Microsatellite assessment of multiple paternity in natural populations of a live-bearing fish, *Gambusia holbrooki*

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Abstract

Three polymorphic microsatellite loci were isolated and employed to examine paternity patterns in two natural populations of live-bearing mosquitofish, *Gambusia holbrooki*. Each locus displayed four to five alleles per population in samples of presumably unrelated adults. Nearly 900 embryos from a total of 50 pregnant females were assayed individually, and paternal alleles in each embryo were identified. Counts of paternal alleles, Mendelian segregation patterns, multilocus allelic associations and genetic relatedness coefficients were employed to estimate the minimum and effective numbers of fathers per brood. At least 90% of the assayed broods were shown to have been fathered by multiple males, a figure substantially higher than previous estimates based on less polymorphic genetic loci. However, the genetic data yield a face-value estimate of only about 2.2 fathers per brood, a number that seems perhaps surprisingly low based on frequencies of attempted copulations by males. Both biological and sampling factors that might bias mean sire counts downward are considered. Although higher sire counts per brood might be obtained from loci with even greater numbers of alleles, little statistical room remains for higher frequency estimates of multiple paternity in *Gambusia*.

Introduction

Females of many species mate with multiple males during the course of a single round of reproduction (Davies, 1991; Birkhead & Møller, 1992). However, secure estimates of the frequency of concurrent multiple paternity in most cases awaited the application of molecular markers (Avise, 1994) that permit documentation of when copulations result in fertilizations such that individual broods are multiply sired. Genetic analyses have documented multiple paternity for a wide variety of animals and plants (e.g. Birdsall & Nash, 1973; Hanken & Sherman, 1981; Griffiths *et al*., 1982; Ellstrand, 1984; Levin, 1988; Travis *et al*., 1990; Parker & Kornfield, 1996). The analyses are facilitated when the mother is known with certainty, as for embryo broods carried internally by pregnant females in live-bearing animals.

The frequency of multiple paternity is important for hypotheses concerning male and female fitness advantages under alternative mating strategies. For example, mating with multiple males may enhance the probability that a female's genes are found in high-fitness genotypic combinations in her offspring, and/or it may provide a selective advantage in variable habitats by promoting genotypic variety among progeny within a brood (Lomann *et al*., 1988; Karron & Marshall, 1990). Access to multiple males could result in enhanced fitness for any females who are sperm limited (Borowsky & Kallmann, 1976). For broods in which offspring interact extensively with one another, frequency-dependent selection might be envisaged (McCauley & O'Donnel, 1984) if multiply fathered broods of half-sibs have a selective advantage over broods of full-sibs (Antonovics & Ellstrand, 1984). Another possibility is that the presence of multiple insemination might not be adaptive either for females or their broods but instead might reflect the outcome of competition among males over reproduction.

The frequency of multiple paternity is potentially important also for its impact on population-level phenomena such as heterozygosity and colonization ability. The number of fathers for broods is expected to have a strong influence on how genetic variation is distributed within species (Chesser & Baker, 1996). Greater sire numbers are expected to increase effective population size and permit retention of higher genetic variation in colonizing species or populations exposed to strong fluctuations in abundance.

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In the fish family Poeciliidae, multiple paternity has been documented genetically in several surveys using genetic-based colour polymorphisms or allozymes (Haskins *et al*., 1961; Borowsky & Khouri, 1976; Robbins *et al*., 1987; Travis *et al*., 1990). However, these studies have been hampered by low levels of genetic variation detected (Greene & Brown, 1991), and by difficulties of scoring genotypes from small embryos. For example, using three di-allelic allozyme loci, Chesser *et al*. (1984) documented a multiple insemination frequency of 56% for natural populations of *Gambusia holbrooki* (then named *G. affinis*) but noted that the true incidence (given the limited diagnostic power of the loci scored) might be closer to 100%.

Microsatellite markers tend to be 'hypervariable' (Tautz, 1989) and, thus, have been employed for refined estimates of kinship and parentage (Queller *et al*., 1993; Bruford *et al*., 1996) in many organisms including fishes (e.g. Colbourne *et al*., 1996; Jones & Avise, 1997a,b). Here we apply microsatellite markers to analyse paternity in natural populations of mosquitofish, *Gambusia holbrooki*. Goals are to assess the proportion of broods multiply sired, the numbers of contributing males and whether these parameters differ between the two populations analysed.

