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ORIGINAL ARTICLE

Mapping QTL for Resistance Against Viral Nervous Necrosis Disease in Asian Seabass

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Abstract Viral nervous necrosis disease (VNN), caused by nervous necrosis virus (NNV), leads to mass mortality in mariculture. However, phenotypic selection for resistance against VNN is very difficult. To facilitate marker-assisted selection (MAS) for resistance against VNN and understanding of the genetic architecture underlying the resistance against this disease, we mapped quantitative trait loci (QTL) for resistance against VNN in Asian seabass. We challenged fingerlings at 37 days post-hatching (dph), from a single backcross family, with NNV at a concentration of 9×10^6 $TCID_{50}$/ml for 2 h. Daily mortalities were recorded and collected. A panel of 330 mortalities and 190 surviving fingerlings was genotyped using 149 microsatellites with 145 successfully mapped markers covering 24 linkage groups (LGs). Analysis of QTL for both resistance against VNN and survival time was conducted using interval mapping. Five significant QTL located in four LGs and eight suggestive QTL in seven LGs were identified for resistance. Another five significant QTL in three LGs and five suggestive QTL in three LGs were detected for survival time. One significant QTL, spanning 3 cM in LG20, was identified for both resistance and survival time. These QTL explained 2.2–4.1 % of the phenotypic variance for resistance and 2.2–3.3 % of the phenotypic variance for survival time, respectively. Our results suggest that VNN resistance in Asian seabass is controlled by many loci with small effects. Our data provide information for fine mapping of QTL and identification of candidate genes for a better understanding of the mechanism of disease resistance.

Keywords QTL · Viral nervous necrosis disease · Nervous necrosis virus · Asian seabass

Introduction

In mariculture, disease is one of the major restricting factors affecting the industry. Genetic improvement of disease resistance is an important task in this industry. In recent years, selective breeding programs for disease resistance have been initiated in several aquaculture species including Atlantic salmon (*Salmo salar*) (Houston et al. 2008; Thodesen and Gjedrem 2006), Atlantic cod (*Gadus morhua*) (Ødegård et al. 2010), rainbow trout (*Oncorhynchus mykiss*) (Dorson et al. 1995; Houston et al. 2010), rohu carp (*Labeo rohita*) (Nguyen and Ponzoni 2006), and Pacific white shrimp (*Litopenaeus vannamei*) (Ødegård et al. 2011b). However, it is not trivial to improve disease resistance through conventional breeding due to difficulties in recording resistance traits in fish (Ødegård et al. 2011a). Marker-assisted selection (MAS) through quantitative trait loci (QTL) mapping is an alternative solution (Ødegård et al. 2011a; Yue 2014).

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QTL mapping for economically important traits has been widely conducted on domestic animals, helping to improve their performance substantially while generating huge economic benefits (Georges 2007). On the other hand, the progress of QTL mapping on aquatic species is still behind that for domestic animals due to various reasons. With the rapid advances of sequencing technologies and continually decreasing costs of genotyping, QTL mapping and association studies for some traits have been conducted in aquaculture species, for example, growth-related traits in clam (*Meretrix meretrix*) (Lu et al. 2013), large yellow croaker (*Larimichthys crocea*) (Ye et al. 2014), and Japanese flounder (*Paralichthys olivaceus*) (Cui et al. 2015); sex-related traits in turbot (*Scophthalmus maximus*) (Vinas et al. 2012) and Atlantic salmon (Gutierrez et al. 2014); and other traits like swimming ability in common carp (*Cyprinus carpio*) (Laghari et al. 2014) and crowding in rainbow trout (Liu et al. 2015). Besides these important traits, QTL and association studies for resistance against viral and bacterial diseases have also been reported in several fish species (Ødegård et al. 2011a; Yue 2014) and more recently in rainbow trout (Vallejo et al. 2014), Japanese flounder (Wang et al. 2014), and catfish (Geng et al. 2015). Major QTL for resistance against infectious pancreatic necrosis (IPN) and lymphocystis disease have been applied in MAS programs for Atlantic salmon (Houston et al. 2008; Moen et al. 2009) and Japanese flounder (Fuji et al. 2006), leading to substantially reduced outbreaks of those diseases.

