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Analysis of Microsatellite Allele That potential as Resistance Marker of Giant Gouramy (*Osphronemus gouramy* Lac.) to *Aeromonas hydrophila*

Meirina Kartika Kusumawardhani[1], Diah Kusumawaty[2,3], and Sony Suhandono[1]

[1] *Department of Biotechnology, School of Life Science and Technology, Bandung Institute of Technology*
[2] *Department of Biology, School of Life Science and Technology, Bandung Institute of Technology*
[3]*Department of Biology, Indonesia University of Education*
Correspondence to: meirinakartika@yahoo.com

Abstract

Meirina Kartika Kusumawardhani, Diah Kusumawaty, and Sony Suhandono. 2014. Analysis of Microsatellite Allele That potential as Resistance Marker of Giant Gouramy (*Osphronemus gouramy* Lac.) to Aeromonas hydrophila. *Aquacultura Indonesiana, 15 (1) : 1-6.* Giant gouramy is one of economical important freshwater fish. However, the production of the fish was declined because of a Motile Aeromonas Septicemia (MAS) disease. Giant gouramy with MAS disease caused an ulcer on their skin, and the worst case infection may cause death. The symptoms is vary widely depends on fish resistance to the disease. The aim of this study is to analyze microsatellite alleles that potential as a resistance marker against *Aeromonas hydrophila*. In previous studies, eleven microsatellite loci were isolated from giant gouramy genome. These loci were tested on DNA from resistant and susceptible giant gouramy that had been treated with *Aeromonas hydrophila*. Resistant giant gouramy was the gouramy that survived at least 50 days post-infection and ssusceptible giant gouramy was the gouramy that died before 50 days post- infection. Three of eleven microsatellite loci were found with unique alleles that appeared only in resistant giant gouramy and potential as resistance marker, which is the 342 bp allele at GE 1.9 locus with (GCA)10 (ACA) 6 motif, the 262 bp allele at GE 2.4 locus with (GCA) 6 (GGA)10 motif, and the 244 bp allele at GE 1.4 locus with (GCT) 9 (TA) 6 motif. All three loci were used to scan 48 giant gouramy broodstock from Tasikmalaya, Singaparna, and Sukabumi. Amplification of the microsatellite loci was performed by PCR with M13 tail sequence in forward primer and fluorescence dye. Successfully amplified alleles were analyzed with GeneAlEx 6.5 software. As a result, fragment 262 bp and 244 bp that contained in 43.75% of 48 gouramy broodstock predicted have a potency as resistance marker to *Aeromonas hydrophila*. For further study, microsatellite motifs have to be screened in gouramy progeny, because microsatellites are inhereted in Mendelian traits.

Keywords: *Aeromonas hydrophila*; Giant gouramy; Microsatellite

Introduction

Freshwater fish farming is one of the prospectively growing agriculture business. Giant gouramy is one of several freshwater fish with the highest demand (Directorate General of Aquaculture 2013), but the production can not meet the market demand. One of the problem is Motile Aeromonas Septicemia (MAS) disease which is caused by infection of *Aeromonas hydrophila* (Noga, 2011). The symptom of the disease is the fish skin appears dark and rough due to lack of mucus. Further caused infection, the fish loosing appetite and an ulcers appears on the skin and organs, such as gills, kidney, liver, pancreas, spleen, and muscle (Swann and White, 1991). The symptoms are vary, depends on the genetic biodiversity of the fish. One of prevention strategy is selection of broodstock that genetically resistance to *Aeromonas hydrophila* with microsatellite analysis. Microsatellites that were systematically closed to coding region plays a role in regulation of gene expression and protein function (Kashi and Soller, 1999), so microsatellites that were link to genes that responsible for MAS resistance may can be used for molecular assisted breeding to develop gouramy resistance to MAS. Variations in microsatellite allele maybe associated with variation of protein function and gene activity. Microsatellites were also suggested to be associated with certain diseases, for that reason microsatellites might act as a marker for some genetic related diseases (Pokhriyal *et al.*, 2012). In previous study, eleven microsatellite loci were isolated from giant gouramy genome. These loci were tested on DNA from resistant and susceptible giant gouramy that had been treated with *Aeromonas hydrophila*. The aim of this study is to predict the microsatellite allele that potential as a resistance marker of giant gouramy against *Aeromonas hydrophila*.

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Materials and Methods

DNA from Resistant and Susceptible Gouramy

In previous study, challenge test by infecting 1.95 x 10^6 cfu/mL *Aeromonas hydrophila* clone 26 to giant gouramy from Tasikmalaya was done by Kusumawaty *et al.* (2005[b]). After 50 days of observation, giant gouramy was grouped into resistant and susceptible fish, which is resistant fish that still alive at least 50 days after treatment and susceptible fish that already died in 50 days after treatment. The DNA from these gouramy was collected as a template for selection the informative loci.

