNA Markers Using NGS Technology

In this study, we obtained a large number of short reads from the red sea bream genome using Illumina HiSeq 2000 sequencing,

Fig. 3 Results of genome-wide quantitative trait loci analysis of the male map of Fam-2014 for RSIVD resistance. Log of odds scores are plotted over 27 linkage groups. Each linkage group is indicated by a bold line with a number. The upper and lower horizontal dotted lines indicate genome-wide significant levels of log of odds scores in 10 and 5%, respectively
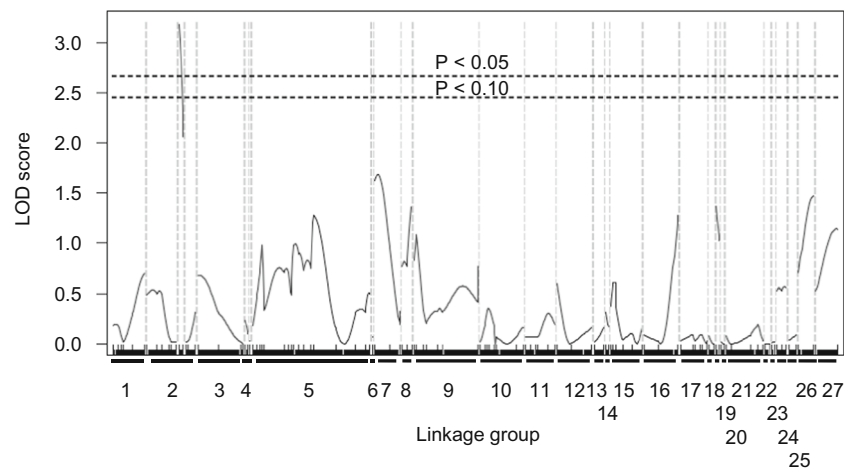
and these were de novo assembled. Approximately 850 Mb of nucleotide sequences of red sea bream were finally assembled. The genome sequences obtained in this study possibly cover over 90% of the red sea bream genome estimated by the C-value of red blood cells of this species (Ojima and Yamamoto 1990). There were only 54 (12%) newly developed microsatellite markers that were not amplified, and this result possibly shows the accuracy of this de novo-assembled sequence of red sea bream.

We designed a massive number of microsatellite DNA markers (172,172 primer pairs) from the de novo-assembled red sea bream genome. Thousands of microsatellite markers have been developed from genomic and transcriptome sequences of teleost fish species using NGS platforms: 1,590 in *Cyprinodon julimes* (Carson et al. 2013), 6,331 in *Rhamdia* sp. (Rodrigues et al. 2015), 14,885 in *Miichthys miiuy* (Che et al. 2014), 40,330 in *Lates calcarifer* (Thevasagayam et al. 2015), 82,794 in

Pelteobagrus fulvidraco (Zou et al. 2015), and so on. These data suggest that the in silico microsatellite mining using de novo-assembled sequences obtained from NGS platforms are also very effective in fish species as described in other vertebrate species (Castoe et al. 2012).

The newly developed microsatellite markers showed clear peaks without stuttering and were easier to genotype than dinucleotide microsatellite markers. The newly developed tri- and tetra-nucleotide markers should be useful for DNA parentage and population genetic analyses instead of the dinucleotide microsatellite markers previously used. Micro satellite markers developed from red sea bream can also be amplified in other sparid fish species such as *Pagrus auratus*, *Evynnis japonica*, *Dentex tumifrons*, and *Sparus macrocephalus* (Takagi et al. 1997; Hauser et al. 2002; Chen et al. 2005), and these markers are possibly useful for population genetics and evolutional studies in other sparid fish species.