Materials and methods

Samples

Mosquitofish (*Gambusia holbrooki*) were collected from two small ponds, Fire and Fisher, about 10 km apart at the Savannah River Site near Aiken, South Carolina (Barnwell County). Frozen fish were sexed and standard body lengths recorded. Fifty pregnant females (25 from each pond) and most of their brooded embryos (890 total) were analysed. Allele frequencies were estimated from independent population samples consisting of 89 and 66 adults not known to be related.

Microsatellite isolation and PCR conditions

Total genomic DNA was isolated from one specimen using a standard proteinase K-phenol-chloroform protocol. Ten micrograms of DNA was fully digested with *Mbo*I and electrophoresed through a 2% agarose gel. Size-selected DNA ranging from 200 to 700 bp was purified using the Prep-A-Gene DNA Purification System (Bio-Rad). Approximately 60 ng of purified DNA was ligated to 100 ng dephosphorylated *Bam*HI digested pBluescript II KS(–) phagemid vector (Stratagene). This ligation mix was used to heat-shock transform competent XL1-Blue *E. coli* host cells (Strategene), which were then plated on standard LB-ampicillin plates and grown overnight.

The partial genomic library then was screened for microsatellites using γ^{32} P end-labelled synthetic probes. Colonies were transferred to Hybond-N filters (Amersham) and DNA was crosslinked by baking at 80 °C in a vacuum for 1.5 h. Filters were alternately hybridized with two oligonucleotide cocktails: $(GACA)_4$, $(GT)_{10}$, $(GGAT)_4$, $(TAG)_6$; or $(GATA)_4$, $(GA)_{10}$, $(TCC)_5$, $(TTA-GGG)_3$. Prehybridization (1.5 h) and hybridization (overnight) were carried out at 42 °C in 6× SSC (from a 20× stock = 3 M NaCl, 0.3 M sodium citrate), 5× Denhardt reagent (from a 50× stock = 1% BSA fraction V, 1% Ficoll and 1% polyvinlypropylene) and 0.1% SDS. Two posthybridization washes (30 min) were performed in a 6× SSC, 0.1% SDS solution. Following overnight autoradiographic exposure, positive recombinants were picked and plasmid DNA extracted using the QIAprep Spin Plasmid Kit (Qiagen). Twenty positive recombinants, all containing microsatellite repeats, were sequenced either manually (fmol DNA cycle sequencing system, Promega) or automatically (fluorescent dye method, Applied Biosystems). From three of these, primers were designed to match the flanking region.

Annealing temperature and $MgCl_2$ concentration were optimized for three primer pairs that reliably amplified the expected size fragment (Table 1). Radioactive PCR was carried out in a 12-μL total volume containing 1× Promega *Taq* buffer, 0.5 or 1 mM $MgCl_2$, 160 μM dNTPs, 0.08 μM γ^{32}P end-labelled and unlabelled primers, and 1 U Promega *Taq* polymerase. Two of the loci (Mf-6 and Mf-13) were multiplexed. Cycling parameters were 94 °C for 1 min, 63 °C or 61 °C (Table 1) for 1 min, and 72 °C for 1 min, repeated through 27 cycles. Amplified fragments were separated on 6% denaturing (sequencing) polyacrylamide gels and visualized by autoradiography.

Table 1 Primers and PCR conditions utilized in the mosquitofish assays.

Locus	Primers	Repeat	Size (bp)*	Annealing temp. (°C)	$MgCl_2$ (mM)
Mf-1	CTGCCCGGAACGTTAGCTGGAGAT† TGCATCTGCCCAGTGTTTGAATGG	GGAT	170	63	0.5
Mf-6	ACGCCTATTGGTCGCCTGAT† TTTGATTTCCTGGATTCTGACTGA	GT	243	61	1
Mf-13	AAAGGCTGCAAACAGTAAAAGTTA† GGTCACAAATATAAAGCCACAGAC	GT	164	61	1

* In base pairs of the cloned sequence.
† End-labelled primer.

