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Zoological Research

# Isolation of CA/GT Microsatellites from the *Paralichthys olivaceus* Genome

CHANG Yu-mei[1,2], SUN Xiao-wen[1,*], LI Shao-wu[1,3], ZHAO Ying-ying[1,4], ZHU Xiao-chen[1,4], LIU Hai-jing[1,*]

(1. *Heilongjiang River Fisheries Research Institute, Chinese Academy of Fishery Sciences, Harbin* 150070, *China;*
2. *College of Life Sciences, Sun-Yat Sen University, Guangzhou* 510275, *China;*
3. *College of Life Sciences, Shanghai Fisheries University, Shanghai* 200090, *China;*
4. *Life Science of Technology Institute, Dalian Fisheries University, Dalian* 116023, *China)*

**Abstract:** A library rich in CA/GT microsatellites was constructed from the *Paralichthys olivaceus* genome by combining biotin capture method and radioactive labeling hybridization. Five hundred and twenty six positive clones were obtained through twice screens. Sequencing confirmed 133 microsatellite loci (number of repeats≥5) in 119 positive clones. Of these microsatellites, two (1.5%) had compound repeat motifs, 63 (47.37%) had perfect motifs and 68 (51.13%) had imperfect motifs. Primer pairs were designed in the flanking regions of 22 microsatelites and subjected to PCR amplification. In 8 artificial gynogenesis families, four pairs failed to amplification, one pair was monomorphic, and the rest were polymorphic with an average of 5.2 alleles per locus. Heterozygosities ranged between 0.375 and 0.846, *PIC* ranged between 0.305 and 0.823. The results suggested that most of the microsatellites we isolated were qualified to be applied to the population genetic studies of *P. olivaceus*.

**Key words:** *Paralichthys olivaceus*; Microsatellite; Biotin capture; Radioactive labeling

# 牙鲆 CA/GT 微卫星标记的筛选

常玉梅[1,2], 孙效文[1,*], 李绍武[1,3], 赵莹莹[1,4], 朱晓琛[1,4], 刘海金[1,*]

(1. 中国水产科学研究院 黑龙江水产研究所, 黑龙江 哈尔滨 150070; 2. 中山大学 生命科学学院, 广东 广州 510275;
3. 上海水产大学 生命科学学院 上海 200090; 4. 大连水产学院 生命科学与技术学院, 辽宁 大连 116023)

**摘要:** 采用生物素选择杂交法与放射性同位素杂交法相结合的技术, 成功地从牙鲆(*Paralichthys olivaceus*)基因组中分离出含有 CA/GT 重复类型的微卫星序列。通过两轮淘选, 共获得 526 个阳性菌落。测序其中的 119 个菌落, 结果获得 133 个含有微卫星座位的序列。除了两个复合型微卫星外(1.5%), 完美型 63 个(47.37%), 非完美型 68 个(51.13%)。设计并合成 22 对微卫星引物, 对 8 个人工雌核发育家系的亲本进行遗传背景分析。PCR 结果表明, 4 对引物无扩增带或者扩增带不是目的条带, 1 对引物表现为单态, 其余 17 对引物均呈多态性, 平均每个座位产生 5.2 个复等位基因, 杂合度为 0.375 ~ 0.846, 多态信息含量为 0.305 ~ 0.823。结果表明, 所筛选的大部分微卫星标记能够用于牙鲆群体遗传学研究。

**关键词:** 牙鲆; 微卫星标记; 生物素选择杂交法; 同位素标记杂交法
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Microsatellites, also known as simple sequence repeats (SSRs), are regions of DNA that exhibit short repetitive sequence motifs (Degnan & Arévalo, 2004). Those motifs are often composed of 1 - 6 bp repeat se-

