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). Nonetheless, 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).
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 presence of a reproductive parasitic morph or evidence of cuckoldry was previously undetermined. Bourgeois males and thousands of embryos from their nests were genotyped at three microsatellite loci (Table 1). Locus RB7 was developed originally for *L. auritus* (DeWoody et al. 1998), whereas loci Lma120 and DS14 were 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 µL of Promega Taq polynucleotide kinase, 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 MgCl2; 50 mM KCl) (Neff et al. 1999), whereas loci RB7 and DS14 were amplified in 10× Promega buffer and 1.5 mM MgCl2. 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.

**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 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 presence of a reproductive parasitic morph or evidence of cuckoldry was previously undetermined. Bourgeois males and thousands of embryos from their nests were genotyped at three microsatellite loci (Table 1). Locus RB7 was developed originally for *L. auritus* (DeWoody et al. 1998), whereas loci Lma120 and DS14 were 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 µL of Promega Taq polynucleotide kinase, 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 MgCl2; 50 mM KCl) (Neff et al. 1999), whereas loci RB7 and DS14 were amplified in 10× Promega buffer and 1.5 mM MgCl2. 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

<table>
<thead>
<tr>
<th>Locus</th>
<th>Primer sequence (5’ → 3’)</th>
<th>Cloned repeat</th>
<th>PCR conditions</th>
<th>No. of alleles</th>
<th>$P_e$</th>
</tr>
</thead>
<tbody>
<tr>
<td>RB7†‡</td>
<td>(F) GTGTTAATAGGAAGCTCTTTC</td>
<td>(GTG)$_4$</td>
<td>94 °C/30 s, 48 °C/30 s, 16 0.84</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(R) TGTTCCTTTAATGTGTTTGA</td>
<td>(GTG)$_3$</td>
<td>72 °C/30 s, for 30 cycles</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(F) TGCCTAACGAAACATTGACC</td>
<td>(GTG)$_2$</td>
<td>94 °C/60 s, 56 °C/60 s, 7 0.85</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(R) TAAAATTCCTCCCATATTCCC</td>
<td></td>
<td>72 °C/30 s, for 7 cycles; 94 °C/45 s, 56 °C/60 s, 72 °C/30 s, for 23 cycles</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(GTG)$_1$</td>
<td>94 °C/30 s, 56 °C/30 s, 8 0.43</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(R) ACTGAGTTAAAAGAAGCTC</td>
<td></td>
<td>72 °C/30 s, for 30 cycles</td>
<td></td>
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</tr>
</tbody>
</table>

*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).
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 #8000 (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 Gamedes 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 gonadal 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$	imes$ higher than that of bourgeois males) normally indicative of the parasitic satellite morphology in other sunfish species (Gross 1982; Taborsky 1998; DeWoody et al. 2000b, 2000c). Indeed, all CSI 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 clutches 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 Gamemes (which uses the most polymorphic locus to estimate the true number of dams) and...
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 for the spotted sunfish (*L. punctatus*) (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 different 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 R87 locus, on the paternal side, is $2.0 \times 10^{-3}$ (two de novo mutations among 1018 paternal gametes assayed in progeny). Similarly, we estimate the paternal mutation rate to be $9.8 \times 10^{-4}$ at the Lm120 locus. Both estimates are within the range of known or suspected microsatellite mutation rates in other vertebrate species (Hancock 1999).

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References


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This study is one chapter in the developing PhD dissertation of Mark Mackiewicz, a graduate student in the Avise laboratory. Andrew DeWoody is a former postdoc in that laboratory, and Dean Fletcher and Dave Wilkins are long-term collaborators interested in fish behaviour.