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Journal
Marine Biology, 148(1)

ISSN
0025-3162

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Publication Date
2005-11-01

DOI
10.1007/s00227-005-0010-9

Marine Biology (2005) 148: 213–221
DOI 10.1007/s00227-005-0010-9

RESEARCH ARTICLE

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Cuckoldry rates in the Molly Miller (*Scartella cristata*; Blenniidae), a hole-nesting marine fish with alternative reproductive tactics

Received: 17 May 2004 / Accepted: 4 April 2005 / Published online: 19 August 2005

Abstract Microsatellite markers were developed and employed to assess genetic maternity and paternity of embryos in nest-tended clutches of the Molly Miller (*Scartella cristata*), a marine fish in which alternative reproductive tactics (ARTs) by males were recently described from behavioral and morphological evidence. Genetic data gathered for 1,536 surveyed progeny, from 23 barnacle-nest holes in a single Floridian population, indicate that on average about 5.5 females (range 3–9) contributed to the pool of progeny within a nest. With regard to paternity, the microsatellite data demonstrate that most of the surveyed nests (82.6%) contained at least some embryos that had not been sired by the nest-tending (bourgeois) male, and overall that 12.4% of offspring in the population had been sired via "stolen" fertilizations by other males. These are among the highest values of cuckoldry documented to date in nest-tending fishes, and they support and quantify the notion that the nest-parasitic ART is reproductively quite successful in this species despite what would otherwise seem to be highly defensible nesting sites (the restricted interior space of a barnacle shell). Our estimated cuckoldry rates in this population of the Molly Miller are compared to those previously reported for local populations in other nest-tending fish species, with results discussed in the context of ecological and behavioral variables that may influence relative frequencies of nest parasitism.

Communicated by P. W. Sammarco, Chauvin

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Introduction

In fish species with external fertilization and extended parental care of offspring in nests (usually by males), intense competition for access to mates and fertilizations has frequently led to the evolution of male alternative reproductive tactics (ARTs) (Gross 1991, 1996; Taborsky 1994, 2001). Such ARTs, as behaviorally defined, are often associated with distinctive morphological features of the fish as well (Gross 1982; Gross and Charnov 1980; Gross and Shine 1981; Taborsky 1997). Probably the most common dichotomy is between bourgeois males (nest tenders) and parasitic sneakers (Taborsky 1998). Bourgeois males typically are large, display colorful secondary sexual characters, actively court females, and tend nests (e.g., fan eggs and defend against predators). Sneaker males can be much smaller and less colorful, show no proclivity to tend nests, and often have much higher testis size relative to body mass (apparently an adaptation for sperm competition associated with nest parasitism; Taborsky 1998). Whenever a parasitic male "steals" some fraction of fertilization events from a bourgeois male, e.g., by sneaking onto his nest and releasing sperm during a spawning episode, the nest-tender then becomes a foster parent and is said to have been cuckolded.

The Molly Miller (*Scartella cristata*; Perciformes; Blenniidae) is an abundant combtooth blenny of temperate and tropical regions (Robins 1986). Found near the shoreline in rocky areas, its range in the tropical western Atlantic extends from Florida, Bermuda, and the northern Gulf through the Caribbean Sea to Brazil (Gilbert 2002; Robins 1986). The Molly Miller is a hole-dwelling species with external fertilization. Large nest-tending (bourgeois) males, whose elongate bodies reach up to 10 cm in length, are highly territorial and also provide extended care to the embryos in their respective nests, which are usually the hollow empty shells of barnacles (McEachran 1998). However, a recent comparative study of body sizes, accessory features of the

reproductive tract involved in sperm production and handling, and testicular glands involved in pheromone synthesis, coupled with behavioral monitoring, documented the presence of two other distinctive male reproductive morphotypes in *S. cristata* (Neat et al. 2003): non-reproducing satellites (medium-sized individuals that remain site-attached to a hole), and sneakers (small vagrant males that appear morphologically and behaviorally specialized for releasing sperm in larger males' nests). In other blennioid species, intrasexual polymorphisms of these sorts have usually (but see also Neat and Locatello 2002) been taken as good indicators for the presence of ARTs and nest parasitism (Goncalves et al. 2003a, b; Miranda et al. 2003; Neat 2001; Oliveira et al. 2000, 2001a, b, 2002).

Although indirect evidence for nest parasitism by males can emerge from morphological assessments and behavioral monitoring, marker-based genetic analyses have made it possible to quantify actual rates and patterns of reproductive cuckoldry in nature. Microsatellite markers, in particular, have proved invaluable for detecting and quantifying reproductive behaviors in fishes, including alternative reproductive tactics and mating systems (Avise et al. 2002; DeWoody and Avise 2001; Jones and Avise 2001). Here we develop and employ a battery of microsatellite markers to examine genetic parentage (maternity as well as paternity) in a Floridian population of *S. cristata*. Of particular interest are male cuckoldry rates, which paradoxically might be hypothesized to be either exceptionally high (because of a high density of Molly Miller nests at the surveyed locale, and the presence of a sneaker ART in this species), or exceptionally low (if the barnacle-hole nests are as physically defensible by bourgeois males as they would appear to be at face value). The genetic findings on cuckoldry rates will be interpreted in the context of earlier behavioral and morphological observations for this species. They will also be compared to similar microsatellite-based reports for local populations in other nest-tending species, in order to address various ecological and behavioral factors that might influence relative frequencies of nest parasitism in fishes.

