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Microsatellite analysis of maternity and the mating system in the Gulf pipefish *Syngnathus scovelli*, a species with male pregnancy and sex-role reversal

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Abstract

Highly variable microsatellite loci were employed to study the mating system of the sexually dimorphic Gulf pipefish *Syngnathus scovelli*. In this species, like others in the family Syngnathidae, 'pregnant' males provide all parental care. Gulf pipefish were collected from one locale in the northern Gulf of Mexico, and internally carried broods of 40 pregnant males were analysed genetically. By comparing multilocus microsatellite fingerprints for the inferred mothers against expected genotypic distributions from the population sample, it was determined that: (i) only one male had received eggs from more than a single female; and (ii) on two separate occasions, two different males had received eggs from the same female. Given the high power to detect multiple matings by males, the first finding indicates that only rarely are individual males impregnated by multiple females during the course of a pregnancy. Conversely, given the lower power to detect multiple matings by females due to sampling constraints, the second finding suggests a high frequency of multiple successful matings by females. Thus, this population of Gulf pipefish displays a polyandrous genetic mating system. The relevance of these genetic findings is discussed with regard to the evolution of secondary sex traits in this species, and in other syngnathids.

Keywords: genetic markers, polyandry, parentage, sexual selection, sexual dimorphism

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Introduction

The family Syngnathidae is characterized by exclusive male parental care of offspring. A female pipefish or seahorse deposits eggs on the male's ventral surface or in his brood pouch. He then fertilizes the eggs and carries them for several weeks until they are born as independent, free-swimming young. The female's investment in offspring is complete after copulation, whereas the male provides the developing embryos with osmoregulation, nutrients and protection (Haresign & Shumway 1981; Berglund *et al*. 1986). To the caregiving male, this mode of fertilization and pregnancy provide assurance of paternity for his brood, probably an important element in the evolution of a high male-investment strategy in progeny rearing (Smith 1996). To the investigator interested in mating behaviours and sexual selection in natural syngnathid populations, this certainty of paternity for brooded embryos facilitates genetic appraisals of maternity, and hence of the mating system.

Extensive paternal care has the ultimate result that in some species of pipefish, sexual selection acts most strongly on females. Indeed, a stronger intensity of sexual selection operating on females than on males constitutes *the* definitional basis of *sex-role reversal* in recent sociobiological theory (Vincent *et al*. 1992). This reversal is thought to result from a female biased operational sex ratio (OSR), such that more females than males are available to mate at any given time (Emlen & Oring 1977). The net effect is that males become a limiting resource in reproduction and females are expected to compete for access to them (Clutton-Brock & Parker 1992). In pipefish, this competition is probably manifested either as male choice for more attractive females, or as dominance relationships among females with regard to mating (Rosenqvist 1990; Berglund 1991).

Whereas OSR appears to predict the sex on which sexual selection will act more strongly (Clutton-Brock &

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Vincent 1991), there is a long-standing tradition in the study of sexual selection that the mating system is also related to the evolution of secondary sex characters. Darwin (1871) first noted that for species in which males compete for access to females, more polygynous species exhibit greater levels of sexual dimorphism than do less polygynous or monogamous species, presumably as a result of more intense sexual selection. Much subsequent work has been devoted to addressing this hypothesis in taxa with traditional sex-roles (Clutton-Brock *et al*. 1977, 1980; Payne 1984; Höglund 1989; Webster 1992; Winquist & Lemon 1994), but little attention has been directed toward the analogous hypothesis in sex-role reversed taxa (but see Jehl & Murray 1986; Andersson 1995).

The family Syngnathidae provides an excellent group to investigate the relationship between the mating system and sexual selection in sex-role reversed species. As a first step, we describe the genetic mating system in nature of the Gulf pipefish *Syngnathus scovelli* using molecular markers. The Gulf pipefish exhibits considerable sexual dimorphism relative to its congeners. Adult female Gulf pipefish are larger than males, have a deep V-shaped abdomen, and display silvery markings along the trunk (Brown 1972; personal observation). Most other species in this genus lack obvious secondary sex traits (Brown 1972; Dawson 1985). Although females of *Syngnathus* species often are larger than males, it is unclear that this evidences sexual selection (for example, it may simply be the consequence of natural selection for increased fecundity). The pronounced gender-specific markings on the Gulf pipefish, however, most likely are the result of sexual selection on females.

The discovery of highly polymorphic microsatellite markers (Tautz & Renz 1984; Tautz 1989) has opened possibilities for study of mating systems in natural populations in species where mating behaviours are difficult to observe directly (Brockmann *et al*. 1994; Craighead *et al*. 1995; Kellogg *et al*. 1995; Primmer *et al*. 1995; Colbourne *et al*. 1996). To this end, we have identified and employed microsatellite markers to study the genetic mating system of the Gulf pipefish. Microsatellite markers are ideal for such analyses in pipefish because they allow detection of multiple mating within a single brood, and because they can be assayed from small tissue samples such as single pipefish embryos. Empirical questions addressed in this study are: (i) how many Gulf pipefish females contribute eggs to a male's brood?; (ii) do individual females mate with multiple males?; (iii) will the microsatellite PCR primers also prove suitable for studies of related syngnathid taxa?

