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TITLE

Molecular Phylogeny and Patterns of Diversification in Syngnathid Fishes

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ABSTRACT

The family Syngnathidae is a large and diverse clade of morphologically unique bony fishes, with 57 genera and 300 described species of seahorses, pipefishes, pipehorses, and seadragons. They primarily inhabit shallow coastal waters in temperate and tropical oceans, and are characterized by a fused jaw, male brooding, and extraordinary crypsis. Phylogenetic relationships within the Syngnathidae remain poorly resolved due to lack of generic taxon sampling, few diagnostic morphological characters, and limited molecular data. The phylogenetic placement of the threatened, commercially exploited seahorses remains a topic of intense interest, with conflicting topologies based on morphology and predominantly mitochondrial genetic data. In this study, we integrate eight nuclear and mitochondrial markers and 17 morphological characters to investigate the phylogenetic structure of the family Syngnathidae at the generic level. We include 91 syngnathid species representing 48 of the 57 recognized genera, all major ocean basins, and a broad array of temperate and tropical habitats including rocky and coral reefs, sand and silt, mangroves, seagrass beds, estuaries, and rivers. Maximum likelihood and Bayesian analyses of 5160 bp from eight loci produced high congruence among alternate topologies, defining well-supported and sometimes novel clades. We present a hypothesis that confirms a deep phylogenetic split between lineages with trunk- or tail-brood pouch placement, and provides significant new insights into the morphological evolution and biogeography of this highly derived fish clade. Based on the fundamental division between lineages - the tail brooding “Urophori” and the trunk brooding “Gastrophori” - we propose a revision of Syngnathidae classification into only two subfamilies: the Nerophinae and the Syngathinae. We find support for distinct principal clades within the trunk-brooders and tail-brooders, the latter of which include seahorses, seadragons, independent lineages of pipehorses, and clades that originated in Southern Australia and the Western Atlantic. We suggest the seahorse genus *Hippocampus* is of Indo-Pacific origin and its sister clade is an unexpected grouping of several morphologically disparate Indo-Pacific genera, including the Pacific pygmy pipehorses. Taxonomic revision is required for multiple genera, particularly to reflect deep evolutionary splits in nominal lineages from the Atlantic versus the Indo-Pacific.

1. INTRODUCTION

Syngnathid evolution has been of interest since Linnaeus' first edition of *Systema Naturae* (1758), where he described several species of *Syngnathus* pipefishes and a Mediterranean seahorse, *Hippocampus hippocampus*. This group of bony fishes displays fascinating and diverse morphologies such as the upright, prehensile-tailed seahorse, and the large, spectacularly ornate seadragons. With their highly unusual morphology, wide variety of forms, and remarkable capacity for camouflage, syngnathids have long fascinated both naturalists and the general public. They are also well known for their unique and specialized reproductive biology. All syngnathids have male brooding structures, and some genera have evolved true male pregnancy, complete with nutrient and oxygen delivery to the developing embryos. This shared character of the group has made them an important model system for studies of mating systems, sex role reversal, and sexual selection (Clutton-Brock & Vincent, 1991; Kvarnemo & Ahnesjö, 2002; Wilson et al., 2003; Jones et al., 2005).

The family Syngnathidae is one of the more biologically diverse and species-rich families of shallow-water fishes. Based on several revisions (detailed in Supplemental Table 2) to the current taxonomy as provided on Fishbase (Froese & Pauly, 2015) and the Catalog of Fishes (Eschmeyer et al., 2016), it contains 57 genera and 300 described species (Dawson 1974; Stiller et al., 2015; Dawson 1985; Supplemental Table 2), and includes the seahorses, pipefishes, pipehorses, seadragons, and one of the latest additions, a pygmy pipedragon (Gomon, 2007). The diversity of syngnathids is nearly comparable to some of the more speciose and better studied fish groups, such as the wrasses with 82 genera and 600 species (Parenti & Randall, 2011), and the groupers with 64 genera and 450 species (Froese & Pauly, 2015). Syngnathid fishes are distributed worldwide in a diversity of temperate and tropical habitats, including rocky and coral reefs, sandy and silty-bottom habitats, mangrove forests, seagrass beds, estuaries, and rivers. In many coastal bays and estuaries, several species of pipefishes may be a dominant component of the ichthyofauna (Allen, 2006; Howard & Koehn, 1985; Pollard, 1984; Whitfield, 1999; York et al., 2006), suggesting an important ecological role in those habitats. In addition to their unique morphologies and reproductive strategy, many syngnathids are exploited, primarily as an ingredient in traditional Chinese medicine (Martin-Smith et al., 2003, Martin-Smith et al., 2006, Vincent et al., 2011) and are threatened from habitat degradation. Concern over conservation status has led to IUCN Red List evaluation for about 40% of syngnathid species. Of the 104 reviewed species, 13 are listed as vulnerable, three are near threatened, one is endangered, and one is critically endangered, while almost 50% are listed as data deficient. Given the many reasons for interest in this charismatic family, surprisingly little is known about their

evolutionary relationships and patterns of diversification.

A suite of synapomorphies support the traditional view of the monophyly of the Syngnathidae (Dunker, 1912; Herald, 1959; Dawson, 1982, 1985). Diagnostic characters that clearly unite the family include the reduction of the gill opening to a small pore in the opercular membrane, dermal plates like body armor arranged in a series of regular rings, the absence of pelvic fins, a fused jaw, and male parental care. Within this monophyletic group, genera have been diagnosed largely based on principal body ridge configurations, particularly in the work of C.E. Dawson, whose highly productive ichthyological career provided the foundation for our current understanding of syngnathid taxonomy. The challenge of elucidating evolutionary relationships among genera has been complicated by the morphological fluidity of important characters, such as combinations of ridge structure configurations that have evolved many times independently. Similarly, counts of fin rays, tail, and trunk rings can be diagnostic for species identification, but vary and overlap widely within and between genera (Dawson, 1982; 1985).

All syngnathids display paternal care, and males brood eggs under their trunk or tail. This unique reproductive strategy has focused attention on male brood-pouch morphology in the classification and interpretation of evolutionary patterns. However, subfamily designations diagnosed by brood pouch characters has led to conflicting opinions regarding the definition and arrangement of major clades (Kaup, 1856; Nelson, 2006; Wilson & Rouse, 2010). Early researchers divided syngnathids into five major subfamilies based on brood pouch morphology (Kaup, 1856), and later into two large unnamed clades based on position of male brood pouch: tail versus trunk (Dunker, 1912). This fundamental division of syngnathids into two clades (with

the exception of genus *Syngnathoides*) was revisited by Herald (1959), who referred to these two groups as the “Urophori” and “Gastrophori”, respectively. This phylogenetic division has been supported in every molecular tree published to date (Wilson *et al.*, 2001; 2003; Teske & Beheregaray, 2009; Wilson & Rouse, 2010). However, the presently recognized subfamily relationships are in direct conflict with this view of the basal split in syngnathid evolution. Currently, there are two subfamilies defined as (i) the Hippocampinae, the seahorses, made up of only one genus, *Hippocampus*, and (ii) the Syngnathinae, made up of all other genera in the family, numbering 56 (Nelson, 2006). More recently, Wilson and Rouse (2010) proposed eight subfamilies based on their mitochondrial molecular phylogeny, which subdivided some long-recognized clades, such as the seadragons (Herald, 1959; Dawson, 1982; 1985). Subfamily designations clearly need to be re-evaluated in syngnathid fishes.

Relationships within these two reciprocally monophyletic clades, the trunk-brooders (consisting of 56 species) and the tail-brooders (244 species; Supplemental Table 2), remain unresolved. Herald (1956) proposed an appealing model of independent parallel evolution of increasing brood pouch complexity in both tail- and trunk-brooders. However, from the first application of molecular phylogenetic approaches, this idea has been refuted, initially by Wilson and collaborators (2001). Mitochondrial and nuclear sequence data reveal multiple independent gains of complex brood pouch structures early in the diversification of both the trunk and tail-brooding lineages (Wilson *et al.* 2003; Wilson & Rouse 2010). Subsequent molecular work has focused on phylogenetic placement and relationships within seahorses (Teske *et al.*, 2004; 2007). Teske and Beheregaray (2009), analyzing five nuclear genes from eight genera,

concluded that the two Pacific and one Atlantic lineages of pipehorses are together the sister group of seahorses, and they cited brood pouch closure as a synapomorphy among these groups to support their view. They suggested all pipehorses should be included in the subfamily Hippocampinae (cf. Herald, 1959; Teske & Beheregaray, 2009). In contrast, Wilson and collaborators (2001) hypothesize a close relationship between *Hippocampus* and *Syngnathus* based on mtDNA analysis, brood pouch morphology, and biogeographic patterns.

