Molecular Ecology Notes (2003) doi: 10.1046/j.1471-8286.2003.00501.x

**PRIMER NOTE**

# Microsatellite loci for paternity analysis in the fathead minnow, *Pimephales promelas* (Teleostei: Cyprinidae)

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**Abstract**

**Many recent studies have employed molecular markers to uncover important aspects of mating systems in teleost fishes. The fathead minnow (*Pimephales promelas*) is a nest-building North American cyprinid that spawns multiply and exhibits exclusive male parental care. A battery of microsatellite markers was developed to analyse paternity in this species. The seven characterized loci possess four to 31 alleles and expected heterozygosities of 0.455–0.974. In combination, they elicit an exclusion probability of 0.999, a desirable level for paternity analysis. In addition, cross-amplifications were conducted to test primer efficacy in 13 other taxa, including two congeners.**

*Keywords*: Cyprinidae, enriched library, microsatellite, paternity analysis, *Pimephales promelas*, primers

*Received 8 April 2003; revision received 13 July 2003; accepted 13 July 2003*

In recent years, alternative reproductive tactics such as sneaking have been described among males in a wide range of taxa (DeWoody & Avise 2001; Taborsky 2001). A better understanding of the evolutionary processes leading to these alternative behaviours will be gained as additional taxa are examined. Among vertebrates, fishes offer some of the best opportunities for investigation because external fertilization is common and large clutch sizes make optimum sampling feasible. Here we describe the isolation and characterization of seven polymorphic microsatellite loci suitable for paternity analysis in the fathead minnow (*Pimephales promelas*), a common North American cyprinid.

Genomic DNA was isolated from hypaxial muscle tissue using a standard phenol/chloroform extraction protocol (Sambrook *et al*. 1989), followed by ethanol precipitation. A microsatellite-enriched genomic library was constructed using a streptavidin-biotin protocol (Hamilton *et al*. 1999). DNA was digested with three restriction enzymes (*Hae*III, *Rsa*I, and *Nhe*I) to produce fragment lengths suitable for cloning (300–800 bp). Subsequently, purified genomic fragments were ligated to linkers (Hamilton *et al*. 1999) using a 50:1 linker to genomic fragment molar end ratio.

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Genomic DNA/linker ligations were hybridized to three biotin-labelled synthetic repeat oligos: GATA, GACA, and AAT. The following hybridization temperatures were determined according to guidelines provided by Hamilton *et al*. GATA = 44 °C, GACA = 48 °C, AAT = 34 °C. Reaction cocktails were incubated for 8–12 h at these temperatures. A second hybridization was conducted to bind products to streptavidin beads. The beads were run through a series of washes of increasing stringency with the final two at 45 °C for AAT and 60 °C for GATA and GACA.

Genomic DNA bound to oligos was eluted by adding 60 µL of preheated T.E (T dot E) buffer to each sample, heating at 95 °C for 10 min, and separating the beads via magnetization. A second elution was collected to recover additional genomic DNA. Repeat-enriched DNA was ligated into pBluescript plasmids which were transformed into Stratagene Epicurian Coli XL1-Blue MRF′ supercompetent cells using a 3:1 molar ratio of insert to vector DNA.

PCR-based screening was performed to determine insert length in approximately 200 positive clones. Inserts were amplified using flanking plasmid primers T7 and T3 according to the following conditions: 96 °C (5 min), 30 cycles of 96 °C (45 s), 60 °C (1 min), 72 °C (1 min) followed by a final extension of 72 °C (5 min). Products were electrophoresed on a 2% agarose gel to visualize insert length. Approximately 100 clones with inserts > 350 bp were selected for sequencing.

