Conserv Genet (2008) 9:1395–1398
DOI 10.1007/s10592-008-9507-3

TECHNICAL NOTE

Development of twenty sequence-tagged microsatellites for the Atlantic cod (*Gadus morhua* L.)

Madjid Delghandi · Mette Serine Wesmajervi · Saskia Mennen · Frank Nilsen

Received: 29 December 2007 / Accepted: 3 January 2008 / Published online: 20 January 2008
© Springer Science+Business Media B.V. 2008

Abstract Fifty-four primer pairs were designed for expressed sequence tag (EST) sequences containing perfect di- and tri-nucleotide motifs and characterised in 96 unrelated fish. Twenty markers were successfully amplified with number of alleles from 2 to 10 per locus and observed and expected heterozygosity ranging from 0.01 to 0.56 and 0.03 to 0.70, respectively. Loci *Gmo*-C213, *Gmo*-C246 and *Gmo*-C247 deviated from Hardy–Weinberg equilibrium. Genetic linkage disequilibrium analysis between all pairs of the loci showed significant departure from the null hypothesis between loci *Gmo*-C213 and *Gmo*-C222, *Gmo*-C233 and *Gmo*-C229, C223 and *Gmo*-C236 and C229 and *Gmo*-C236. The gene identity was determined at 10 of the loci, confirming the associated microsatellites as Type I markers. These microsatellite markers provide useful tools for studies of population genetics, reproductive ecology and constructing linkage maps of Atlantic cod.

Keywords Atlantic cod · *Gadus morhua* · cDNA · Expressed sequence tags (ESTs) · Microsatellites · Type I markers

M. Delghandi (✉) · M. S. Wesmajervi · S. Mennen
Norwegian Institute of Fisheries and Aquaculture Research, Muninbakken 9-13, Breivika, P.O. Box 6122, Tromso 9291, Norway
e-mail: madjid.delghandi@fiskeriforskning.no

M. Delghandi
Department of Marine Science and Fisheries, College of Agricultural and Marine Sciences, Sultan Qaboos University, P.O. Box 34, Al-khoud 123, Muscat, Sultanate of Oman

F. Nilsen
University of Bergen, Institute of Biology, HIB - Thormøhlensgt. 55, Bergen 5020, Norway

Introduction

The Atlantic cod (*Gadus morhua* L.) is a well-known teleost species inhabiting the Atlantic Ocean from North Carolina to Greenland in the west and from the Bay of Biscay to the Arctic Ocean in the east. The species has a long history as an important fish for consumption, but in recent decades stocks have declined dramatically particularly in the eastern Atlantic (Christensen et al. 2003). The species is presently an attractive candidate for aquaculture with commercial farming already underway in Norway, Canada and Scotland. Selection programmes have been established in Norway, Canada and Iceland to develop stock for the aquaculture industry that performs well on commercial traits such as growth rate and disease resistance.

Genetic markers are imperative for proper management of cod, for cod aquaculture, as well as for ensuring a sustainable coexistence of wild and farmed stocks. There is a need for additional genetic markers for refining the stock structure and assigning fish to populations as well as for improvements in aquaculture production both for pedigree tracing and quantitative trait loci (QTL) identification (Nielsen et al. 2006; Westgaard and Fevolden 2007). Recently, we have published a number of microsatellite markers derived from both genomic library enriched for repetitive sequences (Wesmajervi et al. 2007; Westgaard et al. 2007b) and expressed sequence tags (ESTs) (Stenvik et al. 2006; Westgaard et al. 2007a). In contrast to genomic microsatellites, EST-derived microsatellite markers have the potential to be functional markers (Varshney et al. 2005). Here we report the isolation and characterisation of 20 novel microsatellite loci containing di- and tri- nucleotide repeats, which add to those previously reported (Brooker et al. 1994; Miller et al. 2000; O'Reilly et al.

Table 1 Characteristics of 20 microsatellite loci isolated from Atlantic cod (*Gadus morhua* L.)

