

## THE DYNAMICS OF MALE BROODING, MATING PATTERNS, AND SEX ROLES IN PIPEFISHES AND SEAHORSES (FAMILY SYNGNATHIDAE)

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**Abstract.**—Modern theory predicts that relative parental investment of the sexes in their young is a key factor responsible for sexual selection. Seahorses and pipefishes (family Syngnathidae) are extraordinary among fishes in their remarkable adaptations for paternal care and frequent occurrences of sex-role reversals (i.e., female-female competition for mates), offering exceptional opportunities to test predictions of sexual selection theory. During mating, the female transfers eggs into or onto specialized egg-brooding structures that are located on either the male's abdomen or its tail, where they are osmoregulated, aerated, and nourished by specially adapted structures. All syngnathid males exhibit this form of parental care but the brooding structures vary, ranging from the simple ventral gluing areas of some pipefishes to the completely enclosed pouches found in seahorses. We present a molecular phylogeny that indicates that the diversification of pouch types is positively correlated with the major evolutionary radiation of the group, suggesting that this extreme development and diversification of paternal care may have been an important evolutionary innovation of the Syngnathidae. Based on recent studies that show that the complexity of brooding structures reflects the degree of paternal investment in several syngnathid species, we predicted sex-role reversals to be more common among species with more complex brooding structures. In contrast to this prediction, however, both parsimony- and likelihood-based reconstructions of the evolution of sex-role reversal in pipefishes and seahorses suggest multiple shifts in sex roles in the group, independent from the degree of brood pouch development. At the same time, our data demonstrate that sex-role reversal is positively associated with polygamous mating patterns, whereas most nonreversed species mate monogamously, suggesting that selection for polygamy or monogamy in pipefishes and seahorses may strongly influence sex roles in the wild.

**Key words.**—Comparative method, mating patterns, molecular phylogenetics, paternal care, sex-role reversal, sexual selection, Syngnathidae.

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In the vast majority of animals, the male's sole contribution to his offspring is his sperm (Trivers 1972). As a result, even when the observed sex ratio of males to females is equal, the operational sex ratio is often biased toward males and males almost universally compete more strongly for mates, while females typically exert greater mate choice (Darwin 1871; Emlen and Oring 1977). Although most egg-laying fishes leave their eggs unprotected after spawning, sole male care is the predominant pattern in those species that care for their young (Blumer 1982). Paternal care is likely to increase offspring fitness, but may reduce the father's ability to invest in other offspring (i.e., a parental investment *sensu* Trivers 1972).

Sex roles are defined by mating competition (Emlen and Oring 1977). Sex-role reversal occurs when females primarily compete for access to mates, as compared to conventional sex roles with male-male mating competition (cf. mating roles, Gwynne 1991). Traditionally, the concept of parental investment has been used empirically to predict sex roles and intensities of mating competition (Trivers 1972). More recent studies have predicted mating competition by the sexual difference in potential reproductive rates, in which the potential

reproductive rate is the population's mean of offspring production when not constrained by the availability of mates (Clutton-Brock and Vincent 1991; Clutton-Brock and Parker 1992; Kvarnemo and Ahnesjö 1996, 2002; Parker and Simmons 1996; Ahnesjö et al. 2001). If male parental investment reduces their potential reproductive rate below that of females, the operational sex ratio (Emlen and Oring 1977) may become female-biased, resulting in a reversal of sex roles, with females competing more strongly for mates (Clutton-Brock and Parker 1992; Parker and Simmons 1996; Kvarnemo and Ahnesjö 1996, 2002; Parker and Simmons 1996). Recent theoretical work also indicates that parental investment is a primary determinant of sex roles (Kokko and Monaghan 2001; Kokko and Johnstone 2002). Variation in both degree of paternal care and the occurrence of sex-role reversals in pipefishes and seahorses offers opportunities to explore hypotheses concerning the relationship between parental investment, sex roles, and sexual selection (Trivers 1972; Parker and Simmons 1996).

The family Syngnathidae (pipefishes and seahorses) is characterized by remarkable adaptations for paternal care. The female deposits eggs directly onto a specialized brooding area or into a pouch located under the abdomen or the tail of the male (Breder and Rosen 1966). This evolutionary innovation ensures males complete confidence of paternity (Jones and Avise 1997; Jones et al. 1998, 1999), but at a level of paternal investment that exceeds that of most other vertebrates (Breder and Rosen 1966). Thereafter the embryos are nourished, osmoregulated, and protected during a lengthy

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period of male pregnancy (Berglund et al. 1986, 1989; Vincent et al. 1992). The brooding structures vary in complexity in five steps, from: (1) a simple unprotected ventral area for gluing, (2) individual membranous egg compartments, (3) protection of eggs in a pouch with pouch plates, (4) bilateral pouch folds that grow together into a closed pouch, to (5) the most complex and completely enclosed brooding pouch of seahorses (Dawson 1985). There is a further significant difference among species in that brooding may occur on the tail (Urophori: A-type) or on the abdomen (Gastrophori: B-type; Herald 1959). Among the Gastrophori, brooding structures vary in complexity from step 1 to step 3 (Dawson 1985).

Parental investment as defined by Trivers (1972) is extremely difficult to estimate, involving the assessment of ultimate fitness costs from expenditures in different currencies (time, energy, etc.). It is important to recognize that parental expenditures are not necessarily equivalent to parental investment, because expenditures such as parental guarding may not necessarily carry a fitness cost. However, in many cases, time and energy expenditures may be positively correlated and, in general, a large expenditure will often carry larger costs and therefore represent a higher parental investment. In the limited number of syngnathids studied to date, males of species with less complex brooding structures (e.g., *Nerophis ophidion*) spend less energy on their young than do those brooding embryos in enclosed pouches with placental-like structures (e.g., *Hippocampus zosterae*, *Syngnathus typhle*; Berglund et al. 1986; Masonjones 2001). If increasing pouch complexity results in a general increase in male parental investment relative to females, we would expect to see more frequent sex-role reversals in species with more complex brood pouches. Intense mating competition among females (i.e., sex-role reversal) should also favor the evolution of sexual dimorphism, where females are larger and more colorful than males. True to predictions, although some pipefishes retain conventional sex roles (e.g., *Hippichthys penicillus*), several species are sex-role reversed (e.g., *Nerophis ophidion*, *Stigmatopora nigra*, *Syngnathus typhle*), with females that are more vividly colored and striped than males (Table 1). One notable exception to this pattern is the genus *Hippocampus*, in which, although these species have the highest degree of pouch development, sex-role reversal has not yet been documented (Vincent and Sadler 1995; Kvarnemo et al. 2000; Masonjones and Lewis 2000).