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* Corresponding authors (通讯作者), E-mail: xwsun2002@163.com; liuhaijin@hotmail.com
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第一作者简介: 常玉梅, 女, 助研, E-mail: ymchang2002@sohu.com

quences, such as CA, AGA, ATA and the like. In recent decades, microsatelites were widely employed in population genetic studies of numerous species, and such application is continuously expanding (Kohlmann et al, 2005; Mia et al, 2005). This drives the development of methods to isolate microsatellite from the genome. Microsatellites are featured cross species amplification due to the conservativeness among relative species in their flanking region where primers can be designed (add the reference), which reduces the requirements of direct isolation in target species (Mia et al, 2005). However, direct isolation from genome, which avoid drawbacks of cross species amplification, such as allele dropout, null allele etc, is still the main source of microsatellites (Xu et al, 2001). Presently, there are mainly two ways to isolate microsatellites from genome directly, non-enriched and enriched method. The former is not only time-consuming (generally three to eight months) but also low net yield of microsatellites, while the latter is often time-saving (about one month) and high net yield of microsatellites (Dimsoski & Toth, 2001; Chang et al, 2005). Therefore, enriched method gradually plays major role instead of non-enriched method.

*Paralichthys olivaceus* is one of the most importantly cultured fish species around the oceans in Northern China. Because the female grows faster than the male, artificial gynogenetic technology and fry feminization are regarded as effective strategies to approach monosexual reproduction and high productivity (Liu et al, unpublished). However, there are very limited reports concerning the molecular genetic analysis of artificial gynogenesis in *P. olivaceus*. We isolated and characterized a set of microsatellites directly from the *P. olivaceus* genome.

## 1 Materials and Methods

### 1.1 Microsatellite isolation

A modified version of the protocol outlined by Carleton et al (2002) was used in the construction of the microsatellite library of the *P. olivaceus*.

Total genomic DNA from a single individual of *P. olivaceus* was digested with the restriction enzyme Sau 3A I. Fragments ranging from 400 – 800 bp were excised from a 1% low melting point agarose TAE gel and purified using QIAquick Gel Extraction Kit (Qiagen, CA). The double-stranded adaptor molecular A/B was prepared by mixing equal mole amount of oligonucleotides A (5′-GATCGTCGACGGTACCGAATTCT-3′) and B (5′-GTCAAGAATTCGGTACCGTCGAC-3′), heating to 95 ℃ at 10 min, then slowly cooling down to 10 ℃ over a period of 4 h. The recovered DNA fragments were suspended in a 20 μL ligation reaction containing 6.6 μg of A/B double-stranded adaptors and 6 Weiss units of T4 DNA ligase. Ligation was carried out at 16 ℃ overnight.

Excess adaptors were removed by washing on an Ultrafree column (Pall, USA) 2 – 3 times. Two microliter of the recovered adaptor-ligated fragments was used as templates to perform PCR in a volume of 20 μL (oligonucleotide B as primer) following the program: 94 ℃ 3 min; 10 cycles for 94 ℃ 1 min, 58 ℃ 1 min, 72 ℃ 1 min; 72 ℃ 10 min for final extension. The PCR products were also purified 3 times to discard the remaining primers, dNTPs and concentrated to an appropriate volume with Ultrafree column.

For enrichment, the adaptor-ligated DNA fragments was denatured at 95 ℃ for 5 min, then hybridized to a biotinylated probe (5-Biotin-ATAGAATAT $[CA]_{16}$) in 50 μL hybridization solution (6 × SSC, 10 μmol/L oligo B, 0.1% SDS, 0.3 μmol/L probe) at 68 ℃ for one hour. The DNA hybridized to the probe was separated and captured by streptavidin magnetic beads (Dynal Biotech ASA, Norway) at room temperature for 20 min, followed by 4 washing steps, including twice in 6 × SSC/0.1% SDS at room temperature for 10 min, twice in 3 × SSC/0.1% SDS at 68 ℃ for 15 min, twice in 6 × SSC at room temperature, and twice in 0.1 × TE at room temperature quickly. The final step of 95 ℃ for 10 min in 50 μL 0.1 × TE is to denature the single-stranded DNA containing the target microsatellites from the beads. The purified single-stranded DNA was subjected to a second round of PCR according to the same procedure as the first round of PCR. PCR products were also recovered as mentioned above.

The recovered PCR products were ligated into pGEM-T vector (Promega, USA) according to the manufacturer's instructions. The ligation solution was used to transform the competent *E. coli* cells (DH5α), which then plated and cultured on LB agar plate containing 100 μg/mL ampicillin. The recombinants were picked out orderly to nitrocellulose membrane (Promega, USA) in order to identify positive clones by 5′-[$\gamma$-$^{32}$P]ATP labeled probe $(CA)_{16}$ prior to sequencing.

