DNA fingerprinting of bluegill sunfish (*Lepomis macrochirus*) using $(GT)_n$ microsatellites and its potential for assessment of mating success

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Abstract: Variable microsatellite DNA markers are powerful tools to study parentage and gene flow in fish populations. In this work, we cloned and characterized 60 (GT) repeat microsatellite sequences from the bluegill sunfish (*Lepomis macrochirus*) genome. We report the frequency distribution, the lengths, and the proportions of the repeat classes for comparison with microsatellites isolated from other teleost, mammal, and invertebrate genomes. Bluegill microsatellites are similar to those of other fish, strengthening previous reports of significant differences in the organization of microsatellites between teleosts and mammals. Primers for polymerase chain reaction amplification were designed for 17 bluegill microsatellite loci. The allele frequencies, the degree of polymorphism, and heterozygosities were estimated using bluegill from Lake Opinicon, Ontario. Seven loci are potentially highly informative for fingerprinting studies of parentage. A preliminary paternity analysis using two of these loci on brood from a natural nest revealed their potential utility at estimating relative success of the two reproductive life histories, parental and cuckolder, characteristic of male bluegill. Cross-amplification using these microsatellite markers on related fish species showed some degree of locus conservation among taxa, potentially allowing DNA fingerprinting of a variety of centrarchids.

Résumé : Les marqueurs à ADN microsatellite variable sont des outils puissants pour l'étude de la filiation et des mouvements génétiques dans les populations de poissons. Au cours de cette étude, nous avons cloné et caractérisé 60 séquences d'ADN microsatellite répétitives (GT) provenant du génome du crapet arlequin (*Lepomis macrochirus*). Nous indiquons la fréquence, la distribution, les longueurs et les proportions des classes répétitives pour des comparaisons avec des microsatellites isolés du génome d'autres téléostéens, mammifères et invertébrés. Les microsatellites du crapet arlequin sont semblables à ceux d'autres poissons, ce qui semble confirmer des rapports antérieurs faisant état de différences significatives entre les téléostéens et les mammifères dans l'organisation des microsatellites. On a conçu des amorces pour l'amplification de la réaction en chaîne de la polymérase à 17 locus d'ADN microsatellites du crapet arlequin. À l'aide de crapets arlequins du lac Opinicon (Ontario), on a estimé la fréquence des allèles, le degré de polymorphisme et les hétérozygosités. Sept de ces locus pourraient être des sources importantes d'information pour les études de la filiation grâce à l'identification par le code génétique. Une analyse préliminaire de paternité utilisant deux de ces locus avec les petits d'un nid naturel montre leur utilité possible pour l'estimation du succès relatif des deux stratégies de reproduction, par les couples et hors couple, caractéristiques du crapet arlequin mâle. L'utilisation de l'amplification croisée avec ces marqueurs à ADN microsatellite pour des espèces de poissons apparentées indique qu'il y a un certain degré de conservation des locus d'un taxon à l'autre, ce qui rend possible l'identification par le code génétique chez divers centrarchidés.
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Introduction

Polymorphic DNA markers can provide fisheries researchers with new insight into the behaviour, ecology, and genetic structure of fish populations (Wirgin and Waldman 1994). Genetic markers capable of tracking the contribution of individual parents to the recruitment generation can yield valuable information regarding effective population sizes (Withler and Beacham 1994), levels of inbreeding (Gross et al. 1994), disassortive mating, the success of alternative reproductive strategies and life histories (Hutchings and Myers 1988; Rico et al. 1992; Philipp and Gross 1994), and the intensity of natural and sexual selection (Schroder 1981; Fleming and Gross 1994). Furthermore, gene flow studies using neutral DNA markers are relevant to the conservation and management of fish stocks (Hedrick 1994; Wirgin and Waldman 1994). However, the majority of genetic markers that are available to researchers are not ideally suited for investigations of parentage and kinship. Allozymes and restriction fragment length polymorphisms (RFLPs) can be too limited in allelic variation to

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estimate relatedness along recent lineages (Queller et al. 1993). Randomly amplified polymorphic DNA (RAPDs) may be problematic when comparing banding patterns from different gels, and researchers using multilocus fingerprinting may incur high costs (Lewin 1989; Weatherhead and Montgomerie 1991). For these reasons, a powerful class of highly polymorphic genetic markers called microsatellites is increasingly being used in parentage and identity testing (Queller et al. 1993; Wright and Bentzen 1994; O'Reilly and Wright 1995).

