Isolation and Characterization of Tetranucleotide Microsatellites from Atlantic Haddock (*Melanogrammus aeglefinus*)

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Abstract: Five tetranucleotide microsatellite loci developed from Atlantic haddock (*Melanogrammus aeglefinus*) are presented. Loci were isolated using a modified magnetic bead-hybridization selection procedure that enriched for tetranucleotide microsatellites. Loci were polymorphic (3–99 alleles per locus; mean, 33.2) and exhibited high levels of observed heterozygosity (0.07–0.99; mean, 0.73) in a sample of 70 haddock collected from the Scotian Shelf in the Northwest Atlantic Ocean. Primer sets for 5 tetranucleotide microsatellites, originally developed from cod (*Gmo*34), were also tested in Atlantic haddock; one pair yielded readily detectable product and was variable in the population assayed (29 alleles; heterozygosity, 0.96). These loci are suitable for kinship analyses in aquaculture-related applications, and are potentially useful for resolving population structure in the wild.

Key words: Atlantic haddock, tetranucleotide microsatellite, magnetic-bead enrichment.

INTRODUCTION

Atlantic haddock (*Melanogrammus aeglefinus*) is a demersal gadoid fish that is found along much of the North Atlantic continental shelf, as far south as the Bay of Biscay in the east and Rhode Island in the west. Canadian commercial landings of haddock have historically been on the order of 100,000 tonnes on the Scotian Shelf (Dept. of Fisheries and Oceans, Canada, 2000), and haddock remains the most economically important commercial fin-fishery in Atlantic

Canada. However, since the mid to late 1980s, stock biomass in most regions has declined, with concomitant changes in age-structure, reductions in size-at-age, condition, and maturity schedules (Dept. of Fisheries and Oceans, Canada, 2000). The Eastern Scotian Shelf haddock fishery has been closed since 1994.

The decline in the harvest fishery has stimulated development of commercial rearing and aquaculture of haddock for human consumption. As is often the case for marine fish species undergoing aquaculture development, haddock brood stock is collected from the wild and maintained in captivity until spawning time. Frequently, only 10 to 20 males and 10 to 20 females are placed together in a

communal spawning tank, and eggs are collected over the course of days to weeks until spawning is complete (haddock are serial spawners). Little is known about parental (P_1) contributions to the surviving offspring (F_l). P_1 males or females may be suppressing spawning and fertilization success of other P_1 individuals through aggression or hormonal responses. If most P_1 individuals do produce gametes, variation in reproductive success (e.g., via non-heritable variation in the condition of parents, or differential survival among F_1 larvae; see Doyle et al., 1995) may be sufficiently large that trait-based (or random) selection of 20 to 40 F_1 fish for future broodstock may represent very few of the original family lines. Thus, the likelihood for inbred F_2 fish and loss of genetic variation may be high, and may become magnified in successive generations to the point where the aquaculture operation fails.

Highly variable molecular genetic markers based on polymerase chain reaction (PCR) represent important tools to (1) evaluate the inbreeding potential or pedigree outcome of haddock aquaculture populations produced from communal spawning procedures; (2) develop genotype-based methods to avoid or minimize inbreeding; (3) maintain genetic diversity in aquaculture lines; and (4) assist in resolving stock structure in wild populations that may prove critical to protecting intraspecific diversity. Here, we report 5 new tetranucleotide microsatellites from haddock that were isolated using a modification of the magnetic bead-hybridization selection procedure of Hamilton et al. (1991). Markers of this class typically exhibit markedly reduced PCR stuttering relative to dinucleotide microsatellites (O'Reilly and Wright, 1995) and generally exhibit 4-bp differences between adjacent alleles. We also report results of primer tests of recently published cod (*Gadus morhua*) tetranucleotide microsatellites (Miller et al., 2000) to cross amplify in haddock.

