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## MOLECULAR GENETIC DISSECTION OF SPAWNING, PARENTAGE, AND REPRODUCTIVE TACTICS IN A POPULATION OF REDBREAST SUNFISH, *LEPOMIS AURITUS*

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**Abstract.**—Despite a great diversity of reproductive behaviors in fishes, few studies have examined the genetic consequences of alternative reproductive tactics. Here we develop and employ microsatellite markers to assess genetic paternity and maternity of progeny cohorts in a population of redbreast sunfish (*Lepomis auritus*), a species in which males build and tend nests. Nearly 1000 progeny from 25 nests, plus nest-attendant males and nearby adults, were genotyped at microsatellite loci that displayed more than 18 alleles each. The genetic data demonstrate that multiple females (at least two to six) spawned in each nest, their offspring were spatially dispersed across a nest, and more than 90% of the young were sired by the attendant male. However, about 40% of the nests also showed genetic evidence of low-level reproductive parasitism, and two nests were tended by males that had fathered none of the sampled offspring. Genetically deduced reproductive behaviors in this population of redbreast sunfish contrast with those reported previously in bluegill sunfish (*L. macrochirus*) wherein heteromorphic males specialized for parasitism or for parental care coexist in high frequency. Thus, nest-parasitic reproductive behaviors in fishes appear to be evolutionary labile.

**Key words.**—Cuckoldry, maternity, mating system, microsatellites, paternity, polygyny, sexual selection.

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The difficulty of continuous behavioral monitoring of many animals in nature places logistic constraints on ecological studies of mate choice and biological parentage. Even when reproductive hypotheses can be formulated in particular instances, they remain refractory to critical tests in the absence of genetic data on mating systems. Many of these difficulties can be overcome through the use of highly polymorphic genetic markers. For some taxa, such studies have revolutionized thought about reproductive behaviors. For instance, genetic data demonstrate that many passeriform birds thought to be socially monogamous are genetically polygamous (reviews in Birkhead and Møller 1992; Avise 1994). Whereas avian and mammalian taxa have received considerable attention in genetic reanalyses of mating systems, fishes have been largely neglected in such studies (but see Philipp and Gross 1994; Jones and Avise 1997a,b; Jones et al. 1998). This is surprising given the great diversity of mating systems and reproductive tactics suspected in fishes (Taborsky 1994).

Reproductive ecologies of North American sunfish (Centrarchidae) are well documented (Breder 1936; Gross 1982; Lukas and Orth 1993). Typically, a parental (“attendant” or “bourgeois”) male constructs a nest in gravel substrate and then swims in circular patterns above his nest. One or more females approach and spawning is initiated. A female may spawn her entire clutch of several hundred eggs in a few hours or a spawning sequence may involve several successive egg releases. In either case, different eggs could be fertilized by different males within one or more nests. Attendant males continually fan the nests to keep offspring well aerated and free of silt. A bourgeois male (Taborsky 1997) also defends the nest against intraspecific and interspecific intruders until

the young eventually disperse, typically after about one to two weeks.

Intruders to a nest include conspecific “parasitic” males (Taborsky 1997) that may fertilize some of the eggs. From field observations, nest parasitism long has been suspected in centrarchid species (Keenleyside 1972; Gross 1979). Parasitic males frequently intrude upon spawning events by male-female pairs and may steal some fertilizations from the attendant male (Gross 1979; Dominey 1980; Gross and Charnov 1980). In bluegill sunfish (*Lepomis macrochirus*), young parasitic males (sneakers) swim rapidly into the nest and release sperm during spawning, whereas older parasitic males (satellites) gain access to the courtship process by mimicking females in behavior and morphology (Ehlinger et al. 1997). In general, bluegill parasites devote relatively less body mass to somatic tissue and more mass and energy to testes development than do bourgeois males (Gross 1982; Ehlinger et al. 1997). Although the genetic consequences of nest-parasitic behaviors seldom have been addressed in fishes, Gross (1991) suggested that fertilization thievery may be a common phenomenon and an evolutionarily stable strategy in some sunfish.

Here, we employ hypervariable markers at microsatellite loci to dissect the genetic mating system in a population of a related species, the redbreast sunfish, *Lepomis auritus*. Nest-attendant males as well as embryos and larvae of varying developmental stage and spatial position within each nest were assayed. The genetic data reveal the proportion of offspring sired by each nest-attendant male, the numbers of females with which he had spawned, and the frequencies and spatial orientations of fertilization thievery. When compared to those reported previously for the bluegill, the genetic re-

