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Discovery and characterization of a first set of polymorphic microsatellite markers in *Siganus oramin*

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ABSTRACT. Nine microsatellite DNA markers were developed and characterized for *Siganus oramin* by the 5'-anchored polymerase chain reaction technique. A total of 42 alleles were identified in 30 individuals, and the number of alleles per locus ranged from 3 to 7, with an average of 4.7. The observed and expected heterozygosity per locus ranged from 0.5333 to 1.0000 and from 0.5254 to 0.8474, respectively, with an average of 0.7422 and 0.6906, respectively. A significant deviation from the Hardy-Weinberg equilibrium was detected at one microsatellite locus after a Bonferroni's correction ($P < 0.0056$). No significant linkage disequilibrium was found between any of the pairs of the nine loci. The microsatellite loci developed in this study will improve our understanding of the genetic background of *S. oramin*.

Key words: *Siganus oramin*; Microsatellite marker; Polymorphism

INTRODUCTION

The rabbitfish (*Siganus oramin*) is a commercially important fish species, because of its great value as a food resource and in ecological conservation (Zhuang et al., 2008; Fang et al., 2009). It is widely distributed in the coral reefs of the Indo-Pacific region (Du et al., 2008), and the last few decades have seen a significant growth in rabbitfish cultivation in several regions (Lam, 1974). Previous studies on this species have mainly focused on its breeding biology, nutritional evaluation, and ecological monitoring (Wassef and Abdul Hady, 1997; Lai et al., 1999; Zhuang et al., 2008; Fang et al., 2009), but studies on the population genetic diversity and variation of this species are few in number.

Microsatellite markers, also known as simple sequence repeats (SSRs), are co-dominantly inherited, multi-allelic, highly informative, and abundantly distributed throughout the whole genome (Tautz and Renz, 1984; Tautz, 1989; Wright, 1993). Microsatellite markers have consequently become the favored marker in the last few years for genetic studies in fish (O'Connell and Wright, 1997); however, few microsatellite markers have been reported for this important fish species, which has hindered the evaluation of its population genetic structure.

Several strategies have been devised for microsatellite marker isolation (Zane et al., 2002), amongst which the 5'-anchored polymerase chain reaction (PCR) technique is considered to be a rapid and economical protocol (Fisher et al., 1996). In this study, we developed nine polymorphic microsatellite markers from the genomic DNA of *S. oramin*, based on the 5'-anchored PCR method. This set of microsatellite markers will facilitate studies on the genetic differentiation and structure of *S. oramin* and its closely related species.

MATERIAL AND METHODS

Sample collection and DNA extraction

A total of 30 individuals of *S. oramin* were collected from Fujian Province, China. Genomic DNA was isolated from muscle tissue using a traditional phenol-chloroform extraction protocol, as described by Ma et al. (2010a).

5'-anchored primer design and isolation of microsatellite markers

Three 5'-anchored primers with the sequences KKDBDBD$(AC)_6$, KKVRVRV$(CT)_6$, and KKRVRVR$(GT)_6$ were designed for microsatellite isolation, where *K* = G/T, *D* = G/A/T, *B* = G/T/C, *H* = A/C/T, *V* = A/C/G, and *R* = A/G. The primers were synthesized by Sangon Biotech Co. Ltd. (Shanghai, China).

PCR amplification was performed in a 25-µL reaction volume containing 10X PCR buffer, 2.0 mM $MgCl_2$, 0.2 mM dNTP mix, 0.2 µM of each primer, 1 U *Taq* DNA polymerase, and approximately 100 ng template DNA. The thermocycler conditions were as follows: an initial denaturation for 5 min at 94°C, followed by 35 cycles of 45 s at 94°C, 45 s at annealing temperature, and 45 s at 72°C, and a final extra extension at 72°C for 5 min. The amplification products were separated on 1.5% agarose gels (TaKaRa, China). The amplified DNA fragments ranged from 200 to 750 bp in size, and were reclaimed and ligated with a pMD19-T vector (TaKaRa). They were then transformed into *Escherichia coli* DH5a cells (Tiangen Biotech Co. Ltd., China). The positive clones were identified by PCR with vector-specific primers, before being selected for sequencing using an ABI 3730xl sequencer (Applied Biosystems).

The sequences were searched for microsatellites using the SSRHUNTER software (version1.3) (Li and Wan, 2005), and the microsatellite primers were designed using the Primer Premier 5.0 software (http://www.premierbiosoft.com/primerdesign/).

Polymorphism characteristics and assessment

Microsatellite loci polymorphisms were investigated in 30 individuals of *S. oramin*. PCR amplification was performed in a 25-μL total volume containing 10X PCR buffer, 0.4 μM of each primer, 0.2 mM dNTP mix, 1 U *Taq* polymerase (TaKaRa), and approximately 100 ng template DNA. After 5 min denaturation at 94°C, amplification proceeded for 35 cycles (94°C for 30 s, annealing temperature for each pair of primers (Table 1) for 40 s, and 72°C for 45 s), and a final extension for 5 min at 72°C. The PCR products were separated on a 6% denaturing polyacrylamide gel and visualized by silver staining. The ranges of allele size were determined by referring to the pBR322/*Msp*I marker (Tiangen Biotech). All of the methods used for the data analysis were as described by Ma et al. (2010b).

