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PRIMER NOTE

Development and characterization of novel di- and tetranucleotide microsatellite markers in Pacific cod (*Gadus macrocephalus*)

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Abstract

The Pacific cod (*Gadus macrocephalus*) supports large commercial fisheries in the northern Pacific Ocean and the Bering Sea. Here we characterize 10 polymorphic microsatellite loci isolated from enriched genomic libraries. Loci were screened on a sample of 96 spawning adults. The average number of alleles per locus was 25.3 (range 12–44), with expected heterozygosities (H_E) ranging from 0.54 to 0.97. No significant deviations of genotypic proportions from Hardy–Weinberg equilibrium or linkage equilibrium were detected. These markers will be used in future studies of population structure and mixed stock analysis of this important gadid species.

Keywords: enrichment, *Gadus macrocephalus*, microsatellites, Pacific cod

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The Pacific cod (*Gadus macrocephalus*) has become an important commercial fishery target in the US waters since the mid-1980s. From 1999 to 2003, the annual US catch averaged 190 000 metric tons (t) with a mean ex-vessel value of $144 million (Hiatt *et al*. 2004). Two main areas, the Gulf of Alaska (GOA) and the Bering Sea/Aleutian Islands (BSAI), are managed as separate stocks, although relatively little is known regarding genetic stock structure in this species. A survey of allozyme variation (Grant *et al*. 1987) failed to detect significant population structuring within or between these management units. However, levels of population differentiation have not yet been assessed in Pacific cod using microsatellite DNA, a class of highly polymorphic nuclear markers that have proven to be more informative than allozymes in resolving low levels of differentiation typical in marine fishes (e.g. Bentzen *et al*. 1996; Shaw *et al*. 1999; Ruzzante *et al*. 2001; Withler *et al*. 2001). Here we describe 10 novel microsatellite markers isolated from Pacific cod. Future application of these markers in genetic population studies will lead to a better understanding of Pacific cod stock structure, thus, providing an improved basis for rational management of this resource.

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Microsatellite enrichment for *G. macrocephalus* was performed using a variation of the method in Hamilton *et al*. (1999), as described in Keeler-Foster *et al*. (2004). Two restriction enzymes were chosen for use in the protocol, *Hinc*II and *Pvu*II, because they cut genomic DNA to the desired size (200–1000 bp) but did not restrict the linkers or the targeted microsatellite repeat motif. While *Hinc*II had been used with success in constructing previous enrichment libraries (Spies *et al*. 2005; Vu *et al*. 2005), a number of clones in these libraries contained inserts but lacked sufficient flanking sequence to design primers. We attempted to improve the enrichment success by using *Pvu*II, which cuts at a single 6 bp recognition site (CAGCTG), whereas *Hinc*II cuts at four different 6 bp sites. A microsatellite enrichment library was constructed using the biotin-labelled oligonucleotide probe $(GACA)_4$ at a hybridization temperature of 48 °C. Enriched DNA fragments were cloned with the TOPO TA Invitrogen Cloning Kit according to manufacturer's instructions. A total of 200 clones from the enrichment library restricted with *Hinc*II and 100 from the library restricted with *Pvu*II containing inserts within the desired size range (200–1000 bp) were sequenced from

Table 1 Characteristics of 10 microsatellite loci derived from Pacific cod. Abbreviations are: forward and reverse primers annealing temperature (T_a), observed and expected heterozygosities (H_E and H_O) and deviation from expected Hardy–Weinberg genotypic proportions (F_{IS})