Selection of Informative Loci

The DNA from resistant and susceptible gouramy were used for amplification of eleven microsatellite loci that had been found by Kusumawaty *et al.* (2005[a]) (Table 1). Succesfully amplified loci were analyzed by GeneMarker® and GeneAlex software to obtain the information about allele frequency that potential as resistance marker and PIC value. Allele that potential as resistance marker was allele that only appear in resistant gouramy. The informative loci then used to analyzed giant gouramy broodstock from Tasikmalaya, Sukabumi, and Singaparna to see the frequency of allele that potential as resistance marker.

Sampling and Tagging of Giant Gouramy Broodstock

DNA of the broodstock gouramy was collected from Balai Pengembangan Produksi Budidaya Air Tawar (BPPBAT) Tasikmalaya, Balai Besar Pengembangan Budidaya Air Tawar (BBPBAT) Sukabumi, and fish farming PT. Semata, Rancamaya, Singaparna on November 13, 2013 and February 16, 2014. The DNA was collected by cutting approximately 1 cm pelvic fin and preserved at alcohol 96%. Gouramy tagging was done by microchip injection into the muscle near the tail fin of gouramy.

DNA Isolation

DNA from gouramy was isolated by Toonen (1997) method. Pelvic fin samples were rinsed with aqua deion three times . Samples were then added to 750 µL 2x CTAB buffer (100 mm Tris - Cl pH 8.0 , 1.4 m NaCl , 20 mm EDTA , 2% CTAB , 2% PVP , 0.2% β-mercaptoethanol) and 7,5 µL proteinase K (20 mg/mL) at a temperature of 55-65^{o}C. After 2-3 hours, add 5 µL proteinase K and the incubation was continued for 16-18 hours. After incubation, add 7.5 µL RNase (10 mg/mL) and incubated for 1 hour at 37°C. Chloroform : Isoamyl alcohol (24:1) was added as 1/3 volume and centrifuged at 14,000 rpm for 5 minutes. The supernatant was taken and mixed with 1x volume of cold isopropanol, then incubated for 30 minutes at -80^{o}C. Samples were centrifuged at 14,000 rpm for 5 min and the pellet was washed with cold 70% ethanol. Pellets were left to dry and then resuspended in 30-35 mL of TE buffer.

Table 1. Microsatellite motifs from giant gouramy genome

Locus	Motifs	N	∑ Alelle	He	PIC
GE 3.1	(TAA)10	9	1	0.000	0
GE 2.4	(GCA)6(GGA)10	9	4	0.451	0.42
GEb 1.b	GTT)10	14	2	0.459	0.354
GEb 3	(AAG)14	14	2	0.490	0.370
GE 3.3	(GGA)4(CTT)15	14	2	0.408	0.325
GE 1.4	(GCT)9(TA)6	10	4	0.645	0.587
GEb 1.a	(GCT)10	12	1	0.000	0
GE 1.7	(CCA)8(TCA)2	12	4	0.635	0.563
GE 1.10	GCA)5(GTG)7	14	4	0.656	0.604
GE 1.9	(GCA)10(ACA)6	11	4	0.628	0.581
GE 3.1	(TAA)10	Amplification failed			

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PCR with Fluorescence Dye

The informative loci that have an unique allele in resistant gouramy was performed to analyze 48 gouramy broodstock from Tasikmalaya, Singaparna, and Sukabumi. Forward primer was added with M13 sequences that complement with a fluorescence dye, 6-carboxy-fluorescine (FAM) and hexachloro-6-carboxy-fluorescine (HEX). PCR profile used was predenaturation at 95°C for 2 minutes, denaturation at 95°C for 30 seconds, annealing (40°C and 45°C) for 30 seconds, extension at 72°C for 30 seconds and post-extension at 72°C for 7 minutes.

Data Analysis

PCR fluorescence products was analyzed by PT. Genetika Science, Singapore. The electropherogram then processed with GeneMarker® software V 2.6.2 (SoftGenetics, USA) with a standard size dye ROX500 (red). Allele data was analyzed by genetic distance (GD), principal coordinate analysis (PCoA), PIC value, and allele frequency using GeneAlEx 6.5 software.