Materials and methods

Sample collections and microsatellite development

On 12 October 2002, a collection of Molly Millers was made from a single rock surface (148×204 cm) that is part of a rock jetty facing the Gulf of Mexico in St. Andrews State Park, Panama Beach, Florida. By snorkeling, we collected bourgeois males from their barnacle-hole nests and preserved them in ethanol. Each of 38 nest-tenders was captured by placing a hollow plastic tube, closed at one end, over his nest, and inducing the male to swim into the tube by gentle tapping on the tube. Each male's nest was then scraped off the rock surface by chisel and hammer, and the embryos lining its inner wall were preserved in a 20% DMSO/saturated NaCl solution. Care was taken to ensure that each bourgeois male was correctly associated with his nest. An additional 29 individuals (males, females, and juveniles) found swimming on the rock surface were collected for microsatellite development and to estimate population allele frequencies at the loci analyzed.

From a single adult male, genomic DNA was extracted from approximately 25 mg of muscle tissue using a DNeasy Blood and Tissue DNA Extraction Kit (Qiagen). A modified enrichment technique from Hamilton (1999) was used to isolate and sequence microsatellite-containing DNA fragments (Croshaw and Glenn 2003; Hamilton et al. 1999; Hauswaldt and Glenn 2003). Briefly, genomic DNA was digested for 30 min at 37°C with *Rsa*I (New England Biolabs). Fragments were then ligated to double-stranded SuperSNX24 linkers (forward 5′-GTTTAGGCCTAGCTAGCAGAATC-3′; reverse 5′-GATTCTGCTAGCTAGGCCTTAAACAAAA-3′) and hybridized to a cocktail of biotinylated oligonucleotide repeat probes: $(TG)_{12}$, $(AG)_{12}$, $(AAG)_8$, $(ATC)_8$, $(AAC)_8$, $(AAT)_{12}$, $(ACT)_{12}$, $(AAAC)_6$, $(AAAG)_6$, $(AATC)_6$, $(AATG)_6$, $(ACCT)_6$, $(ACAG)_6$, $(ACTC)_6$, $(ACTG)_6$, $(AAAT)_8$, $(AACT)_8$, $(AAGT)_8$, $(ACAT)_8$, $(AGAT)_8$, $(AACC)_5$, $(AACG)_5$, $(AAGC)_5$, $(AAGG)_5$, and $(ATCC)_5$. Hybridized probes were captured on a magnetic block using magnetic streptavidin beads (Dynal).

Enriched DNA was recovered by precipitation and PCR-amplified (25 µl reaction volume) under the following conditions: 10 mM Tris–HCl pH 9.0, 50 mM KCl, 0.1% Triton X-100, 2.0 mM MgCl, 25.0% g/ml BSA, 0.2 mM each dNTP, 0.5 µM SuperSNX24 forward primer, and 0.5 U DNA Polymerase (Promega). The PCR product was ligated into a PCR 2.1-TOPO vector, transformed into One Shot Top10 Chemically Competent *Escherichia coli* cells, and positive colonies were screened for *β*-galactosidase activity using the materials provided in the TOPO TA cloning kit (Invitrogen). To verify insert sizes, inserts from positive colonies were amplified using M13 primers and *Taq*Bead Hot Start Polymerase (Promega).

Inserts ≥500 bp were purified using QIAquick Spin Columns (Qiagen) and sequenced with Big Dye chemistry (version 1.0, Applied Biosystems) on an ABI 3700 DNA Analyzer with 50 cm capillaries. Sequences were edited using Sequence Analysis Software (Applied Biosystems) and primers flanking microsatellite regions were developed using OligoAnalyzer 3.0 (Integrated DNA Technologies). For each successful primer pair developed, tailed PCR was employed for fluorescent screening. In each case, one of the two primers was modified at the 5′ end with the tag 5′-GGAAACAGCTATGACCATG-3′ (Boutin-Ganache et al. 2001; Croshaw and Glenn 2003). In the PCR reaction, a third primer was incorporated that was complementary to this tag and fluorescently labeled at the 5′ end with 6-FAM. All resulting amplified products from this PCR reaction would contain the 6-FAM label and were detected on

the ABI 3700 DNA Analyzer. This method allowed us to check for polymorphism within each locus before investing in the development of fluorescently labeled primers for that marker.

Genotypic assays

All DNA extractions from adult muscle tissue or juvenile fish were performed using the DNeasy Blood and Tissue DNA Extraction Kit (Qiagen). Embryos were removed from the inner cavity wall of the barnacle by peeling away the mucous layer to which they were attached. Embryos of different developmental stages (when present), and also from different locations in a nest, were purposefully chosen in efforts to obtain a robust representation of possible parentage within each brood.

