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Development and characterization of microsatellite markers for an endangered species, *Epinephelus bruneus*, to establish a conservation program

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Kelp grouper (*Epinephelus bruneus* Bloch 1793) is a commercially important fish in Korea. In recent years, the catch of kelp grouper in the coastal waters of Korea has significantly declined. Despite its importance, little is known about its genetic diversity and conservation efforts are hampered. In this study, we isolated and characterized 12 microsatellite loci using an enrichment method based on magnetic/biotin capture of microsatellite sequences from a size-selected genomic library. All loci were readily amplified and contained TG/CA denucleotide repeats. To characterize each locus, 30 individuals from a natural *E. bruneus* population in the coastal waters of Jeju Island, Korea, were genotyped. All loci except three, KEm118, KEm154, and KEm219, were polymorphic, with an average of 8.1 alleles per locus (range 2–18). The mean observed and expected heterozygosities were 0.47 (range 0.19–1.00) and 0.61 (range 0.29–0.92), respectively. A significant deviation from Hardy-Weinberg equilibrium was observed at three loci (KEm134, KEm184, and KEm283). These findings will be useful for effective monitoring and management of genetic variation of kelp grouper as well as for the implementation of a fisheries conservation program.

Keywords: kelp grouper; *Epinephelus bruneus*; heterozygosity; microsatellite; genetic marker

Introduction

The kelp grouper (*Epinephelus bruneus*, Perciformes Serranidae) is narrowly distributed in tropical and temperate oceans of Korea, Japan, China, and Taiwan. It inhabits rocky reefs and mud bottoms. Adults can be found at depths of 20 to 200 m, while juveniles occur in shallow (Heemstra and Randall 1993). *E. bruneus* has been enlisted as Endangered in Earth's Endangered Creatures (EEC) (http://earthsendangered.com) and as Vulnerable in the International Union for the Conservation of Nature and Natural Resources (IUCN) Red List Category & Criteria (www.iucnredlist.org) due to the sharp decline in the catch data. In Korea, *E. bruneus* is an important and very expensive marine fish species. In response to decreases in natural resources and increases in prices, the aquacultural production of kelp grouper has been attempted for a long time. Recently, complete culturing, including reproduction control, captive spawning, hatching, and larval and juvenile rearing, became possible on a small scale.

To develop appropriate recovery and conservation management plans, an understanding of the population structure, gene flow, and genetic diversity within and among populations of a species is crucial. Marine species populations usually have low levels of geographical differentiation because of the relative lack of barriers to gene flow (Ward et al. 1994). However, there is increasing evidence that marine species exhibit greater population differentiation than that expected based on dispersal capabilities alone (Riginos and Nachman 2001; Banks et al. 2007; Hedgecock et al. 2007; Pampoulie et al. 2008). These research findings support a need for more available genetic information about *E. bruneus*.

Despite the urgent need for conservation, little is known about the species genetic diversity, which is of importance for planning a conservation strategy (Frankham et al. 2002). Furthermore, currently no molecular markers are available. Suitable DNA markers are needed to identify single individuals and to assess parentage and genetic relationships. To address this issue, we have attempted to develop species-specific genetic markers for population genetic studies of kelp grouper.

Among available genetic markers, microsatellites are recognized as an essential tool in population studies because of their useful properties, such as high levels of polymorphism, codominant inheritance, and good reproducibility (Tauz 1989; Feral 2002; Liu and Cordes 2004). Over the past decade, microsatellites have produced promising results in studies of genetic variation in many marine species (Castro et al. 2003; Cruz et al. 2004; Chistiakov et al. 2006; An et al. 2010). Recently, numerous microsatellite markers have been identified for endangered species that can serve as

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genetic tools to help us create a more accurate picture of pattern and process in endangered species (DeSalle and Amato 2004). Analysis of the current endangered population state of a population or species using microsatellite markers is helpful in the management of retrieval programs for endangered species.

In this paper, we report the development of primer sets for the 12 microsatellite loci from an *E. bruneus* DNA library enriched for $(CA)_n$ repeats, and the genetic variability at these loci in a wild population of kelp grouper in Korean coastal waters.