Population analysis

DNA was extracted using the method of Gloor & Engels (1992). Females were dissected and those carrying eyed-stage embryos were included in the paternity analyses. Each embryo was ground individually in 100 μL of extraction buffer (10 mM Tris-HCl, 1 mM EDTA, 25 mM NaCl, pH = 8) and digested with 20 μg of proteinase K at 37 °C for 30 min. Following heat inactivation of the proteinase K (90 °C, 5 min), the sample was centrifuged for 3 min at 13 000 r.p.m. One microlitre of supernatant was used for each PCR. DNA from adult males and females was extracted by the same protocol from a small piece of muscle at the base of the caudal fin or from the fin itself.

Data analysis

Population estimates of allele frequencies, heterozygosities and agreement of genotype frequencies to Hardy–Weinberg equilibrium were obtained from the GENEPOP program (Raymond & Rousset, 1995). By comparing offspring genotypes against those of a known mother, paternal alleles in each brood were evident. Initial estimates of minimum sire number for each brood were obtained unequivocally from the number of segregating paternal alleles. For example, a brood displaying five paternal alleles was inferred to have been fathered by at least three males. Occasionally, we could unambiguously infer an additional father for a brood by joint multilocus inspection, as for example when a paternal allele at one locus was paired in particular offspring with more than two paternal alleles at another locus. Finally, for two of the broods in which only two paternal alleles were observed, paternity by two males nonetheless was inferred statistically by significant departures of allele frequencies from Mendelian expectations for a singly sired brood (Hjorth, 1971; Travis *et al.*, 1990).

Also, an indirect estimate of the number of fathers was obtained by calculating genetic coefficients of relatedness between individuals of a brood (program Relatedness 4.2; procedures and rationale described by Queller & Goodnight, 1989). From these values, the effective numbers of fathers were estimated following equation (5) in Ross (1993). This approach assumes an absence of inbreeding, an equal contribution of each participating male to a brood and no variation among males in the number of matings.

Results

Locus behaviour at the population level

The three microsatellite loci resolved well (examples in Fig. 1) but proved to be only moderately variable, with

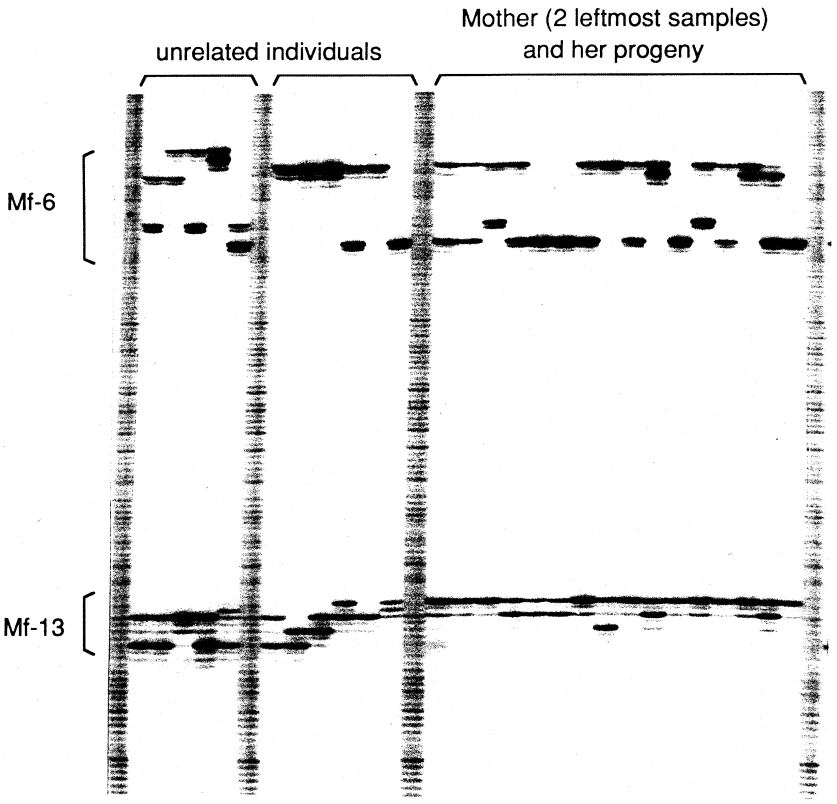

Fig. 1 Autoradiograph showing representative polymorphisms at the Mf-6 and Mf-13 loci. For the brood on the right, note for example the presence of four paternally derived alleles at Mf-6, thereby documenting at least two sires for this progeny array.