Syringe needles (21G) were used to dissect each embryo away from its chorionic membrane as well as to remove its attached yolk sac. Embryos were rinsed in deionized water and placed into separate wells of a 96-well PCR plate (BioExpress) containing either 10, 25, or 50 µl of embryo extraction buffer (10mM Tris, 1 mM EDTA, 25 mM NaCl, pH 8.0). Different volumes were used depending on each embryo's stage of development. DNA extraction was achieved by digesting each embryo with proteinase K (10 mg/ml) for 2 h at 55°C, followed by an inactivation period of 10 min at 95°C. Samples were centrifuged at 4,000*g* for 1 min to pellet cellular debris.

DNA amplifications (Table 1) were performed in a 10 µl reaction volume containing 1 µl of embryonic DNA from the supernatant, PCR Master Mix (Promega), and 0.5 µM of each primer. Products of the MM-9 and MM-D2 loci were pooled, as were those of MM-C and MM-T90, by combining 3 µl of each PCR product with 5.75 µl formamide and 0.25 µl of GeneScan-500 LIZ size standard. Samples were denatured in a 95°C heating block for 5 min and chilled on ice before loading onto an ABI 3700 DNA Analyzer with 50 cm capillaries. The samples were electrophoresed through POP-6 Polymer (Applied Biosystems) at 7,500 V for 83 min. Alleles were sized using the software packages GeneScan and Genotyper (Applied Biosystems).

Genotypic frequencies were tested for Hardy-Weinberg proportions using GENEPOP 3.3 (Raymond and Rousset 1995). Parental exclusion probabilities were calculated for each marker separately, and for all loci jointly, under a model that assumes that one parent (in this case the nest-tending male) is provisionally known (following Selvin 1980). Calculations were performed using an Excel spreadsheet program.

Estimates on the number of female parents that contributed to a half-sib progeny array were carried out using two different methods. A minimum estimate was calculated from the maximum number of different maternal alleles at any locus in a nest, divided by two and rounded upward to the nearest integer (Kellogg et al. 1988). However, in cases when alleles are shared among female parents, the minimum method will underestimate the true number of females that contributed to the brood. The computer simulation GAMETES was employed to estimate this relationship between the number of distinct parental alleles and the number of contributing parents within a brood when alleles are shared among parents (DeWoody et al. 2000). The simulation uses empirical estimates of gene frequencies for a single neutral locus to generate random progeny arrays with a defined reproductive skew. By selecting various parental assemblage sizes consisting of both shared and unshared parents, repeated sampling of the progeny array will produce a distribution of the number of distinct genotypes found within the array. This process can be inverted such that a corrected estimate of the mean assemblage size of parents with a 95% confidence interval is based on the number of different genotypes identified within a brood sample (DeWoody et al. 2000).

Results

Microsatellite markers

The primer pairs listed in Table 1, the first reported for this species, served to amplify four microsatellite loci.

Table 1 Microsatellite loci developed in *S. cristata*

Primer	Sequence (5′ → 3′)	Dye	Annealing conditions[a]	Cloned repeat	No. of alleles	Exclusion probability[b]
MM-D9F	GCGACCAGCCGTTAGCTCTAT	6-FAM	53°C, 30 s	$(GT)_{18}$	9[c]	0.45
MM-D9R	TTTAAGTCCGCGCCAGGATA					
MM-D2F	TGCGCTATTGGCGGGTATTACA	VIC	53°C, 30 s	$(GA)_{28}$	22	0.85
MM-D2R	AGCAGCCATAATGGATTGGCCTTG					
MM-CF	TGTTACTTCAGGGTATTGAGGCT	6-FAM	52°C, 30 s	$(CTAT)_{27}$	20	0.82
MM-CR	GGGCGTCATCTTCTGATGGAAT					
MM-T90F	TGTTGTCGTGATGTCGGTAGA	NED	50°C, 30 s	$(GATA)_{39}$	26	0.87
MM-T90R	CATGTAAGGTTCAGAGGTTAGGTC					

[a]All other cycling conditions are as follows: initial denaturation at 95°C for 60 s, followed by 50 cycles of denaturation at 94°C for 30 s, annealing conditions as above, and extension at 72°C for 30 s

[b]Under the one-parent-known model (Selvin 1980)

[c]Determined only from the population of 29 non-nesting adults and juveniles. The remaining markers are based on a reference sample of 52 fish

Three of these (MM-C, MM-D2, and MM-T90) proved to be highly polymorphic, displaying totals of 20, 22, and 26 alleles, respectively, in a sample consisting of the 23 nesting males and the 29 adult and juvenile non-nesting individuals captured on the sampled rock surface (Fig. 1). Genotypic frequencies at these loci showed no significant departure from Hardy-Weinberg proportions. The fourth locus (MM-D9) was omitted from the parentage analyses because it displayed only a few alleles in high frequency and would have contributed little in terms of exclusionary power. For the three loci that were used, the combined single-parent exclusion probability was 0.97 for any di-locus combination, and 0.99 for all three loci jointly.