Materials and methods

DNA extraction from kelp grouper

For microsatellite isolation and genetic characterization analysis, a non-destructive sampling method was employed and only fin-clips of each sample were collected. For genomic DNA isolation for microsatellite-enriched partial genomic library construction, fin clips were collected from a kelp grouper individual and for genotyping, from a total of 30 individuals. All kelp grouper used in this study were sampled from kelp groupers which were captured near the coastal waters of Jeju Island, Korea, and have been kept for a conservation program by the National Fisheries Research & Development Institute. All the samples were placed in absolute ethanol and kept frozen at $-20°C$ until DNA extraction. The TNES-urea buffer method (Asahida et al. 1996) was used to isolate high-molecular-weight DNA for microsatellite isolation. For genotyping, total DNA was extracted using a MagExtractor-genomic DNA purification kit (Toyobo, Japan) for the automated MagExtractor MFX–2100 DNA extraction system (Toyobo, Japan). The extracted genomic DNA was stored at $-20°C$ until being used for PCR.

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 (Gardner et al. 1999; Hamilton et al. 1999). Extracted DNA (20 μg) was digested with the restriction enzymes *Alu*I, *Rsa*I, *Nhe*I, and *Hha*I (New England Biolabs, Beverly, MA, USA). DNA fragments ranging from 300 to 800 bp were isolated and purified using a QIAquick Gel Extraction kit (Qiagen, Hilden, Germany). The selected fragments were ligated to an adaptor (SNX/SNX rev linker sequences), and the ligated DNA was amplified by PCR using SNX as a linker-specific primer. For enrichment, the DNA was

denatured, and biotin-labeled dinucleotide repeat sequences $((CA)_{12}GCTTGA;$ Li 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 adaptor sequence primer, and the PCR products were purified using a QIAquick PCR purification kit (Qiagen).

Cloning and sequencing of microsatellite loci

The purified PCR products were digested with *Nhe*I and ligated into *Xba*I-digested pUC18 vector (Pharmacia, Piscataway, NJ, USA). The construct was used to transform *Escherichia coli* DH5α competent cells. A small portion of each white colony was screened for the presence of a repeat insert, using PCR with universal M13 primers and the non-biotin-labeled $(CA)_{10}$ primer (Li et al. 2002). The PCR products were checked on 2% agarose gels, and the inserts producing two or more bands were considered to contain a microsatellite locus. Positive clones were cultured and used for purification of plasmid using a QIAprep Spin Miniprep kit (Qiagen) and sequenced using a BigDye Terminator Cycle Sequencing Ready Reaction kit (ver. 3.1; Applied Biosystems, Foster City, CA, USA) and an automated sequencer (ABI Prism 310 Genetic Analyzer; Applied Biosystems). Primer designs were based on sequences flanking the microsatellite motifs, using OLIGO software (ver. 5.0; National Biosciences, Plymouth, MN).

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 for polymorphisms using samples from 30 randomly selected kelp groupers (body length, average 52.5 cm; body weight, average 3.67 kg). PCR amplification was performed in a 10-μl reaction volume containing 0.25 U of Ex*taq* DNA polymerase (TaKaRa Biomedical Inc., Shiga, Japan), $1 \times$ PCR buffer, 0.2 mM dNTP mix, 10 pmol of each primer, and 100 ng of template DNA, using a PTC 200 DNA engine (MJ Research). The forward primer of each pair was 5′ end-labeled with 6-FAM, NED, and HEX dyes (Applied Biosystems). 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.

Table 1. The characteristics of the 12 microsatellite loci isolated from *Epinephelus bruneus*.

