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Dispersal, habitat differences, and comparative phylogeography of Southeast Asian seahorses (Syngnathidae: *Hippocampus*)

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Abstract

Four distinct phylogeographical patterns across Southeast Asia were observed for four species of seahorse (genus *Hippocampus*) with differing ecologies. For all species, genetic differentiation (based on cytochrome *b* sequence comparisons) was significantly associated with sample site (Φ_{ST} = 0.190–0.810, $P < 0.0001$) and with geographical distance (Mantel's r = 0.37–0.59, $P < 0.019$). Geographic locations of genetic breaks were inconsistent across species in 7/10 comparisons, although some similarities across species were also observed. The two shallow-water species (*Hippocampus barbouri* and *Hippocampus kuda*) have colonized the Sunda Shelf to a lesser degree than the two deeper-water species (*Hippocampus spinosissimus* and *Hippocampus trimaculatus*). In all species the presence of geographically restricted haplotypes in the Philippines could indicate past population fragmentation and/or long-distance colonization. A nested clade analysis (NCA) revealed that long-distance colonization and/or fragmentation were likely the dominant forces that structure populations of the two shallow-water species, whereas range expansion and restricted dispersal with isolation by distance were proportionally more important in the history of the two deeper-water species. *H. trimaculatus* has the most widespread haplotypes [average clade distance (D_c) of nonsingleton haplotypes = 1169 km], indicating potentially high dispersal capabilities, whereas *H. barbouri* has the least widespread haplotypes (average D_c = 67 km) indicating potentially lower dispersal capabilities. Pleistocene separation of marine basins and postglacial flooding of the Sunda Shelf are extrinsic factors likely to have contributed to the phylogeographical structure observed, whereas differences among the species appear to reflect their individual ecologies.

Keywords: AMOVA, cytochrome *b*, marine biogeography, nested clade analysis, Sunda Shelf

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Introduction

Identifying patterns among the spatial and temporal distributions of genes and intraspecific lineages that are congruent across a variety of species can imply the influence of common factors such as climatic, tectonic, or oceanographic events (Avise 1994, 2000). Elucidating such patterns can extend our knowledge of the role of biological and nonbiological forces in determining species ranges, (Bernatchez & Wilson 1998; Chen 1999; Hugall *et al*. 2002) driving diversification and ultimately leading to speciation (Losos & Glor 2003). Conversely, dissimilar phylogeographical patterns can shed light on the ecology and the idiosyncratic history of individual species (Zink *et al*. 2001; Rocha *et al*. 2002).

Comparative phylogeography has proven useful in understanding the structure and history of terrestrial and freshwater species (e.g. Bernatchez & Wilson 1998; Taberlet *et al*. 1998; Moritz *et al*. 2001). Generalized phylogeographical patterns among marine species, however, are still poorly known. To date, investigations in the marine realm have been mainly confined to the coasts of North America (Avise 1992; Muss *et al*. 2001; Wares & Cunningham 2001; Dawson *et al*. 2002) the Great Barrier Reef of Australia and

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the Coral Sea (Benzie 1994). In these regions, the role of historical and present-day ocean currents (Benzie 1994; Muss *et al*. 2001), postglacial recolonization (Wares & Cunningham 2001), and ecological differences (Dawson *et al*. 2002) have been implicated in determining present-day patterns.

Southeast Asia is an area of remarkably high marine diversity (Hughes *et al*. 2002) where at least 10 species of seahorse can be found (Lourie *et al*. 1999b; Lourie & Randall 2003). This high diversity is because, in part perhaps, of its complex geological history (Hall & Holloway 1998) and the profound effects that Pleistocene sea level changes had on the configuration of land and sea (Voris 2000). Between about 2.5 million and 10 000 years ago, numerous glacial cycles occurred during which global sea levels fell dramatically (Haq *et al*. 1987). Vast areas of land were exposed where today we find shallow sea (e.g. Sunda Shelf), and present-day islands were connected by land bridges (Heaney 1985; Voris 2000). Although these land bridges would have enabled terrestrial organisms to extend their ranges, they would have acted as barriers for marine organisms, potentially aiding allopatric diversification and possibly even speciation (McManus 1985). The Last Glacial Maximum, when sea levels were at their lowest (about 130 m below present level) occurred approximately 19 000 BP (Yokoyama *et al*. 2000). As the ice melted, sea levels rose, at first gradually, then rapidly, resulting in the flooding of the Sunda Shelf from approximately 14 600 BP (Hanebuth *et al*. 2000).

Phylogeographical studies that span the Indo-Pacific suggest a role for Pleistocene separation of the Indian and the Pacific ocean basins in determining present-day distributions of species (McMillan & Palumbi 1995; Lavery *et al*. 1996; Benzie 1999). Within Southeast Asia itself, however, general patterns have yet to be elucidated. Initial studies indicate that the Coral Triangle region, i.e. eastern Indonesia, the Philippines, and New Guinea is more genetically diverse than the western region, i.e. Sunda Shelf (Arnaud *et al*. 1999; Barber *et al*. 2000, 2002; Nelson *et al*. 2000; Perrin & Borsa 2001; Lourie & Vincent 2004). They also suggest that patterns do not necessarily follow the major ocean currents (Wyrtki 1961; Barber *et al*. 2000).

In addition to extrinsic forces, varying dispersal caused by ecological differences undoubtedly plays an important role in determining a species' phylogeographical structure, the way in which it has responded to historical events and its conservation status. We focus on four species of commercially important seahorses (*Hippocampus barbouri, Hippocampus kuda, Hippocampus spinosissimus* and *Hippocampus trimaculatus*) that are assumed to differ in their dispersal capabilities based on their contrasting habitats. In comparison to many other marine taxa, seahorses are expected to show limited dispersal as a result of their internal brooding, release of fully developed young, and site fidelity as adults (Foster & Vincent 2004).