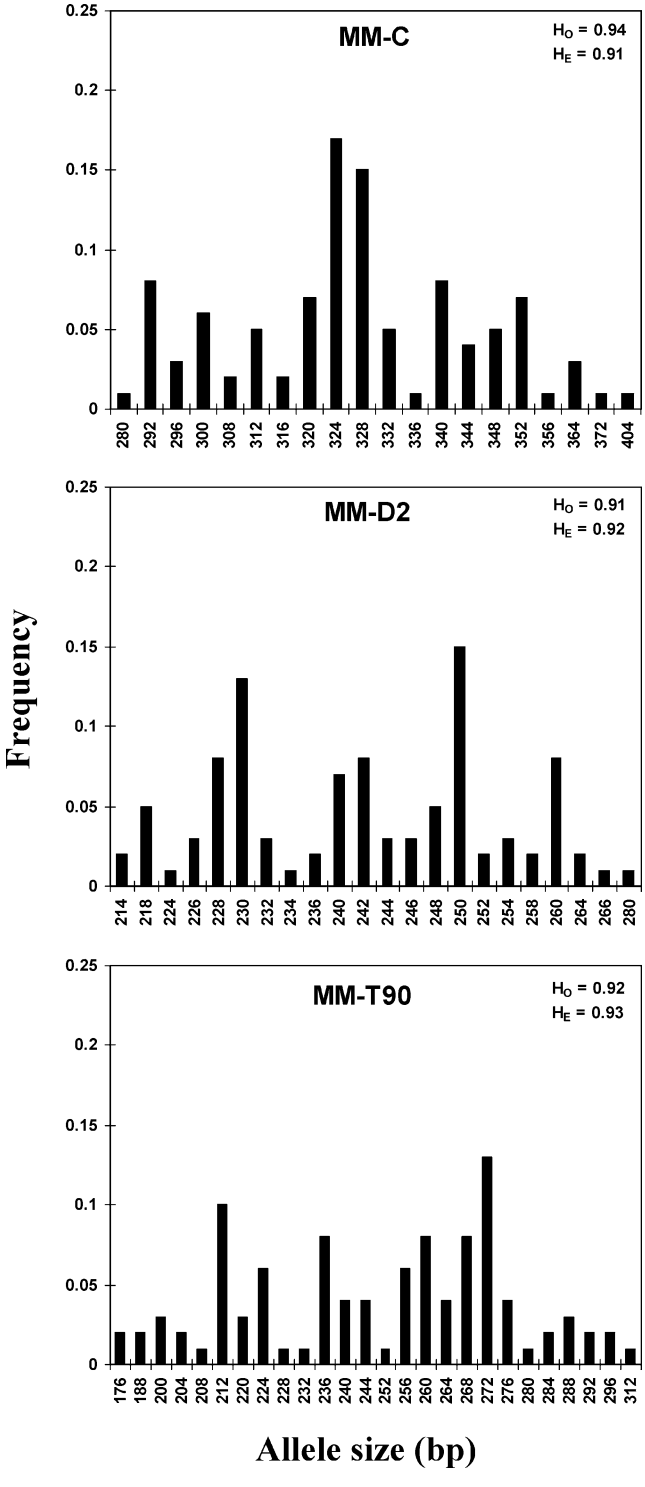

Fig. 1 Histogram of allele frequencies at each of three microsatellite loci employed to assess genetic parentage in the current study. Also shown are observed and HWE-expected heterozygosities

Genetic paternity

Among the 38 collected nests for which the nest-tending males were captured, 23 nests contained embryos suitable for analysis in this study. Among these 23 examined nests, in only three cases (13.0%) did all surveyed progeny display genotypes at all three microsatellite loci that were consistent (at face value) with paternity by their respective nest-tending male. In the remaining 20 nests (87.0%), varying fractions of embryos displayed, at one or more loci, a paternally-derived allele that was different in state from that in its adult nest attendant (Table 2). This is a conservative estimate, because not all progeny from a nest were genetically assayed (roughly 500–1,000 progeny probably occurred in a typical nest, although direct counts were not taken). Altogether, among the total of 1,536 progeny examined, 209 (13.6%) had genotypes of this sort that potentially excluded the nest-guardian male as sire. The estimated frequencies of such embryos in these 20 nests ranged from 1.2% to 41.0% (Table 2).

In a few of these instances (involving 18 progeny, or 1.2% of the total), the inconsistency between a progeny's alleles and that of its nest attendant involved one locus only, whereas allelic matches existed at the other two loci. These single-locus "guardian-inconsistent" alleles invariably differed from one of the nest-guardian's alleles by only one dinucleotide or one tetranucleotide repeat unit (depending on the marker). These observations, being consistent with a stepwise mutation model for microsatellite markers, raise the possibility that these particular guardian-inconsistent alleles might register de novo mutations in the paternal germ line, rather than non-paternity by the resident male. If so, then our estimate of the overall cuckoldry rate in this population is adjusted downward, but only slightly: from 13.6% to 12.4% (i.e., 191 of 1,536 embryos). For this reason, as well as for the fact that genetic exclusion probabilities from the marker loci were inevitably (but only slightly) less than 100%, this latter value should be viewed as a conservative estimate of the overall frequency of foster embryos in this population.

