Male Pregnancy in Seahorses and Pipefishes (Family Syngnathidae): Rapid Diversification of Paternal Brood Pouch Morphology Inferred From a Molecular Phylogeny

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In contrast to the majority of vertebrate species, primary male parental care is common in fishes and encompasses a remarkable diversity of adaptations. Seahorses and pipefishes (Family Syngnathidae) exhibit some of the most specialized forms of paternal care in animals and so are ideally suited to the study of the evolution of male parental care. During mating, female syngnathids transfer eggs to specialized morphological structures that are located on either the abdomen or tail of the male. The male provides all postfertilization parental care and has morphological and physiological adaptations to osmoregulate, aerate, and even nourish the developing embryos. While all syngnathid species are adapted for paternal care, the brooding structure with which this is accomplished varies between species, from simple ventral gluing areas to much more complex structures such as the completely enclosed pouches of the seahorses. Our combined cytochrome *b*-, 12S rDNA-, and 16S rDNA-based molecular phylogeny of syngnathid fishes demonstrates that rapid diversification of male brooding structures has been associated with the major evolutionary radiation of the group, suggesting that development and diversification of structures involved in paternal care may have been key evolutionary innovations of the Syngnathidae. Molecular analyses also highlight geographical centers of biodiversity and suggest interoceanic migration of *Syngnathus* pipefishes from their center of origin in the Pacific.

Evolutionary theory predicts that organisms should attempt to maximize reproductive success by monopolizing resources and mates and optimizing costs and benefits of parental care (Andersson 1994; Clutton-Brock 1991; Darwin 1871; Emlen and Oring 1977). Female parental care far exceeds that of males in many vertebrates, but this pattern is reversed in fishes where, in addition to gametic investment, males often provide the majority of parental care (Blumer 1982). Fish are exceptional in their wide variety of parental care behaviors (Baylis 1981), and have been instrumental in increasing our understanding of the evolutionary origins of parental care (Baylis 1981; Gross and Sargent 1985).

The order Gasterosteiformes includes fishes with a remarkable diversity of reproductive behaviors (Breder and Rosen 1966; Clutton-Brock and Vincent 1991). The family Syngnathidae (pipefishes and seahorses) are characterized by especially pronounced adaptations for male parental care, with the female depositing eggs directly to a specialized incubation area or brood pouch on either the tail (type A: subfamily Urophori) or the trunk (type B: subfamily Gastrophori) of the male (Herald 1959). This key morphological innovation ensures a male complete confidence in the paternity of its offspring (Jones and Avise 1997; Jones et al. 1999), but at a cost of paternal care that exceeds that of most other vertebrates (Clutton-Brock and Vincent 1991).

Primary taxonomic groupings within the family Syngnathidae reflect the location and development of the male brood pouch (Duncker 1915; Herald 1959) (Figure 1): type B1—eggs are loosely attached to the ventral side of the male and are completely unprotected by a brood pouch (*Entelurus, Nerophis*); types A2 and B2—eggs are placed into individual membranous egg compartments (*Solegnathus, Doryrhamphus*); type B3—eggs are incubated in a well-defined pouch and protected by pouch plates (ventral extensions of the lateral plates of the trunk or tail rings) (*Oostethus*); type A4—eggs are placed into a well-defined pouch, with fleshy bilateral pouch folds that meet on the ventral midline of the pouch and partially or fully enclose the eggs (*Syngnathus*); type A5—eggs are incubated in a completely enclosed saclike fleshy pouch, which opens through an anteromesial slit or pore (*Hippocampus*). Al-

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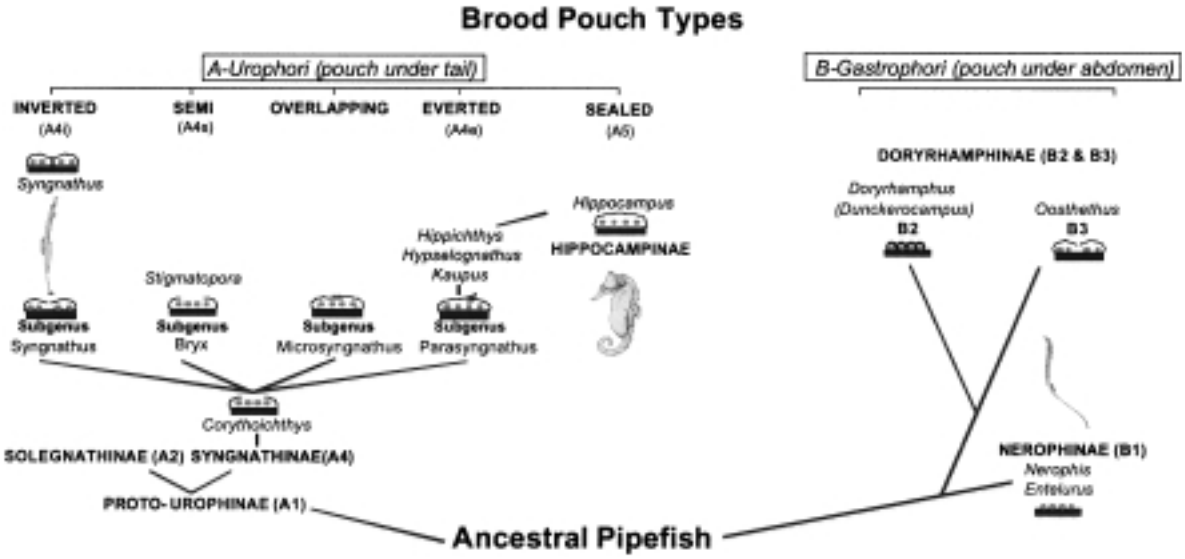

Figure 1. Hypothetical evolutionary development of the syngnathid brood pouch (Herald 1959), detailing independent radiations of Urophori and Gastrophori and the diversification of pouch types. For descriptions of pouch type variation, see text. Generic names (in italics) represent taxonomic sampling of the present study. Shown are schematic cross sections through the male brood pouch (Herald 1959), showing general pouch design and the extent of pouch enclosure.

though neither type A1 or A3 pouch types are known from the fossil record, the subfamilies of both the Gastrophori and Urophori are hypothesized to have evolved through successive development of the brood pouch (Herald 1959; Figure 1).