Materials and methods

Microsatellite characterization

DNA was extracted from a single Gulf pipefish male using a standard phenol : chloroform extraction protocol. Three micrograms of genomic DNA were digested with *Nde*II and electrophoresed through a 2% agarose gel. The region of the gel containing 200–700-bp fragments was excised and the DNA purified using the Prep-A-Gene DNA Purification System (BioRad). The fragments were ligated into *Bam*HI-digested, dephosphorylated pUC19. This ligation was heat-shock transformed into competent DH5α MCR™ *E. coli* (GIBCO BRL), which were subsequently plated on LB-ampicillin plates at a density of 100–150 colonies per plate.

The resulting library was screened with a synthetic GT_{10} oligonucleotide that was end-labelled using γ^{32}P ATP. Bacteria were transferred to nitrocellulose filters and the DNA was crosslinked by heating in a vacuum at 80 °C for 1.5 h. Filters were prehybridized at 42 °C for 1.5 h in a solution containing 6 × SSC, 5 × Denhardts reagent and 0.1% SDS. Hybridization was carried out by adding 33.5 ng of the end-labelled GT_{10} probe to the hybridization bag immediately after the 1.5-h prehybridization. Hybridization took place at 42 °C for 1.5 h. The filters were washed three times in 6 × SSC, 0.1% SDS for 15 min per wash at 42 °C and subsequently were placed on film overnight.

Plasmid DNA was purified from positive colonies using the Wizard Minipreps DNA Purification System (Promega). These potential positives were verified by performing a Southern analysis on the inserts of the plasmids.

Locus	*n*	Number of alleles	Heterozygosity		Exclusion probability
			Observed	Expected	
micro11.1	42	22	0.929	0.922	0.844
micro22.3	42	19	0.952	0.916	0.830
micro25.10	78	28	0.885	0.892	0.796
micro25.22	81	29	0.877	0.947	0.892

Table 1 Microsatellite locus designations, and sample sizes (*n*) of presumably unrelated adults used to establish baseline genetic information from the St Joseph State Park collection of Gulf pipefish. Shown for these baseline data are observed numbers of alleles, observed and expected heterozygosities, and per-locus expected exclusion probabilities (the fraction of females excluded from maternity given a single father-offspring pair)

Hybridization conditions were the same as above. Of ≈ 1200 total colonies screened, 56 potential positive clones were identified. Of these, 36 were verified as true positives by Southerns, five were not positive, and 15 were inconclusive (the miniprep or Southern failed).

Ten of the microsatellite clones were sequenced (fluorescent-dye method) by the Molecular Genetics Instrumentation Facility at the University of Georgia, and nine of these carried inserts which contained GT-rich microsatellites. Two of the sequenced clones proved identical. Primers were designed to amplify six of these microsatellite loci. One of the primer pairs failed to yield consistent amplification products and one has not yet been tested. The other four primer pairs consistently amplified fragments of the expected size and were polymorphic (Table 1).

The amplification of all microsatellite loci was carried out in 10 μL reaction volumes containing 1 × Promega *Taq* buffer, 1.5 mM $MgCl_2$, 0.15 μM each primer, 0.2 mM each dNTP, and 0.25 U Promega *Taq* polymerase. One primer was end-labelled with 1 μCi γ^{32}P ATP per five pmol of primer. The PCR amplification parameters consisted of an initial denaturing step of 3 min at 94 °C followed by 30 cycles of 92 °C for 1 min, an optimal annealing temperature (53–59 °C) for 1 min, and 72 °C for 1 min. The last cycle was followed by a final extension of 8 min at 72 °C. Microsatellites were resolved on standard 6% polyacrylamide denaturing sequencing gels. The primer pairs for two of the loci (micro25.10 and micro25.22) were designed to produce alleles that did not overlap in size. These loci could be coamplified in one PCR reaction and resolved simultaneously on a single sequencing gel. This was accomplished using the same PCR mixture as above, but by adding a second set of primers for the additional locus.

Maternity assessment in the Gulf pipefish

Pregnant Gulf pipefish males were collected on 1, 8 and 9 July 1994, from a single locale in the northern Gulf Coast of Florida (St Joseph State Park, Gulf Co.), using an 2.4-m seine with a 4-mm mesh. Numerous seine hauls taken through a 0.8-ha patch within a broader pasture of shallow seagrass meadows produced 64 pregnant males, eight nonpregnant adult males, and 49 adult females of the Gulf pipefish, which were then frozen in the field on dry ice. In the laboratory, embryos were dissected from pregnant males and rinsed in deionized water before being placed individually into 0.5-mL Eppendorf tubes. They were then placed in 100 μL of Gloor and Engels' extraction buffer (10 mM Tris pH 8.0, 1 mM EDTA, 25 mM NaCl, 200 μg/mL proteinase K freshly diluted from a frozen 20 mg/mL stock) and incubated for 30 min at 37 °C followed by 95 °C for 2 min (Gloor & Engels 1992). This solution was then stored at – 20 °C until needed. One microlitre of the embryo preparation was used for each PCR. A similar procedure was applied to adults. For males, a clip of the caudal fin was prepared in 50 μL of the buffer and a small tissue sample from the brood pouch was prepared in 100 μL, whereas for juveniles and females only the fin clip was used.

