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The genetic mating system and tests for cuckoldry in a pipefish species in which males fertilize eggs and brood offspring externally

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Abstract

Highly variable microsatellite loci were used to study the mating system of *Nerophis ophidion*, a species of pipefish in which pregnant males carry embryos on the outside of their body rather than in an enclosed brood pouch. Despite this mode of external fertilization and brooding, otherwise rare in the family Syngnathidae, the genotypes of all embryos proved to be consistent with paternity by the tending male, thus indicating that cuckoldry by sneaker males is rare or nonexistent in this species. *N. ophidion* is a phylogenetic outlier within the Syngnathidae and its reproductive morphology is thought to be close to the presumed ancestral condition for pipefishes and seahorses. Thus, our genetic results suggest that the evolutionary elaboration of the enclosed brood pouch elsewhere in the family was probably not in response to selection pressures on pregnant males to avoid fertilization thievery. With regard to maternity assignments, our genotypic data are consistent with behavioural observations indicating that females sometimes mate with more than one male during a breeding episode, and that each male carries eggs from a single female. Thus, the polyandrous genetic mating system in this species parallels the social mating system, and both are consistent with a more intense sexual selection operating on females, and the elaboration of secondary sexual characters in that gender.

Keywords: microsatellites, maternity, *Nerophis*, paternity, Syngnathidae

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Introduction

All of the ≈250 species of seahorses and pipefish (Syngnathidae) are characterized by male pregnancy and an absence of female parental care (Breder & Rosen 1966; Dawson 1985; Lourie *et al*. 1999). Depending on the species, eggs are either deposited by females inside a male's enclosed brood pouch, or they are glued externally onto his ventral surface. These modes of egg and embryo brooding by males are unique among fishes, and make seahorses and pipefishes excellent subjects for the study of mating system evolution. Syngnathid mating systems are also of interest because, in at least some pipefish species, females compete for mates more intensely than do males, and sexual selection therefore acts more strongly on females (Berglund *et al*. 1986a,b; Vincent *et al*. 1992). By this definitional criterion, such species are sex-role-reversed (Vincent *et al*. 1992; Rosenqvist 1993).

In genetic paternity analyses based on molecular markers, each male in all syngnathid populations surveyed to date (three pipefish species, *Syngnathus scovelli*, *S. floridae*, *S. typhle*, and a seahorse, *Hippocampus angustus*) has proved to be the exclusive genetic sire of the brood he carries (Jones & Avise 1997a,b; Jones *et al*. 1998a, 1999a, 2000; review in Jones & Avise 2001). Those four species all have an enclosed brood pouch within which egg fertilization takes place, so it is not surprising that a pregnant male is the true sire of all offspring that he bears. This complete assurance of paternity contrasts with the situation in many other fish species with extended male care of offspring, such as sunfish (Gross 1991; DeWoody *et al*. 1998, 2000b; Neff *et al*. 2000), darters (DeWoody *et al*. 2000a), sand gobies (Jones *et al*. 2001) and sticklebacks (Jones *et al*. 1998b). In these nest-tending species with external fertilization, genetic analyses have revealed that paternity by males other than the primary

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attendant (i.e. cuckoldry) is a rather common phenomenon (review in DeWoody & Avise 2001).

Here, we examine genetic paternity in a pipefish species in which males fertilize eggs externally and carry the resulting embryos on the outside of the body. In the straight-nosed pipefish, *Nerophis ophidion*, a female deposits rows of eggs on the ventral surface of a male, who then releases a cloud of sperm through which he swims (Fiedler 1954). This mode of fertilization could provide a window of opportunity for other males to cuckold the egg-carrier by releasing sperm simultaneously with the brooding male. Whether cuckoldry is common in this species is relevant to discussions of the phylogenetic history of brood care behaviour in the Syngnathidae, because conventional wisdom holds that paternity assurance is prerequisite to the evolution of a high investment strategy by males (Clutton-Brock 1991).