A more refined understanding of syngnathid phylogeny has yet to be deeply explored. Most studies have analyzed limited mitochondrial data (Wilson et al 2001, 2003; Wilson & Rouse 2010, Wilson and Orr 2011), or have had limited generic-level taxon sampling (Teske and Beheregaray 2009). To date taxon sampling has been heavily biased toward the two most speciose genera: *Syngnathus* and *Hippocampus*. Despite relatively fast evolutionary rates that are best applied for inferences of shallow evolutionary nodes (e.g. at the generic or species level), mitochondrial markers are often favored over nuclear markers because of higher certainty of homology and relatively constant rates of evolution (Boore & Duerstenberg, 2008). In contrast, there is a lack of information regarding paralogy and evolutionary rates in nuclear genes. A genome duplication occurred shortly before the origin of teleosts (Crow *et al.*, 2006; Hoegg, 2006), making this problem relevant to nuclear marker phylogenetic analyses of Syngnathidae.

To address these potential biases, we sequenced multiple mitochondrial and nuclear markers from a broad diversity of syngnathid genera. Our analysis contains four mitochondrial gene fragments (*12s*, *16s*, *ND1*, *COI*) and four nuclear markers (*18S*, *HoxA13a*, *Myh6*, and *Tmo4C4*), and includes 91 syngnathid species in 48 genera, more than doubling previous generic

sampling, and four outgroup species representing three genera. We included one nuclear locus for which we can infer first order paralogy with confidence; the *HoxA13a* gene. Sampling across all major clades within the family Syngnathidae, we infer evolutionary relationships based on analysis of both single genes and the combined 5160 bp sequence, and 17 morphological characters. We use these results to build a comprehensive generic level phylogenetic hypothesis of syngnathid fishes. Our findings lead us to propose a revised subfamily classification, and allow us to investigate the evolution of brood-pouch complexity, the origin of seahorses, biogeographic centers of endemism and diversity, problematic characters and taxonomic classification, and evidence for convergent evolution across lineages.

2. METHODS

2.1 *Taxon sampling*

We sampled 91 syngnathid species across 48 genera and an additional four outgroup taxa that are closely related within the order Syngnathiiformes (Supplemental Table 1). All specimens were collected for this study by the authors and their colleagues, or were obtained from vouchered museum specimens (i.e. when ethanol-preserved tissues were available). The focus of our study is to provide a generic level phylogeny, so with the exception of large and morphologically diverse genera, we sampled a single individual to represent each genus. Most specimens were collected via SCUBA by hand in nearshore benthic habitats, or by seine net in seagrass beds or sand flats. Each specimen was morphologically identified according to C.E. Dawson (1982, 1985), subsampled for DNA extraction, and then preserved in 70% ethanol as a

voucher. Tissue samples were placed in 100% ethanol and kept at -20°C until DNA extraction.

2.2 DNA extraction and PCR amplification

Total genomic DNA was extracted from fin clippings or small tissue samples taken from the right side of each specimen using the DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA, USA) following the manufacturer's protocol. Partial sequences of the mitochondrial genes 12S ribosomal RNA (*12S*), 16S ribosomal RNA (*16S*), cytochrome c oxidase subunit I (*COI*), and NADH dehydrogenase I (*NDI*), and the nuclear genes 18S ribosomal RNA (*18S*), myosin heavy chain 6 (*Myh6*), anonymous putative titin-like protein (*Tmo4C4*) and homeobox protein transcription factor A13a (*HoxA13a*) were amplified by polymerase chain reaction (PCR) with the primers described in Supplemental Table 3 (Kocher et al. 1989; Palumbi et al. 1991; Folmer et al. 1994; Streelman & Karl 1997; Vandergast & Roderick 2003; Ward et al. 2005; Li et al. 2007). Amplifications were performed in a total volume of 25 µl with 2.5 µl of 10X buffer (10 mM Tris-HCl+15 mM MgCl₂), 2.5 µl dNTPs (200 nM of each), 1 µl of each primer (5 mM), 0.1 µl Taq Gold polymerase (250 units per mL) (Life Technologies, Grand Island, NY, USA), 1 µl template DNA (50 ng), and 17.9 µl ddH₂O. The PCR reactions consisted of 35 cycles, 30 s at 95°C, 45-120 s at 48-58°C (according to primer and species), and 90 s at 72°C. Some samples amplified insufficiently using standard PCR, and were improved by excising, purifying and re-amplifying under the same PCR conditions described above, or, as in the case of *Myh6*, were amplified again with internal primers (Supplemental Table 3).

2.3 *Sequencing and Sequence alignment*

All PCR products were visualized on a 1% agarose gel and purified using Exonuclease-I (20 U/ μ l, New England Biolabs) and Shrimp 185 Alkaline Phosphatase (1 U/ μ l, Roche) according to the manufacturer's instructions. PCR products were cycle-sequenced with the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) in a reaction volume of 10 μ L consisting of 1.5 μ L of 5X Sequencing buffer, 0.5-0.7 μ L of BigDye, 1.2 μ L (10 μ M) of each primer, and 1.0 μ L of PCR product. Sequencing reactions were performed with 50 cycles at 96°C for 10 s, 50°C for 5 s, 60°C for 4 min. Cycle sequencing products were precipitated using a standard ethanol precipitation method (Sambrook & Russell, 2006). Finally, precipitated cycle-sequencing products were re-suspended in 10 μ L of ABI Hi-Di formamide, denatured at 95°C for two minutes followed by 5 min on ice, and then sequenced on an ABI Prism 3130xl automated genetic analyzer (Applied Biosystems, Foster City, CA, USA). Bases were called using Sequencing Analysis v5.2 (Applied Biosystems, Foster City, CA, USA). Consensus sequences for each individual were generated from sequences in forward and reverse directions using Geneious v 8.1.5 (<http://www.geneious.com>, Kears e et al., 2012). All sequences from coding genes were translated to confirm that sequences contained no frameshift, in/dels or stop codons that might indicate pseudogenes.

Multiple sequence alignments were performed using MAFFT 7.0 (Katoh & Standley, 2013) and were manually edited in Geneious. Preliminary analyses were also conducted with the variable regions of ribosomal fragments included and excluded. There were no resulting differences in topology, so all regions of ribosomal fragments were included in subsequent

analyses. Finally, sequences from all genes were trimmed to minimize missing data and combined with Geneious into a concatenated mitochondrial DNA matrix, a concatenated nuclear DNA matrix, and a fully concatenated matrix of all eight loci.

2.4 *Assessing first order paralogy in HoxA13a*

We sequenced both *HoxA13a* and *HoxA13b* paralogs for 14 syngnathid taxa and aligned them with the annotated “a” and “b” paralogs of several teleosts, including fugu, medaka, stickleback, and *Astatotilapia*, as well as with the non-duplicated *HoxA13b* genes of the coelacanth, horn shark, bichir, and bowfin. The *HoxA13a* gene tree recovered well-supported paralog clades, and topologies consistent with the expected relationships among ray-finned fishes. As expected, syngnathids were reciprocally monophyletic with the remaining teleosts fugu, medaka, stickleback, and *Astatotilapia*. Sequencing the *HoxA13a* paralogs was more tractable than *HoxA13b* paralogs (i.e. only one nested PCR with consistent primer combinations was needed), therefore we sequenced the *HoxA13a* marker for the remaining taxa.

2.5 *Phylogenetic Analysis*

MrModeltest 2 (Nylander, 2004) was used to estimate the best model of evolution for each partition of the data for subsequent Bayesian phylogenetic inference analyses selected under the Akaike information criterion (AIC; See Table 1). For coding genes (*ND1*, *COI*, *Myh6*, *Tmo4C4* and *HoxA13a*) substitution models were estimated for each codon position. Bayesian analyses were conducted in MrBayes 3.2.1 (Ronquist & Huelsenbeck, 2003; Altekar et al., 2004)

using the model of sequence evolution recommended by MrModeltest 2 for each partition. Analyses were run for 50,000,000 generations with the Markov chains being sampled every 1000 generations. We determined a burn-in value by examining the sample parameter values using Tracer v1.4. (Rambaut & Drummond, 2007). The distribution of parameter values reached stationarity by 12,500 trees. Maximum likelihood analyses were run in RAxML 7.0.4 (Stamatakis et al., 2006) with the data partitioned as mentioned above, using the GTR-Gamma model. This model optimizes per-site substitution rates for the initial analyses and then evaluates the final tree topology under GTR-GAMMA, yielding stable likelihood values. A 10,000 replicate ML “Fast Bootstrapping” (Stamatakis et al, 2008) analysis was performed to provide branch support values. These methods were used to evaluate topologies among fully concatenated datasets as well as individual markers and genome-specific partitions (i.e. nuclear *versus* mitochondrial).

