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Genetic Variation in Cytochrome b-*Hinf*1 and -*Alu*1 Gene Correlated to Body Size in Soang Gourami (*Osphronemus goramy*) from Single Spawning

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Abstract

Soang gourami fingerling shows variable body sizes eventhough resulted from single spawning. Differences in body sizes among individuals is assumed to be correlated to their genetic component which can be studied using cytochrome b gene PCR-RFLP marker. This study aimed to determine specific PCR-RFLP marker among different sizes of soang gourami collected from single spawning. Genomic DNA was isolated using Chelex method. Cytochrome b gene were amplified and digested using four restriction enzymes. Specific markers were analyzed descriptivelly based on DNA band pattern appear in agarose gel. The result showed that PCR-RFLP markers of Cytochrome b-*Hinf*I of 315 bp, and 210 bp, and also Cytochrome b-*Alu*I of 334 bp and 189 bp are specific markers for large individuals, whereas small individuals are characterized by having Cytochrome b- *Hinf*I 366 bp, and 159 bp and Cytochrome b-*Alu*I 525 bp fragments. It is observed that genetic variation of Cytochrome b-*Hinf*I and -*Alu*I markers are possitively correlated to body size in soang gourami fingerling. Therefore, both cytochrome b-*Hinf*I and -*Alu*I gene can be reffered as specific markers to differentiate among different sizes of soang gourami strain fingerling from single spawning. This result proved that genetic divergences among individuals can be related with certain quantitative characters, such size related. Therefore our study can contribute on fisheries development, especially by providing new technique for fingerling selection to obtain high quality fingerling and also provide new insight the application of molecular technique in fisheries.

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INTRODUCTION

Giant gourami (*Osphronemus goramy* Lacepede, 1801) has been widely cultivated in Indonesia for many years. This species has high economic value due to its taste meat (Azrita, 2015). The high prices of gourami have encouraged the establishment of such fish as one focus of fisheries revitalization in Indonesia for the period of 2009-2025 (Nurdjana, 2008). In terms of cultivation, production of giant gourami is relatively low cost and it species has the ability to adapt to environmental condition with low oxygen levels (Setijaningsih et al., 2007). However, there are two major obstacles that hinder the success of giant gourami cultivation, namely slow growth rate and high mortality due to disease (Achmad et al., 2009).

Several giant gourami strains have been cultivated by the fish farmers, i.e. Soang, Jepang, Paris, Bastar, and Porselen strains (Setijaningsih et al., 2007). It was reported that growth performance of each strain is different (Nugroho et al., 1993) and fish farmers prefer to cultivate soang strain because it is believed to have a better growth rate than other strains. However, if we examined carefully in the fingerling of soang strain from single spawning, their body sizes are varies. These body size variations are suggested because of each individual have different growth rates. Differences in the ability to grow is assumed due to that the fish have different metabolic capabilities, primarily in harvesting energy from the feed (Yurisma et al., 2013), through celular respiration. The performance of cellular respiration assumed directly related to the variations in genetic component of each individual fingerling. The differences in genetic componen profile among individuals within population can be used for genetic diversity analysis in certain species (Millah et al., 2012). According to Kartikaningsih et al. (2001), genetic factors are among various factors that cause giant gourami growth rate is relatively slower than other fish species. Previous study proved that variation on growth hormone gene was possitivelly corelated with individual body size in certain population (Hua et al., 2009).

So far, detection of growth hormone gene 1 (GH 1) from soang gourami resulted incosistence DNA band pattern. Therefore, it could not be used as genetic marker to characterize individuals with different body sizes of that strain (Nuryanto et al., 2014). Meanwhile, cytochrome c oxidase 1 (CO1) which is belived to as the most variable mitochondrial DNA (mtDNA) and commonly use in animal population genetic studies, it was reported that the CO1 gene could not also be used to distinguish large and small individulas soang gourami from single spawning (Azizah et al., 2015). Therefore, it is necessary to seeks other molecular markers to be used as a candidate of specific marker on large and small individuals differentiation. Cytochrome b gene is among mtDNA genes which is familiar in population analysis. Previous studies, either using PCR-RFLP or sequences of cytochorme b gene have proven that this gene is variable enough to discriminate among populations on various fish species (Mesquita et al., 2001; Takehana et al., 2004; Cheng & Lu, 2005; Ma et al., 2010) and from pork in meatballs product (Fibriana et al., 2012). However, those studies were done to evaluate genetic differentiation among populations. So far, there was no study about intra-populational genetic diversity, especially on soang gourami fingerling from single spawning but having variable body sizes. Here we characterized soang giant gourami fingerling population which is consisted of two groups size individuals but originated from single spawning using PCR-RFLP marker of cytochrome b gene.

