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Development of polymorphic microsatellite markers in *Sardinella aurita* by 5'-anchored PCR technique

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Introduction

Sardinella aurita, a member of the family of Clupeidae, lives in the upper and middle layers of warm ocean water (Yang and Qiu 1993). It is a medium-sized pelagic fish widely distributed from Atlantic Ocean to west Pacific Ocean. In Asia, it is naturally found near the southeastern coasts of China and in other countries such as Japan and India (Jiang and Lin 1986; Yang and Qiu 1993). This species is commercially important because of its abundance, fast growth, taste and medicinal value (Yang and Qiu 1993; Khaled *et al.* 2008). It is also an important fish for light seining in China. Some studies are carried out on this fish by experts, which mainly focusses on the eggs and larvae, individual fecundity, ovarian development, molecular phylogeny and species identification and so on (Jiang and Lin 1986; Yang and Qiu 1993; Jérôme *et al.* 2003). However, very few studies on genetic diversity and differentiation of *S. aurita* are available, which has limited the proper management and utilization of *S. aurita* (Kinsey *et al.* 1994).

Microsatellite markers are very popular for the study of molecular phylogeography and population genetics because of high polymorphism, easy genotyping and codominant inheritance (Sun *et al.* 2009). Isolation and utilization of microsatellite markers has been carried out in many species (Wang *et al.* 2009; Ma *et al.* 2011, 2014). Currently, no polymorphic microsatellite markers have been reported for *S. aurita*. Thus, it is essential to isolate polymorphic microsatellite markers for this fish species so as to promote the studies on its genetic background and differentiation. In this study, we developed nine polymorphic microsatellite markers for *S. aurita* by using the 5'-anchored PCR technique.

Materials and methods

Samples collection and DNA extraction

A wild population with 30 *S. aurita* individuals was randomly collected using trawl net in August 2013 from the coast of Fujian province (117.5°E, 23.8°N), China. The body length ranged from 4.21 to 16.00 cm, with an average of 10.02 cm. After collection, the samples were immediately fixed in 75% molecular grade ethanol for subsequent molecular analyses. Total genomic DNA was extracted from the muscle tissue using traditional proteinase K and phenol–chloroform extraction protocols as described by Ma *et al.* (2010).

Designation of 5'-anchored primer and isolation of microsatellites

Four degenerate primers were designed with the sequence KKDBDBD(AC)₆, KKHBHBH(AG)₆, KKVRVRV(CT)₆ and KKRVRVR(GT)₆, where K=G/T, D=G/A/T, B=G/T/C, H=A/C/T, V=A/C/G and R=A/G. In each primer, the repeat parts can anneal to microsatellite loci in genomic DNA and the seven nucleotides at 5' can form the 'anchor'. Then the 5'-anchored PCR was performed as described by Ma *et al.* (2011). The PCR products were separated on 1.2% agarose gel (TaKaRa, Shiga, Japan). The fragments with the size ranging from 200 to 750 bp were reclaimed and ligated with pMD19-T vector (TaKaRa), and then transformed into *Escherichia coli* DH5 α cells (TianGen Biotech, Beijing, China). Positive clones were selected to be sequenced using an ABI3730XL sequencer (Applied Biosystems, Foster City, USA). The sequences obtained were screened using the software SSR Hunter ver. 1.3 for microsatellite sequences (Li and Wan 2005) and microsatellite primers were designed using Primer Premier 5.0 software (<http://www.premierbiosoft.com/primerdesign/>).

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Table 1. Characterization of nine polymorphic microsatellite markers in *S. aurita*.

