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Microsatellite marker distribution pattern in rock bream iridovirus (RBIV) infected rock bream, *Oplegnathus fasciatus*

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Rock bream (*Oplegnathus fasciatus*) is a highly valued aquaculture species in Korea. However, the aquaculture industry suffers huge economic losses due to rock bream iridovirus (RBIV) infection in summer. The objective of this study was to determine genetic diversity and relationships of DNAs isolated from two groups of rock bream after RBIV infection using five microsatellite (MS) markers. The first group of fish died early and the second group of fish died later after RBIV infection. In this experiment, 90 fish (5.1±1.0 cm and 4.1±1.3 g) were injected with 50 μl of RBIV (10^4 $TCID^{50}$/ml) and maintained at 26°C for 15 days. Genomic DNAs were extracted from fins of 20 fish that died earlier or later after RBIV infection. These DNAs were subjected to genotyping using five MS markers (CA-03, CA3-05, CA3-06, CA-10, and CA3-36). Of these markers, CA3-05 (early death group), CA3-06 (late death group), and CA3-36 (both early and late death groups) showed different alleles distribution rates. In-depth studies are needed to provide valuable information for selecting RBIV-resistant fish. In conclusion, microsatellite marker distribution pattern differences between early- and late- death groups of rock bream after RBIV infection showing different RBIV susceptibilities were determined using MS markers and genotyping. Results of this study suggest that MS markers could be used to facilitate the selection of RBIV resistant rock bream.

Key words: rock bream iridovirus, rock bream, microsatellite (MS) marker, disease resistance, genotyping

Introduction

Megalocytivirus isolates from many different fishes have been divided into three major subgroups based on the phylogenetic analysis with the major capsid protein (MCP): genotype I includes rock bream iridovirus (RBIV) (Nakajima and Kurita, 2012). In Korea, an outbreak of RBIV in rock bream (*Oplegnathus fasciatus*) was first reported in the summer of 1998 in southern coastal areas (Jung and Oh, 2000). After the first report, high mortality of rock bream due to RBIV infection occurs annually. Recently, immunological and physical responses of rock bream to RBIV have been evaluated (Jung *et al*., 2014; Jung and Jung, 2017). In addition, a large-scale cDNA shotgun sequencing for transcriptome analysis and a microarray analysis of RBIV infected fish have been performed to find genetic information of RBIV resistant fish (Jung *et al*., 2014; Jung and Jung, 2017). However, immune defensive mechanisms of rock bream against RBIV disease progression remain unclear. Understanding host-RBIV interactions will be useful for developing strategies to control RBIV infections in cultured fish and for developing long-term control and preventive measures against RBIV infections to sup-

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port rock bream aquaculture production.

Molecular markers such as microsatellites (MS) offer new possibilities in plant and animal breeding (Lim *et al*., 2005; Kitamura *et al*., 2009). Disease resistance is one of the most desirable traits in breeding programs because mass mortality due to diseases strongly influences the success of aquaculture. MS markers have been used in analyses of genetic diversity and genetic variations of rock bream (An *et al.,* 2006; Kim *et al.,* 2008; Xu *et al.,* 2008). However, to the best of our knowledge, differences in genetic diversity between susceptible and resistant of rock bream for selective breeding programs have not been reported yet. Thus, the objective of this study was to determine genetic diversity and relationships of DNAs isolated from two groups of rock bream after RBIV infection using five microsatellite (MS) markers. The first group of fish died early and the second group of fish died later after RBIV infection.

Materials and Methods

Experimental infection

The virus used in the present study was originally isolated from RBIV-infected rock bream in 2010 as described previously (Jung *et al.,* 2014). Virus was cultured in a grunt fin (GF) cell line maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA) supplemented with 10% (v/v) fetal bovine serum (Gibco, USA), 100 IU/ml of penicillin G, and 100 μg/ml of streptomycin at 25°C. Virus infectivity was titrated using 96-well microplates seeded with susceptible cells. After 10–14 days of culturing at 25°C, cytopathic effects (CPEs) were evaluated to determine the 50% tissue culture infectious dose ($TCID_{50}$). RBIV having a virus titer of 10^4 $TCID_{50}$/ml.

