Characterization of partially genom library of Gouramy (*Osphronemus gouramy:* La bantidae) which is rich of microsatellite loci: preliminary study

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ABSTRACT

Simple Sequence Repeats (SSRs) or microsatellite have been isolated and characterized from Gouramy fish (*Osphronemus gouramy*) spesific fresh water fish fromIndonesia. The basic protocol of Edward *et al.* (1996) and Giovani *et al.* (1999) followed with minor modifications. DNA Sequences from forty clones were chosen randomly from 200 clones which is rich of microsatellites to be sequenced. Thirty two (80%) clones have microsatellites. These method are interesting because can produced more than one type of microsatellite in one clone with a high percentage. There are 74 microsatellite loci or 81.25% clones that have more than one microsatellite locus. Microsatellite motifs not only appropriate with oligonucleotide bound on Hybond membrane, but also motifs that is not used on the membrane, those are (GC)n, $(TTGC)_{2-6}$, $(TTTC)_5$, (GCCC)n, (CnAn)n and ((CT)nCCT)n. Fifteen motifs obtained is appropriate with 21 types of oligonucleotide used for hybridization. 17 sequences (23 loci) which is wonderfully potential (total repeated motif > 25nt) and 7 sequences (8 loci) which is very potential (total repeated motif 21/25 nt) to be made pairs of specific primer.

Keywords : *Osphronemus Gouramy*, Gouramy, microsatellite, SSRs, fresh water fish, Indonesia

INTRODUCTION

In Indonesia, breeding program of consumption fish which is economically important, such as Gouramy, is routinely conducted through conventional selection and hybridization technique. Selected parental and offspring are only based upon morphological marker. The results achieved remain unsatisfied, because a genetic marker has not been aplied; this is because information of genetic variability of this fish is not available yet. The genetic variability information of Gouramy is needed to select to obtain excellent parental and offspring of Gouramy which have economical value additionally, conventional hybridization is consumable and laborious, because in this method several time backcross is needed (Van Eck, 1995).

In hybridization, accuracy of selection method is a crucial part, and this depends on conspicuous phenotype that is available. The expression gene that is influenced by environmental changes. Therefore, excellent Gouramy strain of Gouramy can be obtained through development of management and breeding strategy.

Genetic emendation program of Gouramy intents to yield excellent Gouramy strain that easily to cultur is characterized by intensively in limited area, performing good development in unsupported waters environmental condition, excellency in economical aspect, and sustainability against disease attack. A good genetic emendation program can be done through applying modern technology, i.e. biotechnology and molecular biology technique, which is followed by generate principals. For insteance, selection os gene marker is applied to accelerate the harvest of the offsprings.

Recently, gene marker or DNA marker widely used among genetic researcher is microsatellite marker or SSRs (Simple Sequences Repeats). Microsatellite marker or SSRs is a short DNA sequence with mononucleotide until hexanucleotides motifs and repeated number not more than 100 times, $(AGG)_{30}$ for instance. This sequence is spread almost in the entire eukariot genom, included fish, with several types of repeated.

The advantage of microsatellite marker is high polimorphism and distinguishing capacity characteristics, especially in very close genetic relationship individu (Xu *et al*, 2001). Those characteristics can be developed as genotype identification tool. Area flanking the microsatellite locus is very spesific and conserved. Microsatellite

locus can be cloned and sequenced. After sequencing, it can be designed a pair of primer that flanking the certain microsatellite locus (Kusumawaty *et al*, 2005a). After spesific primer acquired, polimorphic DNA can be amplified using PCR machine and visualized through electrophoresis on agarose gel, metaphor agarose or poliacrylamid gel (Pancoro *et al*, 2005).

Another advantage of microsatellite is because of a very short sequence of microsatellite locus (≤ 300 base pair), therefore DNA can be multiplied from a small amount of sample, and a bad sampel indeed. This advantage makes it possible to examine juvenile individu. Beside that, microsatellite is stable somatically and can be generated based on Mendelian law (Morgante & Olieveri, 1993). A primer pair that flanked a microsatellite locus is defined as microsatellite marker.