This study aims to develop PCR-RFLP marker to be used as specific marker for large and small individulas differentiation within population of soang gourami fingerling from single spawning.

The result is expected to have contribution in the development of giant gourami cultivation by providing new methods for fingerling selection using molecular character, especially RFLP characters. It is also expected that our study to provide new insight about the possibility of the application of molecular technique in applied sciences such as in aquaculture.

METHODS

This study was conducted from April to November 2016 and used purposive sampling. Fish samples were collected from Purbalingga, Central Java, but the fingerling were bought from Ciamis West Java. The samples of soang giant gourami fingerling were originated from single spawning. Fish samples were divided into two different groups based on their body size; i.e. small individulas with their total length less than 10 centimeter (<10 cm) and large individuals with their total length more than ten centimeter (> 10 cm). For DNA analysis, more or less of 0.5 centimeter of tissue samples were cut off from caudal fins while the individuals were kept alive. Tissues samples were preserved in 96% ethanol.

The DNA was extracted from all samples

using Chelex 5% method (Walsh et al., 1991) with small modification, especially on incubation times. Fragment of cytochrome b gene was amplified using a pair of primers as follow: *forward* 28FOR 5'-CGAACG TTGATA TGAAAA ACCATC GTTG-3' (Meyer et al., 1990) an *reverse* 34REV 5'-AAACTG CAGCCC CTCAGA ATGATA TTTGTC CTCA-3' (Cantatore et al., 1994). The PCR reaction was done in final volume of 25 µl. The final concentration of each reagen was 1X PCR buffer; 0.8 mM of each dNTP; 0.4 pM of each primer; 2 U Taq DNA polymerase, dan 0.8-3.52 ng/µl DNA template. Final volume of 25 µl was reached by adding ultrapure water (ddH_2O) as much as 18.4 µl. Amplification of the cytochrome gene fragments were conducted in thermal condition as follow. Predenaturation at 94 °C for 5 minutes and cotinued by 35 cycles with denaturation at 94 °C for 30 seconds, annealing at 55 °C for 45 seconds, and extension at 72 °C for 1 minute. Final extension was performed at 72 °C for 5 minutes. The amplicons were migrated in 1% of agarose gel and divisualized over UV-light transiluminator.

Consistent and strong amplicons were then digested using *Hinf*I, *Alu*I, *Hind*III, and *Hae*III restriction enzymes to obtain PCR-RFLP marker of the cytochrome b gene. Digestion procedures following the protocol provided by the company (Thermoscientific). Digestive products were migrated in agarose gel 1.2% and visualized over UV-light transluminator. Fragment length of the PCR-RFLP markers were estimated by comparing them to 100 base pair (bp) DNA ladder. Specific PCR-RFLP markers were defined descriptively based on the PCR-RFLP band pattern on agarose gel.

RESULTS AND DISCUSSION

Amplification processes using specific primer resulted amplicons with the size approximately of 525 base pair (bp) length. Similar amplicons sizes were always obtained when amplifications process were repeated. With this consistent result, we are sure that the amplicons are our target products. The obtained amplicons are presented in Figure 1.

The amplicons were then digested using four restriction enzymes, that were *Hinf*I, *Alu*I, *Hae*III and *Hind*III. Restriction fragments are wellknown as PCR-RFLP markers. The size of restriction fragments were estimated using regression analysis after compared to 100 bp DNA ladder. A complete PCR-RFLP markers measurements are presented in Table 1.

It can be seen from Table 1 that digestion of PCR amplicons using *Hinf*I enzymes resulting two different PCR-RFLP pattern from two group sizes. The PCR-RFLP marker from small individuals cosisted of two cytochrome b-*Hinf*I fragments with the sizes of 366 bp and 159 bp length, while from large individuals consisted of 315 bp and 210 bp length fragments. The cytochrome b-*Hinf*I fragments are presented in Figure 2.