In general, if dispersal is low we expect that a significant proportion of the observed genetic variation would be explained by spatial division at particular geographical scales corresponding to the limits of dispersal (Neigel & Avise 1993). Furthermore, if dispersal has been low over a long period (e.g. since the Pleistocene), we expect that population structure might reflect historical arrangements of land and sea. In Southeast Asia, we expect signatures of both ice age isolation of marine basins (McManus 1985; Wallace 1997) and postglacial recolonization of the Sunda Shelf (Voris 2000) (Fig. 1).

Differences in phylogeographical pattern among species may occur based on the specific habitats they occupy. *H. barbouri* and *H. kuda* are shallow-water species generally found in, respectively, seagrass and seagrass/mangrove/estuarine/muddy areas less than 10 m deep (Lourie *et al*. 1999b; Choo & Liew 2003). Such habitats tend to be scattered along coastlines, often in sheltered bays that may be separated from each other by unsuitable habitat. *H. spinosissimus* and *H. trimaculatus*, however, are found at depths of at least 10–15 m, evidently on more open substrates such as sand or gravel and/or in association with octocorals or sponges (Lourie *et al*. 1999a, b; Choo & Liew 2003). Fewer barriers may exist for these habitats, particularly on the contiguous continental Sunda Shelf, and thus populations of deeper-water species may be more highly connected.

As for most seahorses, the dearth of available ecological information hampers accurate conservation assessments and the development of effective management plans (Foster & Vincent 2004). All four species studied here are heavily exploited for use as traditional medicines, aquarium animals, and curiosities which led to their listing as 'vulnerable' on the International Union for Conservation of Nature and Natural Resources (IUCN) Red List (IUCN 2003) and inclusion on the CITES Appendix II (CITES 2003). Increased understanding of their phylogeographical structure could help distinguish evolutionarily significant units (Moritz 1994), elucidate historical and ecological factors that may have determined their phylogeographical structure, and provide an indication of dispersal capabilities.

In this study we (i) examine the phylogeographical patterns, their congruence and their causes, across the four seahorse species, based on mtDNA sequences; (ii) interpret the findings in light of current understanding of geology, oceanography, and ecology; and (iii) highlight possible conservation implications of the results.

Materials and methods

Specimen collection, DNA extraction, amplification and sequencing

A total of 628 specimens of the four species were obtained from across Southeast Asia (Table 1, Fig. 1, Appendix):