Microsatellite studies of genetic mating have revealed a broad array of mating patterns in syngnathid fishes, ranging from strict monogamy in *Hippocampus subelongatus* (Jones et al. 1998) to various forms of multiple mating in many of the pipefish species studied to date (*Nerophis ophidion* [McCoy et al. 2001]; *Syngnathus floridae*, *S. scovelli*, *S. typhle* [Jones and Avise 2001]). Despite a methodological bias towards the detection of multiple mating of male syngnathids (McCoy et al. 2001), all mating patterns studied to date are either polyandrous (multiple mating by females) or polygamous (multiple mating by both males and females; Jones and Avise 2001; McCoy et al. 2001), suggesting that access to multiple mates may be common among female syngnathid fishes. In species in which the potential reproductive rate of females exceeds that of males, the operational sex ratio may

be biased towards females, intensifying female-female mating competition.

To reconstruct the evolution of male brooding structures and study evolutionary patterns of sex-role reversal in the Syngnathidae, we sequenced three mitochondrial genes from a global sampling of syngnathid species, representing all major male pouch types. If the degree of pouch complexity accurately reflects relative paternal investment, theory (Trivers 1972) predicts that sex-role reversal should be most prevalent in syngnathids with more complex brooding structures. In addition to testing this hypothesis, we investigate the association between mating patterns and sex roles in syngnathid fishes (Vincent et al. 1992), explicitly incorporating phylogenetic data in a test of correlated trait evolution in the family Syngnathidae. If mating patterns influence mating competition and thereby sexual selection (see above), we predict a positive correlation between polygamy (where females frequently show multiple matings) and sex-role reversals among syngnathid fishes.

## MATERIALS AND METHODS

### *Sample Collection, Polymerase Chain Reaction Amplification and DNA Sequencing*

We sampled a total of 48 individuals, including 44 specimens from across the entire geographic range of the family representing all major pouch types (Table 1). Morphological work suggests that the Gasterosteiform and Syngnathiform fishes are a monophyletic assemblage (Bowne 1984). With this in mind, we included three outgroup species from the sticklebacks (family Gasterosteidae; order Gasterosteiformes): the threespine stickleback (*Gasterosteus aculeatus*; New York), the blackspotted stickleback (*Gasterosteus wheatlandii*; Rhode Island), and the ninespine stickleback (*Pungitius pungitius*; Scotland, U.K.); and the Japanese tubenout (*Aulichthys japonicus*; Kanagawa, Japan; family Aulorhynchidae; also within the order Gasterosteiformes; Bowne 1984). Specimens were preserved in 70% ethanol and total genomic DNA was extracted by proteinase K/SDS digestion and purified by phenol-chloroform extraction and ethanol precipitation (Kocher et al. 1989).

The polymerase chain reaction (PCR) was used to amplify a 484-bp segment of the large subunit (16S) mitochondrial ribosomal gene, a 352-bp segment of the small subunit (12S) mitochondrial ribosomal gene, and the complete (1149 bp) mitochondrial gene coding for cytochrome *b*: details of the protocol and the primer sequences are published elsewhere (Kocher et al. 1989; Palumbi et al. 1991; Pääbo et al. 1991; Taberlet et al. 1992; Wilson et al. 2001). DNA sequences have been submitted to GenBank (accession numbers: AF354940–AF355033, AF356040–AF356081, AF356539, AY166830–AY166832).

### *Sequence Alignment and Phylogenetic Reconstruction*

The orthologous DNA sequences obtained were aligned, using default settings, by Clustal W (Thompson et al. 1994) and optimized by eye. Congruency of 12S, 16S, and cytochrome *b* mitochondrial DNA datasets was tested with a likelihood-based congruency test ( $\alpha = 0.05$ ; 10000 RELL boot-

TABLE 1. Syngnathid species, sampling locality (for study species), and pouch type with sex role (conventional: male-male competition for mates; reversed: female-female competition for mates) and mating pattern from literature. Pouch type: A, tail brooder; B, abdominal brooder; 1–5, increasing pouch complexity (see text); e, everted pouch folds; s, semi-inverted pouch folds; i, inverted pouch folds (only applicable to the A4 pouch type).

Species	Sampling locality	Pouch type	Sex role	Mating pattern
<i>Hippocampus abdominalis</i>	Tasmania	A5	Unknown	Unknown
<i>Hippocampus barbouri</i>	Philippines	A5	Unknown	Unknown
<i>Hippocampus comes</i>	Philippines	A5	Unknown	Monogamous (Perante et al. 2002)
<i>Hippocampus erectus</i>	Virginia	A5	Unknown	Unknown
<i>Hippocampus fuscus</i>		A5	Conventional (Vincent 1994)	Monogamous (Vincent 1994)
<i>Hippocampus kuda</i>	Philippines/Taiwan	A5	Unknown	Unknown
<i>Hippocampus reidi</i>		A5	Unknown	Monogamous (Dauwe 1993; Nijhoff 1993)
<i>Hippocampus subelongatus</i>		A5	Conventional (Kvamemo et al. 2000)	Monogamous (Jones et al. 1998)
<i>Hippocampus whitei</i>		A5	Conventional (Vincent and Sadler 1995)	Monogamous (Vincent and Sadler 1995)
<i>Hippocampus zosterae</i>	Florida	A5	Conventional (Masonjones and Lewis 2000)	Monogamous (Masonjones and Lewis 2000)
<i>Hippocampus</i> sp.	Kuwait	A5	Unknown	Unknown
<i>Syngnathus abaster</i>	Italy	A4i	Reversed (Fiedler 1954)	Polygamous (Fiedler 1954)
<i>Syngnathus acus</i>	Sweden/Wales	A4i	Reversed (I. Ahnesjö, pers. obs.)	Polygamous (Vincent et al. 1995)
<i>Syngnathus floridae</i>	Virginia/Florida	A4i	Reversed (Jones and Avise 2001)	Polygamous (Jones and Avise 2001)
<i>Syngnathus fuscus</i>	Virginia	A4i	Reversed (Roelke and Sogard 1993)	Unknown
<i>Syngnathus leptorhynchus</i>	California	A4i	Unknown	Unknown
<i>Syngnathus louisianae</i>	Florida	A4i	Unknown	Unknown
<i>Syngnathus rostellatus</i>	Sweden	A4i	Reversed (I. Ahnesjö, pers. obs.)	Polygamous (Vincent et al. 1995)
<i>Syngnathus schlegelii</i>	China	A4i	Reversed (Watanabe et al. 2000)	Polygamous (Watanabe and Watanabe 2001)
<i>Syngnathus scovelli</i>	Florida	A4i	Reversed (Jones and Avise 1997)	Polygamous (Jones and Avise 1997)
<i>Syngnathus taenionotus</i>	Italy	A4i	Unknown	Unknown
<i>Syngnathus typhle</i>	Italy/Sweden	A4i	Reversed (Berglund et al. 1986, 1989)	Polygamous (Jones et al. 1999)
<i>Hippichthys penicillus</i>	Kuwait	A4e	Conventional (Watanabe et al. 1997)	Monogamous (Watanabe et al. 1997)
<i>Hyselognathus rostratus</i>	Australia	A4e	Unknown	Unknown
<i>Kaupus costatus</i>	Australia	A4e	Unknown	Unknown
<i>Urocampus carmirostris</i>	Australia	A4e	Unknown	Unknown
<i>Pugnaso curtirostris</i>	Australia	A4e	Unknown	Unknown
<i>Vanacampus phillipi</i>	Australia	A4s	Unknown	Unknown
<i>Vanacampus poecilolaemus</i>	Australia	A4s	Unknown	Unknown
<i>Stigmatopora argus</i>	Australia	A4s	Reversed (A. Kendrick, pers. comm.) <sup>1</sup>	Unknown
<i>Stigmatopora nigra</i>	Australia	A4s	Reversed (A. Kendrick, pers. comm.) <sup>1</sup>	Unknown
<i>Corythoichthys intestinalis</i>	Australia	A4s	Conventional (Gronell 1984)	Monogamous (Gronell 1984)
<i>Corythoichthys haematopterus</i>	Guam/Indonesia	A4s	Reversed (Matsumoto and Yanagisawa 2001)	Monogamous (Matsumoto and Yanagisawa 2001)
<i>Phycodorus eques</i>	Japan	A2	Conventional (P. Groves, pers. comm.) <sup>2</sup>	Monogamous (P. Groves, pers. comm.) <sup>2</sup>
<i>Phyllopteryx taeniolatus</i>	Australia	A2	Unknown	Unknown
<i>Solegnathus hardwickii</i>	Australia	A2	Unknown	Unknown
<i>Entelurus aequareus</i>	Sweden	B1	Reversed (I. Ahnesjö, pers. obs.)	Polygamous (Vincent et al. 1995)
<i>Nerophis ophidion</i>	Sweden	B1	Reversed (Berglund et al. 1986, 1989)	Polygamous (McCoy et al. 2001)
<i>Dunckerocampus dactylophorus</i>	Philippines	B2	Unknown	Monogamous (Kuitert 2001)
<i>Doryrhamphus excisus</i>	Marquesas	B3	Unknown	Monogamous (Kuitert 2001)
<i>Doryrhamphus japonicus</i>		B3	Conventional (A. Gronell, pers. comm.) <sup>3</sup>	Monogamous (A. Gronell, pers. comm.) <sup>3</sup>
<i>Oosthetus brachyurus</i>	Guatemala	B3	Unknown	Unknown