Results

Amplification of eleven microsatellite loci using resistant and susceptible DNA template was done for selection of informative loci. One of eleven loci was failed to amplified, it might be caused by some mutations in primer annealing region. Based on succesfully amplified alleles that presented in Table 2, there are three loci that have an unique alleles which only appear in resistant gouramy. That three loci are GE. 1.9 which has allele 342 bp, GE. 2.4 which has allele 262 bp, and GE. 1.4 which has allele 244 bp. The appearance of those three alleles in resistant gouramy maybe linked with a genes that responsible with MAS disease, so that alleles have a potency as marker for gouramy resistance against MAS.

Genetic distance analysis for those three loci was visualized with PCoA method that presented in Figure 1. However, using these three loci is not enough to completely distinct the resistant and susceptible gouramy. More loci were needed to distinct the resistant and susceptible gouramy

Those three loci then used for analyzed 48 gouramy broodstock from Tasikmalaya, Singaparna, and Sukabumi. Frequency of unique alleles was analyzed to predict a potency of those three alleles as resistance marker (Table 3). Fragment 342 bp at GE 1.9 locus that appear 29% in resistant gouramy population however did not appear in broodstock gouramy from Tasikmalaya, Singaparna, and Sukabumi, so that GE 1.9 locus had to be tested in another population of gouramy and can not be said as potential marker for resistance yet. Fragment 262 bp at GE 2.4 locus that appear 7% in resistant gouramy population also appear 7% in Singaparna gouramy population. Fragment 244 bp at GE 1.4 locus that appear 42% in resistant gouramy also 26% in Tasikmalaya gouramy population, 50% in Sukabumi gouramy population, dan 3% in Singaparna gouramy population. The appearence of fragment 262 bp and 244 bp at gouramy broodstock could potentially be a marker of resistance. Susceptible gouramy also had a unique fragment that did not appear in resistance gouramy, which is fragment 111 in GE. 2.4 locus and fragment 106 bp and 154 bp in GE. 1.4 locus. However these fragments did not appear in broodstock gouramy from Tasikmalaya, Singaparna, and Sukabumi, so that we could not conclude these fragment as susceptible allele yet. Besides, 71.4% of susceptible gouramy at 2.4 locus also had a null allele phenomenon. Null alleles phenomenon in GE. 2.4 locus appearantly because the primer loss of the annealing ability. This phenomenon where there are mutations (point mutations, insertions, or deletions) in one or both primer annealing sequences that flanking the microsatellite. Mutations at the 3 end primer, where the extension begins, will affect the amplification of microsatellite fragments (Kwok *et al.*, 1990). As a result, fragment 262 bp and 244 bp that contained in 43.75% of 48 gouramy broodstock predicted have a potency as resistance marker to *Aeromonas hydrophila*.

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Table 2. Alleles for ten microsatellite loci (R = resistant, S= Susceptible)

Sample	3.1		2.4		1.b		b.3		3.3		1.4		1.a		1.7		1.10		1.9	
R1	137	137	115	115	161	161	161	161	161	161	244	331	125	125	125	191	161	240	240	342
R2	137	137	115	262	160	160	161	161	160	160	331	331	125	125	191	191	161	161	240	342
R3	0	0	115	115	161	161	162	162	160	160	244	331	125	125	125	191	165	165	165	165
R4	137	137	115	115	161	161	162	162	161	161	0	0	125	125	191	191	165	165	240	342
R5	0	0	115	115	160	160	162	162	160	160	244	331	0	0	191	191	161	240	240	342
R6	137	137	115	115	161	161	161	161	161	161	244	331	125	125	191	247	161	161	136	240
R7	0	0	115	115	161	161	161	161	160	160	244	331	125	125	191	191	161	161	240	240
S1	0	0	0	0	160	160	162	162	160	160	154	154	125	125	0	0	161	161	0	0
S2	137	137	0	0	160	160	162	162	161	161	154	154	125	125	125	125	161	161	0	0
S3	137	137	111	115	161	161	162	162	160	160	0	0	125	125	125	125	160	160	0	0
S4	137	137	0	0	160	160	161	161	160	160	106	331	125	125	125	191	161	161	240	240
S5	137	137	111	115	161	161	161	161	160	160	0	0	125	125	125	125	165	165	165	165
S6	0	0	0	0	161	161	161	161	160	160	331	331	125	125	125	191	165	240	136	240
S7	137	137	0	0	161	161	161	161	160	160	0	0	0	0	0	0	160	160	240	240

