Isolation and Characterization of Polymorphic Microsatellite Markers for the Black Rockfish Sebastes inermis

Hye Suck An¹*, Kwang Soo Kim², Hae Yeong Lee³, En Mi Kim¹ and Mun Gyeong Kwon²

¹Biotechnology Research Institute, National Fisheries Research and Development Institute, Busan 619-902, Korea
²Fisheries Resources Restoration Development and Management Center, National Fisheries Research and Development Institute, Busan 619-902, Korea
³Aquafeed Research Center, National Fisheries Research & Development Institute, Pohang 791-923, Korea

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ABSTRACT

Black rockfish (Sebastes inermis, Scorpaenidae) are a commercially important fisheries resource in Korea. However, no genetic diversity information is available for wild or released hatchery populations. To study the effects of stock enhancement programs and conservation biology, we developed microsatellite DNA markers from S. inermis. We report the isolation and characterization of seven microsatellite loci, designated KSi221B, KSi239A, KSi25A, KSi252B, KSi193A, KSi259B, and KSi234, isolated using an enrichment method based on magnetic/biotin capture of microsatellite sequences from a size-selected genomic library. To characterize each locus, 30 individuals from a natural S. inermis population in Yeosu, Korea, were genotyped. All loci except one, KSi193A, were polymorphic with an average of 9.5 alleles per locus (range 2−20). The mean observed and expected heterozygosities were 0.71 (range 0.43−1.00) and 0.76 (range 0.41−0.90), respectively. Significant deviation from Hardy-Weinberg equilibrium was observed at one locus, KSi221B. Such high variability indicates that these microsatellites should provide useful markers for studies of kinship and population genetics.

Key words: Black rockfish, genetic marker, heterozygosity, microsatellite, Sebastes inermis.

INTRODUCTION

The black rockfish (Sebastes inermis) is a commercially important species in coastal waters from southern Hokkaido to Kyushu, Japan, and along the southern Korean Peninsula (Utaka and Taniuchi, 1991). Planktonic S. inermis larvae and small juveniles recruit to shallow Zostera beds and then gradually migrate to deeper habitats as adults (Harada, 1962; Love, 1991). Black rockfish have long been considered a common fisheries resource in southern Korea. However, production has drastically declined since the late 1990s as a result of several factors, including overexploitation, disease, and deteriorated water quality. To enhance production, large numbers of cultured fry have been released into the southern Korean coastal sea since 2006, and artificial reproduction and cultivation have been practiced since 2000. This has led to an increase in the need for ge-
netic information on black rockfish. In addition, knowledge of the population genetics of black rockfish in these areas is necessary for the adequate management of the genetic resources of wild and cultivated stocks. However, no reports of population genetics of black rockfish have been published to date. Therefore, we sought to develop species-specific markers for population genetic studies.

Microsatellite loci are highly informative genetic markers for studying population genetics because they are evenly dispersed throughout genomes, usually characterized by high length polymorphism, and generally inherited in a Mendelian fashion (Knapik et al., 1998; Cho et al., 2000; Desvignes et al., 2001; Holland, 2001; Bhatia and Arora, 2007). We developed primer sets for seven microsatellites from the S. inermis DNA library enriched with (CA)n repeats and estimated the genetic variability at these loci in a wild population of black rockfish in Korea.

MATERIALS AND METHODS

DNA extraction from black rockfish

To construct the genomic DNA library, the TNES-urea buffer method (Asahida et al., 1996) was used to isolate high-molecular-weight DNA (20 μg) from the mantle musculature of a black rockfish individual captured near the southern coast of Korea.

Isolation of microsatellite-containing DNA fragment

We constructed a partial genomic library enriched for CA repeats using a slightly modified enrichment procedure with pre-hybridization PCR amplification as described elsewhere (Gardner et al., 1999: Hamilton et al., 1999). Extracted DNA was digested with the restriction enzymes AluI, Rsal, NheI, and HhaI (New England Biolabs, Beverly, MA, USA). DNA fragments ranging from 300–800 bp were isolated and purified using the QiAquick Gel Extraction Kit (Qiagen, Hilden, Germany). The selected fragments were ligated to an adaptor (SNX/SNX rev linker sequences). Linker-ligated DNA was amplified using SNX as a linker-specific primer for PCR. For enrichment, the DNA was denatured, and biotin-labeled dinucleotide repeat sequences [(CA)12, GCTTGA; Carleton et al., 2002] were hybridized to the PCR products. The hybridization complex was lifted with streptavidin-coated magnetic spheres (Promega, WI, USA). After washing, the bound enriched DNA was eluted from the magnetic spheres. PCR amplification was performed with an adapter sequence primer. PCR products were purified using a QiAquick PCR purification kit (Qiagen).

