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Development and characterization of polymorphic microsatellite markers in the gray mullet (*Mugil cephalus*)

T.-J. Xu, D.-Q. Sun, G. Shi and R.-X. Wang

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

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

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ABSTRACT. Using an $(AG)_{13}$ enriched genomic library of *Mugil cephalus*, 12 polymorphic microsatellite loci were isolated and characterized in a test population; the number of alleles ranged from 2 to 11. The observed and expected heterozygosities ranged from 0.2593 to 0.8966 and from 0.3047 to 0.8454, respectively. Two loci deviated from Hardy-Weinberg equilibrium; linkage disequilibrium among the 12 loci was non-significant. These polymorphic microsatellite loci will be useful for genetic diversity analysis and molecule-assisted breeding of the gray mullet.

Key words: *Mugil cephalus*; Microsatellite; Molecular marker

INTRODUCTION

The gray mullet, *Mugil cephalus*, commonly referred to as the striped, gray, or black mullet (Nelson et al., 2004), is a species that inhabits tropical and subtropical coastal regions of the world between 42°N and 42°S (Thomson, 1963). *M. cephalus* is an important commercial marine fish species for aquaculture in China (Li et al., 1998; Zhang et al., 2001; Zou and Wu, 2002). This species can survive in both freshwater and sea water, which is significant for artificial cultivation both in coastal and inland regions. Hence, most Chinese studies on gray mullet have focused on its reproductive biology and breeding techniques (Li et al., 1998; Fang et al., 2001; Weng et al., 2001). In addition, to obtain information for the conservation of biodiversity, natural resources, and fishery management, allozyme analysis, biochemical markers and mitochondrial DNA sequences have been used in studies of the gray mullet (Crosetti et al., 1993; Rossi et al., 1998; Rocha et al., 2000, 2005; Huang et al., 2001). These studies mainly focused on the populations of gray mullet from the Mediterranean Sea, Atlantic Ocean and, to a lesser extent, East Pacific and Indian Oceans. Liu et al. (2009) used amplified fragment length polymorphism (AFLP) and the mitochondrial control region to characterize the population genetics of the gray mullet species along the coast of China.

Microsatellites, also known as simple sequence repeats (SSRs), are regions of DNA that exhibit short repetitive sequence motifs (Degnan and Arévalo, 2004). Microsatellites have been widely employed in population genetics studies of numerous species, and such application is continuously expanding (Kohlmann et al., 2005; Mia et al., 2005). These motifs are often composed of 1- to 6-bp repeats and high polymorphism occurs for different repeats. Because they are co-dominant and highly polymorphic, microsatellite DNA markers have been successfully used in revealing population genetic diversity (Sekino and Hara, 2001; Selkoe and Toonen, 2006; Liu et al., 2009). Although it is an important commercial fish species, only 11 polymorphic microsatellite markers have been reported (Miggiano et al., 2005). Lack of sufficient and polymorphic molecular markers has limited the development of molecular phylogeny, population structure and molecule-assisted selective breeding in this fish species. Therefore, in the present study, 12 polymorphic microsatellite DNA markers were developed using fast isolation by the AFLP of sequences containing repeats (FIASCO method) to enrich and develop the molecular information of the gray mullet (Zane et al., 2002).

MATERIAL AND METHODS

Thirty individuals of gray mullet were collected from the East China Sea. 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. An enriched partial genomic library for the repeat motif $(AG)_{13}$ was constructed, essentially using DNA from one individual and following the FIASCO protocol. In brief, genomic DNA was digested using the *Mse*I restriction enzyme (MBI), and DNA fragments between 250 and 1000 bp were isolated from a 1.5% agarose gel. These fragments were ligated to the adapters OligoA (5'-TAC TCA GGA CTC AT-3') and OligoB (5'-GAC GAT GAG TCC

TGA G-3'), and then amplified by polymerase chain reaction (PCR) using *Mse*I-N primers (5'-GATGAGTCCTGAGTAAN-3'). The genomic DNA fragments containing SSR were then captured by hybridization to $(AG)_{13}$ biotin-labeled probes. Captured fragments were ligated to pGEM-T vectors (Promega) and then cloned using the TOP10 compentent cells, following the standard protocol. Some of the positive clones (N = 54) were screened via PCR with T7/SP6 primers, and sequenced using T7 primer on an ABI 3730 automated sequencer. Forty-nine clones were successfully sequenced, and 40 sequences contained sufficient repeat motifs. Some possessed only three to seven repeats, which held less potential for useful polymorphism. Primers for these loci were designed using the PRIMER PREMIER 5.0 software. Thirty primer pairs were designed from 30 sequences, as the remaining SSR were too close to the cloning site to design primers.

