# Evolution of seahorses' upright posture was linked to Oligocene expansion of seagrass habitats

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**Seahorses (Syngnathidae: *Hippocampus*) are iconic marine teleosts that are readily identifiable by their upright posture. The fossil record is inadequate to shed light on the evolution of this trait because it lacks transitional forms. There are, however, extant syngnathid species (the pygmy pipehorses) that look like horizontally swimming seahorses and that might represent a surviving evolutionary link between the benthic seahorses and other, free-swimming members of the family Syngnathidae. Using sequence data from five nuclear loci, we confirm the sister taxon relationship between seahorses and pygmy pipehorses. Molecular dating indicates that the two taxa diverged during the Late Oligocene. During this time, tectonic events in the Indo-West Pacific resulted in the formation of vast amounts of new shallow-water areas and associated expansion of seagrass habitats that would have favoured the seahorses' upright posture by improving their camouflage while not affecting their manoeuvrability negatively. The molecular techniques employed here provide new insights into the evolution of a taxon whose fossil record is incomplete, but whose evolutionary history is so recent that the major stages of morphological evolution are still represented in extant species.**

## 1. INTRODUCTION

Seahorses (Syngnathidae: *Hippocampus*) today are common throughout the world's tropical, subtropical and temperate marine regions (Kuiter 2000), but fossil seahorses are exceptionally rare. The genus is represented at only two well-documented sites, namely Tunjice in Slovenia (Middle Miocene, approx. 13 Myr; Žalohar *et al.* 2009) and Marecchia in Italy (Late Pliocene, approx. 3 Myr; Sorbini 1988). In both cases, the fossil seahorses are considered to be morphologically similar to certain extant species rather than being primitive transitional forms, suggesting that seahorses must have evolved earlier.

The genus *Hippocampus* is one of four genera in the subfamily Hippocampinae, which also includes three genera of pygmy pipehorses (*Acentronura*, *Amphelikturus* and *Idiotropiscis*) that some authors treat as a single genus (Kuiter 2004). Pygmy pipehorses are morphologically very similar to seahorses, but all lack the upright posture. This suggests that they could be a surviving evolutionary link between seahorses and the remaining members of the family Syngnathidae, all of which have a horizontal posture. The species of the temperate Australian pygmy pipehorse genus *Idiotropiscis* are by far the most seahorse-like in appearance (Kuiter 2004), and the time when these shared a common ancestor with the seahorses is therefore likely to be close to the time when the seahorses' upright posture evolved. An Australasian origin of seahorses is supported by the fact that the most basal and second most basal *Hippocampus* lineages occur in the Indo-West Pacific and in Australia, respectively (Teske *et al.* 2004).

Central to understanding why the seahorses' upright posture was favoured by natural selection is to determine what environmental conditions prevailed in Australasia during the time when seahorses and pygmy pipehorses diverged from their common ancestor. To this end, we reconstructed phylogenetic relationships among species of *Hippocampus* and *Idiotropiscis*, determined their placement among six other syngnathid genera representing the major evolutionary lineages of the family Syngnathidae (Wilson *et al.* 2003) and dated the split between seahorses and pygmy pipehorses using a relaxed molecular clock.

## 2. MATERIAL AND METHODS

### (a) Phylogeny reconstruction

Phylogenetic relationships among seahorses and other syngnathids were reconstructed using a dataset 3386 nucleotides in length that comprised four nuclear genes (*RAG1*, *myh6*, *Rhodopsin* and *Tmo4c4*) and a fifth nuclear marker (a region spanning intron 1 of the S7 ribosomal protein) that comprised elements of both exon and intron regions. Details about primers, PCR conditions and sequence alignments, as well as museum collection numbers and GenBank accession numbers, are listed in the electronic supplementary material.

Phylogenetic trees were constructed using maximum likelihood (TREEFINDER; Jobb *et al.* 2004), parsimony (MEGA 4; Tamura *et al.* 2007) and Bayesian inference (MRBAYES 3.1; Ronquist & Huelsenbeck 2003). For the first two methods, support for nodes was assessed by generating 10 000 bootstrap replications. The Bayesian inference was carried out by running four chains simultaneously for 3 million generations and discarding the first 10 per cent of trees as burn-in. Posterior probabilities of nodes were assessed by constructing a 50 per cent majority rule consensus tree. To check for consistency of results, the analyses were repeated three times. For both maximum likelihood analysis and Bayesian inference, the dataset was divided into four partitions: codon positions 1–3 of the nuclear genes, as well as S7. Rates were allowed to vary among partitions, and the GTR + I + G model was specified for each.