Locus	Repeat motif	Primer sequence (5'→3')	Ta (°C)	No. of alleles	Allele size range (bp)	H_o	H_E (P-value)	Genbank accession no.
KEm118	$(GT)_{21}AT(GT)_{11}$	F: GGTGGTTTATGCCTGAGAGT **fam** R: TCCTCTAGCAGAAGCAGTTTT	54	1	240	–	–	JF690181
KEm134	$(CA)_5$-$(CA)_5$	F: ACGTGCGTGCTAAGACAC **hex** R: GGCCGTCTACCTCTTACTGAT	50	3	130–134	0.34	0.40*(0.0000)	JF690186
KEm140	$(GT)_6$-$(GT)_6$-$(GT)_5$-$(GT)_6$	F: ACGATAGGAAAAGGAAGGAAGATGA **fam** R: GGGGATCCTCTAGCAGAAG	58	5	256–266	0.69	0.72 (0.3020)	JF690183
KEm154	$(CA)_{13}$	F: GCACGCACACTTACACAC **hex** R: CTAATGTGCTTCTTCACAAAG	54	1	160	–	–	JF690187
KEm164	$(GT)_{18}$	F: CTGTAACATCAAGCCGTCAT **ned** R: GTATCCTCACAATTGCTCCT	56	2	188–190	0.29	0.44 (0.5415)	JF690190
KEm174	$(GT)_4$-$(GT)_4$-$(GT)_6$	F: GCTCTAGTGTGCTTACCTGTG **ned** R: ATGACCCAGAAGAAACTCCTAC	54	3	178–182	0.26	0.29 (0.4027)	JF690191
KEm184	$(GT)_{10}$-$(GT)_5$	F: AGTCAAACTACACCGCCTAAG **ned** R: GGATCCTCTAGCAGAAGCAC	54	4	180–192	0.19	0.51*(0.0000)	JF690192
KEm210	$(CA)_9$-GA$(CA)_{13}$-GA$(CA)_5$	F: AGTTTGGTCAATGTTATCCTG **fam** R: TCACACCACCTTCATAAGAAAC	54	18	210–250	1.00	0.90 (0.1056)	JF690182
KEm219	$(CA)_7$-TA$(CA)_{13}$-TA$(CA)_{12}$	F: TCAATATTATCTGGTCAGGTG **hex** R: CGGACAGACAGGTGAGAG	58	1	130	–	–	JF690185
KEm228	$(GT)_{18}$	F: GACAACATATGCACACCAAAC **ned** R: GGGGATCCTCTAGCAGAAGC	50	2	110–112	0.34	0.41 (0.5415)	JF690189
KEm276	$(CA)_{20}$	F: CCAGCATGGATATTCTCTCAT **hex** R: AACAAATGCATTCAGTCTCCT	56	22	96–152	1.00	0.92 (1.0000)	JF690188
KEm283	$(CA)_9$-GA$(CA)_{13}$-GA$(CA)_5$	F: AGTTTGGTCAATGTTATCCTG **fam** R: CACCTTCATAAGAACCCTTTA	54	14	204–234	0.70	0.89*(0.0000)	JF690184

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 ($P < 0.01$).

Microsatellite polymorphisms were screened using an ABI PRISM 3100 automated DNA sequencer (Applied Biosystems), and alleles were designated according to the PCR product size relative to molecular size markers (GeneScan-400HD (ROX); Applied Biosystems). Fluorescent DNA fragments were analyzed using GeneScan (ver. 3.7) and Genotyper (ver. 3.7) software (Applied Biosystems).

Statistical analysis

MICRO-CHECKER 2.2.3 (van Oosterhout et al. 2004) was used to detect genotyping errors due to null alleles, stuttering, or allele dropout (1000 randomizations). The number of alleles per locus, expected and observed heterozygosities, linkage disequilibrium among loci, and exact test of deviations from Hardy-Weinberg equilibrium (HWE) were calculated using GENEPOP software (ver. 3.1; http://kimura.univ-montp2.fr/ ~rousset/Genepop.htm) and ARLEQUIN software (ver. 2.0; Schneider et al. 2000). Significance levels were adjusted for multiple tests using the Bonferroni correction technique (Rice 1989).

Results

In total, more than 500 white colonies were obtained from the transformation with the Korean kelp grouper $(CA)_n$-enriched genomic DNA library. Among them, each small portion of about 300 colonies was tested for the presence of a repeat-containing insert by PCR. Finally, 110 insert-containing colonies were obtained. The positive clone rate was 36.7%. All positive colonies were sequenced. Sequencing of the inserts revealed 65 loci containing microsatellite arrays with a minimum of five repeats. These were primarily 2-bp repeat motifs, some of which were combined with other 2-bp repeat motifs. Primers were designed and tested for 65 loci that exhibited adequately long and unique sequence regions flanking the microsatellite array. Of these 65 loci, only 12 primer sets (18.5%; KEm118, KEm134, KEm140, KEm154, KEm164, KEm174, KEm184, KEm210, KEm219, KEm228, KEm276, and KEm283) successfully yielded variable specific PCR products. Although the PCR conditions were optimized by adjusting the dNTP concentrations and using an annealing temperature gradient, the remaining 53 primer sets (81.5%) gave either inconsistent or no clear PCR products. The primer sequences, repeat motifs, annealing temperatures, numbers of alleles, amplified product size ranges, and observed (H_O) and expected (H_E) heterozygosities for the 12 new microsatellite loci are summarized in Table 1.

With the exception of KEm118, KEm154, and KEm219, all loci were polymorphic, with different degrees of variability. The number of alleles per locus ranged from two (KEm164 and Kem228) to 22 (Kem276), with a mean of 8.1 alleles per locus, and all loci were dinucleotide repeats. The mean observed and expected heterozygosities were 0.47 (range 0.19–1.00) and 0.61 (range 0.29–0.92), respectively.