The ability of *Hinf*I enzyme in cutting off the PCR amplicons proved that on cytochrome b gene of soang giant gourami fingerling has specific site which can be recognized by that enzyme. This is because restriction enzyme only able to recognize a specific site so that if there is no restriction site which can be recognized, the sequences will not be cut by the enzyme. The success of *Hinf*I enzyme cutting cytochrome b gene in this study similar to the studies from Hold et al. (2001) on 25 fish species on food products, (Chen & Lu, 2005) in fish *Coilia ectenes*, and Nebola et al. (2010) in several marine fish species, but different to Hold et al. (2001) for 11 other fish species. Similarities and differences among our study and those previous studies indicate that cytochrome b gene varies greatly between species so that some

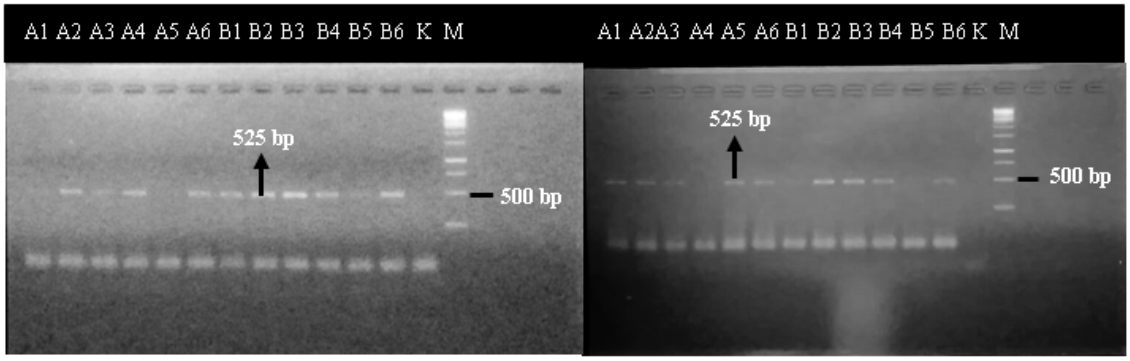

Figure 1. The amplicon of cytochrome b gene from soang giant gourami fingerling
Remarks: A1-A6. Small size fingerling; B1-B6. Large size fingerling; K. Negative control; M. DNA ladder 1 kb (kilo bases)

Table 1. The size of restriction fragment based on regression analysis measurement

Enzymes	Fingerling Size	Migration distance (cm)	y-value	Fragment Length (base pair)	Total Length (base pair)
HinfI	Small	2.17	2.569337	366	525
		2.9	2.20149	159	
	Large	2.31	2.498791	315	525
		2.66	2.322426	210	
AluI	Small	1.87	2.720507	525	525
	Large	2.26	2.523986	334	523
		2.75	2.277075	189	
HindIII	Small and Large	1.87	2.720507	525	525
HaeIII	Small and Large	2.31	2.498791	315	493
		2.8	2.25188	178	

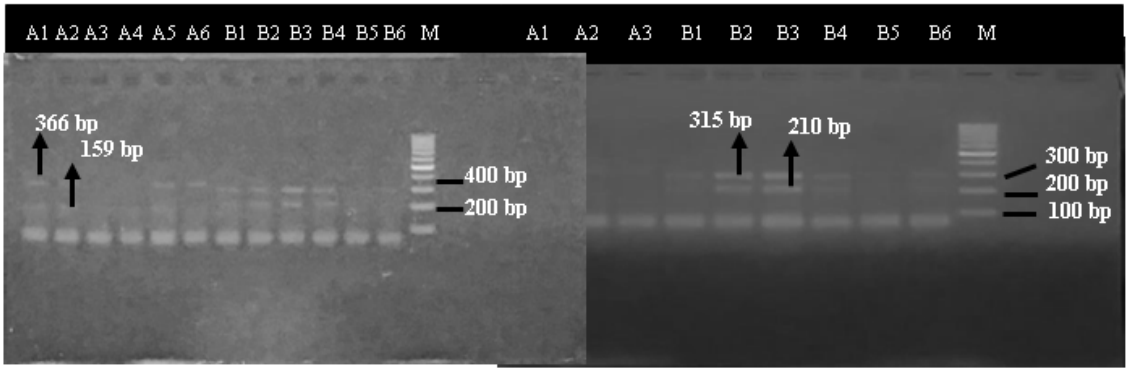

Figure 2. Restriction fragment resulted from *Hinf*I digestion
Remarks: A1-A6 = small individuals; B1-B6 = large individuals; M = 100 bp DNA ladder

species has restriction sites that could be recognize by *Hinf*I enzymes, while the other species has no such sites. The results of this study provide additional information that the cytochrome b gene not only varied between species but also within species, even within population such in this study which used soang giant gourami fingerling from single spawning but has variation in body size among its members.

