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Short Communication

Development and characterization of microsatellite markers for the walking goby (*Scartelaos viridis*; Gobiidae)

D.Q. Sun, H.Y. Li, T.J. Xu and R.X. Wang

Key Laboratory for Marine Living Resources and Molecular Engineering, College of Marine Science, Zhejiang Ocean University, Zhoushan, P.R. China

Corresponding author: R.X. Wang
E-mail: wangrixin1123@126.com

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ABSTRACT. *Scartelaos viridis* (walking goby) is a small edible fish that inhabits warm inshore environments. To provide molecular information of *S. viridis*, we developed and characterized microsatellite markers for this species. Using $(CA)_{15}$-enriched genomic libraries of *Scartelaos viridis*, 44 positive clones were sequenced; 34 sequences contained multiple repeat motifs (di-, tri- and tetra-nucleotide). In all, 23 primer pairs were designed and 15 were successfully amplified. Forty-two *S. viridis* individuals collected from the East China Sea were used to characterize the polymorphism at each locus. Three loci (13%) were polymorphic, with three to six alleles. The observed and expected heterozygosity ranged from 0.1000 to 0.4500 and from 0.4487 to 0.7580, respectively. The polymorphism information content per locus ranged from 0.4214 to 0.7510. Three loci significantly deviated from the Hardy-Weinberg equilibrium (adjusted P value = 0.017); the pairwise tests for linkage disequilibrium between Scvi-1-13 and Scvi-2-11 were significant ($P < 0.05$, adjusted P value = 0.017). The low number of polymorphic microsatellite loci may be due to the close genetic relationship of the individuals that we collected and the large size of the motifs.

Key words: *Scartelaos viridis*; Microsatellite; Molecular marker

INTRODUCTION

Microsatellites, also known as simple sequence repeats (SSRs), are regions of DNA that exhibit short repetitive sequence motifs. These motifs are often composed of 1-6 bp repeats and high polymorphism occurs for different repeats. As microsatellites have some unique characteristics, such as co-dominance of alleles, high allelic diversity, and the relatively simple polymerase chain reaction (PCR)-based screening methods that are reproducible, they have been widely employed in recent years in population genetic studies of numerous species and such application is continuously expanding (Sekino and Hara, 2001; Kohlmann et al., 2005; Mia et al., 2005; Selkoe and Toonen, 2006; Liu et al., 2009). At the same time, microsatellites have been successfully used in genetic mapping and genome analysis (Chen et al., 1997; Li et al., 2000), genotype identification, variety protection seed purity evaluation (Senior et al., 1998), germplasm conservation (Brown et al., 1996), and marker assisted breeding (Weising et al., 1997).

As the microsatellite marker technique is based on specific amplification, the genome DNA of species or a part of the sequence containing microsatellite DNA sequence should be obtained first. Primers are then designed for PCR amplification. At present, microsatellite sequences have been isolated mainly in the following ways: genomic sequencing, and enrichment of the microsatellite separation method. The latter method is widely employed because of its high efficiency of isolation and time savings (Kandpal et al., 1994; Brown et al., 1995).

Scartelaos viridis (walking goby) is a species of small fish that inhabits warm inshore environments around the oceans of China. In recent decades, the walking goby has become popular in some cities in the south of China for its taste and nutrition. The objectives of this study were to design microsatellite primers based on amplified fragment length polymorphism (AFLP) of sequences containing repeats (FIASCO) method (Zane et al., 2002), and to develop and analyze the genetic characterization of microsatellite loci for *S. viridis*.

MATERIAL AND METHODS

DNA extraction and enrichment for microsatellites

Forty-two *S. viridis* individuals were collected from the Zhoushan fishing ground of the East Sea (30°N, 122°E). Total genomic DNA was isolated from the fin clips using the standard phenol-chloroform method with some modification, which was subsequently dissolved in 100 µL TE buffer and then was determined on a 1% agarose gel stained with ethidium bromide using known molecular weight standards. DNA was stored at -20°C. An enriched partial genomic library for the repeat motif $(CA)_{15}$ was constructed essentially using a DNA pool from two individuals and following the modified FIASCO protocol.