There is another reason for interest in biological parentage in *N. ophidion*. Field and laboratory studies (Berglund *et al*. 1986b, 1989; Rosenqvist 1990) have shown that females in this species compete more intensely for mates than do males, and that females possess secondary sexual characters (blue coloration on the head and flank, and appearance-enlarging skin folds) important in mate choice. Does the genetic mating system of *N. ophidion* thus tend toward polyandry, as might be predicted from sexual selection theory for role-reversed species (Jehl & Murray 1986)? Prior behavioural observations have suggested that *N. ophidion* is indeed polyandrous, but caution is required because pronounced differences between the social and genetic mating system are well known in some other organismal groups such as passerine birds (reviews in Birkhead & Møller 1992, 1998; Westneat & Webster 1994; Avise 1996).

The goals of this study were to identify and employ microsatellite markers in *N. ophidion* to determine: (i) if the absence of an enclosed brood pouch leads to occasional fertilization thievery by other males; (ii) how many females deposit eggs on each pregnant male; and (iii) whether individual females deposit eggs on more than one male. Findings on the genetic mating system of this species are evaluated in the context of previous parentage studies of other pipefishes and seahorses, and interpreted with respect to a published phylogenetic sequence for the evolution of different brood structures in the Syngnathidae.

Materials and methods

Collections

Adult *Nerophis ophidion* were collected by pulling a small beam trawl (2-mm mesh) behind a boat through shallow (0.5–6.0 m) eelgrass meadows in Gullmar Fjord on the Swedish west coast (58°15′ N, 11°28′ E). Adult population samples were accumulated from repeated sampling efforts from mid-May to mid-July, 1996. Eleven pregnant *N. ophidion* males were collected on 6 and 8 July and were returned live to Klubban Biological Station where they were frozen at −80 °C with their broods intact.

In addition, ≈ 70–100 fish were placed in an aquarium at a very high density (≈ 0.5 fish/L) to breed. This density of adults is much greater than has been observed in nature (0.02/m^2; Vincent *et al*. 1995) and, hence, should be especially conducive to multiple mating and cuckoldry (because many reproductively active males and females were necessarily in close proximity to any breeding pair). Four pregnant males were obtained from the aquarium and frozen for genetic analysis. Approximately 35 females and non-pregnant males housed in the aquarium were frozen for genetic analysis of population allele frequencies, and the remainder were returned to the collecting site.

Microsatellite characterization

Total genomic DNA from a single *N. ophidion* individual was digested with *Mbo*I, and size selected by gel electrophoresis. The 200–700-bp fragments were ligated into *Bam*HI digested, dephosphorylated pBluescript phagemid (Stratagene). Ligations were heat-shock transformed into competent XL1-Blue *Escherichia coli* (Stratagene) and this partial genomic library was screened with eight ^{32}P-labelled oligonucleotides: $(GT)_{10}$, $(GGAT)_4$, $(GACA)_4$, $(TAG)_6$, $(GATA)_4$, $(GA)_{10}$, $(TCC)_5$ and $(TTAGGG)_3$.

DNA from positive clones was purified using the Wizard Minipreps DNA Purification System (Promega), and was sequenced at the Molecular Genetics Instrumentation Facility at the University of Georgia. Three pairs of primers (Table 1) designed from sequenced clones (GenBank Accession nos AF355588–90) were shown to have adequate polymorphism and resolution. One of each primer pair was labelled with a fluorescent dye for use with the ABI 377 genotyping system.

DNA was extracted from a small clip of tissue from adults, and from entire individual embryos, using a standard proteinase K, phenol–chloroform protocol (Maniatis *et al*. 1982). Genotypes for 15 pregnant males and an average of 24 embryos per male were obtained using all three microsatellite loci, and an additional population sample of 49 adults was genotyped to estimate population allele frequencies. The embryos sampled from each male were spaced evenly along the length of his body.