The different between individual groups with different quantitative characters was also reported by Kusbiyanto et al. (2016) on Soang giant gourami fingerling from single spawning. However, comparison to the study from Kusbiyanto et al. (2016) was not congruence since we used different quantitative characters and different genetic markers. Here we used growth rate as quantitative character which was indicates by body sizes and RFLP of cytochrome b gene as genetic marker, while Kusbiyanto et al. (2016) used individual resistance as quantitative character and major histocompatibility complex (MHC) class II gene as genetic marker. Although not congruence comparison, however, at least we can learn from present study and the study from Kusbiyanto et al. (2016) that genetic divergence might occur among individuals from single spawning but with different quantitative characters, i.e. body size and resistances to diseases.

The results of *Hinf*I restriction generate different cytochrome b-*Hinf*I markers profiles betwen large size and small size of soang gourami fingerling. All large individuals have a uniform cytochrome b-*Hinf*I markers, as well as small fingerling have another uniform profile. This provides evidence that cytochrome b-*Hinf*I markers could be uses as a candidate of specific marker to distinguish fast-growing and slow-growing soang gourami fingerling. These differences are also providing information that there are two haplotypes of cytochrome b gene in Soang gourami fingerling with different body sizes. Each haplotype was only observed in certain group size. This means that genetic variation in cytochrome b-*Hinf*I of soang giant gourami fingerling is possitively related to their body size, especially to total length.

Amplicons digestion using *Alu*I enzyme

produce different pattern of PCR-RFLP markers for small and large individuals. On small individuals, the amplicons were not digested which was indicated by similar size of the DNA fragments between diggested and undiggested amplicons that is 523 bp length fragments. This means that in small individuals no restriction site was recognized by the enzyme, while in large individuals there was. In large individuals, the PCR amplicons were cut into two different Cytochrome b-*Alu*I fragments, i.e. 334 bp and 189 bp length fragments. Digestion poducts of *Alu*I enzyme are presented in Figure 3.

As presented in Figure 3, the *Alu*I enzyme able to cut the amplicons of the cytochrome b gene of large individuals only. This mean that restriction sites were only found in large individuals but not in small individuals, although both groups sizes were collected from single spawning. Our result similar to what were reported by Nebola et al. (2010) in several marine fish species, Cocolin et al. (2005) on rainbouw trout and dentex fish species, and Apostolidis et al. (1996) on *Salmo truta* L. Meanwhile, Ali et al. (2011) also reported that cytochrome b gene of mammal was successfully diggested by *Alu*1 enzyme. Similarities between our study and those previous studies were due to that the cytochrome b gene have specific site that can be recognized by *Alu*1 enzyme although that gene was isolated from different species. Our study and those previous studies proved that *Alu*1 restriction sites of cytochrome b gene are widely distributed acrross animal phyla, starting from fish up to mamalia.

Another information that can be summarized from Figure 3 is that *Alu*1 enzyme only able to digest cytochrome b gene from large individuals. The result shows that cytochrome b gene of soang giant gourami fingerling from single spawning are varies. This variation is possitively correlated with body size. Therefore, Cytochrome b-*Alu*I markers can be selected as a candidate marker for large and small individuals differentiation of soang giant gourami finggerling collected from single spawning.

The *Hae*III enzyme was able to digest PCR products and generated two PCR-RFLP fragments, that are Cytochrome b-*Hae*III of 315 bp and 178 bp length fragments. These PCR-RFLP fragments were obtained from all individuals whatever their total body length. The PCR-RFLP fragments resulted from *Hae*III digestion are presented in Figure 4.