Cloning and sequencing of microsatellite loci

The purified PCR products were digested with the enzyme Nhel, cloned using the XbaI-digested pUC18 vector (Pharmacia, NJ, USA), and transformed into Escherichia coli DH5α competent cells. Each small portion of white colonies was screened for the presence of a repeat insert using PCR with universal M13 primers and the nonbiotin-labeled (CA)10 primer (Li et al., 2002). PCR products were checked on 2% agarose gels, and inserts producing two or more bands were considered to contain a microsatellite locus. Positive clones were cultured and purified. Plasmids from insert-containing colonies were recovered using a QiAprep Spin Miniprep Kit (Qiagen) and sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, version 3.1, CA, USA) and an automated sequencer (ABI Prism 310 Genetic Analyzer, PE Applied Biosystems). Primer designs were based on sequences flanking the microsatellite motifs using the OLG: software package (version 5.0, National Biosciences Inc.).

Assessment of polymorphisms in microsatellite loci

We tested newly designed PCR primer pairs to optimize a distinct amplification using a gradient PCR with a 50–60°C range of annealing temperatures. To characterize each amplified locus, primer pairs were tested on a random sample of 30 black rockfish captured near the southern coast of Korea for polymorphisms in microsatellite loci. PCR amplification was performed in a 10 μl reaction volume containing 0.25 U of ExTag DNA polymerase (TaKaRa Biomedical Inc., Shiga, Japan), 1 × PCR buffer, 0.2 mM dNTP mix, 10 pmol of each primer (the forward primer from each pair was 5’ end-labeled with 6-FAM, NED, and HEX dyes; PE Applied Biosystems), and 100 ng of template DNA using
a PTC 200 DNA engine (MJ Research). PCR reactions were as follows: 11 min at 95 °C followed by 35 cycles of 1 min at 94 °C, 1 min at annealing temperature (listed in Table 1), and 1 min at 72 °C, with a final extension of 5 min at 72 °C. Microsatellite polymorphisms were screened using an ABI PRISM 3100 Automated DNA Sequencer (PE Applied Biosystems), and alleles were designated according to the PCR product size relative to a molecular size marker ([GENESCAN 400 HD [ROX]]; PE Applied Biosystems). Fluorescent DNA fragments were analyzed using the GENESCAN (version 3.7) and the GENOTYPER (version 3.7) software packages (PE Applied Biosystems).

### Statistical analysis

The number of alleles per locus, expected and observed heterozygosities, linkage disequilibrium among loci, and the exact test of deviations from Hardy-Weinberg equilibrium (HWE) were calculated using GENEPOP 3.1 software (Raymond and Rousset, 1995) and ARLEQUIN 2.0 software (Schneider et al., 2000).

### RESULTS

Of 808 white colonies screened, 313 clones initially appeared to be positive by the above PCR-based technique. Of these, 310 clones were sequenced, and 174 loci contained microsatellite arrays with a minimum of five repeats. These were primarily 2-bp repeat motifs, some of which were in combination with other 2-bp repeat motifs. Primers were developed for 52 loci that exhibited long, adequate unique sequence regions flanking the microsatellite array. Only seven primer sets, designated KSi221B, KSi239A, KSi25A, KSi252B, KSi193A, KSi259B, and KSi234, successfully yielded variable profiles consisting one or two bands. With the exception of KSi193A, all loci were polymorphic, although the degree of variability differed at each locus. Primer sequences, repeat motifs, annealing temperature, the number of alleles, amplified product size range, and the observed (H_o) and expected (H_e) heterozygosities for the six polymorphic microsatellite loci are summarized in (Table 1). The number of alleles per locus ranged from two (KSi239A) to 20 (KSi25A), with a mean of 9.5 alleles per locus, and all loci were dinucleotide repeats. The mean observed and expected heterozygosities were calculated as 0.71 (range 0.43–1.00) and 0.76 (range 0.41–0.90), respectively. Significant deviation from HWE for the observed heterozygosities was observed at one locus, KSi221B (P < 0.01). The allele frequency distributions indicated 26 rare alleles (frequency < 5%) of a total of 57 alleles summed over all loci, for a mean of 45.6%. Most rare alleles (22) were detected