Microsatellite marker has been used to analyse many species. Microsatellite marker can distinguish crash and bottleneck population of *Argyroxiphium sanwicense* ssp *sandwicense* (Friar *et al*., 2000), microsatellite is successfully used to make linking map of barley (Liu *et al*, 1996), pollen flow and genetic intrapopulation structure of *Habroisorientalis* (Miyazaki and Isagi, 2000).

Purpose of this study is to produce spesific microsatellite marker kit for Gouramy. This microsatellite marker kit consists of primer pairs that specifically flanking microsatellite loci with repeated number more than 25 nucleotides. Thus, the kit can be used by researchers or breeders for genetic testing, spesifically in Gouramy.

METHODS

Material and DNA preparation

Gouramy fish (*Osphronemus gouramy*) was obtained from Singaparna, Tasikmalaya. DNA isolation was conducted using modified CTAB (Cationic Trimethyl Ammonium Bromide) method (Toonen, 1997). 12 pg of genomic DNA was digested using 15 U of *EcoRV* , 3 U of *AluI*, and 10 U of *RsaI* (Promega) in a tube containing 1 pl 10x buffer, 0.1 pl BSA and deionized sterile water until final volume of 30 pl. The tube was incubated for 4 hours at 37°C. Approximately 500 ng of digested DNA was ligated with 3 pg MluI adapter (consisted of a 21–mer: 5'CTCTTGCTTACGCGTGGACTA3' and a phosphorilated 25-mer: 3'ACACGAGAAGAATGCGCACCTGATp5'. Into the tube was also added 10 pl of 10 mM ATP, 3 U of T4 DNA ligase, 25 pl of 2x rapid buffer (Promega) and deionized sterile water until final volume of 50 pl. One pl of ligated DNA was amplified using 200 ng of 21/ mer primer adaptor in 1.5 pl of 50 mM $MgCl_2$, 200 IM each of dATP, dGTP, dTTP and dCTP, 5 pl of 10x PCR buffer, 2 U of Taq Polymerase (Promega) and deionized sterile water until the final volume of 50 pl. Amplification was allowed using the following steps: Pre Denaturation at 94°C; 30 cycles of denaturation at 94°C, 40 sec, annealing at 60°C for 60 sec, extension at 72°C for 2 min and post extension at 72°C for 5 min. Amplified DNA was runned on 2 % agarose gel in 1 x TAE.

Preparation of reagents for microsatellites enrichment

The following oligonucleotides was used to enrich DNA containing microsatelites such as: (ACT)10, (CTA)10, (TAG)10, (AAG)10, (ACT)10, CT(ATCT)6, TG(TATG)6, (GGA)10, (AGC)10, (GCT)10, (GTG)10, (ACC)10, (ACG)10, (AGG)10, (CAC)5CA and CT(CCT)5. Twentyone oligonucleotides were divided into three groups based on the melting temperature formula stated by Schuller & Zielinski (1989): Tm: 16.6 log [Na+] + 0.41% (G+C) + 81.5 – 500/n – 0.7(% formamide).

Table 1. Oligonucleotide groups based on melting temperature (Tm)

"Melting Temperature" in 5 x SSC					
I	Tm (°C)	II	Tm (°C)	III	Tm (°C)
$(AT)_{15}$	63.45	$(AC)_{15}$	83,95	$(AGC)_{10}$	94.88
$(AAT)_{10}$	63.45	$(AG)_{15}$	83.95	$(GCT)_{10}$	94.88
$(AATT)_{10}$	67.61	$(AAC)_{10}$	76.97	$(GTG)_{10}$	94.88
$(ATTT)_{10}$	67.61	$(AAG)_{10}$	76.97	$(GGA)_{10}$	94.88
$(T)_{25}$	60	$(CTA)_{10}$	76.97	$(GGC)_{10}$	104.45
$(CTAT)_6CT$	72	$(TAG)_{10}$	76.97	$(ACG)_{10}$	94.88
$(TGTA)_6TG$	72	$(CTC)_5CT$	77.35		
		$(CAC)_5CA$	77.35		
- formamide		+ 25% formamide		+ 50 % formamide	