The extreme degree of specialization for paternal care in the Syngnathidae is accompanied by a notable increase in species-level diversity over that of closely related groups. The Syngnathidae are by far the most diverse family in the order Gasterosteiformes, with approximately 230 described species (Dawson 1985), while their close relative, the stickleback family, is comprised of only 7 species (Wootton 1984). However, whereas most species of sticklebacks have a circumpolar distribution concentrated in the northern hemisphere (Wootton 1984), the highest diversity of the syngnathids is concentrated in a relatively small region of the southwest Pacific (Dawson 1985). As the Gasterosteidae are believed to be closely related to the Syngnathidae (Bowne 1984), this striking difference in both species-level diversity and geographic distribution is particularly notable. Evidently lineage-specific factors have been responsible for the clear differences in patterns of speciation observed between these two groups.

Molecular methods have proven useful in delineating the relative significance of intrinsic and extrinsic isolating factors in the speciation process (Barraclough et al. 1998; Howard and Berlocher 1998; Lynch 1989). Molecular markers, and mitochondrial DNA in particular, have also yielded profound insights into the distribution and evolution of a broad array of animal taxa (Avise 2000; Avise et al. 1987) and have given us a better understanding of both the approximate timing and relative rates of diversification in many fish species (Bermingham et al. 1997; Bernatchez and Wilson 1998; Meyer 1993; Meyer et al. 1990). In the present study we use fragments of mitochondrial 12S rDNA and 16S rDNA genes and the complete cytochrome *b* mitochondrial gene to clarify syngnathid phylogeography and investigate the evolution of morphological specializations for paternal care in the Syngnathidae.

Previous morphology-based taxonomic revisions of the family have stressed the importance of the male brood pouch in the syngnathid radiation and have made male reproductive biology a key taxonomic character in defining the group (Dawson 1985; Duncker 1915; Herald 1959). Our molecular investigation investigates this assumption with a suite of three mitochondrial gene fragments. Strong congruency between Herald's (1959) proposed model of paternal care evolution and the present molecular phylogenetic study would provide support for the significance of brood pouch diversification on the evolution of these fishes. Alternatively, conflicts between Herald's morphology-based model and our molecular-based phylogeny might indicate that alternative factors have been responsible for the radiation of the Syngnathidae.

Materials and Methods

Samples

Forty-four samples, including representatives of 34 species, were collected at sites across the entire geographic range of the Syngnathidae (Table 1, Figure 2). Archived syngnathid samples are housed at the Evolutionary Biology Center (Uppsala, Sweden). Specimens used as outgroups (collection locality) were three members of the stickleback family Gasterosteidae [threespine stickleback (*Gasterosteus aculeatus*), New York; blackspotted stickleback (*G. wheatlandii*), Rhode Island; and ninespine stickleback (*Pungitius pungitius*), Scotland], and the Japanese tubenose (*Aulichthys japonicus*) (Kanagawa, Japan), a member of the family Aulorhynchidae.

DNA Extraction/mtDNA Sequencing

Specimens were preserved in 70% ethanol and total genomic DNA was extracted from white muscle or liver tissue by proteinase K/SDS digestion and purified by phenol-chloroform extraction and ethanol precipitation (Kocher et al. 1989). Fragments of 12S rDNA and 16S rDNA genes and the complete cytochrome *b* gene were polymerase chain reaction (PCR) amplified with primers under previously published reaction conditions (Table 2). Approximately 0.2 μg of QIAquick (Qiagen) PCR Purification Kit-purified product from this PCR reaction was cycle-sequenced in both forward and reverse directions with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (V1.0) in 10 μl volumes following manufacturer's instructions (Applied Biosystems), with 5 pmol primer and 2 μl Terminator Ready Reaction Mix. The cycling profile for the sequencing reaction consisted of 25 cycles of 96°C for 10 s, 45°C for 5 s, and 60°C for

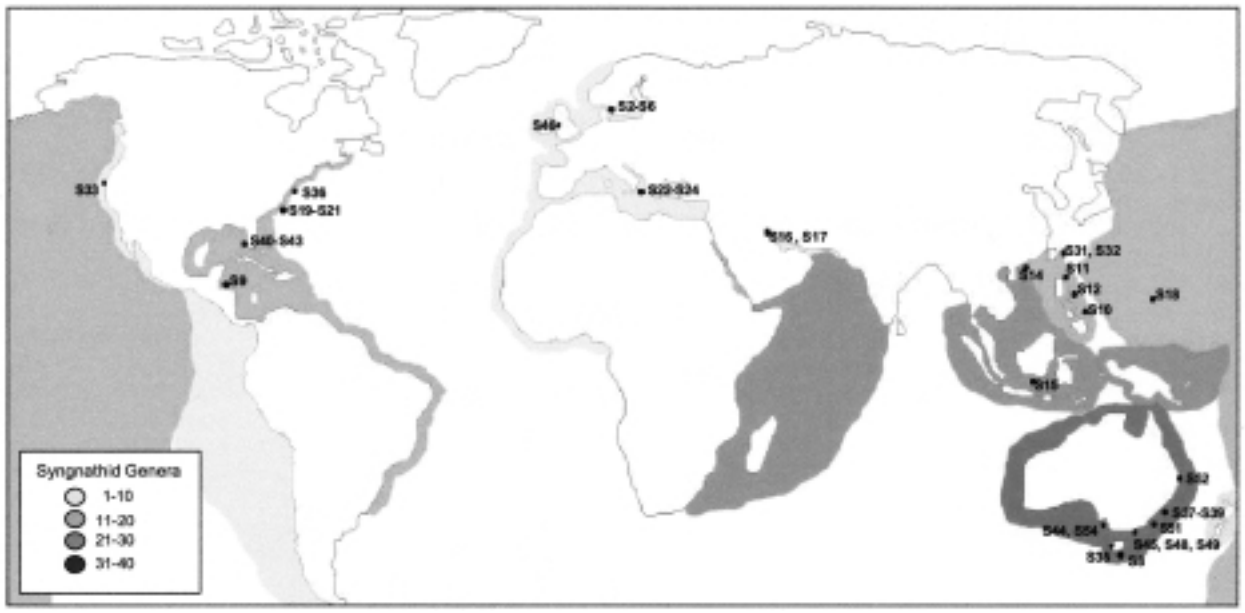

Figure 2. World map detailing seahorse and pipefish sample collection localities and the global distribution of syngnathid genera (Dawson 1985). See Table 2 for species identification and collection information.

