

PRIMER NOTE

Development of microsatellite markers for muskellunge (*Esox masquinongy*) and cross-species amplification in two other esocids

BENJAMIN J. READING, PAUL S. WILLS, ROY C. HEIDINGER and EDWARD J. HEIST
Fisheries and Illinois Aquaculture Center and Department of Zoology, Southern Illinois University Carbondale, Carbondale IL 62901–6511, USA

Abstract

We report the development of seven polymorphic microsatellite loci in muskellunge (*Esox masquinongy*) using an unenriched subgenomic library. Polymorphic loci exhibited 2–11 alleles with observed heterozygosities ranging from 0.190 to 0.917 ($n = 24$). All seven loci amplified by their respective primer pairs resulted in monomorphic products in northern pike (*E. lucius*) whereas three loci amplified monomorphic products in grass pickerel (*E. americanus americanus*); these results imply conservation of flanking sequence but loss of polymorphism between these closely related species. Only one of six microsatellite primers developed in a previous study in northern pike amplified polymorphic products in muskellunge.

Keywords: *Esox*, grass pickerel, microsatellite, muskellunge, northern pike

Received 17 February 2003; revision received 14 May 2003; accepted 14 May 2003

The muskellunge (*Esox masquinongy*) is a large predatory fish common to lakes and large rivers in northern US and Canada. Despite the important ecological role that the muskellunge plays as the apex predator in many freshwater ecosystems and the value of the species to recreational fisheries there has been very little investigation of the genetic population structure of this species. In the only published study of muskellunge genetic variation Koppleman & Philipp (1986) found significant variation at several allozyme markers between muskellunge from widespread localities within North America. The potential for genetic stock structure may be of particular importance considering the widespread stocking of muskellunge throughout North America. Microsatellites have been isolated for the northern pike (*E. lucius*) (Miller & Kapuscinski 1996, 1997; Hansen *et al.* 1999). However, there are no published microsatellite loci for muskellunge, nor have the markers developed in pike been extensively assayed for variation in muskellunge. In this note we report on the development of seven polymorphic microsatellite loci in muskellunge, cross-species amplification in two

other esocids, and on the utility of several of the markers previously developed in northern pike for scoring genetic variation in muskellunge.

Twenty-four adult muskellunge, 15 northern pike and three grass pickerel were collected from Spring Lake in Illinois during March 2002 by trap netting. Pelvic fin clips obtained from each fish were preserved in 1.5 ml Eppendorf tubes containing 95% ethanol. Genomic DNA was isolated from using a Qiagen DNeasy Tissue Preparation Kit (Qiagen) and stored at -20°C . The procedure for isolation of microsatellites from a subgenomic library followed that of Heist & Gold (2000). Isolated muskellunge genomic DNA from a single individual was digested with *Mbo*I restriction enzyme. Resulting fragments were separated using a 1% TAE agarose gel electrophoresis and fragments spanning 300–800 base pairs were excised from the gel using a sterile scalpel. DNA from gel slices was extracted using QIAquick Gel Extraction Kit (Qiagen), ligated into pUC 18 cloning vector (Amersham Pharmacia Biotech), and used to transform DH5 α competent cells (Invitrogen Life Technologies). Approximately 1200 individual colonies were transferred to a 0.45-micron MAGNA nylon transfer membrane (Osmonics) and probed with ^{32}P radio-labelled (GT) $_{10}$ and (GA) $_{10}$ oligo sequences. Thirty-three

Correspondence: Benjamin J. Reading. Fax: (618) 453 6095; E-mail: benhur70@hotmail.com

Table 1 Polymorphic microsatellite loci developed in muskellunge, core sequences and GenBank accession numbers, sample size (n), optimal annealing temperature (T_a), number of alleles per locus (N_A), product size ranges given in base pairs, observed heterozygosities (H_O), heterozygosities expected under Hardy–Weinberg equilibrium (H_E), f value, and 5′–3′ PCR priming sequences