2.6 *Morphological Character Analysis and mapping of Biogeography*

Discrete adult morphological characters were built into a matrix in Mesquite v2.72 (Maddison & Maddison, 2009). All character states were found in the literature where possible (Supplemental Table 4), and otherwise, a minimum of three vouchered museum specimens were used to assign states. While the final morphological matrix contained 17 characters, only 11 of these were parsimony informative (male brood location, pouch plates, pouch folds, pouch closure type, superior ridge configuration, inferior ridge configuration, lateral ridge curve, lateral ridge termination, dorsal fin origin, dorsal fin base, and caudal fin). We applied cladistic tree-building

methods with these 11 morphological characters in RAxML 7.0.4 (Stamatakis et al., 2006). However, topologies retrieved were not congruent with long-recognized, and well supported clades, with splits between close relatives and distant-relatives inferred as sister genera ([Supplemental Figure 4](#)). Therefore, we focused all further analyses on ancestral character state estimation in Mesquite v2.72, using the Maximum Likelihood algorithm with the Markov k-state 1 parameter model (Mk1) implemented. Morphological character and biogeographic character states were assigned to each ancestral node such that they maximized probability of stochastic evolution of the extant state observed (Schluter et al., 1997; Pagel, 1999), and then characters were traced over our final molecular phylogenetic hypothesis using the “trace all characters” option in Mesquite. This allowed us to visualize the likelihood of all morphological and biogeographic states at ancestral nodes across multiple molecular phylogenies.

3 RESULTS

3.1 *DNA sequence variation and individual gene trees*

A total of 95 species in 51 genera (91 syngnathids and four outgroups in three genera of Syngnathiformes) were included in the analysis. The resulting data set is 95% complete, as not all DNA fragments were obtained from all taxa due to poor PCR amplification and putative primer mismatch at the annealing site (Supplemental Table 1). All new sequences were deposited in GenBank (accession numbers XXXXXXXX-XXXXXXX). The final data matrix consisted of 5,160 aligned base pairs (bp): 711 bp of *18S*, 533 bp of *HoxA13a*, 699 bp of *Myh6*, 512 bp of *Tmo4c4*, 439 bp of *12S*, 632 bp of *16S*, 655 bp of *COI*, and 979 bp of *ND1*. This dataset yielded

3,190 variable positions. For each gene, the number of variable sites, the optimal molecular evolutionary models chosen, and the number of nodes with strong support are given in Table 1, where high node support is defined as 0.95 posterior probability for Bayesian inference and 95 for maximum likelihood analysis. The two gene trees that displayed the highest resolution and greatest number of nodes with strong Bayesian posterior and RAxML 7.0.4 bootstrap support were *Myh6* and *ND1*, while the markers with the lowest resolution and fewest well-supported nodes were *I8S* and *COI* (Table 1). Although the tally of resolved nodes was low in *I8S* and *HoxA13a*, these two nuclear markers were successful in resolving deep nodes when used in combination with more variable markers.

Both the concatenated nuclear DNA (nDNA) sequence data ([Figure 1](#)) and the concatenated mitochondrial DNA (mtDNA) sequence data ([Figure 2](#)) produced trees with strong Bayesian posterior and Maximum Likelihood bootstrap support. There was high repeatability of clades derived from our single and combined mitochondrial (2,705 bp) and nuclear (2,455 bp) DNA data sets, with the notable exception of the placement of the seahorse genus *Hippocampus* ([Figure 2](#)). The single-locus trees varied in resolution and support ([Supplementary Figure 1](#)). The individual nuclear and mitochondrial gene trees recovered a suite of principal clades in both tail and trunk brooding lineages that were largely consistent with the combined nuclear gene tree, with mitochondrial genes generally showing lower node support. The full concatenated data set, including all eight gene fragments for 97 taxa and 5% missing data (Supplemental Table 1), recovered highly consistent patterns for the trunk brooders and early clades of tail brooders from both RAxML 7.0.4 *versus* MrBayes 3.2.1 methods (Supplemental Figure 5). However, the two

analyses conflicted in the arrangement of the crown clade of tail brooders, including seahorses (Supplemental Figure 5). We focus on analysis of nuclear and mitochondrial genomes independently, recognizing the potential incongruent evolutionary histories of these two data sources (see discussion below), and because nuclear genes have not yet been thoroughly explored as a source of information for syngnathid phylogeny. The hypothesis of syngnathid evolution that we advance here is based on the nuclear gene topology ([Figure 1](#)), which was the most highly resolved tree, with robust morphological and statistical support.

3.2 *Phylogenetic relationships*

Among sampled outgroups, the genus *Solenostomus* (the ghost pipefishes) is undisputed as sister to the family Syngnathidae (Kawahara et al., 2007; Orr & Fritzche, 1993; Wilson & Orr, 2011). Within Syngnathidae, the most fundamental split is between two major reciprocally monophyletic clades, the trunk- and the tail-brooders ([Figure 1](#)). Trunk brooders are comprised of the Mediterranean-Atlantic genera *Entelurus* and *Nerophis*, the endemic Southern Australian genera *Heraldia* and *Maroubra*, and three widespread Indo-Pacific clades: the genus *Choeroichthys*, the speciose and very widely distributed genus *Microphis*, and the flagtail pipefish *Doryrhamphus* and *Dunckerocampus*. The endemic Southern Australian monotypic genus *Leptoichthys* appears basal to all other trunk-brooders, including the genera *Entelurus* and *Nerophis* in the phylogeny we propose ([Figure 1](#)). However, this position is reversed in the mitochondrial-only analyses (Figure 2), with *Entelurus* and *Nerophis* as the ancestral clade.

The tail-brooders are the more diverse group, with striking examples of novelty and

diversity including the seahorses, seadragons, pygmy pipehorses, and many lineages of pipefish, some quite generic looking, and others that are quite exceptional. Within the seven principal subclades we identify ([Figure 1](#)), the earliest divergence is the Indo-Pacific genus *Corythoichthys* and the endemic Southern Australian genus *Stigmatopora* ([Figure 1](#), Clade i). The next diverging lineage is the seadragon lineage ([Figure 1](#), Clade ii) in which the basal member is the seagrass-associated Indo-Pacific alligator pipefish *Syngnathoides*, followed by the large Indo-Pacific pipehorse genus *Solegnathus*. The kelp-associated, exceptionally ornate, and closely related endemic Southern Australian leafy and weedy seadragons *Phycodurus* and *Phyllopteryx* appear as sister taxa, and are placed at the terminal position in this clade. The endemic and monotypic Southern Australian genus *Stipecampus* forms its own early-diverging branch ([Figure 1](#), Clade iii). These phylogenetic patterns are mirrored in both the mitochondrial and nuclear trees (Figure 2).

The crown clades of tail-brooding syngnathids include highly novel, very well-supported groupings, with surprisingly distinct morphologies appearing among closely related lineages. One large Indo-Pacific lineage (Clade iv) includes a striking diversity of forms, from the very small worm-like pipefish genera *Apterygocampus*, *Nannocampus* and *Siokunichthys* to a monophyletic group of closely related Southern Australian endemics with a bizarre range of snout morphologies ([Figure 1](#)). Another consistently recovered clade appears to share a common ancestor with Atlantic Ocean origins ([Figure 1](#), Clade v), and includes the highly speciose genus *Syngnathus* as well as the Atlantic pygmy pipehorse *Amphelikurus*, demonstrating independent evolution of the diminutive and highly cryptic pygmy pipehorse morphology in both the Atlantic

and the Indo-Pacific. The closely related Indo-Pacific pygmy pipehorse genera *Acentronura* and *Idiotropiscus* appear as sister taxa and were consistently recovered in a clade together with the elongated Indo-Pacific genera *Filicampus* and *Trachyrhamphus*, the ribboned pipefish *Haliichthys taeniophorus*, and the small South Pacific genus *Lissocampus* ([Figure 1](#), Clade vi). This is a surprising set of relationships among highly morphologically distinct genera that has not been inferred previously. Support for many of these novel clades increases when the combined nuclear and mitochondrial data is analyzed for the subset of 75 taxa for which there are no missing data (Supplementary Figure 2).

Finally, the seahorse genus *Hippocampus* forms a globally distributed and well defined clade ([Figure 1](#), Clade vii). The ten sampled seahorse species cluster into pygmy and non-pygmy lineages, with a notable evolutionary distance between these two groups. In the nuclear analysis, the seahorse genus *Hippocampus* is nested within the unusual tail-brooding crown clade “vi” that includes Pacific pygmy pipehorses, ([Figure 1](#)), whereas in the mitochondrial analysis, *Hippocampus* resolves as sister to the Atlantic tail-brooding crown clade “v” (Figure 2).

3.3 *Morphological character analysis*

Although applying cladistic tree-building directly to morphological characters was largely uninformative (Supplemental Figure 4), mapping reconstructed ancestral character states onto the molecular phylogeny allowed us to discriminate between alternative molecular phylogenies. When mapped onto alternative molecular topologies, four morphological characters support the placement of *Hippocampus* described by the nuclear gene analysis (Supplemental

Figure 3g, 3h, 3i, 3j), while not a single character supports the sister relationship between seahorses and the Atlantic clade including *Syngnathus* (Clade v), as suggested by the mitochondrial analysis and previous studies (Wilson et al 2001; Wilson & Orr 2011; Figure 2). Regardless of the genetic markers used to build the phylogeny, our results suggest repeated parallel evolution of the morphological features that make syngnathids so unique, including brood-pouch traits such as the full closure of the male brood structure, body ridge configuration patterns, loss of fins, and grasping features of the tail. Each of these convergences suggests a trend towards the loss of fins and elongation of body plan, and increasing complexity in brood-pouch features.

