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Convergent camouflage and the non-monophyly of 'seadragons' (Syngnathidae: Teleostei): suggestions for a revised taxonomy of syngnathids

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The phylogeny and classification of the charismatic Syngnathidae (e.g. pipefish, seahorses) has not been comprehensively examined to date. In particular, we assessed morphological hypotheses that previously suggested the three 'seadragon' genera (*Phycodurus*, *Phyllopteryx*, *Haliichthys*) do not form a monophyletic group. We used three mitochondrial markers to investigate evolutionary relationships within Syngnathidae, and demonstrated that *Phycodurus* + *Phyllopteryx* formed a clade that excluded *Haliichthys*, indicating the elaborate appendages used for camouflage have evolved independently. A time-calibrated tree revealed the divergence of true seadragons as coincident with other kelp-associated fauna. We found evidence for the resurrection of neglected subfamily names, and recovered Doryrhampinae, Nerophinae, Soleganthinae, Phyllopteryginae, Sygnathoidinae and Haliichthyinae as clades. Even after removing these groups from what is currently recognized as Syngnathinae, we showed that the remaining members of Syngnathinae are not monophyletic. In the light of this information, some conclusions about the diversity of reproductive strategies found within 'Syngnathinae' need to be re-examined and further revision of syngnathid classification is needed.

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Introduction

Syngnathidae contains over 230 species of charismatic fish grouped in 52 genera (Nelson 2006) and includes a range of common names such as pipefish, pipehorses, seahorses and seadragons. Seadragons are among the largest and most spectacular of syngnathids, owing to their elaborate appendages (Fig. 1), and are endemic to Australia. The weedy seadragon *Phyllopteryx taeniolatus* Lacepède 1804, and the leafy seadragon *Phycodurus eques* (Günther 1865) are commonly seen in public aquaria around the world, but both are listed as 'Near Threatened' on the IUCN Red List (Connolly 2006). A third species *Haliichthys taeniophorus* Gray, 1859 has been referred to as either a ribboned pipefish (Dawson 1985), or ribboned seadragon (Kuiter 2003). To date, relationships among these putative 'seadragons' have not been examined closely. Whitley & Allan (1958) suggested that the upper tail ridges of *P. taeniolatus* and *P. eques* were so different that their similar camouflaged appearance must have evolved convergently. On this basis, they inferred *P. taeniolatus* had 'sprang from something like' *H. taeniophorus*, and that *P. eques* 'may have been derived from pipefish like *Leptonotus*'. In an alternative scenario, Kuiter (2003) surmised that *H. taeniophorus* was the sister group to *Phycodurus*.

Because of the unusual condition of male brooding and an array of reproductive strategies, Syngnathidae is considered a model system for investigation of the evolution of mate choice, brooding structures, sex-role reversal, mating systems and sexual selection (e.g. Kvarnemo *et al.* 2000; Sandvik *et al.* 2000; Jones & Avise 2001; Wilson *et al.* 2003; Vincent *et al.* 2004; Monteiro *et al.* 2005). The currently accepted higher classification divides Syngnathidae into two subfamilies, Syngnathinae and Hippocampinae (e.g. Nelson 2006), although a host of subfamily names have been historically used and are nomenclaturally available. Names above the subfamily level were also proposed by Duncker (1915) based on the placement of broods by the males (Urophori and Gastrophori) and these also

Fig. 1 Syngnathids identified as 'seadragons'.—A. Weedy seadragon *Phyllopteryx taeniolatus*.—B. Leafy seadragon *P. eques*.—C. Ribboned seadragon *Haliichthys taeniophorus*. All photographs by Greg Rouse.

remain in use (e.g. Herald 1959; Wilson *et al.* 2003). Wilson *et al.* (2003) carried out the most comprehensive phylogeny of the Syngnathidae to date. Their results supported the division of syngnathids into two clades, comprising tail brooders (Urophori) and abdominal brooders (Gastrophori), but did not discuss implications for subfamily classification. We assess the monophyly of 'seadragons' here, concomitant with examining relationships within Syngnathidae by utilizing and building on the data set of Wilson *et al.* (2003), which included data for *P. taeniolatus*. We expand the sampling for *P. taeniolatus* and the genus *Solegnathus*, and also include data for *H. taeniophorus*, *P. eques* and *Syngnathoides biaculeatus* for the first time in a molecular phylogenetic framework.