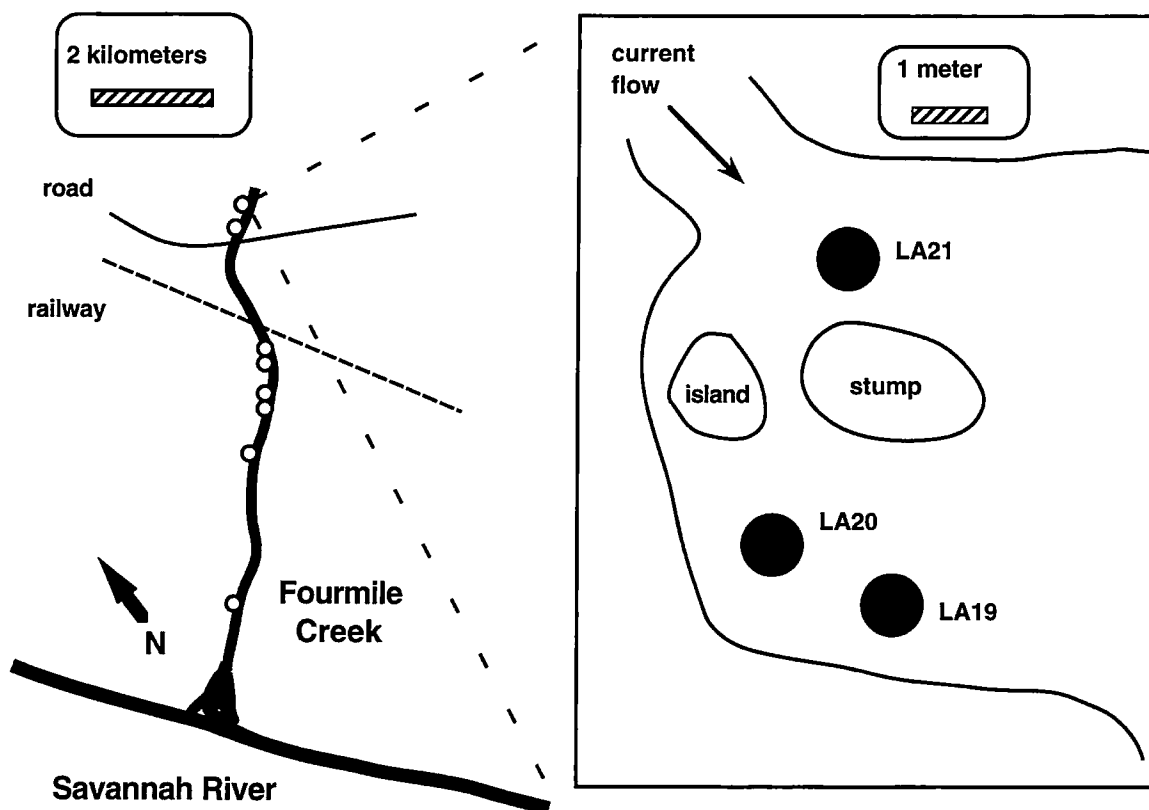


FIG. 1. Map of the eight collecting locales in Fourmile Creek near Barnwell, South Carolina, with a closeup showing the three nest sites within one locale.

sults indicate that a diversity of reproductive behaviors exists and can differ in expression even between closely related fish species.

#### MATERIALS AND METHODS

Nests were collected from Fourmile Creek, a tributary of the Savannah River on the Department of Energy's Savannah River Site, located near Barnwell, South Carolina (Fig. 1). Nest-tending males were identified and captured at night using a spotlight and portable electrofishing devices. Male and female sunfish not attached to a particular nest also were collected, yielding  $N = 64$  adults total. Of these, 48 were males (including three *L. punctatus* captured near a *L. auritus* nest) and 16 were females (including one *L. gulosus* collected within a *L. auritus* nest). Nonnesting adults were collected by electrofishing around woody debris and vegetation within 5–10 m of the sampled nests and probably represent only a small fraction of the redbreast sunfish in the immediate area. Gross morphological inspections as well as examinations of gonadal tissues in dissections of all adult fish revealed no evidence for cryptic satellite males (i.e., female mimics), which have been reported in bluegill sunfish.

A typical nest was about one meter in diameter and contained as many as several thousand young. For genetic assay, samples of nonmobile embryos and larvae were taken from one center and four perimeter locations within each nest. Each sample consisted of all offspring within a 65-mm circle on the nest floor. Substrate samples were scooped into a jar and

returned to the laboratory, where *L. auritus* juveniles were identified under a dissecting microscope (Fletcher 1993). Free-swimming fry were collected from the water column above the nest. Based upon morphological inspection, each offspring was classified as an embryo, hatchling, mid-yolksac fry, or swim-up fry (captured in hover above the nest substrate). Alternative developmental phases within a nest usually were discrete and unambiguous. Offspring were fixed in 100% ethanol, and DNA was extracted up to 18 months later.

To generate a size-selected genomic library for *L. auritus*, total DNA was isolated and pooled from the liver tissue of five adult redbreast sunfish taken from the Oconee River in Athens, Georgia. The library, containing 300–800-bp fragments, was constructed and then screened for  $(GATA)_n$  and  $(GACA)_n$  repeats using an enriched protocol (details in Prodhöl et al. 1996). Fifty positive colonies were isolated using a  $(GATA)_4$  probe, whereas only one proved positive for  $(GACA)_4$ . Positive clones were cycle sequenced using a commercial kit (fmol system; Promega), and PCR primer pairs were designed for six of the  $(GATA)_n$  loci.

DNA was extracted from each embryo using the method of Altschmied et al. (1997). Briefly, small tissue samples were blotted to remove ethanol, macerated in 100  $\mu$ l of extraction buffer (100 mM NaCl, 0.5% sarkosyl), and boiled for 15 min. After cooling to room temperature for 2–3 min, 100  $\mu$ l of 20% Chelex was added and incubated at room temperature for 15 min. Samples were boiled for an additional 15 min and briefly spun in a microcentrifuge to separate DNA from

TABLE 1. Microsatellite locus designations, primer sequences, and product size. Heterozygosity statistics are based on 64 presumably unrelated adult redbreast sunfish from the same river drainage. *P*-values (and associated standard error, SE) represent departures from Hardy-Weinberg equilibrium and are estimated by a Markov chain method (see text). Markov chain parameters were set at 1000 dememorization steps, 1000 batches, and 2000 iterations per batch.

Locus	Primer sequence (5'→3')	Size (bp)	Obs. het.	Exp. het.	<i>P</i> -value	SE
RB7	GTGCTAATAAAGGCTACTGTC TGTTCCTTAATTGTTTGA	117–205	0.891	0.923	0.131	0.007
RB20	GGTCTACTGGTAAATGAGGG GTTGGCTGTCGAGAGTAAAA	200–304	0.844	0.905	0.668	0.008

the Chelex/debris; 1–4  $\mu$ l of this supernatant was used as a PCR template.

Amplifications were performed in 10  $\mu$ l volumes, each containing 1 $\times$  Promega buffer, 1.5 mM MgCl<sub>2</sub>, 200  $\mu$ M dNTPs, 5 pmol of each primer (one of which was radioactively end-labeled with  $\gamma$ <sup>32</sup>P ATP), and 0.5 U Promega *Taq* DNA polymerase. Cycling parameters were as follows: an initial 2 min denaturation at 95°C, followed by 30 cycles of 1 min at 94°C, 30 sec at 47°C (locus RB7) or 60°C (locus

RB20), and 30 sec at 72°C. Reactions were electrophoresed in standard 6% polyacrylamide denaturing gels and exposed to X-ray film for 4–48 h. Alleles were labeled according to molecular size such that, for example, allele “177” was 177 nucleotides in length as judged by relative mobility of the original cloned fragment.

## RESULTS

### Microsatellite Features

We examined six microsatellite loci from the library of potential markers. Two loci (RB7 and RB20; Table 1) proved highly informative. Two other loci were monomorphic in our samples, one failed to amplify, and the sixth was difficult to score for technical reasons. Both of the informative loci also proved to be polymorphic in samples of two other sunfish species, *L. punctatus* and *L. gulosus*. A microsatellite locus *Lma21* from the bluegill (Colbourne et al. 1996) also was assessed, but it was not consistently scorable in redbreast sunfish.