### Table 1. Seven newly isolated microsatellite loci from *Sebastes inermis.*

<table>
<thead>
<tr>
<th>Locus</th>
<th>Repeat motif</th>
<th>Primer sequence (5’→3’)</th>
<th>Ta(°C)</th>
<th>Product size (bp)</th>
<th>No. of Allele size range (bp)</th>
<th>H_o</th>
<th>H_e (P-value)</th>
<th>Genebank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>KSi221B</td>
<td>(TG)_6(TG)_11</td>
<td>F: CATCTGCGGGCGCTGCTG ned R: ACTCCAGCTGCTAACCCTATC</td>
<td>60</td>
<td>229</td>
<td>6</td>
<td>12</td>
<td>0.60*</td>
<td>0.77 (0.000)</td>
</tr>
<tr>
<td>KSi239A</td>
<td>(TG)_5(TG)_4(TG)_7</td>
<td>F: GCATGTGCGTTTGTCTG ned R: GATGAAGGAAAGATCGAACAC</td>
<td>50</td>
<td>132</td>
<td>2</td>
<td>14</td>
<td>0.43</td>
<td>0.41 (1.000)</td>
</tr>
<tr>
<td>KSi25A</td>
<td>(TG)_5</td>
<td>F: AGAGAAAGGAGGGAGCTGAAGATGATAC fam R: TOGATATGTGAAGATGATCAC</td>
<td>55</td>
<td>169</td>
<td>20</td>
<td>56</td>
<td>1.00</td>
<td>0.90 (0.674)</td>
</tr>
<tr>
<td>KSi252B</td>
<td>(CA)_9(CG)(CA)_5</td>
<td>F: CTCATCGCTCTGGCTGCTG fam R: GGGACACACGTGCTACACATC</td>
<td>60</td>
<td>152</td>
<td>7</td>
<td>14</td>
<td>0.63</td>
<td>0.79 (0.159)</td>
</tr>
<tr>
<td>KSi193A</td>
<td>(CA)_9(CA)_9</td>
<td>F: ACTGGCAGCTCTTACCTGCA hex R: CCTCTGTGCTGCTGACCTG</td>
<td>50</td>
<td>110</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>KSi259B</td>
<td>(TG)_5(TA)_10(TG)_5</td>
<td>F: CTGTGCGCTGCTGCTGCTG hex R: ATGTCTCTTTAAACCCGATCACC</td>
<td>63</td>
<td>107</td>
<td>16</td>
<td>16</td>
<td>0.83</td>
<td>0.83 (0.258)</td>
</tr>
<tr>
<td>KSi234</td>
<td>(GT)_7(GT)_8</td>
<td>F: GTGCGGCTGCTGCTGCTG hex R: CTTGCTCTCTTAAACCCGATCATC</td>
<td>50</td>
<td>108</td>
<td>6</td>
<td>20</td>
<td>0.97</td>
<td>0.86 (0.464)</td>
</tr>
</tbody>
</table>

Ta is the optimal annealing temperature; † Primers were 5’ end labeled with the indicated dye. H_o is the observed heterozygosity, H_e is the expected heterozygosity. Exact tests of Hardy–Weinberg equilibrium showed significant heterozygote deviation (*P < 0.01)*
at loci KSi25A and KSi259B. No significant linkage disequilibrium between loci pairs was detected ($P > 0.05$).

**DISCUSSION**

We identified and characterized the first microsatellite markers for the black rockfish, *S. inermis*. We created microsatellite libraries enriched for CA repeat sequences following the protocol of Hamilton et al. (1999) with modifications described by Gardner et al. (1999) and Carleton et al. (2002). Of the positive clones obtained, only 21.5% contained microsatellite repeats (174 of 808), which is lower than that for tilapia (96%; Carleton et al., 2002) and Japanese Spanish mackerel (34%; Yokoyama et al.,

**Figure 1.** Allele frequency (y-axis) and allele base-pair length (x-axis) for six black rockfish microsatellite loci (A–F) in a wild population from Yeosu, Korea.
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2006). In the case of tilapia, a variation of the hybrid capture method was used. Several enriched libraries differed in the selected size of the restricted genomic DNA should be constructed. Nevertheless, we thought the less proper professional judgment when carrying out our procedures might be the reason for the less enrichment efficiency.

Six of seven new microsatellites from *S. inermis* were polymorphic, showing two to 20 alleles per locus, and observed heterogeneities ranged from 0.63 to 1.00. Although high levels of polymorphism were present at all six microsatellite loci, the levels of genetic diversity were slightly lower than those of other marine fishes (see review by DeWoody and Avise, 2000). It is possible that our results represent an underestimate due to the small population of rockfish sampled. It should therefore be noted that the small sample sizes used in our study present clear limitations on the power of the statistical tests used to detect spatial heterogeneity.

One of the six polymorphic microsatellite loci deviated from HWE due to a deficiency in heterozygosity. Relevant heterozygote deficiencies have been reported for many fish species (Waldman and McKinnon, 1993; Hoarau et al., 2002). When using microsatellite markers, it can be difficult to determine whether an excess or deficit of heterozygotes represents a real biological phenomenon or is a technical artifact of PCR amplification or scoring of microsatellite loci (Jones et al., 1998; Li et al., 2002). A deviation from HWE can result from selection, population mixing, or nonrandom mating (Rousset and Raymond, 1995) or from sampling a single population with different allele frequencies in subpopulations (i.e., the Wahlund effect) and selective forces in aquaculture. In addition, presence of null alleles might account for the observed deviation from HWE. Null alleles of microsatellite regions, which occasionally fail to yield an amplification product, may arise through mutations such as point mutations in the primer annealing site (Callen et al., 1993; Pemberton et al., 1995).

The high variability of the microsatellite markers identified in this study will make them to useful genetic markers for monitoring the genetic diversity and structure of *S. inermis* populations. Further studies using these microsatellite markers to investigate genetic differences between wild and cultivated populations of *S. inermis* are in progress.

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