### 1.2 Sequence analysis and primer design

Positive clones were sequenced at Chinese National Human Genome Center (http://www.chgb.org.cn). Software VecScreen (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html) was used to remove

the sequences of T vector, and a computer program developed by us was used to remove the adaptors. The sequences containing motifs repeating more than 5 times were regarded as microsatellites. Twenty two pairs of PCR primers were designed in the flanking regions of the repeating motifs with the software package of Premier Primer 5.0 and subjected to PCR amplification as follow.

### 1.3 Microsatellite genotyping of *P. olivaceus* by PCR

The genomic DNA was obtained from eight mothers of artificially gynogenetic family of *P. olivaceus*. Amplification reactions were carried out in thermocycler 9 700 (Pekin Elmer, USA) in a 25 μL volume containng 1 – 100 ng of template, 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 200 μmol/L each dNTP, 0.1% Triton X-100, 0.1% NP-40, 0.01% gelatin, 0.4 μmol/L of forward and reverse primers, and 1 unit of *Taq* polymerase. Thermal cycling conditions were as follows: 94 ℃ 3 min; 40 cycles for 93 ℃ 30 s, 46 – 59 ℃ 30 s, 72 ℃ 30 s; 72 ℃ 10 min for final extension (Tab. 1). A horizontal agarose gel (2%) and a vertical polyacrylamide gel (8%) electrophoresis system were used to genotype each of these microsatellites as reported (Sambrook et al, 2001; Xu et al, 2002).

### 1.4 Data analysis

The expected heterozygosity (*He*) and the polymorphic information content (*PIC*) were calculated through a computer program written by us according to the following formulae (Nei, 1978; Liang et al, 2004).

$$He = 1 - \sum_{i=1}^{n} P_i^2 \quad (1)$$

$$PIC = 1\sum_{i=1}^{n-1} P_i^2 - \sum_{i=1}^{n-1}\sum_{j=i+1}^{n} 2P_i^2P_i^2 \quad (2)$$

**Tab. 1 Microsatellite primer pairs for *Paralichthys olivaceus*, the number of alleles, the heterozygosity, and the polymorphic information content**