Within the sample of 64 presumably unrelated adults, 22 and 18 alleles, respectively, were observed at RB7 and RB20 (Fig. 2). Conventional exclusion probabilities (Chakraborty et al. 1988) were 0.84 and 0.80 for these loci, yielding a combined parental exclusion probability of 0.97. However, in the case of unknown parentage (not just paternity), modified exclusion probabilities are required (Dodds et al. 1996; Jones et al. 1998). In this case, our combined paternity/maternal exclusion index was greater than 0.90.

Null alleles were not a complication in this study as gauged by two lines of evidence. First, neither locus deviated significantly from Hardy-Weinberg proportions in the population sample of adults (exact test of Guo and Thompson [1992] as conducted in GENEPOP 3.1b [Raymond and Rousset 1995]). Second, null alleles were not observed in any father/progeny array. Most individuals were heterozygous, and in the seven cases in which a nest-attendant male appeared homozygous at one locus, he invariably transmitted this allele to all of his biological offspring. This latter point is illustrated in Figure 3. The attendant male (Fig. 3; second lane from the left) at nest LA35 appeared homozygous, and all 33 assayed offspring within the nest were heterozygous. Twenty-five of these juveniles displayed an allele from the nest-attendant male and thus were likely his offspring, whereas the remaining young (eight of which are marked by arrows in Fig. 3) probably resulted from nest cuckoldry (see below).

### Genetic Paternity

By an extension of the procedures just described, genetic parentage was assessed for all attendant (and other) males.

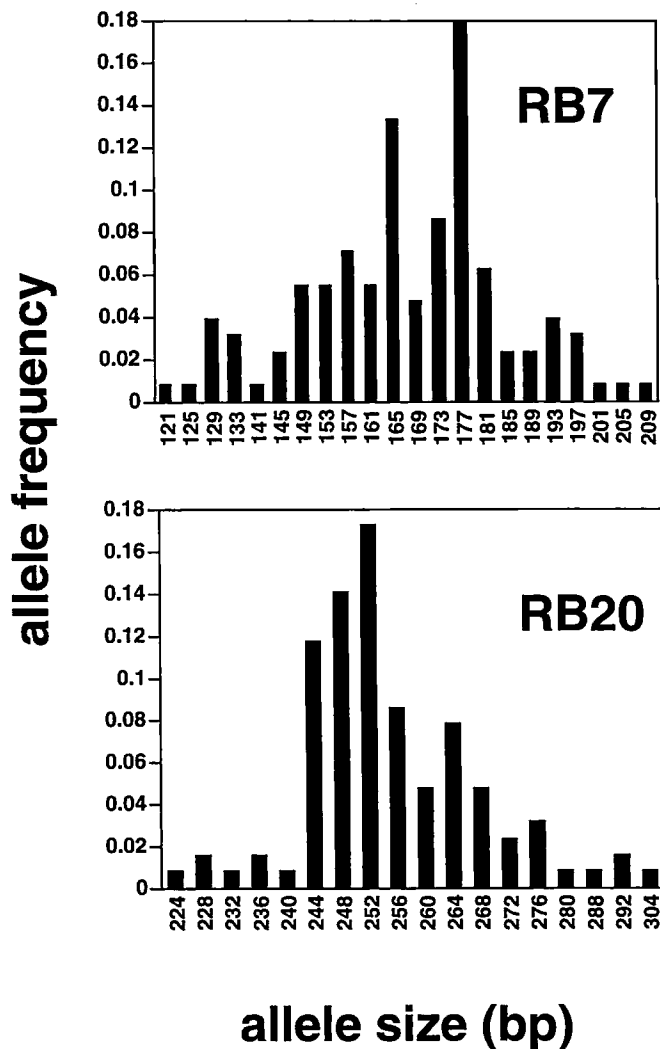


FIG. 2. Allele frequencies for the microsatellite loci RB7 and RB20 in 64 presumably unrelated adult sunfish from the Fourmile Creek site.

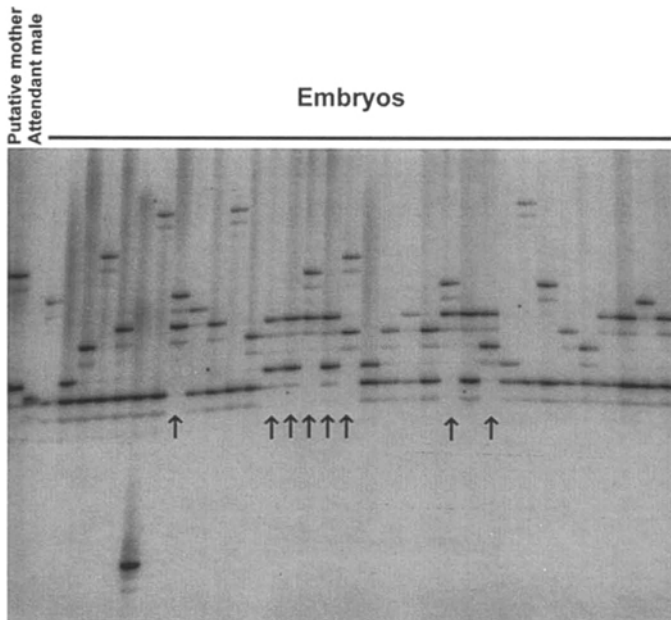


FIG. 3. Microsatellite gel showing the genotypes (RB20 locus, nest LA35) of 33 embryos, the associated nest-attendant male, and a candidate mother (a reproductively mature female captured in close proximity to the nest). On the basis of this genetic information in conjunction with that from the second locus (not shown), the candidate female did not mother more than one of the assayed embryos in this nest. Arrows denote embryos that by genetic evidence were not sired by the nest-attendant male. The attendant male similarly was excluded as sire of one additional embryo by genotypes observed at the second microsatellite locus.

Multilocus genotypes were determined for each attendant male and for all embryos sampled from his nest. By comparing genotypes across half-sib progeny arrays, the genotype of the shared parent became evident. For example, all offspring from nest LA35 (Fig. 3) were heterozygous at RB20. Those sired by the attendant male (which was homozygous for the "236" allele) must have received that paternal allele, such that the other allele was of maternal origin. The same procedure applied to the second locus resulted in assignment of a dilocus maternal gametic haplotype to each juvenile. Note that LA35 was the nest most severely parasitized in our study. In other nests, most of the clutches were sired by a single father, so the maternal haplotypes inherited by offspring were immediately apparent.