Microsatellites are tandemly arrayed noncoding sequences consisting of very short nucleotide motifs and their lengths are usually no larger than 300 base pairs (bp) (Tautz 1989; Wright 1993). Generally, they are abundant and distributed throughout the eukaryotic genome (Tautz and Renz 1984). Within vertebrates, the dinucleotide repeat $(GT)_n$ is believed to be the most common microsatellite (Brenner et al. 1993). DNA fingerprinting using these sequences makes use of the polymerase chain reaction (PCR), whereby tandemly repeated polymorphic segments of minute DNA samples are amplified prior to resolving their lengths. This amplification presents a significant advantage over other non-PCR based methods because it allows the use of relatively small amounts of tissue, including that from preserved otoliths, scales, larvae, and small fry. Allelic variants of a microsatellite locus are codominant and show Mendelian inheritance. Moreover, because microsatellite loci often have many alleles and show high heterozygosity, parentage may be ascertained unambiguously using as few as 6–10 microsatellite markers.

An ongoing research interest of one of our laboratories has been the evolution of alternative reproductive life histories such as that shown by the behaviour of bluegill sunfish (*Lepomis macrochirus*). Bluegill sunfish are found in warm freshwater lakes throughout eastern and central North America (Scott and Crossman 1973). In Lake Opinicon (eastern Ontario), these iteroparous fish spawn from May through July in colonies of about 60 nests (Cargnelli and Gross 1996). The bluegill mating system is characterized by a discrete polymorphism in male behaviour and life history (Gross 1982). Parental males first mature at age 7 or 8 years, then compete for nesting territory in colonies prior to spawning. After spawning, these males remain at their nests for 5–12 days to care for and guard their brood. Other males, called cuckolders, mature precociously at age 2 and successfully steal fertilizations in the nest of parental males (Philipp and Gross 1994), never becoming parental males themselves (Gross 1982). Cuckolders thus leave the care of their offspring to the parental males. Similar alternative life histories are found in many fish species (Gross 1984). The evolution of such alternative reproductive life histories has been hypothesized to be explained in part by negative frequency-dependent sexual selection, whereby the mating success of a life history is inversely related to its frequency in the population (Gross 1985, 1991). Genetic markers are necessary to test this hypothesis by measuring the relative fitness of the alternative life histories. Such genetic markers for bluegill have not previously been available.

This study (*i*) describes the cloning and characterization of microsatellites from bluegill, (*ii*) compares the structure and genomic organization of $(GT)_n$ microsatellites from bluegill to microsatellites isolated from other taxa, and (*iii*) demonstrates the utility of the microsatellites for quantifying fitness by measuring the paternity within a wild parental bluegill's nest.

Materials and methods

Cloning and sequencing of microsatellite loci

A partial genomic library of bluegill sunfish DNA was constructed for the screening of microsatellites. High molecular weight DNA from 0.6 g of liver tissue of a Lake Opinicon bluegill was extracted using the method described in Coen et al. (1982) with few modifications. One hundred micrograms of genomic DNA was digested to completion with restriction endonucleases *Hae*III, *Hin*cII, and *Rsa*I (Pharmacia) according to the manufacturer's instructions. The digestion product was size fractionated by agarose gel electrophoresis and the DNA fragments between 300 and 800 nucleotides in length were excised from the gel. This range of fragment sizes was chosen because DNA fragments of these lengths are short enough to be easily sequenced, yet relatively large enough to provide adequate sequence information up- and down-stream from an average-sized microsatellite region. After electroelution, the DNA was resuspended in 1× TE buffer (10 mM Tris-HCl (pH 8.0); 0.1 mM ethylenediaminetetraacetic acid (EDTA)).

Using excess T4 DNA ligase (Pharmacia), the size-selected DNA fragments were blunt-end ligated into 20 ng of dephosphorylated pUC18 vector cut at the *Sma*I cloning site. Reactions (25 μL) were conducted at three different vector:insert molar ratios (1:0.5, 1:1, and 1:2). After the transformations of DH5α competent cells (Gibco BRL) with 2 μL of the ligation reaction, the plasmids were multiplied according to standard protocols (Sambrook et al. 1989).