Locus	Repeat motif	Primer sequence (5′–3′)	A	Allele size range (bp)	H_O	H_E	FIS	Genebank accession no.
Gmo-C203	GGC	**F-CCCAACAAGCGGAGGAAGC R-GTTTCTTGAAGCTGTTGGCAGGGCTCTT	4	104–113	0.042	0.061	0.324	EU344853
Gmo-C204	CGG	**F-GAAGCCGGCAGAGGCAGAT R-GTTTCTTCCGCTTCCACCACCGTAGC	3	152–161	0.031	0.031	−0.007	EU344854
Gmo-C212	AAT	**F-CAACAGATTGCAACAGAAGACA R-GTTTCTTTAATGTTTAAATGTGGGTGTGC	3	149–155	0.260	0.336	0.225	EU344855
Gmo-C213	AGA	**F-AGGCAAGGTCCACGGTTCTCTG R-GTTTCTTAAGCGCCGGTTGTACTGGATGC	9	259–322	0.425	0.438	0.029*	EU344856
Gmo-C214	TTA	**F-CCTTCACAATCCCATTCTTCTTT R-GTTTCTTAAGGAAACCGATTCTTCTTACAC	6	164–185	0.674	0.697	0.033	EU344857
Gmo-C216	CAG	**F-CCTCGCTCATATGCCCCAGTCC R-GTTTCTTCCGGCAGCTGTGGTAGCCATCT	2	125–128	0.032	0.031	−0.011	EU344858
Gmo-C222	AGA	**F-AGGCAAGGTTCACGGTTCTCTG R-GTTTCTTAAGCGCCGGTTGTACTGGATGC	6	259–313	0.385	0.397	0.032	EU344859
Gmo-C223	TTG	**F-ACTGCGCATCCTCTCAGGAGAC R-GTTTCTTACTTGCTGACGTCGCTAGTATGC	4	204–213	0.297	0.297	0.000	EU344860
Gmo-C224	GCA	**F-CTGCTGCAGCTCCTGATTCAAT R-GTTTCTTGCTCGCGTGCTGCTTTGTG	10	133–160	0.447	0.429	−0.042	EU344861
Gmo-C227	TCA	**F-TTGTTTTGTAAATTACCCCAGACC R-GTTTCTTGTTACAAGCGGGCCCCACCAA	3	192–198	0.010	0.031	0.665	EU344862
Gmo-C229	ACA	**F-TAGTCTGCGCGTCGCGATGTCG R-GTTTCTTACACTGCGCATCCTCTCAGGAGACA	4	189–198	0.302	0.268	−0.125	EU344863
Gmo-C232	CGG	**F-AGAGGCAGATCCGGTGGTTCCAGA R-GTTTCTTCGCCGCCGCTTCTGTATCCAC	4	258–383	0.407	0.530	0.232	EU344864
Gmo-C233	CCT	**F-CTCAACAAAAAGCTTCAGGCAACC R-GTTTCTTTGGACAGGCTATTGGCAGGATG	8	165–186	0.558	0.586	0.048	EU344865
Gmo-C236	ACA	**F-GTCCATTTAGGCCGACATAATCAAT R-GTTTCTTGTGCTTCTAAACGGCGACACTG	4	274–283	0.333	0.292	−0.142	EU344866
Gmo-C246	AT	**F-TTTAAAGTACGTGGACAACCAATGT R-GTTTCTTGAGATAGGAAACGAGAAATGGAATG	2	168–170	0.104	0.204	0.491*	EU344867
Gmo-C247	TC	**F-CTCCCCTGGCCCGTTGATGTAGACC R-GTTTCTTCACCTAATTTTACAATGAAGGAACG	4	174–186	0.099	0.241	0.590*	EU344868
Gmo-C248	GA	**F-AGAATAGAAAGAACAGTCAGCACAA R-GTTTCTTGCCTTGTAATGTTAGCCACTTG	3	151–159	0.104	0.100	−0.040	EU344869
Gmo-C251	TG	**F-GGCGGAGAGGTTTGCAGGTCATTTC R-GTTTCTTCTCGCAGCAGCTCTCAGATGTTCAA	2	181–183	0.135	0.145	0.065	EU344870

Table 1 continued

Locus	Repeat motif	Primer sequence (5′–3′)	A	Allele size range (bp)	H_O	H_E	FIS	Genebank accession no.
Gmo-C255	AC	**F-AATTCTAGTTTACTCCCAGAGATAT R-GTTTCTTATTGTAAAATGTATTCCAACGTAT	2	181–183	0.177	0.162	0.091	EU344871
Gmo-C256	CA	**F-CTAAGAAGGTCTCGTTTCCCGTGAA R-GTTTCTTGCTAGCTATCTGTTGGCACCCATTT	5	239–249	0.422	0.487	0.133	EU344872

A—number of observed alleles; H_O—observed heterozygosity; H_E—expected heterozygosity; F_{IS}—inbreeding coefficient; *depicts significant departure from Hardy–Weinberg equilibrium; Clone sequences are available on GenBank (EU344853 to EU344872); Significance level was adjusted following sequential Bonferroni correction. ($P < 0.05$)

**Fluorescent dye labelled primer; F: forward; R: reverse

2000; Jakobsdottir et al. 2006; Wesmajervi et al. 2007; Westgaard et al. 2007a, b; Stenvik et al. 2006).