In 22 of the 25 nests surveyed (88%), 90% or more of the juveniles sampled displayed genotypes consistent with Mendelian inheritance from the nest-attendant male (Table 2). One exception already has been mentioned: in nest LA35, nine of 33 young (27%) were incompatible with the genotype of the resident adult male. However, genotypes of eight of these latter offspring were consistent with paternity by a single parasitic male, as is the ninth embryo if a single mutation is assumed in the germ line of the hypothesized parasitic father. Thus, we provisionally conclude that the attendant male of nest LA35 sired 24 embryos, and a parasitic male sired the remaining nine embryos, one of which displayed a mutation at locus RB20.

In the two other exceptional nests, LA03 and LA11, few or none of the progeny could have been fathered by the nest-

TABLE 2. Genetically deduced parentage for redbreast sunfish juveniles in 25 nests. Individuals that apparently were not sired by the attendant male are described in Table 3 or, in the case of nest LA35, in Figure 3.

Nest	Total no. young in nest <sup>1</sup>	No. assayed young sired by attendant male	Minimum no. mothers
LA01	161	9 of 10	3
LA03	342	0 of 50	5
LA04	3352	21 of 21	4
LA07	195	10 of 10	2
LA08	7077	15 of 15	4
LA09	4401	10 of 10	2
LA11	219	≤ 5 of 50	4
LA12	2577	47 of 50	3
LA13	1546	58 of 59	4
LA14	4947	9 of 9	3
LA16	2553	15 of 15	2
LA18	175	21 of 22	2
LA19	1339	47 of 48	5
LA21	4390	38 of 38	4
LA22	997	10 of 10	2
LA23	727	60 of 60	3
LA25	4295	20 of 20	4
LA26	1105	174 of 175	6
LA27	1066	22 of 23	4
LA28	4606	99 of 100	6
LA31	1103	29 of 29	4
LA32	6731	44 of 44	4
LA33	684	49 of 49	5
LA35	214	24 of 33	4 with attendant, 2 with cuckold
LA36	317	43 of 46	4

<sup>1</sup> Calculated extrapolation based on size of nest and juvenile densities in the spot samples from sites within the nest (the tremendous task of sorting embryos from substrate prevented absolute counts).

attendant male (Table 2). In each of these cases, nearly all juveniles had genotypes consistent with the possibility that they had been sired by only one nonattendant male (the exceptions occurred in nest LA03, where a second father must be postulated for two of the 50 offspring assayed).

A small proportion of juveniles in several additional nests displayed genotypes that at face value appear inconsistent with paternity by the nest-attendant male (Table 3). The genotypes of three of these offspring indicate that the true sire was not the attendant male because the paternity exclusions involved both loci. However, the remaining 12 "anomalous" offspring (other than those in nest LA35, but including the two embryos from nest LA03 that were not consistent with the half-sib progeny array) had genotypes inconsistent with the putative father at only one assayed locus (Table 3). In seven of these cases, a possible paternal allele in a juvenile differed from a nearest counterpart allele in the resident male by two or more increments in microsatellite repeat count. For example, in offspring LA12A5I (Table 3), allele "189" at locus RB7 differed by three steps in repeat count from the nearest-size allele ("177") in the nest-attendant male. In other species, multi-unit mutations at microsatellite loci are thought to be rarer than single-unit mutations, although the former do occur as well (Jarne and Lagoda 1996 and references therein). Thus, it remains uncertain from our data what fraction of the single-locus paternity "exclusions" are to be attributed to parasitism as opposed to de novo mutations.

TABLE 3. Juveniles provisionally excluded as biological offspring of the corresponding attendant male. Underlined genotypes indicate the genetic exclusions. For those juveniles that differ from the attending male's genotype at a single locus, the number of mutational steps required to generate the difference is listed. "?" indicates missing data. The two juveniles from nest LA03 are included as departures from the hypothetical parasitic male's genotype (see text).

Nest	Embryo	Embryo genotype		Paternal genotype		steps
		RB7	RB20	RB7	RB20	
LA01	B3A	<u>149/149</u>	248/256	<u>133/189</u>	236/256	≥ 4
LA03	A1O	165/181	<u>244/256</u>	149/165	<u>236/248</u>	≥ 1
LA03	A5E	165/169	<u>244/288</u>	149/165	<u>236/248</u>	≥ 1
LA12	A1C	165/?	<u>232/236</u>	177/177	<u>244/252</u>	≥ 2
LA12	A5H	177/193	<u>256/268</u>	177/177	<u>244/252</u>	≥ 1
LA12	A5I	<u>153/189</u>	244/252	177/177	244/252	≥ 3
LA13	C5G	<u>173/177</u>	244/244	129/181	244/292	≥ 1
LA18	A4M	<u>145/161</u>	?	149/177	256/256	≥ 1
LA19	D5B	<u>133/165</u>	252/260	<u>173/177</u>	248/252	≥ 2
LA26	C1T	<u>173/173</u>	<u>248/252</u>	<u>149/157</u>	<u>244/244</u>	both loci
LA27	A5W	<u>153/205</u>	<u>240/240</u>	<u>133/189</u>	<u>236/256</u>	both loci
LA28	A2D	<u>149/161</u>	260/276	<u>157/177</u>	236/260	≥ 2
LA35	A6E	<u>153/169</u>	236/252	<u>145/177</u>	236/236	≥ 2
LA35	A6H	<u>173/181</u>	<u>252/260</u>	<u>145/177</u>	<u>236/236</u>	both loci
LA35	A6M	177/181	<u>240/252</u>	145/177	236/236	≥ 1
LA35	A6N	<u>153/153</u>	<u>240/252</u>	<u>145/177</u>	<u>236/236</u>	both loci
LA35	A6O	<u>149/181</u>	<u>252/264</u>	<u>145/177</u>	<u>236/236</u>	both loci
LA35	A6P	<u>153/153</u>	<u>240/252</u>	<u>145/177</u>	<u>236/236</u>	both loci
LA35	A6Q	<u>153/161</u>	<u>248/268</u>	<u>145/177</u>	<u>236/236</u>	both loci
LA35	A6V	<u>173/181</u>	<u>252/260</u>	<u>145/177</u>	<u>236/236</u>	both loci
LA35	A7A	<u>165/181</u>	<u>244/252</u>	<u>145/177</u>	<u>236/236</u>	both loci
LA36	A6B	<u>145/173</u>	236/252	<u>153/181</u>	252/260	≥ 2
LA36	A7P	<u>149/177</u>	<u>236/248</u>	<u>153/181</u>	<u>252/260</u>	both loci
LA36	A7L2	145/153	<u>236/244</u>	153/181	<u>252/260</u>	≥ 2

Also of interest is the fact that nest LA36 was less than one meter from LA35, yet none of the three "aberrant" young within it displayed a composite genotype compatible with being the offspring of the same individual that parasitized nest LA35. However, all three excluded young in nest LA36 did have genotypes consistent with paternity by the attendant of LA35, suggesting that this nest-attendant bourgeois male was also a nest parasite.

