Signatures of seaway closures and founder dispersal in the phylogeny of a circumglobally distributed seahorse lineage

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Abstract

Background: The importance of vicariance events on the establishment of phylogeographic patterns in the marine environment is well documented, and generally accepted as an important cause of cladogenesis. Founder dispersal (i.e. long-distance dispersal followed by founder effect speciation) is also frequently invoked as a cause of genetic divergence among lineages, but its role has long been challenged by vicariance biogeographers. Founder dispersal is likely to be common in species that colonize remote habitats by means of rafting (e.g. seahorses), as long-distance dispersal events are likely to be rare and subsequent additional recruitment from the source habitat is unlikely. In the present study, the relative importance of vicariance and founder dispersal as causes of cladogenesis in a circumglobally distributed seahorse lineage was investigated using molecular dating. A phylogeny was reconstructed using sequence data from mitochondrial and nuclear markers, and the well-documented closure of the Central American seaway was used as a primary calibration point to test whether other bifurcations in the phylogeny could also have been the result of vicariance events. The feasibility of three other vicariance events was explored: a) the closure of the Indonesian Seaway, resulting in sister lineages associated with the Indian Ocean and West Pacific, respectively; b) the closure of the Tethyan Seaway, resulting in sister lineages associated with the Indo-Pacific and Atlantic Ocean, respectively, and c) continental break-up during the Mesozoic followed by spreading of the Atlantic Ocean, resulting in pairs of lineages with amphi-Atlantic distribution patterns.

Results: Comparisons of pairwise genetic distances among the seahorse species hypothesized to have diverged as a result of the closure of the Central American seaway with those of published teleost sequences having the same distribution patterns show that the seahorses were among the last to diverge. This suggests that their cladogenesis was associated with the final closure of this seaway. Although two other divergence events in the phylogeny could potentially have arisen as a result of the closures of the Indonesian and Tethyan seaways, respectively, the timing of the majority of bifurcations in the phylogeny differed significantly from the dates of vicariance events suggested in the literature. Moreover, several divergence events that resulted in the same distribution patterns of lineages at different positions in the phylogeny did not occur contemporaneously. For that reason, they cannot be the result of the same vicariance events, a result that is independent of molecular dating.

Conclusion: Interpreations of the cladogenetic events in the seahorse phylogeny based purely on vicariance biogeographic hypotheses are problematic. We conclude that the evolution of the circumglobally distributed seahorse lineage was strongly influenced by founder dispersal, and suggest that this mode of speciation may be particularly important in marine organisms that lack a pelagic dispersal phase and instead disperse by means of rafting.
Background

Bifurcations in a phylogeny can be explained by two modes of allopatric speciation: vicariance [1] (the establishment of a dispersal barrier separating regional units of a previously continuously distributed species) and founder dispersal [2-4] (long-distance dispersal of a small number of individuals from a source population followed by founder effect speciation in the absence of additional gene flow). Although most biogeographers consider both vicariance and founder dispersal to be important causes of cladogenesis, speciation as a result of dispersal is sometimes rejected or considered irrelevant noise on the basis of its sporadic nature. It is argued that in the majority of cases in which dispersal has been invoked, it is considered to have affected only individual species rather than the entire fauna of a particular region [5].

Cladogenesis as a result of founder dispersal may be more important in seahorses (genus Hippocampus) than in many other marine organisms studied to date, because of their life-histories and means of dispersal [6]. Seahorses use a prehensile tail to hold on to objects that may serve as rafts, such as floating seaweed [7,8]. As macrobenthic prey tends to be abundant on these rafts [9], displaced seahorses are likely to survive for a considerable amount of time. In most species, pregnant male seahorses can have brood sizes of up to 100–300 individuals [10], suggesting that a sufficiently large number of closely related individuals may arrive simultaneously at a new habitat to establish themselves. As additional recruitment from the source population is unlikely (because long-distance dispersal along the same route is likely to be rare), lack of gene flow coupled with genetic drift may eventually result in speciation. The combination of seahorses rarely dispersing through the open ocean, but surviving well in it and having a high potential of successfully founding new populations, makes them interesting models for studying the relative importance of vicariance and founder dispersal in marine organisms that disperse by means of rafting.

Molecular dating of marine organisms' phylogenies

Recent advances in the field of model-based analyses have considerably improved the level of confidence in time estimates obtained from molecular data [11]. Variations in evolutionary rates can be accounted for, and uncertainties with regard to calibration points can be incorporated by specifying upper and/or lower limits for a particular divergence event. Nonetheless, in recent reviews of molecular biogeography and molecular dating, Heads [12,13] criticized studies whose results supported cladogenesis as a result of founder dispersal on the basis of questionable molecular dating. Molecular dating can be performed using three methods of calibration: a) dating of the root of the phylogeny of a particular taxon by using the age of the taxon’s oldest known fossil; b) dating of the age of a taxon present on a volcanic island by using the age of the island and c) dating of the cladogenic event that gave rise to two lineages present on either side of a geological barrier by using the time when the barrier formed. Heads [13] rejected the first two methods for the following reasons. Firstly, new fossils are often found that are considerably older than the previously known oldest fossil of a particular taxon, and it is thus impossible to be certain whether the oldest fossil of a particular taxon has indeed been found. The method can be considered particularly problematic in the case of shallow water marine organisms, whose fossil record is often fragmented [14,15]. Seahorses present a case in point, because their fossils are known from only two sites in the northern Mediterranean [16,17]. Secondly, dating by means of the age of volcanic islands can be problematic because such islands are located on subduction zones where new islands are created and old ones disappear continuously, suggesting that an extant species occurring on a volcanic island may be older than its habitat.

Molecular dating using vicariance events

Two major types of vicariance events are potentially useful to calibrate molecular clocks of marine organisms: seaway closures and continental break-up. The best documented vicariance event that impacted on the biogeography of marine species is the closure of the Central American Seaway. The rising of the Isthmus of Panama during the Pliocene isolated the tropical western Atlantic and eastern Pacific oceans [18-20] and resulted in the divergence of formerly continuously distributed species, many of which have remained morphologically similar and are thus readily recognizable as sister taxa. For that reason, the majority of studies on marine species have used the closure of the Central American Seaway as a calibration point [21-23]. However, the utility of such geminate species for calibrating molecular clocks can be nonetheless be problematic. Firstly, some of the species that have been identified as sister taxa may have diverged prior to the final closure of the Central American Seaway (3.1 – 3.5 mya [19]), which results in overestimates of mutation rates [22-24]. Such earlier divergence may have been the result of the oceanographic changes associated with the rising of the isthmus before final seaway closure, or originations resulting from changes in carbonate levels as a result of seaway constriction [25,26]. Secondly, incomplete taxon sampling may result in the wrong species being identified as sister taxa [27].

