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## Development of ten microsatellite loci in the marine fish ling (*Molva molva*)

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3 **Development of ten microsatellite loci in the marine fish ling (*Molva molva*)**

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14 **Abstract**

15 **We developed primers for two dinucleotide and eight tetranucleotide microsatellite loci**  
16 **in a marine fish, the ling (*Molva molva*). All markers were obtained from partial**  
17 **genomic DNA libraries and characterized in 55 unrelated individuals from one putative**  
18 **population. The number of alleles ranged from 5 to 24, with an average of 10.5 per locus,**  
19 **and the observed heterozygosity ranged from 0.218 to 0.981 (average 0.643). None of the**  
20 **markers were amplified in two other gadoid species tested, the Atlantic cod (*Gadus***  
21 ***morhua*) and the tusk (*Brosme brosme*).**

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27 The ling (*Molva molva*) is distributed from the Barents Sea in the north down through the NE  
28 Atlantic into to the NW Mediterranean Sea. It is also found in the NW Atlantic off southern  
29 Greenland and Canada. Ling is a demersal fish species that occurs in moderately deep waters  
30 (100-1000 m) with rocky or sandy bottoms (Cohen 1990). It matures at 5-6 years of age and  
31 releases 20 to 60 million eggs per female. Fishing is primarily performed at depths between  
32 200-500 meters (ICES 2008a), both in targeted fisheries and as bycatch in e.g. cod fisheries.  
33 Fishing in European waters in 2007 was estimated at approx. 40 000 tonnes, and the largest  
34 catches were on the continental shelves off Norway, Iceland and the Faeroe Islands. Fisheries  
35 scientists have acknowledged the need for research on the population structuring of this  
36 species (ICES 2008b). To that end, ten polymorphic microsatellite loci are presented here,  
37 intended to contribute towards the study of ling population genetics.

38

39 We employed two different methods to develop the microsatellites: Eight tetrarepeat loci were  
40 developed by the company GIS Genetic Identification Service Inc. and two dimeric loci  
41 (Mmolm1 and Mmolm12) were developed at Durham University, UK. For the tetrarepeat  
42 loci, an enriched subgenomic library was constructed as described in Meredith & May (2002)  
43 and Schwartz & May (2004). Four libraries were screened for the microsatellite motifs  
44 (AAAC)<sub>n</sub>, (CATC)<sub>n</sub> (TACA)<sub>n</sub> and (TAGA)<sub>n</sub>. A total of 100 clones were sequenced and ten  
45 primer pairs were designed. Eight of these were found to be polymorphic and reliably  
46 amplified, and all further tests were restricted to these eight loci.

47

48 For the two dimeric loci we followed the enrichment procedure of Fischer & Bachmann  
49 (1998). Genomic DNA was digested with Sau3A following manufacturer's protocols and  
50 400-800 bp fragments isolated from an agarose gel (cleaned on a Qiagen gel extraction  
51 column). Oligos used to construct linkers (5'GCGGTACCCGGGAAGCTTGG (primer A)  
52 and 5'GATCCCAAGCTTCCCGGTACCGC) were annealed at 68 °C for 5 min. These were  
53 ligated to the size selected DNA fragments in a 30 µl volume, and excess linker cleaned away  
54 using a Qiagen PCR purification column. Constructs were then amplified using primer A in  
55 30 µl, with 1.5 mM MgCl<sub>2</sub>, 100 mM dNTPs, 10X reaction buffer, 300 ng each primer and 0.6  
56 units *Taq*. Incubation at 95 °C for 5 min was followed by the cycle: 94 °C for 45 sec, 68 °C for  
57 1 min, 72 °C for 1 min and, repeated 29 times and followed by a final soak at 72 °C for 10  
58 min. Amplified DNA was then boiled at 100 °C for 10 min, quickly chilled on ice, and 10-15  
59 µg combined with 2.5 µg of the biotinylated probe (B-ATAGAATAT(CA)<sub>16</sub>) in 30 µl. This  
60 was added to a pre-heated solution of 3X SSC, 0.1% SDS at 68 °C and incubated at 68 °C for