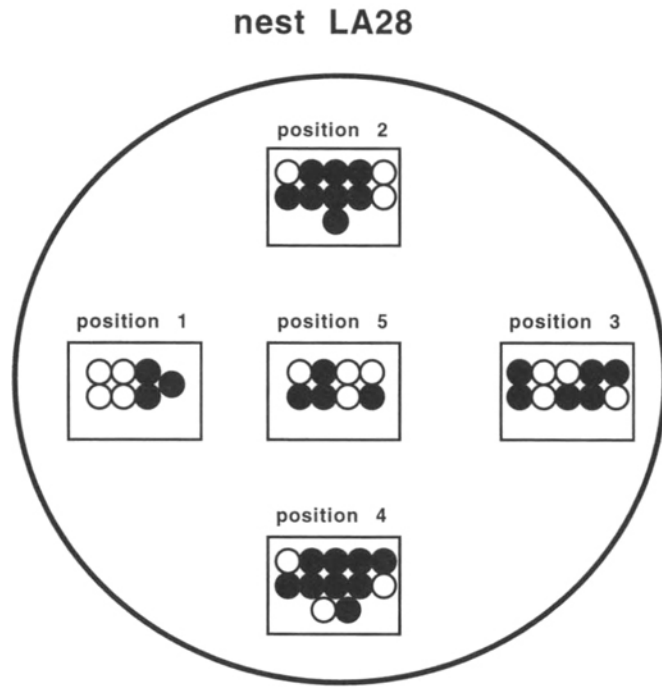
#### Genetic Maternity

Because most juveniles possessed genotypes consistent with inheritance from the nest-attendant male, genetic assignments of maternity were facilitated greatly. Thus, the maternally derived haplotype in each such offspring could be specified. An accumulation of such data across progeny cohorts yielded information on maternal contributions to a nest. In some instances, maternal diploid genotypes could be reconstructed from the progeny arrays given the inferred paternal contribution to each offspring, but this was possible only when a large number of progeny was assayed relative to the number of females spawning in a nest. In more usual situations, maternal gametes could be specified but maternal diploid genotypes at one or both loci remained uncertain. These maternal haplotypes nonetheless provide further information on minimum numbers of females that contributed to progeny cohorts within a nest.

These concepts and procedures can be illustrated by ref-

erence to nest LA28 (Figs. 4, 5). This nest initially was characterized by possession of two developmentally distinct broods, "A" and "B." Examination of maternal genotypes in the progeny revealed that two females contributed offspring to brood "A" (Fig. 4). One of these mothers was deduced to have had the diploid genotypes 145/149 and 236/260 at loci RB7 and RB20, respectively; the other mother had the genotypes 153/177 and 248/248. However, only the haploid maternal genotypes could be deduced securely for brood "B" because some of the mothers must have shared alleles. For example, maternal allele 165 is represented in three distinct gametes and maternal allele 169 is represented in four different gametes. A total of sixteen maternal gametes was deduced for brood "B" (Fig. 5), and these could have stemmed from no fewer than four different mothers (none of which were the same as the two mothers of brood "A," as can be seen by examination of the alleles at locus RB7; Figs. 4, 5). Thus, as judged by maternal gametic contributions, altogether at least six different females contributed to the progeny in nest LA28.

Ten nests contained juveniles of more than one developmental stage. By genetic evidence (deduced female gametic contributions), the different developmental phases within a nest usually represented broods from different mothers (the only exception was nest LA13, where two of three putative developmental broods could have come from the same mother, who released eggs in the nest on two occasions). Thus,



developmental phase "A" embryos

maternal genotypes:

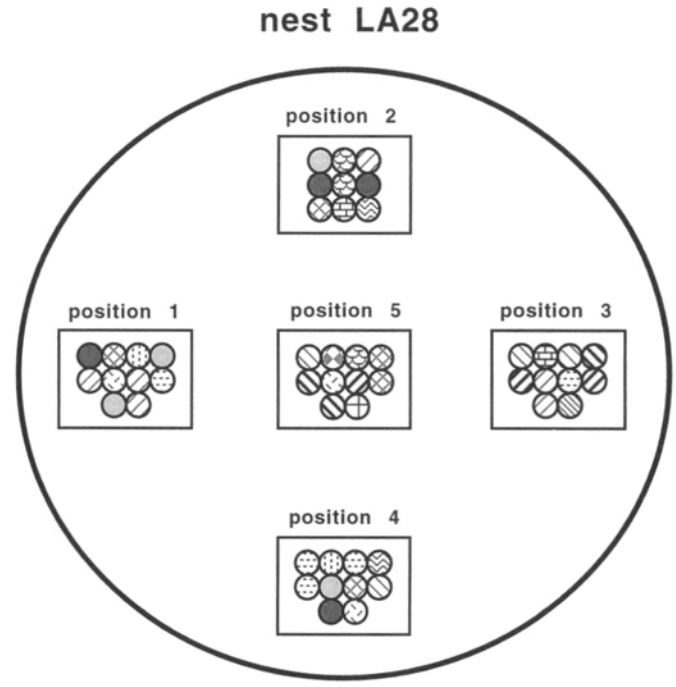
● mother #1	○ mother #2
145/149 at RB7	153/177 at RB7
236/260 at RB20	248/248 at RB20

FIG. 4. Spatial distribution of the maternal genetic contributions to progeny of developmental phase "A" in nest LA28. Each embryo is represented as a circle within a collecting position. For these offspring, maternal diploid genotypes could be deduced (see text).

many of the polygynous matings by a nest-attendant male must have been separated sufficiently in time (at least 12 h) as to be reflected in detectable developmental differentiation among clutches within a nest. However, as for nest LA28, the genetic data also indicate that progeny of a given developmental phase often had multiple mothers. Another example involved nest LA03, where all offspring were indistinguishable in developmental phase but 10 different maternal alleles (at least five mothers) contributed to the nest. Thus, the observed numbers of different developmental phases provide a minimum estimate of the number of females that spawned within a nest.