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strap replicates; Waddell et al. 2000), using maximum likelihood (ML) topologies generated from individual gene analyses as well as the overall ML tree (likelihood parameters estimated by Modeltest Ver. 3.06; Posada and Crandall 1998). Free parameters (including branch lengths) were re-optimized for each dataset on all topologies tested.

Neighbor-joining (NJ) distance and maximum parsimony (MP) analyses were performed with PAUP Ver. 4b10 (Swofford 2000), with indels coded as missing data. Maximum-parsimony trees were constructed with full heuristic searches (500 bootstrap replicates) using random addition (10 replicates), the tree-bisection-reconnection (TBR) branch-swapping algorithm and the MULPARS option. For parsimony analyses, a transversion/transition weighting of two was used. Neighbor-joining analyses (500 bootstrap replicates) applied a HKY model of substitution (Hasegawa et al. 1985), with transition/transversion ratio (2.22), gamma shape parameter (0.57), proportion of invariable sites (0.41), and nucleotide frequencies (A, 0.3092; C, 0.2413; G, 0.1519; T, 0.3031) estimated from the dataset using Modeltest (Posada and Crandall 1998).

Bayesian methods of phylogenetic inference, incorporating posterior probabilities, were calculated using MrBayes Ver. 2.01 (Huelsenbeck and Ronquist 2001), with parameters estimated from the dataset under the HKY + I +  $\Gamma$  model. A Metropolis-Coupled Monte Carlo Markov Chain (MCMCMC) was run for 100,000 generations starting from a random tree (sampling every 25 generations) using four heated chains (temp = 0.2). The MCMCMC reached apparent stationarity at 20,000 generations and trees sampled during this burn-in of the model were not used in further analyses. Bayesian analyses were replicated in four runs with identical results.

The overall ML tree was estimated following a heuristic search on starting tree topologies generated by distance, parsimony and Bayesian analyses. Initial branch lengths were estimated using Rogers-Swofford approximation and optimized following Newton-Raphson optimization (100 branch-length smoothing passes,  $\Delta = 0.000006$ ). Branch swapping followed the TBR algorithm. The optimal ML tree was identical in each of the three replicates.

To investigate whether rates of sequence evolution in the Syngnathidae deviate from the neutral expectation of clock-like behavior, the likelihood of the ML tree was recalculated with the constraint of a molecular clock (Rambaut parameterization for clock optimization). A comparison of the molecular clock tree with the unconstrained topology was tested with a likelihood-ratio test (LRT). Finally, to determine overall support for the ML tree, well-supported internal groupings were shuffled and the deviation of these alternative topologies from the ML tree was tested using a likelihood-based Shimodaira-Hasegawa (SH) test (Shimodaira and Hasegawa 1999).

#### *Reconstruction of Character State Evolution*

Prior to the reconstruction of ancestral character states, duplicated taxa were pruned from the dataset, parameters were re-estimated using Modeltest (Posada and Crandall

1998) and the ML topology was recalculated as outlined above.