Total genomic DNA pool was simultaneously digested with the *Mse*I restriction enzyme (MBI). DNA fragments ranging from 250 to 1000 bp were isolated from a 1.5% agarose gel and purified using the QIAquick Gel Extraction Kit (Qiagen). These fragments were ligated to *Mse*I AFLP adapters OligoA (5'-TAC TCA GGA CTC AT-3') and OligoB (5'-GAC GAT GAG TCC TGA G-3') and then amplified by PCR using adaptor-specific primers (*Mse*I-N: 5'-GATGAGTCCTGAGTAAN-3'). PCR conditions were: 1X *Taq* DNA polymerase buffer, 1.0-1.5 mM $MgCl_2$, 120 ng primer *Mse*I-N, 200 µM each dNTPs, 1.0 U

Taq DNA polymerase (Tiangen) and 5 µL of a 1/10 dilution of digested-ligation DNA. PCR was performed in a volume of 25 µL following the program: 94°C for 5 min; 30 cycles at 94°C for 30 s, 53°C for 30 s, and 72°C for 50 s; 72°C for 5 min for final extension. DNA fragments between 300-1000 bp were isolated and purified.

Genomic DNA fragments containing SSRs were captured by hybridization to $(CA)_{15}$ biotin-labeled probes. For enrichment, the adaptor-ligated DNA fragments were denatured at 95°C for 8 min, and then hybridized to biotin-labeled probes in 70 µL hybridization solution at 65°C for half an hour. The DNA hybridized to the probe was separated and captured by streptavidin magnetic beads at room temperature for half an hour. The microsatellites were obtained following nonspecific and specific washing. Amplifying the microsatellite DNA using the same procedure as the former PCR, the products were ligated to pGEM-T vectors (Promega) according to manufacturer instructions and then cloned to TOP10 cells following the standard protocol.

Primer design and PCR amplification

Some of the positive clones, which were screened via PCR with T7/SP6 and $(CA)_{15}$ primers, were sequenced by T7 primer on an ABI 3730 automated sequencer. Primers were designed in the flanking regions of the repeat motifs (repeating size ≥12 bp) using the PRIMER PREMIER 5.0 software. As some factors will affect the specificity of the amplification, the optimal conditions for PCR amplifications for each primer should be determined by adjusting the annealing temperature and/or Mg^{2+} concentration. Then, polymorphism at each locus was determined using 42 individuals. PCR amplifications were carried out in 25-µL volumes containing 2.5 µL 10X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM of the forward and reverse primers, and 1.5 units *Taq* polymerase (Tiangen). Cycling conditions were 94°C for 4 min followed by 30 cycles at 94°C for 40 s, annealing temperature for 30 s, and 72°C for 40 s, followed by 1 cycle of 72°C for 5 min and then holding at 4°C. PCR amplification was performed on an ABI 9700 thermal cycler.

The products of PCR amplifications were determined on 1.5% agarose gel and then were denatured at 96°C for 8 min using denaturant (98% formamide, 10 mM 0.25% EDTA, 0.25% FF). The denatured amplified products were separated on 6% denaturing polyacrylamide (19:1 acrylamide:bis-acrylamide) gels using silver staining. A denatured pBR322 DNA/*Msp*I molecular weight marker (Tiangen) was used as a size standard to identify alleles (Xu et al., 2009).

Data analysis

The number of alleles, and observed (H_O) and expected (H_E) heterozygosity were estimated using the POPGENE32 software (Yeh and Boyle, 1997), and the ARLEQUIN 3.11 software (Schneider et al., 2000) was used to calculate Hardy-Weinberg equilibrium (HWE) expectations and genotypic linkage disequilibrium. All results for multiple tests were corrected using Bonferroni's correction (Rice, 1989).

RESULTS AND DISCUSSION

From the $(CA)_{15}$-enriched genomic libraries, 44 clones were sequenced successfully and 34 (77.3%) sequences contained sufficient repeat motifs (di-, tri- and tetra-nucleotide). The motif repeats ranged from 3 to 33, and only those repeating more than five times were

regarded as microsatellites. In all, 23 primer pairs were designed as the remaining sequences and were too close to the cloning site to design primers.

Details of the newly developed microsatellite loci are listed in Table 1. Fifteen of the 23 primers were successfully amplified and 3 loci were shown to be polymorphic in *S. viridis*. The variability measures across 42 individuals of walking goby are also summarized in Table 1. Of the 15 pairs amplified primers, 3 polymorphic loci (Scvi-1-13, Scvi-2-11 and Scvi-2-24) showed 3-6 alleles. H_O and H_E ranged from 0.1000 to 0.4500 and from 0.4487 to 0.7580, respectively. The polymorphism information content (PIC) per locus ranged from 0.4214 to 0.7510, of which Scvi-1-13 was middle polymorphic ($0.25 < PIC < 0.5$), while the other two loci were highly polymorphic ($PIC > 0.5$). HWE probability tests showed all three loci significantly deviated from HWE ($P < 0.05$, adjusted $P = 0.017$). And strong deviations from HWE ($P < 0.001$, adjusted $P = 0.0003$) were observed in Scvi-1-13, possibly due to the presence of null alleles. According to the pairwise tests for linkage disequilibrium, only one pairwise test (Scvi-1-13 and Scvi-2-11) was significant ($P < 0.05$, adjusted $P = 0.017$).