Few studies on marine organisms have sampled lineages with circumglobal distributions [28,29]. For these, additional seaway closures could be used as calibration points, which may result in greater precision of molecular dating. The closure of the Tethyan Seaway, which once connected the Atlantic Ocean with the Indo-Pacific via the Mediter-
Table 1: Cytochrome b and 16S rRNA sequences of various teleost lineages whose geographic distributions may have resulted from the closures of the Central American, Indonesian and Tethyan seaways, or from continental break-up and spreading of the Atlantic Ocean. Pairwise Kimura 2-Parameter distances between lineages were plotted in Fig. 3.

| Molecular marker | Letter in Fig. 3 | Lineage 1 | Lineage 2 | Accession numbers | Reference |
|------------------|------------------|-----------|-----------|-------------------|-----------|
| **Cytochrome b** |                  |           |           |                   |           |
| A                 | Selene peruviana | S. setapinnis | AF363743/AF363745 | (see Table 3) | (this study) |
| B                 | Hippocampus ingens/fishei | H. reidi/algiricus | AF821666/AF821670/AF821771 | (see Table 3) | (this study) |
| C                 | Merluccius albidus | M. productus/gayi | AF327455/AF327456/AY786433 | Perez et al., unpubl. |
| D                 | Aulostomus chinensis | A. maculatus | AF018599–AF018629 | 118 |
| E                 | Centropomus viridis | C. undecimalis/poeyi | AF231641/AF231647/AF231653/AF231654 | 28 |
| F                 | Ophioblennius atlanticus | O. steindacheri | AF786433 | 23 |
| G                 | Strongylura marina | S. exilis | AF231641/AF231647/AF231653/AF231654 | 97 |
| H                 | Chaetodon paucifasciatus | C. rhombochaetodon complex | U23585–U23733 | 39 |
| I                 | Chaetodon guttissimus | C. multiscinctus/ puntato/fasciatus | U23585–U23733 | (this study) |
| J                 | Hippocampus kuda (Indian Ocean) | H. kuda (Pacific Ocean) | AF429419–AF429433 | 42 |
| K                 | Pterois miles | P. volitans | (see Table 3) | (this study) |
| L                 | Hippocampus capensis | H. ingens/fishei/ reidi/algiricus | AF3311751–AF3311776 | 121 |
| M                 | Hippocampus guttatus/zosterae/erectus/hippocampus | H. spinassimms/kelda/kuda/fuscus/ capensis/algiricus/ ingens/fishei | (see Table 3) | (this study) |
| N                 | Lethrinus atlanticus | Lethrinus Indo-Pacific lineage | AF812251–AF812271 | 119 |
| O                 | Thalassoma (Atlantic Ocean lineage) | Thalassoma (Indo-Pacific lineage)/Gomphosus | AF328857–AF328885 | 120 |
| P                 | Albula vulpes/glodostental/A/B/C/E | A. neoguinaica/D | AF3311751–AF3311776 | 121 |
| Q                 | Hippocampus reidi | H. algiricus | (see Table 3) | (this study) |
| R                 | Albula sp. B (West Atlantic) | Albula sp. B. (East Atlantic) | AF3311751–AF3311776 | 121 |
| S                 | Strongylura timucu | S. senegalis | AF231653/AF231654 | 97 |
| T                 | Hippocampus erectus | H. hippocampus | (see Table 3) | (this study) |
| U                 | Sparisoma ahlare/rubripinne | S. rubripinne (East Atlantic) | U2457034–U2457036 | 98 |
| V                 | Hippocampus guttatus | H. zosterae/erectus/hippocampus | (see Table 3) | (this study) |
| W                 | Nicholsina usta usta | N. usta calvei | U2457022/U2457023 | 98 |
| X                 | Thalassoma norohanum/bifasciutum | T. newtoni/sancthelenae/ascensionis/pavo | AF328861/AF328863/AF328877/AF328878 | 120 |

**16S rRNA**

| A                 | Holocanthus passer | H. bermudensis | AF350857/AF350867 | 61 |
| B                 | Hippocampus ingens/fishei | H. reidi/algiricus | (see Table 3) | (this study) |
| C                 | Pomacanthus paru/arciatus | P. zonipectus | AF350852/AF350868/AF350874 | 61 |
| D                 | Centropomus ensiferus | C. reabaltio | U85008/U85011 | 61 |
| E                 | Centropomus viridis | C. undecimalis/poeyi | U85012/U85013/U85014 | 23, 23 |
| F                 | Strongylura exilis | S. marina | AF231515/AF231521 | 86 |
| G                 | Aulostomus chinensis | A. maculatus | AF141423 | 122, 123 |
| H                 | Hippocampus kuda (Indian Ocean) | H. kuda (Pacific Ocean) | AF327455/AF327456/AY786433 | (see Table 3) | (this study) |
Raney, has been used comparatively rarely to date phylogenies [30,31]. The reason for this may be that the occurrence of both tectonism and climate change (resulting in cyclic fluctuations in the sea level) makes dating of the vicariance event that severed the link between the Mediterranean and the Indo-Pacific problematic, and the exact date of this seaway's final closure is disputed. Drooger [32] considered it to have taken place during the Late Oligocene (23.8 – 28.5 mya), Adams et al. [33,34] suggested a Late Early Miocene closure (18.4 – 20.5 mya), and Rögl and Steininger [35,36] argued for a temporary re-opening during the Middle Miocene (14.8 – 18.4 mya) followed by complete closure 11.2 – 14.8 mya. Adams et al. [37] rejected the evidence for both the Late Oligocene and Middle Miocene closures on the basis of questionable dating. The Middle Miocene date for a temporary re-opening of the seaway [35,36] is nevertheless widely accepted, although some marine organisms from the Indian Ocean do not seem to have dispersed through the Tethyan seaway during this time [38].

Recent genetic work identified the existence of marine sibling species associated with the Indian and Pacific Oceans, respectively, whose distributions sometimes overlap in Indonesia [6,39-43]. The divergence between some of these has been attributed to temporary closure events of the Indonesian Throughflow, which presently connects the Indian and Pacific oceans. Complete and long-lasting closure events were estimated to have taken place during the Middle Miocene (15 – 17 mya [44]) and during the Late Miocene (7.5 – 9.9 mya [45] or 7.0 – 9.5 mya [46]). A further closure of the Indonesian Seaway took place during the Pliocene (3 – 4 mya [47]). Lastly, tectonic uplifts and lowered sea level during Pleistocene glaciations resulted in restricted exchange between Indian Ocean and West Pacific Ocean faunas [48,49].

In addition to distribution patterns that may have arisen as a result of the three seaway closure events, the presence of sister species on either side of the Atlantic Ocean could be interpreted as being the result of continental break-up. Rosen [50] attributed such patterns to sea-floor spreading and widening of the Atlantic Ocean, following the separation of Africa and South America no later than approximately 84 mya [51].