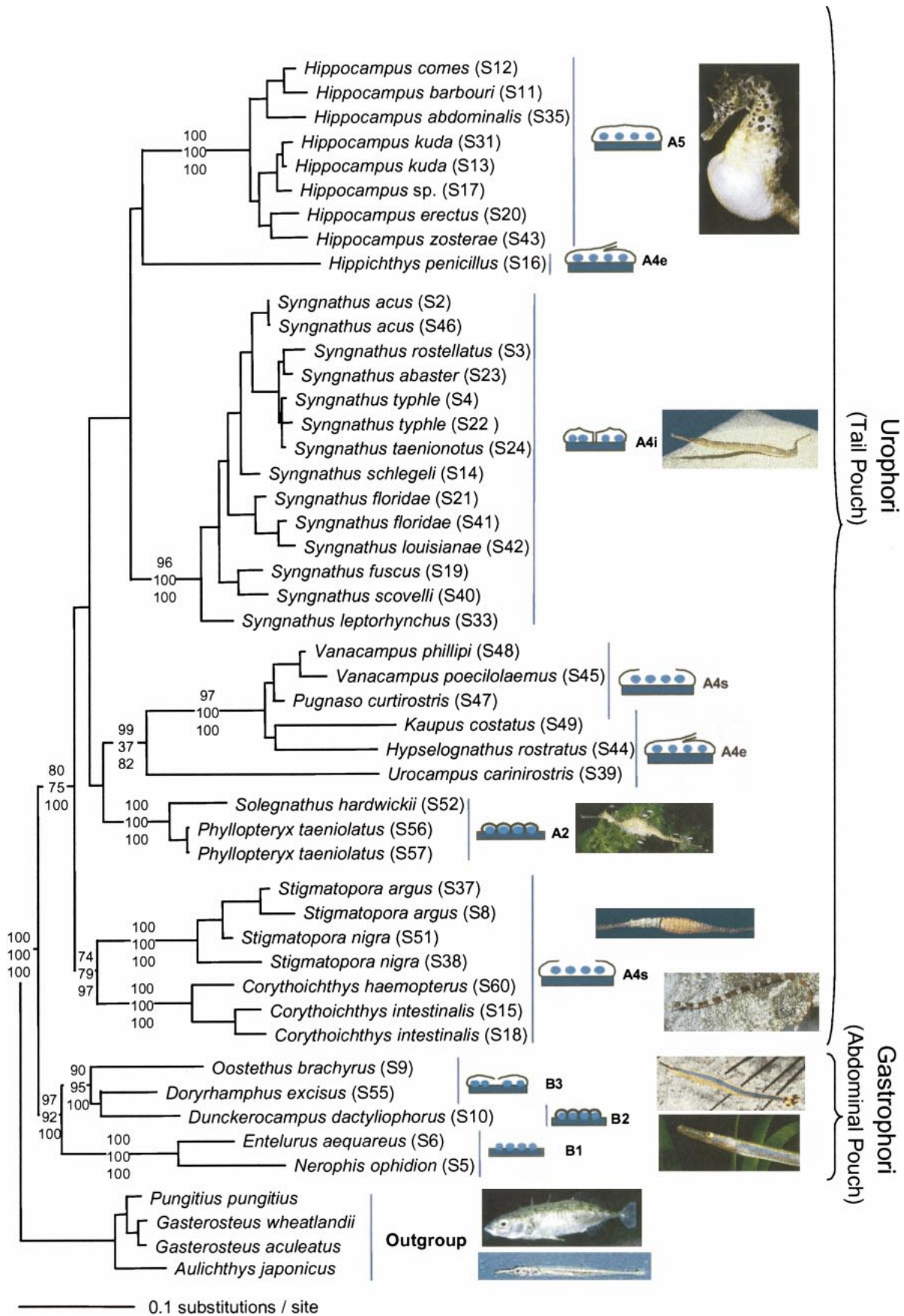
To investigate directional evolution of brooding structures in the Syngnathidae, Multi-state Ver. 0.6 (Pagel 1999a) was used to estimate ancestral character states at each node independently for Urophori and Gastrophori lineages based on brood-pouch morphology for extant taxa (Table 1). A two-parameter forward-backward model of character evolution incorporating independent forward and backward rates of character transformation was initially tested against an unconstrained model of character change. Ancestral character states were estimated by optimizing the overall ML on the phylogeny with individual nodes fixed independently for all possible character states. Preference for one ancestral state over another was deemed significant if the difference in their log-likelihoods was greater than two (Pagel 1999a).

A gradual model of evolution predicts that evolutionary change should accrue slowly and uniformly over the phylogenetic history of a group of organisms. Deviations from this model suggest that character change has been punctuated, possibly indicating lineage-specific selection pressures (Pagel 1999b). To quantify the rate of brood pouch evolution, the likelihood of an unconstrained model incorporating a scaling parameter was compared to a model where the scaling parameter was fixed at 0 (punctuated rate of trait change) for both the Urophori and Gastrophori using Multi-state (Pagel 1999a). The significance of this comparison was tested using the likelihood ratio statistic:  $-2[\log_e(H_0/H_1)] = \chi^2$ ,  $df = 1$ .

Available empirical data on both sex roles and mating patterns for each species of syngnathid included in our phylogenetic analyses (Table 1) were mapped onto our molecular phylogeny using the accelerated transformation (ACCTRAN) feature in MacClade Ver. 3.08a (Maddison and Maddison 1992) in order to determine the most parsimonious character states for each of taxa for which data were unavailable. To test for monophyly of sex roles in the Syngnathidae, the ML tree was recalculated, constraining sex-role reversed, non-role reversed, and outgroup species (Table 1) to be monophyletic groups. This topology was compared with that of the unconstrained ML tree using a SH test (Shimodaira and Hasegawa 1999).

A likelihood-ratio test incorporating branch lengths implemented by DISCRETE Ver. 4.0 (Pagel 1994a) tested the correlation between brood pouch development and sex-role reversal in the family Syngnathidae independently for the Urophori and Gastrophori. Six- and four-state pouch variables were collapsed into a series of binary traits for this comparison as suggested by Pagel (1994b). Sex roles were characterized as conventional (i.e., predominantly male-male competition for mates) or reversed (i.e., predominant female-female competition for mates). Mating pattern was classified as either monogamous (socially and genetically) or polygamous (including both polygamy, in which both males and females mate multiple times for a single brood or clutch; and polyandry, in which only females mate multiple times for a single breeding event.). A comparison of the log-likelihood of an independent model of trait evolution (four parameters) to that of a dependent model of trait evolution (eight parameters) tested the correlation between brood pouch complexity, sex-role reversal, and mating patterns within the Syngnath-





idae. Significance values for phylogenetic tests for correlation (nine tests) were adjusted following sequential Bonferroni correction (Rice 1989).

## RESULTS

### *Molecular Phylogeny of Syngnathid Fishes*

Mitochondrial sequences of 12S rDNA, 16S rDNA, and cytochrome *b* were collected, collated and aligned for 48, 45, and 40 specimens, respectively, resulting in a total sequence length of up to 1985 bp per specimen. Analyses of cytochrome *b* sequence data revealed third codon saturation of transitions for Kimura-2-parameter distances greater than 0.20 (data not shown). A likelihood-based congruency test did not reject the congruency of the dataset ( $P = 0.352$  for overall ML topology). Subsequent analyses were based on up to 1603 bp of sequence data for each individual.

Concatenated DNA sequences analyzed with NJ distance, MP and Bayesian phylogenetic analyses resulted in identical topologies for major groups of syngnathid fishes (Fig. 1). All three analyses support monophyly of the Urophori (tail brooding) and Gastrophori (abdominal brooding) lineages of syngnathid fishes. While phylogenetic resolution was low at the base of the Urophori, monophyly of *Solegnathus* and *Phyllopteryx* (single egg membrane compartments type A2), *Stigmatopora* and *Corythoichthys* (semi-inverted pouch type A4s), *Syngnathus* (inverted pouch type A4i), and *Hippocampus* (completely enclosed pouch type A5) were all supported by our multigene analyses (Fig. 1).