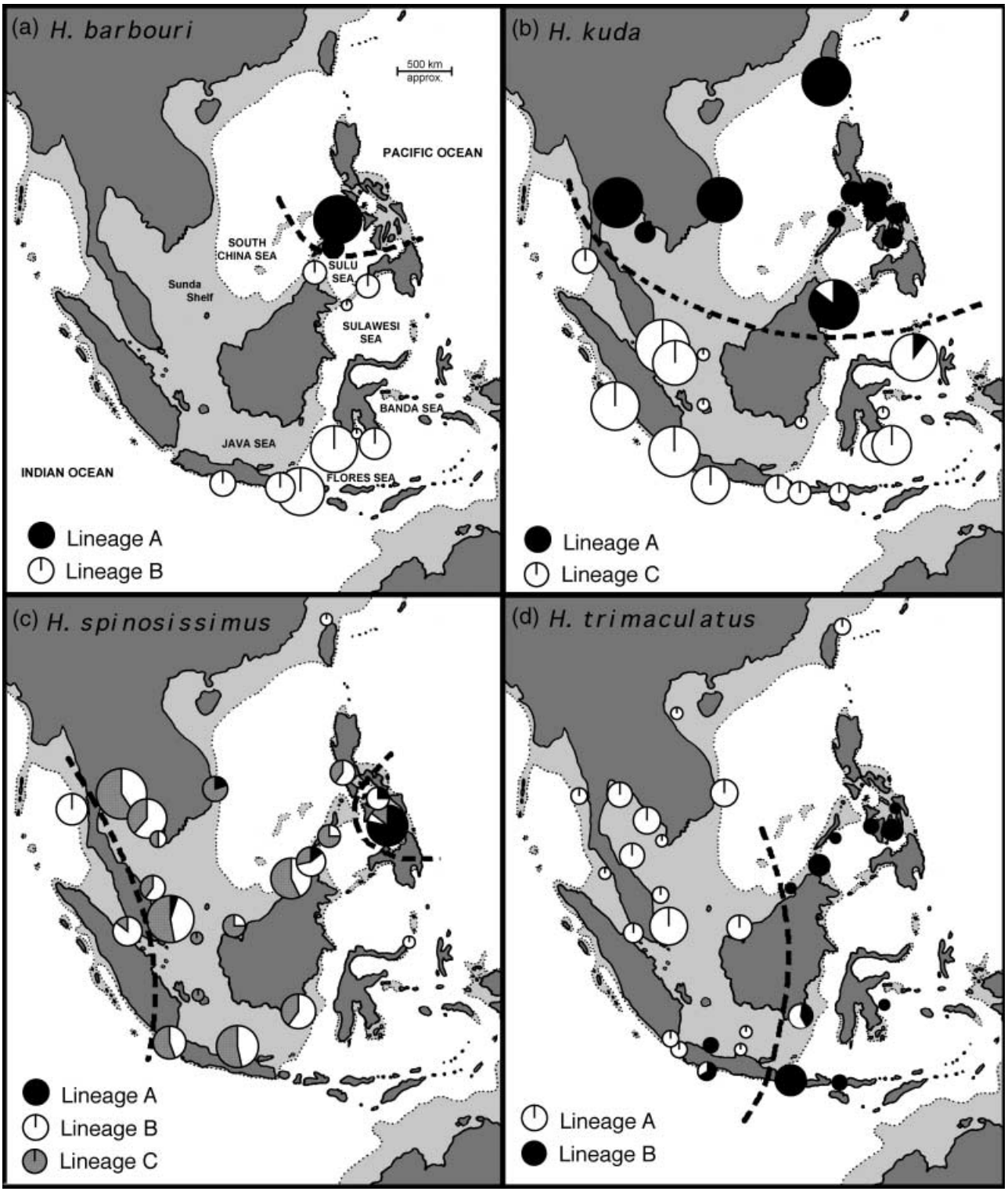


Fig. 1 Geographical distribution of major lineages in Southeast Asia for (a) *Hippocampus barbouri* (b) *Hippocampus kuda* (c) *Hippocampus spinosissimus*, and (d) *Hippocampus trimaculatus*. Pie charts indicate proportions of each lineage, and their area is proportional to sample size (equivalent scale across all boxes). Dotted lines indicate post hoc regionalizations that explain the highest proportion of the total genetic variation. Dark shading, present-day land. Pale shading, exposed land during glacial maxima.

Table 1 Summary of molecular diversity analyses for four species of *Hippocampus* in Southeast Asia

	H. barbouri	*H. kuda*	*H. spinosissimus*	*H. trimaculatus*
n	101	264	172	91
No. of populations	11	28	23	29
Average *n* per population ± SD	9.18 ± 7.77	9.43 ± 8.19	7.48 ± 6.07	3.14 ± 2.57
K (no. of haplotypes)	23	65	87	43
s (no. of polymorphic sites)	29	69	95	59
h (haplotype diversity) ± SD	0.802 ± 0.033	0.946 ± 0.005	0.97 ± 0.006	0.938 ± 0.015
π ± SD	0.005 ± 0.003	0.016 ± 0.008	0.013 ± 0.007	0.018 ± 0.009
Approx. divergence (Tamura) between major lineages (years)	687 579	1 510 343 (within-lineage divergences of geog. localized subclades 252 917–1 550 683)	1 504 316–2 478 304	3 743 907
Φ_{ST} (all populations)	0.890	0.754	0.190	0.677
P value	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Mantel's r ($n > 4$)	0.37	0.56	0.38	0.59
P value	0.019	< 0.0001	0.015	0.01

Hippocampus barbouri ($n = 101$), *Hippocampus kuda* ($n = 264$), *Hippocampus spinosissimus* ($n = 172$) and *Hippocampus trimaculatus* ($n = 91$). Despite extensive questioning of fishers and traders, *H. barbouri* was not found in the Sunda Shelf, and only one individual of *H. spinosissimus* was found in eastern Indonesia (personal observation; C.-K. Choo *et al.* personal communication).

Tissue samples from *H. barbouri* and *H. kuda* were obtained either as fin clips from live animals that were returned to the sea after being measured and photographed, or, as with the majority of specimens of *H. spinosissimus* and *H. trimaculatus*, they were obtained from local fishers, buyers, or retailers (typically for the medicine, aquarium, or souvenir trades) when we had good confidence of their location of origin (for full specimen list, see supplementary material). The DNA sequences for *H. trimaculatus* and *H. kuda* were obtained in the context of previous studies and we retain their original haplotype identifiers in order to facilitate cross-referencing (Lourie 2004; Lourie & Vincent 2004).

DNA was extracted from a small piece of fin tissue (*c.* 1 mm²) or a small piece of tail muscle (*c.* 0.005–0.05 g dry weight) using a standard proteinase K/phenol–chloroform protocol (Sambrook *et al*. 1989) except that no salt was added at the ethanol precipitation step. A section of the cytochrome *b* gene was amplified in a 50-μL reaction using a Perkin-Elmer 9600 Cycle Sequencer, and the following polymerase chain reaction (PCR) mix: 39.5 μL of H_2O, 5.0 μL of Red*Taq* buffer (10X), 1.0 μL of dNTP (10 mM), 1.5 μL of Red*Taq*, 1.0 μL of each primer (10 μM), 1.0 μL (containing about 10–50 ng) of DNA, under the following PCR conditions: 94 °C for 2 min 30 s; 35 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min 15 s; and 72 °C for 5 min. We used the following seahorse-specific primers: forward *shf2* 5′-TTGCAACCGCATTTTCTTCAG-3′ and reverse *shr2* 5′-CGGAAGGTGAGTCCTCGTTG-3′ (Lourie & Vincent 2004) or *1027R* 5′-ACAGGTATTCCCCCAATTC-3′ for some of the *H. kuda* specimens.

PCR products were cleaned using QIAQuick columns (QIAGEN) or Millipore filters according to the manufacturers' instructions and sequenced in both directions in 10-μL reactions using the following reaction mix: 4.5 μL of H_2O, 1.5 μL of buffer (5X), 0.5 μL of DMSO, 1.0 μL BigDye Terminator version 3.0 (ABI), 0.5 μL of primer (20 μM), 2 μL (containing *c.* 40 ng) of DNA, under the following PCR conditions (ABI 9700 Thermocycler) 40 cycles of 96 °C for 30 s, 50 °C for 15 s, 60 °C for 4 min. After adding 20 μL of sterile miliQ H_2O, precipitating with 95% ethanol (68 μL) and sodium acetate (3 μL, 5 M), and adding High-Dye formamide (10 μL), the samples were sequenced with an ABI 3730xl DNA analyser.