**The relative importance of vicariance and founder dispersal**

In the present paper, we investigated the relative importance of vicariance and founder dispersal as modes of allo-

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**Table 1: Cytochrome b and 16S rRNA sequences of various teleost lineages whose geographic distributions may have resulted from the closures of the Central American, Indonesian and Tethyan seaways, or from continental break-up and spreading of the Atlantic Ocean. Pairwise Kimura 2-Parameter distances between lineages were plotted in Fig. 3. (Continued)**

| Lineage | Species | Accession Numbers | Source |
|---------|---------|-------------------|--------|
| I       | *Pterois miles* | AJ429402-AJ429404/AJ429409-AJ429411 | Seyoum et al., unpubl.; 124 |
| J       | *Albula vulpes* | AY857934AP002973 | (this study) |
| K       | *Hippocampus capensis* | (see Table 3) | (this study) |
| L       | *Thalassoma (Atlantic Ocean lineage)* | AY328983-AY329012 | 120 |
| M       | *Holacanthus passer/bermudensis/ciliaris/tricolor* | AY530847/AY530861/AY530864/AY530867/AY530873 | 61 |
| N       | *Hippocampus guttatus/ozerote/erectus/hippocampus* | (see Table 3) | (this study) |
| O       | *Pomacanthus semirubratus/asfuri/sextiactius* | AY530858/AY530852 | 61 |
| P       | *Hippocampus reidi* | AY530874 | (this study) |
| Q       | *Hippocampus erectus* | AY530873 | (this study) |
| R       | *Sparisoma axillare/rubripine (West Atlantic)* | (Not yet available) | 98 |
| S       | *Strongylura timucu* | AF231526/AF231527 | 97 |
| T       | *Nicholsina usta usta* | Not on GenBank | 98 |
| U       | *Thalassoma norohanum/bifasciatum* | AY328988/A328990/A329003/A329004/A329009 | 120 |
| V       | *Hippocampus guttatus* | (see Table 3) | (this study) |
patric speciation that may have impacted on the phylogeny of a circumglobally distributed seahorse lineage (Fig. 1), while accounting for the abovementioned uncertainties regarding molecular dating of the phylogenies of marine organisms. This particular lineage was chosen because its wide distribution may either indicate ancient vicariance or large-scale dispersal, and because it may have been affected by more than one vicariance event. Exact phylogenetic relationships are not fully understood, but all species associated with this lineage have been identified using molecular methods [52,53], ensuring complete taxon sampling. Other seahorse lineages are less useful for this purpose, because they have more restricted distributions, phylogenetic relationships are comparatively poorly resolved, and a recent increase in species descriptions makes it likely that not all species have yet been identified [52-54].

Without knowledge of divergence times, the distribution patterns of several pairs of lineages within the circumglobal clade could be interpreted as being the result of vicariance. These include Eastern Pacific vs. Atlantic species (whose divergence could be the result of the closure of the Central American Seaway), Indian Ocean vs. West Pacific species (Indonesian Seaway closure), Atlantic vs. Indo-Pacific species (Tethyan Seaway closure) and amphitropical Atlantic species pairs (spreading of the Atlantic Ocean).

Vicariance biogeographers have objected to founder dispersal hypotheses because the sporadic nature of such events does not make them falsifiable [55]. Although the establishment of ocean currents has in some cases resulted in repeated directional long-distance dispersal [56,57], whether or not a species becomes established in a new habitat depends considerably on its dispersal abilities and life history characteristics. Even if a large number of species share a pattern resulting from founder dispersal, it is unlikely that they all would have colonized a particular habitat contemporaneously, and molecular dating using founder dispersal is thus less precise than molecular dating using vicariance events. However, if vicariance hypotheses can be rejected, then founder dispersal should be supported by default [5]. In this study, we estimated the ages of divergence events of seahorse lineages with geminate distribution patterns by using the well-documented closure of the Central American Seaway as a primary calibration point. Vicariance biogeographic interpretations of these are challenged if a) divergence time estimates are significantly different from the dates of vicariance events suggested in the literature and b) divergence events in different positions in the phylogeny that have resulted in the same distribution patterns of sister lineages have occurred at different times.

Results
Phylogenetic reconstructions
A maximum likelihood phylogenetic tree reconstructed from sequence data of three mitochondrial and two nuclear markers recovered six major clades that were each associated with a distinct biogeographic region (Fig. 2). A congruent tree was recovered with Bayesian Inference, and most nodes were supported by significant posterior probabilities. The most parsimonious tree, on the other hand, did not recover some of the lineages, but none of the nodes that differed from the other two phylogenies had high support (all jackknife values <56). When a parsi-
mony tree was constructed using the two most rapidly mutating markers only (control region and cytochrome b), then node A was also recovered.

The distribution patterns of several lineages in the phylogeny indicate that they could be the result of seaway closures or spreading of the Atlantic Ocean (indicated by letters A – G). In several cases, the same distribution patterns were recovered in different positions in the tree. Firstly, a split into an Atlantic and an Indo-West Pacific lineage was recovered twice. The first event (node D) resulted in a split into an Atlantic Ocean group comprising Hippocampus hippocampus, H. erectus, H. zosterae and H. guttulatus, and a group whose basal split resulted in an Indo-West Pacific lineage comprising H. spinosissimus and H. kelloggi and a lineage comprising all remaining species (present in the Indo-West Pacific, Atlantic Ocean and East/Central Pacific). The fact that Indo-West Pacific species are present in both of the latter lineages suggests that this group may have originated in this region. The second event that resulted in an Atlantic Ocean and an Indo-West Pacific lineage is the split defined by node C, resulting in the divergence of H. capensis (western Indian Ocean) and the Atlantic/eastern Pacific group comprising H. ingens, H. fisheri, H. reidi and H. algiricus. Amphi-Atlantic distribution patterns were recovered three times, the most basal being a split between the eastern Atlantic H. guttulatus and a group including the western Atlantic species H. erectus.

Figure 2
Phylogeny of the circumglobal seahorse lineage. The phylogenetic tree with the highest likelihood score reconstructed by means of likelihood ratcheting. The data matrix comprised five partitions: mitochondrial control region, cytochrome b and 16S rRNA, and nuclear S7 intron and Aldolase. Associations of lineages with biogeographic regions are indicated. Nodal support is indicated by three numbers; these represent bootstrap values from maximum likelihood searches, jackknife support from parsimony searches, and posterior probabilities from Bayesian Inference. Hyphens indicate clades that were not recovered using parsimony. White circles indicate divergence events that may have resulted from vicariance events. Letters within these correspond to those in Table 2.
Table 2: Divergence time estimates among seahorse lineages whose cladogenesis may have been the result of vicariance events by virtue of the present-day distribution patterns of their species.

| Calibration range | B               | C               | D               | E               | F               | G               |
|-------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| 3.1 – 3.5         | 3.73 ± 0.29     | 3.67 ± 0.42     | 15.12 ± 3.46    | 1.47 ± 0.53     | 5.33 ± 1.79     | 14.60 ± 3.37    |
|                   | (2.26 – 5.84)   | (3.18 – 4.76)   | (9.85 – 23.26)  | (0.54 – 2.61)   | (2.64 – 9.47)   | (9.53 – 22.49)  |
| 3.1 – 4.6         | 4.26 ± 1.15     | 4.16 ± 0.66     | 16.60 ± 4.00    | 1.66 ± 0.63     | 6.01 ± 2.13     | 16.05 ± 3.89    |
|                   | (2.46 – 6.94)   | (3.24 – 5.70)   | (10.50 – 26.05) | (0.60 ± 3.04)   | (2.89 – 11.14)  | (10.09 – 25.22) |
| 3.1 – 8.5         | 5.15 ± 1.92     | 5.03 ± 1.50     | 19.07 ± 5.76    | 2.02 ± 0.94     | 7.18 ± 3.07     | 18.46 ± 5.63    |
|                   | (2.60 – 9.98)   | (3.27 – 8.75)   | (11.04 – 33.05) | (0.66 – 4.31)   | (3.07 – 14.87)  | (10.60 – 32.16) |