A ML model incorporating lineage-specific rates of sequence divergence fit the sequence data significantly better than a model constrained to fit a molecular clock (free model  $-\ln L = -15448.35$ ; constrained model  $-\ln L = -15585.63$ ;  $\chi^2 = 137.28$ ;  $df = 46$ ;  $P < 0.001$ ). Subsequent analyses did not enforce a molecular clock constraint.

A Shimodaira-Hasegawa test supported the monophyly of the Gastrophori and Urophori ( $P < 0.05$  for all topologies tested), but polytomic relationships among well-supported internal groupings within the Urophori could not be rejected as significantly different from the ML topology (Table 2). Although variation in pouch type complexity is evident in a group of syngnathid pipefishes endemic to Australia (A4e and A4s pouch types in *Urocampus/Vanacampus* clade; Fig. 1), specific brooding structures are generally restricted to the monophyletic groups outlined above (see Fig. 1).

### *Reconstruction of Brooding Structure Evolution*

The evolution of brooding structures within the Gastrophori and Urophori was reconstructed using both MP and

ML methods. Parsimony reconstruction suggests six changes in brooding structures within the Urophori and three changes within the Gastrophori (Fig. 2). ML reconstructions using a single forward-backward transition rate did not significantly differ from those with independent transition parameters (Urophori: free model  $-\ln L = -19.2988$ ; constrained model  $-\ln L = -24.0225$ ;  $\chi^2 = 9.4474$ ;  $df = 28$ ;  $P = 1.000$ ; Gastrophori: free model  $-\ln L = -5.7829$ ; constrained model  $-\ln L = -7.5201$ ;  $\chi^2 = 3.4744$ ;  $df = 10$ ;  $P = 0.968$ ). Subsequent analyses were conducted using the two-parameter forward-backward transition model.

Ancestral character states, incorporating branch length information, were estimated using Multi-state (Pagel 1999a). Maximum-likelihood analyses could not reject a directional mode of evolution of brooding structures in either the Urophori or Gastrophori (Fig. 2). Although a punctuated model of brood structure evolution could not be rejected for the Gastrophori ( $\chi^2 = 2.41$ ,  $P = 0.120$ ), brood structure evolution within the Urophori approximates a gradual model of evolution ( $\chi^2 = 7.97$ ,  $P = 0.005$ ; scaling parameter = 3.49).

### *Phylogenetic Reconstruction of Sex Role and Mating Pattern Evolution*

To test the predicted positive association between male parental care (as measured by brood structure complexity) and sex-role reversal, we used parsimony and ML approaches. Mapping sex roles on the pruned ML tree suggests at least four changes in sex roles within the Syngnathidae (Fig. 3a). The log-likelihood estimate of the topology estimated without the constraint of sex-role monophyly is significantly better than that estimated with sex-role-reversed and nonrole-reversed species constrained as monophyletic lineages (Table 2; SH-test ln-likelihoods: unconstrained  $-14667.84$  vs. constrained  $-14867.35$ ;  $\Delta = 199.51$ ; significant at  $P < 0.001$ ).

A likelihood-ratio test (LRT) failed to support a relationship between sex roles and male pouch development within either the abdominal or tail-brooding lineages (Table 3;  $P > 0.05$  for all comparisons after Bonferroni correction). Although our phylogeny indicates that male brooding structures have been highly conserved over the evolutionary history of the group (Fig. 1), sex roles appear to have reversed multiple times, independent of pouch type variation within the family (Fig. 3a).

Parsimony reconstruction of mating pattern evolution on the ML tree indicates that four changes in patterns of mating have occurred within the Syngnathidae (Fig. 3b). We tested for a correlation between sex-role reversal and mating patterns in syngnathid fishes. We performed a likelihood-ratio test, mapping ecological data on sex roles and mating patterns

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FIG. 1. Consensus phylogenetic tree constructed from neighbor-joining distance (Saitou and Nei 1987), maximum-parsimony (Swofford and Olse 1990) and Bayesian phylogenetic analyses (Huelsenbeck and Ronquist 2001) based on the combined dataset of 1603 bp with branch lengths as estimated from maximum-likelihood analysis. Numbers on branches represent bootstrap values from distance and parsimony analyses and consensus support for Bayesian analysis. Archived samples (S accession numbers listed after species names) and data on specimen collections are housed at the Evolutionary Biology Centre (Uppsala Univ.). For descriptions of pouch types, see text and Table 1. *Doryrhamphus* and *Dunckerocampus* are recognized here as separate genera as suggested by Kuitert (1998). Photographs reprinted with permission from Kuitert (2001), top to bottom: *Hippocampus bleekeri* (Rudie Kuitert); *Syngnathus acus* (Charles Hood); *Phyllopteryx taeniolatus*, *Stigmatopora nigra* (Rudie Kuitert); *Corythoichthys* spp. (Rudie Kuitert); *Doryrhamphus excisus* (Phil Woodhead); *Gasterosteus aculeatus* (Rudie Kuitert); *Aulichthys japonicus* (Tomonori Hirata).

TABLE 2. Shimodaira-Hasegawa test of alternate topologies (Shimodaira and Hasegawa 1999). Maximum-likelihood tree, log-likelihood differences, and  $P$ -values for alternative topologies tested.  $L_{\max}$ , maximum likelihood;  $L_{\alpha}$ , likelihood of tree  $\alpha$ . Significantly different topology at \* $P < 0.05$ , \*\* $P < 0.001$ . Groupings tested (pouch type; for legend see Table 1): 1, *Hippocampus* spp. (A5); 2, *Syngnathus* spp. (A4i); 3, Australian endemics (A4e, A4s); 4, *Solegnathus/Phyllopteryx* (A2); 5, *Corythoichthys* (A4s); 6, *Stigmatopora* (A4s); 7, *Oostethus/Doryrhamphus/Dunckerocampus* (B2, B3); 8, *Nerophis/Entelurus* (B1); 9, outgroup.

$\alpha$	$L_{\max} - L_{\alpha}$	$P$	Topology
1	0.0		(((1,2), (3, 4)), (5, 6)), (7, 8)), 9
2	1.8	0.748	((( <i>Hippichthys</i> , 1, 2), (3, 4)), (5, 6)), (7, 8)), 9
3	3.1	0.731	(((1, 2), (3, 4), (5, 6)), (7, 8)), 9
4	21.1	0.066	(((1, 2, (3, 4)), (5, 6)), (7, 8)), 9
5	26.3	0.024*	(((1, 2, (3, 4), (5, 6)), (7, 8)), 9
6	27.9	0.024*	(((1, 2), (7, 8)), (5, 6)), (3, 4)), 9
7	28.3	0.021*	(((1, 2), (7, 8)), (3, 4)), (5, 6)), 9
8	28.3	0.021*	((1, 2), (3, 4), (5, 6), (7, 8)), 9
9 (ML pruned tree)	0.0		(((1, 2), (3, 4)), (5, 6)), (7, 8)), 9
10 (Sex-role reversal monophyly)	199.51	0.000**	((conventional, sex-role reversed), outgroup)

collected from the literature and reconstructed from the parsimony analysis onto our ML phylogeny (Fig. 3b). Results were significant (LRT:  $\chi^2 = 19.3234$ ,  $df = 4$ ,  $P = 0.0007$ ), demonstrating a strong positive association between sex-roles and mating patterns, in which monogamous species have conventional patterns of mating and polygamous species are generally sex-role reversed.