Recombinant clones were cultured on solid Luria-Bertani (LB) medium plates containing 100 μg/mL ampicillin. After the size-selected library was transferred onto Hybond-N nylon membranes (Amersham), the cloned DNA was immobilized onto membranes by baking at 80°C, and then screened for microsatellites by hybridization with a synthetic $(GT)_{15}$ oligonucleotide. The oligonucleotide was 5′ end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (Pharmacia). The filters were hybridized to the oligonucleotide probe at 58°C in 5× SSPE (0.9 M NaCl; 50 mM NaH_2PO_4; 5 mM EDTA); 5× Denhardt's (0.5% ficoll 400; 0.5% polyvinylpyrolidone; 0.5% bovine serum albumin); 0.5% sodium dodecyl sulphate (SDS); and 100 μg/mL RNA after a 9-h prehybridization in the same solution. The membranes were washed once with 2× standard saline citrate (SSC) – 0.2% SDS at room temperature for 15 min and once with 0.2× SSC – 0.2% SDS at 60°C for 5 min (see Sambrook et al. 1989). Clones having DNA inserts containing $(GT)_n$ microsatellites were identified by exposing the membranes to Kodak X-Omat AR film at –70°C with intensifying screens. Positive recombinants were picked from the plates and grown in LB liquid medium. Plasmid DNA was then purified (Goode and Feinstein 1992), alkaline denatured, and sequenced by the dideoxynucleotide chain termination method (Sanger et al. 1977) using T7 DNA polymerase (Pharmacia).

PCR primers (forward and reverse primers) were designed and synthesized complementary to unique sequences flanking 19 microsatellite loci. These microsatellites and the site of primer annealing were chosen to give PCR products in the range of 80–200 bp, a size readily resolved on DNA sequencing gels.

PCR amplification

PCR conditions were optimized for the annealing of each set of primers to the template DNA by systematically increasing or decreasing the temperature. An initial annealing temperature (T_{ann}) of 50°C was chosen. Polymerase chain reactions were conducted in volumes of 10 μL, overlaid with light mineral oil. Standard reaction mixtures contained 10–20 ng of template DNA, 0.6 μM of the forward primer, 0.56 μM of the reverse primer, 200 μM each of dATP, dCTP, dGTP, and dTTP, 1× reaction buffer (10 mM Tris-HCl (pH 8.3), 1 mM $MgCl_2$, and 50 mM KCl), 0.25 units of *Taq* polymerase (Perkin Elmer), and 0.04 μM of reverse primer 5′ end-labeled with [γ-^{32}P]ATP (37 kBq/10 pmol primer) using T4 polynucleotide kinase (Pharmacia). Amplifications were performed using a Perkin Elmer 480 thermal

Table 1. Density of $(GT)_n$ microsatellites in the genomes of various fish, mammal, and invertebrate species, compared with that of bluegill.

Species	Dispersion (kbp^{-1})	Relative dispersion[a]	Source
Fish			
Bluegill (*Lepomis macrochirus)*	14	1.0	The present study
Atlantic cod (*Gadus morhua*)	7	0.5	Brooker et al. 1994
Atlantic salmon (*Salmo salar*)	90	6.4	Slettan et al. 1993
Brown trout (*Salmo trutta*)	23	1.6	Estoup et al. 1993*a*
Zebrafish (*Percina caprodes*)	12	0.9	Goff et al. 1992
Mammals			
Human (*Homo sapiens*)	28	2.0	Stallings et al. 1991; Beckmann and Weber 1992
Mouse (*Mus* sp.)	18	1.3	Stallings et al. 1991
Rat (*Rattus* sp.)	21	1.5	Stallings et al. 1991
Porcine (*Sus* sp.)	46	3.3	Wintero et al. 1992
Invertebrates			
Honeybee (*Apis mellifera*)	34	2.4	Estoup et al. 1993*b*
Snail (*Buliinus truncatus*)	40–45	3	Jarne et al. 1994

[a](Microsatellite dispersion (kbp^{-1}))/(bluegill microsatellite dispersion (kbp^{-1})).

cycler. After a 1-min denaturation at 94°C, samples were subjected to 7 cycles consisting of 1 min at 94°C, 1.0 min at the optimal T_{ann} and 30 s at 72°C, followed by 23 cycles of 45 s at 92°C, 1.0 min at T_{ann}, and 30 s at 72°C. One part deionized formamide stop dye was added to each reaction vessel followed by denaturation at 95°C for 10 min. Two microlitres of each sample was loaded onto a 6% polyacrylamide urea gel and subjected to electrophoresis in 1× TBE buffer (89 mM Tris-borate (pH 8.0); 20 mM EDTA) alongside an M13mp18 sequence to size the PCR products. Once dried, the gel was exposed overnight at room temperature to X-ray film. If adequate PCR product was not obtained by varying the annealing temperature, the $MgCl_2$ concentration was titrated from 2 to 0.5 mM in the reaction buffer to vary the annealing stringency of the primers to the DNA template.