Genotypes for 20 pregnant males and for the embryos in their brood pouches were determined for two loci, micro25.10 and micro25.22. Sampling of the broods was designed to detect any incidence of multiple maternity, and involved a mean of 31 embryos typed per male (an average of 56.4% of each male's brood). For males with less than 43 embryos, PCR was attempted on all individuals in the brood pouch. For males with 43 or more embryos, a sample evenly spaced along the brood pouch was employed. For example, from the male with the largest number of embryos (154) in our sample, every fourth embryo was assayed. The embryos could be sampled evenly because they are arranged in rows within the brood pouch and can be plucked with forceps after the brood pouch is opened.

To provide more power to detect multiple mating by females, broods from an additional 20 pregnant males were added to the analysis. For each of these males, an average of 10 embryos was typed. This reduced our ability to detect multiple mating by these males, but allowed us to infer the genotypes of the mothers of additional broods to determine whether multiple males sometimes had received eggs from the same female.

Results

The microsatellite markers

All four of the microsatellites for which a consistent amplification product was obtained were highly variable. Numbers of alleles observed per locus ranged from 19 to 29 (Fig. 1), and heterozygosities (both observed and expected) were near 90% (Table 1). These loci are ideal for studies of parentage, with expected exclusion probabilities (Chakraborty *et al*. 1988) on the order of 0.8–0.9 for a single locus (Table 1) and > 0.999 for all four loci combined. An example of the gel patterns is presented in Fig. 2.

The loci micro25.10 and micro25.22 were used for the majority of the analyses reported here. In analyses of the 20 males whose broods were sampled most extensively, neither locus deviated from the expectations of Mendelian segregation: In 75 tests, only four showed deviations at $P \leq 0.05$, an expected number of departures for this level of significance.

Null alleles have been noted in many reports which employ microsatellite polymorphisms (Callen *et al*. 1993; Koorey *et al*. 1993; Phillips *et al*. 1993; Pemberton *et al*.

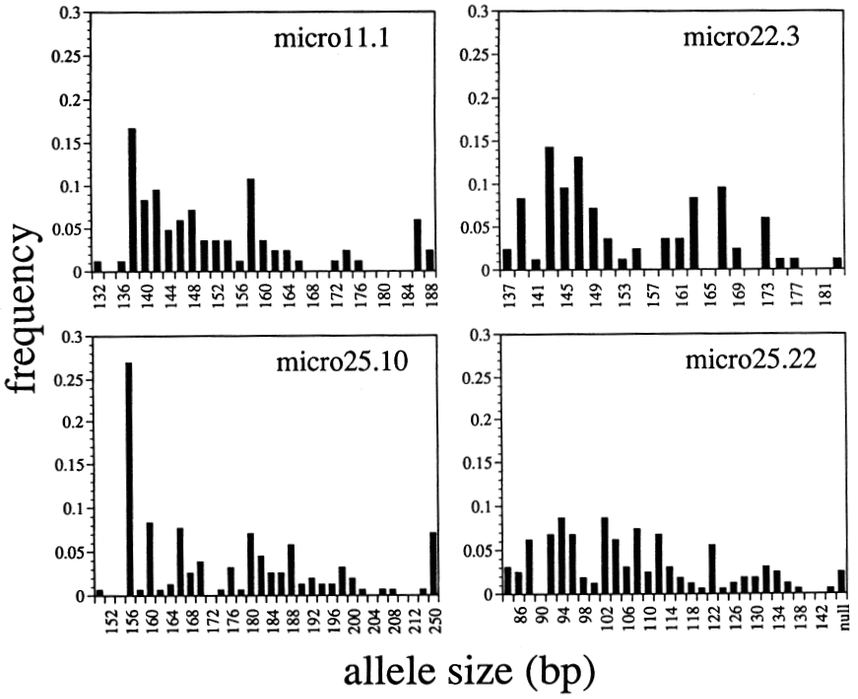

Fig. 1 Histograms of population allele frequencies at each of four microsatellite loci in the northern Florida collection of Gulf pipefish. The samples upon which these frequencies were based involved individual pregnant males, nonpregnant males, and adult females (sample sizes in Table 1). All alleles greater than 220 bp for the locus micro25.10 were lumped under the 250-bp designation. The rightmost column for micro25.22 represents the frequency of null alleles.

1995), and our study also encountered this phenomenon. A null allele at micro25.22 appeared in four of the 40 males for which embryos were genotyped. The presence of a null allele was obvious because the male appeared to be homozygous for micro25.22 yet some of his offspring displayed only a single allele contributed by the mother, with no apparent contribution from the father. The presence of this low frequency null allele probably accounts for the observation of more homozygotes than expected at this locus (Table 1). No null alleles were observed for micro25.10. However, exceptionally large alleles were observed at both loci. These alleles often amplified less efficiently than smaller alleles such that they were not always visible on lighter autoradiograms and, thus, sometimes appeared as nulls. These faint alleles became visible upon longer exposure. All of the detected large alleles segregated in Mendelian fashion, indicating that they were not artefacts.