Amplification of each microsatellite locus was carried out in a total volume of 12 μL containing 1 U of *Taq* DNA polymerase and 0.2 mM of each dNTP. Loci *NO9* and *NO10* used a 1× buffer containing 50 mM KCl, 10 mM Tris, 1.5 mM $MgCl_2$ and 50 μg/mL BSA, whereas locus *NO14.2* used a buffer containing 50 mM KCl, 10 mM Tris–HCl, 0.1% Triton X-100 and 2 mM $MgCl_2$. Reactions for locus *NO9* used 0.125 μM of each primer and 1 μL of concentrated genomic DNA, locus *NO10* used 0.25 μM of each primer and 1 μL of

Table 1 Microsatellite loci assayed from a random sample of *Nerophis ophidion* adults. All three loci used in this study were cloned from *N. ophidion*. Shown are the primer sequences, original cloned repeat sequence, number of adults sampled (*n*), number of alleles, observed and expected heterozygosities, and one-parent-known exclusion probabilities for each locus. The total one-parent-known exclusion probability combined across all three loci is also shown

Locus	Primer sequences (5′ → 3′)	Cloned repeat	*n*	No. of alleles	Heterozygosity Exp.	Heterozygosity Obs.	Excl. Prob.
NO9	CTAATCTGGTTTGTCTCCTGGG GCTTTTTATTCACTCTCTCGCA	$[AC]_{32}$	63	31	0.942	0.889	0.884
NO10	CAGGTGGAGGAAGTGAATAG CTTGGATGAAAGAGATGAGG	$[CA]_5TA[AC]_8TA[AC]_6$	64	5	0.735	0.719	0.503
NO14.2	AGTTGAGGGGTTGAATGGAA TGCGTTGCTGATAAATAGGC	$[TG]_{25}$	62	15	0.755	0.823	0.541
						Total	0.974

Table 2 An example of how maternal genotype was deduced from embryo genotypic counts. Shown are the genotypes of the father and all offspring at each locus. Where both parents are heterozygous, four classes of offspring genotypes are shown, while if one parent is homozygous only two classes are present

Locus	Paternal genotype	Embryo genotypic classes class 1	*n*	class 2	*n*	class 3	*n*	class 4	*n*	Deduced maternal genotype
NO9	168/194	168/196	8	168/214	7	194/196	7	194/214	6	196/214
NO10	170/186	170/186	16	186/186	12					186/186
NO14.2	148/150	148/148	3	148/150	11	148/184	8	150/184	6	148/184

1:10 diluted genomic DNA, and locus *NO14.2* used 0.167 μM of each primer and 1 μL of 1:10 diluted genomic DNA. Cycling conditions for loci *NO9* and *NO10* consisted of an initial denaturing step of 95 °C for 2 min, followed by 30 cycles of 95 °C for 30 s, annealing at 62 °C for 30 s, and extension at 72 °C for 30 s. Locus *NO14.2* used an initial denaturation step of 2 min at 95 °C, followed by 30 cycles of 95 °C for 1 min, annealing at 59 °C for 1 min and extension at 72 °C for 2 min. Polymerase chain reaction (PCR) products were electrophoresed on an ABI 377 machine, and scored using the GENESCAN and GENOTYPER software packages.

Parentage analyses

Genotypes determined for pregnant males and the offspring they carried were used to deduce genetic parentage. For each brood, the genotype of each offspring was examined for genetic consistency with paternity by the potential sire. The maternal allele in each embryo was then determined by subtraction (except for cases in which an embryo had the same heterozygous genotype as the father), and the multilocus genotype of the maternal parent was deduced by an accumulation of such data from multiple progeny within a brood (see Table 2 for an example).

The sample size of progeny (*n*) necessary to detect all nonshared parents contributing to a brood was estimated using the program BROOD (DeWoody *et al*. 2000c), assuming the following parameter conditions: that 200 embryos comprised an average brood to which three females had contributed equally (an overestimate of the anticipated number — only one female per brood). Under these conservative assumptions (in the sense that they are likely to overestimate the required sample size of offspring), the upper 95% confidence limit for *n* was found to be 26.8. Thus, our average sample size of 24 embryos per brood should provide adequate empirical power to determine whether multiple females had spawned with individual males.

Tests for genotypic disequilibria and Hardy–Weinberg equilibrium were conducted using GENEPOP version 1.2 (Raymond & Rousset 1995).