Viral nervous necrosis(VNN) disease, caused by a single-stranded positive RNA virus called nervous necrosis virus (NNV), affects more than 40 fish species including a variety of marine fish species cultured in warm and cold waters (Shetty et al. 2012). Most fish are infected at early developmental stage including both larval and juvenile stages, and the mortality could reach up to 100 % (Shetty et al. 2012). At market size, several fish species are also significantly affected, even if the mortality is lower than that at larval and juvenile stages (Nakai et al. 2009). This disease has been documented on all continents except South America and is a worldwide distributed disease (Nakai et al. 2009). Despite the severity of NNV and huge economic losses caused by this disease, to our best knowledge, no breeding program in aquaculture has been targeted at breeding fish with resistance to VNN.

Asian seabass (*Lates calcarifer* Bloch) is a catadromous fish species belonging to the family Latidae of the order Perciformes (Nelson 2006). It inhabits and spreads in vast areas in the Indo-West Pacific region, from the Persian Gulf, throughout Southeast Asia, to northern Australia (Nelson 2006). Due to its great commercial value, a considerable research effort has been invested in this species since the 1970s, leading to the stably increasing and cost-efficient global production which reached 75,405 tons in 2012 (FAO 2006). Since 1998, we have worked on Asian seabass, focusing on improving growth traits through traditional and molecular breeding programs (Yue et al. 2001). In order to accelerate genetic improvement of Asian seabass, genetic markers such as microsatellites (Xu et al. 2006; Yue et al. 2001) and single nucleotide polymorphisms (SNPs) (Xu et al. 2006) in genes were developed. BAC (Xu et al. 2006) and cDNA (Xia and Yue 2010) libraries were also constructed. Recently, the physical (Xia et al. 2010) and linkage (Wang et al. 2011; Wang et al. 2007) maps of Asian seabass have been developed, leading to the successful mapping of QTL for omega-3 fatty acids (Xia et al. 2014). However, during the rapid expansion of the Asian seabass industry, this fish species suffered from severe NNV infections. The infections often caused more than 90 % mortality of Asian seabass at larval stage and continued to affect seabass at juvenile stage (Azad et al. 2005; Maeno et al. 2004; Parameswaran et al. 2008; Ransangan and Manin 2010), resulting in huge economic losses.

In the present study, we artificially challenged Asian seabass fingerlings with NNV and genotyped 520 fingerlings with 145 microsatellite markers covering the genome of Asian seabass. We mapped five significant and eight suggestive QTL for resistance and five significant and five suggestive QTL for survival time against VNN in Asian seabass. QTL identified in this study could provide valuable information for improving VNN resistance through MAS programs and for understanding the mechanism of disease resistance in Asian seabass.

Materials and Methods

Fish and Ethics Statement

Fish used in this study were from a single backcross family (BC1 hybrid) of Asian seabass. Backcross populations are widely used for genetic mapping (Xu 2010). This approach is frequently employed to introduce a specific trait, such as disease resistance, to an elite line (Xu 2010). The sire used in this study, an offspring of the dam, was a survivor of an outbreak of big belly disease. Fingerlings at 30 days post-hatching (dph) were transferred from the Marine Aquaculture Center (MAC), Agri-food and Veterinary Authority of Singapore (AVA), located on St. John's Island, to Temasek Life Sciences Laboratory. The fingerlings were acclimated in a study tank with about 200 liters of circulated seawater (salinity 30 ppt, pH 7.6) at a stable temperature of 30 °C as well as saturated oxygen, for 7 days prior to challenge. Half of the seawater was replaced with fresh seawater every 2 days. The fingerlings were fed twice a day with a commercial diet (Marubeni Corporation, Tokyo, Japan) during the whole experiment. All handling of fish followed the instructions set up by the Institutional Animal Care and Use Committee (IACUC) of the Temasek Life Sciences Laboratory, Singapore, and the project was approved under the title

"Breeding of Asian seabass resistance to viral diseases" (approval number TLL (F)-13-003).

Preparation of NNV

The NNV strain GGNNV was isolated from the brain, head, kidney, and liver of greasy grouper, *Epinephelus tauvina,* in Singapore; the Asian seabass (SB) cell lines used in the challenge experiment were provided by AVA (Chong et al. 1990; Chong et al. 1987). SB cells were grown on Leibovitz's L-15 media (Life Technologies, Carlsbad, USA) supplemented with 10 % fetal bovine serum (FBS) (Life Technologies, Carlsbad, USA) and 1 % antibiotic antimycotic solution (Sigma-Aldrich, St. Louis, USA), in a 175-cm^2 flask (BD Biosciences, Franklin Lakes, USA) at 28 °C. NNV was propagated in SB cells of 80 % confluence monolayer. When a full cytopathic effect was reached, the NNV-infected cell culture was harvested and stored at −80 °C. Titer $TCID_{50}$ (TCID stands for tissue culture infective dose) of virus stock was subsequently determined on SB cells in a 96-well cell culture plate using the method of Karber (Karber 1931).