Contamination of embryos by paternal DNA was not observed in this study. For the few cases in which the embryo displayed the same genotype as the father, this was parsimoniously explained as resulting from the mother sharing a paternal allele. Invariably, the appropriate genotypic classes expected in progeny were observed.

Mating behaviour of male Gulf pipefish

The logic of deducing a mother's genotype from the microsatellite data is exemplified in Table 2. Given the known father's genotype at a locus, and the genotypes observed among his brooded progeny, the maternal genotype is evident by subtraction. By accumulating such information across loci, multilocus maternal assignments can be made.

The results of these maternity analyses were unambiguous. Among the 20 broods originally examined, no instances of multiple maternity were documented (Table 3). In other words, in no case did a male's brood contain more than two nonpaternal alleles at a locus, nor did these inferred maternal alleles depart in frequency from the expectations of Mendelian segregation. The embryo sampling scheme described above, and the high variability of the markers, make it extremely unlikely that any incidence of multiple maternity would have remained undetected for any of these broods.

However, during the analysis of the additional 20 males for which fewer embryos were assayed per male, a single male (GM 8-2) was found to have mated with two females. This male's multiple mating was first detected in a sample of eight embryos, which together displayed more than two maternal alleles. To examine this situation more fully, an additional 20 embryos from GM 8-2 were assayed for micro25.10 and micro25.22. Eighteen of these embryos also were typed at two additional loci, micro11.1 and micro22.3. In all cases, the data were consistent with this male having mated with two females. Thus, at three of the four loci the brood contained four maternally derived alleles (whereas at the fourth locus, three distinct maternal alleles were evident). Furthermore, the four-locus genotypes of the two mothers could be inferred from the

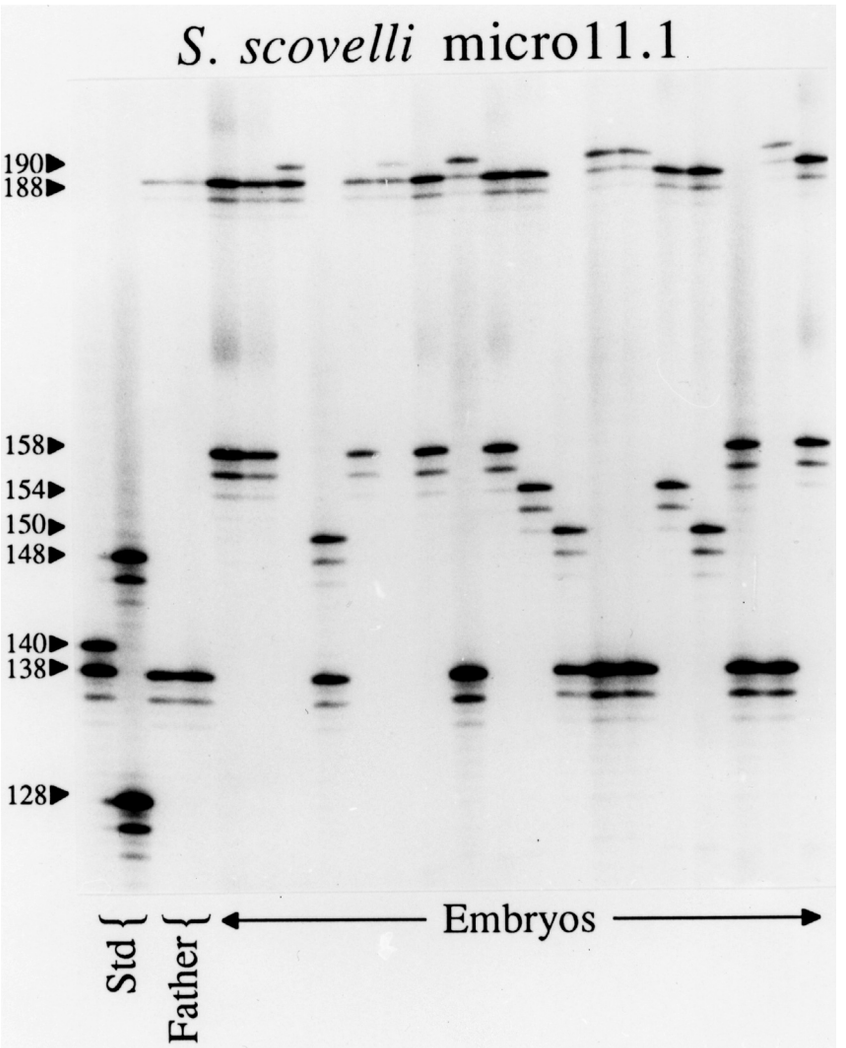

Fig. 2 Examples of gel patterns for the locus micro11.1, showing the male GM 8-2 and 18 embryos dissected from his brood pouch. Allele sizes are indicated in bp. From this progeny array, it was determined that GM 8-2 was exceptional among the males that we sampled in that he had mated with more than one female.

gametic phase disequilibrium in the progeny. Of the 28 embryos assayed, 12 were contributed by one of the mothers and 16 by the other.