MATERIALS AND METHODS

Isolation and Sequencing of Microsatellite Loci

DNA as extracted from a single haddock specimen obtained from the Bay of Fundy and stored in 70% ethanol. Enrichment procedures, following Hamilton et al. (1999), but modified as in McPherson et al. (2001), were used to isolate and clone tetranucleotide microsatellites. Briefly, genomic DNA was digested with the restriction enzyme *Rsa*I. After dephosphorylation using calf intestinal phos-

phatase, genomic DNA was ligated to SNX linkers (Hamilton et al., 1999) in the presence of the restriction enzyme *Xmn*I, which cuts SNX linker dimers, but not SNX linxer–genomic recombinant molecules. This procedure yielded genomic DNA with SNX linkers attached to both ends. Using SNX primers, the recombinant genomic-SNX DNA was PCR amplified and hybridized to $(GATA)_4$ and $(GACA)_4$ oligonucleotides bound to streptavidin-coated paramagnetic beads via a biotin conjugate attached to the 5′ end of the oligonucleotides. Nontargeted DNA was removed from the oligo-bead complex using repeated washes. SNX-genomic recombinant molecules were released from the beads by denaturing at 96°C. In a deviation from the Hamilton et al. (1999) procedure, the recovered SNX-genomic DNA was directly PCR amplified, ligated into a TOPO2.1 T overhang plasmid, (Invitrogen, Carlsbad, Calif.) and transformed into Invitrogen's ONE Shot competent cells following the manufacturers protocol; Hamilton et al. (1999) used restriction enzymes to digest linker DNA from genomic DNA prior to ligation into pUC18. Plasmid DNA was extracted using Qiagen (Valencia, Calif.) plasmid columns, as outlined by the manufacturer. Insert DNA was cycle sequenced using M13 reverse primer (GGA AAC AGC GAC CAT G) and Amersham-Pharmacia (Piseatawarg, N.J.) Thermosequenase Dye Terminator cycle sequencing chemistry. The presence of an identical linker sequence on either side of the genomic insert required the use of a modified thermal cycling regimen to sequence the recombinant clones: 25 cycles of 1 minute at 96°C, followed by 30 seconds at 60°C. The increased annealing temperature prevented or minimized interaction (hybridization) between the SNX sequence and the flanking insert. Sequencing results using the annealing temperature of 56°C (recommended by Amersham) were highly variable. Sequencing reactions were analyzed using an ABI 373 stretch automated sequencer.

Sample Preparation, PCR, Electrophoresis, and Visualization of Microsatellite Alleles

DNA from 70 haddock collected from the Western Bank region of the Scotian Shelf was extracted using Qiagen genomic extraction columns according to the manufacturer's protocol. Loci were amplified individually using a Techne Genius PCR machine. Reactions were carried out in 10-μl volumes, in 50 mM KCl, 20 mM Tris, pH 8.4, 0.2 mM each dNTP, 0.5 μM of unlabeled primer, 0.5 μM of

Table 1. Repeat Array Sequence, Primer Sequence, GenBank Accession Number, Locus Characteristics, Observed Heterozygosity (H_o), Expected Heterozygosity (H_e), P value of test for Deviation from Hardy Weinberg Expectations (HWE) and Annealing Temperature of Five Tetranucleotide Microsatellite Loci Isolated from Atlantic Haddock, and One Locus Isolated from Cod (Miller et al. 2000), Tested in Haddock. Estimates were based on a sample of 70 haddock collected from the Scotian Shelf.