In an ongoing genomics program we are generating ESTs from cDNA libraries of different developmental stages of Atlantic cod (unpublished results). Approximately 1700 ESTs from cod embryos were investigated for the presence of microsatellite motifs using Tandem Repeats Finder software (Benson 1999) after setting the alignment score threshold to 30. A set of 54 flanking primer pairs were designed for EST sequences containing di- and tri- nucleotide motifs using Primerselect (DNASTAR). For fluorescent detection the forward primers were dye labelled (PET, NED, VIC or 6-FAM) (Applied Biosystems; AB). A PIG-tail sequence 5′-GTTTCTT (Brownstein et al. 1996) was included in the reverse primers (Table 1). Genomic DNA from 96 unrelated individuals, originated from randomly selected wild-caught Norwegian coastal- and North-East Arctic cod, was analysed to evaluate the efficiency of amplification and polymorphism of the markers by polymerase chain reaction (PCR) in a GeneAmp PCR system 2720 (AB). In order to utilise a single-step co-amplification of microsatellites, four markers (labelled with different dyes) were combined in a single reaction using Qiagen Multiplex PCR kit (Qiagen, Norway). The amplification was carried out in 10 µl reaction volume containing 10–50 ng template, 1× Qiagen Multiplex PCR Master Mix (3 mM $MgCl_2$) 10 nM of each forward and reverse primer. Thermal cycling conditions were: 15 min at 95°C, followed by 32 amplification cycles at 94°C for 30 s, 56°C for 90 s and 72°C for 60 s and then a final extension at 72°C for 30 min. Genotyping was performed using a 3130*xl* Genetic Analyzer (AB). Data were collected automatically and sized with GeneMapper software (AB) using the GeneScan-500-LIZ size standard (AB). Out of 54 primer pairs 20 were readily scorable and polymorphic (Table 1). Statistical calculations were performed using the program Genepop version 3.4 (Raymond and Rousset 1995). The number of alleles per locus (*A*), observed heterozygosity (H_O), expected heterozygosity (H_E) and inbreeding coefficient (F_{IS}) were calculated. Possible genotyping errors were examined with MICRO-CHECKER version 2.2.3 (Van Oosterhout et al. 2004). The number of alleles varied from 2 to 10 per locus and the observed and expected heterozygosity ranged from 0.01 to 0.67 and 0.03 to 0.70, respectively (Table 1). The loci were further tested for both Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium and significance levels were determined after 100 batches of 5,000 iterations each. Three of the loci (*Gmo*-C213, *Gmo*-C246 and *Gmo*-C247) deviated from HWE after sequential Bonferroni correction (Rice 1989), probably due to the small sample size, genetic drift and the presence of null alleles. Four of 253 pairwise exact tests rejected genotypic equilibrium between loci (*Gmo*-C213

Table 2 Annotation of gene-associated microsatellites by BLAST analysis

Locus	BLASTX similarity match	E-value	Species	GenBank accession no.
Gmo-C203	Ldb4 protein	2.0E–11	*Danio rerio*	AAH97093
Gmo-C213	40S ribosomal protein S30	2.0E–51	*Hippocampus comes*	AAQ63318
Gmo-C222	40S ribosomal protein S30	5.0E–53	*Hippocampus comes*	AAQ63318
Gmo-C223	Fast skeletal muscle alpha-actin	0.0	*Gadus morhua*	AAM21702
Gmo-C227	Similar to high-mobility group box 2	5.0E–66	*Danio rerio*	XP_694681
Gmo-C229	rRNA promoter binding protein	1.0E–41	*Rattus norvegicus*	AAK21974
Gmo-C233	Tropomyosin 3	0.0030	*Ictalurus punctatus*	ABC75556
Gmo-C236	Keratin	1.0E–89	*Carassius auratus*	AAC38008
Gmo-C246	Ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)	0.015	*Homo sapiens*	AAH04247
Gmo-C256	Alpha-1-antiproteinase-like protein	1.0E–143	*Oncorhynchus mykiss*	CAD90255

