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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. floridanus, 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
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 Birkenhead & Meller 1992, 1998; Westneat & Webster 1994; Avise 1996).

The goals of this study were to identify and employ microsatellite loci 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) elagrass 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/m²; 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 32P-labelled oligonucleotides: (GT)₅, (GCG)₅, (GACA)₅, (TAG)₅, (GATA)₅, (GACGA)₅, (GAAG)₅ (TCC), and (TTAGGG)₅.

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 AF355886–89) 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 μM of each dNTP. Loci NO9 and NO10 used a 1× buffer containing 50 mM KCl, 10 mM Tris, 1.5 mM MgCl₂ 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 MgCl₂. 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.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Primer sequences (5′ → 3′)</th>
<th>Cloned repeat</th>
<th>n</th>
<th>Number of alleles</th>
<th>Obs.</th>
<th>Exp.</th>
<th>Excl. exclusivity prob.</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO9</td>
<td>CGTTTTTTAATCTCTGCAGA</td>
<td>[CT]<em>5</em></td>
<td>63</td>
<td>31</td>
<td>0.942</td>
<td>0.889</td>
<td>0.884</td>
</tr>
<tr>
<td>NO10</td>
<td>CAGCTGAGAGCTGAGTAGG</td>
<td>[CA]<em>4</em></td>
<td>64</td>
<td>5</td>
<td>0.735</td>
<td>0.719</td>
<td>0.503</td>
</tr>
<tr>
<td>NO14.2</td>
<td>AGTGGGGGGTGGAGTAGG</td>
<td>[AC]<em>3</em></td>
<td>65</td>
<td>15</td>
<td>0.755</td>
<td>0.823</td>
<td>0.541</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Total</td>
<td></td>
<td></td>
<td>0.974</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Paternal genotype</th>
<th>Embryo genotypic classes</th>
<th>Deduced maternal genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO9</td>
<td>168/194</td>
<td>class 1 n</td>
<td></td>
</tr>
<tr>
<td>NO10</td>
<td>170/186</td>
<td>class 2 n</td>
<td></td>
</tr>
<tr>
<td>NO14.2</td>
<td>148/150</td>
<td>class 3 n</td>
<td></td>
</tr>
</tbody>
</table>

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

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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 ≈ 9.07 × 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
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.

### Table 3

<table>
<thead>
<tr>
<th>Male ID</th>
<th>No. of eggs assayed</th>
<th>Total no. of eggs</th>
<th>Father’s genotype</th>
<th>Deduced mother’s genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>NO9</td>
<td>NO10</td>
</tr>
<tr>
<td>2</td>
<td>18</td>
<td>107</td>
<td>182/188</td>
<td>170/170</td>
</tr>
<tr>
<td>5</td>
<td>16</td>
<td>80</td>
<td>186/194</td>
<td>170/186</td>
</tr>
<tr>
<td>6</td>
<td>15</td>
<td>44</td>
<td>160/190</td>
<td>170/170</td>
</tr>
<tr>
<td>9</td>
<td>18</td>
<td>109</td>
<td>188/238</td>
<td>170/186</td>
</tr>
<tr>
<td>13</td>
<td>21</td>
<td>123</td>
<td>196/198</td>
<td>186/186</td>
</tr>
<tr>
<td>14</td>
<td>24</td>
<td>102</td>
<td>192/204</td>
<td>170/170</td>
</tr>
<tr>
<td>18</td>
<td>19</td>
<td>127</td>
<td>184/194</td>
<td>186/188</td>
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<td>40</td>
<td>94</td>
<td>170/170</td>
<td>182/186</td>
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<td>20</td>
<td>25</td>
<td>83</td>
<td>196/198</td>
<td>170/176</td>
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<td>21</td>
<td>24</td>
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<td>198/198</td>
<td>186/186</td>
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<td>29</td>
<td>29</td>
<td>108</td>
<td>180/188</td>
<td>170/188</td>
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<td>30</td>
<td>29</td>
<td>95</td>
<td>180/192</td>
<td>186/188</td>
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<td>47</td>
<td>27</td>
<td>78</td>
<td>168/190</td>
<td>170/182</td>
</tr>
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<td>58</td>
<td>28</td>
<td>85</td>
<td>168/194</td>
<td>170/186</td>
</tr>
<tr>
<td>Mean</td>
<td>24.1</td>
<td>95.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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 the 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, 12S ribosomal DNA (rDNA) and 16S 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 fifteen-spine 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 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.