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SHORT COMMUNICATION

A genetic assessment of parentage in a natural population of dollar sunfish (*Lepomis marginatus*) based on microsatellite markers

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Abstract

We employ microsatellite markers to assess mating tactics in *Lepomis marginatus*. Genetic assignments for 1015 progeny in 23 nests indicate that about 95% of the offspring were sired by their respective nest-guardians, a finding consistent with the apparent absence of a brood parasitic morphotype in this species. Allopaternal care was documented in two nests, one resulting from a nest takeover, the other from cuckoldry by an adjoining nest-tender. Clustered *de novo* mutations also were identified. About 2.5 females (range 1–7) contributed to the offspring pool within a typical nest. Results are compared to those for other *Lepomis* species.

Keywords: brood parasitism, maternity, mating tactics, paternity

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Introduction

The value of documenting genetic parentage by molecular markers has been demonstrated in many taxonomic groups (e.g. Avise 1994; Birkhead & Möller 1998). Among vertebrate animals, fishes display especially diverse mating behaviours and reproductive tactics (Taborsky 1994, 1997, 2001; Gross 1996; Henson & Warner 1997) whose consequences in terms of genetic parentage have been clarified by the use of such markers (reviews in Avise 2001; Avise *et al*. 2002).

In nearly all Centrarchidae, 'bourgeois' males build and tend nests, court females, and care for eggs and young (Breder 1936; Breder & Rosen 1966; Etnier & Starnes 1993). None the less, the genetic data show that these nest-tenders do not invariably sire all embryos in their respective nests. Instead, they sometimes are cuckolded by other males who 'steal' varying fractions of the fertilization events in a tended nest. Two behavioural avenues to allopaternal care — egg thievery and nest takeovers — have recently been illuminated by microsatellite paternity analyses in several nest-tending fish species (e.g. DeWoody *et al*. 2000c; Jones *et al*. 1998; Largiadér *et al*. 2001; Neff 2001; Porter *et al*. 2002).

Another avenue to allopaternal care in nesting fishes is reproductive cuckoldry via nest parasitism, a phenomenon that genetic paternity analyses have quantified in several North American sunfishes (Gross & Charnov 1980; Philipp & Gross 1994; DeWoody *et al*. 2000a, 2000c; Neff 2001). At least two nest-access routes for reproductive parasites are known: young 'sneaker' males may dart in surreptitiously, or 'satellite' males may gain entry by mimicking females in behaviour and colour (Dominey 1980; Gross 1979; Ehlinger *et al*. 1997). Other bourgeois males also may steal some fertilizations from the resident. In any of these cases, after releasing sperm during a spawning bout, the cuckolder then leaves the nest and, in contrast to the bourgeois male, makes no further investment in rearing the progeny.

In the bluegill sunfish (*Lepomis macrochirus*), satellite males are, on average, significantly smaller than bourgeois males (Gross 1982). In both the bluegill and probably the spotted sunfish (*L. punctatus*), such males mature sexually at an earlier age than resident males (2–3 years as opposed to 7–8 years), lack the bright body and breast colour of nuptial bourgeois males, and show a far higher relative investment in gonadal as opposed to somatic tissue (Gross 1982; Etnier & Starnes 1993; DeWoody *et al*. 2000c).

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Table 1 Microsatellite primers and the resulting background genetic data for dollar sunfish

Locus	Primer sequence (5′ → 3′)	Cloned repeat	PCR conditions	No. of alleles	P_E^*
RB7†	(F) `GTGCTAATAAAGGCTACTGTC` (R) `TGTTCCCTTAATTGTTTTGA`	(GATA)$_4$	94 °C/30 s, 48 °C/30 s, 72 °C/30 s, for 30 cycles	16	0.84
Lma120‡	(F) `TGTCCACCCAAACTTAAGCC` (R) `TAAGCCCATTCCCAATTATCC`	(GT)$_{28}$	94 °C/60 s, 56 °C/60 s, 72 °C/30 s, for 7 cycles; 94 °C/45 s, 56 °C/60 s, 72 °C/30 s, for 23 cycles	7	0.45
DS14	(F) `GCTTATCTTTGTCACCTGTGC` (R) `AATCAAGTAAACGAGCTGGGC`	(GT)$_{13}$	94 °C/30 s, 56 °C/30 s, 72 °C/30 s, for 30 cycles	8	0.43

*Exclusion probability under the one-parent-known model; for all three loci combined, $P_E = 0.95$; †From Neff *et al.* (1999).
‡From DeWoody *et al.* (1998).