**Table 1** Primer sequence, repeat motif, and allelic diversity for 11 microsatellite markers in *Pimephales promelas*. $T_a$, annealing temperature; $n$, sample size; $k$, number of alleles; $H_O$, observed heterozygosity; $H_E$, expected heterozygosity; $P_E$, paternity exclusion probability

| Locus/GenBank Accession no. | Primers (5′→3′) | Repeat motif | Size (bp) | $T_a$ (°C) | $n$ | $k$ | $H_O$† | $H_E$ | $P_E$ |
|---|---|---|---|---|---|---|---|---|---|
| Ppro48<br>AY254350 | F: TGCTCTGCTCTCCTGCGTGTCATT<br>R: CAGCCTCGGCGGTGTTGTTGC | $(TG)_{11}$ | 105–115 | 60 | 50 | 4 | 0.440 | 0.455 | 0.102 |
| Ppro80<br>AY254351 | F: AGCGATTCAACACCTTCAGGA<br>R: GTGGGGAATGGATCGAAACAAT | $(GATA)_{58}$ | 378–736 | 58 | 16 | 22 | 0.750* | 0.974 | 0.795 |
| Ppro118<br>AY254352 | F: CCGGATGCACTGGTGGAGAAAA<br>R: CCAGCAATCATAGCAGGCAGGAAC | $(CTAT)_{11}(CTGT)_{15}$ | 175–339 | 60 | 50 | 31 | 0.900 | 0.949 | 0.785 |
| Ppro126<br>AY254353 | F: CTGCGTGTCTGATAACTGTGACTG<br>R: GTCCCGGGACTTTAAGAAGGTC | $(CA)_{12}$ | 158–180 | 60 | 50 | 10 | 0.720 | 0.758 | 0.369 |
| Ppro132<br>AY254354 | F: GCATTTCCTTTTGCTTGTAAGTCTCAA<br>R: GGTTTAACCCGATCAATGGCTGTGC | $(CT)_{18}$ | 130–184 | 60 | 50 | 19 | 0.840 | 0.856 | 0.553 |
| Ppro168<br>AY254356 | F: CGGCCATACCTTGAGTCCAGG<br>R: CTTTTGCGGTTCGATTGATTTTGT | $(GATG)_{30}$ | 221–427 | 56 | 47 | 13 | 0.447** | 0.533 | 0.165 |
| Ppro171<br>AY254357 | F: GTCGCCGTCGCGTCTGGTTA<br>R: CTCACCCCAATGCCATCCAAGA | $(GAAA)_{27}$ | 226–318 | 66 | 50 | 19 | 0.905 | 0.946 | 0.766 |

Combined exclusion probability: 0.999.
†These loci deviated significantly from Hardy–Weinberg equilibrium: Hardy–Weinberg equilibrium test, CERVUS 2.0 (Marshall *et al*. 1998).
$^*P = 0.148$, $^{**}P = 0.088$.

Samples were sequenced in one direction with BigDye Terminator (Applied Biosystems) cycle sequencing reactions using the T3 primer. Thirty-six contained microsatellite sequences (motifs repeated more than five times). These were sequenced in the opposite direction (with the T7 primer) and compliments were aligned and edited with SEQUENCHER™ 4.1 (Gene Codes Corp.).

PRIMERSELECT© (DNASTAR Inc) was used to design primer pairs for seven microsatellites that possessed adequate flanking regions. PCR optimization was conducted using a gradient thermal cycler (MJ Research PTC-200) with the annealing step set at 50–70 °C to determine optimum annealing temperature. Additional gradients were run using various concentrations of $MgCl_2$ to increase or decrease binding stringency. Optimized reaction conditions were as follows. The reaction mixtures had a total volume of 10 µL and contained 2.0 µL of 1 mM dNTPs, 1.0 µL of 10X PCR buffer, 0.4 µL of 50 mM $MgCl_2$, 0.4 µL 10 mM forward primer, 0.4 µL 10 mM reverse primer, 0.1 µL (0.5 units) of *Taq* DNA polymerase (Invitrogen), 4.7 µL sterile dd$H_2O$, and 1.0 µL (≈100 ng) DNA. Reactions were denatured at 94 °C for 2 min, then carried out for 30 cycles at 94 °C (30 s), annealing temperature (30 s), 72 °C (40 s), followed by a final extension of 72 °C (2 min). Primer sequences and optimum annealing temperatures are listed in Table 1.