Three membranes were prepared corresponding to the groups above. For each membrane was prepared 10 pg of each oligonucleotide in 3x SSC (Standard Saline citrate: 75 mM sodium citrate pH 7, 750 mM NaCl) until the total volume of 1000 pl. The mixture was spotted onto a 0.5 cm of hybond N^+ membrane (Amersham, Arlington Heigh, IL, US) and then the membrane was air-dried for 1 hour and fixed at 65°C for 1 hour. Weakly-bond oligonucleotides were washed off twice from the membrane using 10 ml of hybridization buffer (50% formamide, 3x SSC, 25 mM Na-phoSPhate pH7 and 0.5% SDS) at 45°C for 2 days. The membranes were stored at -20°C until required.

Enrichment of microsatellites

In this research a new protocol for the microsatellites enrichment has been developed on the basis of Edwards *et al.* (1996) and Corderio *et al.* (1999). Microsatellites enrichment was carried out using three tubes. Every tube contained 50 pl denatured DNA in 500 pl of hybridization buffer (5xSSC, 25 mM Sodium phosphate pH7, 0.05% SDS), 2 pg 21-mer oligonucleotide and one membrane. The first tube contained one piece of membrane I and used hybridization buffer without formamide. The second tube contained one piece of membrane II and hybridization buffer with 25% formamide. And the third tube contained one piece of membrane III and hybridization buffer with 50% formamide. All tubes were hybridized at 50°C for 2 days. Following hybridization, one membrane from each tube was washed 5 times (5 minutes for each washing) in 2x SCC containing 0.01% SDS at 50°C, 3 times in 0.5x SSC containing 0.01% SDS at 50°C, and 2 times in deionized sterile water at room temperature. Three tubes filled with 200 pl of deionized sterile water were boiled at 95°C for 5 minutes. The membrane was added into each tube and bound DNA was eluted at 95°C for 5 minutes.

Two hundred pl eluted DNA was transferred into new 1.5 ml tubes, 5 pl of 4M NaCl and 500 pl ethanol absolute was added, and kept for 1 hour at 4°C. Then the mixture was centrifuged at 13,000 rpm for 20 minutes, and liquid phase was poured off. The pellet was vacuumed, and 25 pl deionized sterile water was added to the dry pellet. One pl of eluted DNA was amplified with the addition of 200 ng 21-mer adaptor primer, 25 ul of 1.5 mM $MgCl_2$, 200 IM each of dATP, dTTP, dGTP and dCTP, 10x PCR buffer, and 2 U of Taq DNA polymerase (Promega). Amplification was allowed for 20 cycles. Pre-denaturation at 94°C for 5 min, denaturation at 94°C, for 40 sec, annealing at 60°C for 60 sec, extension at 72°C for 2 min and post extension at 72°C for 5 min. Amplified DNA was runned on 2 % agarose gel in 0.5x TBE.

Transformation and Sequencing DNA

Approximately 3 pl of enriched DNA were ligated into 1 pl of pGEM-T easy vector system I (Promega) using 3 U of T4 DNA ligase and 2x Rapid ligation buffer. Ligation was allowed at 4°C overnight. Plasmid were

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transformed into dH5á (Promega), plated onto LB/agar plates containing 100 pg/ml ampicilin, 20 pl X-gal (50mg/ml), 100 pl 0.1M IPTG, and incubated at 37°C overnight. Plasmids from individual colonies were isolated using procedure stated by Xiang *et al*., (1998) and sequenced at MACROGEN, Seoul, Korea.

Microsatellite locus category

Determination, if the locus or motif found is microsatellite or not, is needed for independent locus, locus with mononucleotide motif and minimum 7 repeats, locus with dinucleotides motif and 4 repeats, trinucleotides and 3 repeats, locus with tetra-/penta-/hexanucleotides and minimum 2 repeats. Determination is also needed if microsatellite motifs is a combination, with minimum number of repeats is 5 for mononucleotide, 2 until 3 repeats for dinucleotides, and 2 repeats or minimum 5 repeats or 10 nucleotides number of repeats for trinucleotides.

Primer that flanking microsatellite locus is designed using Primer3.cgi v 0.2c software (Rozen & Skaletsky, 1997). Analysis primer for the presence of hairpin and dimmer primer is using Primer Premier4 software (Biosoft international, 1996).