Here we quantify genetic parentage in a population of dollar sunfish, *L. marginatus*, a species in which the presence of a reproductive parasitic morph or evidence of cuckoldry was previously undetermined. Bourgeois males construct a nest depression in sand or silt and, through lateral displaying, biting and chasing, defend it against intruders (Etnier & Starnes 1993; Winkelman 1996). Smaller males are typically unsuccessful in maintaining a nest, and this may imply strong nest-site competition. We use microsatellite data to document genetic parentage in more than a total of 1000 embryos from 23 nests. The genetic findings on biological parentage, including cuckoldry rates and numbers of dams per nest, will be interpreted in behavioural terms and compared to outcomes documented previously for other sunfish species.

Materials and methods

Sample collections and molecular techniques

Bourgeois males and thousands of embryos from their respective nests were collected from dollar sunfish nests during May to early July 1998, at three different sections of Fourmile Creek, a tributary of the Savannah River in South Carolina. Nest-tending males were electroshocked whereas embryos were scooped from the nest substrate and preserved in a 20% DMSO/saturated NaCl solution. Additional males and females were collected for morphological appraisals, and a subset of 40 adults total was used to estimate population allele frequencies at the loci analysed.

To address whether the parasitic satellite morph was present, the GSI (gonadosomatic index: the weight of an individual's gonadal tissue expressed as a percentage of its total body weight) was calculated for all 97 adult and juvenile males captured. Gonads were excised from each specimen, dried in an oven at 55 °C and weighed to the nearest 0.1 mg. The remaining soma mass was also dried at 55 °C and weighed. Measurements were performed in triplicate and the average weight was calculated.

DNA from the stomach tissue of adult dollar sunfish was isolated using a standard organic extraction protocol (Maniatis *et al.* 1982). DNA was extracted from embryos by boiling dissected tissue for 15 min in 100 µL Yogi Buffer (0.1 M NaCl, 0.5% N-lauroylsarcosine), followed by the addition of 100 µL of 20% Chelex solution and a 15-min incubation at room temperature. Samples were boiled for an additional 15 min and then centrifuged for 5 min at 14 000 r.p.m. (Altschmied *et al.* 1997).

Embryos and adults were genotyped at three microsatellite loci (Table 1). Locus *RB7* was developed originally for *L. auritus* (DeWoody *et al.* 1998), and locus *Lma120* for *L. macrochirus* (Neff *et al.* 1999). Locus *DS14* was developed in the current study using a dollar sunfish genomic library screened with various radioactively labelled dinucleotide repeat probes (DeWoody *et al.* 1998). Positive clones from this library were identified by autoradiography, and sequenced using ABI PRISM® BIGDYE™ Terminator Ready Reaction Mix on an ABI PRISM® 310 Genetic Analyser.

DNA amplifications (Table 1) were performed in a 12-µL reaction volume containing 0.75 U of Promega *Taq*, 0.2 mM of each dNTP and 5 pmol of each primer. Locus *Lma120* was amplified in 1× Lma buffer (10 mM Tris-HCl, pH 8.3; 1 mM MgCl$_2$; 50 mM KCl) (Neff *et al.* 1999), whereas loci *RB7* and *DS14* were amplified in 10× Promega buffer and 1.5 mM MgCl$_2$. The forward primers for *Lma120* and *DS14* and the reverse primer for *RB7* were end-labelled with different fluorescent dyes (HEX, NED and 6-FAM, respectively) and PCR products were electrophoresed in 4.75% denaturing polyacrylamide gels using an ABI PRISM® 377 Automated DNA Sequencer. Alleles were sized with respect to electrophoretic mobility using GeneScan and Genotyper (ABI) software packages.

Genetic parentage analysis

Forty adults (nesting and non-nesting males as well as females) were used to estimate the population allele frequencies at each microsatellite locus. The combined exclusion probability for these markers was calculated following Selvin (1980).

To estimate the mean number of sampled embryos needed to detect all dams for a given nest, allele frequencies from the sample of 40 adults were used in the computer simulation BROOD (DeWoody *et al*. 2000b). Results indicated that if no more than six females contributed equally to a nest, then a sample of about 44 embryos per nest would be sufficient to detect all of those mothers. We genotyped a mean of 42 embryos per nest in this study.

