Short Paper

Isolation of Microsatellite Loci from Japanese Flounder *Paralichthys olivaceus* and Detection of PCR Fragments with Simple Non-RI Methods

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In recent years, Japanese flounder *Paralichthys olivaceus* has been artificially propagated and released into many of the coastal waters of Japan for stock enhancement. It is thus necessary to investigate the genetic diversity of the wild population and the hatchery stock in relation to the genetic conservation of the wild population. Microsatellites have been shown to be highly polymorphic in nature, and their accuracy for assaying has made them the best of choice for high resolution population analysis.¹⁾ However, the difficulty of the method of detection of PCR amplified fragments with radioisotope (RI), automated-DNA sequencer and chemiluminescent has prevented the microsatellite from being widely used for fish population and breeding studies.

In the present study, we report the isolation and characterization of five microsatellite loci from the Japanese flounder and examine the detection of PCR fragments with formal Non-RI methods (Ethidium bromide and Silver staining) for convenient routine study.

Samples of the Japanese flounder were collected from Nagasaki Prefecture, and DNA samples were extracted from whole blood and fin clip by standard SDS-phenol/chloroform procedures²⁾ and stored at 4°C prior to PCR

analysis.

Primers design to hybridize with unique regions flanking the core sequences of the microsatellites, PCR, PAG electrophoresis, and detection bands with RI were carried out using the methods described by Takagi *et al.*³⁾ Alleles were sized relative to a M13 sequence ladder.⁴⁾ Then, PCR amplified fragments of simple Non-RI methods were electrophoresed with 20 bp molecular markers (Takara Inc.), using long gel (e.g. sequence electrophoresis chamber). The electrophoresis carried on non-denaturized polyacrylamide gel (1×TBE, 7% glycerol, 8% polyacrylamide) at 10 V/cm for 16 hrs. in 1×TBE buffer (0.1 M Tris, 0.1 M boric acid, 0.002 M EDTA, pH 8.3). DNA fragments in the gels were stained by standard protocol of ethidium bromide⁵⁾ (Et.Br.) and photographed on a UV transilluminator. Silver staining was performed according to manufacture's protocol (Wako Inc.).

Primer sequences, annealing temperature, number of alleles, amplified product size range, and heterozygosities for the isolated 5 loci are summarized in Table 1. The 5 microsatellite loci were all highly polymorphic, while the degree of variability was different, at each locus. *Pol-2** had the lowest number of alleles (6) and heterozygosity

Table 1. Primer sequence, allele size, heterozygosity of Japanese flounder microsatellite loci detected by PCR amplification

Locus	motif ^{*a}	Primer sequence (5'-3') ^{*b}	Annealing temp. ^{*c}	No. of samples	No. of alleles	Size (bp) ^{*d}	Heterozygosity (He) ^{*e}
<i>Pol-1*</i>	(GT)	F: TAGGAGGATTGATAGGAT R: CCCGACTTTTAGATGTTC	53	38	36	143–245	0.952
<i>Pol-2*</i>	(GT)	F: AGGATTTAGCCAAGGTCA R: ACATCAAGCCAAGAGAAG	53	40	6	110–124	0.689
<i>Pol-3*</i>	(GT)	F: TTTGTGTTGTAGTTAGTCTG R: GTTGATTGTAGAGTGGTGTG	50	38	22	116–178	0.910
<i>Pol-4*</i>	(GT)	F: GTGTGCTCTGGGCTTTTG R: TTACCTGTCCTGTACTC	50	39	28	91–197	0.935
<i>Pol-5*</i>	(CA)	F: AACTGCTCCTTGAAGTGC R: GGAAGCATCGACTCTCAT	53	39	21	109–175	0.888

^{*a}=Core repeat motif from cloned *Paralichthys olivaceus* sequence. ^{*b}=F and R refer to forward and reverse primer sequence respectively. ^{*c}=PCR annealing temperatures were optimized for *Paralichthys olivaceus*. ^{*d}=Estimated size of the PCR fragment when compared with M13 sequence fragments of known length. ^{*e}=Estimated heterozygosity.

