GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

*Short Communication*

# Development and testing of 13 polymorphic microsatellite markers in *Larimichthys polyactis* (Sciaenidae) using 5' anchored PCR

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Genet. Mol. Res. 10 (3): 1455-1460 (2011)
Received May 2, 2011
Accepted June 21, 2011
Published July 19, 2011
DOI 10.4238/vol10-3gmr1464

**ABSTRACT.** *Larimichthys polyactis* is a commercially important marine fish species in southeast Asia. The population crashed due to overfishing in the 1970s, but has since recovered. We developed 13 novel polymorphic microsatellite markers in *L. polyactis* using 5' anchored PCR. The characteristics of these loci were estimated by analyzing a sample of 30 individuals. A total of 74 alleles were detected, with a mean of 5.7 alleles per locus. There were 2 to 12 alleles, 0.2760 to 0.8247 polymorphism information content, and 0.3214 to 1.000 observed and 0.3097 to 0.8567 expected heterozygosity per locus. The mean observed and expected heterozygosity was 0.6816 and 0.6724, respectively. Three loci deviated significantly from Hardy-Weinberg equilibrium after Bonferroni's correction, and no significant linkage disequilibrum between pairs of loci was found. This information will be useful for the analysis of population genetic diversity, and the management of this important fish resource.

**Key words:** *Larimichthys polyactis*; Microsatellite markers; Polymorphism; 5' Anchored PCR; Redlip croaker

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## INTRODUCTION

The small yellow croaker, *Larimichthys polyactis*, an important fishery resource and marine food fish species, is widely distributed in the Yellow Sea and the East China Sea of the Northwest Pacific Ocean. In recent years, the fishery resource of *L. polyactis* in the Yellow Sea and the East China Sea has been hit hard by overfishing, deterioration of the marine ecosystem and seawater pollution (Froese and Pauly, 2003). The study of genetic diversity and population genetic structure can play an important role in the conservation and sustainable exploitation of this fishery resource. So far, very few studies on genetic diversity, population genetic structure and demographic history of *L. polyactis* have been carried out (Meng et al., 2003; Xiao et al., 2009; Lin et al., 2009).

Microsatellites are widely found throughout the eukaryotic genome (Weber and May, 1989). The advantages of ease of genotyping, high polymorphism and co-dominant inheritance make microsatellite markers very useful (Sun et al., 2009). Isolation of microsatellite markers has been carried out in many important fish species (Sakamoto et al., 2000; Chistiakov et al., 2004; Ma et al., 2009).

Sufficient microsatellite markers are needed to thoroughly understand the genetic population structure, genetic diversity and resource history of a target species. Polymorphic microsatellite markers have been reported for this important species, *L. polyactis* (Li et al., 2006; Wang et al., 2009); however, they are very limited for the study of genetic diversity and conservation for this species. Thus, it is necessary to isolate more polymorphic microsatellite markers for this fish species.

The 5' anchored polymerase chain reaction (PCR) technique offers a number of advantages such as more polymorphism than those from nonanchored primers and cost savings for microsatellite discovery (Fisher et al., 1996). In the present study, we developed 13 polymorphic microsatellite loci in *L. polyactis* by using the 5' anchored PCR technique.

## MATERIAL AND METHODS

### Sample collection and DNA extraction

A total of 30 *L. polyactis* individuals were collected from Zhejiang Province, China. Total genomic DNA was extracted from the muscle tissue using standard proteinase K and phenol-chloroform extraction protocols as described by Ma et al. (2010). The DNA was adjusted to a concentration of 100 ng/μL and stored at -20°C until use.

### 5' Anchored primer design and PCR

The repeat parts of the primers were designed to anneal to microsatellite loci in genomic DNA and the seven at 5' nucleotides in the primers form the ‘anchor’. The 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. The primers were synthesized by Sangon (Shanghai).

PCR amplification was performed in a 25-μL reaction volume containing 2.0 mM

$MgCl_2$, 0.2 mM dNTP mix, 0.2 μM each primer, 1 U Taq DNA polymerase (TaKaRa), 1X PCR buffer, approximately 100 ng template DNA and deionized water. The cycling parameters were an initial denaturation of 5 min at 94°C, followed by 35 cycles of 45 s at 94°C, 45 s at annealing temperature, and 45 s at 72°C, and a final extra-extension at 72°C for 5 min. The PCR products were separated on 1.5% agarose gel (TaKaRa) and visualized by UV-light.