To determine empirically the number of females contributing to each nest, the minimum number of dams was calculated as the smallest integer value greater than or equal to one-half the number of different maternal alleles observed among the progeny within that nest (Kellogg *et al*. 1998). Statistically adjusted estimates of the true number of dams were obtained using the computer simulations GAMETES AND HAPLOTYPES (DeWoody *et al*. 2000b, 2000c).

Results

Population characterization

A strong positive correlation ($r^2 = 0.83$) was observed between somatic tissue weight and gonad weight for the 97 assayed males. Only one male (no. 86) departed noticeably from the regression line in a direction indicative of high gonadal mass, but even he did not possess the extremely high ratio of testes to soma (at least 2× higher than that of bourgeois males) normally indicative of the parasitic satellite morphology in other sunfish species (Gross 1982; Taborsky 1998; DeWoody *et al*. 2000c). Indeed, all GSI values in the dollar sunfish were less than 1.0.

Totals of 16, seven and eight different alleles were observed at microsatellite loci *RB7*, *Lma120*, and *DS14*, respectively, in the genetically assayed sample of adults collected at or near the nests (Table 1). No significant deviations from Hardy–Weinberg proportions were detected (GENEPOP 3.1b software application; Raymond & Rousset 1995). Under a model that assumes that one parent is known with certainty (a reasonable assumption in this case), the combined single-parent exclusion probability was calculated as 0.95; under a neither-parent-known model, the combined exclusion probability was 0.85.

Genetic paternity

In 17 of the 23 nests assayed (74%) all of the surveyed embryos displayed, at all three microsatellite loci, genotypes consistent with paternity by the nest tender (Table 2). In one additional nest (no. 1), the guardian male was not recovered, but the embryos in that nest similarly carried multilocus genotypes indicative of a single sire. Furthermore, in most of the remaining nests, most of the offspring appeared typically to have been sired by the respective resident males. Altogether, among the total of 1015 progeny examined, at least 957 (94.3%) had genotypes fully consistent with paternity by the respective nest-guardian males (Table 2).

In five nests (22%), some of the embryos carried neither paternal allele at one or more loci. From three of these nests (nos 2, 22 and 28), a total of 13 embryos carried a single 'guardian-inconsistent' allele that we deem likely to be of *de novo* mutational origin in the paternal gametic lineage (see Discussion). If so, 12 of these embryos (including 11 from nest no. 28) carried a new mutation at *RB7*, and the other embryo carried a new mutation at *Lma120*. Under this interpretation, the population-wide tally of embryos sired by their respective nest guardians becomes elevated to 970 among the 1015 embryos assayed (95.6%).

The remaining two nests (nos 8 and 9) each contained some embryos definitively inconsistent with paternity by the respective guardian males (i.e. their genotypes could not be explained even by a single *de novo* mutation event in the paternal line). Nest no. 8 consisted entirely of 'swim up' (swim-capable) larvae, 38% of which (19 of 50) could not have been sired by the nest-tender. However, those 19 larvae did possess multilocus genotypes consistent with paternity by the male guarding nearby nest no. 9, situated only 2.2 m away. In nest no. 9, progeny represented two different stages of ontogenetic development and, evidently, two different clutches. Of the 50 progeny surveyed, 24 were swim-up larvae apparently sired by the male guardian of nest no. 9, but the other 26 (52%) were unhatched embryos and early yolk sac larvae evidently sired by another genetically distinctive but unsampled male.

Furthermore, some of the swim up larvae in both nests (nos 8 and 9) displayed identical tri-locus maternal gametotypes, suggesting that the same females had partitioned their clutches between these two males. However, the maternal gametotypes in the early yolk sac larvae from nest no. 9 represented an entirely different set of dams.

Genetic maternity

Within each of four nests (nos 5, 11, 15 and 23), no more than two maternal alleles were observed at a single locus, suggesting that the progeny sets in each case had a single mother (Table 2). In eight nests (nos 1, 4, 13, 14, 17, 18, 21 and 22), progeny appeared to be mothered by just two females each, according to a minimum-number-of-dams estimate. By this same estimation criterion, the remaining 11 nests (48%) each contained progeny from a minimum of three different females.