Paternity application

We tested the utility of 19 microsatellite loci for use in paternity analysis. First, to demonstrate polymorphism and establish allele frequencies for each locus, from 5 to 174 unrelated bluegill were fingerprinted. Second, to demonstrate Mendelian inheritance of the alleles, three males were crossed with three females in containment facilities at the Queen's University Biological Station, Lake Opinicon, and their fry fingerprinted. Third, to demonstrate the utility of microsatellites for field samples, a tissue sample was collected from a nesting parental male bluegill and whole fry were taken from his nest by skin divers after observing cuckoldry (in June 1993). The field samples were preserved in 90% ethanol and later fingerprinted to calculate cuckolder success.

Results and discussion

Density of $(GT)_n$ microsatellites in the bluegill genome

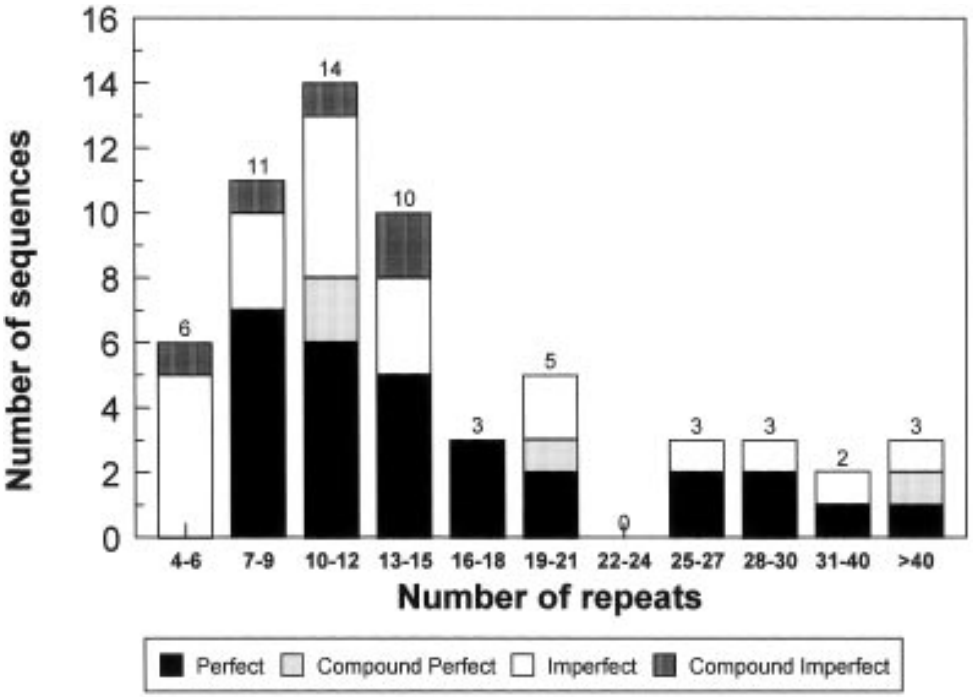
A total of 70 microsatellites were isolated and sequenced from a partial genomic library composed of 2035 clones. Of these 70 microsatellites, 60 were (GT) repeats. The average size of the clonal inserts was about 400 bp. Therefore, the total number of base pairs that were analyzed is estimated to be $400 \times 2035 = 8.14 \times 10^5$ bp. Assuming that the partial genomic library was representative of the bluegill genome, and that the $(GT)_n$ sequences are evenly distributed throughout the genome, a crude estimate of the average distance between neighbouring microsatellites is 14 kilo base pairs (kbp) $((8.14 \times 10^5 \text{ bp})/60)$. On the basis of this estimate, it appears that bluegill $(GT)_n$ microsatellites are more abundant than those reported in mouse, rat, human, honeybee, snail, and pig genomes (Table 1). When compared with other fish, bluegill repeats are about as abundant as those of zebrafish and more abundant than those in brown trout and Atlantic salmon. However, they are less abundant than those in Atlantic cod. Because of the varying stringencies of hybridizations used by investigators during screening of genomic libraries for microsatellites, these comparisons must be interpreted with caution. Nonetheless, the sunfish genome has a relatively large number of $(GT)_n$ microsatellites.