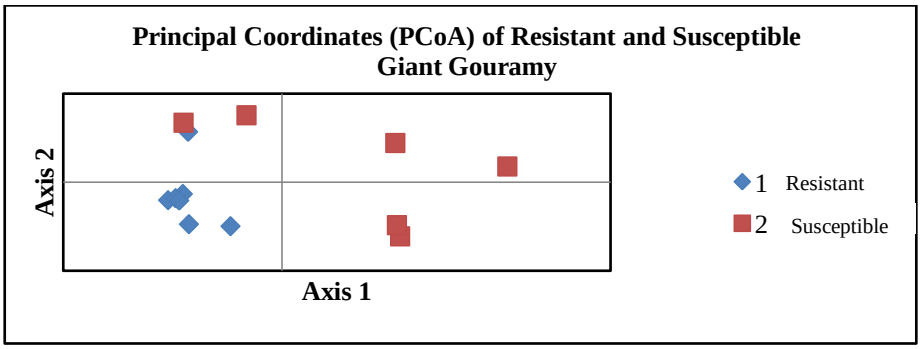

Figure 1. Principle coordinate analysis of resistant and susceptible gouramy using 3 loci (GE 1.9, GE 2.4, and GE 1.4)

Table 3. Alleles frequency for three informative microsatellite loci (Scale 0-1)

Locus	Alelle (bp)	Resistant (n= 7)	Sensitif (n=7)	Tasikmalaya (n=28)	Sukabumi (n=4)	Singaparna (n=16)
GE 1.9	136	0.071	0.125	0.143	0.5	0.5
	165	0.143	0.25	0	0	0
	221	0	0	0.018	0	0
	236	0	0	0.071	0	0
	240	0.5	0.625	0.268	0.5	0.5
	248	0	0	0.268	0	0
	251	0	0	0.143	0	0
	254	0	0	0.089	0	0
	342	0.286	0	0	0	0
GE 2.4	111	0	0.5	0	0	0
	115	0.929	0.5	0.804	0.5	0.7
	256	0	0	0	0	0.033
	259	0	0	0.054	0	0.033
	262	0.071	0	0	0	0.067
	268	0	0	0.054	0	0.1
	331	0	0	0	0	0.067
	407	0	0	0.089	0.5	0
GE 1.4	106	0	0.125	0	0	0
	125	0	0	0.019	0	0
	154	0	0.5	0	0	0
	213	0	0	0.019	0	0
	244	0.417	0	0.259	0.5	0.031
	296	0	0	0.111	0	0.406
	329	0	0	0.019	0	0
	331	0.583	0.375	0.574	0.5	0.531
	419	0	0	0	0	0.031

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Discussion

In previous study by Kusumawaty *et al.* (2005[a]), eleven microsatellite motifs were isolated from giant gouramy genome. Informative loci screening using resistant and susceptible giant gouramy had found three loci that have a unique fragment on resistant gouramy. These primers are GE 2.4 with microsatellite motif (GCA)6(GGA)10, GE 1.9 with microsatellite motif (GCA)10(ACA)6, and GE 1.4 with microsatellite motif (GCT)9(TA)6. Broodstock gouramy from Singaparna showed a highest polymorphism for GE 2.4 locus and broodstock gouramy from Tasikmalaya showed a highest polymorphism for GE 1.9 and GE 1.4 loci. Polymorphism might affect the level of resistance, where the population with higher genetic diversity could survive in a wide range of environmental conditions.

These microsatellite alleles were suggested associated with resistance against *Aeromonas* genotype, the phenomenon of genetic linkage (Cota *et al*., 2010). Genetic linkage is the tendency of two or more alleles at different loci to inherited together as a unit. In this study, microsatellite motifs suggested associated with gouramy immunity system against bacterial pathogens. *Aeromonas* able to express some extracellular enzymes and toxins, one of which is aerolysin. These toxins show the activity of phospholipase A and C. Aerolysin also capable of binding to glicophorin and make pores causing cytoplasm leakage (Gosling, 1996; Howard *et al.*, 1996). Another type of toxin, *Aeromonas* cytotoxic enterotoxin, works by inducing the accumulation of fluid in the intestine and stimulate proinflammatory response by increasing the production of cytokines, including tumor necrosis factor, IL-1 and IL-6 (Chopra *et al.*, 2000). In gouramy immune system, there are three defenses of non-specific immune system, including the integumental defenses, humoral defenses, and cellular defenses. The integumental defenses system were produce mucus that can trap and hold bacteria on the skin surface. In addition, mucus also contains anti-bacterial compounds (anti-bacterial peptides, proteases, lectins, and lysozime). Bacteria that can penetrate the integumental defenses will be faced the humoral and cellular system, which is complement system found in fish blood plasma and phagocytosis by neutophil or macrophag. Blood plasma also contains a variety of compounds that can inhibit the growth of bacteria, such as transferrin and anti-proteases, and also bactericidal compounds, for example lysozime. Besides the non-specific immune system, fish can also produce a specific immune system, including antibody and cellular cytotoxicity involving class I Major Histocompability Complex (MHC) (Uribe *et al.*, 2011).

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