However, the minimum method does not take into account the fact that different parents may share alleles. Thus, the programs GAMETES (which uses the most polymorphic locus to estimate the true number of dams) and

Table 2 Genetic results on biological parentage in 23 dollar sunfish nests

Nest no.	No. young sired by guardian male	Minimum no. of dams	Mean estimated no. of dams	
			(GAMETES)	(HAPLOTYPES)
1	41 of 41	2	2.4	7.3
2	47 of 48*	3	4.2	4.3
3	41 of 41	3	4.3	5.0
4	47 of 47	2	2.4	3.3
5	42 of 42	1	1.0	1.0
6	52 of 52	3	3.4	3.2
7	45 of 45	4	5.8	7.1
8	31 of 50	5	7.6	6.5
9	24 of 50	4	6.8	5.8
10	43 of 43	3	4.2	5.1
11	38 of 38	1	1.0	1.0
12	48 of 48	3	3.4	3.3
13	43 of 43	2	2.4	3.4
14	48 of 48	2	2.0	2.0
15	36 of 36	1	1.0	1.4
17	38 of 38	2	2.4	3.5
18	50 of 50	2	2.0	7.4
20	50 of 50	4	6.8	5.1
21	15 of 15	2	2.8	5.2
22	49 of 50*	2	2.4	3.4
23	47 of 47	1	1.0	1.0
26	48 of 48	3	4.2	4.7
28	34 of 45*	3	3.4	3.4
Mean	41.6 of 44.1	2.5	3.4	4.1
(SD)	(9.0, 7.8)	(1.1)	(1.9)	(2.0)

*Paternity 'exclusion' evidenced at a single locus only (see text).

Table 3 Summary of genetic parentage reported in four species of *Lepomis* sunfish: redbreast (*auritus*), bluegill (*macrochirus*), spotted (*punctatus*), and dollar (*marginatus*)

Species	No. young assayed	No. nests assayed	Approx.% foster young	No. dams per nest, minimum (and adjusted average)	Ref.
Redbreast	996	25	12	3.7 ± 1.2 (not done)	DeWoody *et al*. 1998
Bluegill	1677	38	21	not done	Neff (2001)
Spotted	1434	30	1	4.4 ± 1.4 (7.2 ± 3.5)	DeWoody *et al*. 2000c
Dollar	1015	23	4	2.5 ± 1.1 (3.7 ± 1.8)	Current study

HAPLOTYPES (which takes into account all three polymorphic loci) also were employed. The adjusted number of dams estimated from these programs usually approximated the minimum number, but for some nests HAPLOTYPES estimated two to three times more dams than documented by the minimum count (Table 2).

Discussion

By describing fish reproduction in terms of genetic outcomes, this laboratory study of the dollar sunfish has enabled us to characterize the animals' spawning behaviour in a natural setting. This is the fourth species of *Lepomis* characterized similarly for genetic parentage by microsatellite methods; a comparative summary of major results is presented in Table 3.

Multiple maternity within nests

For *L. marginatus*, the genetic data indicate that on average at least 2.5 and perhaps as many as four dams are responsible for the pool of embryos within a typical nest.

These estimates must be interpreted with some caution because some possible competing biases of unknown magnitude are neglected in the assessments. For example, if some females contribute only small numbers of offspring to a nest, their allelic contributions would probably be missed in our finite samples of embryos, and this would lead to an underestimate of the true number of dams with current data. On the other hand, to the extent that some *de novo* mutations occur in the maternal germ lines (as they evidently do on the paternal side — see below), then the current estimates of dam numbers per nest could be somewhat inflated.

In any event, the current genetic estimates of approximate dam numbers per nest in *L. marginatus* are roughly comparable to estimates previously published for *L. auritus* and *L. punctatus* (Table 3). No such quantitative values have been reported for *L. macrochirus*, but based on the colonial nesting behaviour in this species, several dams probably contribute to an average nest in that species as well (Gross 1991; Henson & Warner 1997). Thus, all indications are that several females normally spawn in the nest of a typical bourgeois male in species of the sunfish genus *Lepomis*.