#### DISCUSSION

Our molecular data demonstrate an extensive morphological divergence in lineages with diverse and highly specialized adaptations for paternal care. In syngnathids, the evolution of increased pouch development has occurred in parallel in both abdominal and tail-brooding lineages, leading to the remarkable diversity of male brooding structures observed today. At the same time, multiple evolution of sex-role reversal within the family Syngnathidae suggests that pouch type variation may not directly reflect relative parental investment, or that the relationship between pouch type and the sexual difference in potential reproductive rates (and thereby the operational sex ratio) is more dynamic rather than being a direct consequence of their brooding structure. In contrast, the strong association between sex roles and mating patterns in syngnathid species highlights the relationship between polygamous mating, in which multiple mating may be more frequent among females than males, and sex-role reversal.

#### *Mitochondrial Phylogeny Supports Parallel Evolution of Major Pouch Lineages*

In accordance with the assumed evolutionary significance of male brooding structures, phylogenetic analyses indicate that distinct pouch types each generally represent monophyletic lineages of species. Our total molecular evidence dataset indicates that most major pouch types arose once early in the evolution of syngnathid fishes, and punctuated evolution of brooding structures in abdominal brooding pipefishes suggests that morphological radiation may have occurred in a relatively short burst of morphological change in this group (Fig. 1). Our molecular data also support Herald's (1959) classification of these fishes into tail- and abdominal brooders, demonstrating the independent radiations of morpholog-

ical structures in the Urophori (A-type: tail pouch) and Gastrophori (B-type: abdominal pouch). Our phylogeny suggests that the primary split between these two lineages occurred at the same time or shortly before the major morphological radiation of male brooding structures and subsequent radiation of species (Wilson et al. 2001). The early diversification of the ancestral syngnathid into tail and abdominal brooders is consistent with results from a karyotypic study, which highlights a possible total-genome duplication in the abdominal-brooding lineage (Vitturi et al. 1998). Brooding structures within these two lineages independently increased in complexity, culminating in the completely enclosed brood pouches located on the tail of seahorses (Herald 1959; type A5) and the well-defined abdominal pouch of *Oostethus brachyurus* (type B3), the most complex abdominal pouch type.

#### *Repeated Shifts in Sex Roles*

Detailed behavioral and ecological studies of a subset of syngnathid species have revealed substantial variation in patterns of sex roles in the family (Vincent et al. 1992). The lack of association between sex roles and pouch type variation demonstrated in our study suggests that the relationship between parental care and sex roles in these fishes may not be as straightforward as predicted. Our conclusions are based on the assumption that pouch type variation accurately reflects relative parental investment. Although it is likely that paternal energy expenditures are positively correlated with increasing pouch complexity (Berglund et al. 1986; Mason-jones 2001), the assessment of true parental investment (sensu Trivers 1972) may be more complex, involving the ultimate fitness costs of both energy and time expenditures in both sexes. Independent of our assumptions, however, it is clear that it is not possible to map sex roles on the consensus phylogeny of syngnathids without allowing multiple appearances of role reversal (Fig. 3a; see results of SH test above).

The lack of correlation between sex roles and brooding structures in our study may be partly explained by results from several recent studies, which demonstrate that sex roles and intensity of competition for mates can be predicted by the differences in potential reproductive rates between the sexes, which are influenced by environmental factors such as