Results

Hardy–Weinberg equilibrium and linkage

The three microsatellite loci displayed 5–31 alleles each (Fig. 1). Expected and observed heterozygosities were moderate to high, yielding a combined exclusion probability (under the 'one-parent-known' model; Chakraborty *et al*. 1988) of 0.974. These values are consistent with the mean numbers of alleles per locus and expected heterozygosities reported for many marine fish (DeWoody & Avise 2000).

Genotypic disequilibria between loci were not detected in the adult population or within broods, and an exact test of Hardy–Weinberg proportions in the adult population was nonsignificant for loci *NO10* and *NO14.2*. However, there was a significant heterozygote deficiency at locus

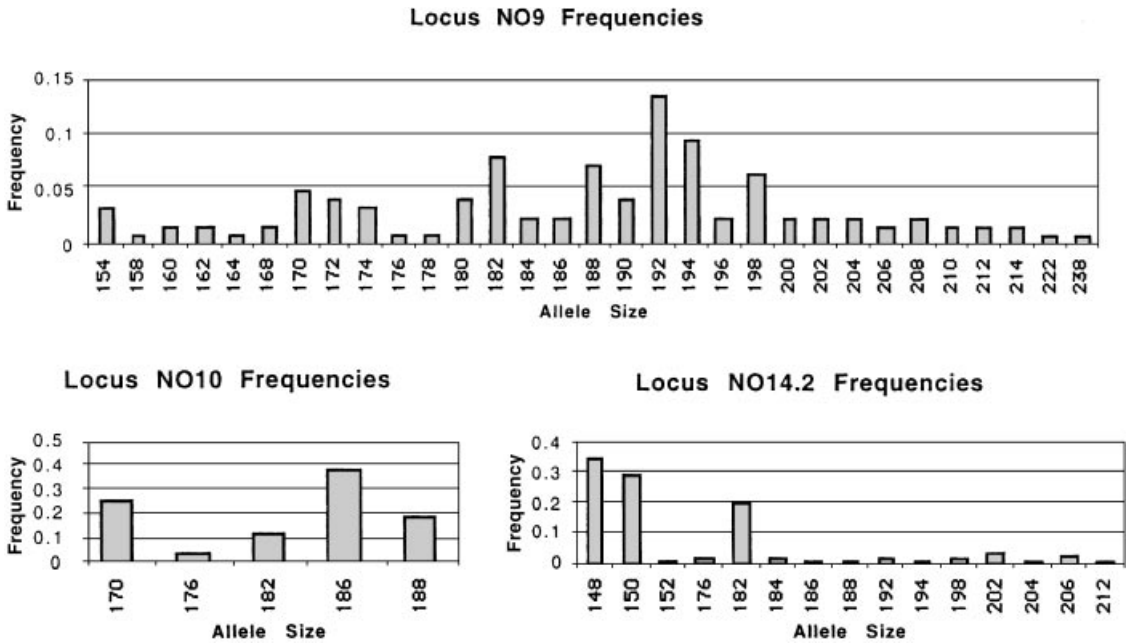

Fig. 1 Allele frequency histograms at each of the three microsatellite loci in the adult population sample of *Nerophis ophidion*. Allelic size refers to the length in base pairs (bp) of the amplified polymerase chain reaction product.

NO9 (exact test $P < 0.04$). This suggests the presence of null alleles, a suspicion further supported by direct examination of genotypes in some of the progeny arrays (see beyond).

Brood paternity

The mean number of embryos carried by the surveyed males was 95.8, of which a mean of 24.1 per pregnant male was genetically analysed. By sampling ≈ 25% of the offspring in each of many broods, even some rare cuckoldry events, if present in the population, should have been encountered in our assays. Yet, essentially all of the 361 total offspring surveyed proved to display genotypes consistent with their having been sired by their respective brooder males (five embryos were possible exceptions to this statement, but as we described below, these do not appear to be attributable to cuckoldry). Thus, we estimate the rate of cuckoldry in *Nerophis ophidion* to be < 1/361 (≈ 0.28%).