Materials and methods

Freshly collected, ethanol-fixed samples were extracted using a DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA), according to the manufacturer's directions. Formalin-fixed and dried specimens were extracted using a modified protocol (Chase *et al.* 1998). We used genomic DNA to amplify three mitochondrial markers: 12S ribosomal RNA (12S rDNA), 16S ribosomal RNA (16S rDNA), and cytochrome *b* (Cyt*b*), (see Table S1 for all primers). Table 1 shows the full list of samples and accession numbers used in our study.

Chromatograms were reconciled and edited in SEQUENCHER V4.8, and sequences compiled in Se-Al v2.0a11 (Rambaut 2002). Our samples were combined with data from GenBank, and preliminary alignments were generated with MUSCLE v3.7 (Edgar 2004). Outgroups were chosen from Solenostomidae and Pegasidae according to Kawahara *et al.* (2008). We used Gblocks (Talavera & Castresana 2007) to explore removing ambiguously aligned regions from the ribosomal data, selecting the least stringent options, which allow smaller final blocks, gap positions within blocks and less stringent flanking regions. This resulted in a 12S rDNA alignment that used 65% of the original data, and 94% for the 16S rDNA partition. We also assessed the protein-coding Cyt*b* data for saturation at the third position with DAMBE V5.0.80 (Xia & Xie 2001) with outgroups included and there was no significant saturation, even for third positions.

We analysed two data sets, the first of which included all data, whereas the second treated ribosomal data with Gblocks. Alignments are available through TreeBASE. Parsimony analyses were executed in PAUP* v4.0b10 (Swofford 2001) using heuristic searches, TBR branch swapping, with 100 random-addition starting tree replicates, and testing support with 100 jackknife replicates (37% deletion according to Farris *et al.* 1996). Single gene analyses were carried out as above but using 1000 jackknife replicates and limiting max trees to 10 000. We carried out phylogenetic analyses with RAxML v7.0.4 (Stamatakis 2006) under a GTR+Γ model (see Yang 2006), with joint branch length parameter estimation and assessed using 1000 rapid bootstrap replicates utilizing the CAT model (Stamatakis *et al.* 2008). MRMODELTEST v2.2 (Nylander 2004) selected a GTR+I+Γ model for all gene partitions using the Akaike Information criterion. We also carried out additional

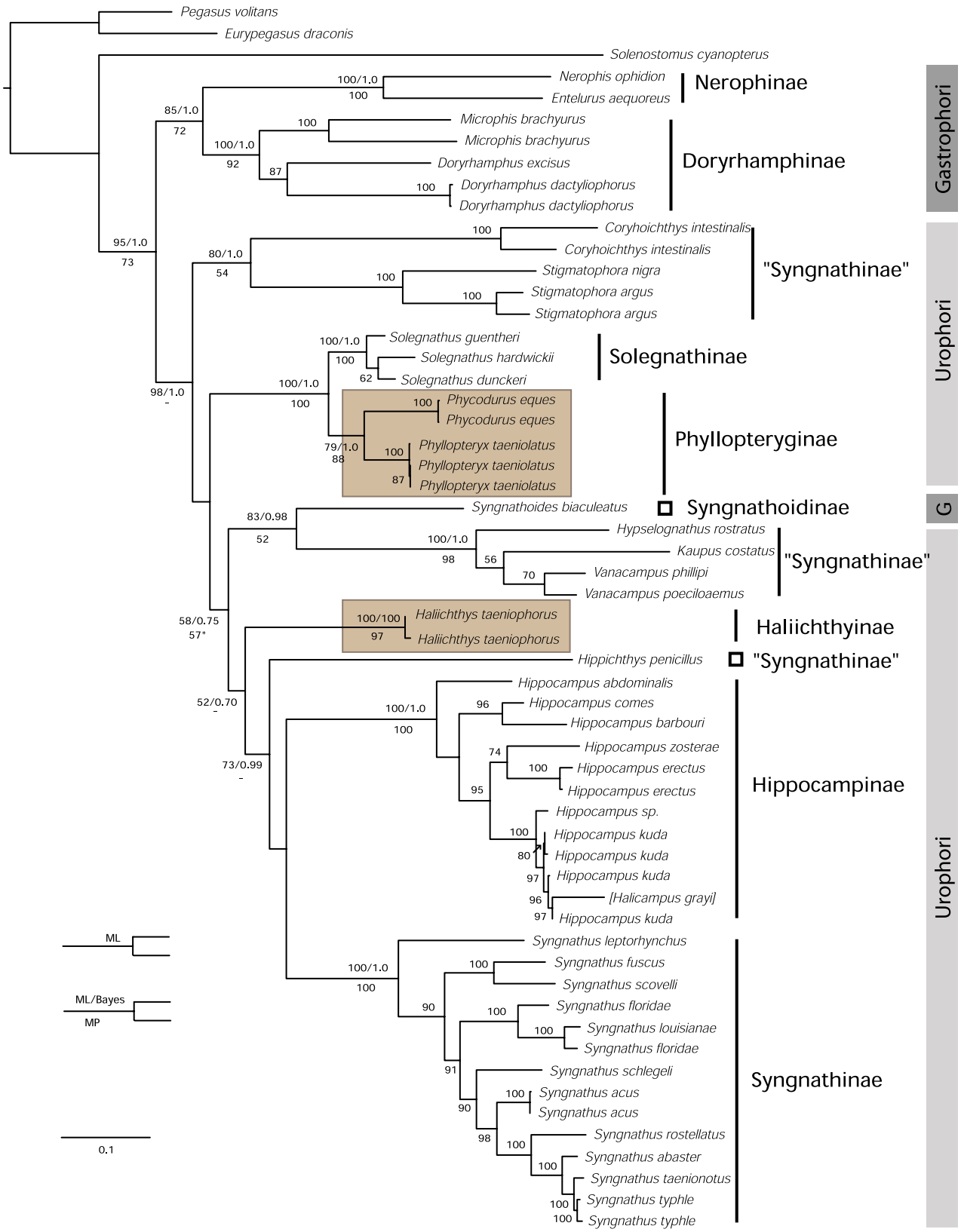
Table 1 Samples and accession numbers used in this study