The NCBI GenBank non-redundant database was used and the reported matches were highly significant. For all loci the microsatellites were located to the 3′untranslated region (3′UTR) of the gene. The accession no. of the matching sequence is given

and *Gmo*-C222, *Gmo*-C233 and *Gmo*-C229, C223 and *Gmo*-C236 and C229 and *Gmo*-C236) after sequential Bonferroni correction. BLAST analysis gave highly significant hits for 10 loci (Table 2). These represent well-characterised genes thus defining the associated microsatellites as Type I markers, all located to the 3′UT region of the genes. In conclusion, these 20 polymorphic microsatellite loci provide useful tools for studies of population genetics, reproductive ecology and for constructing linkage maps of Atlantic cod.

Acknowledgements The present study was supported by the National Programme for Research in Functional Genomics in Norway (FUGE) within the Research Council of Norway (GenoFish). We thank Dr. Marie Cooper for reading of the manuscript and making helpful suggestions.

References

Benson G (1999) Tandem repeats finder: a program to analyze DNA sequences. Nucleic Acids Res 27:573–580

Brooker AL, Cook D, Bentzen P, Wright JM, Doyle RW (1994) Organization of microsatellites differs between mammals and cold-water teleost fishes. Can J Fish Aquat Sci 51:1959–1966

Brownstein MJ, Carpten JD, Smith JR (1996) Modulation of non-templated nucleotide addition by tag DNA polymerase: primer modifications that facilitate genotyping. Biotechniques 20:1004–1010

Christensen V, Guenette S, Heymans JJ, Walters CJ, Watson R, Zeller D, Pauly D (2003) Hundred-year decline of North Atlantic predatory fishes. Fish Fisheries 4:1–24

Jakobsdottir KB, Jorundsdottir OD, Skirnisdottir S, Hjorleifsdottir S, Hreggvidsson GO, Danielsdottir AK, Pampoulie C (2006) Nine new polymorphic microsatellite loci for the amplification of archived otolith DNA of Atlantic cod, *Gadus morhua* L. Mol Ecol Notes 6:337–339

Miller KM, Le KD, Beacham TD (2000) Development of tri- and tetranucleotide repeat microsatellite loci in Atlantic cod (*Gadus morhua*). Mol Ecol 9:238–239

Nielsen EE, Hansen MM, Meldrup D (2006) Evidence of microsatellite hitch-hiking selection in Atlantic cod (*Gadus morhua* L.): implications for inferring population structure in nonmodel organisms. Mol Ecol 15:3219–3229

O'Reilly PT, Canino MF, Bailey KM, Bentzen P (2000) Isolation of twenty low stutter di- and tetranucleotide microsatellites for population analyses of walleye pollock and other gadoids. J Fish Biol 56:1074–1086

Raymond M, Rousset F (1995) Genepop (version-1.2)—population-genetics software for exact tests and ecumenicism. J Heredity 86:248–249

Rice WR (1989) Analyzing tables of statistical tests. Evolution 43:223–225

Stenvik J, Wesmajervi MS, Fjalestad KT, Damsgard B, Delghandi M (2006) Development of 25 gene-associated microsatellite markers of Atlantic cod (*Gadus morhua* L.). Mol Ecol Notes 6:1105–1107

Varshney RK, Graner A, Sorrells ME (2005) Genic microsatellite markers in plants: features and applications. Trends Biotechnol 23:48–55

Wesmajervi MS, Tafese T, Stenvik J, Fjalestad KT, Damsgard B, Delghandi M (2007) Eight new microsatellite markers in Atlantic cod (*Gadus morhua* L.) derived from an enriched genomic library. Mol Ecol Notes 7:138–140

Westgaard JI, Fevolden SE (2007) Atlantic cod (*Gadus morhua* L.) in inner and outer coastal zones of northern Norway display divergent genetic signature at non-neutral loci. Fish Res 85:320–329

Westgaard JI, Tafese T, Wesmajervi MS, Nilsen F, Fjalestad KT, Damsgard B, Delghandi M (2007a) Development of ten new EST-derived microsatellites in Atlantic cod (*Gadus morhua* L.). Conserv Genet 8:1503–1506

Westgaard JI, Tafese T, Wesmajervi MS, Stenvik J, Fjalestad KT, Damsgard B, Delghandi M (2007b) Identification and characterisation of thirteen new microsatellites for Atlantic cod (*Gadus morhua* L.) from a repeat-enriched library. Conserv Genet 8:749–751