Divergence times were estimated using the program MULTIDIVTIME [102] under the assumption that the closure of the Central American Seaway (Node A in Fig. 2) resulted in the divergence of two sister lineages associated with the eastern central Pacific (Hippocampus ingens and H. fisheri) and Atlantic Oceans (H. reidi and H. algiricus), respectively. Phylogeographic distribution patterns may have been the result of the following vicariance events. Node B (Indian Ocean vs. West Pacific): Closure of the Indonesian Seaway. Nodes C and D (Indo-Pacific vs. Atlantic Ocean): Closure of the Tethyan Seaway. Node E, F and G (amphi-Atlantic distribution patterns): continental break-up and spreading of the Atlantic Ocean. Three possible calibration ranges for the closure of the Central American Seaway were specified. Comparisons of the species affected by this vicariance event with other teleosts having similar distribution patterns (Fig. 3) indicate that the seahorses were among the last to diverge. This suggests that their cladogenesis was associated with the final closure of the seaway, i.e. no earlier than approximately 4.6 mya (a hypothesis that is further supported by the finding that marine organisms in nearshore habitats were among the last species to have diverged as a result of Central American seaway closure [20,60,61]). Divergence time estimates are indicated as mean ± S.D. (95% confidence interval). Suggested dates of vicariance events: Central American Seaway closure: 3.1 – 3.5 mya (assuming that the divergence of the transisthmian seahorse lineages took place when a land bridge formed in Central America [18]); 3.1 – 4.6 (taking into consideration that seahorse divergence may have been affected by the reorganisation of ocean currents associated with the closure of the seaway [24]); 3.1 – 8.5 mya (the upper bound being the time when the earliest recorded evolution associated with the closure of the seaway took place in marine corals and foraminiferans [25]); Indonesian Seaway closure: 0.01 – 1.8 [47,48]; 3 – 4 mya [46]; 7 – 10 mya [44,45]; 15 – 17 mya [43]; Tethyan Seaway closure: 11.2 – 14.8 mya [34,35]; 18.4 – 20.5 [36]; 23.8 – 28.5 [31]; complete separation of the land masses on either side of the Atlantic Ocean: 84 mya [50].

and H. zosterae (node G; this group also includes the eastern Atlantic species H. hippocampus in a derived position), followed by a later amphi-Atlantic split between H. erectus and H. hippocampus (node F). The divergence between H. reidi and H. algiricus resulted in a further amphi-Atlantic distribution pattern (node E).

**Molecular dating**

To explore the feasibility that the seahorse lineages present on either side of the Isthmus of Panama diverged as a result of Central American Seaway closure, we compared pairwise genetic distances between them with genetic distances between other geminate teleost lineages present on both sides of the Isthmus of Panama (Table 1, Fig. 3). We hypothesized that if the divergence of the seahorse lineages was linked to the final closure of the seaway, then the pairwise distance between them should be among the lowest of all the teleost lineages investigated. Pairwise distances were also calculated for pairs of teleost lineages that could have arisen as a result of the other three vicariance events relevant to the circumbroadly distributed seahorse lineage by virtue of their distribution patterns. Using relative rate tests, it was found that only one of the teleost genera whose species have distribution patterns identical to those of the geminate seahorse lineages was characterized by a significantly different rate (Lethrinus vs. Hippocampus cytochrome b: difference in the rate of non-synonymous sites = -0.031 ± 0.015 [S.D.], p = 0.039). The rate of 16S rRNA of a number of genera could not be compared with the seahorses, because a different portion of this marker had been sequenced. Pairwise distances between these and the seahorses, as well as those of Lethrinus and Albula (used as outgroup) and the seahorses, are nonetheless shown in Fig. 3 and are indicated with asterisks.

Comparisons of mean K2P distances between pairs of seahorse lineages that may have diverged as a result of vicariance events with those of other teleosts having congruent distribution patterns indicate that the seahorse lineages defined by node A in Fig. 2 (Central American Seaway closure) diverged comparatively recently. We considered this to be evidence for a Pliocene divergence of this lineage as a result of the final closure of the Central American Seaway, an event that is considered to have occurred no earlier than 4.6 mya. The mean genetic distance among cytochrome b sequences of the lineage defined by node B (Indonesian Seaway closure) was slightly lower than that of the lineage defined by node A, whereas the genetic distance among 16S rRNA sequences was distinctly lower. K2P distances among control region sequences (not shown) were also slightly lower for Indonesian Seaway divergence than for Central American Seaway divergence (0.036 and 0.043, respectively), indicating that this event may have taken place during the Late Pliocene or Early Pleistocene. Present-day distribution patterns of several
pairs of lineages in the seahorse phylogeny could be interpreted as having resulted from the remaining two vicariance events (two in the case of Tethyan seaway closure and three in the case of continental break-up during the Mesozoic). Mean pairwise K2P distances between these differed considerably, as did distances between other teleost lineages having the same distribution patterns.

Divergence time estimates among seahorse lineages were obtained using a relaxed molecular clock method [58]. When the final date for the closure of the Central American seaway (3.1 – 3.5 mya) is accepted as the date when the transisthmian seahorse lineages diverged, based on the considerations in the previous paragraph, then only two divergence estimates of nodes defining species pairs whose distribution patterns indicate that they could have resulted from the closures of the Indonesian and Tethyan seaways (Table 2). Asterisks indicate teleost lineages whose evolutionary rate differed from that of the seahorses (A: N), whose rates were not tested because a different portion of 16S rRNA was sequenced (B: A, C, M, O) or which were used as outgroup in relative rate tests (A: P, R; B: J).

![Graphical representation of pairwise distances](image)

**Figure 3**

**Genetic distances among geminate teleost species.** Pairwise Kimura 2-Parameter distances [104] between lineages of seahorses (white squares) and other teleost species (black squares) whose present-day distribution patterns indicate that they may have diverged as a result of the closures of the Central American, Indonesian, or Tethyan seaways, or as result of continental break-up and spreading of the Atlantic Ocean. **A:** Cytochrome b sequences; **B:** 16S rRNA sequences. Letters represent teleost lineages listed in Table 1. The black arrow indicates the pairwise distance between the two seahorses lineages hypothesized to have diverged as a result of closure of the Central American Seaway. These lineages were used to estimate divergence times in Table 2. White arrows indicate distances between lineages whose divergence time estimates matched published dates for the closures of the Indonesian and Tethyan seaways (Table 2). Asterisks indicate teleost lineages whose evolutionary rate differed from that of the seahorses (A: N), whose rates were not tested because a different portion of 16S rRNA was sequenced (B: A, C, M, O) or which were used as outgroup in relative rate tests (A: P, R; B: J).
the species defined by node D were estimated to have diverged 15.12 ± 3.46 mya, which matches the temporary re-opening of the Tethyan seaway during the Middle Miocene (14.8–18.4 mya) followed by complete closure 11.2 – 14.8 mya [34,35]. However, confidence intervals for this estimate are comparatively large (9.85 – 23.26 mya), and the earlier date of 18.4 – 20.5 mya [36] must also be considered feasible.

The establishment of the amphi-Atlantic distribution patterns all post-dated 84 mya, irrespective of the calibration range used for the closure of the Central American seaway. This suggests that these were not the result of continental break-up during the Mesozoic. Because of this, and because 95% confidence intervals of the divergence dates of nodes that have resulted in the same distribution patterns did not overlap when a calibration range of 3.1 – 3.5 mya was specified for the closure of the Central American Seaway (although some overlap was found when wider calibration ranges were specified, Table 2), interpretations of the seahorses’ present-day distribution patterns based exclusively on vicariance biogeographic hypotheses are not well supported.