Sample	GenBank accession numbers		
	Cyt*b*	16S rDNA	12S rDNA
Hippocampinae			
Hippocampus abdominalis	AF356065	AF355013	AF354965
Hippocampus barbouri	AF356048	AF354999	AF354948
Hippocampus comes	AF356049	AF355000	AF354949
Hippocampus erectus	AF356057	AF355007	AF354956
Hippocampus erectus	DQ288341	DQ288359	–
Hippocampus sp.	AF356054	AF355004	AF354953
Hippocampus kuda	AP005985	AP005985	AP005985
Hippocampus kuda	AF356050	AF355001	AF354950
Hippocampus kuda	AF356063	AF355012	AF354962
Hippocampus kuda	–	DQ452301	DQ452299
Hippocampus zosterae	AF356071	–	AF354973
Syngnathinae			
Corythoichthys haemopterus	AY166830	AY166831	AY166832
Corythoichthys intestinalis	AF356052	AF355003	AF354952
Corythoichthys intestinalis	AF356055	AF355005	AF354954
Hippichthys penicillus	AF356053	AF355033	AF354990
Halicampus grayi	AF356062	–	AF354961
Hypselognathus rostratus	AF356072	AF355020	AF354974
Kaupus costatus	AF356074	AF355023	AF354979
Pugnaso curtirostris	–	AF356539	AF354977
Stigmatopora argus	AF356066	AF355014	AF354967
Stigmatopora argus	AF356045	AF354996	AF354945
Stigmatopora nigra	AF356067	AF355015	AF354968
Stigmatopora nigra	–	AF355024	AF354980
Syngnathus abaster	AF356060	AF355010	AF354959
Syngnathus acus	AF356040	AF354991	AF354940
Syngnathus acus	AF356073	–	AF354976
Syngnathus floridae	AF356058	AF355008	AF354957
Syngnathus floridae	AF356069	AF355018	AF354971
Syngnathus fuscus	AF356056	AF355006	AF354955
Syngnathus leptorhynchus	AF356064	–	AF354964
Syngnathus louisianae	AF356070	AF355019	AF354972
Syngnathus rostellatus	AF356041	AF354992	AF354941
Syngnathus schlegeli	AF356051	AF355002	AF354951
Syngnathus scovelli	AF356068	AF355017	AF354970
Syngnathus taeniolatus	AF356061	AF355011	AF354960
Syngnathus typhyle	AF356042	AF354993	AF354942
Syngnathus typhyle	AF356059	AF355009	AF354958
Urocampus carinirostris	–	AF355016	AF354969
Vanacampus phillipi	–	AF355022	AF354978
Vanacampus poecilolaemus	–	AF355021	AF354975
Phyllopteryginae			
Phyllopteryx taeniolatus	**GU182933**	**GU182928**	**GU182920**
Phyllopteryx taeniolatus	AF356076	AF355027	AF354983
Phyllopteryx taeniolatus	AF356077	AF355028	AF354984
Phycodurus eques	**GU182932**	**GU182926**	**GU182918**
Phycodurus eques	**GU182931**	**GU182927**	**GU182919**
Haliichthyinae			
Haliichthys taeniophorus	–	**GU182930**	**GU182922**
Haliichthys taeniophorus	–	**GU182929**	**GU182923**
Solegnathinae			
Solegnathus dunckeri	**GU182935**	**GU182924**	–
Solegnathus guentheri	**GU182934**	**GU182925**	**GU182921**
Solegnathus hardwickii	AY166829	AF355025	AF355025
Syngnathoidinae			
Syngnathoides bimaculatus	AY786432	–	–
Doryrhamphinae			
Doryrhamphus dactyliophorus	AY787231	–	–
Doryrhamphus dactyliophorus	AF356047	AF354998	AF354947
Doryrhamphus excisus	AF356075	AF355026	AF354982
Microphis brachyrus	AF356046	AF354997	AF354946
Microphis brachyrus	AP005986	AP005986	AP005986
Nerophinae			
Entelurus aequareus	AF356044	AF354995	AF354944
Nerophis ophidion	AF356043	AF354994	AF354943
Outgroups			
Eurypegasus draconis	AP005983	AP005983	AP005983
Pegasus volitans	AP005984	AP005984	AP005984
Solenostomus cyanopterus	AB277725	AB277725	AB277725