3.4 *Biogeography*

We explored basic biogeographic patterns by tracing the distributions of all extant species and the maximum likelihood estimated ancestral states calculated in Mesquite v2.72 onto our leading phylogenetic hypothesis (Figure 3). Many clades contain species with distributions that range across the Atlantic, Pacific, and Indian Oceans, including the Red Sea. Regardless of specific topology considered (i.e. mitochondrial *versus* nuclear tree), there is clear evidence of multiple invasions of both the eastern and western Atlantic. Sampled taxa within tail-brooding Clade v share an Atlantic origin, including the pygmy pipehorse *Amphelikturus*, the worm-like pipefish *Penetopteryx*, the freshwater pipefish *Pseudophallus*, and members of the pipefish genera *Anarchopterus*, *Bryx*, *Cosmocampus*, and *Micrognathus*. In the Indo-Pacific, putative conspecifics of these last three genera appear phylogenetically distinct from their Atlantic

counterparts. Multiple lineages have independently dispersed to the eastern Pacific (Figure 3), which has the lowest species diversity of any ocean biome inhabited by syngnathids.

Australia, specifically temperate southern Australia, is confirmed as an important center of origin in several clades, including the seadragons, and one of the principal tail-brooding clades (Clade iv), comprised of six closely related genera of largely sympatric pipefish found in seagrass habitats. In both the trunk-brooding and tail-brooding clades, multiple independent lineages have diversified in Australian waters, with fully six of the seven principal tail brooding clades evolving Australian endemics.

4. DISCUSSION

A phylogenetic foundation allows for deeper understanding of the evolutionary patterns and environmental forces that have shaped the diversity of shallow water fishes. The advancement of DNA sequencing methods has improved capacity for phylogenetic inference by adding a vast number of characters. However, there is growing concern that reliance on molecular data and statistical methods alone prevents objective assessment of alternative phylogenetic topologies (Mooi & Gill, 2010; de Carvalho & Craig, 2011). Simultaneous investigation of both molecular and morphological characters can apply synapomorphies to help distinguish among alternative topologies. Here, we present a phylogenetic hypothesis supported by molecular and morphological data for a family of diverse, derived, and exploited fishes that has previously lacked a comprehensive or consensus view of their evolutionary relationships. Our study confirms the long-recognized and early break between trunk- and tail-brooding

syngnathids, and suggests that complex brooding structures likely evolved multiple times and are not synapomorphic for deep splits in syngnathid evolution.

Our leading hypothesis is based on sequence data from four nuclear genes. We paid special attention to the added resolution from *HoxA13a*, considering the ability of this marker to infer first order paralogy. *Hox* genes have not traditionally been invoked as neutral markers for phylogenetic studies because they are generally constrained and slowly evolving (Hoeg *et al.*, 2007; Crow *et al.*, 2012). However, we find here the *HoxA13a* gene tree generally agrees with the topology retrieved from other unlinked nuclear loci (Supplemental Figure 1). We also find some evidence of nuclear-mitochondrial incongruence, most notably in the placement of the seahorse lineage. Thus, we provide an alternative topology based on four mitochondrial fragments, and map onto both trees 11 morphological characters considered informative in traditional syngnathid systematics (e.g. Herald, 1959; Dawson, 1982; 1985; Figure 2). This approach finds that a suite of morphological synapomorphies supports the topology derived from combined nuclear genes. Our results identify a series of novel and often surprising clades that emphasize the morphological plasticity underlying the syngnathid body plan. We discuss the evolutionary and biogeographic implications of our findings, the need for comprehensive morphological studies given new phylogenetic relationships discovered here, the need for further taxonomic revision in some genera, and propose a revision of subfamily classification.

4.1 *Phylogenetic relationships*

Our proposed topology ([Figure 1](#)) contains groups that are retrieved repeatedly in multiple analyses, and strongly confirms the fundamental division among syngnathids into trunk-brooding versus tail-brooding pipefish. This deep split is also well established in the literature, and suggests the independent evolution of male brood pouch complexity, with various closure types in both major lineages. Character convergence is evident in body ridge configurations, repeated loss of pouch plates, anal fins, and caudal fins, and independent evolution of prehensile tails. These new phylogenetic hypotheses provide opportunities for future studies of character evolution in syngnathids with more comprehensive species sampling.

4.1.1 *Deep evolutionary split in tail vs. trunk brooders*

The deep phylogenetic break between the trunk-brooders (also called abdomen-brooders or Gastrophori cf. Herald 1959) and the tail-brooders (also called Urophori cf. Herald 1959) is the most salient pattern in syngnathid evolution. The one exception is the “alligator pipefish”, *Syngnathoides biaculeatus*, which phylogenetically falls with tail-brooding seadragons (Clade ii) despite simple male brood structures on the trunk (Fig. 3). The primary split between these two major lineages was also recovered by Wilson *et al.* (2003), and is further supported by evidence of a complete genome duplication in early trunk-brooders (Vitturi *et al.*, 1998). We propose a subfamily classification that reflects this well-established break: (i) the subfamily Syngnathinae, the tail-brooders, based on the type genus for the family *Syngnathus* proposed by Linnaeus (1758), encompassing 46 of the 57 valid genera and 244 currently recognized species, and (ii) the subfamily Nerophinae, the trunk-brooders, based on the type species *Nerophis ophidion*, also

described by Linnaeus (1758), including 11 out of the 57 valid genera and 56 species (Supplementary Table 2). In this view of syngnathid evolution, the seahorse genus *Hippocampus* is a derived lineage within the Syngnathinae, and we question the usefulness of retaining *Hippocampus* in its own subfamily Hippocampinae.

4.1.2 *Evolutionary relationships among the trunk-brooders*

Phylogenetic relationships among the diversity of trunk-brooders have never been closely examined with molecular data due to a lack of taxon sampling. Although less speciose than the tail-brooders, these pipefishes possess substantial diversity of form and habit. They exhibit a large range in body-size, from the genus *Leptoichthys*, which is the longest recorded pipefish exceeding 600 mm standard length (SL), to the genus *Choeroichthys*, which is among the smaller pipefishes with adults less than 30 mm SL. The trunk-brooders exhibit a diversity of body plans adapted to a broad range of habitats such as seagrass, macroalgae, rubble, caves and crevices, estuaries, and even freshwater (e.g. in the genus *Microphis*). For example, the genera *Entelurus* and *Nerophis* are worm-like pipefish that have lost the anal, pectoral, and caudal fins, and thus the ability to swim effectively, and live in seagrass and macroalgae just below the low-tide line, while the “upside-down” pipefish and the “flagtail” pipefish (genera *Heraldia*, *Doryrhamphus*, and *Dunckerocampus*) have anal, pectoral, and large caudal fins and are efficient swimmers that navigate overhanging reef structures.

The basal taxon in trunk-brooders is either *Leptoichthys* or a clade containing *Entelurus* and *Nerophis*; this topological position is inconsistent between nuclear and mitochondrial

analyses (Figure 2). In our leading phylogenetic hypothesis ([Figure 1](#)), the genus *Leptoichthys* emerges as the basal taxon, while in the mitochondrial tree, the sister pair *Entelurus* and *Nerophis* are placed as the basal trunk-brooders. Four derived morphological characters support *Leptoichthys* as the basal member of the Nerophinae, consistent with the nuclear topology, while not a single character supports the mitochondrial tree's placement of *Entelurus* and *Nerophis* as the basal lineage (Figure 2). Also within the trunk-brooders, an unexpected grouping of *Microphis* with the flagtail pipefish emerged ([Figure 1](#)), supported by principal ridge configuration patterns that are shared by *Doryrhamphus*, *Dunckerocampus* and *Microphis* (Dawson 1984).

4.1.3 *Evolutionary relationships among the tail-brooders*

There is strong support in all analyses for the division of the tail brooders into a suite of principal clades, with all data sets, including nDNA, mtDNA, and morphological characters identifying three basal clades (Figure 2; Clades i, ii, and iii). A lineage composed of the Indo-Pacific genera *Corythoichthys* and *Stigmatopora* is placed as the most ancestral group (Clade i). Shared morphological traits between these basal sister taxa are thought to represent the ancestral state for tail brooders, and include semi pouch closure, continuous inferior ridges, straight and terminating lateral ridge, and a dorsal fin base that is not elevated ([Supplemental Figure 3](#)). The seadragons (Clade ii) diverge early from the remaining tail-brooders, and include the ornate genera of the leafy and weedy seadragons, *Phycodurus* and *Phyllopteryx*, as well as the large prehensile pipehorse genus *Solegnathus* and the seagrass mimic prehensile pipefish

Syngnathoides. Synapomorphies for this group include the loss of a caudal fin and the absence of brood pouch folds; however, there are differences in placement of the brooding area (i.e. trunk in *Syngnathoides*, and tail in all others), and in the configuration of trunk and tail ridges ([Supplemental Figure 3](#)). Another early diverging lineage is the monotypic Southern Australian endemic genus *Stipecampus* (Clade iii), which appears unique in its placement among sampled taxa.

