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Isolation and characterization of eight novel microsatellite markers in *Acanthopagrus schlegelii*

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ABSTRACT. *Acanthopagrus schlegelii* is a warm temperate demersal fish, which inhabits the sediment substrate or rocky reefs in shallow seas. As this fish is a nutritionally endowed species with good palatability, it is a highly valuable commercial species for aquaculture and has a long historical standing in Western Pacific countries. Because the population of this fish is currently declining in China, studies and measures aimed at addressing this decline are needed. In this study, eight microsatellite markers were screened from 30 wild *A. schlegelii* fishes through the FIASCO method, whereby sequences containing repeats were obtained from amplified fragment length polymorphisms. The allelic number ranged from 3 to 5, with a mean number of 3.625. The average observed heterozygosity was 0.6290, ranging from 0.3214 to 0.8966, while the expected heterozygosity was 0.5435, ranging from 0.3452 to 0.6721. The value for polymorphism information content ranged from 0.313 to 0.666. These results show this population has moderate genetic

variation and low genetic diversity. These novel polymorphic loci will be useful for future genetic studies of *A. schlegelii*.

Key words: *Acanthopagrus schlegelii*; Genetic markers; Microsatellites; FIASCO

INTRODUCTION

Acanthopagrus schlegelii, also known as black seabream or *Acanthopagrus schlegeli*, belongs to the family Sparidae. It is a common species in the countries of East Asia and Viet Nam (Lindberg and Krasyukova, 1969), and usually lives in brackish water or on rocky reefs. In those countries, *A. schlegelii* is a traditional agriculture fish, especially in China and Japan (Fushimi, 2001). As this fish is noted for its good taste and nutritional content, a radical decrease in its population occurred at the end of the 1970s in Japan, and its population is currently declining in China. To recover the population of *A. schlegelii*, a variety of enhancement and release programs have been adopted in Japan (Kitada and Kishino, 2006) and in some areas of China. Furthermore, improvements in living conditions results in an increased volume of domestic sewage and trade effluent being released into the shallow water, which has a negative effect on the population of this fish (Shahidul Islam and Tanaka, 2004).

Microsatellite DNA markers are also named simple sequence repeats (SSRs) and short tandem repeats (STRs), since the unit of repetition is only 2-6 nucleotides long (Field and Wills, 1996). Compared to other genetic markers, SRTs have an advantage over morphological markers due to their ability to reveal co-dominantly inherited multi-allelic products of loci. Furthermore, they are not influenced by epistatic effects or impacted by the environment (Jarne and Lagoda, 1996). Because STRs are useful tools for use in population genetics research, they have been used to study the genetic structure of populations (Dou et al., 2015; Luo et al., 2015), and as an effective tool for mapping programs, population biologists, and paternity tests. STRs have been used to study the genetics of *A. schlegelii* (Gonzalez et al., 2008; Jeong et al., 2007; Yang et al., 2014). Here, eight novel microsatellite markers are presented, which could provide a powerful tool for the study of population genetics in *A. schlegelii*.

MATERIAL AND METHODS

Fish sample collection and DNA extraction

Thirty *A. schlegelii* individuals were collected from the stock enhancement program in Zhangpu, China, and genomic DNA from the musculature was extracted using a standard proteinase K/phenol-chloroform extraction protocol (Sambrook and Russell, 2000). Electrophoresis, on a 1% agarose and under an ultraviolet spectrophotometer, was used to estimate the concentration of DNA in each sample. The samples were then stored at -20°C until use.

Microsatellite-enriched library construction

Microsatellites of *A. schlegelii* were enriched following the technique, which is using the fast isolation by amplified fragment length polymorphism (AFLP) of sequences containing repeats (FIASCO) protocol (Zane et al. 2002). The restriction enzyme FastDigest

*Mse*I (Fermentas, Vilnius, Lithuania) was first used to cut the DNA (100 ng/μL) at 65°C for 10 min, and then products were bound to *Mse*I adapters by T4 DNA ligase at 22°C for 12 h (Fermentas) (adapter A: 5'-ACGATGAGTCCTGAG-3' and adapter B: 5'-TACTCAGG ACTCAT-3'). Subsequently, the concatenated DNA samples were mixed with the bio-labeled oligonucleotide probes $(CT)_{15}$ and $(GT)_{15}$ following denaturation and reacted at 61°C for 1 h to ensure that the linker-ligated fragments were captured by streptavidin-coated magnetic beads (Promega, Madison, WI, USA). The enriched DNA sequence repeats were amplified by polymerase chain reaction (PCR) with the *Mse*I primer. Afterwards, PCR products were pieced together with the PMD^{19}-T vector (TaKaRa, Shiga, Japan) at 16°C for 12 h. After vector DNA samples were transformed into one-shot chemically competent *Escherichia coli* DH5α cells (Tiangen, Beijing, China), and the cells were incubated at 37°C for 1-2 h and then coating the cells on the Luria-Bertani agar plates for 12-16 h. The results of recombination were verified by PCR amplification with the M13 primer and 1% agarose gel electrophoresis, to allow subsequent sequence measurements to be made.