As divergence time estimates are more precise when multiple calibration points are specified [59], we chose a combination of vicariance events that are likely to have impacted on cladogenesis in the seahorse phylogeny to date all other nodes in the phylogeny. The selection of these was based on the assumption that the divergence event that resulted from the closure of the Central American Seaway occurred no earlier than 4.6 mya, and that the
siblings of the Indonesian Seaway 3 – 4 mya resulted in the
divergence of the lineages defined by node B. We also
included node D as a calibration point, assuming that the
Atlantic Ocean vs. basally Indo-Pacific distribution of its
species resulted from the closure of the Tethyan Seaway.
Although the timing of this divergence event is comparat-
ively vague, mean divergence times ± S.D. estimated
when only node A (3.1 – 3.5 mya) and only node B (3.0
– 4.0 mya) were used as calibration points fell within the
ranges of the two most reliable dates for this event sug-
gested in the literature (Node A: 15.12 mya ± 3.46 mya;
Node B: 14.11 mya ± 3.27 mya; Rögl and Steininger
[34,35]; 11.2 – 14.8 mya; Adams et al. [32,33]; 18.4 – 20.5
mya). A phylogenetic tree scaled to geological time was
constructed by specifying divergence times for the three
seaway closure events (Pliocene for the Central American
and Indonesian seaways and Late Early to Middle
Miocene for the Tethyan Seaway) is characterized by the
largest number of divergence events having taken place
during the Pliocene (Fig. 4). The 95% confidence intervals
of older divergence events are large compared to most of
the more recent events, as we allowed for a wide calibra-
tion range for the root node.

Discussion

Seaway closure events as calibration points
In studies of marine organisms that employ molecular
dating, it is common practice to apply the most recent
dates suggested in the geological literature to date diver-
gence events considered to have resulted from the closure
of a seaway [30,60,61]. As the evolutionary histories of
generate species pairs may be more complicated than is
generally acknowledged, we avoided overconfidence in
calibration points by firstly determining which vicariance
events were most likely to have resulted in present-day
biogeographic patterns of seahorse species associated with
the circumglobal clade, and secondly, by specifying upper
and lower bounds for each calibration point to account
for uncertainties concerning the exact dates of vicariance
events. Comparisons with other teleosts showed that the
amphipanamic distribution pattern arose comparat-
ively recently, and is thus likely to have been linked to the
final closure of the Central American Seaway. As the four
seahorse species affected by the closure of the seaway all
occur in shallow water [62], this conclusion is supported
by the hypothesis that divergence events among species
occurring in the nearshore habitat or in freshwater are
linked to the final closure of the Central American Sea-
way, whereas species with significant marine phases are
likely to have diverged earlier [20,63,64]. Estimates of the
vicariance event that resulted in the basally Indo-Pacific
vs. Atlantic Ocean distribution pattern defined by node D
(Figs. 2 and 4) were less certain than those of the other
two vicariance events. However, our estimates indicate
that this basal cladogenic event in the phylogeny is likely
to have occurred some time during the Late Early to Mid-
dle Miocene, which is supported by geological estimates
of the timing of the closure of the Tethyan Seaway [33-
36].

Comparisons with other pairs of generate teleost species
present on either side of the Isthmus of Panama indicate
that K2P distances ~0.05 for cytochrome b, ~0.045 for
control region (domain II), and ~0.02 for 16S rRNA may
indicate that the lineages under investigation are likely
to have diverged as a result of the final closure of the Central
American Seaway. Assuming a conservative estimate of
3.1 – 4.6 mya for the divergence of these lineages, these
values correspond to approximately 1 – 1.5% (Cyto-
chrome b), 0.9 – 1.4% (control region domain II) and 0.3
– 0.5% (16S rRNA) sequence divergence per million
years, and are thus lower than the commonly used value
of 2% per million years for animal mtDNA [63]. Our
results indicate that the closure of the Indonesian Seaway
may also be useful for calibrating molecular clocks. In this
case, a K2P distance of ~0.03 for cytochrome b, ~0.036 for
control region, and a distance below 0.02 for 16S rRNA
may indicate that the lineages under investigation
diverged as a result of Pliocene closure of the seaway.

Causes of other dichotomies in the phylogeny
Apart from the three seaway closures, there are no obvious
vicariance events that could have resulted in any of the
other dichotomies. None of the divergence events result-
ing in sister lineages with amphi-Atlantic distributions
could be linked to continental break-up (84 mya) and
spreading of the Atlantic Ocean on the basis of molecular
dating, even when the unlikely upper bound of 8.5 mya
was specified for the closure of the Central American Sea-
way. This date represents the time when the constriction
of the seaway and the associated increase in carbonate-
content in southern Caribbean deep-sea sediments
resulted in origins in reef corals and carbonate-assoc-
iated benthic foraminifera, which is unlikely to have
affected shallow-water seahorses. A vicariance bioge-
ographic interpretation of these distribution patterns is fur-
ther weakened by fossil data: the oldest known fossil of
the family Syngnathidae (of which seahorses are consid-
ered to be one of the most derived genera by virtue of their
advanced brood pouch morphology [66]) is less than 50
my old [67].

We suggest that the presence of recently diverged sister-
species on both sides of the Atlantic Ocean (H. erectus
[east coast of the Americas] and H. hippocampus [Europe];
H. reidi [east coast of the Americas] and H. algiricus [West
Africa]), is the result of founder dispersal. Divergence time
estimates for these two lineages fall into the Late Miocene/
Early Pliocene and Late Pliocene/Pleistocene, respectively.
Teske et al. [53] hypothesized that the ancestor of H. hip-
Hawai'ian seahorse

persal events in either direction [76]. Our finding that the East Pacific species, and there is evidence for sporadic dis-
the Central Pacific have sister taxon relationships with taxa in the Indo-West Pacific [74,75]. However, many teleost species present in the Atlantic Ocean, as divergence of this lineages was estimated to have taken place after the closure of the Central American Seaway, the founder event that gave rise to H. hippocampus is thus likely to be the indirect result of tectonic changes.

The other East Atlantic species, H. algiricus, is also likely to be the product of long-distance dispersal in an eastward direction, as its sister species, H. reidi, occurs on the east coast of the Americas, and the next basal lineage comprises eastern Pacific seahorses. The divergence event that gave rise to this amphibi-Atlantic gennate species pair took place more recently (Late Pliocene to Pleistocene). There are numerous examples of such recently established amphibi-Atlantic distributions in the literature, and in many cases, the lineages in question are morphologically and genetically so similar that they are considered to be single species [70-73]. Even the oldest amphibi-Atlantic divergence event in the phylogeny, that between the European species H. guttulatus and the lineage comprising basal American species (H. zosterae and H. erectus), is unlikely to be the result of vicariance following the expansion of the Atlantic Ocean, as divergence of this lineages was estimated to have occurred during the Miocene.

Lastly, vicariance models invoked for the presence of species on Hawai'i suggest that because of the geological history of the Pacific plate, endemic Hawai'i taxa should have sister taxon relationships with taxa in the Indo-West Pacific [74,75]. However, many teleost species present in the Central Pacific have sister taxon relationships with East Pacific species, and there is evidence for sporadic dispersal events in either direction [76]. Our finding that the Hawaiian seahorse H. fisheri is closely associated with American/West African seahorses provides a further example of westward dispersal from the Americas, an event that was estimated to taken place after the closure of the Central American Seaway.