Paternity and allopaternal care

In the current study of *L. marginatus*, bourgeois males sired at least 94% of the assayed young within the guardians' respective nests (and this value increases to almost 96% if we assume that the 'aberrant' alleles in 13 progeny from nests nos 2, 22 and 28 reflect *de novo* mutations in the paternal germ lines of the nest-tenders, rather than cuckoldry — see below). Thus, allopaternal care is rare but present in this population. The overall percentages of foster progeny estimated for dollar sunfish nests (about 4–6%) are intermediate to those reported in the spotted sunfish (*c.* 2%) and redbreast sunfish (maximum 11%), and are considerably lower than the value reported (21%) in an assayed colony of bluegill sunfish (Neff 2001).

Male bluegill sunfish (and, perhaps, spotted sunfish; DeWoody *et al.* 2000c) exhibit two distinctive morphological and reproductive phenotypes — bourgeois and satellite — that appear to be maintained by negative frequency-dependent sexual selection (Gross 1991). By contrast, only the typical bourgeois morph has been reported in the redbreast sunfish. From both phenotypic and genetic evidence presented in the current study, a specialized satellite morph (and its associated cuckolding behaviour) appears to be absent in the dollar sunfish as well, at least at this location. Instead, the best-documented instance of nest parasitism detected in the current study of dollar sunfish resulted from stolen fertilizations by a neighbouring bourgeois male.

Apart from the lack of a specialized cuckolder morph, the low levels of allopaternal care in the dollar sunfish may also be related to the low nesting densities within the study population. The dollar sunfish nests were solitary or arranged in small loose aggregations in lotic habitat. Eleven nests had no others within 5 m, and the remaining 17 nests averaged 2.5 m from nearest neighbours. This sparse distribution of dollar sunfish nests contrasts with the dense packing of nests (consistently within one meter of nearest neighbours) in a genetically monitored bluegill colony in which much higher cuckoldry rates were reported (Gross & Charnov 1980; Gross & MacMillan 1981; Gross 1991; Philipp & Gross 1994; Neff 2001). In redbreast sunfish, nest density also appears to be related to cuckoldry rates (Fletcher *et al.* unpublished data). All else being equal, when nests are separated by larger distances, intruder rates by neighbouring males are presumably lower, and in general a nest guardian may be better situated to defend his nest against stolen fertilizations.

Several illegitimate offspring were found in each of two dollar sunfish nests (nos 8 and 9), and the genetic data in conjunction with spatial and morphological considerations permit educated guesses about the behavioural processes responsible. The guardian of nest no. 8 was cuckolded by nest no. 9's guardian male, who apparently stole about 38% of the fertilization events from nest no. 8's guardian. Meanwhile, the guardian of nest no. 9 may have lost his nest temporarily to an unidentified male who spawned in nest no. 9 with a different set of females. The original guardian then returned and, at the time of capture, was tending both his earlier brood and the new embryos produced by the foreign spawners. Of course, other more complicated behavioural scenarios might also be consistent with the genetic data.

Clustered de novo *mutations*

Concerning the unexpected paternal alleles observed in a few of the offspring in nests nos 2, 22 and 28, for two reasons we prefer an explanation involving *de novo* mutation rather than allopaternity. First, in each case the other polymorphic loci were consistent with paternity by the resident male; second, these focal alleles differed by mutation steps consistent with a stepwise mutation model for microsatellite markers. Thus, the novel allele at the dinucleotide-repeat locus *Lma120* was 2-bp (one mutation unit) shorter than one of the unaltered paternal alleles documented in the other progeny from that nest; and similarly, the novel allele at the tetranucleotide-repeat locus *RB7* was 4-bp (one mutation unit) shorter than an unaltered paternal allele documented in the other progeny from that nest.

If these alleles are indeed *de novo* mutations, then at least one of them (at the *RB7* locus in nest no. 28) provides an example of a 'clustered mutation' (see Jones *et al.* 1999; Woodruff & Thompson 1992; Woodruff *et al.* 1996). Such mutations arise premeiotically in the paternal (or maternal) germ line and then are distributed to several of that parent's gametes and thus their progeny. If the clustered mutation detected provisionally in the dollar sunfish is tallied as a single mechanistic event, then the estimated mutation rate at the *RB7* locus, on the paternal side, is 2.0×10^{-3} (two *de novo* mutations among 1018 paternal gametes assayed in progeny). Similarly, we estimate the paternal mutation rate to be 9.8×10^{-4} at the *Lma120* locus. Both estimates are within the range of known or suspected microsatellite mutation rates in other vertebrate species (Hancock 1999).

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