| Locus | Motifs | Sequence(5′-3′) | Annealing temp.(℃) | No.of alleles | He | PIC | GenBank Accession no. |
|---|---|---|---|---|---|---|---|
| Pac2HLJ | $CA_{17}$ | F:TGTAAGTTCTTCTTGGGTCA<br>R:CCTTCTGGTCATCGCTCT | 52 | 5 | 0.753 1 | 0.712 0 | DQ097685 |
| Pac6HLJ | $CA_{34}$ | F:TGCCCTCAAGAAACGATA<br>R:GTGCAAACAGTTACAGACAT | 48 | 4 | 0.745 6 | 0.698 3 | DQ097689 |
| Pac14HLJ | $CA_{20}$ | F:GATCCCAACAACACCAGC<br>R:ACACCACATCCTCCACCT | 52 | 3 | 0.709 1 | 0.655 0 | DQ097688 |
| Pac18HLJ | $CA6N_2CA_{11}$ | F:CCGTTCTTCTGCGTCTTC<br>R:GCTTACTCTGTCGCTCTGC | 55 | 4 | 0.686 4 | 0.637 4 | DQ097687 |
| Pac19HLJ | $CA_{22}N_2CA_3$ | F:TAGTGAGGGAAGCGAGAA<br>R:TGAAGACAGGGAGATGGA | 51 | 7 | 0.826 4 | 0.804 3 | DQ097686 |
| Pac28HLJ | $TG_{23}N_2TG_4$ | F:AAACAACCAGCGAAGAAG<br>R:CATCAGATTTTGGAGTAGC | 46 | 2 | 0.50 | 0.375 | DQ097690 |
| Pac34HLJ | $CA_{14}N_2CA_8$ $N_2CA_3$ | F:ATCATCTCCTTGTCCGTTTA<br>R:AACCCAACCTGTCATTACTC | 59 | 8 | 0.845 7 | 0.826 8 | DQ097694 |
| Pac38HLJ | $GT_{13}$ | F:ACGACACGACGGACACCA<br>R:GAAGCAGCGGAGGAAACG | 51 | 5 | 0.753 1 | 0.712 0 | DQ097693 |
| Pac39HLJ | $CA_{42}$ | F:CACATCTATCACGCACCC<br>R:AGCCAACGAAACTCCACT | 52 | 4 | 0.704 1 | 0.649 9 | DQ097692 |
| Pac44HLJ | $GT_{15}$ | F:TGATGCTGAGGGATGATTG<br>R:GCAGAGGCAGATTAAACCA | 51 | 7 | 0.828 4 | 0.805 9 | DQ097691 |
| Pac50HLJ | $CA_{28}N_2CA_3$ $NCA_{20}$ | F:TTGGATGACAGGAAGTGG<br>R:AACCAAAGGAAACGCACT | 46 | 5 | 0.763 9 | 0.726 0 | DQ097695 |
| Pac55HLJ | $CA_{21}$ | F:GCTGTCCATTGTCCTGAA<br>R:CAACCAACTCCACCTTTC | 51 | 5 | 0.764 4 | 0.727 3 | DQ097699 |
| Pac78HLJ | $GT_{31}$ | F:TCGGAGTGGAGCTGGTGT<br>R:GGATTGTCAAGATTCGGTA | 46 | 1 | 0 | 0 | DQ097698 |
| Pac88HLJ | $GT_{52}$ | F:TGACTACGCCTTCCACAT<br>R:TAGCCATCAACCAGCAAA | 51 | 4 | 0.613 3 | 0.536 9 | DQ097697 |
| Pac99HLJ | $TG_{22}N_2TG_8$ | F:CGACGATCAGAAGACAGA<br>R:TCAGGTGGGACATAAAGA | 51 | 6 | 0.789 1 | 0.758 8 | DQ097696 |
| Pac106HLJ | $GT_{11}$ | F:CGTAGGCAGCTCCAGAGT<br>R:CCACAGCGAGGTGAAACA | 47 | 2 | 0.375 | 0.304 7 | DQ097700 |
| Pac110HLJ | $CA_{13}N_2CA_3$ | F:TTCATTCTGGGTTCTGCC<br>R:AAGCCTCTACCAACTCCTCA | 51 | 2 | 0.375 | 0.304 7 | DQ097702 |
| Pac130HLJ | $TG_2N_6TG_{41}$ | F:TGTTGCTCATGCCCTTGT<br>R:GAGTCCATCGCTTCCTGT | 49 | 6 | 0.786 4 | 0.751 4 | DQ097701 |

Where *He* is the expected heterozygosity in a population; $n$ is the number of alleles at one locus; $p_i$, $p_j$ are the frequencies of $i$th, $j$th allele at one locus, $j = i + 1$.

## 2 Results

### 2.1 Microsatellite isolation and characteristics

Prior to double check with the radioactive labeling method, a total of 2000 clones in the first round screening were obtained. The positive clones were identified by PCR method with the universal primers. Approximately 100% clones were positive showed on the PCR results (Fig. 1). However, only 526 positive clones (26.3%) were found after the second round screening with the radioactive labeling method. Sequencing the 119 positive clones confirmed that 111 positive clones contain 133 microsatellite loci (numbers of repeats ≥ 5), 79 of which have unique flanking sequences where primers were designed. Of these sequences, only two contained compound repeat motifs (1.5%); of the remaining sequences there involved 63 perfect (47.37%) and 68 imperfect (51.13%) repeat motifs. The most frequent motifs were CA/GT repeats (93.23%) as anticipated. Though the motif CT/GA was not screened, there was a small proportion (6.77%) found in association with the target motifs.

Fig. 2 showed that the repeat length of CA/GT microsatellite loci frequently occurred in 11 – 30, and when the length of repeat motif increased, the number of microsatellite loci was on the decline.