61 17 h. Streptavidin coated beads (Rao et al. 2003) in 160  $\mu$ l were washed four times in 10 mM  
62  $\text{Na}_2\text{HPO}_4$  pH7, 0.1% SDS, 0.1 M NaCl (1 ml), and then resuspended in 160  $\mu$ l of the same  
63 buffer. This was combined with the 300  $\mu$ l of hybridised DNA, and rotated for 48 h at room  
64 temperature. A magnet was used to separate the beads while pipetting off the supernatant and  
65 washing as above six times. DNA was eluted in 60  $\mu$ l 0.1 x TE for 5 min at 95 °C. One- $\mu$ l  
66 aliquots were then re-amplified in 30  $\mu$ l in six tubes on a gradient PCR machine with  
67 annealing temperatures ranging from 58 °C to 68 °C for 15 cycles. Reactions with product in  
68 the desired size range (400-800 bp) were pooled and the final concentration adjusted to 30-40  
69  $\text{ng } \mu\text{l}^{-1}$ . This was used for cloning into pGEM-T Easy Vector (Promega) according to  
70 manufacturer's instructions with the clones transformed in XL1-Blue (Stratagene) using blue /  
71 white selection. Positive colonies were transferred onto new plates (with AMP & TET  
72 selection) in a pattern suited to picking using a 6-channel multipipette, which was used to  
73 transfer cells into 96-well plates for PCR amplification. This screening step used the vector  
74 primers T7 and SP6 together with a microsatellite-specific primer  
75 (5'TGTGGCGGCCGC(TG)<sub>8</sub>) so that two bands would be seen on the agarose gel if the clone  
76 was positive for a microsatellite DNA locus. TC repeats were detected when they were near  
77 TG repeats (in each case primers were designed from that clone to amplify only the TC  
78 repeat). The PCR conditions were 2.5 mM  $\text{MgCl}_2$ , 100 mM dNTPs, 10X reaction buffer, 135  
79 ng of each primer and 0.4 units *Taq*. Incubation at 96 °C for 2 min followed by the cycle: 55  
80 °C for 40 sec, 72 °C for 1 min and 94 °C for 40 sec repeated 30 times, and followed by a final  
81 soak at 72 °C for 10 min. A total of 410 clones were screened and 25 positives selected for  
82 further assessment.

83

84 Population screening was conducted using 55 individuals of ling caught in Norwegian waters  
85 outside Bergen (66.44 N, 12.99 E). Genomic DNA was isolated with Viogene's blood and  
86 tissue extraction kit (Viogene Inc.). PCR amplifications were carried out in 10- $\mu$ l reaction  
87 volumes, containing 1  $\mu$ l of template DNA (20-40  $\text{ng } \mu\text{l}^{-1}$ ), 1X PCR buffer ( $\text{Mg}^{2+}$  free), 0.2  
88 mM of each dNTP, 1.1-1.5 mM  $\text{MgCl}_2$ , 0.125 mM of forward and reverse primers (Sigma)  
89 and 0.025 units  $\mu\text{l}^{-1}$  of *Taq* polymerase (TaKaRa Bio. Inc.). Fluorescently (CY-5) 5'-tagged  
90 forward primers were used. Thermal cycling conditions were for the tetra loci were as  
91 follows: an initial denaturation step at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s,  
92 30 s of primer specific annealing temperature (Table 1) and 72°C for 1 min. A final extension  
93 step at 72°C for 5 min completed amplification. For the locus MmolA6, a touchdown program  
94 were used. The initial annealing temperature was 64°C for 30 s, 11 cycles followed when

95 decreasing the annealing temperature 0.5 degrees per cycle until 59°C was reached.

96 Annealing at 59°C was held for 29 cycles.

97

98 We used a touchdown PCR procedure for the dimeric loci Mmolm1 and Mmolm12: initial  
99 denaturation at 94 °C for 3 min, 94 °C for 30 s, first annealing temperature for 30 s and 72 °C  
100 for 1 min. Eleven cycles followed where the annealing temperature decreased by 0.5 degrees  
101 per cycle. Cycling with the final annealing temperature for 18 cycles was followed by 72 °C  
102 for 5 min.