The crown group of tail-brooding syngnathids is comprised of several principal clades that contain the remaining sampled taxa; we define four major well-supported groupings ([Figure 1](#); Clades iv - vii). The tail-brooders again demonstrate the fluidity underlying the syngnathid body plan, and display remarkable convergence in morphological evolution. For example, the proposed topology reveals a monophyletic Indo-Pacific clade of small, worm-like pipefish united by loss of the anal fin that includes *Nannocampus*, *Siokunichthys*, and *Apterygocampus*. Their relationships reveal a progressive pattern of fin loss: where *Nannocampus* and *Apterygocampus* have lost the pectoral and anal fins, *Apterygocampus* has also lost the dorsal fin, making it an extreme worm-like pipefish. Loss of three sets of fins is rare in syngnathids, and is only found in three genera of the 48 sampled here, including *Nerophis*, which is a trunk-brooder. Our phylogeny suggests the evolution of this extreme worm-like body plan arose at several times, independently in the eastern Atlantic within the trunk-brooders, and at least twice in the tail-brooders with the Atlantic *Penetopteryx* and the Indo-Pacific worm-like clade ([Figure 1](#)). The similarity between Atlantic *Penetopteryx* and Indo-Pacific *Apterygocampus* was noted by Dawson & Allen, 1978, and the latter was only removed from the former recently. We did not

sample Indo-Pacific *Bulbonaricus* or the monotypic, southern Australia endemic *Notiocampus*. We hypothesize that *Bulbonaricus* falls within the Indo-Pacific worm pipefish clade, and that *Notiocampus* represents a fourth independent evolution of a worm-like morphology in syngnathid fishes.

The pygmy pipehorse body plan (diminutive size, prehensile tail, loss of anal and caudal fins, angled head, and complete closure of brood pouch) appears to have evolved independently in both the Indo-Pacific and in the Atlantic. These two lineages exhibit remarkable similarities in their morphologies, to the extent that Dawson (1984) suggested all pygmy pipehorses shared a common ancestor. Both have angled heads, similar complex brood pouch morphology, an elevated dorsal fin base, shared lateral ridge configurations, and a prehensile tail. What is most surprising, however, is that none of the closest relatives of the Atlantic pipehorse share these specific traits. Several morphological differences support our findings of independent evolution of Atlantic and Pacific pygmy pipehorses. The Indo-Pacific genera *Acentronura* and *Idiotropiscus* have their dorsal fin origin on the trunk and do not have a caudal fin. In contrast, the Atlantic pipehorse *Amphelikturus* has its dorsal fin origin on the tail, and retains a rudimentary caudal fin (Dawson, 1985). Despite their superficial similarities, we find no support for the conclusion that pygmy pipehorses represent an evolutionary link between seahorses and other free-swimming benthic pipefish (Teske and Beheregaray 2009).

With significantly more thorough taxon sampling among Atlantic syngnathids than any previous study, we identify a large and morphologically diverse clade united by common ancestry in the Atlantic ocean that includes the genus *Syngnathus*, the most speciose of all genera

(Clade v). The Atlantic clade contains lineages currently recognized as the genera *Cosmocampus* and *Micrognathus*, which both have conspecifics found in the Indo-Pacific. However, our phylogeny finds the Indo-Pacific lineages of *Cosmocampus* and *Micrognathus* are entirely independently evolved, suggesting their putatively diagnostic body ridge configurations are a result of convergence. More complete sampling of species within these genera from both the Atlantic and Pacific is needed to support taxonomic revision and address their apparent polyphyly.

While many revealing evolutionary patterns are well-supported by our analyses, the question of the placement of seahorses is not unambiguously resolved. Two alternatives were found, one of which places the genus *Hippocampus* within the novel Indo-Pacific lineage “vi”, which includes, the large, highly elongate pipefish *Trachyrhamphus*, the ornate Australian “ribboned pipefish” *Haliichthys*, and the Pacific pygmy pipehorses *Acentronura* and *Idiotropiscus* ([Figure 1](#)). Alternatively, the analyses completed with only mtDNA characters placed *Hippocampus* sister to the Atlantic clade (v), which includes the speciose and well studied genus *Syngnathus*, an arrangement that has been proposed in previous molecular studies (Wilson *et al.* 2001, 2003; Wilson & Rouse 2010; [Figure 2](#)). When morphological characters are included to test support among these two interpretations, the placement of *Hippocampus* within the Indo-Pacific Clade “vi” has significantly more support, with four synapomorphies among ridge configuration patterns and dorsal fin morphology ([Figure 2](#); see [Supplemental Figure 3](#) for details). Not a single examined morphological character supports the position of *Hippocampus* as sister to the Atlantic clade. We advocate the evolutionary pattern as reflected in the nDNA

topology ([Figure 1](#)). In this view of *Hippocampus*, seahorses are a highly derived lineage of pipefish that originated in the Indo-Pacific and successfully diversified into a unique, speciose, widely-distributed lineage of tail-brooding pipefish.

4.1.4 *Mitochondrial - nuclear incongruence*

Until recently (i.e. Teske & Behrer, 2009), nDNA sequences had not been used in phylogenetic inference of syngnathids, and even this work was highly limited in taxon sampling, with only 8 of 57 recognized genera included. All other molecular work to date has focused exclusively on mtDNA (Wilson *et al.* 2001, 2003; Wilson & Rouse 2010). Given that mtDNA and nDNA gene trees are often incongruous (Shaw, 2002; Chan & Levin, 2005; Currat *et al.* 2008; Toews *et al.* 2012; Shaw 2002; Rubinoff & Holland, 2005; Fisher-Reid & Wiens, 2011), and that mtDNA markers are essentially a single locus (Madison, 1997; Springer *et al.*, 2001, Bossu & Near, 2009), previous mtDNA based phylogenies are vulnerable to misleading topologies. We employ two strategies to recover a well-supported phylogenetic hypothesis with molecular techniques: (i) simultaneous analysis of multiple unlinked nDNA and mtDNA markers, and (ii) targeted use of genes that exhibit short coalescence times relative to the speciation rate.

Our results uncovered several instances of incongruence between mtDNA and nDNA gene trees, including the placement of the seahorse clade, the identity of the basal lineage of trunk brooders, and the monophyly of *Halicampus*. Incongruence can arise because of incomplete lineage sorting (Maddison, 1997; Sota & Vogler, 2001; Funk & Omland, 2003;

Hoelzer, 1997) and introgression (Currat et al. 2008; Busso & Near, 2009; Moore, 1995; Hoelzer, 1997; Keck and Near, 2010; Shaw, 2002; Chan and Levin, 2005; Toews et al. 2012). When all molecular data were combined for a single analysis, the results of the RAxML 7.0.4 *versus* Bayesian reconstructions produced consistent evolutionary patterns for the Gastrophori and the basal Urophori; however, the two methods produced conflicting topologies among the principal clades of the tail brooders crown group (Supplemental Figure 5). Although alternative hypotheses need to be explicitly tested, we posit that hybridization and mtDNA introgression is the most likely explanation for mt/nDNA incongruence in syngnathids. Hybridization is common in animals (Mallet et al 2005; Schwenk *et al.* 2009), and is long recognized as an important evolutionary force in fishes (Hubbs *et al.* 1955; Scribner *et al.* 2000). Hybridization is thought to be responsible for mt/nDNA incongruence in multiple fish lineages (Scribner *et al.* 2000; Willis *et al.* 2013), and has been documented in syngnathids (Wilson *et al.* 2006; Moser *et al.* 2013; Otero-Ferrer *et al.* 2015; Hablützel 2009, 2010). In light of mt/nDNA incongruence and suspected past transfer of mtDNA through hybridization, we present the nDNA and mtDNA trees separately, and assess support between these alternative hypotheses by mapping morphological characters used in traditional syngnathid systematics. Overall, the nDNA tree had substantially greater morphological support and higher statistical support over the mtDNA tree (Table 1; Figure 2), and we present this as our leading hypothesis of syngnathid evolutionary relationships ([Figure 1](#)).

Another incongruence is in the monophyly of the genus *Halicampus*. In the nDNA tree *Halicampus macrorhynchus* alone comes out as sister to the *Filicampus/Trachyrhampus* clade

with moderate support (.98 MrBayes 3.2.1 posterior probability, 75 RAxML 7.0.4 bootstrap value; Figure 2, Clade vi). In contrast, in the mtDNA tree, all other sampled *Halicampus* species group with the large, “ornate pipefish” *Halicampus macrorhynchus* with moderate support (.99 MrBayes 3.2.1 posterior probability, 65 RAxML 7.0.4 bootstrap value), and are placed sister to the *Filicampus/Trachyrhampus* clade with very low support (.68 MrBayes 3.2.1 posterior probability, 25 RAxML 7.0.4 bootstrap value). The polyphyly of *Halicampus* was originally suggested by Dawson (1985), and is supported by several morphological characters of the snout. There are at least a dozen additional species of *Halicampus* not sampled here, most of which are small and cryptic. Pending further taxon sampling, the monophyly and placement of *Halicampus* remains unresolved.