Conclusion

Since the validation of plate-tectonic theory and the development of cladistic methods, vicariance events have been primarily invoked to explain disjunct species distributions throughout the world. However, improvements in molecular dating techniques have resulted in increased support for recent dispersal hypotheses over more ancient vicariance events, as in many cases, molecular divergences were considered too small to be explained by vicariance [5]. Despite these developments, Heads [12,13] rejected dispersal hypotheses in favor of vicariance hypotheses, and considered studies on the phylogeographic history of cichlid fishes [77,78] (a teleost family of Gondwanan origin that occurs exclusively in freshwater) to be exemplary in terms of their convincing conclusions (and lack of molecular dating). Given that long-distance dispersal between continents is impossible for obligate freshwater fishes, vicariance hypotheses are appropriate to explain observed distribution patterns of freshwater organisms. However, this is completely different in marine organisms, many of which can readily disperse in the oceans over great distances [27,70,71,76,79], and to whom the barrier is thus not absolute. Heads [12] acknowledged that many marine organisms can readily reach far outside their established ranges, but argued that they do not establish themselves because of competition from congeneric vicariants already established in such habitats. Likewise, Briggs [80] suggested that competition may prevent the colonization of high diversity habitats. The "competitive exclusion principle" [81] is now considered to be controversial [82]. Although a number of recent experiments do support the notion that high diversity may increase a community's invasion resistance [83-86], its effect may be difficult to discern from other factors such as predation, inappropriate climate and disease [87]. Nonetheless, the successful establishment of a founder population may be inhibited to some degree by the presence of interspecific competition [88-90], or facilitated by a lack of resource competition (i.e. the existence of an "empty niche" [91]), as the available resources allow the founders to rapidly increase their numbers. This may explain why a) the lineage defined by node A (Fig. 2) is absent from the species-rich West Pacific, despite the high dispersal potential of its species and b) the colonization of West Africa and Hawai'i (two regions with low seahorse diversity) was possible for H. algiricus and H. fisheri, respectively. The European species H. guttulatus and the more recent arrival H. hippocampus are sympatric, but they do not compete directly because of different microhabitat preferences [92]. The establishment of human-introduced marine species in new habitats is well documented [93-95], and it seems unreasonable to reject the notion that such colonization events may occur naturally, albeit at a lower rate. Both vicariance and founder dispersal thus have to be considered plausible in marine organisms, and our study indicates that molecular dating is a useful tool to determine when a divergence event is likely to have occurred and what may have caused it.

Even if one does not consider genetic differentiation of molecular markers to be at least roughly correlated with time, one must nonetheless concede that the establishment of the same phylolgeographic patterns in clades nested within each other in the same phylogeny cannot have occurred simultaneously. Hence, if the divergence of H. guttulatus vs. H. zosterae, H. erectus and H. hippocampus was the result of continental break-up and spreading of...
the Atlantic Ocean (vicariance), then the split between *H. erectus* and *H. hippocampus* must have been caused by subsequent long-distance dispersal. This is further supported by the fact that even if the unlikely upper limit of 8.5 mya is specified for the closure of the Central American Seaway, the upper 95% confidence limit for the divergence of *H. erectus* and *H. hippocampus* postdates even the most recent estimate for the spreading of the Atlantic Ocean. Likewise, if the Atlantic biome clade comprising the above species and the basally Indo-Pacific clade comprising all other species diverged as a result of Tethyan Seaway closure, then node A cannot define a western Tethyan lineage that arose at the same time (again, the upper 95% confidence limit using the upper bound of 8.5 mya considerably postdates the most recent geological estimate for this event). Furthermore, the fact that the basal split in the seahorse phylogeny into an Atlantic Ocean lineage and a basally Indo-Pacific lineage predated all of the divergence events resulting in amphi-Atlantic distribution patterns (which could be interpreted as Tethyan Seaway closure 11.2 – 28.5 mya, and continental break-up 84 mya, respectively) suggests that none of the amphi-Atlantic distribution patterns are the result of vicariance. As none of the pairwise genetic distances between amphi-Atlantic sister lineages of other teleosts investigated were substantially greater than the distance between the two most divergent pair of seahorse lineages, we conclude that these did not diverge as a result of vicariance either. Our results thus support other recent studies on Atlantic Ocean marine organisms that identified long-distance dispersal as the cause for the establishment of amphi-Atlantic sister lineages [70,96-98].

The impression that vicariance hypotheses are increasingly being invoked to explain biogeographic patterns in the sea [12] may to some extent have been created by the increased use of molecular dating in genetic studies of marine organisms, which relies on well-documented vicariance events to use as calibration points. Founder dispersal events are less useful for this purpose, but we conclude that in the circumglobally distributed seahorse lineage, divergence events that resulted from founder dispersal are likely to outnumber divergence events that resulted from vicariance. We hypothesise that founder dispersal is thus of particular importance in species that disperse by means of rafting.

**Methods**

**Taxon sampling and sequencing**

The total sample used to reconstruct the phylogeny of the monophyletic circumglobally distributed seahorse lineage (clade 4 in Teske et al. [53]) consisted of 26 individuals from 13 species (Table 3). We also included a single individual each of species from the circumglobal clade’s three sister lineages (clades 1, 2 and 3 in Teske et al. [53]) as outgroup taxa. We attempted to obtain tissue material from more than one specimen of each ingroup species to account for intra-specific variation. This was considered particularly important in the case of species with wide distribution ranges. The Hawai’ian seahorse that was previously referred to as *Hippocampus hilonis* [6] or *H. fisheri* [53] is correctly referred to as *H. fisheri* in this study. Morphologically, this species resembles *H. trimaculatus* (a species closely related to the outgroup species *H. comes*), suggesting that it is not part of the circumglobal clade and that specimens that group genetically with *H. ingens* are likely to be *H. hilonis* (a possible synonym of *H. kuda*, SA Lourie, pers. comm.). We have now confirmed that our Hawai’ian specimens are morphologically very different from *H. kuda* and that they fit the description of *H. fisheri* [62] well. All five genetic markers used in this study confirm that our specimens are the sister taxon of *H. ingens*, that they are not part of the *H. kuda* complex, and that they are genetically very different from *H. trimaculatus* (Fig. 2). This suggests that the morphology of *H. fisheri* was misleading.

For phylogenetic reconstruction and molecular dating, we used sequence data of three mitochondrial markers (control region, cytochrome *b* gene and 16S rRNA) and one nuclear marker (the first intron of the S7 ribosomal protein, hereafter referred to as S7 intron). Sequences of a second nuclear marker, Aldolase, were used for phylogenetic reconstructions only. Mitochondrial markers often fail to resolve deeper relationships at the taxonomic levels of family and order in various teleosts [99,100], but generally provide good resolution at and below the genus level [101,102]. For that reason, they can be considered ideal to study the phylogeny of the circumglobal seahorse clade (one of five major genetic lineages comprising the genus *Hippocampus* [53]). DNA extraction and amplification of molecular markers followed previously described protocols [43,53,103]. A total of 61 new sequences were generated for this study (GenBank accession numbers starting with DQ, Table 3). These were complemented with 82 previously published seahorse sequences (accession numbers starting with AF and AY [6,43,52,53]). For phylogenetic reconstructions, complete cytochrome *b* sequences generated by Casey et al. [52] were used whenever available, which are 424 bp longer than the partial cytochrome *b* sequences generated in this study and in Lourie et al. [43]. Aldolase sequences were obtained for a single individual of each species only. As this marker was characterized by comparatively little variation, it was not used for molecular dating.

