Isolation of novel microsatellite markers from *Paralichthys lethostigma* (Paralichthyidae) and their cross-species application in Pleuronectiformes


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Received January 25, 2013
Accepted July 17, 2013
Published December 16, 2013
DOI http://dx.doi.org/10.4238/2013.December.16.2

**ABSTRACT.** We investigated the genetic diversity of the southern flounder *Paralichthys lethostigma*. Microsatellite-enriched libraries were constructed and novel microsatellite markers were developed and applied for genetic detection of wild populations. Cross-species amplification was also conducted in five pleuronectiforme species. Of 45 randomly selected and sequenced clones, 43 contained a CA or GA repeat motif. Fourteen pairs of primers were designed to investigate the polymorphism and genetic structure of a wild population collected from North Carolina State coastal waters. Two loci were monomorphic and 12 loci were polymorphic. The number of alleles per polymorphic locus ranged from 2 to 16, with an average of 7.3, and the expected heterozygosity per locus ranged from 0.10 to 0.92, with an average of 0.58. Cross-species amplification showed that most of the markers could successfully amplify *Paralichthys olivaceus* DNAs, few markers amplified in...
Verasper variegatus and Verasper moseri, and none of them could amplify Scophthalmus maximus and Cynoglossus semilaevis DNAs. The isolated polymorphic markers would be useful for the genetic breeding and assessment of genetic variation within the genus Paralichthys.

Key words: Paralichthys lethostigma; Microsatellite marker; Cross-species amplification; Pleuronectiformes

INTRODUCTION

Southern flounder, Paralichthys lethostigma, is an ecologically and commercially important large benthic flatfish inhabiting coastal waters of United States, which supports recreational and commercial fisheries (Froeschke et al., 2011; Renshaw et al., 2012). Farming southern flounder for food and recreational purposes started in the 1990s (Smith et al., 1999a,b; Jenkins and Smith, 1999), and now a stable spawning and seedling production technique has been established (Watanabe et al., 2006). We introduced southern flounder to China from North Carolina, USA, in 2002 (Liu et al., 2007). Its unusual ability to grow well in freshwater (Bai and Lin, 2006) and wide temperature tolerance made it a promising species for aquaculture.

To maintain long-term resource sustainability and provide a sustainable development of the aquaculture of this species, resource enhancement and genetic management of southern flounder should be carried out to assess reductions in population sizes and to aid efforts in marker-assisted selection and broodstock improvement. On the other hand, this species exhibits a sex-biased growth pattern, in which the females show better growth than males (Liu et al., 2007). Thus, all-female production by chromosome set manipulation or sex control is both of theoretical interest and economic importance. Therefore, the basic genetic information of southern flounder populations is highly required.

To study the genetic composition and variation of populations, one needs polymorphic molecular markers, such as microsatellites. Of all the molecular marker types, microsatellite markers have proven to be an extremely valuable tool for genetic study and the conservation and management of genetic resources. To date, few microsatellite sequences have been made available in GenBank and reported by other researchers (Shao et al., 2008; Renshaw et al., 2012), which has limited the assessment of the population structure, molecular phylogeny and molecular-assisted selective breeding in this marine fish species. Thus, the development of new microsatellite markers in southern flounder is very important for analyzing genome organization and evolution and for developing molecular breeding techniques.

In the present study, we developed 12 novel polymorphic microsatellite loci for southern flounder. The level of polymorphism for these markers was evaluated. Additionally, the cross utility of these markers was tested in five other flatfish species.

MATERIAL AND METHODS

DNA extraction

Thirty individuals of P. lethostigma were collected from the Center for Marine Science, University of North Carolina Wilmington, USA (34°08’N, 77°52’E). Fin clips were
sampled and genomic DNA was extracted from alcohol-preserved muscle tissues using the phenol-chloroform method as modified by Taggart et al. (1992). The extracted genomic DNA was stored at -20°C until genotyping.

**Microsatellite-enriched library construction**

Microsatellite-enriched library was conducted using the “fast isolation by amplified fragment length polymorphism of sequences containing repeats” method described in detail by Zane et al. (2002), with minor modification. Genomic DNA was digested with MseI at 37°C for 3 h and ligated with a synthesized MseI adaptor (5’-TAC TCA GGA CTC AT-3’/5’-GAC GAT GAG TCC TGA G-3’) using T4 DNA ligase (Sangon, Shanghai, China). The digestion-ligation mixture was amplified using the adaptor-specific primer (5’-GAT GAG TCC TGA GTA A-3’). Microsatellite-containing fragments were selectively enriched, captured and washed using biotinylated (CA)12 or biotinylated (GA)12 and Streptavidin Magne Sphere® Paramagnetic Particles (Promega, USA). Fragments containing microsatellites were ligated with the pMD18-T (TaKaRa, Dalian, China) vector and transformed into competent *Escherichia coli* JM109 cells (TaKaRa) by electroporation.