The identity of the basal lineage of trunk-brooders differs between the nuclear and mitochondrial topologies. In the nDNA tree, the Australian monotypic *Leptoichthys* is basal, while in the mtDNA tree, the lineage shared by the Mediterranean *Entelurus* and *Nerophis* is basal. As with the placement of *Hippocampus*, there are a suite of morphological characters supporting the topology produced by the nuclear tree. Three different body ridge configuration characters and two pouch characters, including pouch closure type ([Supplemental Figure 3](#)), offer morphological evidence supporting the nuclear hypothesis. While *Entelurus* and *Nerophis* appear “primitive” due to their lack of an anal fin and possession of only rudimentary caudal fins, loss of fins are thought to be the derived state (Dawson & Allen, 1978). *Leptoichthys* shares the presence of an anal fin and well developed caudal fins with all other trunk-brooders. Not one of the 11 examined morphological characters supports the mtDNA topology placing *Entelurus* &

Nerophis in the basal trunk-brooder position. At the generic level, it is unlikely further taxon sampling will help resolve this issue, as *Doryichthys* is the only genus of trunk-brooding syngnathid not included in this analysis. Analysis of expanded genetic markers are needed to confirm our hypothesis of the basal trunk-brooding lineage.

4.2 *Morphological evolution*

Our analysis incorporated the most widely used morphological characters for diagnosing syngnathid genera and species (Herald, 1959; Herald & Hanna, 1965; Dawson, 1983; Dawson, 1985; Randall & Earle, 1994; Gomon, 1997; Browne & Smith, 2007; Allen & Erdmann, 2008). These traits include principal body ridge configurations, brood pouch structures, trunk and tail ring counts, the presence or absence of anal fin, and fin ray counts. Our results demonstrate that many of these characters accurately distinguish between closely related genera and species at shallow evolutionary time scales. However, there is evidence of extensive homoplasy at deeper time scales, limiting the utility of these characters for resolving deeper nodes. There is need for a description of new homologous morphological traits, such as perhaps osteological characters, to better resolve the phylogeny of this group. Here we focused on the application of morphological traits to distinguish among alternative molecular hypotheses in the arrangement of crown clades, and to distinguish patterns of phenotypic evolution suggested by our leading phylogenetic hypothesis. This new phylogeny provides an opportunity for future, more rigorous morphological studies of syngnathid fishes.

Previous studies of phenotypic evolution in syngnathids pointed to repeated convergence in brood-pouch traits and body plan, which has left researchers uncertain of the evolutionary origins of complex brood-pouches (Wilson & Meyer, 2001), the origin of seahorses (Casey *et al.*, 2004; Teske *et al.*, 2004; Wilson & Rouse, 2010), and taxonomic classification. Our analyses confirm a clear phylogenetic split in syngnathids based on brood pouch placement, but also point to a high degree of morphological fluidity in syngnathids, and multiple examples of homoplasy in body ridge configuration, head angle, loss of fins, snout and tail morphology, body size, and development of skin filamentation. In particular, there are numerous similarities between several Atlantic and Pacific forms, such as the worm-like pipefish and pygmy pipehorses. Complex brood pouch structures and fully prehensile tails appear multiple times across the tree, and are discussed in details below.

4.2.1 *Brood structures*

All syngnathids display paternal care in the form of male brooding, and the diversity found in brood structures has both fascinated and puzzled researchers. Some species have a simple ventral area for egg-gluing, while others have protective pouch plates, or skin folds with no closure, everted, semi-everted, or inverted pouch closure, or complete closure with just a small slit or pore for receiving eggs and delivering hatched juveniles. Given the paucity of morphological characters for informing patterns of syngnathid evolution, brood pouch structure has been frequently utilized for phylogenetic inference. Historically, the evolutionary pathway from simple to complex brood structures was thought to have progressed linearly in both tail-

and trunk-brooding lineages, culminating in tail-brooders with completely enclosed brood pouches in the genus *Hippocampus*, and in the trunk-brooders with the well-defined pouches in *Microphis* and *Doryrhamphus* (Dunker 1912; Herald 1956; Dawson, 1985).

More recent molecular approaches call this idea into question, and the evolutionary pathway of brood pouch complexity remains unclear (reviewed in Wilson & Orr, 2011). The mtDNA phylogeny of Wilson *et al.* (2001, 2003) suggested that most major pouch types arose once early in the syngnathid evolution. They also suggest a sister relationship between *Hippocampus* and *Syngnathus*, a proposal that was reinforced with additional mtDNA data by Wilson and Rouse (2010). An alternative arrangement based on a multi-marker analysis by Teske & Beheregaray (2009) concluded the seahorse genus *Hippocampus* is sister to the Pacific pygmy pipehorse *Idiotropiscus*, with both genera sharing the synapomorphy of fully enclosed brood pouches that open proximally through a pore. Given the limited taxon sampling in previous studies, brood pouch morphology remains a problematic synapomorphy for elucidating higher-order relationships. The significantly broader taxon sampling in this study supports a completely novel hypothesis that places seahorses within a previously unrecognized grouping of Indo-Pacific genera, and definitively refutes the sister relationship of *Hippocampus* and *Idiotropiscus*.

Our results suggest complexity in the male brooding structure has arisen multiple times independently, with the possibility of convergent evolution of complex brood structures across many lineages of syngnathids (Supplemental Figure 3d, 3e). There appear to be multiple pathways to complex brood pouch morphology. We suggest that full closure of the brood pouch

has evolved four times in syngnathids: in the seahorse *Hippocampus*, the Pacific pygmy pipehorse lineage (*Acentronura* + *Idiotropiscis*), in the tiny, worm-like pipefish *Apterygocampus*, and in the Atlantic pygmy pipehorse *Amphelikturus* (Supplemental Figure 3d, 3e). Four of the five genera with advanced brood pouches have prehensile tails; only *Apterygocampus* does not. We speculate that this suite of traits may have evolved in association with taxa that occur at low densities, and exhibit a putatively monogamous mating system, at least in *Hippocampus*. The genera *Acentronura*, *Idiotropiscus*, and *Amphelikturus* are poorly known and require further study to distinguish their mating systems.

4.2.2 Tail morphology

Our results also provide evidence for a remarkable convergence in tail morphology. The caudal fin is lost multiple times independently (Supplemental Figure 3m). The basal trunk-brooders (*Nerophis* and *Entelurus*) share a loss of all fins but the dorsal fin (Supplemental Figure 3n), while the seahorses, the Pacific pygmy pipehorses, the ribboned pipefish *Haliichthys taeniophorus*, the primitive trunk brooder *Stigmatopora narinosa*, and the seadragons (*Solegnathus*, *Syngnathoides*, *Phyllopteryx*, *Phycodurus*) all have lost the caudal fin, but occur in various clades throughout the phylogeny (Supplemental Figure 3m). Loss of the caudal fin may be correlated with prehensile tails, however there are species with grasping tails that retain a rudimentary caudal fin (e.g. *Amphelikturus*).

Some genera of syngnathids are unique in being able to grasp substrate with their tail. Examples of syngnathids with prehensile tails include the seahorses, Pacific pygmy pipehorses,

the ribboned pipefish *Haliichthys taeniophorus*, and the seadragon genera *Syngnathoides* and *Solegnathus* (Supplemental Figure 3I). Neutens *et al.* (2014) conducted a detailed study of the musculoskeletal organization of the tail in these groups, and found that through alternate types of skeletal modifications there is more than one way to achieve a prehensile tail. In the typical “pipefish” (which is assumed to be the ancestral state), dermal plates are large and articulate tightly with vertebral segments to create a rigid structure. Instead, in seahorses, Pacific pygmy pipefishes, and *Haliichthys*, the size of dermal plates gradually reduce from the head to the tip of the tail and modified joints achieve a gradual bending pattern. In complete contrast, the prehensile members of the seadragons and several basal genera (e.g. *Nerophis*, *Entelurus*, *Stigmatopora*) create flexibility in only the last 30% of the length of the tail with simple loss of dermal plates (Neutens *et al.*, 2014).

Our topology supports multiple pathways of achieving a prehensile tail in syngnathid fishes, and further suggests that prehensile tails may have evolved not just twice, but as many as nine times. In the trunk-brooders, the *Nerophis* and *Entelurus* lineage has a prehensile tail with half of their body involved in grasping. In the basal tail-brooders, a single species, *Stigmatopora narinosa* has a tail grasping behavior. Interestingly, this species is in the same clade as the pipefish *Corythoichthys*, which was chosen by Neutens *et al.* (2014) as the ancestral state of syngnathid musculature. In the prehensile seadragons, grasping is confined to the last 30% of the tail. In the Indo-Pacific and Australian clade “iv” ([Figure 1](#)), *Urocampus* has the distal half of its body involved in grasping. The Atlantic clade “v” has evidence of two independent acquisitions of grasping tails, once in *Amphelikturus* and again in *Syngnathus abaster*, a species not sampled

here. In the Indo-Pacific clade “vi”, many genera have grasping tails (Supplemental Figure 3l). The musculoskeletal anatomy of these genera needs to be examined to determine if it functions as a synapomorphy for this group. In this assemblage, *Lissocampus caudalis* (with only loose grasping) and the Pacific pygmy pipehorses *Acentronura* and *Idiotropiscus*, the ribboned pipefish *Haliichthys taeniophorus*, and *Hippocampus* all have various levels of tail grasping. Finally, several other examples of tail grasping exist in genera, including *Kyonemichthys rumengani* and *Notiocampus ruber* (which only has loose grasping), for which phylogenetic relationships remain unknown. In all, our phylogeny strongly suggests multiple independent evolution of grasping tails in syngnathid fishes (Supplemental Figure 3l) using several alternative biomechanical configurations. Additional species sampling and more comprehensive morphological studies will likely reveal further diversity in prehensile tail forms among syngnathids.