Length and sequence of bluegill microsatellites

The cloned microsatellites were partitioned into three categories, perfect, imperfect, and compound as defined by Weber (1990), giving the results presented in Fig. 1 and Table 2. Perfect microsatellites are uninterrupted series of a repeat unit. Imperfect microsatellite sequences differ from perfect ones by the presence of no more than three bases that arrest an alternating tandem repeat, such that its terminal ends on either side are at least three full repeats in length. Compound repeat sequences consist of several different repeat types and are separated by less than three intervening bases. Compound repeats are further subdivided as either perfect or imperfect sequences, depending upon the condition of the individual microsatellite units.

The predominant category of bluegill $(GT)_n$ microsatellites are the perfect repeats, while the most common size-class including all categories contains sequences with lengths of 10–12 repeats. Imperfect and perfect microsatellites have approximately the same size distribution, regardless of whether one measures the longest run of uninterrupted repeats, or the overall number of repeats within their sequences. Only one compound microsatellite locus had more than two different repeated motifs (two of these motifs had over 40 repeats). In all, this microsatellite comprised over 90 dinucleotides. Although only 10 compound repeats were sequenced, a wide variety of associated microsatellites were observed. Of these, $(GT)_n$ microsatellites were found with neighbouring tandem

Fig. 1. Frequency of different size-classes measuring the longest uninterrupted microsatellite arrays from bluegill sunfish. Size-classes are labeled as number of repeat units.



repeats of one to six nucleotides. This variety was also noted in the honeybee (Estoup et al. 1993*b*).

Brooker et al. (1994) found that representative teleost microsatellites are significantly larger and show greater degeneracy in their tandem repeats than do mammalian microsatellites. They suggested that this difference in repeat sequences may be due to more frequent slippage events during DNA replication in a cold-water environment. The bluegill microsatellites share many similarities with the three fish species, Atlantic cod (*Gadus morhua*), brown trout (*Salmo trutta*), and Atlantic salmon (*Salmo salar*), studied by Brooker et al. (1994). First, the proportions of bluegill microsatellites falling within Weber's (1990) three categories closely resemble those of Atlantic cod and brown trout. Among these fish, almost 50% of the microsatellites are categorized as being imperfect and thus degenerate in repeat sequence. Second, bluegill and the other three fish species share their most frequent microsatellite size-class (which is moderately shorter than the most frequent size-class in mammals). Third, the microsatellites of all four species are on average longer than those of mammals. Furthermore, the fish microsatellites can be quite large, while those of mammals rarely exceed 30 repeats. Fourth, all four fish species tend toward a bimodal distribution of microsatellite size-classes (although in bluegill the second peak is not as dominant).

Recently, dinucleotide microsatellite repeats have also been identified in invertebrates. Like fish, some of the $(GT)_n$ microsatellites in a snail (*Buliinus truncatus*) are large, exceeding 50 repeats (Jarne et al. 1994). Honeybees *(Apis mellifera*; Estoup et al. 1993*b*) have the same proportions of microsatellites in perfect and imperfect categories as do the fish, and some exceed 85 repeats in length. Therefore, the difference in size and degeneracy of microsatellites between fish and mammals may be a characteristic not restricted to cold-water environments, but shared by many poikilotherms.

Table 2. Number of microsatellite sequences in different repeat categories (perfect, imperfect, and compound; see text) in bluegill.

Category	$(GT)_n$/$(AC)_n$	$(CT)_n$/$(GA)_n$ and $(AT)_n$	Polynucleotides	Total No.	Total %
Perfect	29	2	2	33	47
Imperfect	22	2	3	27	39
Compound (perfect)	4	1	0	5	7
Compound (imperfect)	5	0	0	5	7

Note: Perfect dinucleotide microsatellites have ≥6 motifs, and the longest runs of dinucleotide repeats in close association with other microsatellites have ≥4.

Variability of bluegill microsatellites

PCR primers were designed for 19 loci. Of these primer sets, 17 provided scorable product. When the primer sets were used to amplify loci from unrelated individuals, 7 loci were found to be polymorphic with 2–8 alleles. The heterozygosities of these loci range from 27 to 63% (Table 3).

Many microsatellites (71%), although large, are not polymorphic. Weber (1990) noted that the degree of microsatellite polymorphism in humans is positively correlated with the size of the microsatellite array. However, bluegill microsatellites vary in the extent of their polymorphisms, apparently independently of size. Even sequences that are of the same category and size-class as other polymorphic loci may or may not exhibit polymorphism. This finding suggests that factors other

Table 3. Nucleotide and primer sequences, optimal annealing temperature, and $MgCl_2$ concentration for PCR amplification of the 17 bluegill sunfish microsatellite loci.

