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Ten new microsatellite markers in cutlassfish *Trichiurus lepturus* derived from an enriched genomic library

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Cutlassfish (*Trichiurus lepturus* Linnaeus 1758) is a commercially important fish in Korea. In recent years, the catch of cutlassfish in the coastal waters of Korea has significantly declined. Its genetic characterization has been little studied. To assist conservation and management efforts, we isolated and characterized 10 microsatellite loci 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 *T. lepturus* population in the coastal waters of Jeju Island, Korea, were genotyped. All loci except two, KTh9B and KTh22A, were polymorphic, with an average of 14.3 alleles per locus (range, 10–22). The mean observed and expected heterozygosities were 0.80 (range, 0.50–0.97) and 0.82 (range, 0.68–0.95), respectively. A significant deviation from Hardy-Weinberg equilibrium was observed at three loci (KTh6B, KTh10, and KTh16). This high variability indicates that these microsatellites may be useful for high-resolution studies of population genetics.

Keywords: cutlassfish; heterozygosity; microsatellite; *Trichiurus lepturus*; genetic marker

Introduction

The cutlassfish (*Trichiurus lepturus*) is found in tropical and temperate waters throughout the world, including the Yellow Sea, East China Sea, South China Sea, and west and south coastal waters of Korea (Froese and Pauly 1997). As an economically important demersal species caught year-round off the coast of Korea, cutlassfish are intensively harvested by a variety of methods such as bottom trawling, longline, handline, gillnet, drift net, and purse seine. The annual catch of cutlassfish in Korea reached its peak at 166,390 metric tonnes (t) in 1974, before making an abrupt decline to approximately 72,313 t in 2008 (Kang 2007). Owing to the severe decline of its population in recent years, the cutlassfish has been listed as a species for resource recovery in Korea.

Coastal nations of the world recognize the urgent need to ramp up protection of marine fisheries, using both recovery and conservation management strategies. 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 1994; Banks et al. 2007; Hedgecock et al. 2007; Pampoulie et al. 2008). These research findings support a need for more available genetic information about the cutlassfish.

Although cutlassfish are important commercially, little is known about the genetics of stocks in Korean waters, and currently no molecular markers are available. To address this issue, we have attempted to develop species-specific genetic markers for population genetic studies of cutlassfish.

Microsatellite loci are increasingly replacing or complementing other markers for numerous applications in evolutionary and conservation genetics (Jarne and Lagoda 1996). Their high level of polymorphism provides the potential to define unique multilocus genotypes. This, coupled with their relative ease of identification using PCR and the reliability of allelic determination as compared with other methods, makes microsatellites particularly useful in population genetic studies. In this paper, we report the development of primer sets for 10 microsatellite loci from a *T. lepturus* DNA library enriched for (CA)n repeats, and the genetic variability at these loci in a wild population of cutlassfish in Korean coastal waters.

Materials and methods

DNA extraction from cutlassfish

The TNES-urea buffer method (Asahida et al. 1996) was used to isolate high-molecular-weight DNA (20 μg)

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from the mantle musculature of a cutlassfish individual captured near the coastal waters of Jeju Island, 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 (Gardner et al. 1999; Hamilton et al. 1999). Extracted DNA 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 purified. Plasmids from insert-containing colonies were recovered using a QIAprep Spin Miniprep kit (Qiagen) and sequenced using a BigDye Terminator Cycle Sequencing Ready Reaction kit (ver. 3.1; PE Applied Biosystems, Foster City, 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 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 in microsatellite loci using samples from 30 randomly selected cutlassfish. 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 × 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 (PE 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. 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 molecular size markers (GeneScan-400HD (ROX); PE Applied Biosystems). Fluorescent DNA fragments were analyzed using GeneScan (ver. 3.7) and Genotyper (ver. 3.7) software (PE Applied Biosystems).

Statistical analysis

Genotyping errors due to null alleles, stuttering, or allele dropout were detected using Micro-Checker software (ver. 2.2.3; van Oosterhout et al. 2004). 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; Raymond and Rousset 1995) and ARLEQUIN software (ver. 2.0; Schneider et al. 2000). A sequential Bonferroni correction was applied to derive significance levels for analyses involving multiple comparisons (Rice 1989).

Results

More than 250 white colonies were obtained from the transformation with the cutlassfish $(CA)_n$-enriched genomic DNA library. Of those, 200 colonies were screened by PCR for the presence of a repeat-containing insert, and 96 colonies initially tested positive. Sequencing of the inserts from these 96 colonies revealed 80 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 31 loci that exhibited adequately long and unique sequence regions flanking the microsatellite array. Only 10 primer sets (KTh1B, KTh5, KTh6B, KTh9B, KTh10, KTh11A, KTh13B, KTh16, KTh22A, and KTh32)

Table 1. The characteristics of the ten microsatellite loci isolated from *Trichiurus lepturus*.

