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Development and characterization of microsatellite markers of the eastern keelback mullet (*Liza affinis*)

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ABSTRACT. Twenty-four polymorphic microsatellite loci were isolated and characterized for *Liza affinis* using a (GT)13-enriched genomic library. The number of alleles per locus ranged from 3 to 9, with a mean number of 6.250. The observed and expected heterozygosities ranged from 0.417 to 1.000 and from 0.550 to 0.861, with an average of 0.859 and 0.779, respectively. Deviation from Hardy-Weinberg proportions was detected at three loci. Evidence of null alleles was found at two loci. These markers will be useful in further studies investigating the genetic variation and population structure of this species, and may provide insights into the maintenance and efficient management of eastern keelback mullet resources.

Key words: *Liza affinis*; Microsatellite loci; Population structure; Genetic diversity

INTRODUCTION

Eastern keelback mullet (*Liza affinis*) belongs to the family Mugilidae, and is widely distributed in Japan (except for northern Hokkaido) and along the coasts of China, from the Yellow Sea to the South China Sea (Senou et al., 1987). With the decline of many traditional economic fish resources, *L. affinis* is gradually becoming a new kind of important economic fish for the global fisheries industry. In order to prevent the reduction of *L. affinis* populations, it is important to characterize the population structure, which may provide insights into the maintenance and efficient management of *L. affinis* resources. Because of their high polymorphism, reproducibility, relative abundance, and multi-allelic and co-dominant nature, microsatellite loci are considered to be powerful genetic markers and have been widely used in population structure analyses. Here, for the first time, we isolated microsatellite loci for *L. affinis*, which will provide a powerful tool for analyzing population structure of *L. affinis*. A limitation of this study was that the number of microsatellite loci developed using streptavidin-coated magnetic beads was lower than the number developed using a next-generation sequencing platform.

MATERIAL AND METHODS

Twenty-four individuals were collected from coastal waters near Zhanjiang, China, and preserved at -20°C until DNA extraction. Genomic DNA was extracted from muscle tissue of *L. affinis* using the standard phenol-chloroform method. A microsatellite-enriched genomic library was constructed following the enrichment protocols described by Ma and Chen (2009). Genomic DNA was digested with *Mse*I restriction enzyme (New England Biolabs, USA). The adapters (5'-TAC TCA GGA CTC AT-3'/5'-GAC GAT GAG TCC TGA G-3') were ligated to the DNA fragments. The ligated products were then pre-amplified in a 25-μL reaction system using an adapter specific primer (5'-GAT GAG TCC TGA GTA A-3') to verify the ligation and to increase the DNA concentration (Lin et al., 2015). Subsequently, the ligation products were hybridized to the biotin-labeled probe, (GT)13. Next, fragments containing simple repeat sequences were captured by streptavidin-coated magnetic beads (Promega, USA), washed, and recovered by heating and ethanol precipitation. The enriched fragments were amplified by polymerase chain reaction (PCR) using the adapter specific primer. The final amplification products were ligated into the pMD 18-T vectors (TaKaRa, Japan) and transformed into *Escherichia coli* DH5α competent cells. A total of 142 positive clones were selected using blue/white screening, and were sequenced by an ABI Prism 3730 automated DNA Analyzer (Applied Biosystems, USA). Following sequence analysis, PCR primer pairs were designed to amplify 119 microsatellite loci with suitable flanking regions using the PRIMER PREMIER 5 software (Premier Biosoft International, USA).

The designed primers were analyzed using 24 *L. affinis* individuals from the coastal waters near Zhanjiang, China. The PCR was carried out in a 25-μL reaction mixture containing 17.25 μL ultrapure water, 2.5 μL 10X PCR buffer, 2 μL dNTPs, 1 μL each primer (5 μM), 0.25 μL Taq polymerase, and 1 μL DNA template. The PCR program consisted of an initial step for 2 min at 95°C followed by 35 cycles of 0.5 min at 94°C, 1 min at the locus-specific annealing temperature (Table 1), and 1 min at 72°C, and a final step for 10 min at 72°C, after which the reaction was held at 4°C. Negative controls were included in all PCR amplifications

to confirm the absence of contaminants. PCR products were purified with a Gel Extraction Mini Kit (Watson BioTechnologies Inc., China), separated on 6% denaturing polyacrylamide gel, and visualized by silver staining (Lin et al., 2011).

Table 1. Characteristics of microsatellite loci in *Liza affinis*.