Locus	Repeat array sequence	Primer sequence	Accession No	Size range (bp)	Number of alleles	H_o	H_e	HWE	T_a (°C)
Mae2	$(AGAC)_1(AGCC)_1(AGAC)_4$	F: TGG GAC TTT CAC CAC TCG R: TGC ATT CAT TCA TTC GTC	AF417601	60–72	3	0.071	0.070	1.0	54
Mae7	$(GACG)_1(GACA)_5$	F: CAC ACC GAG ATA AAC CAA R: AAA GCC TCT TGT ACT AAC TG	AF417602	192–220	9	0.657	0.699	0.7350	50
Mae9	$(GACA)_{21}$	F: GGA TGC CCA GCT TAG GAT R: TGG AAC TGG GTA GTG TAA C	AF417603	152–308	37	0.971	0.969	0.1567	50
Mae13	$(GTCT)_{17}(GCCT)_1$ $(GTCT)_5$	F: GCT ATT GGG TTG GAA CA R: GCA GGC GTT TCA ATT AGA	AF417604	123–481	99	1.0	0.994	1.0	54
Mae46	$(ATAG)_{27}$	F: AAA TAA TGC CGC TAT CAG R: TAT AAC AAC CAA ACC AAA CAA	AF417605	124–196	18	0.843	0.915	0.1235	52
Gmo34*	$(GACA)_n$	F: GGT TGG ACC TCA TGG TGA A R: TCC ACA GAA GGT CTC CTA A	AFI159234	120–236	29	0.986	0.956	0.8910	50

*From Miller et al., 2000.

Hex or Fam labeled primer (Table 1), 0.8 U *Taq* DNA polymerase. Thermal cycling conditions were as follows: 1 cycle of 94°C for 2 minutes; 3 cycles of 94°C for 1 minute, T_x°C for 30 seconds; 72°C for 30 seconds; 35 cycles of 90°C for 30 seconds, T_x°C for 30 seconds; 72°C for 30 seconds (where T_x is given in Table 1); and 1 cycle of 72°C for 15 minutes. Five tetranucleotide microsatellite loci *Gmo*3, *Gmo*8, *Gmo*19, *Gmo*34, and *Gmo*37) of the 7 developed from cod (Miller et al., 2000) were tested using conditions specified by the authors, and at annealing temperatures 4°C below the recommended annealing temperatures. Following PCR, an equal volume of deionized formamide (containing 0.05% wt/vol bromophenol blue) was added to each sample, denatured for 10 minutes at 94°C, and size fractionated on 6 M, 6% polyacrylamide gels for 1 to 2 hours (depending on the size of the expected product; Table 1). Microsatellite alleles or bands were visualized using an FMBIO II (Hitachi, Tokyo, Japan).

Population Analyses

Observed heterozygosity (H_o) was calculated as the proportion of individuals exhibiting 2 different alleles. Expected heterozygosity (H_e) was calculated using GENEPOP Version 3.1d (Raymond and Rousset, 1995). The number of alleles observed was based on a sample size of 70 fish, and conformation of genotypic proportions to Hardy-Weinberg expectations was tested using GENEPOP Version 3.1d.

RESULTS

More than 80% of the clones isolated contained one or more microsatellite arrays, typically consisting of primarily 4-bp repeat motifs. Many microsatellite-bearing clones were either identical or very similar to others isolated here. Primers were developed for 12 loci that exhibited unique sequence at either or both of the 5′ and 3′ regions flanking their respective arrays. Five primer sets yielded variable profiles consisting of 1 or 2 bands (Table 1). All were polymorphic (3–99 alleles per locus; mean, 33.2) and exhibited low to very high H_e (0.07–0.99; mean, 0.73). Only 1 of 6 tetranucleotide microsatellites originally developed for cod, *Gmo*34, cross amplified and was found to be a useful codominant marker in haddock (29 alleles, $H_e = 0.96$; Table 1). The remaining cod loci either failed to amplify or were invariant. None of the loci showed evidence of sig-

nificant departure from Hardy-Weinberg expectations (Table 1).

DISCUSSION

These 5 new tetranucleotide microsatellites from haddock provide a suite of loci that exhibit minimal PCR stutter and, with the exception of *Mae*13, all have alleles with primarily 4-bp differences. Thus, the various loci are relatively easy to identify and size using manual or automated systems. In addition, we have shown that 1 of the 5 cod tetranucleotide microsatellites tested, cross amplifies in haddock. Five of these 6 loci are highly variable and clearly suitable for use in aquaculture development, kinship analyses, and resolving population structure in the wild. The sixth locus (*Mae*2) exhibited only 3 alleles among the 70 individuals surveyed, and it may be suitable for population analyses.

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