## Isolation of microsatellite markers

The DNA fragments, ranging from 200 to 750 bp, were reclaimed and ligated with pMD19-T vector (TaKaRa), and then transformed into *Escherichia coli* DH5α cells (TianGen Biotech Co., Ltd.). The positive clones were selected randomly and cultured overnight. The vector-specific primers were used for the identification of bacteria. PCR amplification was performed in a 25-μL reaction volume containing 2.0 mM $MgCl_2$, 0.2 mM dNTP mix, 0.2 μM each primer, 1 U Taq DNA polymerase (TaKaRa), 1X PCR buffer, and 1 μL bacteria cultured overnight. The cycling parameters were an initial denaturation of 5 min at 94°C, followed by 35 cycles of 45 s at 94°C, 45 s at 55°C, and 45 s at 72°C, and a final extra-extension at 72°C for 5 min. The PCR products were separated on 1.5% agarose gels (TaKaRa). After identified by PCR, the positive clones were randomly selected for sequencing using an ABI3730XL sequencer (Applied Biosystems).

Microsatellite sequences were screened using the SSRHUNTER 1.3 software (Li and Wan, 2005). Microsatellite primers were designed using the Primer Premier 5.0 software (http://www.premierbiosoft.com/primerdesign/).

## PCR amplification and polymorphism assessment

Polymorphisms of the microsatellite primers were tested in 30 individuals of *L. polyactis*. The PCR amplification was performed in a 25-μL volume containing 1X PCR buffer (TaKaRa), 0.4 μM of each primer, 0.2 mM dNTP mix, 1 U Taq polymerase (TaKaRa) and 50 ng template DNA. After denaturation for 5 min at 94°C, amplification was allowed to proceed for 35 cycles (94°C for 30 s, annealing temperature for each pair of primers (Table 1) for 40 s, 72°C for 45 s) and a final step at 72°C for 5 min. The PCR products were separated on a 6% denaturing polyacrylamide gel and visualized by silver staining. Allele size ranges were determined by referring to the pBR322/*Msp*I marker (TianGen Biotech Co., Ltd.).

## Data analysis

Genetic diversity indexes including observed number of alleles ($N_a$), observed heterozygosity ($H_O$) and expected heterozygosity ($H_E$), polymorphism information content (PIC), and chi-square tests for Hardy-Weinberg equilibrium (HWE) were calculated using the POPGENE version 1.31 software (Yeh et al., 1999). Significance values for all multiple tests were corrected by sequential Bonferroni's procedure (Rice, 1989). The null allele frequency was estimated by the MICRO-CHECKER version 2.2.3 software (Van Oosterhou et al., 2004).

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**Table 1.** Locus name, repeat sequence, primer sequences, annealing temperature ($T_a$), number of observed alleles ($N_a$), allele size range, observed ($H_O$) and expected heterozygosity ($H_E$), polymorphism information content (PIC), and GenBank accession number for 13 polymorphic microsatellite loci in *Larimichthys polyactis*.

| Locus (GenBank) | Repeat motif | Primer sequences (5'-3') | $T_a$ (°C) | Size range (bp) | Zhejiang population (N = 30) | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | $N_a$ | $H_O$ | $H_E$ | PIC |
| Lap1 (HQ678298) | $(TG)_{29}$ | F: 5' TCTTGAGTAATCTGAAAATAAAT 3'<br>R: 5' CTAAAGATGTCTCTCAGAAAAGT 3' | 47 | 192-206 | 5 | 0.6000 | 0.7731 | 0.7172 |
| Lap2 (HQ678299) | $(GA)_5…(AG)_4$ | F: 5' GAGAGGGGAGGAAGGACAAGAAG 3'<br>R: 5' GTGTGTGTGGGTGGATGGGTTTA 3' | 57 | 193-205 | 4 | 1.0000* | 0.5735 | 0.4679 |
| Lap3 (HQ678300) | $(GAA)_8…(TG)_{10}$ | F: 5' GCGAGACGAGAGACAGAGGAGGT 3'<br>R: 5' GTTTGCTTAGAGGTTATCACACACG 3' | 57 | 137-155 | 7 | 0.6154 | 0.7526 | 0.7043 |
| Lap4 (HQ678301) | $(CA)_6$ | F: 5' GGCTACCAGTCTACCATACG 3'<br>R: 5' AGAGGACGGTGTGTGTAAG 3' | 54 | 101-125 | 8 | 0.5833 | 0.8254 | 0.7807 |
| Lap5 (HQ678302) | $(TG)_4…(TG)_5$ | F: 5' TTGATAGGCAAGAGATGGGAAAG 3'<br>R: 5' CCTGCTGGTGCTGAAACAGAGAA 3' | 54 | 171-187 | 4 | 0.3214* | 0.3097 | 0.2760 |
| Lap6 (HQ678303) | $(TA)_5$ | F: 5' GGAAAACACTGGTAGTAAACAT 3'<br>R: 5' GAGAAGGAGGAGGACACGG 3' | 52 | 135-157 | 7 | 0.7241 | 0.7858 | 0.7374 |
| Lap7 (HQ678304) | $(CT)_5$ | F: 5' AGAGAGACAGAGAGGGAGAACAC 3'<br>R: 5' TGAGAGGAAAATACACAGAACAG 3' | 54 | 159-177 | 2 | 0.7917 | 0.5098 | 0.3746 |
| Lap8 (HQ678305) | $(AC)_7AG(AC)_7$ | F: 5' GCAGGTAGATAAAAATGGGAATGAG 3'<br>R: 5' ACGGGTGTCATAGGGCTGGC 3' | 56 | 127-149 | 6 | 0.5385 | 0.7994 | 0.7494 |
| Lap9 (HQ678306) | $(CTC)_6$ | F: 5' CTGCTAAACTCGCCAGTGATT 3'<br>R: 5' GATGGAGGATGGAGATGGAGA 3' | 56 | 101-117 | 6 | 0.7308 | 0.7647 | 0.7109 |
| Lap10 (HQ678307) | $(TC)_4…(GA)_4$ | F: 5' CAGTTCAAGGGGGTTCATTTAGA 3'<br>R: 5' GCCAAACAACCAGGGTCAGCT 3' | 54 | 126-138 | 4 | 1.0000* | 0.5442 | 0.4259 |
| Lap 11 (HQ678308) | $(GA)_8$ | F: 5' TCCTTCCCCCCAATAGACAGTAA 3'<br>R: 5' TGAGTCATAGATTTTCTCGGCTT 3' | 54 | 163-185 | 12 | 0.8148 | 0.8567 | 0.8247 |
| Lap 12 (HQ678309) | $(AC)_5$ | F: 5' GGACCCACAACCGACACTGACT 3'<br>R: 5' ACCCATGCAAAGCTGGTGAGTC 3' | 57 | 236-244 | 2 | 0.3333 | 0.4088 | 0.3208 |
| Lap 13 (HQ678310) | $(AC)_{24}$ | F: 5' TGGATGGAGTTTCCGTGTAGTT 3'<br>R: 5' CTGTTTCAGGATTCAGGCGTGT 3' | 54 | 226-248 | 7 | 0.8077 | 0.8379 | 0.7983 |
| Average | - | - | - | - | 74 | 0.6816 | 0.6724 | 0.6068 |