4–5 alleles segregating per population. The Fire and Fisher Pond populations were distinct genetically (Table 2): some common alleles were present at only one location and these as well as some shared alleles contributed to the significant allele frequency differences ($P < 0.01$, exact test for population differentiation in GENEPOP). Alleles were not observed in the more-or-less continuous array of size classes that might be anticipated under a simple step-wise mutation model. This overtly discontinuous pattern of length variation (Table 2) suggests either that the step-wise mutation model does not apply well, and/or that intermediate alleles have been lost historically or remained unsampled from these populations.

Genotypic frequencies within each population were in Hardy–Weinberg equilibrium (exact tests as implemented in GENEPOP; P values invariably greater than 0.10). With one exception involving a null allele (see below), maternal alleles segregated in all broods in accord with Mendelian expectations. Observed (and expected) single-locus heterozygosities ranged from about 53% to 78% (Table 3). Single-locus exclusion probabilities (the probability of exclusion of a random individual from the population as being the father of an embryo) ranged from 0.29 to 0.54, and multilocus exclusion probabilities were 84% in both populations (Table 3).

Null alleles sometimes are present in microsatellite studies and can complicate parentage assessments (Pemberton *et al*., 1995). However, only one instance was evident here. The brood of one female displayed a pattern of segregation of maternal alleles (at locus *Mf-13*) that departed from Mendelian expectations and suggested the presence of a null allele. This allele cannot be in high frequency in the populations because it did not cause detectable departures from Hardy–Weinberg expected genotypic frequencies.

Table 2 Allele frequencies at three microsatellite loci in field collections of individuals not known to be related from the Fisher Pond ($n = 89$) and Fire Pond ($n = 66$) populations of mosquitofish.

Locus	Allele size (bp)	Allele frequencies	
		Fisher Pond	Fire Pond
Mf-1	178	0	0.256
	170	0.212	0.244
	162	0.394	0.055
	150	0.280	0.274
	138	0.114	0.171
Mf-6	247	0	0.460
	245	0.061	0.182
	243	0.167	0.068
	241	0.197	0.102
	233	0.075	0.188
	229	0.500	0
Mf-13	164	0.447	0.045
	163	0.023	0.017
	162	0.197	0.343
	160	0.076	0.011
	156	0.257	0.584

Paternity analysis

Multiple paternity of broods was common in the two mosquitofish populations (Table 4). Based on the straightforward criteria of the number of paternal alleles segregating in a brood, 43 of the 50 broods analysed (86%) were sired by more than one male. By adding the criteria of Mendelian segregation of paternal alleles, the estimate of multiply sired broods increased to 45 (90%). In both the Fire and the Fisher Pond populations, triply inseminated females also were evident, in frequencies of 44% and 24%, respectively. The mean numbers of fathers per brood were 2.36–2.12 in the two populations. Due to the limited resolution afforded by these loci (different parents sometimes may share alleles that camouflage their genetic contributions to a brood), these values clearly represent minimal estimates of the true sire numbers. Effective numbers of sires per brood estimated by the coefficient of relatedness approach were slightly lower: 2.12 and 1.60 for the Fire and Fisher Pond populations (Table 5). However, due to the wide confidence intervals on these estimates, the difference between the population means was not statistically significant (Table 5).

Table 3 Heterozygosities and exclusion probabilities in the mosquitofish populations.

Locus	Fisher Pond			Fire Pond		
	Heterozygosity		Exclusion probability*	Heterozygosity		Exclusion Probability*
	Observed	Expected†		Observed	Expected†	
Mf-1	0.621	0.714	0.482	0.780	0.772	0.544
Mf-6	0.687	0.679	0.410	0.682	0.709	0.499
Mf-13	0.728	0.694	0.466	0.528	0.542	0.291
Total	0.679	0.696	0.837	0.663	0.674	0.838

* As in Weir (1990).