For the artificial infection experiments, pathogen-free rock bream samples were obtained from a local hatchery; the absence of RBIV by virus gene copy numbers. To measure the absolute virus copy number, genomic DNA was extracted from whole spleen (5~15 mg) samples using an AccuPrep®Genomic DNA extraction kit (Bioneer, Korea) according to the manufacturer's protocol. A standard curve was generated to determine the major capsid protein (MCP) gene copy number by quantitative real-time polymerase chain reaction (qRT-PCR) as previously described (Jung *et al*., 2014). The virus copy number was determined from 1 μl of genomic DNA taken from 100 μl of total genomic DNA.

Fish (5.1 ± 1.0 cm and 4.1 ± 1.3 g) were intra-peritoneally (i.p.) injected with either RBIV (10^4 $TCID_{50}$/50 μl/fish) or phosphate-buffered saline (PBS) (50 μl/fish). Each groups of 90 fish were maintained in a circulating aquaria containing 250 L of UV-treated seawater at 26°C for 15 days. Overall daily seawater exchange rate was 10% of system volume per day (25 L/day). To determine distribution patterns of MS markers in rock bream after RBIV infection, all dead fishes were sampled for fin and preserved in 100% ethanol.

Genotyping

Fins have been used to evaluate the DNA genetic markers (Norris *et al*., 1999; Alam and Islam, 2005). Genomic DNAs were isolated from fins (15~20 mg) of each rock bream using an AccuPrep®Genomic DNA extraction kit (Bioneer, Korea) according to the manufacturer's instructions. Polymerase chain reaction (PCR) reactions were performed using Accu Power® PCR PreMix (Bioneer, Korea) in a final reaction volume of 20 μl containing 1 μl of each primer (10 μM), 13 μl of DEPC-treated water, and 5 μl of the template (genomic DNA) on an ABI 2720 Thermal Cycler (Applied Biosystem, Foster City, CA, USA). PCR amplification conditions were as follows: 95°C for 5 min, 35 cycles of 95°C for 1min, 54 or 60°C for 1 min, and 72°C for 1 min, followed by a final extension at 72°C for 5 min. PCR products were electrophoresed on an ABI 3730xl Genetic Analyzer (Applied Biosystems, USA) using a POP7 gel matrix with GeneScan™–500 LIZ® Size Standard

(Applied Biosystems, USA). GenSeScan Analysis software (V.3.7, Applied Biosystems, USA) was used to score microsatellite alleles. Allele size was manually verified. Five MS marker (CA1-03, CA1-10, CA3-05, CA3-06, and CA3-36) were selected from our previous research (Kim *et al.,* 2011). Microsatellite loci are summarized in Table 1.

Results and Discussion

To prevent disease, attempts have been made to develop genetic markers for selecting disease resistant individuals recently. Accumulation of genetic information of a particular species (individual) is necessary. MS markers have been used to analyze genetic diversity within species, population genetic structure, and genomic mapping of fish (McConnell *et al.,* 1995; Perez- Enriquez *et al.,* 1999; Moen *et al.,* 2004). In our previous studies, we have obtained a large quantity of mRNA information using GS-FLEX (Jung *et al*., 2014; Jung and Jung, 2017). The aim of this work was to develop markers for detecting specific RBIV-resistant individuals with the available genetic information.

In the present study, genotyping was performed using MS markers to find alleles associated with resistance to RBIV using two groups of fish (20 fish in the group with early death and 20 fish in the group with later death after RBIV infection). Mortality was observed at 4 days post infection (dpi). The infected fish showed a dark body colour with typical signs of RBIV infection, such as enlarged basophilic cells in the spleen and kidney imprints. The mortality reached 100% mortality at 15 dpi (Fig. 1). None of the control fish died throughout the experimental period. Excessive hypertrophy of the spleen and kidney of virus infected rock bream was found, leading to mass mortality. Fish DNAs were used for genotyping using five MS markers (CA1-03, CA1-10, CA3-05, CA3-06, and CA3-36). Of these markers, CA3-05, CA3-06, and CA3-36 showed different allele distributions in both early and late death groups (Table 2; Fig. 2, 3, and 4). For the CA3-05 marker, the allele distribution had 156 bases to 167 bases, with high

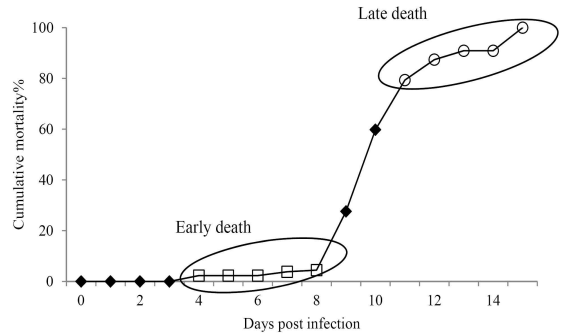

Fig. 1. Cumulative mortality of rock bream intra-peritoneally (i.p.) injected with RBIV (10^4 $TCID_{50}$/50 μl/fish) at 26°C. Early death and late death groups of fish were used for microsatellite marker analysis.