Among the 19 nests that showed unambiguous genetic evidence for cuckoldry, six (31.6%) contained fewer than 5% foster young, eight (42.1%) contained between 10% and 30% foster offspring, and 5 (26.3%) contained more than 30% foster embryos. In no case had most or all of the embryos in a nest been sired by a foreign male, as might otherwise have been expected if

Table 2 Genetic parentage deduced for embryos in 23 barnacle-hole nests of *S. cristata*

Nest no.	Paternity by bourgeois male[a]	Percentage of offspring not sired by nest-tending male	No. of dams	
			Minimum	Estimated[b]
1	52 of 56	7.1	5	6.5 ± 0.9
3	33 of 35	5.7	4	4.3 ± 0.8
5	54 of 54	0.0	5	5.8 ± 0.9
6	88 of 88	0.0	7	8.1 ± 0.8
9	52 of 80	35.0	5[c]	6.1 ± 0.9
10	64 of 65	1.5	6	7.4 ± 0.9
11	59 of 72 (2)	18.1	5	5.9 ± 1.0
15	23 of 39	41.0	5[c]	5.0 ± 1.0
16	58 of 88	34.1	3	4.1 ± 0.6
17	81 of 82 (2)	1.2	5	5.3 ± 0.9
18	37 of 39	5.1	5	5.6 ± 0.9
19	38 of 50	24.0	4[c]	4.3 ± 0.8
20	90 of 90	0.0	7	7.5 ± 0.9
21	33 of 44	11.0	4	4.4 ± 0.7
23	64 of 75 (5)	14.7	4	4.4 ± 0.8
24	66 of 69	4.3	5	5.3 ± 0.9
25	56 of 66	15.2	5	5.7 ± 0.8
27	62 of 64 (3)	3.1	5	5.0 ± 0.9
29	84 of 86 (3)	2.3	8	9.4 ± 1.1
30	83 of 84	1.2	3	2.8 ± 0.7
31	88 of 91	3.3	3	2.8 ± 0.9
33	47 of 68	30.9	5	6.3 ± 0.8
35	33 of 52 (3)	36.5	4	5.1 ± 1.1
Mean (SD)	58.5 of 66.8 (20.2, 17.9)	12.8 (13.9)	4.9 (1.3)	5.5 (1.6)

[a]Values in parentheses could be interpreted as single-locus de novo mutations, rather than evidence of cuckoldry (see text)
[b]Calculated from the GAMETES computer program, which records the most likely number of unshared parents for a given number of different gametes observed within a brood (DeWoody et al. 2000)
[c]Nest contained progeny sired by multiple cuckolder males, rendering this value approximate because it is difficult to distinguish female alleles from those of cuckolding males

nest takeovers (rather than cuckoldry per se) had been involved. In each of three nests (numbers 9, 15, and 19), an examination and tally of paternal-origin alleles made it evident that more than one nest-parasitic sire (in addition to the resident nest-holder) had contributed to the collection of progeny.

Genetic maternity

Maternal contributions to each nest were deduced by allelic subtraction, i.e., by specifying at each locus the maternal allele in each progeny after identifying that embryo's probable paternal allele. (For nests 9, 15, and 19, where more than one parasitic male contributed to paternity, determining the maternal allelic contribution was admittedly more difficult because female alleles could not be discriminated from male alleles with 100% confidence through allelic subtraction, but a "best guess" nonetheless could be made; see Table 2.) Across all 23 nests, the minimum estimate of the mean number of dams per nest was 4.9 ± 1.3. A statistically upward-adjusted estimate based on the GAMETES computer simulation (see Table 2) yielded a mean count of 5.5 ± 1.6 dams per nest.

Discussion

Genetic maternity and paternity

For the Florida population of Molly Miller blennies surveyed, our genetic data document that multiple (often at least five) dams contributed to the collection of embryos within a typical nest. So, almost invariably, several females had spawned within the barnacle-hole enclosure of each bourgeois male during the current reproductive period. Roughly similar levels of successful multiple mating by bourgeois males have likewise been reported in genetic parentage analyses of approximately a dozen other nest-tending fish species (Avise et al. 2002). Thus, an emerging generality for most (albeit not all; DeWoody et al. (2000c) fishes is that a typical nest tended by a bourgeois male normally includes mixtures of half-sib embryos stemming from roughly half-a-dozen different mothers.

Our current genetic data for the Molly Miller also document high rates of cuckoldry: at least 82.6% of the surveyed nests clearly had been parasitized, and approximately 12.4% of the offspring in this Florida population had been sired via "stolen" fertilizations. These genetically deduced rates of nest parasitism and cuckoldry are among the highest such values yet reported in nest-tending fish species (Figs. 2, 3).