Locus	Repeat motif	Primer sequence (5′→3′)	*T*a(°C)	No. of alleles	Allele size range (bp)	H_O	H_E	P-value for HWE	Genebank accession no.
KTh1B	$(TG)_{30}$	F: CACAGAGACCTGGTAGACG **fam** R: TTTAAGTTCCCAAGAGAGCAG	52	12	182–268	0.80	0.68	0.2542	EF157601
KTh5	$(TG)_{44}(TGCG)_9$	F: AACACATGCACACCAAACTC **fam** R:GGCTCTAAGTCACCCATAATG	54	17	198–298	0.91	0.87	0.3020	EF157602
KTh6B	$(TG)_{22}TA(TG)_{19}$	F: TACTTTTATTCAAGGCAGCAT **fam** R: CAGAAGCACTCGACAGTCTTA	52	17	156–246	0.97	0.82	0.0000	EF157603
KTh9B	$(TG)_{14}$-$(TG)_7$ $TA(TG)_{18}TC(TG)_7$	F: CTACACTCACAAAGGCAGAAG **fam** R: GATCCTCTAGCAGAAGCACA	52	1	250	–	–	–	EF157604
KTh10	$(CA)_5CG(CA)_6$	F: AGGAGTAAAGACCACAGCCA **hex** R: CCGAGCACGAGAGCAA	55	11	140–164	0.50	0.81	0.0000	EF157605
KTh11A	$(TG)_{11}$	F: CACTCTAACGTGACTGGACAA **hex** R: ATGTATCGTCACCACAAACTG	55	14	120–178	0.97	0.90	0.4027	EF157606
KTh13B	$(TG)_5GG(TG)_{27}$	F: CACTGCTGGACGACACTG **hex** R: CCTCTAGCAGAAGCACATACA	52	11	108–164	0.73	0.74	0.1633	EF157607
KTh16	$(TG)_5TT(TG)_4TT(TG)_{11}TT$ $(TG)_8$-$(TG)_{12}$	F: TTGAAGAATCAGGGAGAGAAC **hex** R: CCTGCCTCCAGAGACAGT	54	22	62–142	0.67	0.95	0.0000	EF157608
KTh22A	$(TATG)_8.(TG)_{44}$	F: AGTAATATACGCATGCACGAC **fam** R: GCACAGCAGGGGTTTATG	52	1	222	–	–	–	EF157610
KTh32	$(CA)_{13}CG(CA)_4$	F: ATAAAAGAAGAAACCCATCCC **hex** R: CGAGCCACATCACATCACTA	54	10	102–126	0.88	0.75	0.5415	EF157612

*T*a 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 ($P < 0.01$).

successfully yielded variable profiles consisting of one or two bands. The remaining 21 primer sets gave either inconsistent or no PCR products, despite adjusting the dNTP concentrations and using an annealing temperature gradient. With the exception of KTh9B and KTh22A, all loci were polymorphic, with different degrees of variability. 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 10 new microsatellite loci are summarized in Table 1.

The number of alleles per locus ranged from 10 (KTh32) to 22 (KTh16), with a mean of 14.3 alleles per locus, and all loci were dinucleotide repeats. The mean observed and expected heterozygosities were 0.80 (range, 0.50–0.97) and 0.82 (range, 0.68–0.95), respectively.

Significant deviation from HWE after Bonferroni correction was observed at three loci, KTh6B, KTh10, and KTh16 ($P < 0.01$); owing to their heterozygosity deficit condition, these should be used with caution. Micro-Checker analysis revealed that the loci KTh6B, KTh10, and KTh16 may be affected by null alleles. The allele frequencies of the 10 microsatellites are shown in Figure 1. Allele frequency distributions indicated 67 rare alleles (frequency $< 5\%$) out of a total of 116 alleles summed over all loci, for a mean of 57.8%. The rare alleles were detected at most loci and were never associated with a particular locus. No significant linkage disequilibrium between loci pairs was detected ($P > 0.05$).

Discussion

Microsatellite DNA is suitable for examining the demographic and genetic structures of fish populations, because their high mutation rate results in a large number of alleles (Kilger and Schmid 1994). Microsatellites can provide information on the degree of separation between stocks and differences in the genetic structure between populations on oceanic and regional geographic scales (Ruzzante et al. 1998; Hutchinson et al. 2001). Understanding the genetic diversity of cutlassfish populations is vital for recovery and conservation management planning. To this end, we identified and characterized the first microsatellite markers for cutlassfish. 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, about 48% contained microsatellite repeats (96 of 200); this number is lower than that for tilapia (96%; Carleton et al. 2002), but higher than that for Japanese Spanish mackerel (34%; Yokoyama et al. 2006). 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. Nevertheless, we conclude that the enrichment procedure using magnetic bead hybridization is an efficient protocol for the isolation of highly informative microsatellite loci.

Two of the 10 primer pairs that yielded variable profiles exhibiting one or two bands were monomorphic. The high proportion of monomorphic microsatellites was probably the result of the isolation of clones containing predominantly shorter repeats. Brandström and Ellegren (2008) observed a relationship between microsatellite length and degree of polymorphism, and were able to quantify this relationship for lengths of a few to several repeat units. They 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 (O'Reilly et al. 2004).