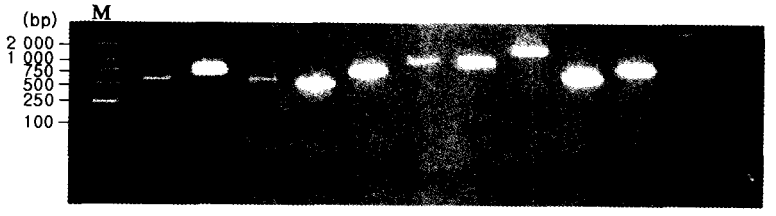


Fig. 1 Ten positive clones obtained in the first round screening

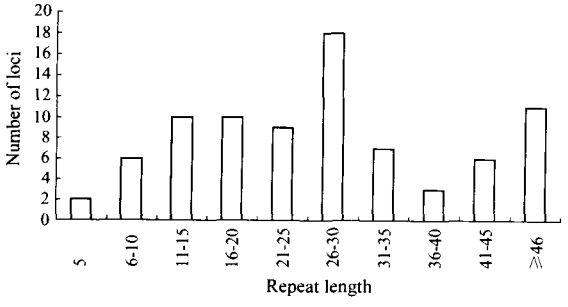


Fig. 2 Numbers of CA/GT microsatellite loci in accordance with repeat length

### 2.2 Genetic analysis of 18 microsatellite loci in *P. olivaceus*

Among 22 primer pairs designed at microsatellite loci, 18 of which amplified the target bands from the genomic DNA of 8 female *P. olivaceus*. Locus Pac78HLJ was proved monomorphic and all other loci were polymorphic. The data analysis showed that the number of alleles per locus averaged 5.2, the heterozygosity of each locus ranged between 0.375 and 0.846, and PIC ranged between 0.305 and 0.823 (Tab. 1).

## 3 Discussions

A variety of methods to isolate microsatellites were reported. However, the enriched method was the most frequently employed by many research groups (Zhang et al, 2002; Chang et al, 2005). In our lab, microsatellites have been isolated from more than 20 aquaculture species by the biotin-capture method, and detailed information of these studies is available at our web site, www.fishbreeding.org.

Theoretically, all of the clones contain the target microsatellites by the method of biotin capture (Reddy et al, 2001). However, only 10% – 90% of the clones are positive according to the results obtained in our lab and some other groups (Zhang et al, 2002; Chang et al, 2005). As shown in Fig. 1, if simply screening the library with the biotin capture method, the positive rate of clones was 100%, whereas only 26.3% were really positive through the double check with radioactive-labeled probe. This may be due to three reasons: Firstly, after DNA hybridized to biotinlated probe, a series of subsequent washing steps didn't remove the non-specific fragments completely. Secondly, the streptavidin-coated magnetic beads have been stored too long (more than six months) before they were used, which seemed a key factor impacting the positive rate (Reddy et al, 2001). Finally, the screen with the radioactive-labeled probe led to the loss of some small fragments containing microsatellites, and only a single set of oligos restricted

the potential range of microsatellite species being detected (Chang et al, 2005; Degnan & Arévalo, 2004).

Of 22 microsatellite loci, 17 were highly polymorphic, with an average of 5.2 alleles per locus, heterozygosities ranging between 0.375 and 0.846, and *PIC* varying between 0.305 and 0.823. The results indicated that a majority of the loci isolated in our research were potentially valuable for population genetic studies, marker densification on genetic linkage map (Maria et al, 2003), and marker-assisted selection (MAS) in gynogenetic families.

As shown in Fig. 2, the vast majority of microsatellite alleles were less 45 repeat units in length, which was similar to the results reported (Carleton et al, 2002; Webster et al, 2002; Garza et al, 1995). This may be caused during the evolutionary course of microsatellite by factors such as selection, biased mutation, gene conversion, and random mutation in repeat units (Garza et al, 1995; Zhang & Zhang, 2001). Moreover, the results suggested that the random mutation in repeat units (i.e., imperfection type) played an important role in constraining the divergence of average repeat numbers in different species. Mutation events of microsatellites not only involve the changes in the number of repeat units, but also involve additional types of mutations, such as insertions or deletions of non-repeat sequences, as well as single base substitutions. Especially, the base substitutions are most likely to drive the evolution of imperfect microsatellites from perfect ones, such mutations generally reduce the numbers of contiguous repeats and the propensity of slippage (Angers & Bernatchez, 1997; Garza et al, 1995; Ellegren et al, 1997).

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