Locus	Core Sequence/ Accession Number	n	T_a	N_A	Product Size Range	H_O	H_E	f	Priming Sequence (5′–3′)
<i>Ema3</i>	(CA) ₉ N ₄ (CA) ₃ N ₂ (CA) ₃ N ₂₉ - (TC) ₄ N ₃ (CA) ₁₅ AY220522	24	60 °C	6	197–209	0.708	0.769	0.081	F: CAGTCCATTTCAGGGGGTATG R: CACCTGTGTGAGTGTGTGACC
<i>Ema9</i>	(TG) ₁₀ (CG) ₂ (TG) ₂ CG(TG) ₄ - (CG) ₂ (TG) ₇ AY220523	24	64 °C	2	167–169	0.502	0.291	0.425	F: GACCATACAGATTACTGTTCCAG R: ACACACACCTGACGCATCC
<i>Ema13</i>	(TG) ₁₇ AY220524	21†	60 °C	7	104–124	0.190	0.850	0.780*	F: CACGCTCTAGTGAACACGTCTCC R: TGTGTGCCCTCAACTTCCATTAAAC
<i>Ema15</i>	(TG) ₁₃ C(TG) ₁₂ AY220525	24	64 °C	3	149–153	0.708	0.616	-0.153	F: GAGCCTCTGAAGGAATCAGGATG R: TAGTGACTCCAACCTCTCTCTCCG
<i>Ema30</i>	(TG) ₁₄ AY220526	24	64 °C	3	123–129	0.625	0.582	-0.075	F: TATGCCAAATGGCTCCTCTAATG R: AAGTGCCCGCAGGAACGTCAAC
<i>Ema31</i>	(TC) ₇ (AC) ₁₂ AY220527	24	64 °C	5	155–179	0.917	0.733	-0.257	F: CAGAACACGCTTTACAAAGCAGG R: AGTCTCAAATCCACAGTGGAC
<i>Ema32</i>	(TG) ₂₂ (TCTG) ₆ (TG) ₁₁ - CTTT(GT) ₆ AY220528	24	60 °C	11	206–266	0.917	0.822	-0.117	F: TACCCCATTTGGTCTGGTGG R: AAGTTCTAACAGTAGCCTCTCC
<i>Elu51</i>	(AC) ₁₆	24	60 °C	3	147–153	0.208	0.194	-0.074	F: GTGGGCATTTCAGCCGATATAGC R: CTGTCTCATTTACTGCTGGCTC

*Indicates a significant deviation from Hardy–Weinberg expectations.

†Locus *Ema13* failed to amplify in 3 of 24 muskellunge.

positive clones were isolated using Wizard Plus Minipreps DNA Purification Systems (Promega) and manually sequenced by the dideoxynucleotide chain termination method using M13 primers and *fmol* DNA Cycle Sequencing System (Promega). Sequences flanking the microsatellite core repeat regions were used to design forward and reverse PCR primers with MACVECTOR 6.5.3 software (Oxford Molecular Group Ltd. 1998).

Primers were screened using 24 muskellunge samples and the cloned fragment as a size standard. For locus *Elu51*, products were sized in relation to a standard of 10 ng control pGEM-3zf(+) DNA (Promega) that was manually sequenced by the dideoxynucleotide chain termination method using M13 primers and *fmol* DNA Cycle Sequencing System (Promega). Forward primers for each microsatellite locus were radiolabelled with [γ -³²P]ATP using T4 polynucleotide kinase. PCR reactions (10 μ L) contained 1–15 ng DNA template, 1 \times PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 9.0, and 0.1% Triton X-100), 200 mM each dNTP, 2 mM MgCl₂ and 0.2 units *Taq* polymerase. Amplifications were initially conducted in an Eppendorf Master Cycler Gradient at annealing temperatures ranging from 50 °C to 64 °C to identify optimal annealing temperatures. Amplification consisted of a two-minute denaturing step at 94 °C, followed by 25 cycles of 94 °C for 30 s, 60–64 °C for 30 s, and 72 °C for 30 s. PCR products were separated with a 6% denaturing polyacrylamide gel electrophoresis.