Fig. 4 Results of chromosome-wide quantitative trait loci analysis for RSIVD resistance in the male LG2 of Fam-2015. Log of odds scores are plotted over 3 linkage groups comprising LG2. The upper and lower horizontal dotted lines indicate chromosome-wide significant levels in 10 and 5% of log of odds scores, respectively
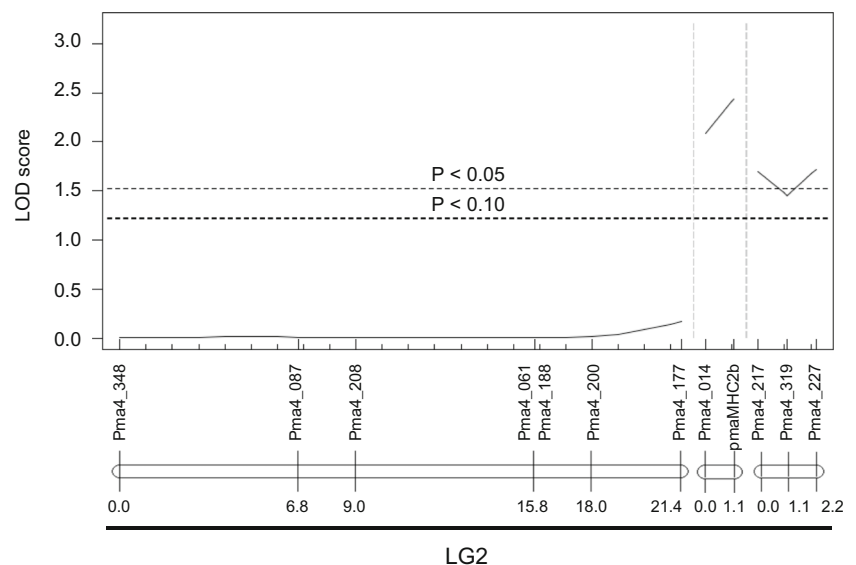
Table 2 Summary of quantitative trait loci for resistance to RSIVD in two test families

Test family	Linkage group	Position (cM)	Locus marker	LOD score	PVE (%)
Fam-2014	LG2-2M	0	*Pma4_014*	3.19	31.1
Fam-2015	LG2-2M	1.1	*pmaMHC2b*	2.43	12.4
	LG2-3M	1.7	*Pma4_227*	1.72	8.8

PVE (%): percentage of the phenotypic variance explained by QTL

Construction of Linkage Maps and Its Coverage

In the female linkage map, 148 markers were mapped on 23 linkage groups, whereas 197 markers were assigned to 27 LGs in the male linkage map. Red sea bream has 24 haploid chromosomes (Imajo et al. 2007) and was not equal to the linkage groups constructed in this study. The male/female marker density of our maps was 3.0/4.8 cM between framework markers. In addition, the genome coverage of the linkage map was approximately 65% in both sexes, a little lower than linkage maps of other fish species (Nomura et al. 2011; Jiang et al. 2013; Guo et al. 2013; Ye et al. 2014; Kessuwan et al. 2016; Uchino et al. 2016). This difference is possibly caused by low recombination rates from a small number of individuals and a small number of microsatellite DNA markers used for genotyping. Moreover, the parental fish used for the mapping family are domesticated and passed selective breeding for over 50 years (approximately through 10 generations). Moreover, several economic traits such as growth have been fixed (Sawayama and Takagi 2016), which may cause selective sweep around genes involved in economic trait and cause the low recombination rate in some chromosome regions.

Uchino et al. (2016) demonstrated constructing genetic linkage maps with whole genome sequences of bluefin tuna (*Thunnus orientalis*) based on comparative information of medaka chromosome structure and suggested that fish in Percomorpha have a similar chromosome constitution to medaka. Mining of additional microsatellite markers to compare de novo-assembled sequences of red sea bream and medaka genomes would be useful to increase genome coverage of the linkage map. Further study is needed to construct high coverage linkage maps of red sea bream based on a comparative genome approach.

The male/female recombination ratio of the red sea bream was 1:1.6. Different male/female recombination ratios in fish species are often observed: 1:2–2.03 in grass carp (Xia et al. 2010), 1:3.18 in channel catfish (Waldbieser et al. 2001), 1:1.68–4.31 in rainbow trout (Sakamoto et al. 2000; Danzmann et al. 2005; Rexroad et al. 2008), 1:1.48 in European sea bass (Chistiakov et al. 2005), 1:1.2 in gilthead sea bream (Franch et al. 2006), 1:1.3–1.6 in turbot (Bouza et al. 2007; Ruan et al. 2010), and 1:1.2–1.5 in kelp grouper (Liu et al. 2013; Kessuwan et al. 2016). There is no sex-related difference in recombination rate in protogynous and protandrous hermaphrodite species. Red sea bream is a protogynous hermaphrodite species, and our results also show evidence that determining the recombination rates between sexes is not as important as its genetic sex.