Mating behaviour of female Gulf pipefish

For two of the males (GM 12-4 and GM 10-19) whose broods were analysed initially, the inferred genotype of the mother was identical at both micro25.10 and micro25.22 (Table 3), despite the fact that the expected frequency of this genotype in the population under random mating is only 6.1×10^{-4}. This finding suggested that these males may have mated with the same female. To investigate this possibility more fully, two additional microsatellite loci (micro11.1 and micro22.3) were scored for these two males and 10 embryos from each. The inferred genotypes of the mothers again matched, thus yielding a perfect four-locus identity overall (Table 4). The expected frequency of this four-locus genotype in the population is 3.2×10^{-8} (Table 4). Thus, the most plausible explanation is that these two males had mated with the same female. Both males carried embryos of about the same stage of development, suggesting that the female mated with both individuals within a reasonably short span of time and had split a single batch of eggs. One male carried 81 embryos and the other carried 49.

In an attempt to document additional cases of multiple mating by females, another 20 pregnant males were examined with an average of 10 progeny assayed per brood (Table 5). This sampling scheme allowed us to infer two-locus genotypes for the mothers of the broods at micro25.10 and micro25.22. In some cases, only a partial two-locus genotype was obtained, but this was sufficient to establish the female either as unique to one brood or as

Male ID	Father's genotype	Progeny genotypic classes and number in each class: class 1	*n*	class 2	*n*	class 3	*n*	class 4	*n*	Inferred maternal genotype
GM 12–4	166/182	156/166	13	156/182	14					156/156
GM 12–8	176/182	176/190	11	176/194	2	182/190	5	182/194	9	190/194
GM 11–1	156/180	156/176	15	176/180	22					176/176
GM 11–2	156/180	156/170	8	156/180	8	170/180	10	180/180	6	170/180
GM 10–3	166/166	158/166	19	166/166	10					158/166
GM 10–5	186/202	180/186	4	182/186	8	180/202	5	182/202	4	180/182
GM 11–3	156/200	156/162	4	156/192	4	162/200	3	192/200	1	162/192
GM 10–18	156/168	156/196	10	156/204	12	168/196	5	168/204	5	196/204
GM 10–9	156/182	156/176	7	156/182	7	176/182	5	182/182	8	176/182
GM 10–6	156/174	156/160	19	160/174	19					160/160
GM 10–10	192/194	182/192	8	192/196	12	182/194	7	194/196	2	182/196
GM 10–17	198/250	180/198	7	198/198	10	180/250	10	198/250	11	180/198
GM 10–4	170/198	170/180	11	170/192	11	180/198	6	192/198	10	180/192
GM 10–11	180/182	156/180	11	180/186	12	156/182	9	182/186	10	156/186
GM 10–19	160/250	156/160	14	156/250	27					156/156
GM 9–7	186/190	186/188	8	186/190	9	188/190	14	190/190	7	188/190
GM 9–1	180/196	156/180	10	162/180	7	156/196	8	162/196	11	156/162
GM 9–4	156/176	156/156	6	156/160	8	156/176	8	160/176	7	156/160
GM 9–2	170/188	170/180	11	170/186	9	180/188	5	186/188	5	180/186
GM 9–5	156/182	156/156	14	156/180	5	156/182	2	180/182	9	156/180

Table 2 An example of genotypic counts for micro25.10 for offspring within each of 20 broods of pregnant Gulf pipefish males. In most cases, four genotypic classes of offspring were observed, but in cases in which one of the parents was homozygous, only two classes were seen

a possible mother of multiple broods. All broods except two proved to have unique mothers in this additional sample. For the two broods (GM 8-5 and GM 8-8) for which the inferred mothers had identical two-locus genotypes, additional loci revealed another perfect four-locus match (Table 4). In this case, the expected frequency of the four-locus genotype was 1.8×10^{-11}, thus providing strong evidence for multiple mating by females. The embryos of these two males were also at about the same developmental stage, indicating again that the female probably split a single batch of eggs between them. GM 8-5 and GM 8-8 carried 99 and 90 embryos, respectively.

Male ID	No. of embryos: Total	Assayed	Father's genotype: micro25.10	micro 25.22	Inferred mother's genotype: micro 25.10	micro 25.22	exp. freq. maternal genotype
GM 12-4	*81*	*27*	*166/182*	*112/130*	*156/156*	*104/112*	*6.1×10^{-4}*
GM 12-8	39	27	176/182	98/102	190/194	88/118	5.0×10^{-7}
GM 11-1	57	37	156/180	94/112	176/176	112/200	8.6×10^{-7}
GM 11-2	154	32	156/180	102/104	170/180	112/118	9.1×10^{-6}
GM 10-3	48	28	166/166	106/null	158/166	94/128	3.2×10^{-6}
GM 10-5	34	21	186/202	106/112	180/182	88/128	1.4×10^{-5}
GM 11-3	12	11	156/200	102/108	162/192	112/112	1.1×10^{-6}
GM 10-18	122	32	156/168	94/122	196/204	96/110	5.5×10^{-7}
GM 10-9	35	27	156/182	110/110	176/182	96/100	4.8×10^{-6}
GM 10-6	117	37	156/174	86/92	160/160	96/126	1.2×10^{-5}
GM 10-10	63	29	192/194	108/124	182/196	92/104	9.6×10^{-6}
GM 10-17	104	38	198/250	92/132	180/198	86/92	1.5×10^{-5}
GM 10-4	86	38	170/198	86/114	180/192	92/92	1.2×10^{-5}
GM 10-11	47	42	180/182	92/94	156/186	108/112	1.4×10^{-4}
GM 10-19	*49*	*41*	*160/250*	*108/108*	*156/156*	*104/112*	*6.1×10^{-4}*
GM 9-7	92	38	186/190	84/92	188/190	94/108	1.9×10^{-5}
GM 9-1	63	36	180/196	94/116	156/162	96/102	4.0×10^{-5}
GM 9-4	30	29	156/176	88/120	156/160	96/106	1.9×10^{-4}
GM 9-2	55	30	170/188	96/132	180/186	88/106	1.4×10^{-5}
GM 9-5	38	30	156/182	104/106	156/180	92/94	4.5×10^{-4}