Challenge of Fingerlings with NNV

The immersion challenge method was adopted to provide data for genetic analysis based on a previously published paper (Fenner et al. 2006). On the day of challenge, 18 fingerlings were randomly selected as a subgroup for assessing the average body weight of the whole population. We estimated it to be 1.00±0.20 g. Subsequently, 700 fingerlings were transferred to a 30-liter tank with clean seawater containing 9×10^6 $TCID_{50}$/ml of NNV and immersed for 2 h under close monitoring. For the control, similar to the NNV challenge tank, 700 fingerlings were sham-challenged in clean seawater by adding a volume of used L-15 media equivalent to that of the NNV containing cell culture used in the challenge tank. After that, each group of 700 fingerlings was transferred back to a tank with clean seawater.

Measurement of Traits

The behavior of the fingerlings was closely observed during the whole experiment. Twice a day, mortalities were recorded and removed, stored in pure ethanol, and kept at −80 °C for further analysis. The whole experiment was terminated when there were less than three mortalities for two consecutive days. All the survivors from the mock and 200 survivors among the NNV challenged fingerlings were killed by overdose of AQUI-S® and stored in pure ethanol at −80 °C. Two disease-related traits, resistance and survival time, were recorded. Resistance was a dichotomous trait with binary data defined as survival (trait value of 1) versus non-survival (trait value of 0) status of each individual at the end of the experiment. Thus, this model excluded time of survival from trait analysis. Such data can be analyzed using a linear model, and a precise prediction of breeding value can be achieved (Ødegård et al. 2011a). Resistance was based on whether each challenged fish was dead/alive at a certain time point and ignored the lifespan of the fish. On the other hand, to some extent, survival time may also reflect the degree of resistance. For example, the fish that died later might have been more resistant than the fish that died early. Thus, survival time was also recorded. It was defined as the number of days-post challenge (dpc) for which each individual lived (e.g. give trait value of 10 for fish that died at 10 dpc). Consequently, the survival time for all the survivors was scored with the same value (e.g., give trait value of 25 for all the surviving fish). A survival score model has been proposed to generate the data (Veerkamp et al. 2001), which can be analyzed using a threshold model with a more accurate prediction of breeding value in some cases (Ødegård et al. 2011a).

Examination of NNV

The NNV was examined on mortalities and survivors from both control and challenged groups. From each group, five fingerlings were randomly selected from pools of mortalities and survivors and subsequently dissected to collect the brain tissues. Total RNA was extracted from the collected brains using TRIzol reagent (Life Technologies, Carlsbad, USA) following the manufacturer's instructions. Subsequently, 2 μg of total RNA from each sample was converted to cDNA with M-MLV Reverse Transcriptase (Promega, Madison, USA) using 0.5 μg of random hexamer oligo following the manufacturer's instructions. Two pairs of primers (RNA1-4 and RNA2-4, Table 1) specifically and respectively targeting RNA1 and RNA2 of NNV were used in multiplex PCR amplification to detect the presence of NNV in the examined samples. The expected lengths of PCR products were 826 and 469 bp. A PCR reaction of 25 μl included 1× PCR buffer, 500 μM of each dNTP, 1.5 μM of primers of RNA1-4F and RNA1-4R, 2.5 μM of primers of RNA2-4F and RNA2-4R, 2.5 units of Taq polymerase, and 8 ng of cDNA. The thermal cycling conditions were as follows: 94 °C for 3 min, followed by 36 cycles of 94 °C for 30 s, 59 °C for 30 s, and 72 °C for 45 s, with a final extension at 72 °C for 10 min. Water was used as a negative control and cDNA from NNV was used as positive control. α-Tubulin was also amplified as an assessment of the RNA using a pair of primers of Lca-tub shown in Table 1 (5 μM of each in 25 μl PCR reaction) and annealing temperature of 55 °C. PCR product of 5 μl stained with ethidium bromide (EB) was electrophoresed on 2 % agarose gel, and DNA fragments were visualized using Gel Doc XR (Bio-Rad, Hercules, USA).

