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Development of novel polymorphic microsatellite markers in *Siganus fuscescens*

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ABSTRACT. Rabbitfish, *Siganus fuscescens*, is widely distributed in the Indo-Pacific regions and eastern Mediterranean. Its dwelling place includes reef flats, coral reef regions, and seagrass meadows in tropical area and reef areas or shallow waters in locations at high latitudes. In the present study, 10 new polymorphic microsatellite markers were screened from 30 wild *S. fuscescens* individuals, using a method of fast isolation protocol and amplified fragment length polymorphism of sequences containing repeats. The number of polymorphic alleles per locus was 3 to 5 with a mean of 4.3, while the value of polymorphic information content ranged from 0.283 to 0.680. The values of the observed and expected heterozygosities were in the range 0.3333-0.8462 and 0.3011-0.7424, respectively. Deviation from Hardy-Weinberg equilibrium was not observed in this study. These polymorphic loci are expected to be effective in evaluating the genetic diversity, population structure, and

gene flow and in determining the paternity in *S. fuscescens*, as well as for conservation management.

Key words: *Siganus fuscescens*; Microsatellites; Genetic markers; FIASCO

INTRODUCTION

The mottled spinefoot, *Siganus fuscescens*, classified in the order Perciformes, family Siganidae (rabbitfishes), genus *Siganus*, is characterized by having three soft rays between their inner and outer spine, and seven anal fin spines. Its body is olive green or brown with a silver belly and small spots. The fish displays a mottled color and projects its venomous spines, when frightened. Therefore, it has different names, such as dusky or sandy spinefoot in the United States, dusky rabbitfish in Australia, and *barangen* in Philippines. *S. fuscescens* is widely distributed in the Indo-Pacific and eastern Mediterranean region, from Korea to Australia. The habitat of *S. fuscescens*, an omnivorous fish, ranges from reef flats, coral reef regions, and seagrass meadows in the tropical area to reef areas or shallow waters in high latitude regions. *S. fuscescens* is a species with high economic value. It is a popular mariculture species (Bryan and Madraisau, 1977) with a good taste, is a high protein fodder, and can be harvest throughout the year (Alcala, 1979). As a result, its production increased sharply, causing a situation of overfishing. To counter this urgent situation, appropriate measures are needed to protect and make sustainable development of *S. fuscescens*.

Microsatellite DNA or simple sequence repeats (SSRs) are tandem repeats ranging from 1 to 6 nucleotides, found at a high-frequency in the nuclear genomes. Different individuals exhibit variations in these, which are manifested as repeat number differences (Guichoux et al., 2011), high-mutation rates of SSRs (Selkoe and Toonen, 2006), and mutational mechanisms (Li et al., 2002) resulting in the appearance of length polymorphisms. Microsatellite DNA has an advantage over other morphological markers as it reveals co-dominantly inherited multi-allelic products of the loci. Moreover, it is free from the consequences of epistasis and other issues because of environmental conditions. In the study of population genetics, population differentiation, and evolution, SSRs are considered to be effective tools for genetic conservation. In the present study, 10 microsatellite markers were screened to obtain information useful for population genetic studies and protection of species resources. Microsatellite markers have already been used as a genetic tool for population studies in *S. fuscescens* (Li et al., 2013; Ning et al., 2015).

MATERIAL AND METHODS

Fish sample collection and DNA extraction

Wild *S. fuscescens* (30 individuals) were captured in Xiamen, China and preserved in alcohol at -20°C. Genomic DNA was extracted from single individuals using the standard phenol-chloroform extraction method (Sambrook et al., 2000). Concentration of the extracted DNA was estimated by electrophoresis on a 1% agarose gel and detection using an ultraviolet spectrophotometer. The DNA sample was finally stored at -20°C.