Identification of a Novel QTL for RSIVD Resistance

We mapped 250 markers on the linkage maps, and each marker was placed at an interval of 3.2 cM based on the genome size of red sea bream (approximately 800 Mbp). It is known that marker density does not affect the power to detect QTL at an interval between two markers of less than 10 cM (Massault et al. 2008). In addition, we combined selective genotyping with interval mapping for focusing on a single trait, which is also known to increase the power to detect QTL (Massault et al. 2008). Therefore, the linkage maps developed in this study require sufficient resolutions to detect QTL. However, red sea bream is an important aquaculture fish species, and there is a large demand for genetic improvement, so the linkage maps of red sea bream still need to be improved in the future with more DNA markers and individuals for high resolution.

One major QTL was identified from a candidate male that was significantly associated with a convalescent population after an outbreak of RSIVD at an aquaculture farm in 2012 (Sawayama and Takagi 2017). Using this male, we produced two paternal sib-families (Fam-2014 and Fam-2015) and conducted challenge tests twice using different origins of RSIV samples. The results (survival rate) of two challenge tests conducted in 2014 and 2015 largely differed. The body weight of the two test families (Fam-2014 and Fam-2015) could not be adjusted to similar sizes because of facility limitation. In addition, the viral titer of samples used for Fam-2015 was 10 times higher than the viral samples used for Fam-2014. These experimental conditions for RSIVD-resistant trait evaluation were unfortunately not normalized, and this possibly caused the different mortality rates between the two challenge tests conducted in 2014 and 2015. However, one QTL obtained in Fam-2014 was still significantly associated with survival in Fam-2015. Based on these experiments, the single QTL linked to the RSIVD-resistant trait was reproducibly identified in the candidate male. We also found a large value of PVE in this QTL (31.1%) in Fam-2014. This QTL is equally effective compared with other disease-resistant QTL observed in other aquaculture fish species (Das and Sahoo 2014). The QTL

should be useful for MAS to develop the RSIVD-resistant strain of red sea bream.

The RSIVD-resistant QTL found in the candidate male was closely located to *MHC class IIβ*. MHC genes encode proteins of the innate or adaptive immune systems, which have been considered candidates for molecular marker association between polymorphism and disease resistance. MHC class II, consisting of one alpha chain and one beta chain, presents foreign peptides derived from extra cellular pathogens and present to T cells (Kjøglum et al. 2006); polymorphisms in the MHC are known to associate with disease resistance in several fish species (Kjøglum et al. 2006; Wynne et al. 2007; Xu et al. 2008). The QTL found in this study possibly links mutation-caused amino acid substitutions of *MHC class IIβ*. Several studies have shown significant association between the SNPs in the region formed from pocket 4 of the antigen binding groove in this gene (Zerva et al. 1996; Nagaoka et al. 1999; Sharif et al. 2000), which plays an instrumental role in antigen binding and recognition by T-lymphocytes. We already identified non-synonym mutation in the same region of the red sea bream *MHC class IIβ* of exon 2, which causes a structural change in pocket 4 (data not shown). In addition, a scaffold containing the *Pma4_014* sequence was identified. We searched for homologous sequences in other model fishes and found that this marker was closely located beside one apoptosis-related gene (data not shown). The chromosome region is located on MHC cluster immune-related genes (Sambrook et al. 2005; Lukacs et al. 2007), and further studies such as comparative genomics and expression analysis of immune genes in a strain selected by the QTL-linked markers are required to identify a causative gene.

Conclusion

In the present study, we identified a novel QTL linked to an RSIVD-resistant trait in a candidate male of red sea bream using newly developed linkage maps composed of tri- to hexa-nucleotide microsatellites and a minisatellite located in the *MHC class IIβ* gene. One marker that was closely linked to *MHC class IIβ* was found to be associated with resistance to RSIVD in the first trial. The same marker and *MHC class IIβ* were also significantly linked to the RSIVD-resistant trait in the second trial, and the effect of QTL was reproducible in two different families. This QTL should be useful for a further MAS program with the candidate male.

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