Sequence analyses

The primers *shf2* and *shr2* amplified a fragment of the cytochrome *b* gene 780 bp long and *shf2* and *1027r* a fragment 855 bp long. For *H. barbouri*, *H. kuda* and *H. spinosissimus*, 696 bases between bases 219 and 914 (with reference to the entire gene, Casey *et al*. 2004) were unambiguously edited using SEQUENCHER version 3.0.1 (Gene Codes Corporation) and BBEDIT LITE version 3.0 (Felciano 1994) and manually aligned using SEQPUP version 0.6f (Gilbert 1996). Polymorphic sites were rechecked with the original sequence trace files. Haplotype definitions have been submitted to GenBank (see supplementary material). Accession nos: *H. barbouri* AY495716–AY495738 and *H. spinosissimus* AY495739–AY495825). The previously published *H. trimaculatus* samples (AF192699–AF192703, AY322434–AY322476 excluding AY322436, AY322451, AY322460) were 692 bp long. To make them comparable to those for the other three species, four 'n' were added to the end of each sequence. The *H. kuda* samples (AY422091–

AY422115, AY422126–AY422166, excluding AY422157) were 688 bp long. We re-edited (and submitted to GenBank) the missing eight bases at the start of each sequence to make them comparable to the other species. Fourteen sequences in the *H. kuda* data set were missing the first 2–8 bases. However, since these bases were invariable in the remaining 250 specimens sequenced, we assumed that they would be identical in these specimens.

Haplotype and nucleotide diversity indices (Nei 1987) were calculated using ARLEQUIN version 2.000 (Schneider *et al*. 2000). We tested for differences among the species using Student's *t*-test (Zar 1996) and the parameter estimates and their standard deviations given in ARLEQUIN.

Intraspecific cladogram estimation

We estimated intraspecific relationships using TCS version 1.13 (Templeton *et al*. 1992; Clement *et al*. 2000). This method uses coalescence theory (Hudson 1990) to determine the limits of parsimony, and maximum parsimony to define a set of plausible connections among haplotypes that have a cumulative probability of > 95% of being true (Templeton *et al*. 1992). This method is considered more appropriate than traditional phylogenetic approaches for closely related sequences. It also provides a way to visualize alternative connections (i.e. 'loops') that are otherwise collapsed into unresolved polytomies (Crandall *et al*. 1994; Posada & Crandall 2001).

We defined nested sets of haplotypes for geographical analysis (see succeeding section) according to standard rules (Templeton *et al*. 1987). Ambiguities in the networks, such as closed loops or 'stranded' clades, were resolved using published rules and predictions based on coalescence theory (Templeton & Sing 1993; Crandall *et al*. 1994).

Geographic analyses

Population structure was estimated using Φ_{ST}, an analogue of conventional *F*-statistics that takes into account both haplotype frequency and sequence divergence. We used a model of sequence divergence (Tamura 1992) that accounts for multiple substitutions, and a transition–tranversion ratio and nucleotide proportions estimated from the data. We also included a gamma correction of 0.2159 based on results from MODELTEST version 3.06 (Posada & Crandall 1998).

To test for associations between genetic and geographical variation, we used an analysis of molecular variance (AMOVA) to determine the proportion of genetic variation (based on Φ_{ST}) that could be explained by partitioning the samples into populations or geographical regions (Excoffier *et al*. 1992), and a Mantel test (Mantel 1967) to test for a correlation between pairwise Φ_{ST} vs. geographical distance, which was estimated as the shortest straight-line distance between pairs of populations without crossing present-day land.

To assess the congruence of phylogeographical patterns across species, we determined (for each species) a regional partitioning of populations that could explain the greatest genetic variation using post hoc hierarchical AMOVA (Liebers & Helbig 2002). The regionalization for each species was then used as a hypothesis against which the genetic variation of each of the remaining three species was tested (in the areas where they overlapped), again using hierarchical AMOVA. If the model regions could explain a significant proportion of the observed variation in each tested species, we concluded that the phylogeographical distribution of the tested species could be consistent with that of the model species. In addition to the comparisons among species, a similar analysis assessed the possibility that the observed patterns could reflect a single separation into Indian Ocean and Pacific Ocean basin populations (Benzie 1998). The location of the division between the ocean basins was based on oceanographic data which separated the oceans by a line running east–west from the Malay Peninsula, through the Java and Flores seas (Wyrtki 1961).

To separate the possible roles of historical events vs. on-going limitations to gene flow, we conducted a nested clade analysis (NCA) (Templeton 1998) using GEODIS version 2.0 (Posada *et al*. 2000). NCA tests for associations between populations or geographical distance (measured as above) and haplotypes or clades at various nested levels within the phylogenetic network (Templeton *et al*. 1995; Templeton 1998) allowing inferences to be made at different temporal scales. The resultant significant clade and nested clade values were interpreted with a revised version of the original inference key (Templeton 2004). Furthermore, we used Tajima's *D* (Tajima 1989), Fu's *F* (Fu 1997) and mismatch analyses (Rogers 1995), as implemented in ARLEQUIN, to test for evidence of population expansion within clades that were inferred by NCA as having undergone long-distance colonization and/or range expansion. Evidence of predicted demographic changes obtained from independent analyses can lend extra support to NCA inferences (Masta *et al*. 2003).