Ecological and behavioral factors affecting cuckoldry rates in the Molly Miller

We had no clear a priori expectations about cuckoldry rates in the Molly Miller blenny, because diametrically opposed proximate influences might be at work. On the one hand, the barnacle-hole nests of bourgeois males are relatively enclosed and spatially confined, so they would seem at face value to be highly defensible against nest parasitism (especially when compared, for example, to the wide-open nests of sunfish, which are merely broad shallow depressions fanned into the substrate of a pond or stream). Bourgeois male blennies are often nearly as large as the openings to the nests they guard. Like bourgeois sunfish, these nest-holding blenny males are highly territorial and aggressively repel approaching intruders, but given the nest architectures it might seem that the blenny's defensive tactics could be relatively more effective.

On the other hand, ARTs (including spawning behaviors by suspected sneaker males) were recently reported from field observations in *S. cristata* (Neat et al. 2003). Furthermore, the density of potentially breeding individuals in the Molly Miller population that we surveyed was extremely high ($\sim$13 nesting males/m^2

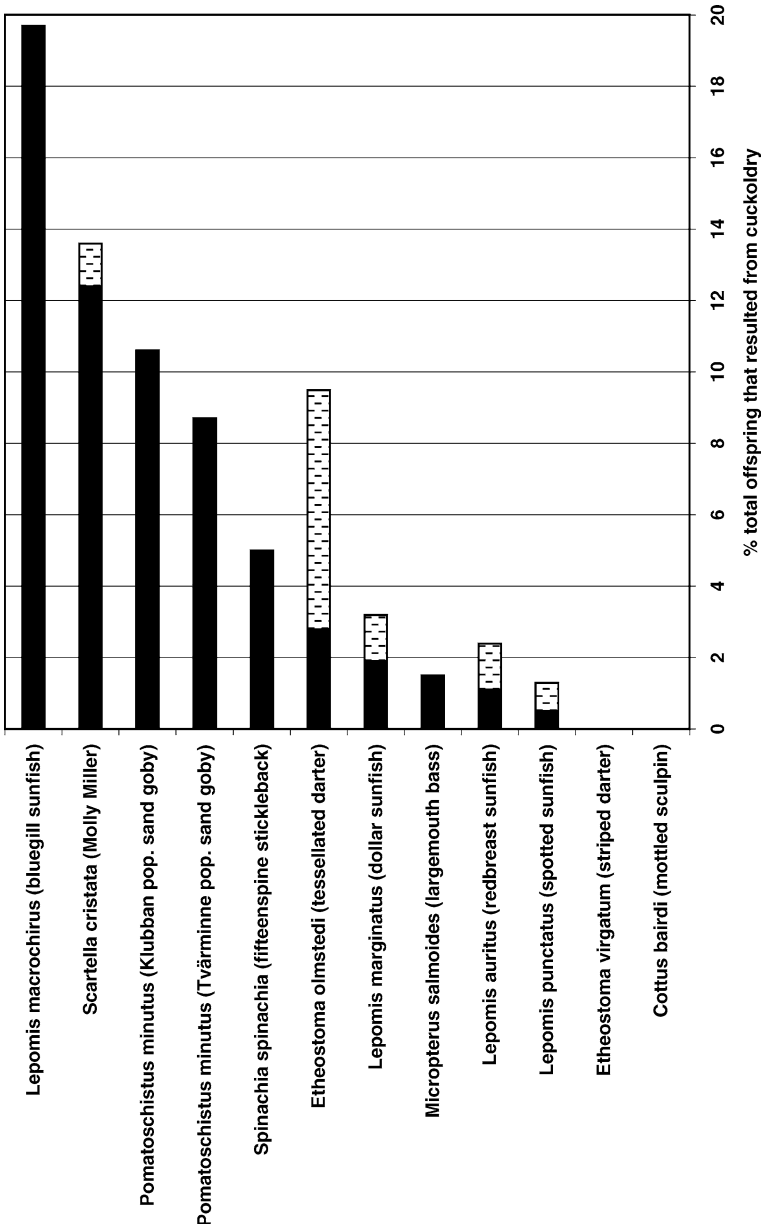

Fig. 2 Microsatellite-deduced cuckoldry rates (total percentages of progeny arising from sneaked fertilizations in a given population) gleaned from previous reports on fish species with male nest-tenders (DeWoody et al. 1998, 2000a, 2000b, 2000c; Fiumera et al. 2002; Jones et al. 1998, 2001a, 2001b; Mackiewicz et al. 2002; Neff 2001; Porter et al. 2002). *Black bars* are minimum estimates of cuckoldry rates in the sense that fertilizations were deemed to be sneaked only if the relevant progeny had guardian-inconsistent alleles at two or more loci. *Gray bars* indicate the higher such estimates when single-locus guardian-inconsistent alleles are provisionally interpreted to indicate sneaked fertilization events as well (rather than de novo mutations in the bourgeois male's germ line). Probable nest-takeover events (as evidenced by all or nearly all progeny within a nest being foster embryos to their guardian) were not counted as cuckoldry events via sneaking in the estimates summarized here

on average), perhaps yielding many opportunities for cuckoldry (by sneakers and/or other nest-tending males). At the Florida collection site, bourgeois males typically nested in close proximity (often within 10 cm of several neighbors) in their adopted barnacle-shell homes, and much of the remaining surface area was inhabited by additional adults and juveniles. In these dense, bustling communities, a single rock surface sometimes contained dozens of tended nests per square meter, with many additional individuals swimming about the rock face as well.