Table 3 Summary of the genotypes and embryo numbers of the 20 males for which broods were extensively sampled. Also given are the two-locus genotypes of the inferred mothers of these males' broods, and the expected frequency of these genotypes under random mating. Shown in italics are the two males for which the inferred mothers had an identical two-locus (and four-locus, not shown) genotype. For micro25.22, the allelic designation 200 represents an allele greater than 150 bp for which the exact size was not determined

Table 4 Inferred four-locus genotypes for the presumably shared mothers of broods from males GM 10-19 and GM 12-4, and from GM 8–5 and GM 8–8. The expected population frequencies of these genotypes under random mating are also presented

Male ID	Inferred genotype of mother at microsatellite locus….				Expected freq. of four-locus genotype
	micro25.10	micro25.22	micro11.1	micro 22.3	
GM 10-19	156/156	104/112	156/158	143/143	3.2×10^{-8}
GM 12-4	156/156	104/112	156/158	143/143	3.2×10^{-8}
GM 8-5	188/250	84/128	156/164	141/143	1.8×10^{-11}
GM 8-8	188/250	84/128	156/164	141/143	1.8×10^{-11}

Amplification of the microsatellite loci in other taxa

The capacity of all four PCR primer pairs to successfully amplify these microsatellite loci was also examined in several other gasterosteiform taxa: five congeners of *Sygnathus scovelli* (*S. floridae, S. louisianae, S. leptorhynchus, S. californiensis* and *S. typhle*), one species of pipefish (*Microgrnathus crinigerus*) and two species of seahorses (*Hippocampus erectus* and *H. zosterae*) also in Syngnathidae, and the threespine stickleback (*Gasterosteus aculeatus*) representing Gasterosteidae. The evaluation of PCR conditions in these taxa was not extensive in that only the annealing temperature was optimized, so the possibility remains that with further effort better amplification results might be obtained.

None the less, all four primer pairs yielded the expected PCR amplification products in all assayed *Syngnathus* species (example in Fig. 3). Outside this genus, amplification became more problematic. Some of the primers yielded products of the expected size in the pipefish and seahorses not in the genus *Syngnathus*, but in most cases the amount of product was reduced. None of the primers yielded expected amplification products in the stickleback (Fig. 3), suggesting that the primers may not be useful outside Syngnathidae.

Discussion

Pipefish mating systems

Despite the power to detect multiple maternity with the highly polymorphic microsatellite markers employed, in only one case in our sample of 40 paternal broods could a male be shown to have received eggs from more than one female. To bolster the conclusion of single mating by males, it is instructive to consider three ways in which a

Table 5 Numbers of embryos assayed and deduced maternal genotypes for the offspring of 20 subsequently assayed males for which broods were sampled less extensively. Blank entries indicate missing data. Shown in italics are the males (GM 8-8 and GM 8-5) whose inferred mates shared the same two-locus genotype. The pregnant male GM 8-2 had received eggs from two females

Male ID	No. of embryos assayed	Father's genotype		Inferred mother's genotype	
		micro25.10	micro 25.22	micro 25.10	micro 25.22
GM 12-2	30	156/180	– / –	184/250	– / –
GM 12-3	17	156/250	92/104	156/166	86/92
GM 8-4	4	160/176	88/122	156/180	90/96
GM 8-8	*15*	*160/170*	*102/null*	*188/250*	*84/128*
GM 8-3	5	156/188	102/null	156/168	104/116
GM 6-5	2	156/188	96/102	156/ –	96/122
GM 7-9	7	156/166	96/132	160/250	92/128
GM 7-7	3	170/190	108/108	160/ –	122/132
GM 7-6	7	160/166	94/104	156/196	106/200
GM 7-10	8	160/180	98/128	160/250	94/106
GM 8-5	*18*	*194/198*	*84/102*	*188/250*	*84/128*
GM 8-6	16	156/162	108/132	160/194	108/112
GM 6-6	7	208/250	92/106	168/180	130/ –
GM 9-10	3	156/156	102/130	164/206	110/ –
GM 7-11	8	156/156	100/null	180/194	110/112
GM 6-2	8	156/200	94/122	156/160	110/ –
GM 8-2	28	198/250	104/112	176/250	102/110
				180/198	120/null
GM 6-1	6	156/166	86/108	198/214	112/136
GM 7-4	8	164/168	88/102	166/176	96/200
GM 6-4	8	168/180	92/96	194/250	94/102