All individuals have the same PCR-RFLP patterns (Figure 4). This fact proved that cytochrome b gene of soang giant gourami has restriction site for *Hae*III enzyme. This result is similar to the result from Nebola et al. (2010) on various marine fish species, Cheng & Lu (2005) on *Coilia ectenes*, Takehana et al. (2004) on *Oryzias latipes*, Lin et al. (2002) on freshwater eels, Hold et al. (2001) on 36 fish species, and Cocolin et al. (2000) on four marine fish species filet. This similarities could be due to that cytichrome b gene on our research object and those previous studies (Cheng & Lu, 2005; Takehana et al., 2004; Lin et al., 2002; Hold et al., 2001; Cocolin et al., 2000) have restriction sites which can be recognized by *Hae*III. This phenomenon indicated that *Hae*III restriction sites on cytrochrome b gene are widely distributed on many fish species, either on freshwater or marine water species.

If we look into detail on Figure 4, it can be observed that cytochrome b gene was digested. However, the Cytochrome b-*Hae*III markers shows similar pattern between small and large individuals. This means that only one allele of Cytochrome b-*Hae*III was resulted. Therefore, the Cytochrome b-*Hae*III marker could not be used as candidate marker to differetiante between fast-

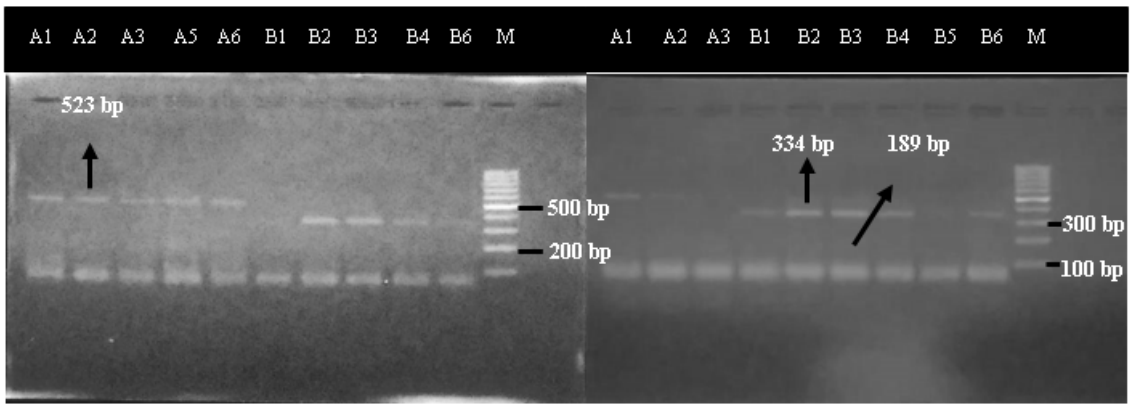

Figure 3. Restriction fragment resulted from *Alu*I digestion
Remarks: A1-A6 = small individuals; B1-B6 = large individuals; M= 100 bp DNA ladder