Table 1 Primers used for NNV test in Asian seabass fingerlings

Primer name	Primer sequence (5′–3′)	Target genome/gene	Product length (bp)
RNA1-4F	CGTTACAGCGGGCTCGGAATG	RNA1	826
RNA1-4R	TTAGCCCAGCCAATGTCGTCAATC		
RNA2-4F	GCTGGGGCATCTGGGACAACTTT	RNA2	469
RNA2-4R	TCACTGCGCGGAGCTAACGGTAAC		
Lca-tub-F	GGCACTACACAATCGGCAAAGAGA	α-Tubulin gene	144
Lca-tub-R	TCAGCAGGGAGGTAAAGCCAGAGC		

DNA Isolation, Markers, and Genotyping

A total of 330 out of 347 mortalities and 190 randomly selected survivors from the challenged group were used as a panel for data collection. Caudal fins of the panel were taken. DNA was isolated using a method developed by our lab (Yue and Orban 2005). A total of 149 microsatellite markers (supplementary Table S1), including one (LcaRTP3) recently developed, were selected based on a published genetic map (Wang et al. 2011) to achieve a wide coverage within and across linkage groups and were used to genotype the panel. Either forward or reverse primers were labeled with either FAM or HAX fluorescence. PCR was conducted as described in (Wang et al. 2011). PCR products were detected using capillary electrophoresis by a 3730xl DNA analyzer (Applied Biosystems, Waltham, USA), and allele sizes were determined by comparison with size standard GS-ROX-500 (Applied Biosystems, Waltham, USA) using the software GeneMapper (Applied Biosystems, Waltham, USA).

Linkage Mapping

A total of 149 microsatellite markers (supplementary Table S1) were used for linkage mapping. The criteria for marker selection were that the number of markers per linkage group (LG) would be evenly distributed as much as possible, and the average distance between markers should not be too large, with recommended 20 cM for QTL detection (Dekkers and Hospital 2002). The assessment for Mendelian segregation distortion of each marker using chi-squared test was performed on the software JoinMap 4.1 (Van Ooijen 2011). The score of deviation from the expected ratio 1:1 of each marker was checked, and the marker was removed from further analysis if it was deemed unreliable for determining genotype. Linkage among markers was evaluated using JoinMap 4.1. Marker order and position in the genetic map were determined if a minimum logarithm of odds (LOD) score exceeded 3 as an indication of significant linkage.

QTL Mapping

QTL mapping was conducted using MapQTL 6 (Van Ooijen and Kyazma 2009) through interval mapping (IM) with a maximum likelihood (ML) test. The Kruskal-Wallis analysis in MapQTL 6 was used to determine the association between marker and QTL. Under the hypothesis that underlying VNN resistance is a continuous variable, the phenotypic expression is dependent on an appreciation threshold. The significance threshold for IM was determined by a simulation with a permutation test of 10,000 iterations for each LG (Churchill and Doerge 1994). QTL were considered as suggestive if the significance level was between 5 to 1 % at chromosome-wide level, and significant if the significance level was below 1 % at chromosome-wide or between 5 to 1 % at genome-wide level (Center 1995). Confidence intervals for the estimated positions of QTL were calculated by bootstrap analysis with ≥1000 iterations (Visscher et al. 1996).

Results

Challenge Experiment and Trait Measurement

Mass mortality (more than four a day) was observed in the NNV-challenged tank after 10 dpc with a peak of 37 mortalities a day at 20 dpc. A slight rising mortality had also occurred in the control tank at approximately the same time (20 dpc), with unclear reasons. The mortalities decreased to close to baseline (less than three mortalities for two consecutive days) at 24 dpc (Fig. 1). Hence, the experiment was terminated at that time. The overall mortality rates of the mock group and the challenged group were 15.29 and 49.57 %, respectively. Termination of experiment when cumulative mortality rate approaches about 50 % is a typical practice in challenge experiments.

Examination of NNV

There were amplifications of two expected bands (826 bp for RNA1 and 469 bp for RNA2) from survivors (Fig. 2, lanes 11–15) and mortalities (Fig. 2, lanes 16–20) in the NNV-challenged group, and the bands of mortalities were more intensive than those from survivor samples. There was no amplification from survivors (Fig. 2, lanes 1–5) and mortalities (Fig. 2, lanes 6–10) in the mock group.