To infer the relative dispersal capability of each species, we estimated, using GEODIS as previously discussed, the geographical spread or clade dispersion (D_c) of haplotypes that were represented by more than a single individual (nonsingleton haplotypes). We calculated the mean D_c for interior and tip haplotypes separately, since tip haplotypes are, by definition, younger than interior ones within a nested series (Crandall & Templeton 1993) and theoretically have had less time to spread out from their origin (Templeton 1998). However, because there was no significant difference between the average D_c for tip vs. interior haplotypes for any species (Mann–Whitney *U*-test, $P = 0.059$ for *H. barbouri*, and $P \geq 0.266$ for the other species), we combined them. We

tested for differences in average clade dispersion among the four species using Kruskall–Wallis and nonparametric multiple comparisons post hoc tests (Zar 1996) in STATVIEW version 4.51 (Roth *et al*. 1992–95).

Estimation of divergence times

Timings of major splits within the species' lineages were roughly estimated using a molecular clock calibration for *Hippocampus* cytochrome *b* sequences of 1.4% divergence (corrected for within lineage diversity) per million years (Myr) based on the split between *Hippocampus ingens* and *Hippocampus reidi* on either side of the Isthmus of Panama (data from Casey *et al*. 2004). We were justified in our application of a molecular clock based on log-likelihood ratio tests for each species ($-2 \log \Lambda = 19.9–76.8$, $P > 0.217$). However, given the uncertainties involved in this method of calibration (Knowlton & Weigt 1998) and molecular clocks as a whole, and the fact that we are applying this calibration to related species, our estimates should be interpreted cautiously.

Results

Sequence analyses

Across all four species, 628 individuals, and 696 bp sequenced, a total of 218 different haplotypes were identified defined by 239 polymorphic sites. No insertions, deletions or unexpected stop codons were encountered. Haplotype diversity ranged from 0.802 (*Hippocampus barbouri*) where over 60% of the individuals had the same haplotype, to 0.967 (*Hippocampus spinosissimus*) where over 77% of the individuals had unique haplotypes (Table 1). Average nucleotide diversity ranged from low ($\pi = 0.005$) in *H. barbouri*, consistent with shallow overall evolutionary structure, to relatively high in *Hippocampus kuda* ($\pi = 0.016$) and *Hippocampus trimaculatus* ($\pi = 0.018$) indicating deep phylogenetic divisions (Table 1). All species differed significantly from one another in terms of the depth of their phylogenetic structure (as measured by π) and their haplotype diversity (as measured by h) (t-tests, $P \leq 0.027$ and $P < 0.001$, respectively).

Intraspecific cladogram estimation

The nesting designs resulted in a four-step hierarchy for *H. barbouri*, five-step ones for *H. spinosissimus* and *H. trimaculatus*, and a six-step one for *H. kuda* (Fig. 2a–d). The 95% limits of parsimony were exceeded in two of the four species of seahorse: *H. kuda* and *H. trimaculatus* indicating deep phylogenetic divisions. Some ambiguities occurred (especially in *H. kuda* and *H. spinosissimus*) but they did not affect the results or conclusions.

Geographic analyses

A significant percentage of the total genetic variation was explained by population differentiation in all species: Φ_{ST} estimates ranged from 0.190 (0.202 when only populations with $n \geq 4$ were used) for *H. spinosissimus*, to 0.810 (0.813 when $n \geq 4$) for *H. barbouri*, with $P < 0.0001$ in all cases (Table 1). The correlation between population pairwise Φ_{ST} and geographical distance was also significant in all species (Mantel's $r = 0.37–0.59$, $P \leq 0.019$ in all cases, only $n \geq 4$ used) indicating possible isolation by distance. The major sublineages within each species were generally geographically localized (Fig. 1a–d). Since deep genetic

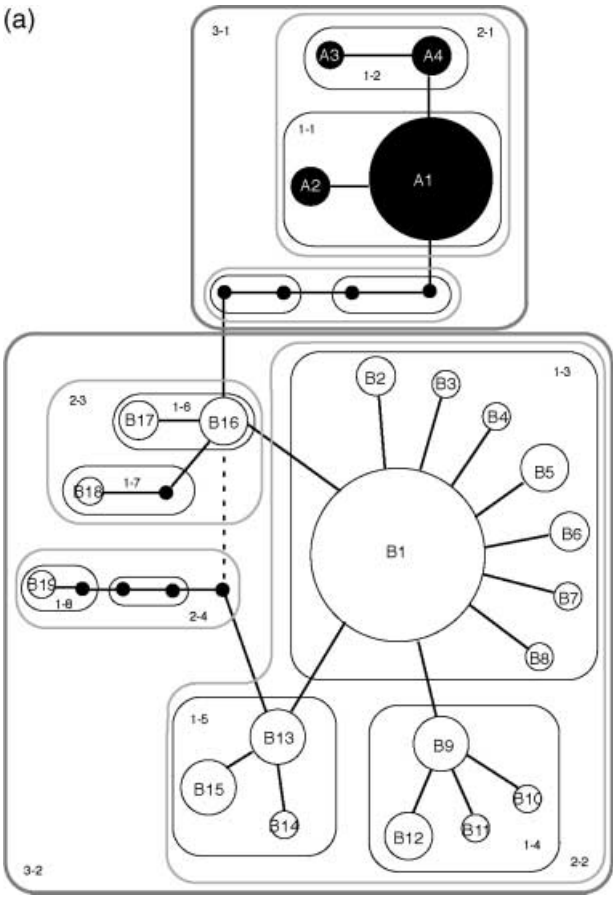


Fig. 2 Haplotype networks and associated nesting designs for four species of seahorse in Southeast Asia: (a) *Hippocampus barbouri* (b) *Hippocampus kuda* (c) *Hippocampus spinosissimus* and (d) *Hippocampus trimaculatus*. The areas of the circles are proportional to the number of individuals sharing each haplotype (scale consistent across all species). Solid lines represent single nucleotide mutations. Dotted lines represent alternative connections consistent with the 95% limit of parsimony. Small black dots represent haplotypes that were not observed in the sample, but are hypothesized to connect observed haplotypes. For *H. kuda* and *H. trimaculatus*, two unconnected networks were observed indicating that the 95% limit of parsimony has been exceeded.