Table 1. Locus name, repeat sequence, primer sequence, annealing temperature (Ta), number of observed alleles (N_A), locus size, observed (H_O) and expected heterozygosity (H_E), and GenBank accession No. for nine polymorphic microsatellite loci in *Siganus oramin*.

Locus (GenBank accession No.)	Repeat motif	Primer sequence (5'-3')	Ta (°C)	N_A	Expected size (bp)	H_O	H_E
Sigo1 (KM246735) (KJ655521-KJ655529)	$(CA)_{19}$	F: ACTGTGAGGAAGCCTTGGTTAA R: TGTGCGTCTTTGTGTATGTTGT	53	5	124	0.8000	0.7972
Sigo2 (KM246736)	$(TG)_9$	F: ACAAGAAGGTTAGAAAGACGGC R: TTAAAGGCAAATGTTTGGAAAT	48	4	222	0.6000	0.5254
Sigo3 (KM246737)	$(TG)_{16}$	F: TGTGCGTCTTTGTGTATGTTGT R: ACTGTGAGGAAGCCTTGGTTAA	53	7	124	0.9667	0.8379
Sigo4 (KM246738)	$(CA)_{17}$	F: ACTGTGAGGAAGCCTTGGTTAA R: TGTGCGTCTTTGTGTATGTTGT	53	6	120	1.0000*	0.8474
Sigo5 (KM246739)	$(TCC)_6$	F: GCAGAATGCCTTCGCCCCTC R: CCGTGCTTCGCCACAGTAACA	59	5	235	0.6786	0.7552
Sigo6 (KM246740)	$(CTG)_9$	F: TAAAAGCATAGGAAAGAAAAAGA R: CCAAGTCACGACAAGACAAAATA	49	5	189	0.5333	0.6299
Sigo7 (KM246741)	$(CT)_7CC(CT)_4$	F: TCTCGCCTTGTTTCCCTGTTC R: TGTCTGCACCCTGTTGCATGT	55	3	115	0.8667	0.6198
Sigo8 (KM246742)	$(CA)_{37}$	F: CCCTCTCTTTAACATACCCACA R: GACCACATCTTTCCAGCTCTAC	55	3	222	0.5556	0.5737
Sigo9 (KM246743)	$(AC)_8$	F: TGTCCTACCTCGTATTCTTGGC R: CTTCACTGACCGTTTTCTGTT	54	4	164	0.6786	0.6292

*Significant deviation from the Hardy-Weinberg equilibrium after a Bonferroni's correction ($P < 0.0056$).

Data analysis

The observed number of alleles (N_A), observed heterozygosity (H_O), expected heterozygosity (H_E), polymorphism information content, chi-square tests for the Hardy-Weinberg equilibrium (HWE), and the linkage disequilibrium (LD) were calculated using POPGENE (version 1.31) (Yeh et al., 1997). Significance values for all multiple tests were corrected through a sequential Bonferroni procedure (Rice, 1989). MICRO-CHECKER was used to evaluate genotyping errors, the presence of null alleles, and allelic dropout (Van Oosterhout et al., 2004).

RESULTS AND DISCUSSION

High variability and relative ease of scoring are the two main features that make

microsatellites very powerful markers for genetic studies. However, a major disadvantage of microsatellites is the high cost of their development. In this study, the discovery process was relatively easy, since the genomic background was not required. In addition, the screening effort was greatly reduced by the construction of genomic libraries enriched for $(AC)_6$, $(CT)_6$, and $(GT)_6$ repeats. PCR profiles from genomic DNA with primers containing microsatellite repeats were employed, in order to capture the polymorphisms associated with microsatellite loci. This strategy is highly suitable for non-model species whose genomic information is frequently unknown. A sequencing analysis of 113 clones from the genomic libraries yielded 82 clones that contained microsatellite marker loci. Based on sequences with sufficient flanking regions, 31 pairs of primers were successfully designed using the Primer Premier 5.0 software. The polymorphisms of these loci were assessed using 30 individuals of *S. oramin* from Fujian Province, China.

As a result, nine pairs of primers amplified specific and polymorphic PCR products, and a total of 42 alleles were identified in the 30 individuals. N_A per locus ranged from 3 to 7, with an average of 4.7. The H_O and H_E per locus ranged from 0.5333 to 1.0000 and from 0.5254 to 0.8474, respectively, with an average of 0.7422 and 0.6906, respectively. One microsatellite locus (Sigo4) significantly deviated from the HWE after a Bonferroni's correction ($P < 0.0056$), which may have been due to the small sample size or the presence of null alleles. MICRO-CHECKER analysis showed no evidence of scoring errors, or technical or statistical artifacts. There was no significant genotypic LD between any of the pairs of the nine loci after a Bonferroni's correction ($P > 0.0056$). These nine sequences were further searched in GenBank using the BLASTn program, and similar sequences were not found. The characteristics of these primers are listed in Table 1.

In conclusion, nine polymorphic microsatellite markers were developed for *S. oramin* using the 5′-anchored PCR technique. These microsatellite primers are the first batch to be published for *S. oramin*, and will provide a valuable tool for studies on the genetic differentiation, structure, and effective management of *S. oramin*.

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