103

104 Sizing of PCR products were performed on a Beckman Coulter's CEQ 8000 automated  
105 sequencer where all lanes included a 400-bp ladder. Allele sizes were scored with the  
106 software CEQ 8000 Genetic Analysis System (version 8.0.52). We tested the loci for all  
107 individuals to assess gene diversity and evidence for linkage disequilibrium or deviation from  
108 Hardy-Weinberg expectations.  $F_{IS}$  was estimated and tested using the probability tests within  
109 GENEPOP on the web (<http://wbiomed.curtin.edu.au/genepop/>). The software  
110 MICROCHECKER (Van Oosterhout et al. 2004) was used to investigate the presence of null  
111 alleles or other technical artifacts. One locus, MmolmC5, showed significant deficiency of  
112 heterozygotes (Table 1), and was estimated to contain 23% null alleles. We also tested for  
113 presence of linkage disequilibrium (LD) between pairs of loci using GENEPOP, but no  
114 evidence for LD was detected. Finally, we tested all loci for cross species amplification on  
115 eight individuals in each of two other gadoids, the Atlantic cod (*Gadus morhua*) and the tusk  
116 (*Brosme brosme*): no useful amplification was found for any of the loci.

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ocus	GenBank Accession no	$T_a$ (°C)	Repeat motif	Primer sequences (5'-3')	Size range (bp)	$N_A$	$H_E$	$H_O$	$F_{IS}$	$P$ -value
olm1	xxx	61-56	(GT) <sub>3</sub> (AT) <sub>2</sub> (GT) <sub>14</sub> (GC) <sub>3</sub> (GT) <sub>3</sub> CC(GT) <sub>4</sub>	F: CAGCACTGGAGCTCTCAC R: TTTTGGTCAGCAGACTG	289-321	9	0.674	0.691	-0.025	0.708
olm12	xxx	60-55	(AC) <sub>6</sub> CC(AC) <sub>16</sub>	F: TGCTCCATGTTCTCTCCATC R: TATTAGCCTGAGCTGGAA	225-271	8	0.665	0.691	-0.039	0.285
olmA6	xxx	64-59	(TTTG) <sub>7</sub>	F: GTCCAAGACGATCCAGACC R: CCAATGAACCAATGAACCA	238-290	12	0.671	0.667	0.007	0.542
olmB2	xxx	56	(GTAG) <sub>9</sub> GTTGGTAGGTTG(GTAG) <sub>6</sub>	F: ATTTGGAGATACAGGGCAGAG R: CATTGATGGGTGGATGAATAG	242-266	6	0.496	0.473	0.047	0.496
olmC1	xxx	56	(ATGT) <sub>19</sub>	F: TCACTGCCTATTTCTGGTATTC R: CAAAGGAGATTGGGTTGTG	241-297	14	0.909	0.981	-0.080	0.948
olmC5	xxx	56	(ATGT) <sub>3</sub> ATG(ATGT) <sub>24</sub>	F: CCTCGTACTCGGCAAACA R: GGGACCTCAGTCTCACTGG	166-326	24	0.938	0.574	0.390***	0.000
olmB115	xxx	58	(GTAG) <sub>3</sub> ATAG(GTAG) <sub>10</sub>	F: TCCATCCATCCACAGATTC R: TGAGAAGACTCCACCATAAGAC	186-202	5	0.234	0.218	0.068	0.266
olmD131	xxx	56	(ATCT) <sub>26</sub>	F: ATGGGAAGCATACTGTTTTCT R: ATGGCTATCAGACAGACGG	230-278	13	0.861	0.836	0.029	0.459
olmD132	xxx	58	(ATCT) <sub>7</sub> AT(ATCT) <sub>3</sub>	F: CCAATGTTCTCCGTTCTC R: AGTTTCCTCAGACAGGTCACA	158-190	7	0.558	0.527	0.056	0.349
olmD137	xxx	58	(ATCT) <sub>14</sub>	F: CCCCCAATCTCTCTCCCTA R: CCTGTCCACCTCCACATTC	163-195	7	0.754	0.774	-0.026	0.263

**Table 1.** Primer sequences and characteristics of ten ling (*Molva molva*) microsatellite loci. Size range of fragments (bp), number of alleles ( $N_A$ ), expected ( $H_E$ ) and observed ( $H_O$ ) heterozygosity and deviation from Hardy-Weinberg expectations ( $F_{IS}$ ), are based on a sample of 55 individuals.  $P$ -values for two-sided tests, \*\*\* $P < 0.001$ .

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