At face value, the genotypes of five embryos (1.4%) were problematic. In one case, one embryo from the brood of male 47 had a three-locus genotype identical to that of its brooding male. At two loci, this genotype conflicted with the inferred genotype of the mother (based on the other 26 embryos sampled from the brood). Thus, most likely, the assay itself was contaminated by the father's tissue. For three embryos from another male (number 19), provisional *de novo* mutations at the *NO9* locus probably account for an aberrant genotype. These embryos displayed a genotype that appeared homozygous for an allele inferred to be present only in the mother of the brood. One possibility is that a premeiotic mutation in the father resulted in a null allele distributed to multiple offspring. Such 'clustered mutations' (Woodruff & Thompson 1992) have been documented in another pipefish species, *Syngnathus typhle* (Jones *et al*. 1999b). Alternatively, a second male might have fertilized these eggs. The expected frequency of an individual with a multilocus genotype consistent with siring these three embryos is $\approx 9.07 \times 10^{-5}$. Because both the frequency of this genotype and the (conventional) rate of *de novo* mutation at microsatellite loci are comparably low, we cannot decide between these competing possibilities. Finally, in one embryo from male 9, both alleles at locus *NO9* were inconsistent with either parent (despite the fact that the genotype of this embryo was consistent with the rest of the brood at the other two loci). We have no simple explanation for this observation, but one possibility is that a mutation at *NO9* occurred in both the maternal and paternal gametes.

Female mating patterns

No more than two maternal alleles per locus were present in any male's clutch of embryos, indicating that only one mother had contributed to each brood (Table 3). Also, based on the observed genotypes of brooded embryos, in no case had a female contributed embryos to the broods of two (or more) males collected from the wild. However, in

Table 3 Three-locus genotypes of the 15 males for which offspring were genotyped. Also shown are the deduced maternal genotypes at each locus. Note that the only case in which the same female genotype (female 53) was deduced is between males 47 and 58, both of which were kept in an aquarium that also housed that female. The notation of 170/– for the deduced locus *NO9* genotype for the female who mated with male 23 indicates that this individual appeared to be heterozygous for an allele of 170 bp and a nonamplifying (null) allele

Male ID	No. of eggs assayed	Total no. of eggs	Father's genotype			Deduced mother's genotype		
			NO9	*NO10*	*NO14.2*	*NO9*	*NO10*	*NO14.2*
2	18	107	182/188	170/170	148/182	142/186	186/186	182/182
5	16	80	186/194	170/186	150/182	170/182	170/170	148/150
6	15	44	160/190	170/170	148/182	170/186	168/188	148/148
9	18	109	188/238	170/186	150/182	170/192	170/186	148/150
13	21	123	196/198	186/186	150/182	196/206	186/188	148/210
14	24	102	192/204	170/170	148/148	164/204	186/186	148/148
18	19	127	184/194	186/188	148/148	188/204	170/182	150/176
19	40	94	170/170	182/186	182/186	184/192	170/182	182/182
20	25	83	196/198	170/176	148/182	162/208	170/170	148/150
21	24	98	198/198	186/186	148/150	192/192	170/186	148/150
23	28	104	198/202	170/186	148/148	170/–	170/170	148/182
29	29	108	180/188	170/188	150/150	160/172	170/186	148/176
30	29	95	180/192	186/188	192/202	188/206	182/188	150/150
47	27	78	168/190	170/182	148/148	196/214	186/186	148/184
58	28	85	168/194	170/186	148/150	196/214	186/186	148/184
Mean	24.1	95.8						

the aquarium breeding experiment, an instance of multiple mating by a female was detected in the broods carried by males 47 and 58 (Table 3). In both of these broods, the deduced maternal genotype matched female 53, who possessed a three-locus genotype with an expected frequency of only 2.5×10^{-6}. These two males had been allowed to breed in an aquarium in which this female (and many others) was present, so it is almost certain that female 53 was indeed the true mother of both sets of embryos. The other pregnant males sampled from the aquarium experiment (males 29 and 30) evidently had mated with different females. However, because some females from the aquarium were released before the genetic assay, the inferred maternal genotypes from these broods did not match any of the female genotypes directly sampled.