4 min. Ethanol/sodium acetate-purified cycle sequencing products were analyzed on an ABI 377 Automated Sequencer (Applied Biosystems). Sequences have been deposited in GenBank (see Table 1 for accession numbers).

Phylogenetic Analysis of Sequence Data

The orthologous DNA sequences obtained were aligned, using default settings, by CLUSTALW (Thompson et al. 1994) and optimized by eye. Preliminary sequence analysis by DAMBE version 4.0.17 (Xia 2000) was used to investigate the transition : transversion ratio as a measure of sequence saturation. PAUP version 4b3b (Swofford 2000) and MODELTEST version 3.0 (Posada and Crandall 1998) were used to estimate rate heterogeneity (as estimated by the gamma parameter Γ) and to determine phylogenetic models appropriate for both single gene and combined analyses.

Preliminary sequence analyses indicated that all three gene fragments best fit an HKY model of substitution (Hasegawa et al. 1985) (Figure 3). Neighbor-joining distance (100 bootstrap replicates) and maximum parsimony analyses were performed with PAUP version 4b3b (Swofford 2000), with indels coded as missing data. Parsimony minimal trees were determined with full heuristic searches (100 bootstrap replicates) using random addition (10 replicates), the TBR branch-swapping algorithm, and the MULPARS option. Quartet puzzling maximum-likelihood estimates (10,000 puzzling steps) were calculated by TREE-PUZZLE version 4.02 (Strimmer and von Haesler 1999) with transition:transversion ratio and nucleotide frequencies estimated from the dataset.

Results

Preliminary sequence analyses were conducted on aligned 12S rDNA (339 bp) and 16S rDNA (497 bp) gene fragments and the complete cytochrome *b* gene (1149 bp) to investigate base composition bias, distribution of sequence variation, and possible saturation of transitions and transversions. While frequencies of bases were approximately equal for 12S rDNA and 16S rDNA gene fragments, cytochrome *b* exhibited significantly lower frequencies of glycine ($P < .0001$), a widespread pattern among vertebrate taxa (Johns and Avise 1998), and fishes in particular (Meyer 1993). While significant rate heterogeneity was detected for all three genes (Table 3), no taxon-specific base compositional biases were detected for any of the three gene fragments (chi-squared test: $P > .1$ for all genes). Although 12S and 16S did not exhibit saturation of transitions, cytochrome *b* gene transitions at the third codon positions were saturated (Figure 3). Third codon positions of cytochrome *b* were removed from subsequent phylogenetic analyses.

Single-Gene Analyses

While sequence data for all three genes were collected for the majority of specimens, a subset of species failed to amplify at one or more gene fragments due to the poor quality of the collected material. In an effort to include as broad an array of taxa as possible and to identify possible differences in rates of molecular evolution across different genes, we conducted separate neighbor-joining, maximum parsimony, and maximum-likelihood analyses on each of the three mitochondrial gene fragments.

All three gene fragments provide good within-pouch-type resolution and indicate that the initial origin of pouch types occurred extremely rapidly, as evidenced by the low level of resolution of basal relationships between pouch types (Figure 4). High levels of resolution both above and below this point on the phylogeny (Figure 4) strongly suggest that the polytomy inferred at all three gene fragments reflects an increased rate of diversification during this time period. Judging from brooding structures of extant species, morphological diversification appears to have evolved early in the evolution of each major lineage and has been strongly conserved over subsequent within-lineage evolution (but see A4e/s pouch-type transitions discussed below; Figure 4). In addition to this extremely rapid diversification of pouch varieties, all single-gene analyses indicate a clear split between the Urophori (pouch under tail) and Gastro-

Table 1. Syngnathid specimens included in this study

Species (ID#)	Pouch	Collection locality	Collector
Urophori (type A: tail pouch)			
Solegnathus hardwickii (*S52*)	A2	Australia	C. Linaker
Syngnathus abaster (S23)	A4i	West Sicily, Italy	P. Franzoi
S. acus (S2)	A4i	Sweden	I. Ahnesjö
S. acus (S46)	A4i	North Wales, Britain	C. Linaker
S. floridae (S21)	A4i	Virginia, USA	R. L. Teixeira
S. floridae (S41)	A4i	Florida, USA	R. Ruiz-Carus
S. fuscus (S19)	A4i	Virginia, USA	R. L. Teixeira
S. leptorhynchus (S33)	A4i	Humboldt, USA	R. Fritzsche
S. louisianae (S42)	A4i	Florida, USA	R. Ruiz-Carus
S. rostellatus (S3)	A4i	Sweden	I. Ahnesjö
S. schlegeli (S14)	A4i	Pearl River Estuary, China	F. Leung
S. scovelli (S40)	A4i	Florida, USA	R. Ruiz-Carus
S. typhle (S4)	A4i	Sweden	I. Ahnesjö
S. typhle (S22)	A4i	West Sicily, Italy	P. Franzoi
S. taenionotus (S24)	A4i	Po delta, Italy	P. Franzoi
Corythoichthys intestinalis (S15)	A4s	Ambon, Indonesia	A. Vincent
C. intestinalis (S18)	A4s	Tumon Bay, Guam	C. Dayton
Stigmatopora argus (S8)	A4s	Dunalley Bay, Tasmania	A. Jordan
S. argus (S37)	A4s	Botany Bay, Australia	C. King
S. argus (S50)	A4s	Australia	C. Linaker
S. nigra (S38)	A4s	Botany Bay, Australia	C. King
S. nigra (S51)	A4s	Australia	C. Linaker
Urocampus carinirostris (S39)	A4s	Botany Bay, Australia	C. King
Vanacampus phillipi (S48)	A4s	Australia	C. Linaker
V. poecilolaemus (S45)	A4s	Australia	C. Linaker
Halicampus grayi (S29)	A4e	Vietnam	I. Ahnesjö
H. grayi (S30)	A4e	Vietnam	I. Ahnesjö
Hippichthys penicillus (S16)	A4e	Kuwait Bay, Kuwait	A. Vincent
Hypselognathus rostratus (S44)	A4e	Australia	C. Linaker
Kaupus costatus (S49)	A4e	Australia	C. Linaker
Trachyrhampus serratus (S54)	A4e	Australia	C. Linaker
Hippocampus abdominalis (S35)	A5	Hobart, Tasmania	A. Vincent
H. barbouri (S11)	A5	Philippines	A. Vincent
H. comes (S12)	A5	Philippines	A. Vincent
H. erectus (S20)	A5	Virginia, USA	R. L. Teixeira
H. kuda (S31)	A5	Taiwan	Fang
H. kuda (S32)	A5	Taiwan	Fang
H. sp. (S17)	A5	Kuwait Bay, Kuwait	A. Vincent
H. zosterae (S36)	A5	USA	H. Masonjones
H. zosterae (S43)	A5	Florida, USA	R. Ruiz-Carus
Gastrophori (type B: abdominal pouch)			
Entelurus aequareus (S6)	B1	Sweden	I. Ahnesjö
Nerophis ophidion (S5)	B1	Sweden	I. Ahnesjö
Doryrhamphus dactyliophorus (S10)	B2	Philippines	A. Vincent
Oostethus brachyrus (S9)	B3	Puerto Barrios, Guatemala	D. Reznick