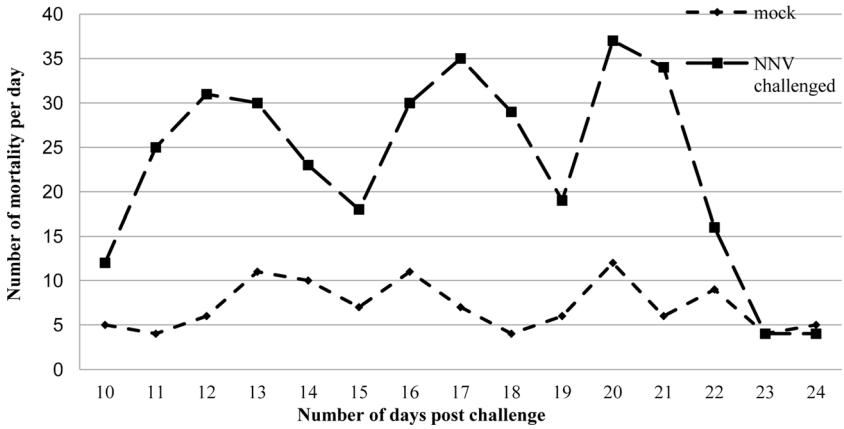

Fig. 1 Daily mortality curve of mock and NNV-challenged Asian seabass fingerlings 173–×–100mm (300×300 DPI)

Linkage Mapping

A total of 145 out of 149 markers were successfully mapped on the genetic map consisting of 24 LGs spanning 994.06 cM. The lengths of each LG varied from 11.09 to 82.76 cM with an average length of 41.42 cM (supplementary Table S2). The number of markers in each LG varied from 3 to 11 with an average number of 6.04 markers per LG (supplementary Table S2). The average distance between markers was 6.86 cM (supplementary Table S2).

QTL Mapping

Thirteen (Table 2) and ten (Table 3) QTL for resistance and survival time were detected, respectively. Four significant QTL at genome-wide level located in LGs 20, 21, and 24, were detected for resistance. Phenotypic variance explained (PVE), in percentages, attributed to those QTL were 4.10, 3.60, 3.50, and 3.70 %, respectively. Moreover, one significant QTL at chromosome-wide level, located in LG 10, was also detected for resistance with a PVE of 3.5 %, which was slightly lower than the PVE of three of the significant QTL at genome-wide level. In addition, eight suggestive QTL were identified in seven LGs with PVE ranging from 2.2 to 2.9 % (Table 2).

For the survival group, five significant QTL (one in LG 9, two in LG 20, and two in LG 21) were identified at genome-wide level (Table 3). They explained 3.00–3.30 % of the phenotypic variance. Additionally, five suggestive QTL (located in LGs 4, 10, and 24) were identified at chromosome-wide level with PVE varying from 2.20–2.90 %. There was no QTL detected for survival time at genome-wide level.

Besides one significant QTL identified for both resistance and survival time, five other QTL were detected at approximately the same location in different LGs and were considered significant or suggestive for both resistance and survival time (Tables 2 and 3). For example, two QTL located in LGs 10 and 24 were identified as significant for resistance and suggestive for survival time; two QTL located in LGs 20 and 21 were suggestive for resistance and significant for survival time. One consistent QTL in LG 4 was identified as suggestive for both resistance and survival time. Seven significant and suggestive QTL in seven LGs were found only for resistance and four QTL in three LGs were only for survival time.

Discussion

VNN is a major challenge in mariculture. In order to promote MAS for resistance against VNN and understanding of the genetic architecture of disease resistance in Asian seabass, we conducted a whole genome scan for QTL for resistance to VNN. To the best of our knowledge, this is the first report of QTL for resistance against VNN in Asian seabass. Five significant QTL and eight suggestive QTL for resistance, and five significant and five suggestive QTL for survival time, were detected. The phenotypic variance explained by the detected QTL in this paper ranged from 2.2 to 4.1 %. Similarly, ten QTL with up to 27 % PVE were found for resistance against the parasite *Gyrodactylus salaries* in Atlantic salmon