Overall, for this population of *L. auritus*, estimates of the minimum number of mothers per nest ranged from two to six, with a mean of 3.7 (Table 2). Somewhat surprisingly, by genetic evidence no adult female captured in the vicinity of a particular nest had spawned in that nest.

Maternity also was examined with respect to the microspatial positions of juveniles within each nest. Although our sample sizes of progeny were modest, maternal alleles characteristically proved to be dispersed widely across a nest such



developmental phase "B" embryos

maternal gametes (RB7/RB20 alleles):

● 161/236	○ 165/256	○ 181/240	⊕ 205/240
○ 169/248	○ 153/224	○ 161/248	○ 169/272
○ 193/248	○ 153/226	○ 205/272	○ 209/272
○ 165/248	○ 169/240	○ 165/224	○ 169/236

FIG. 5. Spatial distribution of the maternal genetic contributions to progeny of developmental phase "B" in nest LA28 (see legend to Fig. 4). For these offspring, only the maternal haploid (gametic) genotypes could be deduced (see text).

that juveniles from one site could not be distinguished consistently from those at others (e.g., Figs. 4, 5). This lack of evident spatial clustering of progeny may not be surprising given that adult pairs typically swim and deposit eggs in a circular pattern over the entire nest during the spawning process (Lukas and Orth 1993).

DISCUSSION

The picture that emerges from this genetic dissection of a redbreast sunfish population is one in which multiple females (at least two to six) spawn in a nest and more than 95% of the young are sired by the nest-attendant male. However, small fractions of progeny (< 10%) in a moderate proportion of nests (40%) were the result of parasitism, and in rare cases males tended nests composed almost exclusively (> 95%) of offspring that they did not sire. These conclusions will be examined for caveats and interpreted in the light of reproductive behaviors previously suspected for other sunfish species.

### Parasitism or Mutation?

*Disparities at Two Loci.*—Although most of the offspring displayed genotypes consistent with their having been sired by the nest-attendant male, a small fraction of young carried alleles not present in the respective attending male. In each of three cases (involving nests LA26, LA27, and LA36), the single “aberrant” offspring almost certainly was the product of reproductive parasitism because paternity exclusions for the nest-attendant male occurred at both loci. Whether the parasitism resulted from stolen fertilization or by immigration of foreign offspring cannot be decided by the genetic data alone, but immigration seems highly unlikely given that many of the juveniles assayed were embryos that lack “self propulsion.” In any event, such progeny were rare, comprising only 0.6%, 4.4%, and 2.2% of the offspring sampled from these respective nests.

*Disparities at One Locus.*—In other possible instances of nest parasitism (Table 3), the paternity “exclusions” involved one locus only. Thus, an alternative interpretation is that an offspring received a de novo germ-line mutation from the nest-attendant male which was, therefore, its true father. This scenario is most tenable for juveniles carrying an allele that was only one putative “mutation step” (a 4-bp insertion or deletion in this case) removed from an allele in the nest-attendant male. This is because, in other species, more than 80% of microsatellite mutations reportedly involve incremental rather than saltational changes in size (Weber and Wong 1993; Jin et al. 1996; Primmer et al. 1996).

However, for seven of the 12 redbreast sunfish young involved in single-locus paternity exclusions (not including those in parasitized nest LA35), the alleles in question differed in size from those in the nest-attendant male by at least two repeat units (8-bp). If 80% of new microsatellite mutations in *L. auritus* involve single stepwise changes, the probability that seven of these 12 putative mutations involve more than one step is less than 0.05 (binomial probability,  $P = 0.004$ ), suggesting that at least some of these juveniles also resulted from parasitism. If all of the suspect juveniles in Table 2 resulted from parasitism, then the stolen-fertilization success rate within parasitized nests was about 3.9% (22 of 566 offspring). For the population at large (including non-parasitized nests, but excluding takeover nests LA03 and LA11), the frequency of offspring from parasitic matings was about 2.5% (22 of 896 offspring).

These values can be interpreted as providing relative success rates (i.e., reproductive fitnesses to the juvenile stage) of males with alternative mating tactics. Clearly, the bourgeois strategy greatly predominates over parasitic strategies in the Fourmile Creek population. Nonetheless, the evolutionary mechanism(s) that maintain these behaviors and govern their relative frequencies cannot be decided by these kinds of genetic data. This discussion assumes that the two mating strategies are indeed distinct and there is no parasitism by bourgeois males. However, our data suggest that the two reproductive strategies are not mutually exclusive and that bourgeois males may parasitize other nests if the opportunity arises.

Alternatively, if we postulate that de novo mutation is responsible for all of the single-locus paternity exclusions, then

TABLE 4. Probabilities of genetic identity at two microsatellite loci for nest-attendant males that were provisionally excluded as biological fathers of some offspring in a nest. The probability of identity represents the likelihood that the genotype of a nest-attendant male is shared by another individual drawn at random from the population. Calculations assume that the genotype frequencies are in Hardy-Weinberg equilibrium.

Bourgeois male	RB7	RB20	Combined
LA01	$1.47 \times 10^{-3}$	$2.01 \times 10^{-2}$	$2.95 \times 10^{-5}$
LA03	$7.81 \times 10^{-2}$	$1.10 \times 10^{-2}$	$8.58 \times 10^{-4}$
LA012	$3.23 \times 10^{-2}$	$4.03 \times 10^{-2}$	$1.30 \times 10^{-3}$
LA013	$4.88 \times 10^{-3}$	$3.66 \times 10^{-3}$	$1.78 \times 10^{-5}$
LA018	$1.97 \times 10^{-2}$	$7.38 \times 10^{-3}$	$1.45 \times 10^{-4}$
LA019	$3.09 \times 10^{-2}$	$4.83 \times 10^{-2}$	$1.49 \times 10^{-3}$
LA026	$7.69 \times 10^{-3}$	$1.37 \times 10^{-2}$	$1.06 \times 10^{-4}$
LA027	$1.47 \times 10^{-3}$	$2.01 \times 10^{-2}$	$2.95 \times 10^{-5}$
LA028	$2.52 \times 10^{-2}$	$1.10 \times 10^{-2}$	$2.78 \times 10^{-4}$
LA035	$8.42 \times 10^{-3}$	$1.37 \times 10^{-2}$	$1.16 \times 10^{-4}$
LA036	$6.83 \times 10^{-3}$	$1.61 \times 10^{-2}$	$1.10 \times 10^{-4}$

mutation rates can be estimated. A total of 996 embryos was assayed, and 12 were excluded as half-sibs of their respective progeny arrays at a single locus (i.e., were not sired by the attendant male). Considering only the paternal side, locus RB7 would have undergone seven mutational events, yielding a mutation rate of  $7.0 \times 10^{-3}$ ; and locus RB20 would have undergone five mutational events for an estimated mutation rate of  $5.0 \times 10^{-3}$ . These values agree well with tetranucleotide mutation rates in other organisms (Weber and Wong 1993; Ellegren 1995; Primmer et al. 1996).