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Table 2. Allele frequencies of five microsatellite loci of Japanese flounder, *Paralichthys olivaceus* from Nagasaki Prefecture

Allele	<i>Pol-1*</i>	Allele	<i>Pol-2*</i>	Allele	<i>Pol-3*</i>	Allele	<i>Pol-4*</i>	Allele	<i>Pol-5*</i>
143	0.026	110	0.350	116	0.013	92	0.013	109	0.013
145	0.026	112	0.400	122	0.026	118	0.026	121	0.244
147	0.013	114	0.075	128	0.026	122	0.026	123	0.128
149	0.026	116	0.150	130	0.171	124	0.013	125	0.051
151	0.105	122	0.125	132	0.132	126	0.026	127	0.103
153	0.013	124	0.125	134	0.079	128	0.026	129	0.077
155	0.040			140	0.066	132	0.026	131	0.013
157	0.026			142	0.118	134	0.180	133	0.103
159	0.066			146	0.079	138	0.013	135	0.026
161	0.026			148	0.040	140	0.026	139	0.026
163	0.040			150	0.013	142	0.051	141	0.026
165	0.013			152	0.026	144	0.013	145	0.013
167	0.066			154	0.040	148	0.064	147	0.026
169	0.026			156	0.013	152	0.051	151	0.013
171	0.053			158	0.026	154	0.064	153	0.013
173	0.053			160	0.056	156	0.051	155	0.013
175	0.013			162	0.013	158	0.064	157	0.026
177	0.013			164	0.013	160	0.039	163	0.013
179	0.053			170	0.013	162	0.026	167	0.026
181	0.026			172	0.013	164	0.026	171	0.039
183	0.013			178	0.013	166	0.026	175	0.013
185	0.238			184	0.013	168	0.147		
{				{					
245						198			

(0.689) of the five loci. *Pol-1**, *Pol-3**, *Pol-4** and *Pol-5** were highly polymorphic in both allele number and heterozygosity, as observed in the total number of alleles per locus (21–36), and a high degree of heterozygosity ranging from 0.888 to 0.952. Allele frequencies of the five microsatellites for samples from Nagasaki Prefecture are shown in Table 2. At locus *Pol-2**, the frequencies of major alleles 110 and 112 were 0.350 and 0.400. At loci *Pol-1** and *Pol-4**, allele frequencies were overall low with the most common allele only having frequency under 0.100. At loci *Pol-3** and *Pol-5**, were somewhat intermediate with the major allele having a frequency at 0.171 and 0.244. These microsatellites will be used widely in further population analysis and monitoring genetic change of brood stocks in fisheries and aquaculture due to their highly polymorphic nature.

Figure 1 shows the Microsatellite electropherogram obtained using the primer Pol 2 locus with RI labeled, Et.Br. and Silver staining methods. Allele identification was easy for each of the alleles detected every 2 bp between 20 bp molecular markers according to Et.Br. and Silver staining methods. Et.Br. and Silver staining methods were compared with band patterns and allele size of RI method. When using the Et.Br. method, density of homozygous bands were more distinct than that of the heterozygous bands. Furthermore, the silver staining method showed higher resolution than the Et.Br. method. As a result of the high resolution of the silver staining method, shadow bands were detected. Moreover, real bands were very distinct, so misreading could be avoided in allele identification. These results suggest that the Et.Br. and the silver staining methods are not only useful in detection of microsatellite PCR fragment under Non-RI conditions for high resolution but also economical reasons.

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(A) Radio Isotope

(B) Etidium Bromide

(C) Silver Stain

Fig. 1. Banding pattern of electropherogram of *Pol-2** microsatellite locus.

(A) Radioisotope detection. The size standard is a sequencing ruler of M13 mp18. (B) Ethidium bromide staining. (C) Silver staining. The size standard of (B) and (C) are 20 bp molecular markers.

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