**Isolation of microsatellite-containing DNA fragments and primer design**

Each recombinant was subjected to three individual polymerase chain reaction (PCR) screenings using two universal sequencing primers and (CA)12DN oligonucleotide to check whether the microsatellite motif was located in the middle of the insert. In the first reaction, universal forward and universal reverse sequencing primers were used; in the second reaction, universal forward sequencing primer and (CA)12DN oligonucleotide were used; in the third reaction, universal reverse sequencing primer and (CA)12DN oligonucleotide were used. Recombinant clone-producing products of obviously different length between the first reaction and the second or the third reaction were sequenced and trimmed. The SSRHunter V1.3 software was used to scan the sequencing data (Li and Wan, 2005). Sequences with microsatellite motifs and flanking regions were selected for PCR primer design using the Primer Premier software (PREMIER Biosoft International, 2009).

**PCR amplification and genotyping**

The microsatellite primers designed were used to amplify genomic DNA of 30 *P. lethostigma* individuals, 10 *Paralichthys olivaceus* individuals, 10 *Verasper variegatus* individuals, 10 *Scophthalmus maximus* individuals, and 10 *Cynoscion nebulosus semilaevis* individuals. The PCR mixture contained 0.25 U Taq DNA polymerase (TaKaRa), 1X buffer, 1.5 mM MgCl2, 200 µM dNTP (each), 200 µM primers (each direction), and about 50 ng genomic DNA. The PCR conditions were denaturation at 94°C for 1 min, followed by 30 cycles of 1 min at 94°C, 1 min at annealing temperature, and 1 min at 72°C, with a final extension for 5 min at 72°C. The optimized annealing temperatures of different primer pairs are listed in Table 1. The PCR product was separated on 6% denaturing polyacrylamide gel and visualized by silver staining. Allele size was determined using the Quantity One V4.62 software (Bio-Rad, USA) by referring to a 20-bp DNA ladder marker (TaKaRa).
Genetic data analysis

The Popgene version 1.32 software (Yeh et al., 1999) was used to calculate the number of alleles ($N_A$), the number of effective alleles ($N_E$), observed heterozygosity ($H_O$), and expected heterozygosity ($H_E$). Polymorphism information content (PIC) of each locus was calculated according to Botstein et al. (1980). Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium tests were conducted using the Genepop online version (Rousset, 2008). Significance criteria of all multiple tests were corrected following the sequential Bonferroni correction (Rice, 1989).

RESULTS AND DISCUSSION

Two microsatellite-enriched libraries, (CA)$_n$ enriched and (GA)$_n$ enriched, were constructed using this method. One hundred randomly selected recombinant clones were selected from each library. A total of 48 clones (48%) in the (CA)$_n$ library and 38 clones (38%) in the (GA)$_n$ library were found to contain inserts with a microsatellite motif in the middle position indicating that the enrichment was highly effective. Of the 86 clones surviving PCR screening, 45 clones were sequenced, which revealed that 43 (95.5%) contained a microsatellite motif in the middle position (Table 2). According to classification rules from Weber (1990), the sequences were divided into three categories: 26 perfect repeat sequences without interruptions in the runs of CA or GA dinucleotides (60.5% of total), 14 imperfect repeat sequences with one or more interruptions in the run of repeats (32.6%), and 3 compound repeat sequences.
with adjacent tandem simple repeats of a different sequence (6.9%). When classified using the repeat sequence type, the repeat number of 15 clones was between 5 to 9, and for 28 clones, it was higher than 10.

<table>
<thead>
<tr>
<th>Criterion</th>
<th>Category</th>
<th>No. of sequences</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weber (1990)</td>
<td>Perfect</td>
<td>26</td>
<td>60.5</td>
</tr>
<tr>
<td></td>
<td>Imperfect</td>
<td>14</td>
<td>32.6</td>
</tr>
<tr>
<td></td>
<td>Compound</td>
<td>3</td>
<td>6.9</td>
</tr>
<tr>
<td>Repeat motif</td>
<td>Two bases 5 ≤ n ≤ 9</td>
<td>15</td>
<td>34.9</td>
</tr>
<tr>
<td></td>
<td>Two bases n ≥ 10</td>
<td>28</td>
<td>65.1</td>
</tr>
</tbody>
</table>

Table 2. Classification of microsatellite DNA sequences obtained from Paralichthys lethostigma in this study.