4.3 Biogeography

Maximum likelihood mapping of geographic distribution patterns demonstrates syngnathids are highly successful at dispersal and colonization. They occur in all major ocean provinces except polar regions, they have invaded freshwater multiple times, and some genera have circled the globe (e.g., *Hippocampus*, *Microphis*). Representatives of all genera exclusive to east Atlantic, west atlantic, east and central pacific are included here, with only two missing genera from Australia (*Kimblaeus* and *Notiocampus*), and seven missing genera from the

Indo-Pacific (*Bulbonaricus*, *Campichthys*, *Doryichthys*, *Ichthyocampus*, *Kyonemichthys*, *Leptonotus*, and *Minyichthys*).

Both subfamilies we designate here - Nerophinae and Syngnathinae - have lineages distributed in all ocean biogeographic regions in which syngnathids occur (Figure 3). A pattern is evident of tropical Indo-Pacific taxa that have temperate Australian sister lineages, and this occurs multiple times in distant parts of the tree. Radiation is evident of specific tail-brooding lineages in the Atlantic (clade v) and in Australia (clade vi).

These patterns demonstrate that two hypothesized evolutionary processes for fish diversification are occurring contemporaneously in syngnathids. The “center of origin” hypothesis suggests diversity arises through in situ speciation (Rocha et al., 2008), which is supported here by the recovery of clades originating from a common ancestor in both the Atlantic (Tail-brooders clade v) and in Australia (Tail-brooders clade iv). In the “center of accumulation” hypothesis, species originate in other oceanic provinces then accumulate in certain regions via major patterns of dispersal (Rocha et al., 2008), which is demonstrated here by independent invasions in several Atlantic lineages and especially in multiple, diverse southern Australian lineages. Australia clearly stands out as possessing an extraordinarily diverse syngnathid fauna, with 43% of all syngnathid species occurring in Australian waters, derived from lineages distributed broadly across the phylogeny (Figure 3).

4.3.1 *Atlantic radiation*

Extensive taxon sampling of almost all currently recognized Atlantic genera (missing only the genus *Minyichthys*) demonstrates both multiple Atlantic invasions from within Indo-Pacific clades, as well as an Atlantic radiation from a common ancestor (Syngnathinae clade v). Recent invasions from the Indian Ocean via southern Africa, e.g. in the genus *Microphis*, may also be contributing to syngnathid diversity in the Atlantic (Casey et al., 2004, Floeter et al., 2008). These patterns indicate multiple processes have shaped the diversity of Atlantic syngnathids, including both the “center of origin” and the “center of accumulation” hypotheses of evolutionary diversification (Rocha et al., 2008).

The near complete generic-level taxon sampling in this study provides the first evidence for a speciose, morphologically and ecologically diverse Atlantic clade (Tail brooders clade v), including poorly known syngnathids such as the pygmy pipehorse *Amphelikturus*, the diminutive *Anarchopterus*, the worm-like *Penetopteryx*, the obligate freshwater species *Enneacampus* and *Pseudophallus*, and the second most diverse genus in the family, *Syngnathus* ([Figure 1](#), Figure 3). Within this Atlantic group, two subclades are identified, one of which is formed by *Pseudophallus* and *Syngnathus*. *Pseudophallus* (4 species) is restricted to freshwater drainages in Central and South America, while *Syngnathus* comprises 32 species (Froese & Pauly; 2015) that are widely distributed in the Greater Caribbean, the Eastern Atlantic, and the Mediterranean. The second sub-clade contains the eastern Atlantic genus *Enneacampus* and a rich diversity of western Atlantic species, suggesting that ancestors of this sub-clade dispersed across major biogeographical barriers, including the mid-Atlantic barrier and the outflow of the Amazon river (Floeter et al., 2008). Members of both Atlantic sub-clades demonstrate ecological tolerance of a

wide range of salinities, and interestingly, the basal lineages of each sub-clade, *Enneacampus* and *Pseudophallus*, are found in coastal rivers and streams. *Syngnathus*, despite being mostly marine, has several euryhaline species distributed in freshwater lakes, rivers, and upper estuaries (*S. abaster*, *S. fuscus*, *S. schmidtii*, and *S. scovelli*; Cakić et al., 2005; Edward et al., 2002; Enghoff and Ediger, 2015; Tutman et al., 2012; Whatley 1969).

Some Atlantic syngnathids, such as in the highly speciose genera *Hippocampus* and *Syngnathus*, reveal a pattern of wide-ranging trans-Atlantic colonization via west-to-east dispersal and large-scale diversification that follows the major Atlantic currents: the North and South Atlantic Currents, the Gulf Stream, and the Brazil and Benguela Currents (Boehm *et al.* 2013; Mwale, 2005). This may be due to their ability to sporadically disperse long distances through rafting on floating mats of *Sargassum* and other macroalgae (Casazza and Ross 2008; Woodall *et al.*, 2009; Boehm *et al.*, 2013). Additionally, these two genera may have benefited from invasion of the extensive seagrass habitats that occur in the both temperate and tropical Atlantic (Short *et al.*, 2007). In contrast, the speciose trunk-brooder genus *Microphis* is depauperate in the Atlantic with only two valid subspecies, *M. brachyurus lineatus* and *M. brachyurus aculeatus*, which are distributed in the Western and Eastern Atlantic, respectively, compared to the 18 recorded species of *Microphis* in the Indo-Pacific. This disparity in species diversity in *Microphis* between ocean basins might reflect effective soft biogeographical barriers (e.g. the cold Benguela current) to east-west founder dispersal and colonization from the Indian to the Atlantic oceans (Floeter et al., 2008; Rocha, 2008).

There are remarkable convergences in highly derived morphologies between members of this newly recognized Atlantic radiation and phylogenetically distant Indo-Pacific counterparts. In some cases, the morphological convergence is so great that species were originally assigned to genera that we find to be distantly related. Examples include the pygmy pipehorse *Amphelikurus dendriticus*, which was originally classified in the Pacific pygmy pipehorse genus *Acentronura*. Similarly, the diminutive, finless Atlantic genus *Penetopteryx* was originally classified as the morphologically similar Indo-Pacific genus *Apterygocampus*. Our results indicate that both these genera have independently evolved extreme worm-like morphology, with shared losses of the anal, pectoral, and caudal fins, and identical configuration of principal body ridges. A convergence in snout morphology can be seen in the seagrass-associated Atlantic species *Syngnathus typhle* and the Southern Australian species *Leptoichthys fistularius*, which both have similar long, flat, and broad tube snouts.

There are five additional genera with species found in both the Pacific and Atlantic. For two of these, *Cosmocampus* and *Micrognathus*, we find Atlantic and Indo-Pacific lineages to be only distantly related, with each genus appearing in the Atlantic clade v and independently in the Indo-Pacific clade vi ([Figure 1](#), Figure 3). Significant taxonomic revision is clearly required, highlighting the prevalence of convergent morphologies between ocean basins, and the challenge of diagnosing syngnathid genera with characters such as body ridge configuration patterns. The remaining three genera putatively found in both ocean basins have not been genetically sampled in either the Pacific (*Bryx*), the Atlantic (*Penetopteryx*), or both (*Minyichthys*). It is likely that as

additional species of these 3 genera are investigated, more polyphyletic lineages may be identified.

4.3.2 *Australian diversity and endemism*

While the center of diversity for the majority of marine fish families is in tropical regions such as the Greater Caribbean and the Indo-Pacific coral triangle, (Rocha et al., 2008; Allen, 2007), in Syngnathidae, diversity is extremely high in Australian waters, with 129 out of 300 species occurring on Australian coastlines (43%), including 30 seahorse species, or 59% of all those currently recognized (Froese & Pauly, 2015). Every major clade of syngnathids we identify, with the exception of the Atlantic clade, includes at least one Australian member. These lineages are very broadly distributed throughout the phylogeny in both the trunk- and tail-brooders. In addition, an Australian endemic radiation occurred within one major tail-brooding clade (Figure 3, clade iv).

Endemism is also very high, particularly in the temperate waters of southern Australia. Sixteen syngnathid genera are endemic to Australia, 15 of which are endemic to southern Australia, including the two Australian genera not sampled here (*Kimblaeus* and *Notiocampus*). Part of this diversity arose as an endemic radiation of temperate southern Australian syngnathids, comprised of six sampled genera (Figure 3, clade v). There is strong morphological support for the monophyly of this clade, including pouch closure type and body ridge configurations. All species in this clade are associated with or near seagrass (Browne et al., 2008).