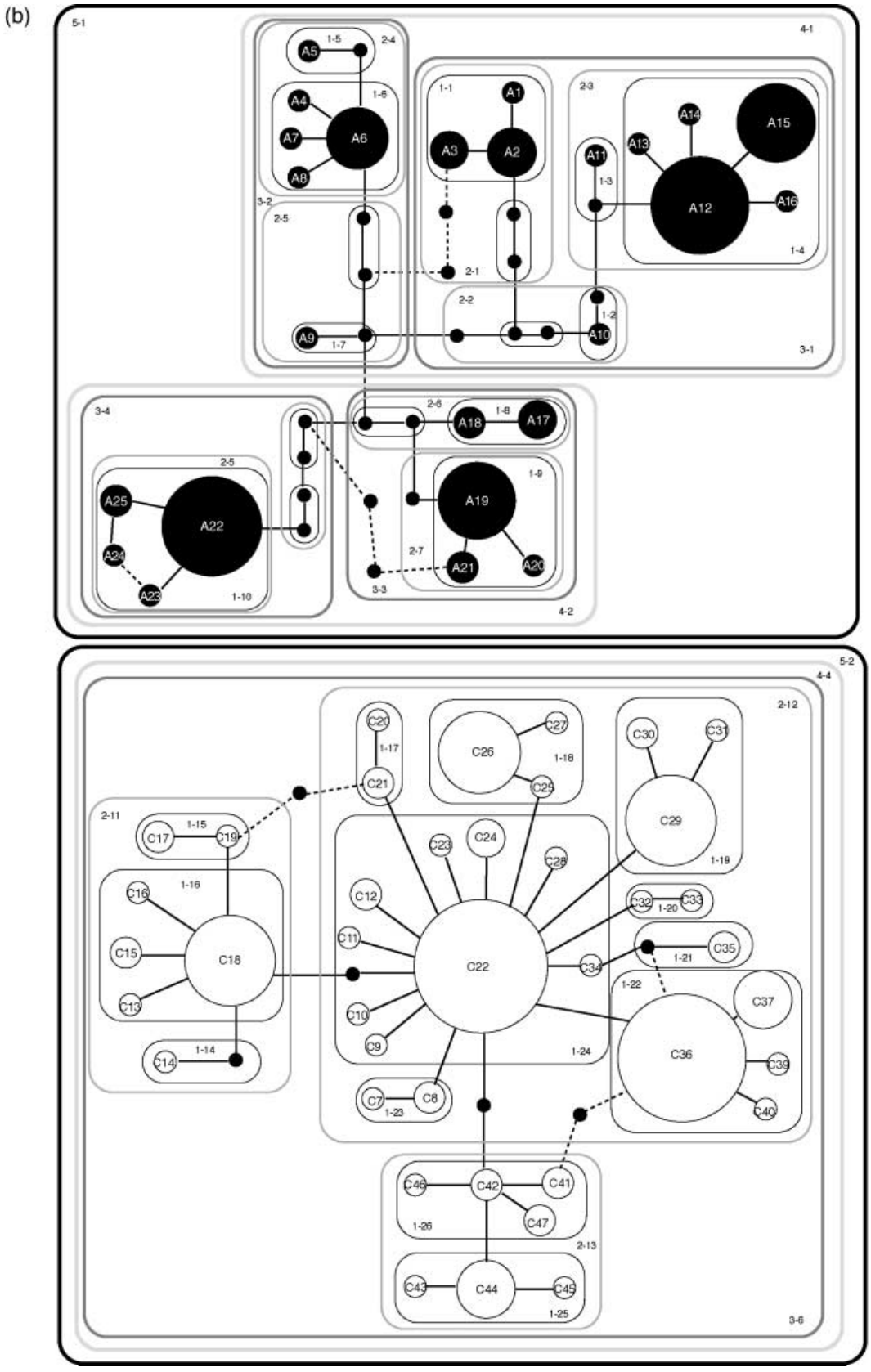
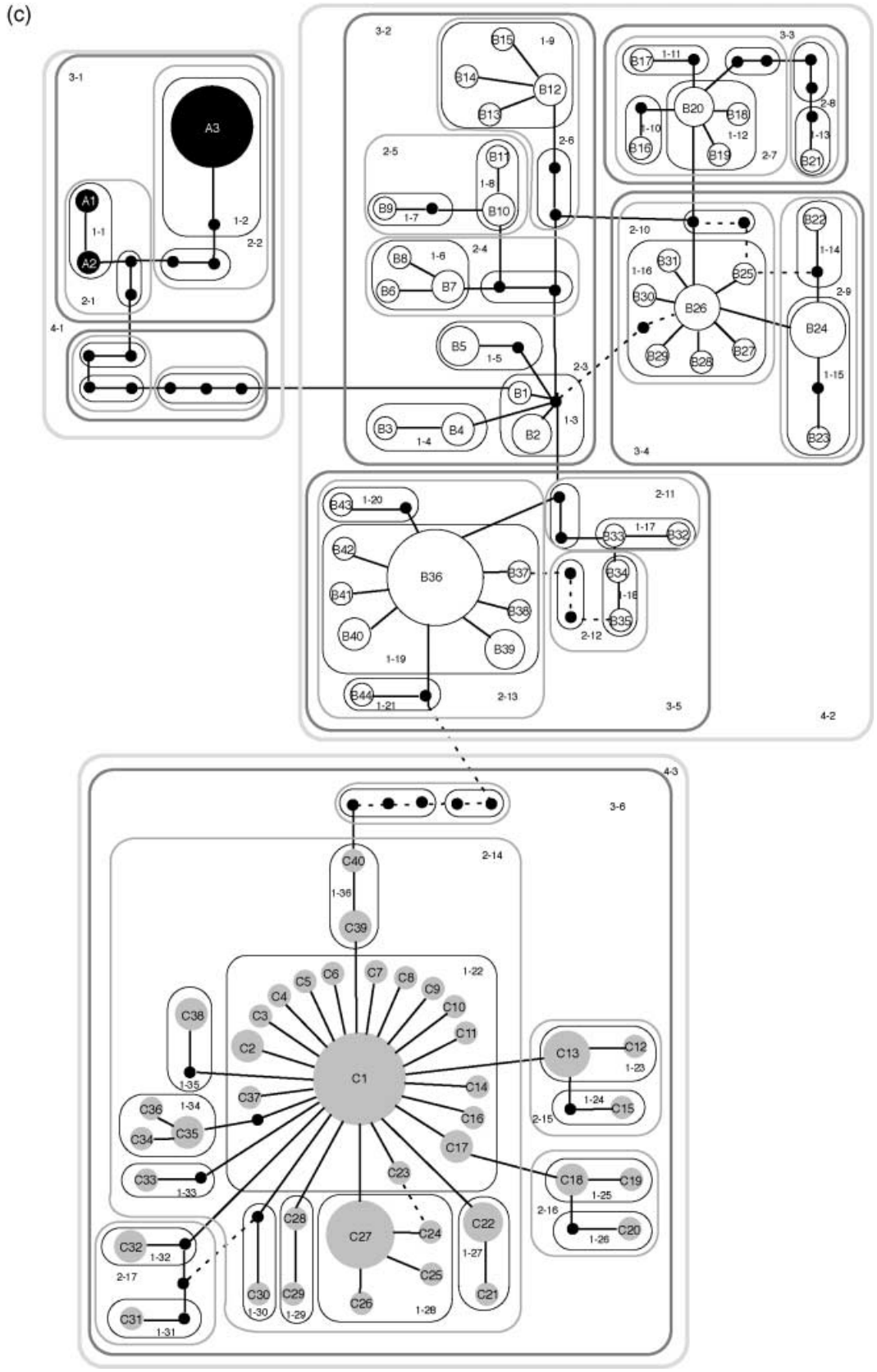