RESULT AND DISCUSSION

Forty plasmid DNA consisted was selected from 200 plasmids. Plasmids were sequenced at Macrogen, Seoul, Korea, using ABI 3730 XL machine. Sequencing process has been done in one direction using T7 primer.

The result of sequencer machine reading still consists of cloning vector sequence. Therefore, to know the inserted DNA sequence it is needed to know the adaptor 21-mer sequence as a marker on 5'-end and 25/ mer sequence on 3'-end of the inserted DNA. Inserted DNA sequence is nucleotide sequence at the middle of both oligomer (Figure 1). Sequence analysis indicated that inserted DNA fragment of Gouramy fish obtained is ranged between 148 nt – 667 nt. Sequence length acquired is variable, from 40 sequences no one sequence is similar with another sequence, although several sequence showed similar length but have different DNA sequence. Microsatellite motifs found in Gouramy genom have a high variation, from mono/ until tetranucleotides.

GAATTACTATCCAGCTCCGGCCGCATGGCGGCCGCGGGAATTCGATTCTCTT
GCTTACGCGTGGACTAGACGCTGTGGCACCTGATAAAATTTCCT*GCTGCTGC*
*TGCTGCTGCTGCTGCTGCTGCT*ACTGTGGCCACTGAATTGGCAGCGTTTGCC
GCTGCTGACATCGTGTCTG*GTTGTTGTTGTTGTTGTTGTTGTTGTTGTT*GCT
GCAGTTGCTGCTGTATTTGCAGCACTGGCTGATGTTAATGCTGATGCTGCTA
TGTGTGCTGCTTGGAGTGCTGCTGATGCTGGTGACACTGTTGATGCTTGTTG
TAC*TGCTGCTGCTGCTGCTGCTGCTGCTGCTGC*TTCAGATGCTTGATTTGCT
GCATTTACTGCCAGTTCCACTGGTGCTGCACCGGCTGCGACTAAAGTGGCAG
TAGTCCACGCGTAAGCAAGAGAA**TCACTAGTGAATTCTCGGCCGCCTGCAGG**
TCTACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCT
ATAGTGTCACCTAAATAGCTTGGCGTAATCATGGCCATAGCTGTTTCCTGTG

Figure 1. Recombinant plasmid DNA sequence after colony pG-b1sequencing.
Note: red sequence is 21-mer (adaptor), green sequence is 25-mer (adaptor), black sequence is plasmid sequence, and violet sequence is microsatellite motifs obtained, $(GCT)_{10}$, $(GTT)_{10}$ and $(TGC)_{10}$.

that is widely used on plants, except $(TGTA)_6TG$, $(CTAT)_6CT$ and $(CAC)_5CA$. Total microsatellite locus acquired from 32 sequence are 74 loci.

Sequence analysis also showed that 26 from 32 recombinant DNA samples (81.25%) consists more than one microsatellite locus. It is clearly confirmed by Edwards *et al* (1996), that almost 50 - 70% clones yielded from enrichment method will consist of more than one microsatellite locus. The most motifs appeared in Gouramy fish genom are (AC)n and (AG)n. The average of the most motifs appeared are motifs that contain 25/30% G/C (48.15%), 70% G/C (24.1%), and the least motifs appeared are motifs that only contain A/T (11.1%).

In this research, is used 21 microsatellite motifs to find microsatellite locus in Gouramy genom (Table 1). Fifteen motifs are appropriate with motifs used in hibridisation. Seven motifs are not found,i.e: $(T)_{25}$, $(AT)_{15}$, $(AATT)_{10}$, $(ATTT)_{10}$, $(CTAT)_6CT$ and $(TGTA)_6TG$, but there are six motifs that are not appropriate with the motifs used or motifs bound on membrane, i.e: (GC)n, $(TTGC)_{2\_6}$, $(TTTC)_5$, (GCCC)n, (CnAn)n and ((CT)nCCT)n. This condition was also found in microsatellite motif isolation in *Phaseolus vulgaris* by Solis *et al* (2002), mangrove by Maguire *et al* (2000) and in manggo by Mulyani (2002).