A unique combination of biogeographic and ecological features contribute to the exceptional endemism and diversity of temperate Australian syngnathids. These include a long east-west, ice-free coastline with an extensive continental shelf, the relatively stable and isolated geologic history of the region, and limited fluctuations in sea temperature throughout the late Cretaceous (Poore, 1995; Shepherd & Edgar, 2013). Cyclic changes in sea level led to periodic emergence of the Bassian Isthmus, which historically connected Tasmania with mainland Australia, alternately separating and connecting southern and eastern Australian coastal waters (Lambeck & Chappell, [2001](#)). These conditions have generated some of the highest levels of marine endemism of any oceanic province across a broad range of taxonomic groups. For example, in temperate southern Australian waters, approximately 85% of fish species, 95% of molluscs and 90% of echinoderms are endemic (Hoese et al., 2006; Poore, 1995). In contrast, approximately 13%, 10% and 13% of fish, molluscs and echinoderms, respectively, are endemic in Australia's tropical marine regions (Hoese et al., 2006; Poore, 1995). The diversity of temperate Australian endemic macroalgae is also very high, with almost 1200 species, nearly 800 of which are red algae (McCarthy & Orchard, 2007; Millar, 2007; Phillips, 2000; Womersley, 1990). Syngnathids display a similar pattern, with 46 of 64 Australian endemic species distributed in temperate Australia, where most of them are associated with seagrass ecosystems. Southern Australia boasts the largest temperate seagrass ecosystems in the world, with 22 species of seagrasses that collectively cover over 10,000 km² (Shepherd & Robertson, 1989). The diversity and extensive coverage of seagrass and macroalgae in southern Australia have certainly contributed to the variety of syngnathid lineages found there.

Particularly notable is the state of South Australia, whose coast falls completely within the Great Australian Bight. In this region several Australian endemic syngnathids, such as *Stigmatopora narinosa* and *Vanacampus vercoi*, have known distributions that are restricted to portions of the two large coastal bays - Spencer Gulf and the Gulf of St Vincent (Browne *et al.*, 2008). Other Australian fish lineages which share disjunct east-west distributions have isolated populations in these two bays, including *Filicampus tigris*. The saline and subtropical conditions in the gulfs are unique to temperate Australia and have probably enabled these bays to act as refugia for species commonly recorded further north (Byrne, 2008, Fraser *et al.*, 2009, Naughton, 2013).

Given the phylogenetic breadth represented in southern Australian endemics, a mechanism is needed for repeated invasions into this vast, stable diverse seagrass habitat. On both sides of the Australian continent, prevailing currents flow south. Many southern Australian species have distributions that include coasts of eastern and southern Australia or western and southern Australia, suggesting the west coast Leeuwin current and the East Australian current intermittently transport ocean biota, including syngnathids, into southern Australian waters, where they encounter stable and extensive coastal seagrass habitat.

5. CONCLUSION

Based on broad taxonomic sampling, the inclusion of both mitochondrial and nuclear markers, and the evaluation of morphological characters, the evolutionary hypothesis presented here represents a significant advance in our understanding of syngnathid relationships. We find

basic patterns that accord well with previous phylogenetic studies based on more limited molecular and taxonomic data sets. Many clades representing novel groupings are identified, and the overall picture is one of astonishing morphological fluidity. The flexibility of the syngnathid body plan has produced a large and diverse array of sizes and shapes, some of which are quite spectacular.

We confirm multiple lineages with independent evolution of a fully enclosed male brood pouch, representing one of the most extreme forms of male parental care among vertebrates. With at least nine independent origins, the ability to evolve a prehensile tail has occurred through multiple pathways of skeletal modification within both the tail and trunk-brooder subfamilies. Convergent evolution of miniaturization, a prehensile tail, and body ridge configuration has resulted in strikingly similar pygmy pipehorse genera in both the Atlantic and the Pacific. Similarly, the extent of convergence in morphological traits across several syngnathid genera in the Atlantic and in the Indo-Pacific has not previously been recognized. Adaptability to different aquatic environments has led multiple lineages to evolve freshwater and estuarine species. Many clades demonstrate circumglobal distribution, revealing high dispersal capacity. Taken together, our phylogenetic hypothesis reveals that the morphological and ecological plasticity of syngnathid fishes are major contributing factors to the evolutionary success of this charismatic group of fish.

The proposed definition of two subfamilies, the Nerophinae and the Syngathinae, is justified given it is the fundamental division within syngnathid evolution. Both subfamilies independently evolved a range of brood pouch structures, ecological habits, and morphological

innovations, and both successfully colonized global temperate and tropical oceans. Within the Syngnathinae, several important questions remain, including clearer resolution of the sister group to seahorses, and multiple instances of polyphyly for several lineages with Pacific and Atlantic conspecifics. While the taxonomic focus here is on generic level diversity, with denser species sampling, we would expect some repeats to patterns we are already seeing, for example, Australian endemic members of Indo-Pacific clades.

Further insights into the evolution of seahorses and pipefish can be gained with both additional taxon sampling and the application of high throughput genomic sequencing strategies such as Ultra Conserved Elements (McCormack et al. 2012, Faircloth et al. 2012, Crawford et al. 2015). Given the polyphyly identified here for several genera with both Atlantic and Pacific species, a priority is investigating the three other genera that have putative congeners in both ocean basins. Only nine currently recognized syngnathid genera are not represented in this study, but they include some of the most enigmatic and poorly known pipefish, including the recently described and highly derived Indo-Pacific “pygmy pipedragon” genus *Kyonemichthys*, and yet another worm-like pipefish, the monotypic southern Australian endemic *Notiocampus*. As new genetic techniques and additional taxon sampling provide further resolution to our understanding of syngnathid evolution, our appreciation is likely to grow for the evolutionary diversity of these remarkable fishes.

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FIGURE LEGENDS

Figure 1. Leading phylogenetic hypothesis using nuclear gene sequences (*Myh6*, *Tmo4c4*, *18S*, *HoxA13a*), retrieved with Bayesian and maximum likelihood inference; MrBayes 3.2.1/RAxML 7.0.4 support values are indicated. Hash (#) indicates support values of 1 for Bayesian and 100 for maximum likelihood. Star (*) indicates a disagreement between the nuclear and mitochondrial analyses.

Figure 2. Comparison of phylogeny with (A) nuclear loci only (*18S*, *HoxA13a*, *Myh6*, *Tmo4c4*), and (B) mitochondrial loci only (*12S*, *16S*, *COI*, *ND1*). Both trees were retrieved with Bayesian and maximum likelihood inference; MrBayes 3.2.1/RAxML 7.0.4 support values are indicated. Hash (#) indicates support values of 1 for Bayesian and 100 for maximum likelihood. Star (*) indicates a disagreement between the two analyses. Major clades are shaded to highlight topological patterns. Red branches lead to species in the genus *Hippocampus*, those leading to species in the genus *Halicampus* shown in blue, those leading to species in the genus *Leptoichthys* shown in orange, all others in black. Pie charts indicate the number of 11 examined morphological characters that support certain nodes in the alternative topologies (i.e. are synapomorphic).

Figure 3. Leading phylogenetic hypothesis using nuclear gene sequences (*18S*, *HoxA13a*, *Myh6*, *Tmo4c4*), with geographic origin traced as branch colors corresponding to shades in global map. Both subfamilies we designate here - Nerophinae and Syngnathinae - have lineages distributed in all biogeographic regions. For Nerophinae, this includes 2 putative subspecies of western

Atlantic *Microphis brachyurus*, and a *Doryrhamphus* subspecies (*D. e. paulus*) endemic to eastern Pacific that are not represented in our phylogeny.

Figure 1

Figure A

B. mtDNA

Figure 3

Aulostomus chinensis
Aulostomus maculatus

Fistularia petimba

Table 1

Gene	Size in base pairs	No. of variable sites	Optimal model (position 1; 2; 3 for coding genes)	Nodes with Bayesian posterior >95	Nodes with ML bootstrap >95
nDNA combined	2455	1267		78	54
<i>18S</i>	711	173	SYM + I + gamma	25	11
<i>HOX-A13a</i>	533	433	(SYM + gamma; HKY + gamma; SYM + I + gamma)	47	17
<i>Myh6</i>	699	333	(SYM + I + gamma; SYM + I + gamma; SYM + gamma)	47	24
<i>TMO-4c4</i>	512	328	(SYM + I + gamma; SYM + I + gamma; SYM + gamma)	46	25
mtDNA combined	2705	1923		70	49
<i>12S</i>	439	272	SYM + I + gamma	44	23
<i>16S</i>	632	444	SYM + I + gamma	47	22
<i>CO1</i>	655	330	(SYM + I + gamma; SYM + I; SMY + gamma)	40	18
<i>ND1</i>	979	640	(SYM + I + gamma; SYM + I + gamma; SYM + gamma)	50	20
Full concatenated	5160	3190		71	51

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Graphical Abstract