Locus (GenBank acc. no.)	Repeat sequence	Primer sequences (5'–3')	T_a (°C)	Size range (bp)	N_a	H_o	H_E
Sara1 (KJ655521)	(TG) ₁₀	F: 5'-ATGTTATGCAGTTCTCGTGGAC-3' R: 5'-ACACAAAATCAAAACAAGCAA-3'	53	224–260	4	1.0000	0.7162
Sara 2 (KJ655522)	(CA) ₅ ...(CA) ₅	F: 5'-GCTTACTCTTGCCCTCACA-3' R: 5'-TCTGCTATTACAATACGCCAT-3'	52	180–196	2	0.4667	0.3638
Sara 3 (KJ655523)	(AG) ₃₃	F: 5'-AGCGGGAGAAGAAGAAAAG-3' R: 5'-AAAAAGAAAAACAGAAAAATGC-3'	52	238–258	5	1.0000	0.7579
Sara 4 (KJ655524)	(GA) ₇	F: 5'-CGACTGCATTCATTTTTGTCTC-3' R: 5'-CATTCTTTCTAACCCCCACC-3'	51	139–157	5	1.0000	0.7813
Sara 5 (KJ655525)	(TC) ₅ C(CT) ₉ ...(TG) ₆	F: 5'-TGGATCTAATGTCCCTGCTC-3' R: 5'-GTTTTTCTTCCCCCTGTT-3'	52	198–210	6	1.0000	0.8281
Sara 6 (KJ655526)	(AC) ₉ ...(GGA) ₈	F: 5'-CCCCCAAACACATAACAC-3' R: 5'-CCCTGAACCTCCAGAAGC-3'	52	193–207	2	0.2143	0.4442
Sara 7 (KJ655527)	(AC) ₃₄	F: 5'-AGCCATTTGTGTGACCATATC-3' R: 5'-AGCTCTCTTTGTGAAAGTGCA-3'	52	196–210	5	1.0000*	0.7861
Sara 8 (KJ655528)	(AG) ₉ G(GA) ₅	F: 5'-TGTTTTTCTTCCCCCTGTT-3' R: 5'-TAATGTCCCTGCTCCATCTGC-3'	55	195–209	6	0.8519	0.8288
Sara 9 (KJ655529)	(TGTC) ₅ (TG) ₁₈	F: 5'-TCATACACATATCACACAGTAAGCA-3' R: 5'-GAGCAAAATAATCTCTCACGAATAG-3'	53	246–264	6	1.0000	0.8179
Average	–	–	–	–	4.56	0.8370	0.7027

T_a , annealing temperature; N_a , number of observed alleles; H_o , observed heterozygosity; H_E , expected heterozygosity, *significant deviation from HWE after Bonferroni correction ($P < 0.0056$).

Evaluation of microsatellite markers and data analysis

The characteristics of the microsatellite primers were tested in 30 individuals of *S. aurita*. The PCR amplification was performed in a 25 μ L volume which was composed of 1 \times PCR buffer, 0.4 μ M of each primer, 0.2 mM dNTP mix, 1 U *Taq* polymerase and 50 ng of template DNA. Thermocycler conditions were as follows: one cycle of denaturation at 94°C for 5 min; 35 cycles of 30 s at 94°C, 40 s at a primer-specific annealing temperature (table 1), and 45 s at 72°C; and one cycle of extension at 72°C for 5 min for a final step. 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/*MspI* marker (TianGen Biotech).

The indices about genetic diversity were calculated using the software PopGene ver. 1.31 (Yeh et al. 1999). Significance values for all multiple tests were corrected by sequential Bonferroni procedure (Rice 1989). The software Micro-Checker was used to estimate the null allele frequency (Van Oosterhou et al. 2004).

Results and discussion

In this study, a total of 233 clones were tested and 110 positive clones were sequenced. After aligning sequences and excluding similar sequences, 78 unique sequences containing microsatellite repeats were found and 32 pairs of primers were designed successfully based on the flanking sequences of microsatellites. The polymorphism of these primers was assessed using a wild population of *S. aurita*. Although the PCR conditions were optimized, 23 pairs of primers were

monomorphic, smears or invalid. Finally, we isolated nine novel polymorphic microsatellite markers in *S. aurita*.

A total of 41 alleles with size ranging from 139 to 264 bp were identified in 30 individuals. The number of alleles per locus ranged from two to six, with an average of 4.56. The observed and expected heterozygosity per locus ranged from 0.2143 to 1.0000 and 0.3638 to 0.8288, with an average of 0.8370 and 0.7027, respectively. Of the nine loci, one (Sara 7) showed significant deviation from Hardy–Weinberg equilibrium after Bonferroni correction ($P < 0.0056$). It may result from the small sample size or the presence of null alleles. No significant linkage disequilibrium was found between pairs of loci.

Determination of genetic structure of an organism can provide essential information for the proper utilization and resource management (Han et al. 2008). As an ideal and popular molecular marker, microsatellites have been used successfully to investigate genetic diversity, constructing genetic maps for species (Dudaniec et al. 2010; Ma et al. 2012; Song et al. 2012). We developed nine polymorphic microsatellite markers in *S. aurita* by the 5'-anchored PCR technique and all loci showed considerable variation in the Fujian population. These loci will provide useful information for the study of genetic structure of *S. aurita* and for the effective management of this fish resource.

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