Motifs from each locus were then grouped based on (1) its potential level and primer pairs that flanking microsatellite locus area were designed and (2) Weber (1990). The first category are stated on Table 2. Primer for microsatellite motif locus grouped in Lower Potential (LP) category will not be designed, or will be the last choice to be designed. Primer for microsatellite motif locus grouped in WP category is the prominent choice to be designed.

Table 2 Category of potential level of microsatellite locus

Category	Number of Number
Lower Potential (LP)	< 15 nt
Potential (P)	16/20 nt
Very Potential (VP)	21/25 nt
Wonderfully Potential (WP)	> 25 nt

The second category based on Weber (1990), as followed: 1. Perfect: there is no other nucleotides interval among microsatellite motifs; 2. Imperfect: there is nucleotide interval that is not microsatellite motif among microsatellite motifs; 3. Perfect compound: compound of two or more microsatellite motifs and there is no nucleotide that is not microsatellite motif among microsatellite motifs; and 4. Imperfect compound: compound of two or more microsatellite motifs and there are nucleotides that is not microsatellite motifs between microsatellite motifs. Compound two or more microsatellite motifs and between two motifs consist nucleotide that is not microsatellite motif. Categorizations part of motifs based on Weber (1990) can be seen on Table 3.

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Table 3 Microsatellite data after recombinant DNA plasmid analysis
(Number of repeat > 21 nt or minimum 8 nucleotides in tetranucleotide)

NO	DNA Sample	MICROSATELLITE				
		size (nt)	E locus	Motif length (nt)	Category	Motif
1	E1.2	400	2	37 28	WP/PC WP/PC	*(CCT)3[(CT)3(CCT)]2. *[(GGT)3(GT)]2(GGT)2.
2	E1.3	530	2	40 40	WP/P WP/IC	*(GCTT)10. *(TTGC)6(TC)4T(TTGC)2
3	E1.4	466	2	28 39	WP/IC WP/PC	*(GA)4G(TTTC)5 *(GCT)9(AT)6
4	E1.5b	217	1	14	VP/IC	*(GA)3GCA(CAGA)2
5	E1.7b	284	2	27 30	WP/PC WP/PC	*(CCA)4(TCA)3(CAA)2 * (CCA)8(TCA)2
6	E1.9	285	1	42	WP/IC	*(GCA)10A(CCA)4
7	E1.10	265	1	36	WP/PC	*(GCA)5(GTG)7
8	B1.9	334	1	26	WP/PC	(GT)9(GA)4
9	B1.1	349	3	30	WP/P	*(GCT)10;*(GTT)10;*(GCT)10
10	B1.3	148	1	33	WP/P	*(GAA)13
11	B1.13	357	1	24	VP/I	*(GCG)3G(GCG)5, (GCG)8
12	B2.3	335	1	33	WP/I	*(C)12(A)2(C)7(A)5C(A)6,4(CnAn)3
13	E2.2b	273	1	38	WP/P	*(TG)19
14	E2.4b	285	1	48	WP/PC	*(GCA)6(GGA)10
15	E2.5	241	1	21	VP/P	*(TTA)7.
16	E2.6b	390	1	30	WP/P	*(GCA)10
17	E2.7b	225	1	32	WP/IC	*(GAT)3(GGT)5TC(GT)4
18	E2.8b	285	1	8	VP/P	* (GCCC)2
19	F3.1	342	2	30 22	WP/I VP/I	*(TTA)5TTA(TTA)4, (TTA)10 *(GA)3GA(GA)4GA(GA)2, (GA)11
20	E3.2	164	1	40	WP/IC	*(CTT)6CTT(CTT)2(CT)2CC/TT (CTT)2,4 (CTT)9(CT)2(CTT)3
21	E3.3	169	1	57	WP/IC	*(GGA)3(CTT)11(CC/TT)(T/CC/TT)(CC/TT)(CTT)2,4 (GGA)3(CTT)16
22	E3.9b	443	1	8	VP/P	* (GCCG)2

Note:
VP: Very Potential, WP: Wonderfully Potential, P: Perfect, PC: Perfect Compound
I: Imperfect, IC: Imperfect Compound

There are twentytwo sequences grouped in VP and WP category. Six sequences have more one microsatellites locus. Seventeen sequences consist twenty one microsatellites locus group on WP categories and five sequences group in VP categories. Microsatellite motif grouped in VP and WP categories was chosen to be primer designed.