Microsatellite-enriched library construction

Microsatellite-enriched library of *S. fuscescens* was constructed by the fast isolation by amplified fragment length polymorphism of sequences containing repeats (FIASCO) protocol (Zane et al., 2002). The DNA samples were digested with the restriction endonuclease FastDigest *Tru*1I at 65°C for 10 min. Blunt-ended fragments ranging from 500 to 1000 bp were selected and ligated to *Mse*I adapter A (5'-ACGATGAGTCCTGAG-3')/*Mse*I adapter B (5'-TACTCAGGACTCAT-3') using T4 DNA ligase (Fermentas, Vilnius, Lithuania) at 37°C for 3.5 h. The product was denatured at 95°C for 10 min and enriched for microsatellite repeats by hybridization to the biotin-labeled oligonucleotide probes $(CT)_{15}$ and $(GT)_{15}$ at 61°C for 1 h. The linker-ligated fragments were captured via streptavidin-coated magnetic beads (Promega, Madison, WI, USA). Enriched single stranded repeats were amplified by polymerase chain reaction (PCR) using *Mse*I adapter A. Subsequently, the isolated fragments with potential SSRs were ligated into PMD19-T vector (Takara, Shiga, Japan) at 16°C for 3.5 h and transformed into One Shot chemically competent *Escherichia coli* DH5α cells (Tiangen, Beijing, China). The cells were then plated on Luria-Bertani agar plates containing 60 mg/mL ampicillin and grown overnight at 37°C. The recombinant clones were detected by PCR amplification using universal M13 primers and the PCR products were visualized on 1% agarose gels. A total of 150 positive clones with fragments ranging from 400 to 1000 bp were chosen, for further sequencing by Life Technologies (Carlsbad, CA, USA).

Primer design and polymorphism test

A total of 107 positive clones containing the SSRs were obtained by using the software SSRhunter1.3 (Li and Wan, 2005); these clones were sufficient to provide for the needs of the present study. Meanwhile, 68 pairs of microsatellite amplification primers were designed through the Primer Premier version 5.0 software (Clarke and Gorley, 2001). To test for polymorphism, 30 genomic DNA samples, extracted earlier from the 30 wild *S. fuscescens* individuals, were used for optimizing the amplification conditions for each primer pair; 33 primer pairs were successfully selected for testing by amplification of genomic DNA. Subsequently, individual loci were amplified in 10-μL reactions containing 50 ng/mL genomic DNA templates, 10X Taq buffer, 0.4 M each primer, 0.2 mM each dNTPs, and 0.25 U Taq DNA polymerase (Fermentas). Cycling parameters were set as follows: denaturation at 94°C for 5 min, followed by 30 cycles at 94°C for 40 s, a primer-specific annealing temperature (Table 1) for 40 s, and extension at 72°C for 60 s. This was followed by a final extension at 72°C for 10 min. The PCR products were electrophoresed on 6% denaturing polyacrylamide gels using a Sequi-Gen Sequencing Cell system (Bio-Rad, Hercules, CA, USA) and visualized through silver staining.

Data analysis

Allele size were scored according to the 10-bp DNA ladder (Fermentas), while estimation of allelic diversity, tests for zygotic equilibrium (Hardy-Weinberg), and linkage disequilibrium (LD) as well as the observed and expected heterozygosities were conducted using POPGEN 32 (version 1.32; Yeh et al., 2000). Furthermore, CERVUS 3.0 (version 3.0) was used to calculate the polymorphic information.

Table 1. Characterization of 10 microsatellite primers in Siganus fuscescens (N = 30 individuals).