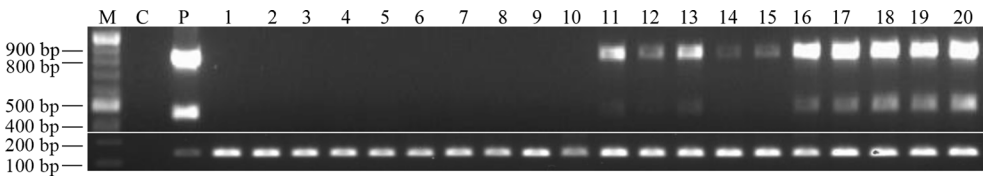

Fig. 2 PCR detection of NNV in the brains of sampled Asian seabass fingerlings. *Upper panel*: fragments amplified by RNA1-4 and RNA2-4, *lower panel*: α-Tubulin; M: marker, C: negative control, P: positive control, Lanes 1–5: survival fingerlings (mock), Lanes 6–10: mortalities (mock), Lanes 11–15: survival fingerlings (NNV-challenged), Lanes 16–20: mortalities (NNV challenged) 173×25mm (300×300 DPI)

Table 2 QTL detected for resistance against VNN in Asian seabass fingerlings

LG	Interval (cm)	Sig.	Threshold LOD	Peak LOD	Peak position (cm)	PVE (%)	Nearest marker	Marker position	K*	Sig.
2	0.00–6.73	suc	c0.05=2.6	3.02	6.73	2.60	LcaTe0360	6.73	6.63	**
2	11.59–11.59	suc	c0.05=2.6	2.65	11.59	2.30	LcaTe0359	13.58	10.18	**
4	26.45–32.30	suc	c0.05=2.4	2.76	29.30	2.40	LcaTe0075	29.30	12.08	****
6	34.34–36.60	suc	c0.05=2.5	2.56	36.60	2.20	LcaTe0409	36.60	11.86	***
10	8.52–8.52	sic	c0.01=3.4	3.98	8.52	3.50	Lca590	8.52	18.32	******
14	0.00–4.00	suc	c0.05=2.1	2.45	0.00	2.20	Lca147	0.00	10.63	**
15	20.33–29.37	suc	c0.05=2.4	2.66	24.37	2.30	Lca163	20.37	13.30	****
20	0.00–3.00	sig	g0.05=4.0	4.69	0.00	4.10	LcaTe0441	0.00	19.52	*******
20	27.13–34.13	suc	c0.05=2.4	2.88	30.13	2.50	Lca957	23.13	6.22	**
21	18.71–31.32	suc	c0.05=2.6	3.33	24.32	2.90	Lca421	20.32	15.58	****
21	58.12–58.37	sig	g0.05=4.0	4.16	58.37	3.60	LcaTe0545	58.37	18.21	******
24	33.30–43.30	sig	g0.05=4.0	4.08	38.30	3.50	Lca231	43.61	4.99	**
24	44.61–47.77	sig	g0.05=4.0	4.20	47.20	3.70	LcaTe0157	47.77	6.06	**

QTL are suggestive at chromosome-wide (suc) or significant at chromosome-wide (sic) or significant at genome-wide (sig)

Sig. significant level

<0.05; *<0.01, ****<0.005; *****<0.001; ******<0.0005; *******<0.0001

(Gilbey et al. 2006). Additionally, resistance against the parasite *Perkinsus marinus* in eastern oyster was controlled by 12 QTL (Yu and Guo 2006). Consistent with those findings, our data also suggest that VNN disease resistance in the examined family of seabass is polygenic and controlled by many loci with small effects. The vast majority of evidence seems to favor the notion that disease resistance is a complex trait. However, contrary to these findings, several pieces of evidence challenged that disease resistance could also be a result of major QTL with large effect. For example, two independent groups reported that resistance against IPN in Atlantic salmon was affected by two major QTL with PVE of more than 23 % (Houston et al. 2008; Moen et al. 2009). One of the two groups further demonstrated 50.9 % of PVE for susceptibility to freshwater IPN could be explained by a single QTL in Atlantic salmon (Houston et al. 2010). Other evidence also pointed to a major QTL contributing 50 % of PVE for resistance against the viral lymphocystis disease in Japanese flounder (Fuji et al. 2006). Besides viral diseases, resistance against bacteria was also mapped to a major QTL with a PVE of 50–86 % across families in rainbow trout (Baerwald et al. 2011). Furthermore, a major QTL with a PVE of 32.9–35.5 % for resistance against parasitic disease was recently identified in yellowtail (*Seriola quinqueradiata*) (Ozaki et al. 2013). Altogether, these data suggest that disease resistance is a complicated genetic issue. The locations and effects of QTL for