See Figure 2 for geographic distribution of the family and the origin of individual samples (sample ID (S#) after species name). GenBank accession numbers AF354940–AF355033, AF356040–AF356081, AF356539.

Table 2. Polymerase chain reaction (PCR) primers for seahorse 12S rDNA, 16S rDNA, and cytochrome *b* mitochondrial genome fragments

Primer	Sequence	Reference
12S		
L1091	5′-AAACTGGGATTAGATACCCCACTA-3′	Kocher et al. 1989
H1478	5′-GAGGGTGACGGGCGGTGTGT-3′	Kocher et al. 1989
H2001	5′-AACCAGCTATCACCAGGCTCG-3′	
16S		
L2510	5′-CGCCTGTTTATCAAAAACAT-3′	Palumbi et al. 1991
H3058	5′-CCGGTCTGAACTCAGATCACGT-3′	Palumbi et al. 1991
Cytochrome *b*		
L14725	5′-CGAAGCTTGATATGAAAAACCATCGTTG-3′	Pääbo et al. 1991
L15162	5′-GCAAGCTTCTACCATGAGGACAAATATC-3′	Taberlet et al. 1992
H15240	5′-TTRTCTACNGARAANCCNCCTCA-3′	
H15915	5′-TCATCTCCGGTTTACAAGAC-3′	Irwin et al. 1991
H15926	5′-AAGGGKGGATTTTAACCTCCG-3′	This study

Primer names follow the convention of naming the primer by the most 3′ position of the primer in the human mtDNA sequence (Kocher et al. 1989).

phori (pouch under abdomen) (Figure 4), consistent with predictions from Herald's (1959) postulated evolution of syngnathid brooding structures and supporting the taxonomic division of the Syngnathidae into two separate subfamilies (Figure 1).

Maximum-likelihood phylogenetic analyses were conducted both with the assumption of constant rates of molecular evolution (i.e., molecular clock) and with rates of molecular evolution free to vary across lineages. In all three cases, the log-likelihood of the trees estimated without a molecular clock constraint was significantly greater than those assuming clocklike behavior (likelihood ratio test, $P < .001$), indicating that all three gene fragments exhibit significant rate heterogeneity in these fishes. However, the addition or removal of additional taxa did not affect the basic tree topology, which was consistent across all three gene fragments (Figure 4), demonstrating congruency of molecular data. The three gene fragments were therefore combined for further analyses.

Total Molecular Evidence

Neighbor-joining distance, maximum parsimony, and quartet puzzling maximum-likelihood phylogenetic analyses conducted on the combined dataset of 1602 bp supported the single-gene analyses (Figure 5). As expected (de Queiroz et al. 1995), while the tree topology of the combined analysis remained essentially identical to the analysis of individual gene fragments, the larger number of characters present in the combined dataset provided greater confidence of inference, as measured by bootstrap support for critical branches (Figure 5). Once again, the rapid diversification of the Syngnathidae is clear from the combined analysis, and this analysis clarifies the basal branching of the abdomen-brooding pipefishes (type B: Gastrophori) from tail-brooding lineages (type A: Urophori) (Figure 5). All three methods of phylogenetic analysis indicate that the division of the ancestral pipefish into abdomen- and tail-brooding forms preceded a rapid diversification of pouch types, and that an increase in pouch complexity occurred independently in the Urophori and Gastrophori (Figure 5).

The total evidence molecular analysis also provides insight into the evolution of *Hippocampus* seahorses. In contrast to Herald's (1959) model of pouch evolution, which suggested a transition from the everted abdominal pouch (type A4e) to the completely enclosed pouch of the seahorses (type A5) (Figure 1), the total evi-

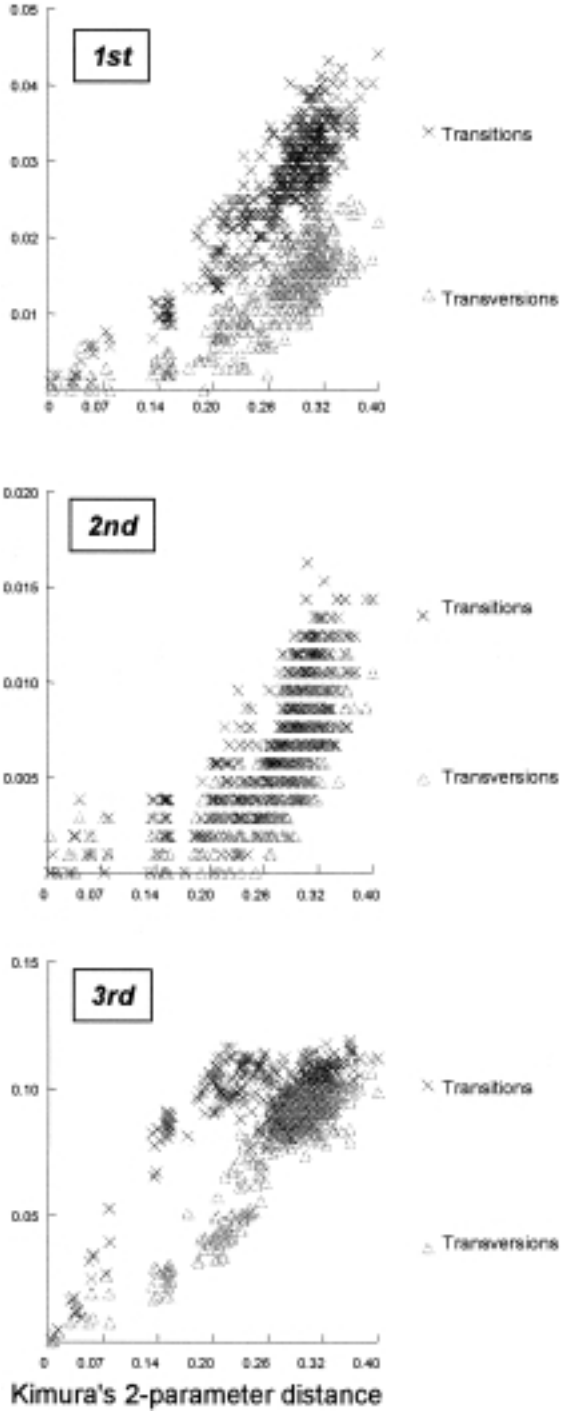

Figure 3. Transitions/transversions plotted against Kimura-2-parameter distance for the complete cytochrome *b* gene (1149 bp). Plots were generated by DAMBE version 4.0.17 (Xia 2000). Transitions at the third codon positions of cytochrome *b* are saturated.