Discussion

The genetic mating system

There is an inherent asymmetry in the genetic power to detect multiple mating by male vs. female syngnathids in any natural (or captive) population that has been sampled less than exhaustively. Because offspring cohorts come 'prepackaged' on a male's body, if he has mated successfully with multiple females this will be readily apparent in any genetic assay with sufficient resolution (i.e. with high polymorphism, as in this study). However, if a particular female has mated with multiple males, this will be revealed in the genetic assays only if more than one of her pregnant mates happen to have been included in the sample examined. Thus, if the population is large or sparsely sampled, multiple mating by females may seldom be detected even if the phenomenon is relatively common. In this study, we failed to detect multiple mating by males (a behaviour that would be easy to document), whereas in aquarium studies we did detect an instance of multiple mating by females (a behaviour that in principle is otherwise far more difficult to uncover). These findings imply that multiple mating by males is rare or nonexistent, and that multiple mating by females can and does occur in this species. Thus, our genetic findings are not inconsistent with previous behavioural observations of polyandry in *Nerophis ophidion* (Berglund *et al*. 1986a,b, 1989).

Our genetic results agree with earlier behavioural observations that each male straight-nosed pipefish carries the eggs of only one female. Those previous studies (Berglund *et al*. 1986a,b, 1989) further demonstrated that, although females in this species have a greater overall energetic investment in young, males limit total reproduction because the duration of brooding by a male is longer than the time taken by a female to produce a clutch of eggs. Indeed, a female given access to multiple males can lay 1.8 times more eggs than a typical *N. ophidion* male can accommodate (based on his observed mean clutch size; Berglund *et al*. 1989). Furthermore, males already carrying eggs have not been observed to remate, possibly because mating with a second female could dislodge the earlier clutch. From these observations, the behavioural or social mating system of *N. ophidion* was also determined to be polyandrous.

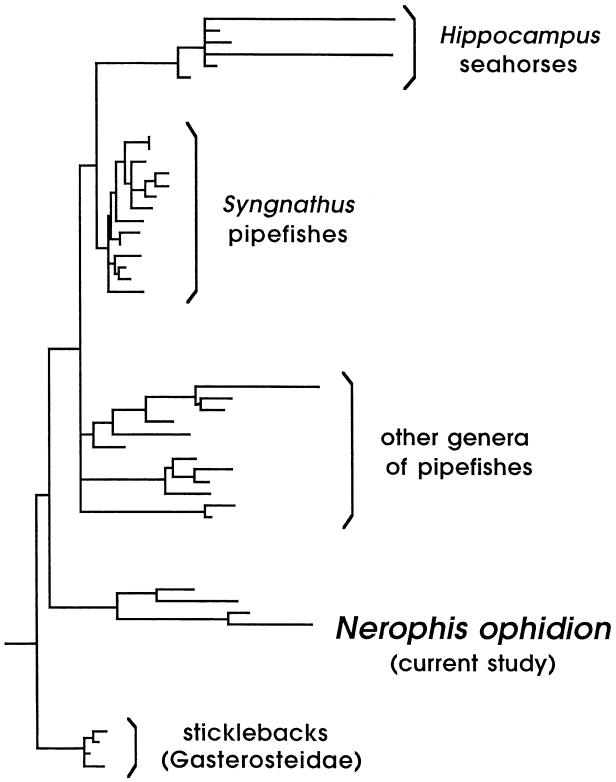

Fig. 2 Phylogenetic tree for the Syngnathidae based on mitochondrial DNA sequence data (redrawn from Wilson *et al*. 2001). Each terminal node represents a different species genetically assayed. Note that *Nerophis ophidion*, the subject of our study, belongs to a basal syngnathid lineage, and that all prior molecular studies of parentage in the group have focused on species representing the more derived genera *Hippocampus* and *Syngnathus*.