GenBank accession No.	Locus ID	Primer sequences (5'-3')	Repeat motif	Allele size (bp)	Ta (°C)	N_A	PIC	H_O	H_E
KT367794	HLZY-3	F: TAACGGTTCTATCAGGG	$(TC)_5C\ (CT)_7$	200-240	55	4	0.483	0.4333	0.5701
		R: TGCTTCGGATTCAGG							
KT367795	HLZY-5	F: TTCATCACTGCCTGTCCTT	$(CTT)_3$	260-300	62	4	0.283	0.3333	0.3011
		R: AGCGTGTCATTGTTGGGT							
KT367796	HLZY-7	F: CCCATAGATACAGAGGAAGGA	$(GT)_4...(GA)_4GG(GA)_{10}...(TG)_{16}$	300-330	62	4	0.646	0.4815	0.6450
		R: GGCAGAAACCAGCAGAAC							
KT367797	HLZY-12	F: CGGACCTGCTTGAGT	$(AGC)_3$	240-260	47	5	0.670	0.5926	0.6730
		R: AACCTGTTTGGTGGAA							
KT367798	HLZY-13	F: TGGCAGTGATGAGGTG	$(ATT)_3$	200-240	57	5	0.671	0.7500	0.6994
		R: CATTTGGAATCGCAGT							
KT367799	HLZY-18	F: CCGTCTAAACATTGGC	$(CAA)_8...(CAA)_4...(CAA)_3$	300-340	60	4	0.680	0.8000	0.7424
		R: ACCTCCCTGTCGTTCT							
KT367800	HLZY-21	F: CTGACTCCCAACTTCC	$(CTT)_3(CTG)_5$	290-320	52	5	0.542	0.3667	0.6124
		R: CAGACCTGTTTCCAATC							
KT367801	HLZY-24	F: GCTGGTAAAGGTGGTGG	$(CTG)_3$	150-180	62	3	0.356	0.4333	0.4062
		R: GTGATAGTGATGCCTCTGG							
KT367802	HLZY-29	F: CTGCCAAATCTCGTT	$(AC)_{41}...(TG)_4$	170-190	54	5	0.628	0.8462	0.6146
		R: TTTGCTTTCCACCTT							
KT367803	HLZY-100	F: ACCGCACCTGACACTCT	$(CTTGTC)_3...(CT)_5$	200-240	62	4	0.382	0.4667	0.4186
		R: ACGGGAACATCACCATC							

Ta = annealing temperature; NA = number of polymorphic alleles per locus; PIC = polymorphic information content; HO = observed heterozygosity; HE = expected heterozygosity.

RESULTS AND DISCUSSION

Characterizations of the 10 polymorphic loci are shown in Table 1. Error analysis was carried out using MICRO-CHECKER (Van et al., 2004). None of the locus exhibited significant departure from the Hardy-Weinberg expectations ($P > 0.005$). The number of polymorphic alleles per locus was 3-5, with a mean of 4.3. The value of polymorphic information content (PIC) ranged from 0.283 to 0.680, which is commonly used in genetics as a measure of polymorphism for a marker locus used in linkage analysis. Four microsatellite loci appeared to be intermediately polymorphic ($0.25 < PIC < 0.5$) and the rest were considered to be highly polymorphic ($PIC > 0.5$; Botstein et al., 1980). The values of the observed heterozygosities (H_O) were from 0.3333 to 0.8462, with an average 0.5504, whereas the range of the expected heterozygosities (H_E) was from 0.3011 to 0.7424, with an average of 0.5683.

DISCUSSION

Among the 107 positive clones, more than 90% were dinucleotide repeats, especially $(CT)_n$, although $(GT)_n$ had an advantage in terms of the number of repeats, which ranged from 4 to 84. Moreover, 71.3% positive clones contained the SSRs, 66% could be designed for microsatellite amplification primers, and only 10 of the 68 pairs (14.7%) could be amplified to polymorphic loci by PCR amplification, without the effects of null allele, stutter bands, short allele dominance, and allelic dropout, caused on genotyping of microsatellite. This demonstrates that enrichment was highly effective.

The value of H_O higher than H_E revealed the high genetic diversity of this population. All the 10 characteristics of the microsatellite loci indicated their potential utility in examining genetic diversity, variability, and demographic connectivity among the highly exploited *S. fuscescens* populations.

Conflicts of interest

The authors declare no conflict of interest.

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