Table 1. Microsatellite markers used for analysis in this study

Locus	Sequence	Ta	Repeat motif	Size(bp)
CA1-03	F CATCATGTTGGCAGGTTCAT R GTTGAATCTCAGGGCTGCTC	54	$(AC)_{40}$	146-224
CA1-10	F CAGAGGTGGAAACGACAGTG R GCCTGCATGTGACTATGTGAA	60	$(CA)_{34}$	120-155
CA3-05	F AAGGCACCGACTCACGTAGGG R CGGATCACTTTCAGCCTCACATC	60	$(TG)_{32}$	147-167
CA3-06	F TGAAAGACAAGAAGCAACAGTGCAA R TGGAAAGGACAGATCCCTGCAT	60	$(TG)_{28}$	143-173
CA3-36	F TTGCTTGCTGCTTGTTCGTTTC R GGGCTTCCCTGCTTTGCTTG	60	$(TG)_{24}$	159-194

Table 2. Results of microsatellite marker analysis for RBIV-infected rock bream in early death group (sample E) and late death group (sample L)

Sample Name	CA1-03		CA1-10		CA3-05		CA3-06		CA3-36	
	Size 1	Size 2	Size 1	Size 2	Size 1	Size 2	Size 1	Size 2	Size 1	Size 2
E_01	165	201	138	138	162	166	162	163	0	0
E_02	179	192	137	137	156	162	156	162	169	186
E_03	177	182	138	151	156	162	158	162	161	179
E_04	179	182	126	152	160	160	162	169	179	184
E_05	178	190	151	151	158	160	162	171	169	181
E_06	154	216	134	148	156	166	158	162	173	196
E_07	177	201	140	151	162	162	156	158	171	179
E_08	154	156	134	148	156	166	156	169	173	196
E_09	200	201	126	142	161	167	156	169	171	194
E_10	165	178	146	150	160	160	154	154	175	184
E_11	177	184	139	151	162	162	158	158	171	179
E_12	154	190	126	151	158	166	158	162	169	171
E_13	188	200	134	135	162	166	158	162	158	196
E_14	182	216	137	142	160	160	157	163	171	194
E_15	154	154	126	151	162	166	158	162	169	171
E_16	154	156	134	148	156	160	162	169	173	196
E_17	154	156	134	142	156	160	158	162	170	194
E_18	154	154	134	142	160	166	162	169	194	196
E_19	180	203	126	144	158	162	162	169	171	177
E_20	196	205	152	152	156	160	162	169	159	175
L_01	178	182	137	151	160	162	158	162	169	179
L_02	178	178	146	151	162	162	154	158	179	181
L_03	196	207	137	139	156	160	162	162	169	186
L_04	179	179	137	137	156	162	154	156	168	168
L_05	179	188	138	138	157	163	154	158	171	186
L_06	177	179	138	142	156	160	154	158	169	186
L_07	155	155	128	154	160	162	154	154	169	171
L_08	152	152	137	144	156	162	156	168	165	177
L_09	154	154	142	151	161	161	154	162	158	169
L_10	167	179	126	151	160	162	154	156	159	179
L_11	192	228	137	146	163	165	154	162	165	171
L_12	152	152	137	137	160	160	150	154	159	159
L_13	165	198	146	152	159	165	162	171	177	194
L_14	179	196	130	138	156	156	158	162	169	186
L_15	179	218	126	151	154	162	158	162	159	179
L_16	177	177	138	151	157	163	154	156	171	179
L_17	180	196	126	152	159	163	154	162	177	184
L_18	165	165	144	146	156	160	154	166	169	169
L_19	167	177	137	137	161	163	156	162	159	186
L_20	177	181	128	148	161	167	154	162	169	173

distribution alleles having 156, 160, 162, and 166 bases in the early and late death groups (Fig. 2A, 2B, and 2C). Furthermore, the high distribution allele having 166 bases was observed only in the early death group (Fig. 2B). For the CA3-36 marker, the allele distribution had 158-196 bases, with high distribution alleles having 169 and 171 bases in the early and late death groups (Fig. 4A, 4B, and 4C). In the early death group, high distribution allele (196 bases) rates were observed (Fig. 4B). For the CA3-06 marker, the allele distribution had 150-171 bases, with high distribution alleles having 158 and 162 bases in both early and late death groups (Fig. 3A, 3B, and 3C). Interestingly, high distribution allele having 169 bases in the early death group while high distribution allele had 154 bases in the late death group (Fig. 3B and 3C). This indicates that the CA3-06 marker distribution rates were positively co-related with RBIV resistance or susceptibility. A similar observation has been reported for infectious hematopoietic necrosis