Previous molecular studies of several syngnathid species have revealed genetic mating systems ranging from monogamy (in *Hippocampus angustus*; Jones *et al*. 1998a), to polyandry (in *Syngnathus scovelli*; Jones & Avise 1997a), to polygynandry (in *S. floridae* and *S. typhle*; Jones & Avise 1997b; Jones *et al*. 1999a). With regard to any relationship between the genetic mating system and the degree of sexual dimorphism, these studies have also revealed an apparent trend generally consistent with conventional sexual selection theory (Darwin 1871; Bateman 1948; Trivers 1972; Williams 1975; Andersson 1994) as applied to sex-role-reversed species (Jehl & Murray 1986). Namely, the more polyandrous of the syngnathid species genetically surveyed to date are those displaying the greater degree of dimorphism in secondary sexual characters (Jones & Avise 2001). In these sexually dimorphic species, females are invariably the more adorned sex, presumably due to the greater intensity of sexual selection operating on that gender. The genetic and behavioural observations on *N. ophidion* further support this trend; this species appears to be polyandrous, and, as mentioned previously, is strongly dimorphic in secondary sexual characters.

Paternity assurance and the evolution of male brooding

We found no evidence for genetic cuckoldry in the broods of *N. ophidion* males. From an evolutionary perspective, it is perhaps not surprising that males of this (and other) syngnathid species typically father all of the offspring that they carry. For such extensive paternal care to have evolved, males must have gained a considerable assurance of paternity (absence of fertilization thievery) to help offset their energetic and other costs of caring for eggs and embryos over an extended time (Baylis 1981; Clutton-Brock 1991).

Recently, a molecular phylogeny of Syngnathidae was estimated using nucleotide sequences from 1602 bp of mitochondrial DNA sequence from the cytochrome *b*, 12*S* ribosomal DNA (rDNA) and 16*S* rDNA genes (Wilson *et al*. 2001). Based on that phylogeny (a skeletonized version of which is presented in Fig. 2), the seahorses (*Hippocampus*) and pipefishes (*Syngnathus*) analysed previously for genetic mating systems (Jones & Avise 1997a,b; Jones *et al*. 1998a, 1999a) are closely related groups and share the most advanced types of enclosed brood pouches. By contrast, the lineage leading to *Nerophis* was apparently an early offshoot in the family tree, and *N. ophidion* is thought to represent the unelaborated brooding configuration more closely representative of the presumed ancestral condition in the Syngnathidae: external fertilization and brood care on the outside of the male's body (Herald 1959; Wilson *et al*. 2001).

In the four members of the stickleback family (Gasterosteidae) used as outgroups in the Wilson *et al*. (2001) phylogenetic analysis, males build nests and guard eggs. The only genetic study to date of mating behaviours in sticklebacks (Jones *et al*. 1998b) showed that both male and female fifteenspine sticklebacks (*Spinachia spinachia*) mate with multiple partners, and that ≈ 18% of the male-tended nests contained embryos resulting from sneaked fertilization events. Thus, regular male cuckoldry attendant with external fertilization and nest construction was probably a characteristic feature also of other fishes ancestral to the modern Syngnathidae. Our genetic results for *N. ophidion* indicate that the evolution of a body-carrying mode of egg-tending by males was probably sufficient to eliminate most such opportunities for cuckoldry.

Thus, the fact that we failed to detect any cuckoldry in *N. ophidion*, an external brooder, also suggests that the evolutionary elaboration of more fully enclosed brood pouches in most other pipefishes and seahorses may not have been primarily in response to selection pressures for cuckoldry avoidance (see Baylis 1981; Wootton 1984), but rather as a fitness-enhancing means of providing increased protection and nutrition for developing embryos.

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This study constitutes the first chapter in the dissertation of Beth McCoy, currently a graduate student in the Avise laboratory interested in the evolution of fish mating systems and reproductive tactics. As a former a PhD student who graduated from the Avise laboratory in 1998, Adam Jones pioneered the use of molecular markers in the comparative study of genetic mating systems and sexual selection theory in the male-pregnant Syngnathidae.