Table 1. Characterization of 15 microsatellite loci of *Scartelaos viridis* developed from an enriched genomic library.

Locus	Motif	Type*	Primer sequence (5'-3')	Size (bp)	Tm (°C)	Mg^{2+} (mM)	N_A	H_O H_E	PIC	Accession No.
Scvi-1-8	$(AG)_{12}(TG)_8$	C	CGGCCGCGGGAATTCGAT TGACCCTGGCCCTGCACA	197	56	1.5	1			HQ128584
Scvi-1-10	$(CGTG(TG)_4)_2$ $(CGTG)_4$	C	ATGAAGCAACGGACCCTC AGCGCTACGTCAAACTGATA	241	54	1.5	1			HQ128585
Scvi-1-13	$(GAG)_5$	P	GTCTGGGCTGTATCTCATT CTAGCGTCATAAACACCAA	144-156	50	1.5	4	0.1000 0.4487**	0.4214	HQ128586
Scvi-2-4	$(GA)_6$	P	TCTCATACAGTGCCAGTT TCACTACTCTGCAAATACAC	128	48	1.5	1			HQ128587
Scvi-2-10	$(GAC(AG)_4)_5$	C	ACGAACATTTAAGACCAG TTCCAAACAGGAAGTGAT	188	50	1.5	1			HQ128588
Scvi-2-11	$(TC)_9$	P	CACCCACCTGTTGGTACTATTT TCAAACGCACTCCGATGA	244-254	52	1.5	6	0.4500 0.7580**	0.7510	HQ128589
Scvi-2-12	$(AG)_{18}(TG)_{24}$	C	CAACGGACTCAGGAAAGG TGTCACGAGGACGCAGAA	216	52	1.5	1			HQ128590
Scvi-2-20	$(TG)_{10}$ $TACG(TG)_8$	I	GCTACAAGGCTCTACAGG GCTAGTTTGACATGACCC	131	43	1.5	1			HQ128591
Scvi-2-24	$(TG)_{19}$	P	GGATTCACAGGAAAAGGA CAAACCAGAAGAAGACCC	262-266	54	1.5	3	0.2778 0.6373**	0.5602	HQ128592
Scvi-3-17	$(TG)_{15}(TGG)_3$	C	CCATCTCCTCCCTGTGGCT ACCGTAGAGGGCGCTGTTG	245	45	1.5	1			HQ128593
Scvi-3-19	$(TG)_{33}$	P	AACAGAAAGCTGAAACCCAT ATCTTGCAGGCCCAATCC	155	52	1.5	1			HQ128594
Scvi-4-10	$(AG)_{23}(TG)_{26}$	C	AACAATGAAGCAACGGAC AGCGCTACATCAAACTGA	211	50	1.5	1			HQ128595
Scvi-4-17	$(AG)_8$	P	GAGATTGGCGCAAAACAA CCCATGTTCCCCTTTCTA	150	54	1.0	1			HQ128596
Scvi-4-18	$(TG)_{29}$	P	AGGAAAGGATAGAAGAGTC CTGAGCTTGGCTACTAAA	167	43	1.5	1			HQ128597
Scvi-4-20	$(AG)_6$	P	ATTGGGACAAAACAGGAG TCTTTGTCCTTCTCACCC	141	50	1.0	1			HQ128598

*The types P, I and C stand for perfect, imperfect and compound, respectively. Tm = annealing temperature; N_A = number of alleles; PIC = polymorphism information content; H_O = observed heterozygosity and H_E = expected heterozygosity. **Denotes significant deviation from HWE after Bonferroni's correction ($P < 0.016$).

Although the number of sequences surveyed in this study was limited, our results still certainly suggest that the *S. viridis* genome has an abundance of microsatellites (77.3%), but the polymorphism of these loci was low, since only 13% of the loci showed polymorphism.

Maybe the individuals used in this study are too closely related, affecting the test for polymorphism. Besides, the polymorphism per locus is positively related to the motifs repeat size (Weber, 1990; Brandström and Ellegren, 2008). Some microsatellites found in *S. viridis* repeated more than 20 times, which may be too large, limiting the mutation rates.

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