Tests of Hardy–Weinberg equilibrium and linkage disequilibrium were performed using GENETIC DATA ANALYSIS Version 1.0 (alpha = 0.05) (Lewis & Zaykin 2001). Six of the most polymorphic northern pike microsatellite loci developed by Miller & Kapuscinski (1996, 1997) and Hansen *et al.* (1999) were selected to test in 24 muskellunge. PCR using developed muskellunge microsatellite primer sequences was performed on DNA samples from 15 northern pike and three grass pickerel. Conditions and procedure for PCR were the same as previously described.

From 33 positive clones, 13 PCR primer pairs were developed. Two of these loci were monomorphic in a sample of 24 muskellunge and seven of the loci produced polymorphic DNA products (Table 1). The remaining four primer pairs failed to amplify interpretable products. The two monomorphic loci had dinucleotide repeat blocks of only 9 or 10. The seven polymorphic loci exhibited 2–11 alleles with observed heterozygosities ranging from 0.190 to 0.917. Locus *Ema13* failed to amplify in three of 24 muskellunge and exhibited a significant deficit of heterozygotes based on Hardy–Weinberg expectations after a sequential Bonferroni adjustment of alpha. All loci paired with *Ema13* were significant for linkage disequilibrium after sequential Bonferroni adjustments of alpha. These results are consistent with the presence of one or more null alleles at locus *Ema13* with a cumulative frequency of approximately 0.35.

Table 2 Microsatellite loci, product sizes in relation to muskellunge clones and optimal annealing temperatures (T_a) for northern pike, product sizes in relation to muskellunge clones and optimal annealing temperatures (T_a) for grass pickerels. *Ema13* generated a product of equal size to the cloned muskellunge fragment in grass pickerels. Product sizes indicated 'similar' fell within the range of observed muskellunge alleles. Product sizes indicated 'smaller*' were markedly smaller in comparison to muskellunge products and did not fall within the observed size range of muskellunge alleles

Locus	Northern Pike Product Size Relative to Clone	Northern Pike T_a	Grass Pickerel Product Size Relative to Clone	Grass Pickerel T_a
<i>Ema3</i>	Similar	58 °C	No Product	50–64 °C
<i>Ema9</i>	Smaller*	58 °C	No Product	50–64 °C
<i>Ema13</i>	Smaller*	58 °C	116 bp	55 °C
<i>Ema15</i>	Smaller*	63 °C	Smaller*	55 °C
<i>Ema30</i>	Smaller*	58 °C	No Product	50–64 °C
<i>Ema31</i>	Smaller*	58 °C	Similar	60 °C
<i>Ema32</i>	Similar	50 °C	No Product	50–64 °C

Only one of the six northern pike loci amplified polymorphic products in muskellunge (Table 1). This locus, *Elu51*, has been previously reported to amplify a monomorphic product in muskellunge (Miller & Kapuscinski 1996). Primers for northern pike loci *Elu19* and *Elu276* amplified products in muskellunge, but were monomorphic. This indicates that the priming sequences for these three loci were evolutionarily conserved; however, the microsatellite regions expressed no variability. Primers for *Elu2*, *Elu6*, and *Elu252* failed to amplify interpretable products in muskellunge.

Primer pairs for all seven microsatellite loci developed in muskellunge amplified monomorphic products in northern pike and three of the seven primer pairs amplified monomorphic products in grass pickerel (Table 2). Northern pike and grass pickerel exhibited alleles at five and one of the loci, respectively, that was diagnostically different in size from the range of alleles found in muskellunge. While none of these markers were polymorphic in northern pike or grass pickerel, the amplification of products of very different sizes in these two species may prove useful as species diagnostic markers or for identification of hybrids within the genus *Esox*. For example, northern pike are often crossed with muskellunge to produce hybrid 'tiger muskies' which would be genotypically distinct from either purebred muskellunge or northern pike.

Acknowledgements

Funding for this research was provided by grant number F-141-R from Federal Aid in Sport Fish Restoration Act (Dingell-Johnston Program) and Illinois Department of Natural Resources. We thank the staff and faculty of Southern Illinois University Fisheries and Illinois Aquaculture Center, Jake Wolf Memorial State Fish Hatchery, and IDNR biologists Wayne Herndon, Rob Hilsabeck, and Shawn Hirst.

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