New sequences are in bold.

likelihood analyses constraining the monophyly of 'seadragons' and assessed the change in likelihood by applying the Shimodaira & Hasegawa (1999) test implemented in PAUP* v4.0b10, using re-estimated log-likelihood approximation. Bayesian analyses were executed in MRBAYES v3.0b4 (Huelsenbeck & Ronquist 2001), partitioned by gene (unlinked) with multiple runs of 5 million generations, sampling every 1000 generations. Stationarity and convergence of runs were assessed using TRACER 1.4.1 (Rambaut & Drummond 2007) and the appropriate burn-in (250 000 generations) removed before constructing majority-rule consensus trees.

To assess the question of the time to the most recent ancestor for Phyllopteryginae and then to their most recent common ancestor with *Solegnathus*, a minimum age estimate was applied to the Syngnathidae following the parameters in the analysis of Teske & Beheregaray (2009). They used the oldest syngnathid fossils (Patterson 1993) dating to the Lutetian ages (48.6 ± 0.2 Ma and 40.4), but allowed for possibility that Syngnathidae is older by using a mean age of 52.2 Ma with 95% confidence interval spanning 48–56 Ma. We did not use the other two calibration points that were used by Teske & Beheregaray (2009), as they were based on divergences within *Hippocampus* species pairs that were not compatible with our taxon sampling. Major nodes (subfamilies, Gastrophori and Urophori including *Syngnathoides*) that were well supported (>80 bootstrap or >0.95 PP) in the MRBAYES and RAxML analyses were constrained to be monophyletic. The phylogeny and divergence times were then estimated using the BEAST 1.5.4 package (Drummond & Rambaut 2007) that implements a Bayesian relaxed molecular clock method (Drummond *et al.* 2006). This was run with the complete data set and as with the MRBAYES and RAxML analyses, the



partitions were Cyt*b*, 16S, and 12S, with a GTR+G+I model unlinked across partitions. The following parameters were set in the BEAST 1.5.4.xml file; uncorrelated log-normal prior model of rate change, a Yule prior process to model divergences and the divergence date for use with normal distributions with the standard deviation set to cover a central 95% range of the age estimates. A Newick tree based on one of the maximum parsimony analysis trees was used as a starting tree following 'initial model is invalid' errors with random starting trees. Three separate MCMC analyses of 50 million generations were run to provide independent parameter samples (saved every 1000 generations) that were checked in TRACER v1.5 to check convergence and stationarity. Based on these results, the last 10 000 trees of each of the runs were combined with LOGCOMBINER 1.5.4 and then analysed with TREEANNOTATOR 1.5.4 to give the maximum-clade credibility tree, posterior probabilities, and to estimate divergence times and corresponding 95% confidence intervals for mean posterior densities.