Locus	Locus sequence	Primer sequences	*N*	No. alleles	Size (bp)	% H	T_{ann} (°C)	[$MgCl_2$] (mM)
Lma 10	$(TG)_{10}(TATGTG)_4$	5′ GTCTGTAAGTGTGTTTGCTG 5′ GAAACCCGAAACTTGTCTAG	57	4	117–134	56	50	1.5
Lma 12	$(ACACAT)_2(ACACAC)(ACACAT)_4$	5′ CTGCTCAGCATGGAGGCAG 5′ TTCTTCCACAATATTCTCGCG	171	8	89–135	27	58	1.5
Lma 20	$(AC)_4N(CA)_{25}$	5′ GGCACTAATCTAATTGTAGCC 5′ TTGTGTGTCTGCATTGGAATC	155	8	85–117	62	64	1.0
Lma 21	$(TC)_{19}(AC)_{11}$	5′ CAGCTCAATAGTTCTGTCAGG 5′ ACTACTGCTGAAGATATTGTAG	174	6	154–182	60	58	0.5
Lma 25	$(GT)_{18}$	5′ CATTGGAAGTGGCTGCAAGC 5′ CAGACAGGTCAGACCTGCTC	36	1	129	0	50	1.5
Lma 26	$(TG)_{12}$	5′ TAATATACCAAACCTGCATGTC 5′ CTCACAGGGAATAAACCGTC	15	1	124	0	54	1.0
Lma 29	$(GT)_{30}$	5′ CCCTGTTACTTGTGTATTC 5′ ATTCAGAGGCAAGCATTATC	138	5	85–118	63	54	1.0
Lma 35	$(TG)_7N(GT)_{20}$	5′ CAGCCCCATGTATGAAGAC 5′ CCTACACCAATAGGAGGTGC	15	2[a]	113, 145	—	50	1.5
Lma 44	$(CA)_{10}N(AC)_4(ACC)_3$	5′ CAGTGATTTCCATTCCTCATC 5′ CTGCTGCCAGAGACAATGAC	15	2	98, 102	28	50	1.5
Lma 45	$(AAG)_4N_{11}(GT)_{17}$	5′ CACGAACCACTGAAGCTCAG 5′ ATAGCAGGTCGGGTTCACTG	5	1	106	0	50	1.5
Lma 54	$(TG)_{30}$	5′ CGCCTCGCCATGCTCAGTG 5′ AGTGTTTCAGGGACAGTAACAG	10	1	132	0	57	1.0
Lma 55	$(TG)_{13}NN(TG)_6N_6(GT)_5(GCGT)_3$	5′ TCAACATGCCAGCACTCAG 5′ CTGTATTTTGACTCTAGAGG	5	1	123	0	50	1.5
Lma 57	$(CA)_{20}NN(CA)_3$	5′ AAGATGTGGATGGCCTCAAC 5′ ACATGGCAGAGGGTTGTGAC	5	1	102	0	50	1.5
Lma 71	$(TG)_9$	5′ TGCGGGCAGCTCGGTCTGG 5′ TCTCTCTGCCTGCATGTAGCTG	30	2[a]	146, 167	—	50	1.5
Lma 78	$(TCA)_5NNN(TCA)_5$	5′ TAATGCTCTGACTGAAGGAG 5′ CAGTTAAAGGCATGGCGAAG	20	2[a]	113, 122	—	50	1.5
Lma 86	$(CA)_{39}(GA)_n(TG)_n$	5′ CAGAGTTACTGCAATCAGCTC 5′ CAGATGCAGGTGTGCTACTC	10	1	240	0	50	1.5
Lma 87	$(AC)_{15}(A)_5$	5′ ATGACACAGACTCACCATGC 5′ CTCCTGCCCATAAATCAGAC	172	4	118–151	53	58	0.5

Note: Number of alleles, sizes of PCR products, and percent heterozygosity (calculated from allele frequencies) were determined from *N* unrelated individuals collected from Lake Opinicon, Ontario.

[a] Primers amplify two microsatellite loci that are each fixed for one allele.

than length may be responsible for allelic variation at microsatellite loci (see also Wright 1994).

Paternity application to bluegill sunfish

Several of the microsatellites isolated from bluegill were evaluated for diagnosing paternity in Lake Opinicon bluegill sunfish. The test crosses revealed Mendelian inheritance of alleles for the five polymorphic loci that were tested. Each fry from the test crosses had an expected allele from each parent (Table 4).

The polymorphic microsatellite loci, Lma 21 and Lma 87, were amplified from a parental male and 10 randomly selected fry from a natural nest where cuckoldry had been observed (Fig. 2).