Table 3 QTL detected for survival time against VNN in Asian seabass fingerlings

LG	Interval (cm)	Sig.	Threshold LOD	Peak LOD	Peak position (cm)	PVE (%)	Nearest marker	Marker position	K*	Sig.
4	24.45–35.30	suc	c0.05=2.4	2.82	29.30	2.50	LcaTe0075	29.30	13.67	****
9	21.00–24.31	sic	c0.01=3.1	3.44	22.31	3.00	Lca301	22.31	10.17	**
10	0.00–6.86	suc	c0.05=2.6	3.15	4.00	2.70	LcaB201	6.86	17.31	*****
10	8.52–9.52	suc	c0.05=2.6	3.33	8.52	2.90	Lca590	8.52	17.31	*****
10	14.76–17.87	suc	c0.05=2.6	2.81	16.76	2.50	LcaTe0070	16.87	13.05	****
20	0.00–3.00	sic	c0.01=3.2	3.77	0.00	3.30	LcaTe0441	0.00	15.62	*******
20	28.13–32.13	sic	c0.01=3.2	3.44	30.13	3.00	Lca957	23.13	9.19	**
21	20.32–30.32	sic	c0.01=3.3	3.71	25.32	3.20	Lca421	20.32	17.81	******
21	38.12–50.12	sic	c0.01=3.3	3.61	45.12	3.1	Lca228	33.12	12.33	****
24	46.20–47.77	suc	c0.05=2.4	2.49	47.20	2.20	LcaTe0157	47.77	4.88	**

QTL are suggestive at chromosome-wide (suc) or significant at chromosome-wide (sic)

Sig. significant level

<0.05; *<0.01; ****<0.005; *****<0.001; ******<0.0005; *******<0.0001

disease resistance depend on species, types of pathogen, and environmental factors. However, we do notice the limitations of our study. First, phenotypic divergence of parents might be a major concern affecting the detection power of QTL against VNN in Asian seabass. The survivors from either artificial challenging experiments or natural outbreaks of disease, especially viral diseases, are commonly excluded as stock brooders for fear that they might be disease carriers having the potential to transmit diseases (Vike et al. 2009). Absence of trait evaluation about founder parents could pose a challenge to Asian seabass by compromising the detection power of QTL against VNN. Second, using a single family may have higher power to detect family-specific or rare QTL, but could also trade off the detection power of common QTL (Li et al. 2011). In contrast, approach of multi-family mapping permits cross-evaluation and validation of QTL in multiple genetic backgrounds. Thus, it could enhance the detection power for shared QTL and improve the resolution preciseness of QTL positions and estimation accuracy of QTL effects (Sneller et al. 2009). Consequently, efforts will be made to challenge multi-family of Asian seabass with NNV and verify our detected QTL in these families as well as map common QTL against VNN in multi-family. Given the limitations in the implementation of QTL with small effects in MAS in Asian seabass, genomic selection (GS) as a variant of MAS with the potential to capture all the QTL of large or small effects might overcome those restrictions (Meuwissen et al. 2001). In the vast majority of aquatic species, however, GS is still in its infancy, because one of the fundamental features of GS is the large amount of high-density genome-wide markers, despite the availability of SNP chips for Atlantic salmon (Houston et al. 2014) and catfish (Liu et al. 2014). Nonetheless, a recently developed platform of genotyping-by-sequencing (GBS) with simultaneous marker discovery and genotyping (Peterson et al. 2012) could have the potential to overcome the difficulties of Asian seabass by generating thousands of genome-wide SNPs markers (Gorjanc et al. 2015). Genotyping the multi-family of Asian seabass challenged with NNV by GBS could be the future direction for disease resistance study in Asian seabass.

In this study, we found that one significant and several suggestive QTL for both resistance and survival time were located in similar positions within the same LGs. Similarly, in other fish species, several QTL for resistance and survival time, in close positions within the same LGs, were also reported in turbot against furunculosis caused by bacteria pathogen *Aeromonas salmonicida* (Rodríguez-Ramilo et al. 2011), scuticociliatosis caused by a parasite *Philasterides dicentrarchi* (Rodríguez-Ramilo et al. 2013), and viral haemorrhagic septicaemia (VHS) caused by novirhabdovirus (Rodríguez-Ramilo et al. 2014). The possible reasons for concordance include (1) the high correlation between both traits as the resistance trait includes survival time and (2) the same genes being involved in the underlying mechanism for disease resistance. It is also possible that the two measurements may actually reflect different aspects of disease resistance (Ødegård et al. 2011a). However, our data suggest that it is impossible to differentiate between the resistance and survival time. Further fine mapping of QTL, followed by identification and characterization of genes located in those QTL, may resolve the issue.