### (b) Molecular dating

Molecular dating was performed using a Bayesian method (BEAST 1.4.8; Drummond & Rambaut 2007; see electronic supplementary material for methodological details). Two to three calibration points were specified. In each case, a normal prior was used, and its mean and standard deviations were set in such a way that 95 per cent confidence intervals corresponded to the upper and lower bounds of each calibration point. In this way, uncertainty concerning the exact dates of the calibration points could be accounted for. The age of the oldest syngnathid fossils (Monte Bolca formation, Early Eocene) (Patterson 1993; Bellwood 1996) was used as the first calibration point. These fossils date from the boundary between the Ypresian and Lutetian ages (approx. 48–50 Myr). To account for the possibility that they are younger than the origin of the family, we specified the beginning of the Eocene as an upper bound (mean = 52.2, s.d. = 2.3, 95% confidence interval: 48–56). The other two calibration points were based on genetic evidence for divergence events in a seahorse phylogeny that resulted from the
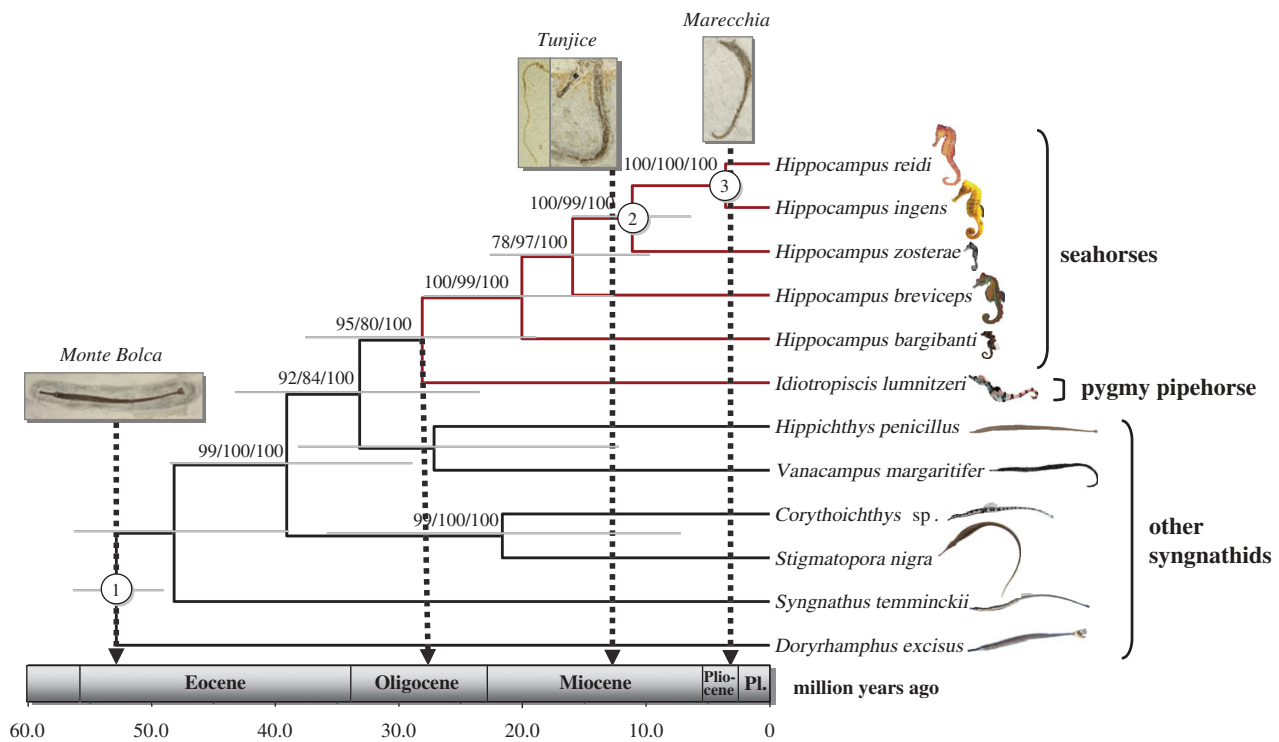
Figure 1. A chronogram scaled to geological time reconstructed from DNA sequence data of seahorses, and other species representing the major genetic lineages of the family Syngnathidae. Nodal support is indicated as bootstrap values from maximum likelihood analysis, bootstrap values from parsimony analysis and posterior probabilities from Bayesian inference. Numbers in white circles indicate nodes used as calibration points and include the age of the oldest syngnathid fossils (1) and the closures of the Tethyan and Central American seaways (2 and 3). Light grey bars are 95 per cent highest posterior density intervals of estimated node ages. Grey arrows indicate the age of the oldest syngnathid fossils, the ages of the only two well-documented fossil sites that contain seahorses and the time when seahorses and pygmy pipehorses diverged from their common ancestor (Pl. = Pleistocene).

