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

Journal:	*Molecular Ecology Resources*
Manuscript ID:	draft
Manuscript Type:	Permanent Genetic Resources Note
Date Submitted by the Author:	n/a
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Keywords:	Stock structure, North Atlantic, Conservation Genetics, Fisheries Management

1   PERMANENT GENETIC RESOURCES

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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)n, (CATC)n (TACA)n and (TAGA)n. 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.

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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'GATCCCAAGCTTCCCGGGTACCGC) 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 $MgCl_2$, 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$(CA)_{16}$) 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 µl were washed four times in 10 mM

62   Na_2HPO_4 pH7, 0.1% SDS, 0.1 M NaCl (1 ml), and then resuspended in 160 µl of the same

63   buffer. This was combined with the 300 µl 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 µl 0.1 x TE for 5 min at 95 °C. One-µl

66   aliquots were then re-amplified in 30 µl 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   ng µl^{-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)$_8$) 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 $MgCl_2$, 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-µl reaction

87   volumes, containing 1 µl of template DNA (20-40 ng µl^{-1}), 1X PCR buffer (Mg^{2+} free), 0.2

88   mM of each dNTP, 1.1-1.5 mM $MgCl_2$, 0.125 mM of forward and reverse primers (Sigma)

89   and 0.025 units µl^{-1} of Taq polymerase (TaKaRa Bio. Inc.). Flourecently (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.

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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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Locus	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	$(GT)_3(AT)_2(GT)_{14}(GC)_3(GT)_3CC(GT)_4$	F: CAGCACTGGAGCTCTCAC R: TTTTGGTCAGCACGACTG	289-321	9	0.674	0.691	-0.025	0.708
olm12	xxx	60-55	$(AC)_6CC(AC)_{16}$	F: TGCTCCATGTTCTCTCCATC R: TATTAGCCTGAGCTGGAA	225-271	8	0.665	0.691	-0.039	0.285
olmA6	xxx	64-59	$(TTTG)_7$	F: GTCCAAGACGATCCAGACC R: CCAATGAACCAATGAACCA	238-290	12	0.671	0.667	0.007	0.542
olmB2	xxx	56	$(GTAG)_9GTTGGTAGGTTG(GTAG)_6$	F: ATTTGGAGATACAGGGCAGAG R: CATTGATGGGTGGATGAATAG	242-266	6	0.496	0.473	0.047	0.496
olmC1	xxx	56	$(ATGT)_{19}$	F: TCACTGCCTATTTCTGGTATTC R: CAAAGGAGATTGGGTTGTG	241-297	14	0.909	0.981	-0.080	0.948
olmC5	xxx	56	$(ATGT)_3ATG(ATGT)_{24}$	F: CCTCGTACTCGGCAAACA R: GGGACCTCAGTCTCACTGG	166-326	24	0.938	0.574	0.390***	0.000
olmB115	xxx	58	$(GTAG)_3ATAG(GTAG)_{10}$	F: TCCATCCATCCACAGATTC R: TGAGAAGACTCCACCATAAGAC	186-202	5	0.234	0.218	0.068	0.266
olmD131	xxx	56	$(ATCT)_{26}$	F: ATGGGAAGCATACTGTTTTCT R: ATGGCTATCAGACAGACGG	230-278	13	0.861	0.836	0.029	0.459
olmD132	xxx	58	$(ATCT)_7AT(ATCT)_3$	F: CCAATGTTCTCCGTTCCTC R: AGTTTCCTCAGACAGGTCACA	158-190	7	0.558	0.527	0.056	0.349
olmD137	xxx	58	$(ATCT)_{14}$	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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