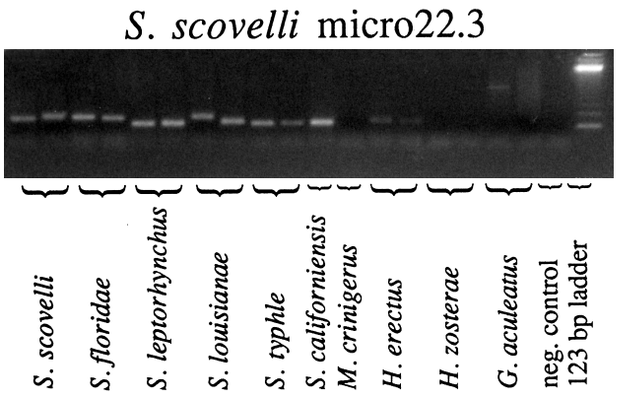

Fig. 3 Results of PCR amplification efforts for other species of Gasterosteiformes using the micro22.3 primer pair developed for the Gulf pipefish *Syngnathus scovelli*. The other three loci yielded similar results.

single female might in principle be incorrectly ascribed as the sole mother of a brood whose father truly had been mated successfully by multiple females. First, perhaps nonexhaustive sampling of brood pouches caused us to overlook embryos contributed by later-mating females. However, at least for the first 20 males in which large samples were typed, this is most unlikely. For example, the adult with the smallest proportion of embryos genotyped (21%) carried a brood of 154 offspring, of which 32 were typed. Even in this extreme case, if a second female had contributed as few as 13 embryos to random locations throughout the brood pouch, we would have sampled *at least* one of them with a probability greater than 0.95. If the embryos were clumped together by maternity, as they well may be, our sampling strategy for brood pouches (minimally, every fourth egg assayed) would nearly ensure the detection of genetic contributions by a second female.

Second, perhaps an occasional male had mated with two females that shared the same di-locus genotype, resulting in an incorrect assessment of maternity. This is unlikely because based on a model of random mating as applied to the observed microsatellite allelic frequencies, the expected population frequencies of the two-locus genotypes assigned to the mothers were low (ranging from 6×10^{-4} to 5×10^{-7}; Table 3). Third, perhaps an occasional male had mated with two females that were homozygous for different alleles. This too is unlikely given the rarity of observed and expected homozygotes at these loci (Table 1). In summary, only rarely (< 5%) do single-male broods of *S. scovelli* at this north Florida locale have multiple mothers. The factors that limit most males to a single genetic mate remain unknown.

Conversely, the genetic data suggest that females often distribute their eggs among multiple males. Demonstrably, two pairs of assayed males carried broods originating from the same mother. Given the severe sampling biases operating against the detection of multiple mating by females in nature, such instances probably are common. Collections were made within an extensive seagrass meadow such that no doubt only a minute fraction of an enormous population was examined. Indeed, of 37 adult females collected from this locale and typed at micro25.10 and micro25.22, all but two were excluded (based on two-locus genotypes) as possible mothers of the broods assayed, making it evident that we sampled only a small fraction of the breeding adults at this locale. Thus, females that appeared to have mated only once based upon our sample of males may well have mated with additional males that we failed to collect. In other words, although we documented only two instances of multiple mating by females, these genetic findings suggest that females routinely mate with multiple males.

Given the current genetic observations on maternity and paternity in *S. scovelli*, and in consideration of the inherent asymmetry in the power to genetically document multiple matings by males vs. females in large natural populations of a species with male pregnancy, we conclude that the mating system of these Gulf pipefish can best be described as tending toward genetic polyandry. In the course of a single male pregnancy, most fathers have only one successful mate, whereas females commonly distribute eggs to more than one male.

This study involves a single point sample from one locale, and thus fails to address possible temporal or geographical variation in the Gulf pipefish mating system. However, a comparison with Brown's (1972) study of a Gulf pipefish population from Cedar Key, Florida, gives no indication that our sample was atypical in certain demographic parameters that might influence the mating system. Numbers of embryos carried per male in our sample (mean = 66.3; max. = 154; $n = 20$) are similar to those reported by Brown (mean = 55.6; max. = 175; $n = 346$). Among adults, Brown found that the ratio of female to male Gulf pipefish fluctuated throughout the year from 0.51 to more than 1.5; our observed ratio of 0.68 is within this range. However, given that the OSR is considered a key factor affecting the strength of sexual selection (Emlen & Oring 1977; Vincent *et al*. 1994), the temporal variation in sex ratio documented by Brown could be of interest for future genetic investigations into possible variation in the mating system of *S. scovelli*.

Among other *Syngnathus* species, detailed behavioural information about the mating system is available only for a European pipefish, *S. typhle* (Berglund & Rosenqvist 1993; Berglund 1994; Vincent *et al*. 1994; Rosenqvist & Johansson 1995). In this species, it appears that during the course of a male pregnancy, males mate with multiple females and females mate with multiple males. This

conclusion is based on direct observations of mating behaviour in the laboratory, and on the consideration that some field-caught males contain eggs of distinct colours believed to have originated from different females (Berglund *et al*. 1988). However, the frequency of multiple mating in natural populations of *S. typhle* has yet to be determined.