Primer design and polymorphism test

After sequencing, polymorphic primers were designed on the basis of STRs via Primer Premier 5.0 software (Clarke and Gorley, 2001). Thirty genomic DNA samples were used to confirm the conditions for each primer pair. The total volume of the reaction mix for PCR was 10 μL, and cycling parameters were set according to the following conditions: a) 94°C for 10 min to ensure the DNA was denatured; b) 28-32 cycles for PCR amplification acted as 94°C for 40s, annealing temperature (Table 1) for 40 s, 60s at 72°C, for extension; c) 72°C for 10 min (Ning et al., 2015). Finally, polymorphism was acknowledged by the Sequi-Gen Sequencing Cell machine (Bio-Rad, Hercules, CA, USA) using 6% denaturing polyacrylamide gels and, ultimately, makes it visible through the silver staining.

Table 1. Characterization of eight microsatellite primers in *Acanthopagrus schlegelii* (N = 30).

GenBank accession No.	Locus ID	Primer sequences (5'-3')	Repeat motif	Allele size (bp)	Ta (°C)	N_A	PIC	H_O	H_E
KT898193	HJD-4	TGTTGAAGGTGTGGAGGTG	$(GAG)_8$	220-230	61.2	3	0.440	0.6667	0.5034
		GAATCAGCAGGAGAGGAAAC							
KT898194	HJD-5	CACGAAGGCAGGAAAGAT	$(GGA)_8$	330-360	62.8	4	0.502	0.6071	0.4825
		TTACTACGCTCCCACCAAG							
KT898195	HJD-34	AACACTCAGGGTGAACTGGG	$(AGC)_3(GT)_4$	240-280	62.8	3	0.460	0.7000	0.5237
		CACACACACGCTGCTGCTAT							
KT898196	HJD-73	GCTTCATGTTTCTCGCCCTG	$(GGT)_4$	210-250	62.8	4	0.630	0.8966	0.6721
		ATATCTTGCGTCACCAGTCG							
KT898197	HJD-97	ATAGGTGGATGAACAGGG	$(CT)_{38}$	180-200	45.4	3	0.313	0.4000	0.3452
		AGAGTGATGAGGGCAGAT							
KT898198	HJD-118	GGACGACTTTAGGGACTGG	$(AC)_{20}$	180-200	48.5	3	0.482	0.3214	0.5253
		AGAGCAGAATGGAATGGG							
KT898199	HJD-120	GATGTATTTCTCCTGTCTGCC	$(GA)_5N(GT)_8N(GT)_{11}$	190-210	64.0	4	0.666	0.6400	0.6392
		TCTGTCTCTGTTTCTCCCTCTC							
KT898200	HJD-157	GCTCTCCTTGCTTCCACTC	$(GT)_{19}$	190-220	63.0	5	0.595	0.8000	0.6565
		ATGACGAAGAAGAGGACGAT							

Ta = annealing temperature; N_A = number of polymorphic alleles per locus; PIC = polymorphism information content; H_O = observed heterozygosity; H_E = expected heterozygosity.

Data analysis

Allelic information was obtained by referring to the 10-bp DNA ladder

(Fermentas); additionally, the software POPGEN (version 1.32) was applied to estimate allelic diversity and to test for zygotic equilibrium (Hardy-Weinberg), linkage disequilibrium (LD), observed heterozygosities (H_O) and expected heterozygosities (H_E); and polymorphic information content (PIC) was obtained by CERVUS 3.0 software (version 3.0).

RESULTS

In this study, 30 specimens were used to investigate polymorphisms of *A. schlegelii*. The eight polymorphic loci are characterized in Table 1. MICRO-CHECKER was used to check for errors (Van Oosterhout et al., 2004). All loci conformed to Hardy-Weinberg expectations ($P > 0.005$). The allelic number (NA) varied from 3 to 5 (HJD-157) for each locus, while the allelic size distribution ranged from 180 (HJD-97) to 360 (HJD-5) bp. The PIC value ranged from 0.313 to 0.666, and four of the eight polymorphic loci were shown to be moderately polymorphic ($0.25 < PIC < 0.5$), while the rest were considered to be highly polymorphic ($PIC > 0.5$) (Botstein et al., 1980). Furthermore, the observed heterozygosities (H_O) and expected heterozygosities (H_E) were in the range 0.3214-0.8966 and 0.3452-0.6721, respectively.

DISCUSSION

A total of 71 clones were randomly selected for sequencing among all 168 positive clones tested by M13 primers, and up to 60 of the sequenced DNA clones were found to contain STRs. These data show that FIASCO is a more efficient method of STR enrichment when compared to other methods, as only 36 microsatellites were found from 70 positive clones (Liu, et al., 2007). Furthermore, 40 (66.67%) STRs were imperfect in the present study, whereas 33% were perfect and compound (Weber, 1990).

A survey of the literature confirmed these eight loci to be newly identified and most of these eight loci were perfect loci (Jarne and Lagoda, 1996). The average PIC value in this study was 0.5112, which is almost triple that obtained in a previous study of the same species (Yang et al., 2014). Meanwhile, each value for H_E was much lower than the value for H_O, revealing low genetic diversity of this population.

In summary, these data suggest that the germplasm resources of *A. schlegelii* are declining and future studies should aim to protect the genetic resources of *A. schlegelii*.

Conflicts of interest

The authors declare no conflict of interest.

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