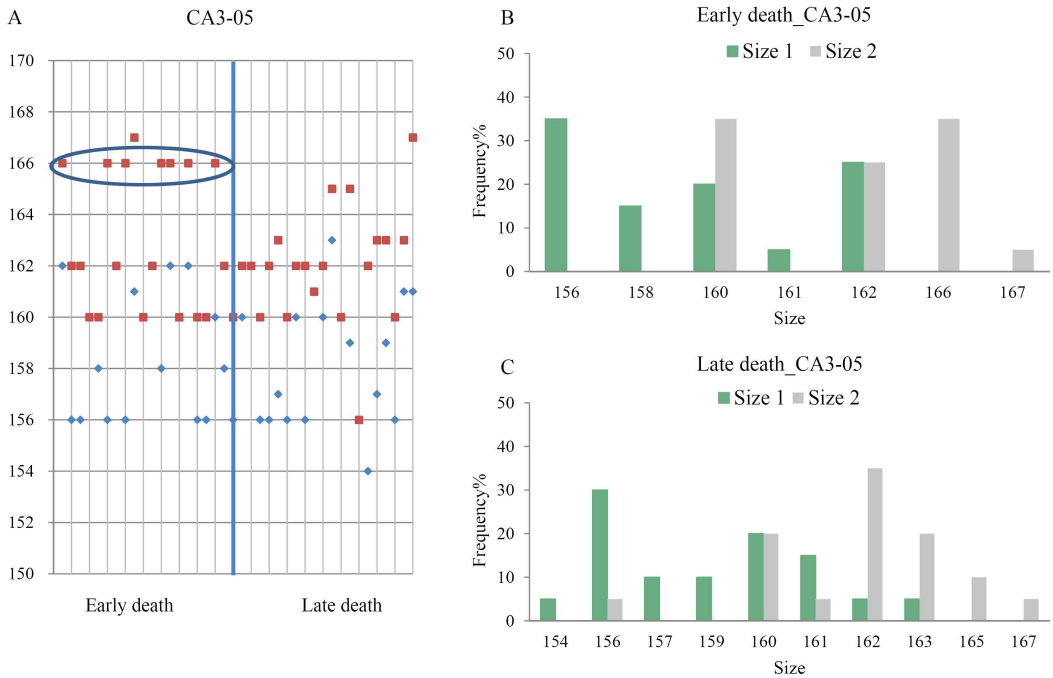

Fig. 2. Distribution patterns of microsatellite marker (CA3-05) in RBIV-infected rock bream. Circles indicates high distribution alleles (◆ Size 1, ■ Size 2).