Although the unusually high cuckoldry rates we have documented in the Molly Miller are generally consistent with behavioral and morphological observations indicating the presence of a sneaker ART in this species (Neat et al. 2003), we cannot determine from current genetic data precisely which males had stolen fertilization events in the cuckolded nests. In eight nests, we were able to deduce without ambiguity the multi-locus genotype of the parasitic sire, but in no instance did it perfectly match any genotype observed in our collection of nesting and non-nesting adults (or juveniles). Failure

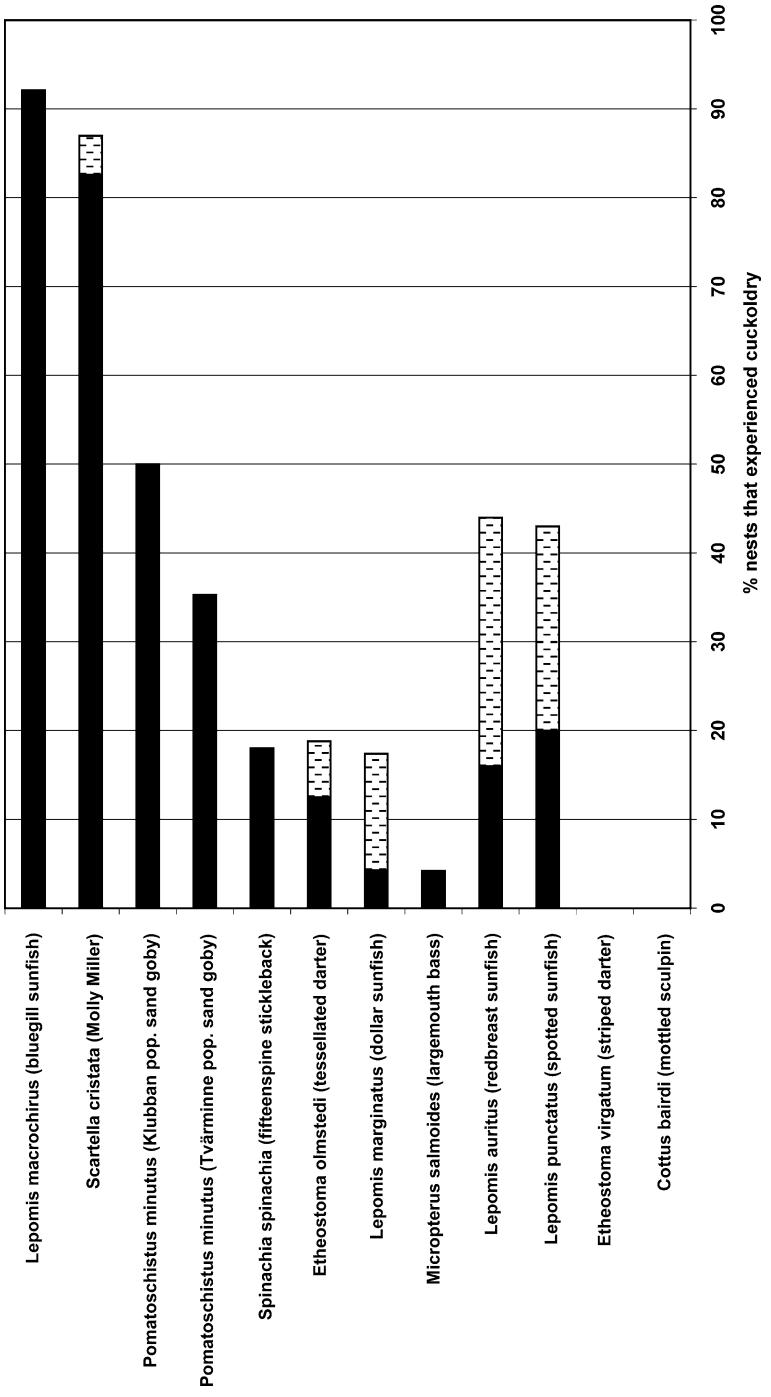

Fig. 3 Microsatellite-deduced rates of nest parasitism (total percentages of nests containing at least some progeny arising from sneaked fertilizations in a given population) gleaned from previous reports on fish species with male nest-tenders (see legend to Fig. 2 for references and additional comments)

to detect a match simply means that the true sires of those progeny remained unsampled in our study. This is not particularly surprising because the local population of adults was clearly much larger than the size of our collection, and also because, during the collection effort, we noticed that many non-nesting individuals left the rock or hid in crevices where they escaped capture.