In the first batch, 14 pairs of primers (Table 1) were designed according to 20 sequences and used to investigate the polymorphism of 30 wild southern flounder individuals collected from the Center for Marine Science, University of North Carolina Wilmington, USA. All of the tested 14 primer pairs showed clear band patterns. Two loci (BCA65 and GA13) were monomorphic and twelve markers were polymorphic (Table 3). A total of 87 alleles were detected at the 12 polymorphic loci and the total effective allele numbers was 52. The \( N_A \) at each locus ranged from 2 (BCA65 and GA13) to 16 (BCA27) with an average of 7.3. The \( N_E \) ranged from 1.1 (BCA65 and GA13) to 10.3 (GA94) with an average of 4.3. The difference between \( N_A \) and \( N_E \) was caused by the uneven frequency of each allele. The \( H_O \) of each locus ranged from 0 (GA13) to 0.67 (BCA27) with an average of 0.35, and the \( H_E \) of each locus ranged from 0.10 (BCA65) to 0.92 (GA94) with an average of 0.58. According to the PIC value of each locus, two loci (BCA65 and GA13) were low polymorphic loci (PIC < 0.25), four loci (BCA68, BCA83, GA5, and GA31) were moderately polymorphic (0.25 < PIC < 0.5), and the other six markers (BCA27, BCA52, BCA70, BCA84, GA34, and GA94) were highly polymorphic (PIC > 0.5). None of the loci showed significant linkage disequilibrium. After sequential Bonferroni correction for multiple tests, three loci were found to depart significantly from HWE. Further tests indicated that heterozygote deficiency at these loci was responsible for the departure. Another possible explanation for the departure from HWE could be a dramatic decline in spawning populations and non-random mating or genetic bottlenecks.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Size range (bp)</th>
<th>( N_A )</th>
<th>( N_E )</th>
<th>( H_O )</th>
<th>( H_E )</th>
<th>PIC</th>
<th>P</th>
<th>Paralichthys olivaceus</th>
<th>Verasper variegatus</th>
<th>Verasper moseri</th>
<th>Scophthalmus maximus</th>
<th>Cynoglossus semilaevis</th>
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</thead>
<tbody>
<tr>
<td>BCA27</td>
<td>261-298</td>
<td>16</td>
<td>9.0</td>
<td>0.67</td>
<td>0.90</td>
<td>0.88</td>
<td>0.922</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BCA52</td>
<td>186-226</td>
<td>12</td>
<td>8.9</td>
<td>0.41</td>
<td>0.90</td>
<td>0.88</td>
<td>0.013</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BCA65</td>
<td>146-152</td>
<td>2</td>
<td>1.1</td>
<td>0.10</td>
<td>0.10</td>
<td>0.09</td>
<td>0.746</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BCA68</td>
<td>110-138</td>
<td>6</td>
<td>2.1</td>
<td>0.47</td>
<td>0.53</td>
<td>0.49</td>
<td>0.363</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BCA70</td>
<td>320-378</td>
<td>9</td>
<td>7.3</td>
<td>0.60</td>
<td>0.88</td>
<td>0.85</td>
<td>0.003*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BCA83</td>
<td>156-164</td>
<td>3</td>
<td>2.1</td>
<td>0.47</td>
<td>0.54</td>
<td>0.47</td>
<td>0.105</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BCA84</td>
<td>102-122</td>
<td>6</td>
<td>2.3</td>
<td>0.28</td>
<td>0.57</td>
<td>0.53</td>
<td>0.011</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>GA5</td>
<td>211-221</td>
<td>4</td>
<td>1.4</td>
<td>0.13</td>
<td>0.27</td>
<td>0.25</td>
<td>0.002*</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>GA13</td>
<td>90-96</td>
<td>2</td>
<td>1.1</td>
<td>0.00</td>
<td>0.13</td>
<td>0.12</td>
<td>0.000*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>GA31</td>
<td>180-194</td>
<td>3</td>
<td>1.6</td>
<td>0.23</td>
<td>0.39</td>
<td>0.32</td>
<td>0.113</td>
<td>-</td>
<td>-</td>
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<tr>
<td>GA34</td>
<td>121-146</td>
<td>10</td>
<td>4.8</td>
<td>0.32</td>
<td>0.80</td>
<td>0.76</td>
<td>0.017</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GA94</td>
<td>129-154</td>
<td>14</td>
<td>10.3</td>
<td>0.53</td>
<td>0.92</td>
<td>0.90</td>
<td>0.463</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

\( P = P \) value for exact test for Hardy-Weinberg equilibrium (HWE); \(*=\) departure from HWE after Bonferroni’s correction; (+) = success in cross-species amplification; (-) = unsuccess in cross-species amplification.

Table 3. Characterization of microsatellite markers developed for Paralichthys lethostigma.
Cross-amplification of the southern flounder markers was examined in five other flatfish species. The results showed that six markers (50%) successfully amplified *P. olivaceus* DNAs, two markers (16.7%) yielded amplifications in *V. variegatus*, while none of them amplified *S. maximus* and *C. semilaevis* DNAs (Table 3). The results indicated that half of the microsatellite markers could successfully amplify in different species within the genus *Paralichthys*, only a few markers could obtain cross-species amplifications in the family Pleuronectidae, and all microsatellite markers were unable to cross-amplify in species from the families Scophthalmidae and Cynoglossidae. This indicated that the microsatellite markers developed in *P. lethostigma* could be used for related species in the genus *Paralichthys*. All of the polymorphic markers would be useful for the future genetic breeding and the assessment of genetic variation within *Paralichthys*.

ACKNOWLEDGMENTS

Research supported by the China Agriculture Research System (#CARS-50) and the National High Technology Research and Development Program, China (#2012AA10A413). We thank Dr. Wade O. Watanabe for providing fin clip samples of wild southern flounder population at the Center for Marine Science, University of North Carolina Wilmington, USA.

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