Results

The leafy and weedy seadragons (*Phycodurus* and *Phyllopteryx)* formed a monophyletic group (recognized as the subfamily Phyllopteryginae) (Fig. 2). The third 'seadragon' species (*H. taeniophorus*) consistently fell outside a Phyllopteryginae + Solegnathinae clade (Fig. 2, Fig. S1), although its position could not be confidently determined. In the most optimal trees it was the sister to a polytomy containing Hippocampinae, *Syngnathus* and *Hippichthys*. Constraining the three 'seadragon' taxa to be monophyletic resulted in an increase of 48.608 log-likelihood units, making the constrained tree significantly less likely ($P = 0.00015$) under a Shimodaira–Hasegawa test. Incorporation of a relaxed clock approach dated the divergence of true seadragons, Phyllopteryginae, from their sister group, Solegnathinae, at 7.59–21.66 Ma (within 95% of the highest posterior density, mean 14.35 Ma) (Fig. S2). This places the origin of Phyllopteryginae at a mean of 8.27 Ma (3.64–13.68 Ma).

Despite the combined data set showing unambiguous support for the monophyly of Phyllopteryginae, single gene analyses under maximum likelihood and parsimony did identify some conflict regarding a *Phycodurus* + *Phyllopteryx* clade (Table 2). Cyt*b* data strongly supported this sistergroup relationship. 12S rDNA data also supported this topology after removal of ambiguously aligned regions with Gblocks, although strong support was lacking. In conflict with these results (whether intact or Gblocked), 16S rDNA data strongly supported a *Phyllopteryx* + *Solegnathus* relationship.

We found support for Doryrhamphinae + Nerophinae as the sister group to the rest of Syngnathidae (see Fig. 2). Within the remaining Syngnathidae, Solegnathinae, Phyllopteryginae, Hippocampinae and Haliichthyinae were recovered as well-supported clades. Syngnathinae was not monophyletic, but instead formed four different clades scattered across the tree (Fig. 2). Our optimal trees also show the position of *S. biaculeatus* (Gastrophori) inside a clade containing other members of the Urophori, rather than with other Gastrophori such as Nerophinae + Doryrhamphinae. The rooting of the analyses with Pegasidae, and then Solenostomidae, which exhibit female brooding of young, suggests brooding is an apomorphy for Syngnathoidea (=Solenostomidae + Syngnathidae).

Discussion

The monophyly of seadragons and the position of Halicampus

Our phylogenetic analyses indicated that the three genera that have been referred to as seadragons (*Phycodurus*, *Phyllopteryx* and *Haliichthys*) did not form a clade (Fig. 2, Fig. S1). Rather *Phycodurus* and *Phyllopteryx* were sister taxa in the optimal multi-gene trees of all analytical methods employed here and formed the sister group to *Solegnathus*, a group of pipefish that are sometimes also referred to as spiny seadragons (Whitley & Allan 1958). Our combined data support the hypothesis that *Phyllopteryx* and *Phycodurus* are closely related (=Phyllopteryginae) and hence do not support the hypothesis of convergent evolution in their anatomy, as previously proposed (Whitley & Allan 1958; Kuiter 2003). The argument for leafy and weedy seadragons not being close relatives was based primarily on differences in tail ridge morphology, and re-interpretation of the anatomy is now required within this phylogenetic framework.

Interestingly, examination of single gene analyses identified conflict in the data, with 16S rDNA supporting an alternative *Phyllopteryx* + *Solegnathus* relationship (Table 2). The source of this incongruence is not immediately clear, as the veracity of sequences was checked by using multiple specimens, and other explanations such as long-branch

Fig. 2 Maximum likelihood phylogeny of syngnathids based on three mitochondrial genes with ambiguously aligned regions of 12S rDNA and 16S rDNA data removed, with support tested by 1000 bootstrap replicates. Support below 50 is not shown unless it appears alongside greater support at a node representative of relationships among subfamilial rankings. These nodes also show support values under Bayesian inference (posterior probabilities) and maximum parsimony (jackknife). *Support is for a node that does not include *Haliichthys*. Square brackets surround *Halicampus grayi*, indicating likely contamination of Cyt*b* in NCBI.