The genetic mating system of the Gulf pipefish is, perhaps, more similar to that of another pipefish species (*Nerophis ophidion*) in which a receptive male receives an entire brood from a single female, and females reportedly often mate with multiple males within the timeframe of a male pregnancy (Rosenqvist 1993). However, males of *N. ophidion* do not have a fully enclosed brood pouch as Gulf pipefish do; rather, eggs are glued to the male's ventral surface. This is thought to have promoted an evolutionary tendency for male behavioural reluctance for matings with multiple females, because later matings could dislodge previously attached eggs (Rosenqvist 1993). *N. ophidion* females also differ from Gulf pipefish females in that they are thought to deposit a full load of eggs at each copulation, before preparing another batch for a different male (Berglund & Rosenqvist 1993). In contrast, our observations suggest that Gulf pipefish females are capable of splitting a single batch of eggs among more than one male.

The mating system of *N. ophidion* has been described as sequential polygamy (Vincent *et al*. 1992): during the timeframe of a single male pregnancy, males mate with one female only, whereas females mate sequentially with multiple males. This is similar to the definition of polyandry that we suggest also for the Gulf pipefish, and is in distinction to the polygynandrous system of *S. typhle* in which both males and females mate multiple times during the course of a male pregnancy. All three species can be considered polygamous, however, because at least one sex in each has multiple mates during a short and well-defined breeding interval.

Mating systems and sexual selection

Syngnathids have been proposed to fall into two separate categories with regard to sexual selection (Vincent *et al*. 1992): (i) monogamous pipefishes and seahorses which are not sex-role reversed (under the definition of sex-role reversal discussed in the introduction); and (ii) polygamous pipefishes which are. The former category includes the seahorses *Hippocampus* spp. and the pipefish genus *Corythoichthys*, both of which appear to consist of monogamous species in which competition for mates is greater among males than among females (Gronell 1984; Vincent 1994; Vincent & Sadler 1995). The latter category includes *S. typhle*, *N. ophidion*, and probably *S. scovelli*, in which the relative intensity of competition for mates by the two genders is thought to be the reverse. In these species, the stronger operation of sexual selection on females than on males can in some cases lead to the elaboration of female secondary sex traits. As already mentioned, *S. scovelli* females have a deep, enlarged abdomen and V-shaped silvery stripes along the trunk region. In *N. ophidion*, females have intense blue coloration on the trunk and appearance-enlarging skin folds (Rosenqvist 1993). In *S. typhle*, on the other hand, although females tend to be larger than males, only weak sexual dimorphism and little development of female secondary sex traits are displayed (Svensson 1988; Berglund *et al*. 1989). This is true also of many other species of *Syngnathus* (Brown 1972; Dawson 1985).

Why do some species of pipefish exhibit conspicuous secondary sex traits, whereas others do not? Perhaps there is a relationship in this family not only between the direction of sexual selection and the mating system (as suggested by Vincent *et al*. 1992), but also between the mating system and the strength of sexual selection. In species with traditional sex-roles, a simple prediction originally inspired by Darwin (1871) is that polygyny results in more intense sexual selection. Although this prediction has received general empirical support (Stamps 1983; Björklund 1990; Oakes 1992; Webster 1992; Winquist & Lemon 1994), the correlation between the extent of polygyny and the evolution of sexual dimorphism is far from perfect (Clutton-Brock *et al*. 1977; Leutenegger & Cheverud 1982; Payne 1984; Höglund 1989). It must be emphasized, however, that almost all studies conducted previously have examined social (behavioural) as opposed to genetic (realized) mating systems. A frequently observed discrepancy between the social and genetic mating systems (Birkhead & Møller 1992; Searcy & Yasukawa 1995) no doubt contributes to the difficulty of testing associations between the mating system and the evolution of sexual dimorphism (Møller & Birkhead 1994).

In any event, the analogous hypothesis for sex-role reversed species is that sexual dimorphism and expression of secondary sex traits in females may be more pronounced in species that are more strongly genetically polyandrous than in species that are less so (Jehl & Murray 1986). Thus, in the future the comparative mating systems of multiple syngnathid species might profitably be examined genetically. After grouping species into those that are genetically monogamous and not sex-role reversed, vs. those that are genetically polygamous and sex-role reversed, it may be informative to further subdivide the latter category based on the degree of polyandry vs. polygynandry. The hypothesis to be further evaluated is whether increased levels of polyandry, and hence in the presumed disparity in the intensity of sexual selection on females vs. males, are associated with increased gender dimorphism in secondary sexual characters. Although available information for *S. typhle* (a polygynandrous species

lacking dimorphism) and for *N. ophidion and S. scovelli* (polyandrous species with considerable dimorphism) appears to be consistent with (and indeed motivated) this hypothesis, further evaluation will require comparative study of genetic mating systems across additional syngnathid and other species with sex-role reversal.

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The work described here is the first instalment of a dissertation project by Adam Jones that involves genetic examination of maternity and the mating system in species in which males (rather than females) provide parental care. Such species afford unusual perspectives on possible interrelationships between the mating system, sex-role reversal, intensities of sexual selection, and degrees of sexual dimorphism. This work is part of a broader research programme in John Avise's laboratory on the use of molecular markers in ecology and evolution.