Locus name	Primer sequence (5'-3')	Repeat motif	Ta (°C)	Allele size range (bp)	Sample size	N_A	H_O	H_E
La77	F: AAAGGGCACAAGGTGAGTAAA R: GCGTCCCATATCTGAGCAGT	$(AC)_{10}$	52	240-280	24	5	1.000	0.789
La79	F: AGACGGGAAAGGAGTGGAAGA R: CCAGGGCACAGCATATTGAGA	$(TG)_8…(TG)_8$	49	220-270	24	6	0.999	0.826
La83	F: CTGTCCCCTGCTGACCTAA R: GTTACCAAACATTAAAAGCCAT	$(TG)_{15}$	48	254-380	24	6	1.000	0.803
La89	F: ATCACATCTACCTCCAGT R: ATTTCCATACAGTCTAATG	$(TG)_9$	48	208-232	24	3	0.667	0.582
La106	F: TAAGGGATGGGCTGTATTTG R: CATTGATGGCATTATCATTAGTA	$(AC)_{14}$	55	220-320	24	8	1.000	0.861
La112	F: CTGGATCATGGGGCTTAC R: TACTGGCTGCTGCCTTCT	$(TC)_9…(TC)_7…$ $(CA)_6…(CA)_7$	58	220-240	24	7	0.667	0.782
La138*	F: ATTGTTCATACCGTAGTGC R: GATTAAGCCAAGAAGAGG	$(CTCCT)_5$	51	201-242	24	7	0.500	0.793
La139*†	F: CTTTATGTCTCCTCCAGCGTT R: TGTGCCTTTTCTAATGTCAGC	$(CTCCT)_5…(GT)_5$	60	210-240	24	5	0.417	0.794
La140	F: CACTCCACCATACACCTCC R: CACTGGCTGCTTTACCTTA	$(AC)_{34}$	51	210-250	24	8	1.000	0.806
La153	F: GGCTTTACTTAATTGTTTTGTTC R: GCTTCACTTTCTCAGGCTCT	$(TG)_5…(GA)_6…$ $(AG)_5…(GA)_6$	51	200-230	24	3	0.833	0.550
La165	F: CAGTTTGTGCTTTTAGGT R: CTTTTGTAGGAGCGTTAT	$(GA)_7…(GA)_{18}$	51	280-380	24	9	1.000	0.828
La170	F: TCTCCTCCTACGGGTCAG R: CAGCCAAACGATCACAAA	$(TG)_{12}$	55	205-240	24	8	1.000	0.840
La172	F: CACTGGTAAATTCCTTTCAGA R: CGTTACATACGACGCATACA	$(CA)_{10}$	55	180-220	24	5	0.833	0.796
La173	F: TAGACTGCCGCTAATTTCACT R: ACGAGGAGACTTATCCCTGTA	$(CA)_9…(CA)_{17}$	56	220-280	24	8	1.000	0.849
La188†	F: TTGCCAAATTGCTAGATC R: GGAGGAATAGTGGGAGAA	$(AC)_{10}$	48	210-240	24	6	0.958	0.789
La195	F: ACTATTCCCGAGGAGTCT R: TTCCAGAATTTTGTCTTAAC	$(CA)_5…(CA)_{17}…(CA)_5$	48	190-250	24	7	0.875	0.824
La200	F: TCCCTGACAGAATACCAAC R: GGTGTAGTAGAGCGAGGC	$(GT)_{11}$	50	240-270	24	7	0.958	0.785
La201	F: GGTTAGGGTTGCTTTATC R: GGTGTAGTAGAGCGAGGC	$(TG)_5…(GT)_{11}$	51	280-320	24	8	0.833	0.848
La214	F: GAATCATACATCCACCAGC R: AACCCTAAACCCGTTCTC	$(TG)_{11}$	48	220-270	24	5	0.667	0.670
La217	F: ATCAGTCATTATTGCCTTTT R: ACACGCATGAGTAAACTACAT	$(AC)_6…(TG)_8$	48	250-300	24	8	0.870	0.854
La227	F: CATCCCACATCACCAAAG R: GATTACTAAAGGCTACTGCTAC	$(AC)_{10}$	53	200-320	24	5	0.792	0.785
La241	F: GAGTTTGGTATTTATCTTGT R: ATGCACGTTCATTTTAGT	$(TG)_{27}$	54	210-245	24	5	0.792	0.691
La251	F: AGACCCTCAGCACCAACG R: TCCGCCTATTGACTGTAGTTTT	$(CA)_{14}$	54	220-285	24	6	0.958	0.816
La261†	F: ACTGAGGCTTGTGAATGG R: CTATCAGGGAAGGGTTGG	$(TG)_{10}$	55	230-305	24	5	1.000	0.740

Ta = optimized annealing temperature; N_A = number of alleles; H_O = observed heterozygosity; H_E = expected heterozygosity. *Locus may harbor null alleles (null allele frequency >5%). †Deviation from Hardy-Weinberg proportions (adjusted P value < 0.0021).

The observed and expected heterozygosities and Hardy-Weinberg disequilibrium were calculated by GENEPOP 4.0 (Rousset, 2008). Null allele frequencies (Brookfield, 1996) were calculated by MICRO-CHECKER 2.2.3 (Van Oosterhout et al., 2004). All results for multiple tests were corrected using Bonferroni's correction (Rice, 1989).

RESULTS

Twenty-four out of 44 loci were clearly amplified and shown to be polymorphic. The number of alleles per locus ranged from 3 to 9, with an average of 6.250. The observed and expected heterozygosities ranged from 0.417 to 1.000 and from 0.550 to 0.861, respectively,

with an average of 0.859 and 0.779 (Table 1). Three loci showed significant deviation from Hardy-Weinberg proportions, except for the loci La 139, La 188, and La 261 after Bonferroni correction ($P < 0.0021$). Two loci (La 138, La 139) showed evidence of null alleles (null allele frequency >5%). No genetic disequilibrium was detected in each locus pair.

DISCUSSION

According to the results showed above, these 24 new microsatellite markers could be used to analyze the genetic structure of *L. affinis* and to generate important genetic data for the conservation and recovery of *L. affinis* resources.

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