† Under Hardy–Weinberg equilibrium for a random mating population.

Table 4 Parentage inferences for the two populations.

mother ID	No. of offspring					Inferred no. of fathers	
	Total	Analysed	No. paternal alleles*			Allelic counting	Total (see text)
Fire Pond							
1	17	17	3	5	2	3	3
2	21	19	4	3	3	2	3
3	23	19	4	2	2	2	3
4	20	14	3	5	2	3	3
5	25	15	3	2	2	2	2
6	22	15	4	3	2	2	2
7	20	17	4	4	3	2	3
8	21	18	5	4	2	3	3
9	20	16	3	3	3	2	2
10	18	16	3	3	3	2	2
11	17	16	4	3	1	2	2
12	12	12	3	3	2	2	2
13	10	10	2	2	1	1	1
14	12	12	4	4	3	2	3
15	23	19	4	3	2	2	2
16	13	13	2	2	1	1	2
17	20	17	3	3	2	2	2
18	23	15	3	3	2	2	3
19	17	14	2	2	1	1	1
20	26	23	4	3	2	2	2
21	15	13	2	3	2	2	2
22	17	13	3	4	1	2	3
23	26	18	3	4	2	2	3
24	18	17	3	4	2	2	3
25	18	15	2	4	1	2	2
Mean	19.00	15.72				2.00	2.36
SD	4.34	2.84				0.50	0.64
Fisher Pond							
1	42	18	2	1	1	1	1
2	45	18	3	3	3	2	2
3	27	18	1	3	2	2	2
4	21	15	3	3	3	2	2
5	22	19	2	2	3	2	2
6	36	18	3	4	2	2	2
7	33	19	3	2	3	2	2
8	27	21	3	2	3	2	2
9	32	20	3	3	5	3	3
10	17	16	2	2	3	2	2
11	18	15	1	2	1	1	1
12	26	19	3	4	4	3	3
13	32	17	3	5	3	3	3
14	46	20	2	4	2	2	2
15	19	19	3	3	4	2	3
16	20	18	3	2	2	2	2
17	18	15	3	2	2	2	2
18	30	19	2	1	3	2	2
19	26	14	4	4	3	2	2
20	26	20	2	4	3	2	3
21	29	18	2	1	2	1	2
22	25	17	2	1	2	1	1

continued

Table 4 (contd.)

	No. of offspring					Inferred no. of fathers	
ID	Total	Analysed	No. paternal alleles*			Allelic counting	Total (see text)
23	13	11	3	4	3	2	3
24	21	15	3	2	1	2	2
25	12	11	3	3	2	2	2
Mean	26.52	17.2				1.96	2.12
SD	9.09	2.63				0.54	0.60

* Number of alleles detected at *Mf-1*, *Mf-6* and *Mf-13*.

Female size, fecundity and number of fathers

A positive correlation has been reported between size and fertility of *Gambusia* females (Krumholz, 1948). This correlation holds also in the Fire and Fisher Pond populations (Fig. 2). A significant relationship also has been reported in poeciliid fishes between female size and the probability of multiple paternity for her brood (Travis *et al*., 1990; Greene & Brown, 1991). However, this relationship was not evident in the current study: mothers' sizes were not significantly associated in either population with the probability that offspring had been multiply sired (Fig. 3).

Discussion

The microsatellite loci afforded considerable improvement over allozyme methods used previously to estimate frequencies of multiple paternity in live-bearing fishes. Our analyses based on tetra- and penta-allelic loci indicate that at least 90% of the mosquitofish broods are multiply sired. Earlier studies based on assays of small numbers of di- and tri-allelic allozyme loci (Chesser *et al*., 1984; Greene & Brown, 1991) had detected multiple paternity in other wild *Gambusia* populations in about 55–65% of assayed broods. Allozyme assays of an experimental population of *Gambusia* detected multiple paternity in 62% of the broods surveyed (Robbins *et al*., 1987).