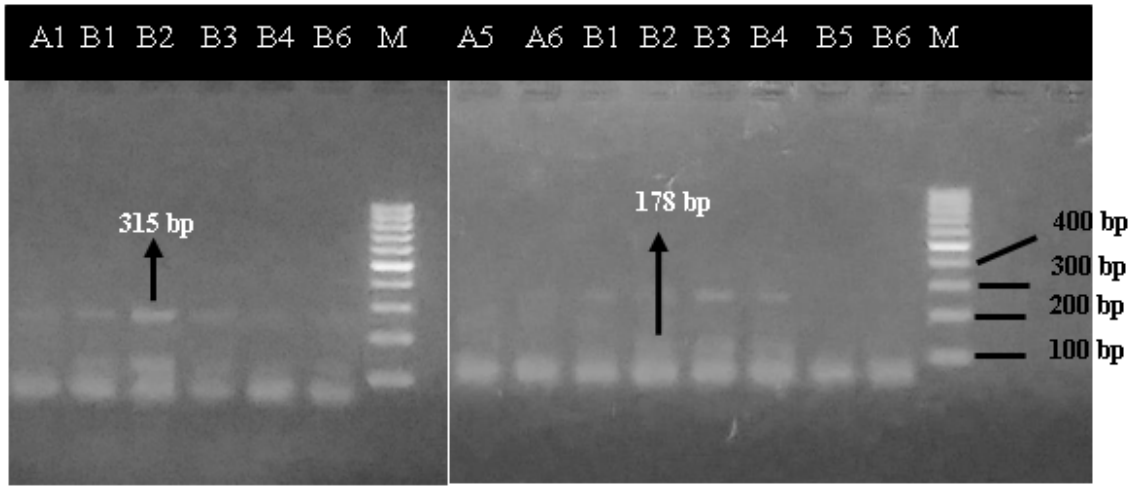

Figure 4. Restriction fragment resulted from *Hae*III digestion
Remarks: A1-A6 = small individuals; B1-B6 =large individuals; M. 100 bp DNA ladder

growing and slow-growing individuals of soang gourami fingerling collected from single spawning.

The *Hind*III enzyme was unable to digest cytochrome b gene. This was proven by a fact that digested products have similar sizes to undigested PCR products, i.e. 525 bp length fragments. The cytochrome b-*Hind*III fragments are presented in Figure 5.

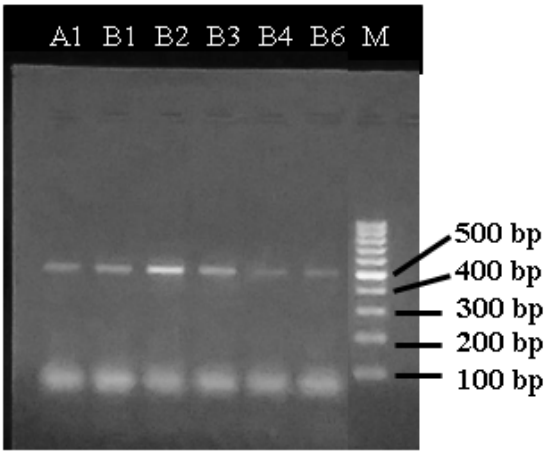

Figure 5. Restriction fragment resulted from *Hind*III digestion
Remarks: A1-A6 = small individuals; B1-B6 = large individuals; M = 100 bp DNA ladder

Inability of *Hind*III enzyme to cut the PCR product was due that on cytochrome b gene no restriction site available which can be recognized by the enzyme. We could not compare our study to previous studies because so far there were no studies in fish species that utilized *Hind*III enzyme to digest cytochrome b gene (Nebola et al., 2010; Cheng & Lu, 2005; Takehana et al., 2004; Lin et al. 2002; Hold et al., 2001; Cocolin et al., 2000). The reason could be due that those researchers realized that there were no *Hind*III restriction sites on cytochrome b gene on various fish species. Therefore, it was not surprising that we could not observed any *Hind*III restriction sites on cytochrome b gene of soang gourami fingerling so the gene was not digested by HindIII enzyme. Therefore, the cytochrome b-*Hind*III can not be used as specific marker for size selection on soang giant gourami fingerling collected from single spawning.

It was common that peoples studied growth variation among individuals from a single spawning using growth hormone gene or growth factor gene as genetic markers. However, we were unsuccessful to amplify those gene from soang giant gourami fingerling (Nuryanto et al., 2014). Different to previous studies, in this study, we used indirect estimation on observing genetic variation among individuals with different body sizes but resulted from single spawning using an alternative gene that is cytochrome b. This gene was selected due to its function as co-enzyme in cellular metabolisms. Therefore, it was expected that the variation in that gene will result in variation of metabolism rates among individuals which lead to different growth rate. The final result would be differences in body sizes among individuals. Therefore, the utilization of cytochrome b gene in such study is a novelty.

Our result proved that RFLP markers of cytochrome b-*Hinf*I and –*Alu*I were variable among individuals and their variations were related to body sizes of each individual. According to this result, those two RFLP markers can be used as a fundament for developing a new technique

for fingerling selection to obtain high quality fingerling. This is a new contribution of our study on the development of giant gourami cultivation. From scientific view, our study provide new insight about the application of molecular technique in applied sciences, e.g. in aquaculture.

CONCLUSIONS

During the study, it was observed that Cytochrome b-*Hinf*I and –*Alu*I variation in soang giant gourami fingerling population were correlated to their body size. Therefore, both PCR-RFLP markers can be used as specific markers to differentiate large and small individuals of sowang giant gourami fingerling from single spawning.

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