Eight of the 10 new microsatellite loci from cutlassfish were polymorphic, showing 10 to 22 alleles per locus, with expected heterozygosities ranging from 0.68 to 0.95 ($H_E = 0.82$). The levels of genetic diversity were higher than those of most other marine fishes (see review by DeWoody and Avise 2000). However, higher levels of microsatellite polymorphism have been demonstrated in Atlantic herring ($H_E = 0.91$; Shaw et al. 1999), Pacific herring ($H_E = 0.89$; O'Connell et al. 1998), and walleye pollock ($H_E = 0.85$; O'Reilly et al. 2004).

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). The departure from HWE due to a deficit of heterozygotes may be the result of a technical artifact of PCR amplification, mis-scoring of heterozygotes for homozygotes, small sample size, presence of null alleles, or sampling of a single population with different allele frequencies in subpopulations (i.e. the Wahlund effect). 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

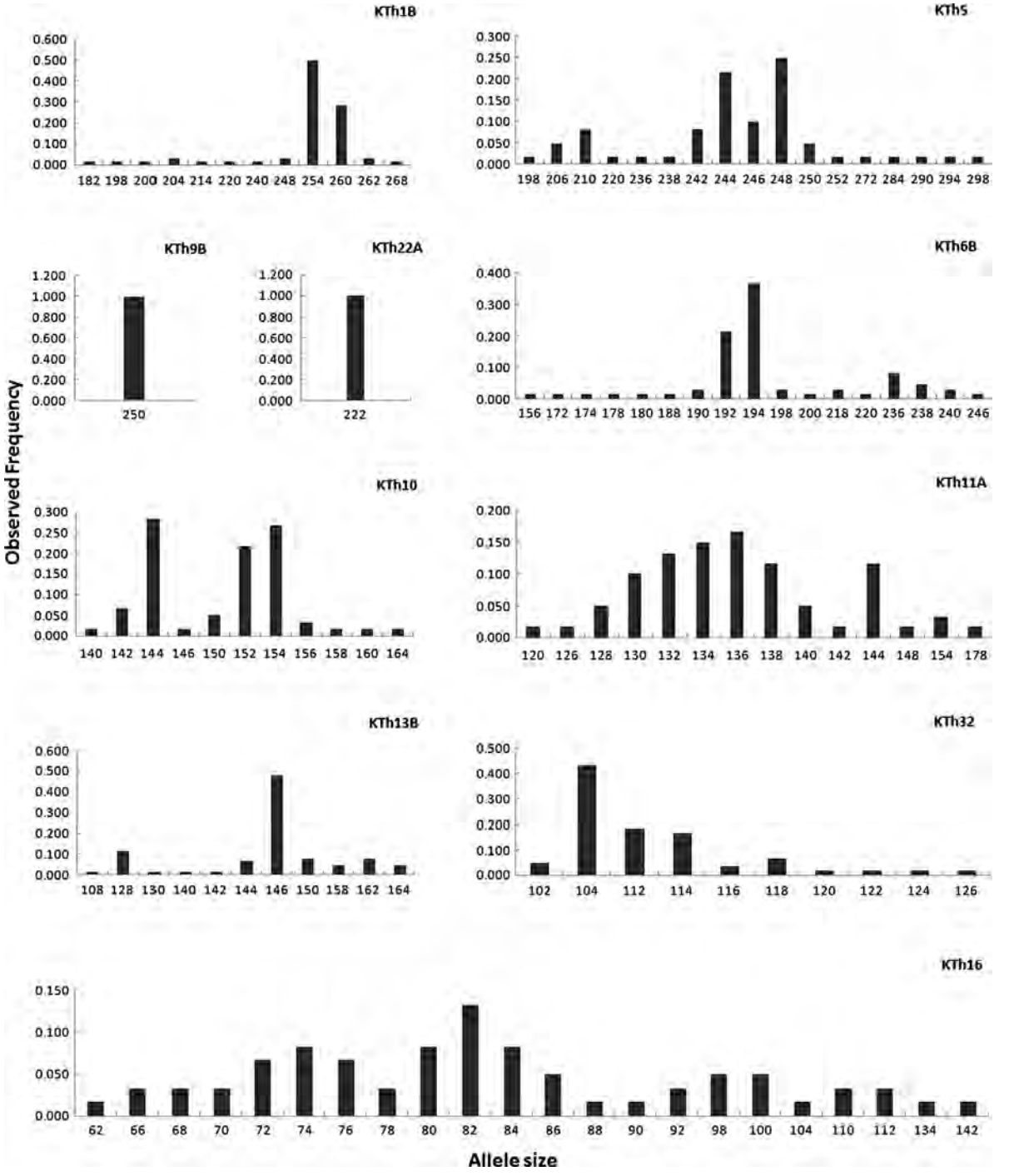

Figure 1. Allele size frequency distributions of the ten microsatellite loci of *Trichiurus lepturus* used in this study.

analyses that rely on HWE, as false homozygotes would be common (Pemberton et al. 1995).

The high variability of the microsatellite markers identified in this study makes them very useful for monitoring the genetic diversity and structure of cutlassfish populations. Further studies using these microsatellite markers to investigate genetic differences among wild populations of cutlassfish in Korea are ongoing.

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herein are those of the authors and do not necessarily reflect the views of NFRDI.

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