Fig. 2 *Continued*



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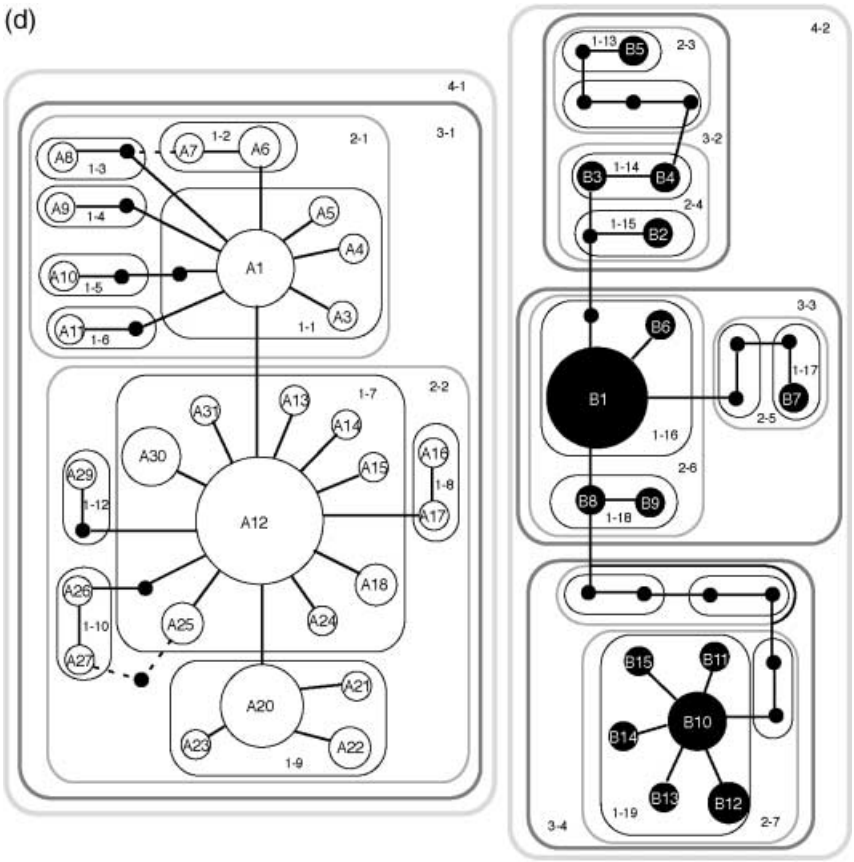

Fig. 2 *Continued*

divisions may bias the Mantel test, we repeated the analysis for *H. kuda* and *H. trimaculatus* separating populations by the sublineage to which most of their members belong. For *H. kuda*, the relationship stayed strong for populations consisting primarily of lineage A ($r = 0.69$, $P = 0.006$), but the relationship became marginally nonsignificant for populations consisting predominantly of lineage C ($r = 0.24$, $P = 0.071$). For *H. trimaculatus* the relationship disappeared when we tested predominantly A and B populations separately (for lineage A: $r = -0.42$, $P = 0.940$; for lineage B: $r = 0.94$, $P = 0.343$). Thus, simple isolation by distance was insufficient to account for the observed results.