*Show significant deviation from HWE after Bonferroni's correction ($P < 0.0038$).



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## RESULTS AND DISCUSSION

*L. polyactis* is a commercially important fish species in China, but it has been over-exploited and its natural resource has severely declined over the last few decades (Froese and Pauly, 2003). Research on the genetic background of *L. polyactis* has been carried out (Meng et al., 2003; Xiao et al., 2009; Lin et al., 2009). A more thorough understanding of *L. polyactis* population genetics will be helpful for the development of sustainable fishing strategies, and for the protection of this important resource. Microsatellite markers have many advantages in this regard.

In this study, four anchored primers were selected for isolating microsatellites. The length of PCR products, ranging from 200 to 750 bp, was recovered from agarose gels and ligated into a pMD19-T vector and transferred into DH5α competent cells. A total of 74 clones were tested and 48 positive clones were selected to be sequenced using an ABI Prism 3730 automated DNA sequencer. Forty-six of 48 sequences, which contained microsatellite repeats at the middle and/or at the flanking region of the sequences, were found. And 18 pairs of primers were designed successfully using the Primer Premier 5.0 software. The polymorphism of these primers was assessed using 30 individuals of *L. polyactis*. Although the PCR conditions were optimized, five pairs of primers had either amplified monomorphic PCR products of the expected size or smears. Finally, we isolated 13 novel polymorphic microsatellite markers in *L. polyactis*.

A total of 74 alleles were identified in 30 individuals. Allele sizes ranged from 101 to 248 bp. The $N_a$ per locus ranged from 2 to 12, with an average of 5.7. The PIC value for the 13 microsatellite loci ranged from 0.2760 to 0.8247. The $H_O$ and $H_E$ per locus ranged from 0.3214 to 1.000 and from 0.3097 to 0.8567, with an average of 0.6816 and 0.6724, respectively. Compared with the heterozygosity of *L. polyactis*, the heterozygosity in our study was higher than that of the Dalian population (Wang et al., 2009), but lower than that of another population (Li et al., 2006). Compared with the heterozygosity of other species, such as *Epinephelus awoara* (0.598; Dong et al., 2007) and *Verasper moseri* (0.60; Ma and Chen, 2009), the heterozygosity in our study was higher. Although the genetic diversity of *L. polyactis* was relatively high, the population structure and resource have been destroyed. So we should protect the genetic diversity by reducing pollution and controlling fishing. Ten loci were in HWE, while three loci (Lap2, Lap5, Lap10) showed significant deviation from HWE after Bonferroni's correction ($P < 0.0038$). This may be the result of the small sample size or the presence of null alleles. No linkage disequilibrium was found to be between loci pairs.

In conclusion, we developed 13 polymorphic microsatellite markers in *L. polyactis* by the 5' anchored PCR technique. All loci showed considerable variation in the Zhejiang population. These loci will provide useful information for the study of genetic diversity and structure of *L. polyactis* and for the effective management of this fish resource.

## ACKNOWLEDGMENTS

Research supported by National Non-Profit Institutes (East China Sea Fisheries Research Institute) (#2008M04).

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