The parental male's genotype at loci Lma 21 and Lma 87 was 154/160 and 128/128, respectively. All progeny sired by this male should inherit one of his alleles at each of these loci. Therefore, his progeny should have either the 154 or the 160 allele at locus Lma 21 and the 128 allele at locus Lma 87. Of the 10 fry, 3 can be excluded as progeny of the parental male. These are individual 3 (genotype 164/164 and 118/118 at loci Lma 21 and Lma 87, respectively) and individual 6 (164/164 and 118/133), which do not share an allele with the parental male at either locus, and individual 8 (160/164 and 118/133), which shares an allele with the parental male at locus Lma 21 but does not share an allele with the parental male at locus Lma 87.

Examining the genotypes for the fry at Lma 87 in Fig. 2, and assuming that in addition to the parental male (128/128) there were only one female and one cuckolder male contributing to the fry, it is possible to predict the genotypes of that female and cuckolder. Fry 1 is 128/128 and therefore received the 128 allele from both the mother and father. Fry 3 is 118/118 and by a similar argument the mother must possess

Table 4. Inheritance data of five polymorphic bluegill microsatellite loci tested with full-sib families.

Full-sib family	Locus	Female	Male	Offspring					
				AA	AA′	A′A′	AA″	A′A″	A″A″
1	Lma 10	119/128 (AA′)	128/128 (A′A′)	—	20	18	—	—	—
1	Lma 12	128/128 (AA)	128/128 (AA)	35	—	—	—	—	—
2	Lma 10	117/119 (AA′)	128/128 (A″A″)	—	—	—	10	16	—
2	Lma 44	102/102 (AA)	102/102 (AA)	31	—	—	—	—	—
3	Lma 21	160/164 (AA′)	164/164 (A′A′)	—	12	10	—	—	—
3	Lma 87	128/128 (A′A′)	118/118 (AA)	—	21	—	—	—	—

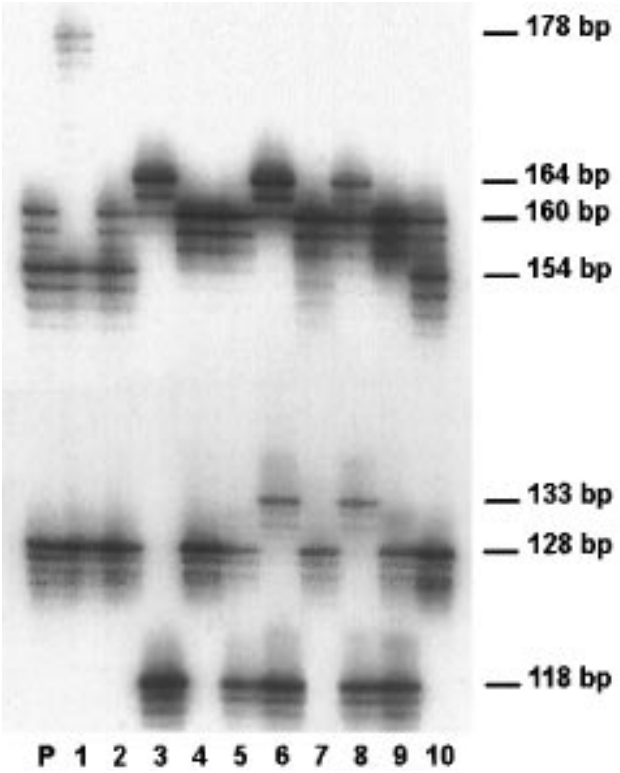
the 118 allele. Having assessed the mother's genotype as 118/128, it is possible to determine the genotype of the assumed single cuckolder male. Since fry 3 was identified previously as unrelated to the parental male, the cuckolder male must be the father and possess an 118 allele. Fry 6 and fry 8 were also identified previously as unrelated to the parental male, and since the mother does not possess the 133 allele, the 133 allele in their 118/133 genotype must come from the cuckolder. The cuckolder male's genotype is therefore 118/133.

The assumption of only one mother is not sustained when considering the second locus. Examining the genotypes for the fry at Lma 21 in Fig. 2, it is possible to determine that there was an additional female contributing to the fry in this brood. Fry 3 and fry 4 are homozygous for the 164 and 160 alleles, respectively. If there was only one mother she must possess both of these alleles (160/164). But fry 1 (154/178) does not possess either the 160 or 164 allele. Thus, at least three maternal alleles exist (160, 164, and either 154 or 178) and therefore there are at least two mothers. Because fry 3 (164/164) has already been identified as fathered by a cuckolder male, allele 164 is also a cuckolder's allele. Finally, if the 154 allele is maternal then the 178 allele from fry 1 must also be a cuckolder's as the parental male (154/160) does not possess this allele.