Table 5 Relatedness estimates within mosquitofish broods.

	Relatedness		Effective no. of fathers	
Population	mean*	95% interval*	mean†	95% interval‡
Fire Pond	0.368	0.302	2.12	4.77
		0.433		1.36
Fisher Pond	0.406	0.326	1.60	3.29
		0.487		1.06
t-test	1.132	ns		

* Calculated from the program Relatedness 4.2.
† Calculated by substituting the mean relatedness estimates into equation 5 of Ross (1993), and solving for effective number.
‡ Calculated as in †, using the 95% confidence limits from Relatedness 4.2.

The presence of Hardy–Weinberg equilibrium, lack of substantial inbreeding and the rarity of null alleles all suggest that these microsatellite markers have not distorted population genetic parameters that otherwise might bias against the detection of multiple paternity. However, the genetic variation detected in this study was

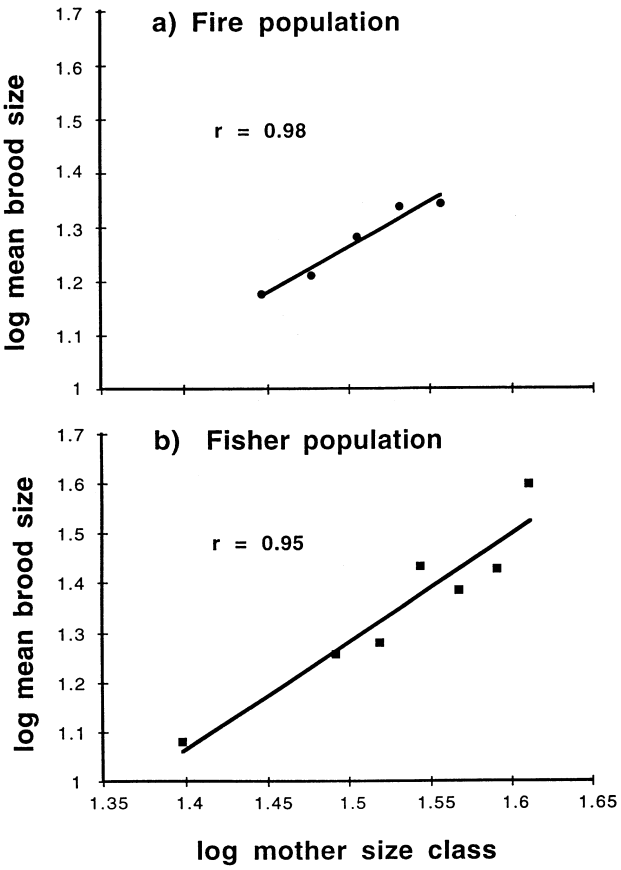

Fig. 2 Linear regression between size class and mean brood size (both log transformed) in the Fire and Fisher populations. In this presentation, the fish were grouped into size classes to be consistent with the procedure employed in an earlier study of this relationship by Krumholz (1948). For the brood data considered individually ($n = 25$ families per population), the correlations were lower ($r = 0.52$ and 0.69 for the Fire and Fisher Pond populations) but nonetheless significant ($P < 0.01$).

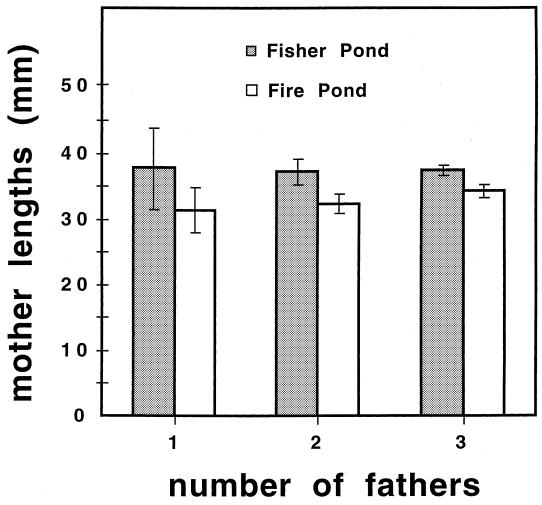

Fig. 3 Mean sizes of mothers who were inferred genetically to have had one, two and three mates. Shown are the 95% confidence intervals. These means were not significantly different ($P > 0.38$; single-factor ANOVA).