An ABI Prism 310 Genetic Analyser (Applied Biosystems) was used to score allele lengths in 50 randomly chosen individuals from a single, large population in Roberts County, South Dakota, USA. The most variable locus possessed 31 alleles. Average paternity exclusion probabilities ranged from 0.102 to 0.795. Two loci (Ppro80 and Ppro168) deviate significantly from HW equilibrium and probably have null alleles. These seven loci elicit a combined paternity exclusion probability of 0.999, indicating they are suitable for paternity analysis in this taxon. All aforementioned statistical analyses were performed in CERVUS 2.0 (Marshall *et al*. 1998).

Additionally, cross-taxon amplification attempts for all seven primer pairs were conducted to determine efficacy in 13 taxa (Table 2). A more detailed screening is given for the two congeners, *Pimephales notatus* and *P. vigilax*. In these taxa, six of the seven primer pairs successfully amplified microsatellites and the amplified loci exhibited moderate to high variation. In other taxa, reactions were conducted in single individuals. As expected, some loci are well-conserved within Cyprinidae. Indeed, the only noncyprinid amplification occurred in *Ictalurus punctatus* (locus Ppro48). Note that all products were verified by genotyping as described previously. In conclusion, these novel microsatellite loci may be useful for comparative mating system analyses within the genus and may have broader utility within the family Cyprinidae.

## Acknowledgements

This research was supported by grants from the Initiative for Ecological and Evolutionary Analysis, the Center for Great Plains Studies, and Special Funds from the University of Nebraska School of Biological Sciences.

**Table 2** Cross-taxon amplification to determine priming site conservation. For *P. notatus* and *P. vigilax*. *n*, sample size; *k*, number of alleles. For others, X indicates successful amplification in a single individual. All reactions were conducted under conditions optimized for *P. promelas*

| | | Locus | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Taxon | | Ppro48 | Ppro80 | Ppro118 | Ppro126 | Ppro132 | Ppro168 | Ppro171 |
| Congeners | | | | | | | | |
| *Pimephales notatus* | *n* | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| | *k* | 5 | 13 | 10 | 8 | 1 | * | 2 |
| | size range (bp) | 127–141 | 300–586 | 168–212 | 155–171 | 118 | | 293–301 |
| *Pimephales vigilax* | *n* | 10 | 10† | 10 | 10 | 10 | 10 | 10 |
| | *k* | 1 | 8 | 14 | 4 | 3 | 1 | 1 |
| | size range (bp) | 103 | 252–506 | 165–313 | 164–180 | 131–147 | 208 | 291 |
| Others | | | | | | | | |
| *Notropis stramineus* | | X | | X | X | X | | |
| *Cyprinella lutrensis* | | | | X | X | X | X | |
| *Semotilus atromaculatus* | | | | | X | X | | |
| *Campostoma anomalum* | | X | | X | X | | X | |
| *Cyprinus carpio* | | | | X | X | | | |
| *Ictalurus punctatus* | | X | | | | | | |

*no amplification; †5 of 10 individuals failed to amplify, presumably due to null allele.
Additional taxa for which all amplifications failed: *Catostomus commersoni*, *Fundulus kansae*, *Culaea inconstans*, *Lota lota*, and *Prochilodus lineatus*.

## References


DeWoody J, Avise J (2001) Genetic perspectives on the natural history of fish mating systems. *Journal of Heredity*, **92**, 167–172.

Hamilton MB, Pincus EL, Di Fiore A, Fleischer RC (1999) Universal linker and ligation procedures for construction of genomic DNA libraries enriched for microsatellites. *Biotechniques*, **27**, 500–507.

Marshall TC, Slate J, Kruuk L, Pemberton JM (1998) Statistical confidence for likelihood-based paternity inference in natural populations. *Molecular Ecology*, **7**, 639–655.

Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, New York.

Taborsky M (2001) The evolution of bougeois, parasitic, and cooperative reproductive behaviors in fishes. *Journal of Heredity*, **92**, 100–110.