dence phylogeny instead supports a sister-group relationship between the seahorses and *Syngnathus* pipefishes (inverted pouch type A4i) (Figure 5).

Phylogeography of Syngnathidae

We included geographical data on our consensus phylogenetic tree in an effort to infer historical biogeographic partitioning which might be partially responsible for the diversification of these fishes. Several interesting patterns are observed in the within-lineage diversification of the subfamily Urophori. There is a clear split of *Syngnathus* pipefishes into North American and European lineages (Figure 5), and while significant species-level diversification has occurred, there has been no corresponding diversification of pouch types within the genus.

Although male pouch types appear to have been established during the formation of most major syngnathid lineages, there is a significant amount of variability in pouch structure in a small group of pipefishes endemic to southeastern Australia. While *Solegnathus hardwickii* (type A2) is widespread in southeast Asia, *Urocampus carinorostris* (type A4s) and the other species on this branch of the tree are all Australian endemics that have apparently evolved both the semi (A4s) and everted (A4e) pouch types multiple times (Figure 5). A Kishino–Hasegawa test rejected the null hypothesis of pouch monophyly (*t* test: $P < .001$), supporting the replicated evolution of A4s and A4e pouch types in this lineage.

Discussion

Rapid Diversification and Independent Evolution of Syngnathid Brooding Structures

Both single-gene and total molecular evidence analyses indicate a rapid radiation of major lineages and, concomitantly, also of pouch types in the Syngnathidae. Tail- and abdomen-brooding lineages evolved independently, with remarkable increases in pouch morphological complexity in both subfamilies, an evolutionary series that reached its greatest development in the completely enclosed pouches of the seahorses (subfamily Urophori). While our study provides some evidence for repeated evolution of a number of pouch types (pouch types A4e and A4s; Figure 5), the morphological variation between these pouch types represents a relatively minor proportion of the total brood pouch diversity found in these fishes (Figure 1). Our molecular data confirm much of Herald's (1959) morphological scenario of brood pouch evolution (Herald 1959), suggesting that the evolution of the paternal brood pouch has been closely associated with the rapid diversification in both subfamilies of the family Syngnathidae.

Given the nest building, maintenance, and considerable territorial guarding behavior of the Gasterosteidae (Breder and Rosen 1966), the exceptional development of the syngnathid brood pouch may not be entirely unexpected (Baylis 1981). As gasterosteoid nests face predation and attacks by neighbors, and protected eggs may be fertilized by sneaky males (Wootton 1984), the evolution of male brooding in the Syngnathidae presumably resulted

Table 3. Hierarchical likelihood ratio test of phylogenetic model as implemented by MODELTEST version 3.0 (Posada and Crandall 1998)

Model	Parameters	–ln(likelihood)	Gamma (Γ)	Proportion of invariable sites (I)
12S				
JC	1	4832.9736		
F81	4	4806.0513		
HKY	5	4692.2627		
HKY + Γ	6	4285.8623*	0.4329	0
HKY + Γ + I	7	4285.7329		
16S				
JC	1	5792.5630		
F81	4	5781.4595		
HKY	5	5670.0010		
HKY + Γ	6	5021.7520		
HKY + Γ + I	7	5015.7070*	0.6219	0.3880
Cytochrome *b*				
JC	1	5992.3228		
F81	4	5941.6152		
HKY	5	5747.3623		
HKY + Γ	6	5197.8252		
HKY + Γ + I	7	5185.4873*	0.4590	0.3946
Combined analysis				
JC	1	17239.4961		
F81	4	17176.9199		
HKY	5	16765.9062		
HKY + Γ	6	14996.7080		
HKY +Γ + I	7	14981.1475*	0.4333	0.2755

*Selected model of evolution ($P < .01$).

in a net increase in reproductive success, as measured by the proportion of fertilized offspring that survive to reproduce. One may speculate that the benefits of carrying embryos away from predators and sneakers may have led to the simple attachment of embryos to the ventral surface in a pre-pipefish ancestor (Baylis 1981). Subsequent within-lineage diversification and closure of the brooding structure could help to explain the diversity of pouch types observed today.

Evolutionary Origin of *Hippocampus*: The "Birth" of Seahorses

Total molecular analyses provide evidence for a sister-group relationship between *Syngnathus* and *Hippocampus,* suggesting that the A4i (inverted pouch type) pipefishes and A5 (sealed pouch) seahorses have a common ancestor. While Herald (1959) believed that the A4e (everted pouch) pipefishes gave rise to *Hippocampus* seahorses, our molecular analyses unequivocally group *Syngnathus* and *Hippocampus* (Figure 5).

In his study on the evolution of syngnathid brooding structures, Herald (1959) paid particular attention to the evolution of the seahorse brood pouch, and based on a suite of shared characters, proposed a hypothetical evolutionary sequence from an everted pouch ancestor to the completely sealed pouch of the seahorse. Unfortunately many of the characters used in his analyses (raised dorsal fin base, absence of caudal fin, prehensile tail) have multiple independent origins in the Syngnathidae (Dawson 1985; Duncker 1915), possibly confounding his attempts to identify the ancestors of the sealed-pouch seahorses. Herald (1959) believed that an Atlantic subspecies of *Acentronura* was the closest extant relative of *Hippocampus,* yet acknowledged several important differences between the genus *Acentronura* and *Hippocampus,* including absence of lateral tail ridges in *Acentronura* and a complete lack of protecting pouch plates in *Hippocampus.* Based on these differences, Herald (1959) concluded that although these two genera resemble each other superficially, they did not evolve in the same manner. Further morphological comparisons of reproductive characters within this family are clearly necessary in light of our molecular phylogenetic analyses.