Nonetheless, we can conclude that few if any of the documented instances of cuckoldry were attributable to nearby barnacle-nesting bourgeois males, because most if not all of these (but not necessarily other bourgeois males that may have nested directly in rock crevices) had been successfully collected from the rock surface that we examined. We can also conclude that few if any documented instances of "cuckoldry" were attributable instead to very recent nest takeovers or nest piracy events (such as when a large male might have evicted and replaced a bourgeois male already tending embryos). Presumably, the genetic footprint of any such recent nest takeover would have been 100% (or nearly so) foster offspring in a nest, an observation that we did not encounter.

Finally, each multi-locus collection of alleles deciphered for a cuckolder sire was also unique to the progeny within a given nest. This means that no parasitic male had demonstrably stolen fertilization events in more than one nest that was included in our collection.

Ecological and behavioral factors influencing cuckoldry rates in other fish

Figures 2 and 3 summarize cuckoldry rates as previously estimated through comparable genetic analyses in local populations of other fish species with extended nest care by males. Cuckoldry rates in the Molly Miller (more than 12% of total offspring, involving more than 80% of surveyed nests) are to our knowledge the highest such values yet documented in any population of a nest-tending fish species, with only one exception: In a nesting population of bluegill (*Lepomis macrochirus*) in Ontario, Canada, genetic data indicated that more than 90% of nests had been parasitized and approximately 20% of total offspring were the result of cuckoldry (Neff 2001; Philipp and Gross 1994).

Bluegill are colonial nesters, and are also well known to have satellite and sneaker males specialized for the ART of nest parasitism (Gross 1979; Gross and Macmillan 1981). By contrast, populations of four other sunfish (Centrarchidae) species that have been subjects of genetic paternity analyses have exhibited cuckoldry rates about an order-of-magnitude lower than in the bluegill (typically ca. 2%; DeWoody et al. 1998, 2000a, 2000c; Mackiewicz et al. 2002). In these species, nests at the surveyed sites were much more sparsely distributed than in the dense bluegill colony mentioned above. Furthermore, with the possible exception of *L. punctatus* (see DeWoody et al. 2000a), these other sunfish species are not known to include distinctive sneaker-male phenotypes that are morphologically or behaviorally specialized for nest parasitism. Thus, in the sunfish, both the density of nests and the presence versus absence of specialized sneaker morphs might predict the observed trends across species in relative cuckoldry rates, so these two influences cannot be teased apart with available information.

Apart from the bluegill population mentioned above, genetically deduced cuckoldry rates in the Molly Miller are approached only in previous reports for the sand goby, *Pomatoschistus minutus* (Jones et al. 2001a, 2001b). In two Scandinavian populations of that species, microsatellite data revealed that approximately 9–11% offspring in about 35–50% of surveyed nests were the result of cuckoldry (Figs. 2, 3). Interestingly, among all nest-tending fish species considered to date, the sand goby is probably most similar in general nesting ecology and behavior to the Molly Miller blenny. Like the blenny, the sand goby is a shallow-water marine species that nests in protected cavities, often in or under mussel shells or stones. These nests might also seem at face value to be highly defensible by bourgeois males, but cuckolders nonetheless were documented to have achieved considerable reproductive success.

Nevertheless, when nest defense is exceptionally easy, cuckoldry rates should tend to be low. The epitome of "nest" defensibility occurs in male-pregnant pipefishes and seahorses in which fertilization normally is internal (rather than external) to the male's body. Females lay unfertilized eggs into the brood pouch of a male who then releases sperm therein. In extensive genetic surveys of many hundreds of embryos in several syngnathid species, absolutely no instances of cuckoldry by males have yet been documented (Jones and Avise 1997; McCoy et al. 2001). This observation stands in sharp contrast to the routine occurrences of cuckoldry documented in nearly all surveyed fish species with "external male pregnancy" (i.e., with nest-tending habits by males). It is also strong testimony to the notion that defensibility of the "nest site" can, at least in extreme cases, be a key factor influencing cuckoldry rates and patterns.

In the Molly Miller blenny (as well as in sunfish, sand gobies, and other fish that tend eggs outside the body), higher nesting densities (all else being equal) could potentially make nest-guarding more difficult and thereby increase opportunities for stolen fertilizations. However, in two separate populations of the sand goby, *P. minutus*, that differed dramatically with respect to nest-site density, cuckoldry rates were surprisingly similar (Jones et al. 2001b), suggesting that nesting density was not a deciding factor in that species.

Although the current study is certainly less than definitive with regard to the proximate factors affecting nest parasitism rates in fishes, it does contribute to a growing body of literature documenting that cuckoldry and resulting foster parentage are widespread and often remarkably common phenomena in nest-tending species, including those in which nesting modes would seem to be highly defensible.

Acknowledgements We thank Judith Mank and DeEtte Walker for useful comments on the manuscript. We are grateful to John Barnes, Joe Nairn, and Travis Glenn for suggestions in preparing the genomic library and in microsatellite isolation. Work was carried out at the Genome Analysis Facility at the University of Georgia, and was supported by University funds and by a Pew Foundation fellowship (to John C. Avise). All experiments were performed in the United States and comply with the laws therein.

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