Interestingly, these inferred mutation rates are similar in general magnitude to the probabilities of genetic identity at individual loci (Table 4). This means, for example, that the paternity “exclusion” at the RB7 locus in embryo C5G from nest LA13 (Table 3) may have resulted from a mutational event in the nest-attendant male’s germ line (probability  $7.0 \times 10^{-3}$ ) or, with similar likelihood, from a parasitic male hypothesized to share a genotype with the nest-attendant male at locus RB20 (probability  $3.7 \times 10^{-3}$ ).

Regardless of the precise contribution of parasitism versus de novo mutation to the paternity “exclusions,” the following conclusions hold for this population: (1) the success rate of nest parasitism as gauged by the fraction of offspring produced is low ( $< 5\%$ ); and (2) mutation rates at the microsatellite loci assayed were too low ( $< 10^{-2}$  per generation) to have seriously compromised the mating system analysis.

### Nest Takeovers

The attendant male at LA03 did not sire any of the 50 juveniles surveyed from that nest. One possibility is that he and the true attendant male were mislabeled in the collections. However, the data indicate that a single hypothetical male of the appropriate two-locus genotype could have sired 48 of the 50 juveniles in that nest. A male with this genotype was not represented in our collection of adult sunfish, indicating that the problematic male was not confused with one of our other samples. Thus, a more likely explanation is that a foreign male appropriated the nest site after the parental male departed, was expelled, or died.

The same phenomenon was observed in nest LA11. There,



the attendant male could have sired no more than five of the 50 young sampled from the nest. Yet, genotypes in all of the assayed progeny could be attributed to a single hypothetical male not represented in our collection of adults. Again, the nest site probably was appropriated by another male. Nest takeovers are likely an opportunistic response to nest-site availability. Perhaps a takeover male increases his chances of siring a brood through acquisition of a desirable nest site, even if it already contains embryos. Another possibility is that such males may profit by eating offspring in the takeover nest because cannibalization is a common phenomenon in *L. auritus* (pers. obs.).

#### *Numbers of Spawning Females per Nest*

The mean number of mothers that at face value contributed progeny to a nest was 3.7 (range 2–6). These values must be interpreted as minimal estimates of maternal numbers in this population because only two hypervariable loci were employed in the genetic assays, and only modest numbers of progeny were sampled genetically from among the hundreds or thousands of juveniles within most nests. Unfortunately, statistical estimation of the number of females contributing to a half-sib progeny array is not trivial. Simulation and maximum-likelihood approaches currently are under development in our lab and elsewhere (JAD, DeWoody, Fuimera, and Avise, unpubl.; K. L. Kichler, pers. comm.), but to our knowledge no model has been published that can account for as many as seven parents contributing to a half-sib progeny array (as is the case in our dataset). Such models are needed to provide more accurate estimates of the number of mothers to spawn in a nest.

Earlier, we examined potential mutation rates in the paternal germ line. These estimates were sufficiently high as to raise the question of whether similar mutations in the maternal germ line might seriously affect our estimates of the number of females to spawn in a nest. If the highest of the possible mutation rates deduced in males ( $7.0 \times 10^{-3}$ ) is assumed, then by analogy seven new maternal alleles might be expected in our sample of 996 embryos, meaning that the original estimates were inflated *at most* by seven additional mothers. Even in this most extreme situation, this number of females distributed over the 25 surveyed nests would decrease the mean estimated number of mothers per nest only from 3.7 to 3.4.

#### *Interspecific Comparisons*

A life-history analysis of bluegill sunfish (*L. macrochirus*) in Lake Opinicon, Ontario, indicated that 85% of the reproductively active males were reproductive parasites (Gross 1982). The remaining males (15%) built nests and provided parental care. Genetic estimates of cuckoldry rates in bluegill subsequently were provided by allozyme data (Philipp and Gross 1994). Proportions of offspring not sired by the nest-attendant males ranged from 0% to 59% across four colonies in Lake Opinicon and were correlated significantly with densities of reproductive parasites. Microsatellites also were used to document cuckoldry in Lake Opinicon bluegill (Colbourne et al. 1996), albeit in a sample from a single nest.

Bluegill sunfish exhibit three distinct male phenotypes—

parental, sneaker, and satellite—that presumably are maintained through negative frequency-dependent sexual selection (Gross 1991). By contrast, in the pumpkinseed sunfish (*L. gibbosus*), parasitic males appear to be sneakers only (Gross 1982); satellite behavior has not been observed. Pumpkinseed sunfish are not colonial breeders, and reproductive parasites appear to be rarer than in most bluegill populations (Gross 1982). However, the current genetic data on redbreast sunfish suggest that group nesting is not invariably associated with a high rate of male nest parasitism in sunfish. The low levels of nest parasitism in the current study when compared to those for bluegills from Lake Opinicon are perhaps not surprising given that all of the redbreast sunfish in our sample were sexually dimorphic (i.e., no morphologically distinguishable “male reproductive parasites” as defined by Taborsky [1997] were detected). Another factor perhaps promoting the lower rate of nest parasitism in redbreast sunfish was the fact that *L. auritus* in Fourmile Creek are not as densely colonial as are *L. macrochirus* in Lake Opinicon, although in Fourmile Creek the proximity of nearby nests does influence parasitism rates of individual nests more than any physical characteristic of the parasitized male such as size, age, or coloration. Parasitism rates are also affected by aggressiveness, at least in captive populations. The most aggressive males (regardless of size) are the best nest defenders (D. Fletcher, pers. obs.).

Behavioral reports of attempted parasitism via male mimicry of females has been reported in a Virginia population of redbreast sunfish (Lukas and Orth 1993). However, the current genetic data for redbreast sunfish in the Savannah River system are consistent with a predominant reproductive strategy of parental care by biological fathers. Levels of nest parasitism are known to vary widely among bluegill colonies (Gross 1991) and to depend largely on the density of parasitic males. The lack of distinctive parasitic male morphs in our sample, together with a probable assignment of paternity for offspring in one nest (LA36) to the nest-attendant male of an adjacent nest, suggest that much of the nest parasitism in redbreast sunfish from Fourmile Creek results from extrapair fertilizations by bourgeois males rather than by specific cuckolding morphs.