formation of land bridges that separated formerly continuous marine habitats (Teske *et al.* 2007). The best documented of these is the closure of the Central American Seaway during the Late Pliocene, which resulted in the divergence of *Hippocampus reidi* (West Atlantic) and *Hippocampus ingens* (East Pacific) from a common ancestor (Teske *et al.* 2007). The final closure of this seaway occurred approximately 3.1–3.7 Myr (Duque-Caro 1990), but to account for the possibility that these two seahorse species diverged prior to this date as a result of ocean current reorganization in the region (approx. 4.6 Myr; Haug & Tiedemann 1998), we specified the older date as an upper bound (mean = 3.85, s.d. = 0.45, 95% confidence interval: 3.1–4.6). The date of the third calibration point, the closure of the Tethyan Seaway that once connected the Atlantic Ocean with the Indian Ocean, is comparatively vague because there were several phases of closing and reopening prior to complete closure. Previous analyses indicate that both Late Early Miocene (Adams *et al.* 1983) and Middle Miocene (Rögl & Steininger 1983) closures may have resulted in divergence of seahorse lineages (Teske *et al.* 2007). We therefore allowed for a wide calibration range that included both these dates (mean = 15.85, s.d.= 2.85, 95% confidence interval: 11.2–20.5), but also dated the syngnathid phylogeny without this calibration point.

## 3. RESULTS
Phylogenetic reconstruction confirmed the sister taxon relationship between the seahorses and the pygmy pipehorse genus *Idiotropiscis* (figure 1). A divergence time estimate of 28 Myr (three calibration points; s.d.= 0.4, 95% confidence beherval: 18–37 Myr; figure 1) or 25 Myr (two calibration points; s.d. = 0.25 Myr; 95% confidence interval: 16–34 Myr) indicates that the seahorses' upright posture most likely evolved during the Late Oligocene (confidence intervals range from the Early Oligocene to the Early Miocene).

This estimate considerably predates the age of the oldest known seahorse fossils.

## 4. DISCUSSION
A Late Oligocene estimate of the divergence of seahorses and the endemic Australian pygmy pipehorse genus *Idiotropiscis* indicates that the evolution of the seahorses' upright posture was likely to have benefitted from geological changes that occurred in the Indo-West Pacific during this time. Following climatic cooling and lowering of sea levels during the Early Oligocene approximately 34 Myr (Miller *et al.* 2008), tectonic events in the Indo-West Pacific (the most important one being the collision of Australia/New Guinea with the Eurasian plate; Hall 1998) during the Late Oligocene and Early Miocene (approx. 25–20 Myr) resulted in the formation of vast areas of shallow-water habitat between Australia and Indonesia (Wilson & Rosen 1998). This facilitated expansion of seagrass habitats (Brasier 1975).

All three species of *Idiotropiscis* described to date occur in temperate Australian waters (Kuiter 2004), suggesting that the common ancestor of *Hippocampus* and *Idiotropiscis* may have originated in this region. Off northeastern Australia, conditions were temperate during the Late Oligocene (Davies *et al.* 1991), indicating that this species must have occurred in close proximity to the Indo-West Pacific during the Oligocene–Miocene period of tectonic changes. The earliest seahorses would have greatly benefited from the expansion of seagrass habitats. Not only can

seahorses manoeuver exceptionally well in such a habitat (Flynn & Ritz 1999), but the vertical seagrass blades would also have provided good camouflage for their upright bodies, and in that way afforded them both protection from predators and an improved ability to ambush prey. Pygmy pipehorses would not have benefited from the seagrass radiation, and for that reason probably remained restricted to the macroalgal reefs in which they still occur today. Divergence of the two taxa may initially have been driven by different selection pressure in seagrass and algal reef habitats. When water temperature increased during the Early Miocene (Davies *et al.* 1991), adaptation to higher temperatures in the case of the seahorses that by now would have been widespread in the Indo-Pacific may then have resulted in divergence from their sister taxon.

The molecular techniques employed here provide new insights into the evolution of a taxon whose fossil record is uninformative because it lacks transitional forms, but whose evolutionary history is so recent that the major stages of morphological evolution are still represented in extant species.

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