Table 2 Topological conflict identified by comparing single gene analyses to combined analyses

Data set	Solegnathinae topology	Maximum parsimony jackknife support	Maximum likelihood bootstrap support
12S	((*Phycodurus*, *Solegnathus*) *Phyllopteryx*)	66, 97	37, 92
12S Gblocked	((*Phycodurus*, *Phyllopteryx*) *Solegnathus*)	69, 98	33, 84
16S	((*Phyllopteryx*, *Solegnathus*) *Phycodurus*)	98, 99	93, 98
16S Gblocked	((*Phyllopteryx*, *Solegnathus*) *Phycodurus*)	97, 99	91, 99
Cyt*b*	((*Phycodurus*, *Phyllopteryx*) *Solegnathus*)	99, 100	99, 100
Three genes	((*Phycodurus*, *Phyllopteryx*) *Solegnathus*)	90, 100	70, 100
Three genes Gblocked	((*Phycodurus*, *Phyllopteryx*) *Solegnathus*)	88, 100	79, 100

attraction are unwarranted. It may be that ancient lineage sorting or an unrecognized gene duplication event contributed to a different evolutionary history for genes that appear to share a locus.

The origin of Phyllopteryginae is estimated to range from the Pliocene to mid-Miocene, with the mean estimate occurring in the late Miocene. Other kelp-associated taxa also first appear as fossils in the late Miocene, such as the sirenians *Dusisiren dewana* (Takehashi *et al.* 1986) and probably *Hydrodamalis cuestae* (Domning & Deméré 1984). This is consistent with the idea that kelps originated in the late Miocene (Estes & Steinberg 1988). Some authors argue for an earlier origin of kelps, with a late Miocene diversification (Domning 1989), largely based on a putative kelp fossil occurring in the mid-Miocene (Parker & Dawson 1965). Our results depict the earliest part of the confidence interval in the mid-Miocene, and are congruent with both scenarios. Either way, it seems likely that the evolution of the fleshy appendages characteristic of the Phyllopteryginae was influenced by habitat association, as has been invoked for the acquisition of an upright posture of sea horses (Teske & Beheregaray 2009).

The convergent acquisition of appendages in *Haliichthys* and *Halicampus* requires further data to accurately date that node. In Fig. 2 *Halicampus grayi* (Gray's pipefish or Mud pipefish) is nested inside *Hippocampus kuda* (a seahorse species), yet *H. grayi* is clearly a pipefish and does not show any of the apomorphic features of *Hippocampus*. On exploration of single gene data sets, it was clear that Cyt*b* data were responsible for that signal. The available partial Cyt*b* sequence (AF356062) shows 99% similarity to several sequences from *H. kuda*, and almost certainly represents a contamination event. Removal of the Cyt*b* sequence results in *H. grayi* being strongly supported as the sister to *Haliichthys* (e.g. Gblocked data set, likelihood bootstrap 86, data not shown), despite being represented only by 12S rDNA data. In any case it is clear that *H. taeniophorus* should not be referred to as a seahorse or 'seadragon', and is more reasonably referred to as the 'ribboned pipefish'.

Systematics and classification

Herald's (1959) syngnathid classification is based primarily on Duncker's (1915) arrangement and recognizes six subfamilies: Nerophinae, Syngnathoidinae, Doryrhamphinae, Solenognathinae, Syngnathinae, Hippocampinae. A year earlier, Whitley & Allan (1958) ignored Doryrhampinae and Nerophinae, split Phyllopteryginae (weedy and leafy seadragons = *Phycodurus* + *Phyllopteryx*) from Solegnathinae, and erected Haliichthyinae (for *Haliichthyes*), Acentronurinae and Leptoichthyinae as separate groups from Syngnathinae. Confusingly, even though they placed *Haliichthyes* in a separate subfamily from *Phycodurus* and *Phyllopteryx*, Whitley & Allan (1958) hypothesized that *Haliichthyes* and *Phyllopteryx* were more closely related than *Phyllopteryx* was to *Phycodurus*.