In this research, there are minor modification of microsatellite enrichment method based on Edwards *et al*. (1996) and Giovani *et al.*, (1999), which is efficient because 80% of sequenced recombinant DNA sample contain microsatellite motifs. This result is supported by Solis *et al*. (2002), who compared enriched library with unenriched library, and the enriched library method is more efficient compared to unenriched library method. Zane *et al*. (2002) stated that efficiency using of enrichment method in microsatellite motif isolation and characterisation ranged between 50% - 90% for plant and animal genom.

The result of sequence analysis and characterisation showed that Gouramy microsatellite motifs have high variation. But several motifs have very small number of repeats, those are 6/8 repeats of mononucleotide, 3 repeats for dinucleotides, and 2/3 repeats for trinucleotides. This is a worried condition, because it can affect primers to produce polimorfism. But Treuren *et al.* (1997) stated that minimum number of microsatellite repeats for

nucleotide is 6 times, whereas for dinucleotides is 3 times. Lefort *et al* (1997) selected trinucleotides with minimum 3 repeats and tetra nucleotides with minimum 2 repeats as micorsatellites. Beside that, microsatellite hasa unique character in the number of repeats that ranged between 6-100 nt. Based on the above theory, microsatellite motifs obtained in DNA Gouramy genom are the shortest repeats motifs. Microsatellite motif with minimum number of repeats was also obtained by Santos *et al* (2003) from *Carica papaya* genom and by Dayanandan *et al* (1997) from *Pithecellobium elegans* genom. Therefore, primers designed in this research are expected to produce polymorphism when it is used for SSRs amplification of another Gouramy species. To prevent obtaining monomorphic locus, specific primers will only be only designed for sequences in VP and WP categories.

Primer sequence of microsatellite flanking area is designed to produce primer with length of 17-22 nucleotides, contains approximately 50% GC and Tm about 60°C. Primer is designed using Primer3.cgi 0.2c software (Rozen & Skaletsky, 1997). After primer design using Primer3.cgi 0.2c software, it is yielded 25 primer pairs of microsatellite motifs from VP and WP categories, with GC percentage ranged between 50 - 55% and Tm ranged between 57 - 59°C. Primer length ranged between 18-22 nucleotides. Primer for microsatellite motif contained in the 3' end or 5' end will not be designed. Jakse & Javornik (2001) suggested, microsatellite motif should be minimum 30 nucleotides from the beginning or from the end of microsatellite sequence to produce a good primer. Another cause a primer cannot be designed is if the sequence is rich with T/A. As foreknown, hidrogen bound between T and A is less than between G and C. If primer of sequence rich with T/A is produce, the primer will be unspesific to acquaint its bound site.

To know whether the primer pair flanking the microsatellite locus can be used to amplify microsatellite locus of gouramy fish spesifically, it is needed to do further research on DNA amplification using several different gouramy individu.

CONCLUSION AND SUGGESTION

From this research result, it can be concluded that although enrichment process using microsatellite motifs that is usualy used for plants, those motifs can also be used to isolate microsatellite locus in Gouramy fish (*Osphronemus gouramy* Lac.) with 80% of efficiency. The motifs that often appear is (AC)n and (GC)n. In Gouramy genom isvery rare found microsatellite motifs that only contain A/T or AT, whereas microsatellite motifs that often appear is microsatellite motifs that contain GC and AT compounds.

Suggestion for further research is that it is needed to do further testing on primers that is succesfully producing polimorphism on Gouramy genom. To obtain microsatellite motifs that can more represent Gouramy genom, it is necessary to sequence more recombinant plasmid DNA.

ACKNOWLEDGEMENT

This research is fully funded by DIKTI, DEPDIKNAS, with Research Grant Agreement Letter No: 032/SP2H/PP/DP2M/III/2007, 31 December 2006.

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