The genera *Hippocampus* and *Syngnathus,* with some of the most developed male brooding structures in the Syngnathidae, are by far the most species-rich genera in the family (Dawson 1985). The close phylogenetic relationship between *Hippocampus* and *Syngnathus* as inferred from our molecular phylogeny suggests that shared characteristics, possibly in addition to complex paternal care structures, have played an important role in the diversification of these two groups. *Syngnathus* and *Hippocampus* have also been especially successful at achieving wide geographic distributions (Dawson 1985; Lourie et al. 1999), indicating that the long-distance dispersal capability in these two genera may be higher than that of other members of the Syngnathidae (see *Syngnathus* discussion below).

Syngnathus Biogeography: A Pacific Origin?

Due to the broad geographic distribution of many of the genera analyzed in the present study, it remains difficult to elucidate centers of origin of the species-rich clades, including *Hippocampus* and *Syngnathus.* Although *Syngnathus* has a cosmopolitan distribution and species are found not only in marine, but also in freshwater and estuarine environments, Atlantic Ocean lineages of *Syngnathus* are clearly disjunct from the less speciose Indo-Pacific lineage (Dawson 1985). Moreover, two distinct configurations of body ridges distinguish the European species from those found in the western Atlantic and eastern Pacific (Dawson 1985). Our total evidence molecular results clearly separate European and western Atlantic species and demonstrate a close relationship between *Syngnathus leptorhynchus,* an eastern Pacific pipefish, and both the Atlantic Ocean *Syngnathus* pipefishes and *Syngnathus schlegeli,* a representative of the disjunct Indo-Pacific lineage. Interoceanic dispersal of a syngnathid ancestor may be responsible for seeding the Atlantic radiation of *Syngnathus,* a pattern that has also been detected in *Gasterosteus aculeatus* (Orti et al. 1994), a species whose center of molecular diversity is found in the Pacific Ocean. Whereas body ridge configuration has varied over the history of the genus *Syngnathus* (Dawson 1985), brood pouch structure has been remarkably conserved.

Parental Investment and Sexual Selection: Insights from the Syngnathidae

It is now commonly believed that relative parental investment of sexes in their young is a key factor responsible for sexual selection (Clutton-Brock and Parker 1992; Trivers 1972). Given the complete, but still variable, paternal care of the Syngnathidae, pipefishes and seahorses offer an exceptionally well-suited system to investigate sexual selection in relation to parental investment. In contrast to predictions from parental investment theory (Trivers 1972), the variation in sexual dimorphism among syngnathids (Berglund et al. 1986; Vincent 1994) does not appear to be associated with the degree of pouch development (Berglund et al. 1986; Vincent et al. 1992).

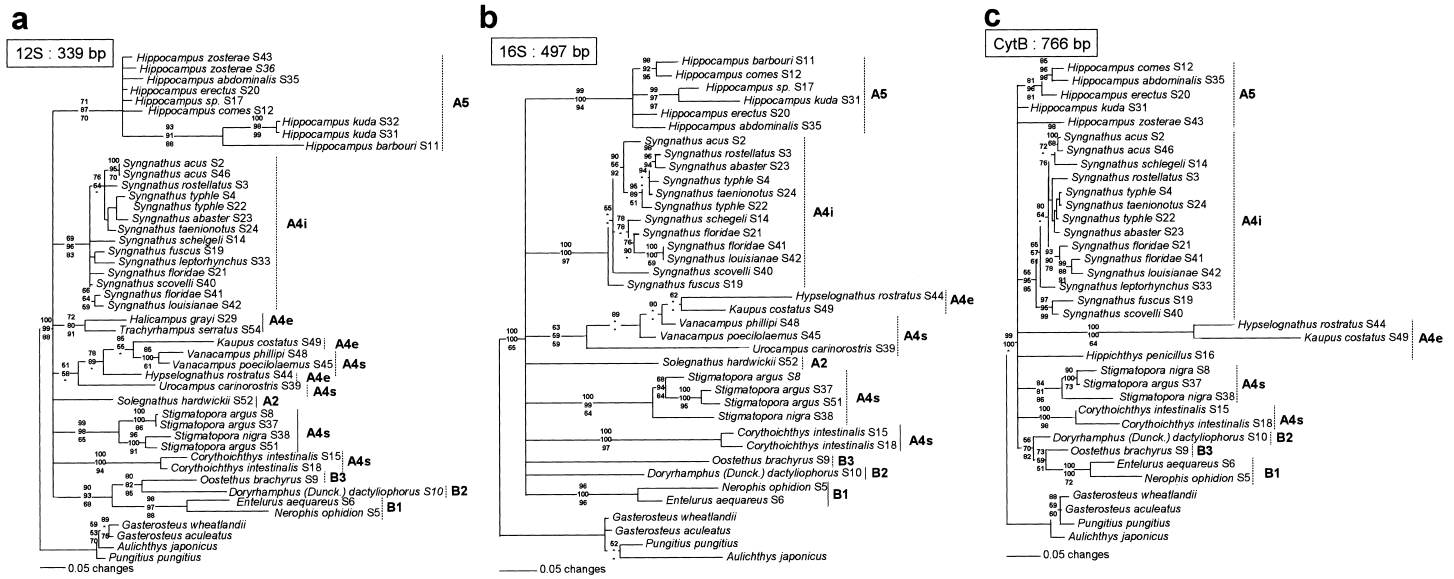

Figure 4. Consensus phylogenetic tree for **(A)** 12S rDNA, **(B)** 16S rDNA, and **(C)** cytochrome *b* sequence data with branch lengths as estimated from neighbor-joining HKY distances. Numbers on branches represent bootstrap values/puzzling support from distance/parsimony/likelihood analysis (asterisks indicate collapsed branches).

Instead, accumulating evidence indicates that environmental variables as well as anatomical and physiological constraints may strongly influence differences in potential reproductive rates between the sexes (Ahnesjö 1995; Kvarnemo and Ahnesjö 1996; Vincent et al. 1992), thereby influencing mate competition and ultimately sexual selection. Furthermore, ongoing molecular studies suggest that sex role reversal has had multiple independent origins uncorrelated with brood pouch development (Wilson AB, et al., submitted), indicating that environmental factors have played a significant role in influencing sex role reversal and sexual selection in syngnathid fishes.