Significant deviation from HWE after Bonferroni correction was observed at three loci, KEm134, KEm184, and KEm283 ($P < 0.01$); owing to their heterozygosity deficit condition, these should be used with caution. Micro-Checker analysis revealed that the loci KEm134, KEm184, and KEm283 may be affected by null alleles. The allele frequencies of the 12 microsatellites are shown in Figure 1. Allele frequency distributions indicated 33 rare alleles (frequency < 5%) 5%) out of a total of 73 alleles summed over all loci except three monomorphic loci, for a mean of 45.2%. The rare alleles were detected at most loci and were never associated with a particular locus. In total, 36 pair-wise tests for linkage disequilibrium among nine polymorphic loci were not significant ($P > 0.05$) except for two pairs of loci (KEm184 and KEm283; KEm140 and KEm283).

Discussion

Monitoring the genetic variance of successive generations of kelp grouper populations is essential for the successful implementation of a recovery and conservation program. Microsatellite DNA is suitable for monitoring population-level phenomena. The basic requirement of any culture program is the existence of genetic variation because a reduction in genetic variation can diminish the ability of a population to respond to other stressors. Therefore, an important aspect of cultured populations is that exposure to inbreeding could be induced even in large stocks by behavioral, physiological and other factors. Random drift and loss of variability can occur if renewal of stocks is practiced using related individuals, if a few individuals monopolize the sperm or egg pool or if the sex ratio becomes strongly biased. Hence, continued genetic monitoring of the cultured populations is warranted. It seems that the development of molecular markers and especially microsatellites has provided the solution to this problem, because it is now possible, by using a small number of microsatellite loci, to give a unique genetic identity to each parent in a mass spawning and to identify its progeny and to assess parentage and genetic variation. To this end, we identified and characterized the first microsatellite markers for kelp grouper.

We created microsatellite libraries enriched for CA repeat sequences following the protocol of Hamilton et al. (1999), with modifications described by Gardner
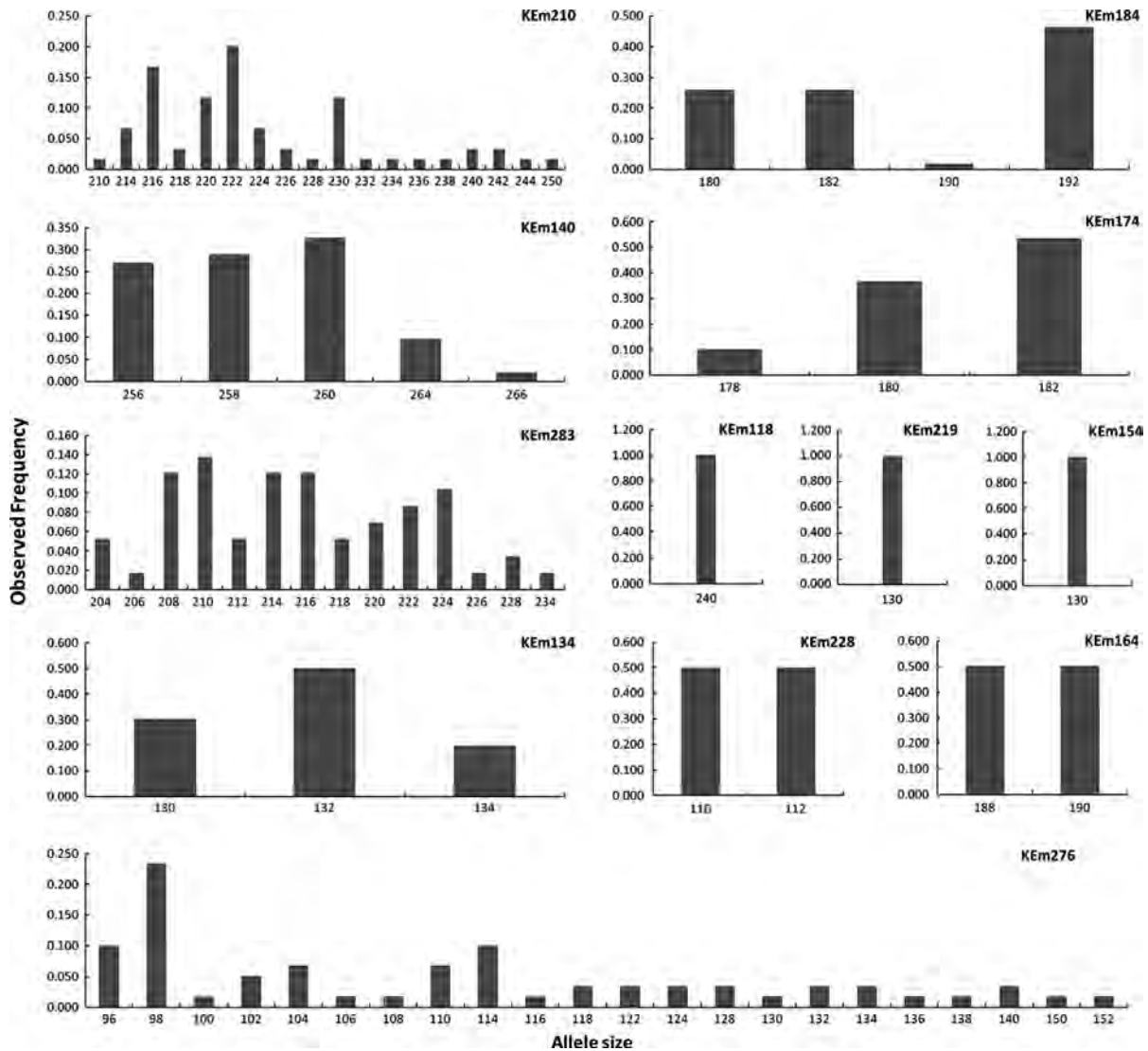
Figure 1. Allele distributions of the 12 microsatellite loci of *Epinephelus bruneus* used in this study.