The current study is among the few currently available that have employed genetic markers to examine the incidence and relative success rates of alternative mating tactics in fishes. Results suggest that the magnitude and pattern of reproductive parasitism are evolutionarily labile features in species with extended male parental care of offspring. Many more studies of this sort will be needed before the true scope of heterogeneity in reproductive strategies in fishes will be uncovered.

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## LITERATURE CITED

- ALTSCHMIED, J., U. HORNUNG, I. SCHLUPP, J. GADAU, R. KOLB, AND M. SCHARTL. 1997. Isolation of DNA suitable for PCR for field and laboratory work. *Biotechniques* 23:228–229.
- AVISE, J. C. 1994. *Molecular markers, natural history and evolution*. Chapman and Hall, New York.
- BIRKHEAD, T. R., AND A. P. MØLLER. 1992. *Sperm competition in birds*. Academic Press, New York.
- BREder, C. M., JR. 1936. The reproductive habits of North American sunfishes (family Centrarchidae). *Zoologica* 21:1–47.
- CHAKRABORTY, R., T. R. MEAGHER, AND P. E. SMOUSE. 1988. Parentage analysis with genetic markers in natural populations. I. The expected proportion of offspring with unambiguous paternity. *Genetics* 118:527–536.
- COLBOURNE, J. K., B. D. NEFF, J. M. WRIGHT, AND M. R. GROSS. 1996. DNA fingerprinting of bluegill sunfish (*Lepomis macrochirus*) using (GT)<sub>n</sub> microsatellites and its potential for assessment of mating success. *Can. J. Fish. Aquat. Sci.* 53:342–349.
- DODDS, K. G., M. L. TATE, J. C. MCEWAN, AND A. M. CRAWFORD. 1996. Exclusion probabilities for pedigree testing from animals. *Theor. Appl. Genet.* 92:966–975.
- DOMINEY, W. J. 1980. Female mimicry in male bluegill sunfish—a genetic polymorphism? *Nature* 284:546–548.
- EHLINGER, T. J., M. R. GROSS, AND D. P. PHILIPP. 1997. Morphological and growth rate differences between bluegill males of alternative reproductive life histories. *N. Am. J. Fish. Mgt.* 17:533–542.
- ELLEGREN, H. 1995. Mutation rates at porcine microsatellite loci. *Mammol. Genome* 6:376–377.
- FLETCHER, D. E. 1993. Nest association of dusky shiners (*Notropis cummingsae*) and redbreast sunfish (*Lepomis auritus*), a potentially parasitic relationship. *Copeia* 1993:159–167.
- GROSS, M. R. 1979. Cuckoldry in sunfishes (*Lepomis*: Centrarchidae). *Can. J. Zool.* 57:1507–1509.
- . 1982. Sneakers, satellites, and parentals: polymorphic mating strategies in North American sunfishes. *Z. Tierpsychol.* 60:1–26.
- . 1991. Evolution of alternative reproductive strategies: frequency-dependent sexual selection in male bluegill sunfish. *Philo. Trans. R. Soc. Lond. B Biol. Sci.* 332:59–66.
- GROSS, M. R., AND E. L. CHARNOV. 1980. Alternative male life histories in bluegill sunfish. *Proc. Natl. Acad. Sci. USA* 77:6937–6940.
- GUO, S. W., AND E. A. THOMPSON. 1992. Performing the exact test of Hardy-Weinberg proportion for multiple alleles. *Biometrics* 48:361–372.
- JARNE, P., AND P. J. L. LAGODA. 1996. Microsatellites, from molecules to populations and back. *Trends Ecol. Evol.* 11:424–429.
- JIN, L., C. MACANBAS, J. HALLMAYER, A. KIMURA, AND E. MIGNOT. 1996. Mutation rate varies among alleles at a microsatellite locus: phylogenetic evidence. *Proc. Natl. Acad. Sci. USA* 93:15285–15288.
- JONES, A. G., AND J. C. AVISE. 1997a. Microsatellite analysis of maternity and the mating system in the Gulf pipefish *Syngnathus scovelli*, a species with male pregnancy and sex-role reversal. *Mol. Ecol.* 6:203–213.
- . 1997b. Polygynandry in the dusky pipefish *Syngnathus floridae* revealed by microsatellite DNA markers. *Evolution* 51:1611–1622.
- JONES, A. G., S. OSTLUND-NILSSON, AND J. C. AVISE. 1998. A microsatellite assessment of sneaked fertilizations and egg thievery in the fifteen-spine stickleback. *Evolution* 52:848–858.
- KEENLEYSIDE, M. H. 1972. Intraspecific intrusions into nests of spawning longear sunfish (Pisces: Centrarchidae). *Copeia* 1972:272–278.
- LUKAS, J. A., AND D. J. ORTH. 1993. Reproductive ecology of redbreast sunfish *Lepomis auritus* in a Virginia stream. *J. Fresh. Ecol.* 8:235–244.
- PHILIPP, D. P., AND M. R. GROSS. 1994. Genetic evidence for cuckoldry in bluegill *Lepomis macrochirus*. *Mol. Ecol.* 3:563–569.
- PRIMMER, C. R., H. ELLEGREN, N. SAINO, AND A. P. MØLLER. 1996. Directional evolution in germline microsatellite mutations. *Nature Genet.* 13:391–393.
- PRODOHL, P. A., W. J. LOUGHRY, C. M. McDONOUGH, W. S. NELSON, AND J. C. AVISE. 1996. Molecular documentation of polyembryony and the micro-spatial dispersion of clonal sibships in the nine-banded armadillo, *Dasypus novemcinctus*. *Proc. R. Soc. Lond. B Biol. Sci.* 263:1643–1649.
- RAYMOND, M., AND F. ROUSSETT. 1995. GENEPOP: population genetic software for exact tests and ecumenicism. *J. Hered.* 86:248–249.
- TABORSKY, M. 1994. Sneakers, satellites, and helpers: parasitic and cooperative behavior in fish reproduction. *Adv. Study Behav.* 23:1–100.
- . 1997. Bourgeois and parasitic tactics: do we need collective, functional terms for alternative reproductive behaviors? *Behav. Ecol. Sociobiol.* 41:361–362.
- WEBER, J. L., AND C. WONG. 1993. Mutation of human short tandem repeats. *Human Mol. Genet.* 2:1123–1128.

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