We detected several QTL specific to resistance and survival time. Similar results were also reported in QTL for resistance against *A. salmonicida* (Rodríguez-Ramilo et al. 2011), *P. dicentrarchi* (Rodríguez-Ramilo et al. 2013), and VHS (Rodríguez-Ramilo et al. 2014) in turbot. In addition, it should be noted that several LGs contain more than one QTL for both resistance and survival time. The possible reason could be that those disease-resistant genes underpinning the QTL might be enriched in several chromosomes and involved in the same pathway against viral infection. However, without the sequence information of those chromosomes, this remains as a speculation.

One significant QTL with the highest PVE, spanning 3 cM in LG 20, was detected for both resistance and survival time, suggesting that this QTL might have an effect on both

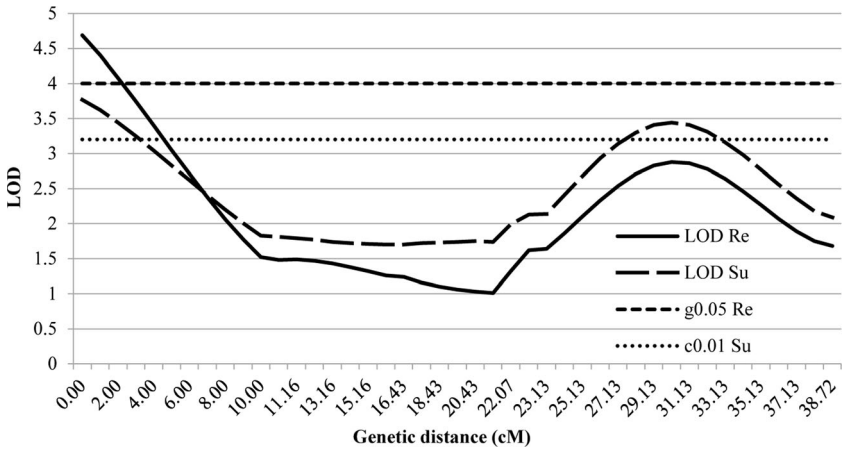

Fig. 3 QTL for resistance and survival time of Asian seabass fingerlings in LG 20. Threshold of LOD value, g0.05 for genome-wide significance at 0.05 level, c0.01 for chromosome-wide significance at 0.01 level for resistance (Re) and survival time (Su) 128×64 mm (300×300 DPI)

resistance and survival time (Fig. 3). The marker LcaTe0441 located in this QTL may be useful in the selection of fish with resistance to VNN. Certainly, before utilization of the marker in MAS, it is essential to examine the detected QTL in other families and populations. On the other hand, dissection of this region may lead to isolation of the causative genes for disease resistance. Based on estimation of a reference linkage map (Wang et al. 2011), the genetic distance approximately equals 0.97 Mb in the physical map and could contain dozens or even hundreds of genes. Therefore, future studies should focus on fine-mapping this QTL by genotyping more markers in larger populations, which could increase the power to detect more recombination in this region.

Interestingly, a detectable amount of NNV was present in the surviving fish from the challenged group, although they did not show any symptoms of VNN. This asymptomatic state has long been recorded in several fish species including Asian seabass (Azad et al. 2006), European seabass (Haddad-Boubaker et al. 2013; Hodneland et al. 2011), seabream (Haddad-Boubaker et al. 2013; López-Muñoz et al. 2012), and turbot (Olveira et al. 2013), in farmed and wild populations. This phenomenon has two implications. On one hand, those fish are the carriers of the virus and could be the potential sources for horizontal and vertical transmission of VNN. Thus, development of a feasible and efficient method to detect the virus and separate asymptomatic individuals deserves further investigation. On the other hand, we speculate that the genes in the mapped QTL in this paper may not be involved in host resistance of preventing virus infection, but could be involved in pathways limiting virus replication. Hence, more investigation of those genes will be carried out.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that there is no conflict of interest.

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