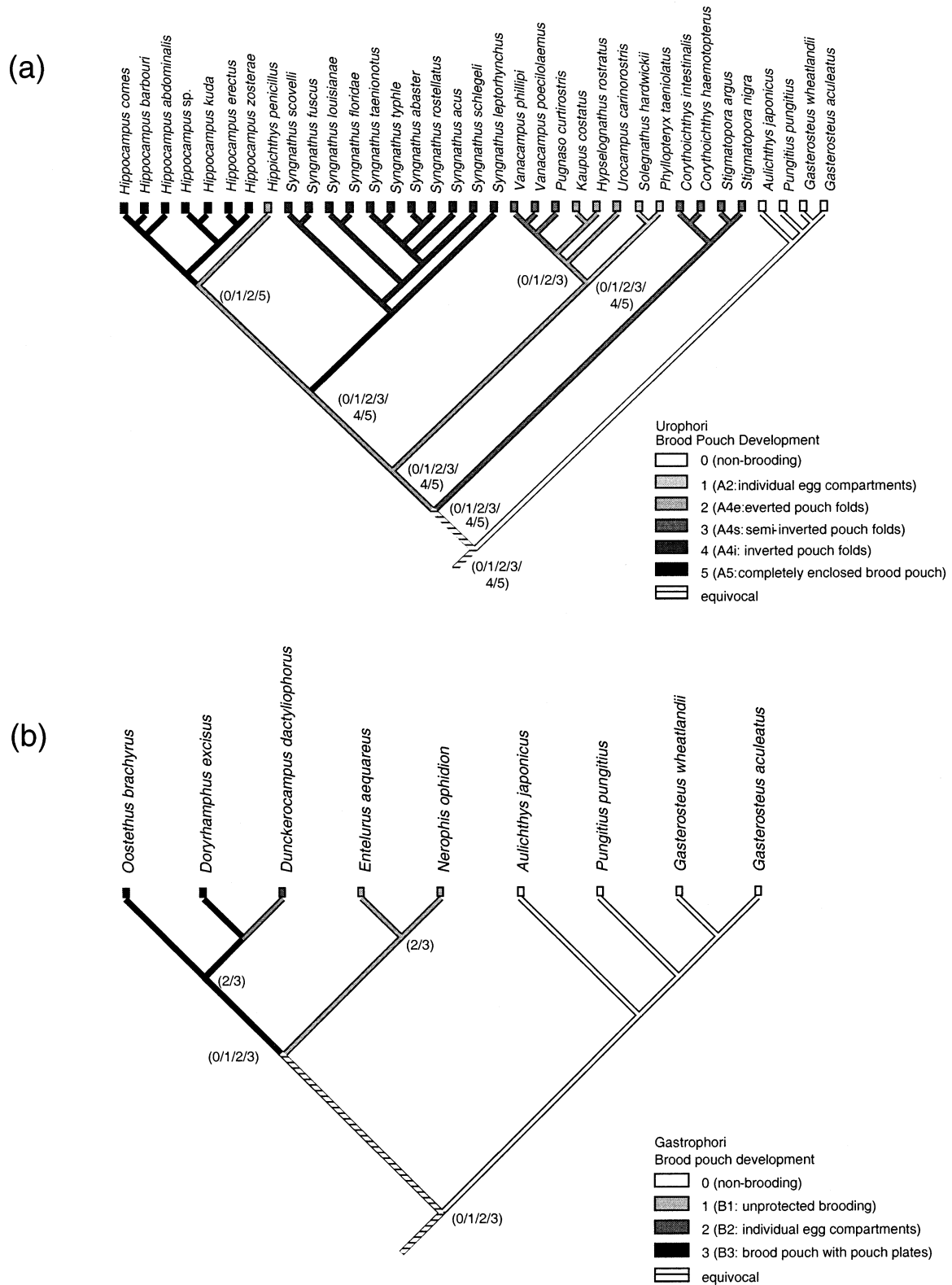


FIG 2. Most parsimonious reconstruction of the evolution of brooding structures in the (a) Urophori and (b) Gastrophori mapped on the pruned maximum-likelihood phylogenetic trees for each group using MacClade Ver. 3.08a (Maddison and Maddison 1992). Maximum-likelihood ancestral character states were also estimated at each node using Multi-state Ver. 0.6 (Pagel 1999a). Possible character states at nodes in which more than a single ancestral state is equally likely are indicated in parentheses.



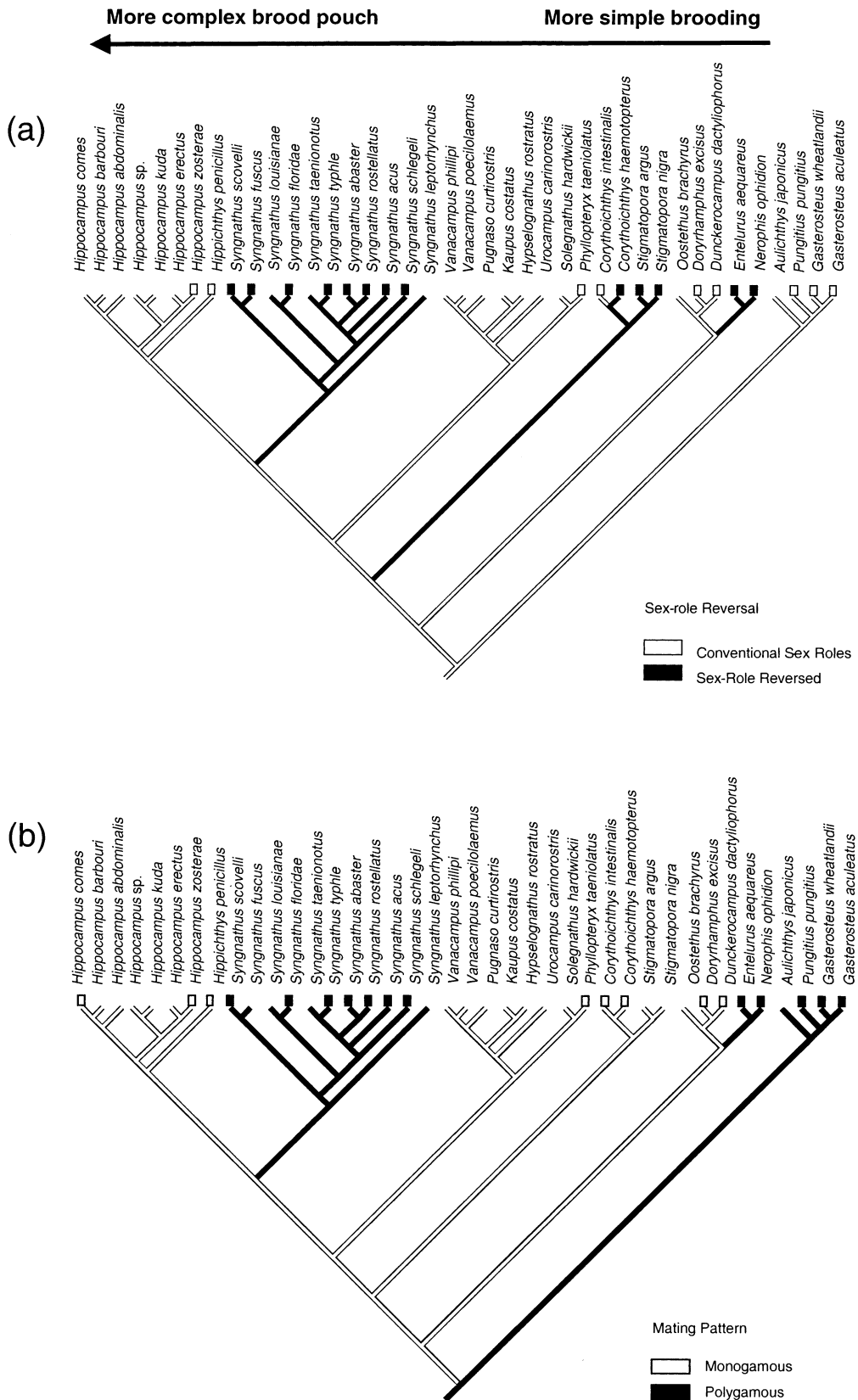


FIG. 3. Most parsimonious reconstruction of (a) sex-role reversal and (b) mating pattern mapped on the consensus phylogenetic tree (Fig. 1) using MacClade Ver. 3.08a (Maddison and Maddison 1992) with empirical data coded as boxes where available. Character states

temperature and food availability (Kvarnemo and Ahnesjö 1996, 2002; Ahnesjö et al. 2001). In sand gobies (*Pomatoschistus minutus*; Kvarnemo 1994), temperature influences sexual differences in the potential reproductive rate, causing variation in the intensity of mating competition. Mating competition in the sex-role reversed pipefish *Syngnathus typhle* is also strongly influenced by fluctuations in temperature (Ahnesjö 1995). During a warm breeding season, males have shorter pregnancies and are available more frequently for mating than under colder ambient water temperatures (Vincent et al. 1994). Even though females always compete for access to mates in *Syngnathus typhle*, they will compete even more intensely when temperatures are reduced, due to a more female-biased operational sex ratio caused by the extended period of male pregnancy (Vincent et al. 1994; Ahnesjö 1995).

Sex roles may also shift depending on food availability as demonstrated in the bush cricket (Gwynne and Simmons 1990; Simmons 1992). While the energetic intake and reproductive investment of female bush crickets on a limited diet was significantly lower than that for those allowed unlimited access to food, no significant difference in either energetic intake or reproductive investment could be detected in males raised under same the two food regimes (Gwynne and Simmons 1990; Simmons 1992). As a result, male parental investment in bush crickets became even more important when food resources were scarce and female competition for mates was intensified under these conditions (Gwynne and Simmons 1990; Simmons 1992).