These early classifications have fallen out of favour in recent years, and current classification schemes typically recognize only Syngnathinae and Hippocampinae (e.g. Nelson 2006), or ignore subfamily classifications (Wilson *et al.* 2001, 2003) in favour of the tribe names Urophori and Gastrophori, erected by Duncker (1915). Our phylogeny, largely congruent with that of Wilson *et al.* (2003), lends support for some neglected subfamily names (Doryrhampinae, Nerophinae, Soleganthinae, Phyllopteryginae, Sygnathoidinae, Haliichthyinae) as they are recovered as clades and provides evidence that Syngnathinae is not monophyletic (see Table 3 for resulting classification). All the species in *Syngnathus*, the type genus for this subfamily, formed a clade that was sister group to Hippocampinae and so we propose the name Syngnathinae only be applied to this group. The remaining three 'Syngnathinae' groups (Fig. 2) will likely require new names. The placement and status of Acentronurinae (pygmy pipehorses, e.g. *Acentronura*, *Idiotropiscis*) could not be assessed here owing to a lack of data, although it has been suggested that they are the sister group to Hippocampinae (Teske & Beheregaray 2009). The division of Syngnathidae into only two subfamilies is untenable and further revision is clearly needed.

Wilson *et al.* (2001, 2003) rooted their analyses with members of the sticklebacks, Gasterosteidae. Since that

Table 3 Classification derived from phylogenetic analyses. Acentronurinae remains untested

Syngnathidae	Common names
Nerophinae	Marsupium-lacking pipefish
Doryrhamphinae	Flagtailed pipefish
Solegnathinae	Spiny seadragons
Phyllopteryginae	Weedy and leafy seadragons
Syngnathoidinae	Alligator pipefish
Haliichthyinae	Ribboned seadragons
Hippocampinae	Seahorses
"Syngnathinae"	Pipefish

time, whole mitochondrial genome evidence has suggested that ghost pipefish (Solenostomidae) form the sister group to Syngnathidae, and seamoths (Pegasidae) are the sister-group to these (Kawahara *et al.* 2008), and we rooted our trees accordingly. A Solenostomidae + Syngnathidae sister-group arrangement (=Syngnathoidea) suggests that the acquisition of brooding may have preceded male brooding, as in solenostomids it is the females that brood the young (Orr & Fritzsche 1993; Sado & Kimura 2006), and Pegasidae use broadcast spawning (Herold & Clark 1993). However, it appears equally parsimonious that either female brooding or male brooding is the plesiomorphic condition for Syngnathoidea. Interestingly, the change in outgroup here did not result in a change in the root position for Syngnathidae, but it did remove the signal of saturation in the third codon positions for Cyt*b*. In contrast to Wilson *et al.* (2001, 2003), we found no statistical evidence for saturation in Cyt*b*, either within the ingroup, or with respect to outgroups and so included these data.

Tail brooding vs. abdominal brooding

In addition to the subfamilial arrangements, syngnathids have long been divided into two taxa (Urophori and Gasterophori) depending on the location of eggs on the males and subsequent developing young. This arrangement was first outlined by Duncker (1915), and the corresponding evolutionary hypotheses expanded upon by Herald (1959), and the two clades were subsequently supported as reciprocally monophyletic in a molecular phylogenetic context (Wilson *et al.* 2001, 2003). However, the inclusion of *Syngnathoides* in a phylogeny for the first time revealed a conflict in the division of syngnathids with tail brooding (Urophori) from those with abdominal brooding (Gastrophori). Our optimal trees show the *S. biaculeatus* (Gastrophori) inside the clade containing other members of the Urophori, rather than with other Gastrophori. Another exception to this simple two-group hypothesis had already been noted by Lourie & Randall (2003) who reported abdominal brooding had been secondarily acquired in some pygmy seahorses, likely driven by reduction in body size.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Maximum likelihood phylogeny of syngnathids based on three mitochondrial genes, with support tested by 1000 bootstrap replicates. Support below 50 is not shown unless it appears alongside greater support at a node representative of relationships among subfamilial rankings. These nodes also show support values under Bayesian inference (posterior probabilities) and maximum parsimony (jackknife). *Support is for a node that does not include *Haliichthys*. Square brackets surround *H. grayi*, indicating likely contamination of Cyt*b* in NCBI.

Figure S2. BEAST (Bayesian evolutionary analysis by sampling trees)-generated chromogram showing relationships among Syngnathidae. Values given at nodes indicate the mean value of the 95% highest posterior density interval, which is highlighted by grey bars. Scale bar indicates time in millions of years.

Table S1. Primer combinations used in this study

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