Utilizing both loci we have determined that (*i*) in addition to the parental male there were at least one cuckolder and at least two females contributing to the brood; (*ii*) the mothers must possess the 118 and 128 alleles at Lma 87, and the 160, 164, and either 154 or 178 alleles at Lma 21; (*iii*) the cuckolder(s) must possess the 118 and 164 alleles; and (*iv*) the 178 allele also belongs to the cuckolder(s) if the 154 allele is maternal. The ability to detect progeny not sired by a putative parent, and to determine the number and genotypes of the unknown parents, increases with the number of polymorphic microsatellite loci utilized.

In rare circumstances, a fry may be falsely assigned as a cuckolder's offspring for having none of the paternal parent's alleles at a single locus because of the relatively high rates of mutation at microsatellite loci. Dallas (1992) estimated microsatellite mutation rates at 10^{-4} to 10^{-3}.

Fig. 2. PCR-amplified microsatellite alleles of a parental male (P) and 10 randomly selected fry from a Lake Opinicon bluegill nest using Lma 21 (alleles 154–178 bp) and Lma 87 (alleles 118–133 bp). The genotypes at loci Lma 21 and Lma 87 are as follows: (P) 154/160, 128/128; fry (1) 154/178, 128/128, (2) 154/160, 128/128, (3) 164/164, 118/118, (4) 160/160, 128/128, (5) 160/160, 118/128, (6) 164/164, 118/133, (7) 154/160, 128/128, (8) 160/164, 118/133, (9) 160/160, 118/128, and (10) 154/160, 128/128. Fry numbers 3, 6, and 8 do not share alleles with the parental male at one or both loci; thus, they were sired by a cuckolder. Sizes of alleles were estimated using an M13mp18 sequence ladder (not shown).



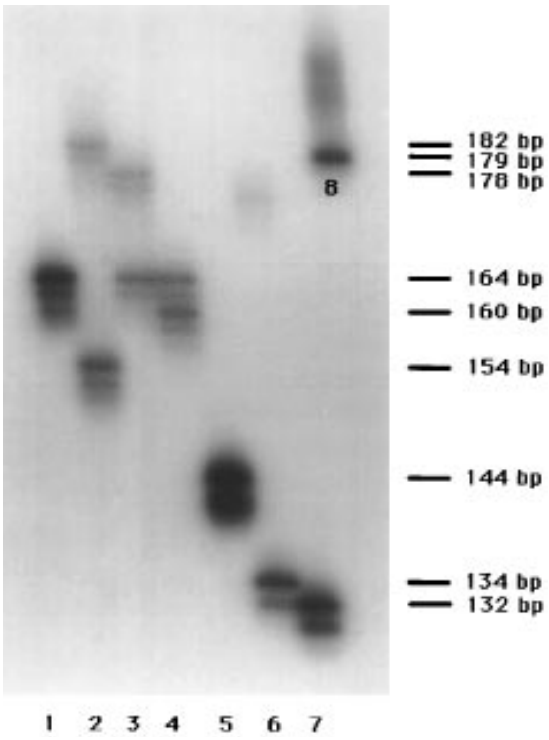
Cross-amplifications using bluegill primers

Selected primer sets that we developed for bluegill were tested on four other species. Primer sets for bluegill microsatellite loci Lma 20, Lma 21, Lma 29, and Lma 87 amplified DNA from pumpkinseed sunfish (*Lepomis gibbosus*); Lma 21 and 87 primers amplified DNA from largemouth bass (*Micropterus salmoides*) and rock bass (*Ambloplites rupestris*), and Lma 21 primers amplified DNA from yellow perch (*Perca flavescens*; Fig. 3).

For all four sets of primers, no changes to the PCR conditions were required. Thus, there has been evolutionary conservation of these loci, which may indicate their utility in phylogenetic and broader paternity studies.

In summary, the microsatellites presented here provide a useful comparison with those of other organisms, and also a

Fig. 3. PCR products of various alleles at the Lma 21 locus from bluegill (1–4), a pumpkinseed (5), a largemouth bass (6), a rockbass (7), and a perch (8). Sizes of the PCR products were estimated using an M13mp18 sequence ladder (not shown).



potential tool for parentage analyses of fish mating systems in nature.

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