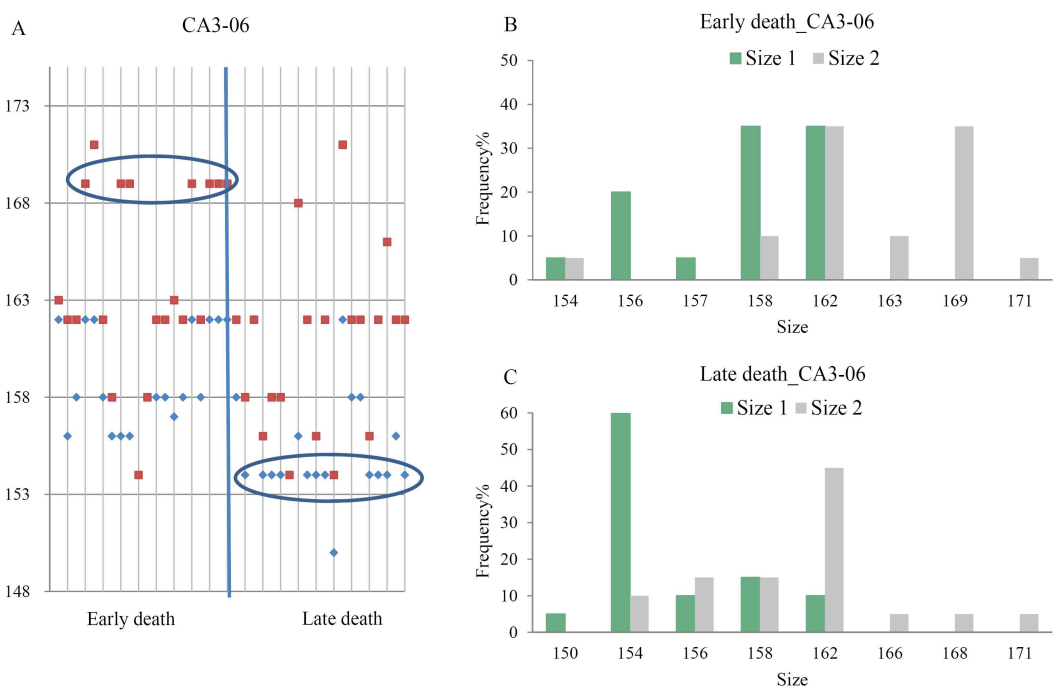

Fig. 3. Distribution patterns of microsatellite marker (CA3-06) in RBIV-infected rock bream. Circles indicates high distribution alleles (◆ Size 1, ■ Size 2).

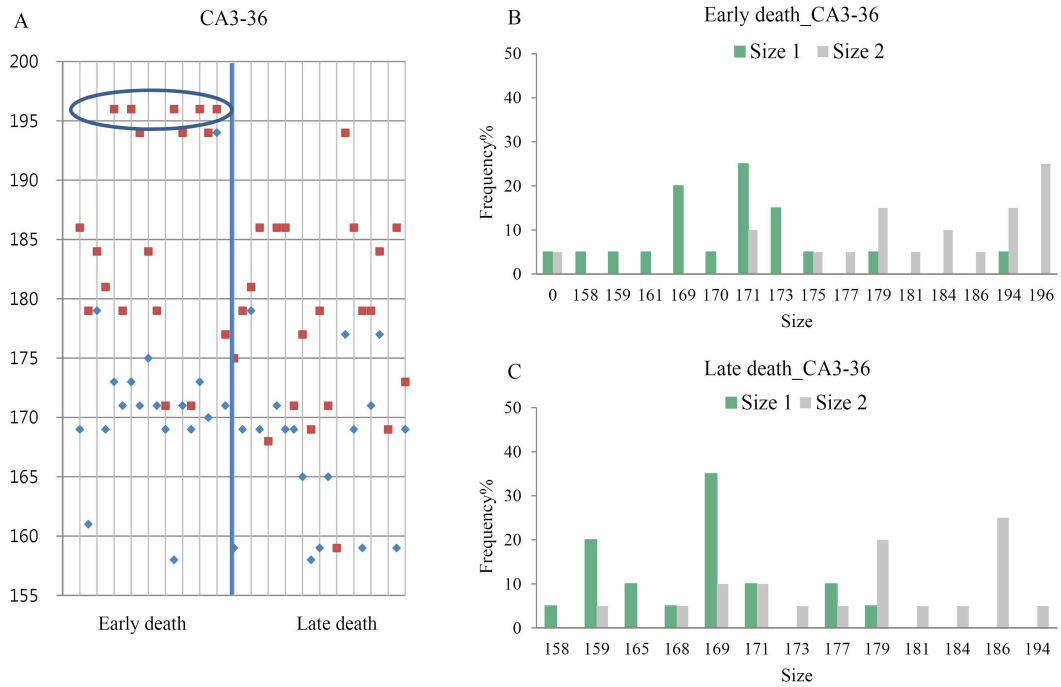

Fig. 4. Distribution patterns of microsatellite marker (CA3-36) in RBIV-infected rock bream. Circles indicates high distribution alleles (◆ Size 1, ■ Size 2).

virus (IHNV)-infected steelhead trout (*Oncorhynchus mykiss*) (i.e. They found six microsatellite markers distributed in three linkage groups (Sire LG11, 20, and 25) showing association with IHNV resistance in one of ten families studied) (Rodriguez *et al*., 2004).

When the three MS markers (CA3-05, CA3-06, and CA3-36) were analyzed together, the two groups (early and late death) were not separated perfectly using these markers. However, CA3-06 marker showed different allele distribution for fish died early and late after RBIV infection. Therefore, more detailed studies are required to find a MS marker that can facilitate the selection of RBIV resistant rock bream.

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