Given that sex roles can shift and the intensity of mating competition can vary in relation to environmental factors both within and between species, one might expect variation in these characters independent of phylogenetic relationships. However, in the Syngnathidae, although a strict correlation between sex-role reversal and pouch development is rejected, there is, at the same time, an intriguing consistency in the similarity of sex roles and pouch type at higher taxonomic levels (e.g., within the genera *Syngnathus* and *Hippocampus*; Table 1; Figs. 1, 3a).

#### *Correlated Evolution of Sex Roles and Mating Patterns*

Although male pouch development does not predict the presence of sex-role reversal in the Syngnathidae, analysis of our data highlights a strong correlation between sex-role reversal and mating patterns in syngnathid species, as suggested by Vincent et al. (1992). Syngnathids exhibiting sex-role reversal are generally polygamous, whereas those species with nonreversed patterns of mating competition are monogamous. Among seahorses (*Hippocampus* spp.), the monogamous mating pattern is well documented both socially (Vincent and Sadler 1995; Kvarnemo et al. 2000; Masonjones and Lewis 2000; Perante et al. 2002) and genetically (Jones and

Awise 2001) and social monogamy has been confirmed among *Corythoichthys* spp. pipefishes (Gronell 1984; Matsumoto and Yanagisawa 2001). In both of these genera, the female transfers a full clutch to her mate, either into the male's brooding pouch (seahorses) or as a sheet of eggs onto the brooding structure of *Corythoichthys* spp.

Polygamy occurs in the genus *Syngnathus*, where both males and females mate multiple times during a single breeding period. As a result, males brood eggs from several females concurrently (Berglund et al. 1989; Vincent et al. 1995; Jones and Awise 2001). In contrast, in species with more simple brooding structures, males receive their entire brood from a female during a single mating (e.g., *Nerophis* and *Entelurus*) but females are capable of mating with additional males well before the previous male has finished brooding the clutch (i.e., polyandry; Berglund et al. 1989; Vincent et al. 1995).

The pattern of monogamous species showing conventional sex roles has, so far, only a single exception. The pipefish *Corythoichthys haematopterus* clearly mates monogamously, but in a wild population where the operational sex ratio was consistently skewed toward an excess of females, behavioral observations indicated female-female competition (Matsumoto and Yanagisawa 2001).

The association between sex roles and mating patterns is intriguing and its causes, constraints, and consequences warrant further attention. One may, however, speculate on ecological differences associated with the pattern. Several of the polygamous and sex-role reversed species live in eelgrass habitats and tend to occur at relatively high densities, have no home ranges, and are comparatively mobile (e.g., *Syngnathus typhle*, *S. rostellatus*, *S. scovelli* and *Nerophis ophidion*; Vincent et al. 1995; Jones and Awise 1997). In contrast, monogamous species with a conventional mating pattern tend to occur at lower densities, have reduced mobility, and fixed home ranges (i.e., *Corythoichthys* spp., *Hippocampus* spp.; Gronell 1984; Vincent and Sadler 1995; Kvarnemo et al. 2000; Perante et al. 2002). These differences in population densities may influence opportunities for remating. A similar ecological association has been found in polyandrous birds, where species with male-only care are generally found at lower densities compared to species with female-only care (Owens 2002).

Although a skewed operational sex ratio or differences in quality of potential mates may create competition for mates in monogamous species, considerable mating competition in polygamous animals generally results in more extensive sexual dimorphisms as compared to monogamous species (Andersson 1994). When a polygamous mating pattern results in more frequent opportunities for female multiple mating (i.e., polyandry), intensified mating competition among females may lead to a reversal of conventional sex roles. This close correlation between sex-role reversal and polyandry has also

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for *Doryrhamphus excisus*, *Dunckerocampus dactyliophorus*, and *Phyllopteryx taeniolatus* derived from closely related taxa. Character states for outgroups from references in McLennan et al. (1988). The most parsimonious reconstruction of these characters on this phylogeny involves multiple independent origins of sex roles (four steps) and mating patterns (three steps) within the Syngnathidae. Sex roles are uncorrelated with male brood pouch development (LRT:  $P > 0.05$  for all comparisons; Table 3). In contrast, there is a strong correlation between sex roles and mating pattern in the Syngnathidae (LRT:  $P < 0.001$ ).

TABLE 3. Comparison of independent versus dependent (correlated evolution) maximum-likelihood models (Pagel 1994a) of the evolution of brooding structures and sex roles within the Urophori and Gastrophori. Brooding structures were sequentially dichotomized to facilitate testing with DISCRETE Ver. 4.0 (Pagel 1994a). Significance of these comparisons was tested with a likelihood ratio test ( $df = 4$ ). For pouch type legend, see Table 1.

Comparison	Brooding structures		Independent	Dependent	Likelihood ratio ( <i>P</i> -value)
	Character 0	Character 1			
<b>Urophori</b>					
1	no pouch (outgroup)	A2/A4e/A4s/A4i/A5	-11.0088	-10.1906	1.6364 (0.802)
2	no pouch/A2	A4e/A4s/A4i/A5	-14.9650	-13.6909	2.5482 (0.636)
3	no pouch/A2/A4e	A4s/A4i/A5	-21.2781	-17.5515	7.4532 (0.114)
4	no pouch/A2/A4e/A4s	A4i/A5	-16.4685	-15.1471	2.6428 (0.619)
5	no pouch/A2/A4e/A4s/A4i	A5	-13.8033	-12.9921	1.6224 (0.805)
<b>Gastrophori</b>					
1	no pouch (outgroup)	B1/B2/B3	-4.5257	-3.5041	2.0432 (0.728)
2	no pouch/B1	B2/B3	-5.8226	-4.6460	2.3532 (0.671)
3	B1/B2	B3	-6.1092	-5.0823	2.0538 (0.726)

been identified in several species of the avian orders Gruiformes and Charadriiformes (Emlen and Oring 1977), highlighting the potential significance of mating patterns on sex roles in natural populations.

In reconstructing complex ecological traits, there is a possibility of introducing systematic error due either to biased taxonomic sampling and/or lack of data for taxa included in the study. Although our molecular phylogenetic study includes the widest taxonomic sampling of syngnathid species presented to date, and syngnathid mating behaviors are arguably some of the best studied, ongoing ecological studies on syngnathid fishes continue to be critical to further explore the findings presented here. The low level of statistical power in such reconstructions also decreases the possibility to detect significant correlations between ecological variables, adding additional confidence to the significant correlation observed between sex-role reversal and polygamy, but suggesting that the identification of more frequent transitions of ecological characters on the syngnathid phylogeny could potentially lead to significant correlations between pouch development and sex-role reversal. Additional behavioral study of syngnathid fishes and a more highly resolved molecular phylogeny of the family Syngnathidae will clearly help to address these issues.

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