**Alignments and phylogenetic reconstructions**

Sequence alignments were generated using BALI-PHY [104]. This program estimates alignment and phylogeny
Table 3: Samples used in this study, including species names, collection localities, collectors or museums that contributed samples and GenBank accession numbers.

| Species          | Collection locality | Collector/ Museum | Control region | Cytochrome b | 16S rRNA | S7 intron | Aldolase |
|------------------|---------------------|-------------------|----------------|-------------|----------|-----------|----------|
| **Ingroup:**     |                     |                   |                |             |          |           |          |
| Hippocampus      |                     |                   |                |             |          |           |          |
| algiricus        | Benin (*Ghana)       | Z. Sohou, J. Macpherson | DQ288337 | AF192642 | AY277302 | AY277328 | AY277366 |
| H. capensis      | South Africa        | P. Teske          | DQ288338 | DQ288353 | AY277302 | AY277328 | AY277352 |
| H. erectus       | USA (Gulf of Mexico) | FM                | DQ288325 | AF192662 | AF355007 | AY277339 | AY277354 |
| H. fisheri       | Colombia            | H. Hamilton       | DQ288326 | DQ288341 | DQ288359 | DQ288378 |          |
| H. fuscus        | Egypt (Red Sea)     | H. Gabr           | DQ288327 | DQ288354 | DQ288371 | AY277335 | AY277359 |
| H. guttulatus    | Italy (*Portugal)   | PS, *J. Curtis    | DQ288328 | AF192666 | AY277304 | AY277337 | AY277348 |
| H. hippocampus   | Italy (Portugal)    | PS                | DQ288323 | AF192666 | AY277306 | AY277338 | AY277348 |
| H. ingens        | Ecuador             | H. Hamilton       | DQ288333 | DQ288346 | DQ288365 | DQ288383 |          |
|                  | Mexico (East Pacific)| J. Baum          | DQ288347 | DQ288354 | DQ288371 | AY277335 |          |
|                  | Peru                | PS                | DQ288331 | AF192672 | AY277303 | AY277333 | AY277365 |
|                  | USA (East Pacific)  | H. Hamilton       | DQ288332 | DQ288344 | DQ288363 | DQ288382 |          |
| H. kellogii      | Vietnam             | PS                | DQ288329 | AF192655 | AY277298 | AY277325 | AY277350 |
| H. kuda          | Fiji                | H. Hamilton       | DQ288333 | DQ288357 | DQ288374 | DQ288388 |          |
|                  | India               | A. Sreepada       | DQ288345 | AF192679 | DQ288372 | AY277324 | AY277355 |
|                  | Indonesia           | S. Lourie         | DQ288349 | AF192654 | DQ288376 | DQ288387 |          |
|                  | Philippines         | M. Santos         | DQ288369 | AF192683 | DQ288375 | AY277329 | AY277356 |
| H. reidi         | Brazil              | L. Smith          | DQ288336 | AF192692 | DQ288368 | DQ288386 |          |
|                  | Honduras            | H. Hamilton       | DQ288335 | DQ288348 | DQ288367 | DQ288385 |          |
|                  | USA (Gulf of Mexico)| PS                | DQ288334 | DQ288347 | DQ288366 | DQ288384 | AY227367 |
| H. spinossissimus| Philippines         | S. Lourie         | DQ288329 | AF192695 | AY277296 | AY277323 | AY277364 |
| H. zosterae      | USA (Gulf of Mexico)| FM                | DQ288327 | AF356071 | DQ288360 | DQ288379 | AY277371 |
|                  | USA (Gulf of Mexico)| FM                | DQ288328 | AF356071 | DQ288361 | DQ288380 |          |
| **Outgroup:**    |                     |                   |                |             |          |           |          |
| H. breviceps     | Australia           | AM                | DQ288319 | AF192647 | AY277287 | AY277320 | AY277342 |
| H. comes         | Philippines         | N. Perante        | DQ288321 | AF192656 | AY277289 | DQ288376 | AY277352 |
| H. coronatus     | Japan               | T. Mukai          | DQ288320 | AF192658 | AY277293 | AY277319 | AY277348 |

Sequences in boldface were used to reconstruct phylogenies. All sequences except those of Aldolase were used for molecular dating. Cytochrome b sequences whose accession numbers start with AF are 423 bp longer than those starting with DQ and were used to reconstruct phylogenies. Control region, cytochrome b and 16S rRNA are mitochondrial markers, S7 intron and Aldolase are nuclear markers. In some cases, samples from two different localities were used to represent a particular species. FM = Florida Museum, PS = Project Seahorse, AM = Australian Museum.

*Collection locality, collector, and accession number of the less frequently used sample.
simultaneously in a Bayesian framework, and in this way avoids the problems associated with poor guide trees in the more widely used alignment program CLUSTALX [105]. Confidence of the results is assessed using posterior probabilities, and the indel model implemented allows indels several characters in length and also allows these to nest or overlap if they lie on separate branches. Three partitions were characterized by length differences (control region, 16S rRNA and S7 intron). These were aligned individually because of computational constraints when aligning combined data-sets. Prior to generating BALI-PHY alignments, we explored which models of sequence evolution were most appropriate for each partition by generating CLUSTALX [105] alignments of ingroup sequences using default settings, and then using the Akaike information criterion [106] as implemented in MODESTEST version 3.7 [107]. As all models selected were fairly complex, we specified the most complex model presently implemented in BALI-PHY (the Tamura-Nei model [108]) for simultaneous estimation of alignment and phylogeny. A gamma distribution parameter and an assumed proportion of invariable sites were also specified if these were selected as model components by MODESTEST. For each partition aligned in BALI-PHY, 500 iterations were performed, and the final alignment was based on all alignments recovered excluding the burn-in. The procedure was repeated five times to ensure consistency of results. BALI-PHY’s algorithm has very short burn-in times, and in the case of our comparatively small data-sets, convergence was complete by the tenth generation for all three partitions characterized by length differences. Maximum a posteriori alignments used for further analyses were thus based on the remaining 490 iterations.

A single representative of each species was used for alignment and phylogenetic reconstruction, because exploratory alignments using the program CLUSTALX [105] followed by phylogenetic reconstructions using the neighbour-joining method [109] revealed that the geographically distant representatives of each species were monophyletic. An exception was made in the case of the Indo-Pacific species Hippocampus kuda, which was represented by one individual from India (Indian Ocean lineage) and one individual from north Sulawesi, Indonesia (West Pacific lineage). Control region sequences indicated that these two lineages may not be monophyletic and might be considered to be different species [6], a result that was, however, not strongly supported. In addition to 14 specimens representing the ingroup, we also included the three outgroup species H. breviceps, H. cones and H. coronatus (representatives of clades 1, 2 and 3 in Teske et al. [53]). As the alignment method was strongly influenced by the presence of missing data, a section of missing data 50 bp in length in the S7 intron sequence of H. erectus was temporarily replaced with corresponding characters from its sister species H. hippocampus (the surrogate characters were removed for subsequent analyses). The sister-taxon relationship of the two species was strongly supported by previous studies [52,53].