Conclusions

The rapid diversification of male pregnancy in the Syngnathidae and increasing complexity of pouch structure in both major lineages of the family indicate that highly developed male parental care has been closely associated with syngnathid radiation. Although much of previous morphological analyses are supported by our molecular data, significant discrepancies between molecular and morphological work suggest that further examination of pouch development and/or taxonomic revision of the group may be necessary. Our molecular results shed new light on the phylogeography of the family, suggesting a Pacific origin for *Syngnathus* pipefishes and indicating regional concentrations of genetic biodiversity in the western Indo-Pacific.

With the present molecular phylogenetic framework in place, future studies of syngnathid species should aim to further characterize behavioral and morphological variation within the family and to clarify this variation in relation to established phylogenetic relationships. At the same time, more detailed species-level phylogenetic studies will help to increase our understanding of the influence of mating systems on the evolution of these fascinating creatures. The marriage of population genetic (Jones and Avise 2001) and phylogenetic data will continue to broaden our perspective on the relationship between the evolution of mating and parental care systems and the diversification of syngnathiform fishes.

References

Ahnesjö I, 1995. Temperature affects male and female potential reproductive rates differently in the sex-role reversed pipefish, *Syngnathus typhle.* Behav Ecol 6:229–233.

Andersson M, 1994. Sexual selection. Princeton, NJ: Princeton University Press.

Avise JC, 2000. Phylogeography: the history and formation of species. Cambridge, MA: Harvard University Press.

Avise JC, Arnold J, Ball RM, Bermingham E, Lamb T, Neigel JE, Reeb CA, and Saunders NC, 1987. Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. Annu Rev Ecol Syst 18:489–522.

Barraclough TG, Vogler AP, and Harvey PH, 1998. Revealing the factors that promote speciation. Philos Trans R Soc Lond B 353:241–249.

Baylis JR, 1981. The evolution of parental care in fishes, with reference to Darwin's rule of sexual selection. Environ Biol Fish 6:223–251.

Berglund A, Rosenqvist G, and Svensson I, 1986. Reversed sex roles and parental energy investment in zygotes of two pipefish (Syngnathidae) species. Mar Ecol Prog Ser 29:209–215.

Bermingham E, McCafferty SS, and Martin AP, 1997. Fish biogeography and molecular clocks: Perspectives from the Panamanian isthmus. In: Molecular phylogenetics of fishes (Kocher TD, Stepien CA, eds). New York: Academic Press; 113–128.

Bernatchez L and Wilson CC, 1998. Comparative phylogeography of Nearctic and Palearctic fishes. Mol Ecol 7:431–452.

Blumer LS, 1982. A bibliography and categorization of bony fishes exhibiting parental care. Zool J Linn Soc 76:1–22.

Bowne PS, 1984. Systematics and morphology of the Gasterosteiformes. In: The evolutionary biology of the threespine stickleback (Bell MA and Foster SA, eds). Oxford: Oxford University Press; 28–60.

Breder CM and Rosen DE, 1966. Modes of reproduction in fishes. New York: Natural History Press.

Clutton-Brock TH, 1991. The evolution of parental care. Princeton, NJ: Princeton University Press.

Clutton-Brock TH and Parker GA, 1992. Potential reproductive rates and the operation of sexual selection. Q Rev Biol 67:437–456.

Clutton-Brock TH and Vincent AC, 1991. Sexual selection and the potential reproductive rates of males and females. Nature 351:58–60.

Darwin C, 1871. The descent of man, and selection in relation to sex. London: J. Murray.

Dawson CE, 1985. Indo-pacific pipefishes (Red sea to the Americas). Ocean Springs, MS: Gulf Coast Research Laboratory.

de Queiroz A, Donoghue MJ, and Kim J, 1995. Separate versus combined analysis of phylogenetic evidence. Annu Rev Ecol Syst 26:657–681.

Duncker G, 1915. Revision der Syngnathidae. Mitt Hamb Zool Mus Inst 32:9–120.

Emlen ST and Oring LW, 1977. Ecology, sexual selection, and the evolution of mating systems. Science 197:215–223.

Froese R and Pauly D, 2000. FishBase (visited/last modified September 5, 2000) ‹www.fishbase.org›.

Gross MR and Sargent RC, 1985. The evolution of male and female parental care in fishes. Am Zool 25:807–822.