modest or low by microsatellite standards. We do not know whether this low variability is due to demographic factors (such as historical bottlenecks) in the populations analysed, or alternatively to intrinsic low variation in our microsatellites. The two ponds studied were small and their populations apparently isolated from one another (from genetic as well as geographical evidence). A paucity of genetic variation is not a species- or genomic-wide feature of *Gambusia* because mosquitofish populations often display high heterozygosities and substantial geographical variation by allozyme standards (Wooten *et al*., 1988; Scribner & Avise, 1993). The low variation in this study also is not related evidently to the number of nucleotides in the repeat units: the tetranucleotide locus and the two dinucleotide loci displayed similar levels of genetic variation.

Our current, higher estimate of the frequency (> 90%) of multiple paternity in mosquitofish from microsatellite data might itself still be low for several reasons. First, two males that fathered a brood might have shared one or both alleles at a locus and hence remained undetected in our assays. We can estimate the incidence of this event by assuming that each female mates with exactly two males, and then by considering the cumulative probability that these males share one or two alleles at a locus (McCauley & O'Donnel, 1984). For the three loci considered together, this probability is about 10% in both populations. By this reasoning, the binomial probability of seeing three or more single-sired broods in a sample of 25 broods is more than 14%. Three and four single-sired broods were genetically deduced for the Fire and Fisher populations, respectively. Thus, we cannot exclude the possibility that all mosquitofish broods sampled from these populations are multiply sired.

Second, the paternal allelic pool sampled from a brood might not represent completely the allelic pool of the sperm that contributed to its production, particularly if some fathers produced very few offspring and not all brood members were surveyed genetically (Birdsall & Nash, 1973; Merritt & Wu, 1975). Any factor that causes a departure from equal paternity for participating fathers is expected to bias downward the number of alleles segregating in a brood, and hence to bias similarly the frequency estimates of multiple paternity. In our case, however, many of the broods were sampled completely or nearly so.

A third limitation on the current appraisal of multiple paternity is perhaps the most important for the design of future research efforts with these fishes. Although the minimum number of fathers could be estimated for each clutch, we are unable with available data to set an upper bound on the number of sires for several reasons. First, with the maximum of five alleles per locus observed, the counting method permits documentation of at most three fathers per brood. Second, the likelihood that different males share alleles becomes higher with increasing numbers of fathers, a nontrivial difficulty given the observed heterozygosity levels. Finally, if males contribute differentially to the progeny, finite numbers of offspring from incompletely sampled broods could cause additional fathers to be overlooked. To our knowledge, no fully appropriate statistical procedures to correct for these factors are available. However, application of a statistical method by Levine *et al*. (1980) to our data suggests that broods with three or more fathers might be in the majority in both of the ponds.

From the available microsatellite data, our face-value estimates suggest that somewhat more than two fathers on average contribute to a mosquitofish brood. It is interesting to compare this finding to the mating behaviour of *Gambusia* in nature. Martin (1975) showed that each male has on average 0.93 'sexual acts' per min. Of these, at least 10% consist of gonopodial thrusting (Itzkowitz, 1971). The great majority of the copulations may result from males forcibly inseminating females (Bisazza *et al*., 1989). The impression of a high rate of mating seems to contrast with the modest number of fathers per brood estimated genetically. Several possibilities exist. First, gonopodial thrusting need not indicate successful insemination. Second, post-insemination barriers (including various mechanisms of sperm competition and/or post-mating sperm utilization by females) might lower the number of fathers whose sperm actually fertilized eggs and produced assayable embryos. Third, the available genetic data may have underestimated the true number of sires.

Of these three possibilities, the last may be the easiest to address in future research. We have demonstrated that the incidence of multiple paternity in at least some mosquitofish populations can be greater than 90%, but uncertainties remain as to the mean number of brood

sires. This latter issue too should yield eventually to genetic approaches, provided that microsatellite (or other) loci with far larger numbers of alleles per locus can be identified in local *Gambusia* populations.

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