The data matrix used for phylogenetic reconstructions included the three partitions aligned using BALI-PHY, as well as three additional partitions. Firstly, complete cytochrome b sequences were used, whenever available (resulting in 424 bp of missing data in H. fisheri and H. fuscus). Secondly, indels in the three partitions that were characterized by length differences were coded as presence/absence characters in some analyses if they were present in more than one species, had a length of more than one nucleotide, and had clearly defined alignment boundaries. Thirdly, Aldolase sequences [53] were included (Table 3). These contained only two indels, each of them one nucleotide in length, and were aligned by eye. The aligned sequence lengths of control region, 16S rRNA and S7 intron were 389, 532 and 596 nucleotides in length, respectively. Eight indels were coded as presence/absence data, and cytochrome b and Aldolase sequences were 1140 and 188 nucleotides in length, respectively, resulting in a total sequence length of 2853 characters.

This data matrix (excluding the partition comprising indels) was used to recover a maximum likelihood tree by generating 200 iterations of the ‘likelihood ratchet’ [110], which is a model-based procedure analogous to the parsimony ratchet [111]. Tree searches that employ ratcheting are less likely to become stuck on suboptimal tree islands than any other method of phylogenetic reconstruction presently available. Runs were repeated five times to ensure that the tree space was adequately explored, as indicated by the tree topology with the highest likelihood score being consistently recovered. Nodal support for this tree was obtained by means of bootstrap resampling (1000 replicates) using maximum likelihood in PAUP* version 4.0b10 [112], with a single most appropriate model being specified for the whole data-set as determined using the Akaike Information Criterion in MODESTEST. The heuristic search was limited to a maximum of 10 000 saved trees. Secondly, we used the heuristic parsimony analysis in PAUP* to recover the most parsimonious tree using the same data-set and including indels as presence/absence characters. Default parameters were specified, with 100 random addition replicates and 1000 trees retained at each step. Nodal support for this topology was obtained by means of jackkniﬁng (100 000 replicates, 50% deletion) using default parameters in PAUP*. Thirdly, MRBAYES version 3.1 [113] was used to determine posterior probabilities for each node. The Markov chain Monte Carlo process was set for four chains to run simultaneously for 2 000 000 generations, with trees being sampled every 100 generations. In addition to
examining posterior probabilities of the resulting trees to
determine when burn-in was complete, we also compared
standard errors of posterior probabilities between simulta-
nous runs. As these tended to decrease for some time
after the burn-in phase, only trees were used once the dif-
ference in standard errors had also stabilized (i.e. the first
4 000 out of a total of 20 000 trees were excluded). Baye-
sian analyses were repeated three times to ensure that
chains had converged. We specified unique model priors
for each partition as determined using MRMODELTEST
version 2.2 [114]. An exception were the 16S rRNA
sequences, which were aligned to the secondary structure
model of the teleost Pygocentrus nattereri [115] to identify
stem and loop regions, and the doublet model was
invoked for complementary stem regions. Model priors
from MRMODELTEST were, however, specified for stem
regions for which no complementary regions were availa-
ble for our partial 16S rRNA sequences, as well as for loop
regions.

**Molecular dating**

We compared genetic divergence between seahorse line-
ages whose distribution patterns may have arisen as a
result of the closures of the Central American, Tethyan or
Indonesian seaways, or spreading of the Atlantic Ocean,
with those of various other teleost lineages having the
same distribution patterns. Following previous studies
that estimated genetic distances of transisthmian sister
species [22,23], we calculated genetic distances under the
Kimura 2-Parameter (K2P) model [116]. As cytochrome b
and 16S rRNA sequences are most frequently used for
phylogenetic reconstructions in teleosts, we limited our
comparisons to these two markers.

To ensure that evolutionary rates between seahorses and
other teleosts were not significantly different, we applied
the relative rate test implemented in RRTREE [117]. This
test takes into account phylogenetic relationships and cor-
rects for sampling imbalances. All relative rate tests were
conducted in a pairwise fashion by comparing rates in the
relative rate test implemented in RRTREE [117]. This
same three species that comprised the out-
group in phylogenetic reconstructions were also used as
reference taxa in these analyses. Significant rate differ-
ences were identified in the case of three of the partitions
(16S rRNA, control region and S7 intron), which
prompted us to estimate the ages of divergence events
among different seahorse lineages by means of the Baye-
sian relaxed clock method for multiple genes imple-
mented in the programmes ESTBRANCHES and
MULTIDIVTIME [58]. The maximum likelihood tree
topology was specified, and to incorporate within-species
differences, we included all specimens available for each
species and specified phylogenetic relationships among
these as unresolved polytomies. Again, the three species
Hippocampus breviceps, H. coronatus and H. comes were used
as outgroup taxa. MULTIDIVTIME was used to estimate
the ages of divergence events and their 95% confidence
intervals. The Markov chain was sampled 50 000 times,
with 100 cycles between each sample and a burn-in of 50
000 cycles. The process was then repeated by sampling
100 000 times and specifying a burn-in of 100 000 cycles.
Differences in the results of these two runs would indicate
that the program has not been run for sufficiently long for
Markov chains to converge. This was found not to be the
case (divergence time estimates differed by no more than
0.0005).

Settings of the parameters rttm, rbrate, brownian and big
time followed suggestions in the MULTIDIVTIME manual.
The prior expected number of time units between tip and
root (rttm) was set to 22 mya (Early Miocene), as this date
was between the most recent (Middle Miocene) and old-
est (Late Oligocene) divergence times specified for the
root node. As the value for rttm should be between 0.1 and
10, we set it to 2.2. All subsequent values expressing time
in million years were also multiplied by 0.1.

The mean of the prior distribution for the rate at the root
node (rbrate) was estimated by dividing the median of all
branch lengths from root to ingroup tips by rttm. A value
of 0.7 was specified for brownian, as brownian multiplied
by rttm should be between 1 and 2 (2.2 × 0.7 = 1.5). The
highest possible number of time units between tip and
root (big time) was set to 50 (i.e. 500 million years). The magnitude of standard errors was set equal to that of each of the parameters rttm, rtrate and brownian. We examined the effect of the choice of parameter priors on divergence time estimates by doubling all of them. The fact that time estimates obtained in this way differed by no more than 0.1 (1 million years; mean difference: 0.03 or 300 000 years) suggests that the choice of priors had little effect on time estimates.

Molecular dating was done in two ways. Firstly, to date divergence events that may have resulted from vicariance events, we specified only the well-documented closure of the Central American Seaway as a calibration point and specified three alternative calibration ranges for the timing of this event: a) 3.1 – 3.5 mya [19], i.e. assuming that the divergence of the transisthman seahorse lineages took place during the final closure of the seaway; b) 3.1 – 4.6 mya, with the upper limit representing the onset of a marked reorganization of ocean circulation in Central America that was associated with the rising of the Isthmus of Panama [35], and c) 3.1 – 8.5 mya, to account for the possibility that seahorses were affected by the earliest possible evolution associated with the closure of the seaway [26]. Secondly, we dated all nodes in the phylogeny by using a well-supported divergence range of the transisthman seahorse lineages in combination with other divergence events whose divergence time estimates matched the timing of vicariance events suggested in the literature in the first analysis.

Authors' contributions
PRT designed the study, obtained the majority of the samples, carried out the laboratory work, did the analyses and prepared the manuscript. HH provided additional samples and helped with the laboratory work. CAM and NPB participated in the design of the study and contributed to the preparation of the manuscript. All authors read and approved the final version.

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