Hasegawa M, Kishino H, and Yano T, 1985. Dating of the

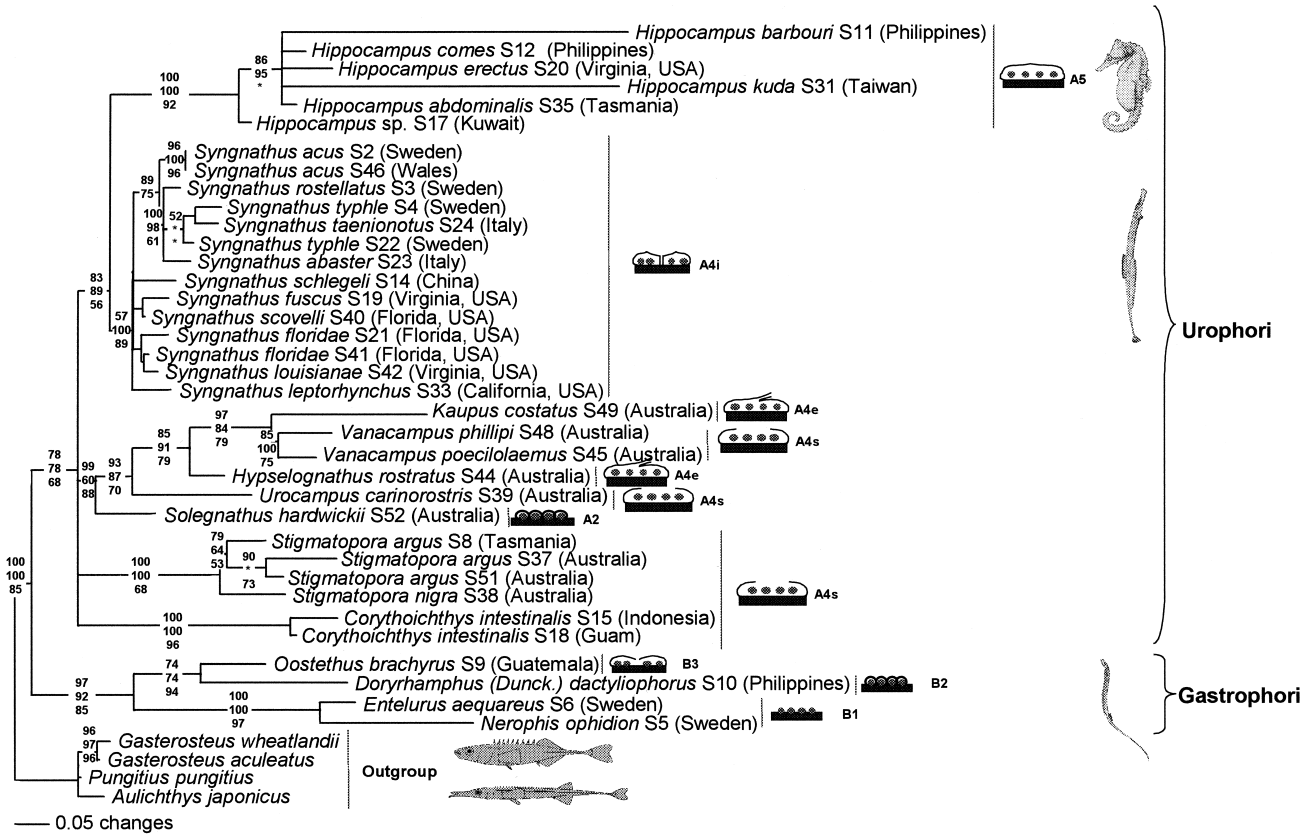

Figure 5. Consensus phylogenetic tree constructed from neighbor-joining distance, maximum parsimony, and quartet puzzling maximum-likelihood analyses based on the combined dataset of 1602 bp with branch lengths as estimated from neighbor-joining distances. Numbers on branches represent bootstrap values/puzzling support from distance/parsimony/likelihood analysis (asterisks indicate collapsed branches). Syngnathid diagrams adapted from Froese and Pauly (2000), Nelson (1994), and Vincent et al. (1992).

human-ape splitting by a molecular clock of mitochondrial DNA. J Mol Evol 22:160–174.

Herald ES, 1959. From pipefish to seahorse—a study of phylogenetic relationships. Proc Calif Acad Sci 29:465–473.

Howard DJ and Berlocher SH, 1998. Endless forms: species and speciation. New York: Oxford University Press.

Irwin DM, Kocher TD, and Wilson AC, 1991. Evolution of the cytochrome *b* gene of mammals. J Mol Evol 32: 128–144.

Johns GC and Avise JC, 1998. A comparative summary of genetic distances in the vertebrates from the mitochondrial cytochrome *b* gene. Mol Biol Evol 15:1481–1490.

Jones AG and Avise JC, 1997. Microsatellite analysis of maternity and the mating system in the Gulf pipefish *Syngnathus scovelli,* a species with male pregnancy and sex-role reversal. Mol Ecol 6:203–213.

Jones AG and Avise JC, 2001. Mating systems and sexual selection in male-pregnant pipefishes and seahorses: Insights from microsatellite-based studies of maternity. J Hered 92:150–158.

Jones AG, Rosenqvist G, Berglund A, and Avise JC, 1999. The genetic mating system of a sex-role-reversed pipefish (*Syngnathus typhle*): a molecular inquiry. Behav Ecol Sociobiol 46:357–365.

Kocher TD, Thomas WK, Meyer A, Edwards SV, Pääbo S, Villablanca FX, and Wilson AC, 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. Proc Natl Acad Sci USA 86:6196–6200.

Kvarnemo C and Ahnesjö I, 1996. The dynamics of operational sex ratios and competition for mates. Trends Ecol Evol 11:404–408.

Lourie SA, Vincent ACJ, and Hall HJ, 1999. Seahorses: an identification guide to the world's species and their conservation. London: Project Seahorse.

Lynch JD, 1989. The gauge of speciation: on the frequencies of modes of speciation. In: Speciation and its consequences (Otte J and Endler JA, eds). Sunderland, MA: Sinauer; 527–553.

Meyer A, 1993. Evolution of mitochondrial DNA in fishes. In: Biochemistry and molecular biology of fishes (Hochachka PW and Mommsen TP, eds). Leiden, Netherlands: Elsevier; 1–38.

Meyer A, Kocher TD, Basasibwaki P, and Wilson AC, 1990. Monophyletic origin of Lake Victoria cichlid fishes suggested by mitochondrial DNA sequences. Nature 347:550–553.

Nelson JS, 1994. Fishes of the world. New York: John Wiley & Sons.

Orti G, Bell MA, Reimchen TE, and Meyer A, 1994. Global survey of mitochondrial DNA sequences in the threespine stickleback: evidence for recent migrations. Evolution 48:608–622.

Palumbi SR, Martin AP, Romano SL, McMillan WOD, Stice L, and Grabowski G, 1991. The simple fool's guide to PCR. Honolulu: University of Hawaii.

Pääbo S, Thomas WK, Whitfield KM, Kumazawa Y, and Wilson AC, 1991. Rearrangements of mitochondrial transfer RNA genes in marsupials. J Mol Evol 33:426–430.

Posada D and Crandall KA, 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics 14:817–818.

Strimmer K and von Haesler A, 1999. Tree-puzzle. Oxford: University of Oxford.

Swofford D, 2000. PAUP*. Phylogenetic analysis using parsimony (*and other methods). Sunderland, MA: Sinauer.

Taberlet PA, Meyer A, and Bouvet J, 1992. Unusual mitochrondrial DNA polymorphism in two local populations of blue tit (*Parus caeruleus*). Mol Ecol 1:27–36.

Thompson JD, Higgins DG, and Gibson TJ, 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680.

Trivers RL, 1972. Parental investment and sexual selection. In: Sexual selection and the descent of man (Campbell B, ed). London: Heinemann; 136–179.

Vincent A, Ahnesjö I, Berglund A, and Rosenqvist G, 1992. Pipefishes and seahorses: Are they all sex role reversed? Trends Ecol Evol 7:237–241.

Vincent ACJ, 1994. Seahorses exhibit conventional sex roles in mating competition, despite male pregnancy. Behaviour 128:135–151.

Wootton RJ, 1984. A functional biology of the sticklebacks. London: Croom Helm.

Xia X, 2000. DAMBE (data analysis in molecular biology and evolution). Boston: Kluwer.

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