Locus	GenBank Accession nos	Repeat motif	Primer sequences (5′–3′)	T_a	Size range (bp)	No. of alleles	H_E	H_O	F_{IS}
Gma100	DQ027806	$(GACA)_{18}$	F: CGGTATCGTCATTGCTGACA R: TCGCCCTTCGACTAAGTGTT	55	223–393	44	0.969	1.000	–0.022
Gma101	DQ027807	$(CTGT)_{19}$	F: ATTGTTGCTGGTGGTGTTTG R: AACCCTTTATATCTACG	55	119–241	22	0.921	0.942	–0.013
Gma102	DQ027808	$(CTGT)_{17}$	F: TGGTTTCATTCGGTTTGGAT R: GGGCTCAGGTAAAGCCTCTT	55	221–275	12	0.873	0.911	–0.034
Gma103	DQ027809	$(GACA)_{16}$	F: TGGATGTGTGCGTCTACATTG R: AATCGCAACTGAGGTGAGTCT	55	190–394	38	0.893	0.802	+0.107
Gma104	DQ027810	$(GA)_8(CAGAGACA)(GAGACA)_4(GACA)_{16}$	F: AAAGAGAGCCACAGCCAGAT R: ATTCAACTGTTGGCCTCTGC	55	168–230	28	0.935	0.949	–0.009
Gma105	DQ027811	$(CA)_7(GA)(CA)_7$	F: CAAAGAGAGTGATCGCATCG R: CTGCACCCCTAGGAAGAGTG	55	189–367	41	0.890	0.835	+0.067
Gma106	DQ027812	$(GTCT)_5 \ldots (GTCT)_5$	F: TCACCATCACCTAGCAACCA R: GCGGAGATGGAGGATTACTG	55	179–225	13	0.807	0.804	+0.008
Gma107	DQ066622	$(CTGT)_{12}$	F: GGGAGTGGAGTACAGGGTGA R: CCATTGTTTAACATCTGGGACA	55	195–243	15	0.869	0.914	–0.042
Gma108	DQ027813	$(GACA)_7$	F: AAGTCCCAACACACCAAAGC R: CTCCTCTCTCGCGCTCTTTA	55	210–280	15	0.540	0.549	–0.006
Gma109	DQ066623	$(GTCT)_7G(GTCT)_{18}$	F: CATTTTACCTTTTGCTGAGGTG R: AAATTAAATTAGTTAGATGGAAAGA	55	257–373	25	0.925	0.955	–0.025

plasmid minipreps (QIAprep Spin Miniprep Kit, QIAGEN) using the Thermosequenase II cycle sequencing kit (Amersham Biosciences). Forward and reverse DNA sequences were obtained with a LI-COR 4300S DNA analyser (LI-COR) and analysed using E-SEQ software (LI-COR). Aligned sequence contigs were constructed using SEQUENCHER software version 4.2 (Gene Codes Corp.). A total of 36 sequences, 19 from the *Hin*cII-restricted library and 17 from the *Pvu*II-restricted enrichment, contained microsatellites with sufficient flank to design primers for polymerase chain reaction (PCR) amplification. Primers were designed using the PRIMER3 software program (Rozen & Skaletsky 2000). Four loci from the *Hin*cII-restricted enrichment and six from the *Pvu*II-restricted library amplified robustly, suggesting that the enrichment success using the *Pvu*II digest was higher than when using *Hin*cII.

These 10 loci were screened on a sample of 96 Pacific cod collected from the eastern side of Kodiak Island, Alaska in 2003 (Table 1). Genomic DNA was extracted using the QIAGEN DNeasy tissue extraction kit (QIAGEN) according to the manufacturer's protocol. Loci were amplified using a nested PCR method (Schuelke 2000). An M13F (–21) sequence was added to the 5′ end of each forward primer. DNA fragments produced via PCR were subsequently labelled for automated genotyping by adding labelled M13F(–21) to the PCR. PCR amplifications were conducted in 10 μL volume containing *c*. 100 ng template DNA, 10 mM Tris-HCl (pH 8.3), 50 μM KCl, 2.5 mM $MgCl_2$, 1.5 mM of each dNTP, 5 pmol of each primer (F primer 5′ end M13 tag), 0.5 U Biolase *Taq* polymerase (Bioline) and 0.5 pmol of fluorescently labelled M13 primer, either IRD700 or IRD800 (LI-COR), complimentary to the M13 sequence on the 5′ end of the forward primer. A 'touchdown' thermal cycle was performed in an MJ Research PTC-100 Thermalcycler. The PCR profile consisted of an initial denaturation step at 95 °C (2 min), 5 cycles at 95 °C (1 min), 60 °C [1 min (–1°/cycle)], 72 °C (1 min), followed by 20 cycles at 95 °C (30 s), 55 °C (30 s) and 72 °C (30 s). Automated genotyping was performed on the LI-COR 4300S and analysed using LI-COR SAGA GT software.

Single locus statistics for the Pacific cod sample was calculated using GENETIX version 4.02 (Belkhir *et al*. 2000) (Table 1). Loci were tested for conformance to expected Hardy–Weinberg equilibrium (HWE) genotypic frequencies and linkage equilibrium using exact tests implemented in GENEPOP (Raymond & Rousset 1995). Locus polymorphism was moderate to high, with expected heterozygosities (H_E) from 0.54 to 0.97. The number of alleles per locus ranged from 12 to 44 with a mean of 25.3 over all loci. There was no evidence for linkage disequilibrium among loci nor were there significant departures from HWE expectations observed at any locus. The lack of significant homozygote excesses in the screened samples indicated that null alleles were absent or occurred at low frequencies, and thus do

not pose a significant source of bias for estimates of genetic differentiation. Preliminary results presented here suggest that these loci may prove to be useful neutral markers for examining genetic population structure of Pacific cod and related gadid species.

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