et al. (1999) and Carleton et al. (2002). Of the positive clones obtained, about 36.7% contained microsatellite repeats (110 of 300); this number is lower than that for tilapia (96%; Carleton et al. 2002), but similar to that for rock carp (34%; Wang et al. 2010). In the case of tilapia, a variation of the hybrid capture method was used, which is probably a reflection of the relative complexity of several enriched libraries with different size selection of the restricted genomic DNA. The professional judgment we used in conducting some analytical procedures may explain the lower enrichment efficiency. Although the number of sequences surveyed in this study was limited, our analysis showed that the relative abundance of microsatellites in the *E. bruneus* genome was useful as a general source of microsatellite markers.

Three of the 12 primer pairs were monomorphic. Nine of the 12 new microsatellite loci from kelp grouper were polymorphic, showing two to 22 alleles per locus, with expected heterozygosities ranging from 0.29 to 0.92 ($H_E = 0.61$). The levels of genetic diversity were lower than those of most other marine fishes (see review by DeWoody and Avise 2000). However, lower levels of microsatellite polymorphism have been demonstrated in wolf fish, *Hoplias malabaricus* ($H_E = 0.14–0.51$; Gondim et al. 2010) and armored catfish, *Hypostomus gymnorhynchus* ($H_E = 0.18–0.90$; Telles et al. 2010). It is interesting to note that the locus KEm276 has the most alleles and the observed heterozygosity of 1 is very extreme, but most loci show lower levels of polymorphism due to lower mutation rate. Brandström and Ellegren (2008) observed a

relationship between microsatellite length and degree of polymorphism, and concluded that most repeats are 'perfect' repeats with no interruptions; however, when interruptions were present in more than 15% of the loci, polymorphism was significantly lower compared with that associated with perfect repeats.

Three of the eight polymorphic microsatellite loci deviated from HWE because of a deficiency in heterozygosity. Other researchers have shown that microsatellite markers deviating from HWE were usually attributable to deficiencies of heterozygotes (Hoarau et al. 2002; An et al. 2005, 2009). Heterozygote deficiency can increase because of several factors, including the presence of unrecognized null alleles, inbreeding, admixture of independent populations (the Wahlund effect), artificial and natural selection during seed production and artificial or natural selection acting on the genetic markers (Bohonak 1999; Li et al. 2002). Heterozygote deficiency has been reported for many other marine invertebrates (Evans et al. 2004; Zhang et al. 2005). However, in many cases, its cause remains unknown (Addison and Hart 2005). Based on an analysis of our Micro-Checker results, null alleles appear to be a likely explanation. Null alleles of microsatellite regions, which occasionally fail to yield an amplification product, can arise through mutations such as point mutations in the primer annealing site (Callen et al. 1993). A high frequency of null alleles may complicate many types of population genetic analyses that rely on HWE (Pemberton et al. 1995).

The microsatellite primers developed in this study would greatly benefit the understanding of genetic diversity of the endangered *E. bruneus*. Moreover, they may be very useful for planning management of the captive stocks of *E. bruneus* that are now reared in NFRDI, Korea, as a part of the conservation program.

Acknowledgements

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