Polymorphism at each locus was determined using 30 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 $dNTP_S$, 0.2 µM of the forward and reverse primers, and 1.5 U *Taq* polymerase (Takara). Cycling conditions were 94°C for 4 min followed by 30 cycles of 94°C for 40 s, annealing temperature for 45 s (see Table 1), 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 thermalcycler. 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. POPGENE32 (Yeh and Boyle, 1997) and ARLEQUIN 3.11 softwares (Schneider et al., 2000) were used to estimate the number of alleles, observed (H_O) and expected (H_E) heterozygosity, violation of 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

All thirty primer pairs were successfully amplified, except for two pairs (Muce-24 and Muce-25), and 12 of these loci were shown to be polymorphic in the gray mullet. Details of the 12 newly developed microsatellite loci and variability measures across 30 individuals are summarized in Table 1. The number of alleles per locus ranged from 2 to 11, with an average of 5.9167 and H_O and H_E ranged from 0.2593 to 0.8966 and from 0.3047 to 0.8454, respectively. The polymorphism information content (*PIC*) per locus ranged from 0.2582 to 0.8339, of which five loci indicated intermediate polymorphic information ($0.25 < PIC < 0.5$), while the other five loci, being highly polymorphic ($PIC > 0.5$), could be useful for further study of population diversity.

Hardy-Weinberg equilibrium probability tests showed that the majority of the 12 loci were at HWE, Muce-14 (adjusted P = 0.0042) being the exception. Strong deviations from HWE ($P < 0.01$) were observed in Muce-37, possibly due to the presence of null alleles. In total, 60 pairwise tests for linkage disequilibrium among 12 loci were non-significant ($P > 0.05$, adjusted P = 0.0008). These polymorphic microsatellite loci in the gray mullet will enable studies of genetic variation, population structure, conservation genetics, and molecule-assisted selective breeding of the gray mullet in the future.

Table 1. Characteristics of 12 polymorphic microsatellite loci in gray mullet.

Locus	GenBank accession No.	Repeat motif	Primer (5'-3')	Tm (°C)	Size range (bp)	n	*PIC*	H_O H_E
Muce-9	HM060969	$(TG)_9(AG)_6$	F:ATAAAGACTTGAAGGGAA	48	100~134	9	0.8037	0.8148
			R:GTTGAGGTAGTTAGGAGC					0.8258
Muce-14	HM060970	$(TC)_9$	F:AGTGACACCGTATCTGGTC	50	284~334	3	0.4111	0.2593*
			R:CTCCGTAGTAGTAACAATGAAA					0.4582
Muce-16	HM060971	$(GA)_9A(AG)_3$	F:TGGCTGGGTCCGTTAGAT	48	166~178	4	0.5731	0.5357
			R:TGGCGTCACAAGAACATTAG					0.6486
Muce-26	HM060972	$(ACAA)_3$	F:TGCGGAGACAATGTAAAC	50	244~274	3	0.3415	0.2667
			R:AAATGAAACAATCCACCC					0.4150
Muce-37	HM060973	$(AC)_7$	F:TACTCAGCCAGCAGGTGT	47	222~248	9	0.6683	0.8966**
			R:AATACAGGGTTGTTGTCG					0.7093
Muce-38	HM060974	$(GA)_5(GGAGA)_4$	F:GCACCAACATCTCACCTG	50	259~337	6	0.8339	0.5517
			R:CCTACCATTTACCCCTCT					0.8454
Muce-44	HM060975	$(CA)_5(GA)_4$ (CA)11	F:GCTTCGGAGGACCAAC	51	183~203	5	0.4612	0.4667
			R:CGACAGCCACTGTTATG					0.4856
Muce-51	HM060976	$(GA)_7$	F:TGTCCGTTTTGGTAAGC	49	159~169	4	0.4603	0.3793
			R:TCGCCTTTTCATCTCA					0.5024
Muce-55	HM060977	$(TC)_8$ $(GCTC)_5$	F:AGAAGAAGACAGGGACTC	47	74~136	11	0.6214	0.6667
			R:AGAAATACTCTGCTAACCT					0.6444
Muce-57	HM060978	$(G)_{13}$	F:GCGATCATCTCCACAATA	50	117~121	2	0.2582	0.2917
			R:CGTTCACAGTGCGTAACAG					0.3047
Muce-74	HM060979	$(CT)_{13}$	F:GACCCGTCGGCTATGTAA	50	111~241	6	0.6153	0.5714
			R:GATTTGTTGCTCCGTATCT					0.6767
Muce-80	HM060980	$(AG)_{12}$	F:ACTGGGTTCAGATAGAAAT	49	198~238	9	0.7003	0.5333
			R:CTCGTGGAGGAAACATAA					0.7267

F = forward primer; R = reverse primer; Tm = annealing temperature; n = number of alleles; *PIC* = polymorphism information content; H_O = observed heterozygosity; H_E = expected heterozygosity. *Significant departure from HWE ($P < 0.05$). **Highly significant departure from HWE ($P < 0.01$).

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