Distinct phylogeographical patterns were seen for three of the four species. *H. barbouri* showed a division between the Sulawesi Sea vs. the Sulu Sea vs. the Flores Sea/Indian Ocean (Fig. 1a, Table 3a). The major division in *H. kuda* was between the Pacific Ocean/South China/Sulu Seas vs. the Indian Ocean/Java/Flores/Banda/Sulawesi Seas (Fig. 1b, Table 3b). At a smaller scale, divergent sublineages were restricted to the Gulf of Thailand (clade 3-4), the South China Sea (clade 3-3), the South China/Sulu Seas (clade 3-2) and the Sulu/Sulawesi Seas (clade 3-1) (Table 3). In *H. spinosissimus*, three major lineages occurred that were sympatric over much of their range (Fig. 1c, Table 3c). Only one of the lineages (A) was geographically restricted, primarily to the central Philippines. For *H. trimaculatus*, the major division separated the Sulu/Sulawesi/Banda/Flores Seas from the Indian Ocean Andaman/Java/South China Seas at right angles to that predicted by a simple Indian–Pacific ocean basins separation (Fig. 1d, Table 3d).

Concordance among phylogeographical patterns across species was tested by defining post hoc regionalizations (Fig. 1a–d). Two major regions were defined for *H. barbouri* (eastern Sulu Sea vs. western Sulu/Sulawesi/Banda/Flores Seas/Indian Ocean), two for *H. kuda* (Pacific Ocean/South China/Sulu Seas vs. Indian Ocean/Java/Flores/Banda/Sulawesi Seas), three for *H. spinosissimus* (Andaman Sea vs. Java/South China/Sulu/Sulawesi Seas vs. central Philippines), and two for *H. trimaculatus* (Indian Ocean/Andaman/Java/South China Seas vs. central Philippines/Sulu/Sulawesi/Banda/Flores Seas). These highest level groupings explained 81% (*H. barbouri*), 67% (*H. kuda*), 38% (*H. spinosissimus*), and 72% (*H. trimaculatus*) of the total genetic variance in each species (Table 2). Using the same regionalizations to test each of the other species in turn, we found that the exact geographical location of phylogeographical breaks were generally inconsistent across species (7/10 comparisons). However, some congruence was noted, especially between *H. kuda* and *H. barbouri*, and between *H. spinosissimus* and *H. trimaculatus* (Table 2). A

Table 2 Post hoc analyses of congruence among phylogeographical patterns shown across four species of *Hippocampus*

test species	based on … *H. barbouri* 2 groups	*H. kuda* 2 groups	*H. spinosissimus* 3 groups	*H. trimaculatus* 2 groups	Ocean basins 2 groups
H. barbouri					
among groups percentage	**80.770**	54.530	v. small overlap	all in one group	0.91
among populations within groups percentage	**8.800**	31.170	all in one group		80.10
within populations percentage	**10.430**	14.300			19.01
H. kuda					
among groups percentage	89.180	**66.800**	–4.630	–5.120	39.91
among populations within groups percentage	1.540	**16.500**	84.170	79.660	39.96
within populations percentage	9.280	**16.700**	20.460	25.470	20.13
H. spinosissimus					
among groups percentage	3.470	1.450	**38.260**	5.500	2.01
among populations within groups percentage	0.310	21.140	**0.380**	15.850	17.82
within populations percentage	96.220	77.410	**61.360**	78.650	80.16
H. trimaculatus					
among groups percentage	–43.460	–0.320	35.330	**72.270**	5.14
among populations within groups percentage	40.080	61.050	39.720	**9.160**	62.79
within populations percentage	103.380	39.270	24.950	**18.570**	32.06

Bold figures on the diagonal indicate the percentage of variation explained for each species by the post hoc division into regions for that species. Other figures represent the percentage variation explained based on post hoc regions defined by each of the other species (listed in the first row) in turn. For the comparisons across species, only areas where the sampled ranges overlapped were included in the analysis. The last column represents a post hoc hypothesis defined by a simple separation of the Indian/Pacific ocean basins.

single Indian–Pacific ocean basins partitioning explained little genetic variation except for *H. kuda* (40%).

In all species, the NCA revealed significant associations between haplotypes (or higher level lineages or clades) and sample sites (i.e. 'populations'), and all showed significantly large and small clade and nested clade distances (Table 3a–d). The patterns of significant associations led to inferences of past fragmentation and/or long-distance colonization with subsequent isolation in all species, particularly at the highest clade levels. For *H. barbouri*, such

Table 3 Summary of nested clade analysis results for (a) *Hippocampus barbouri* (b) *Hippocampus kuda* (c) *Hippocampus spinosissimus*, and (d) *Hippocampus trimaculatus*. See end of table for explanation of abbreviations

(a) *H. barbouri*			Total hierarchy: 4-steps. Total clades: 11				
Signif. clades	χ^2	sig.	Signif. subclades	D_c	D_n	Chain of inference	Inferred scenario
Total cladogram	101.00	0.000	3-1 (T) 3-2 (I) I–T	–– –– ++	++ –– ––	1-19-20-2-3-5-15-NO	FRAG/LDC between NW Sulu Sea vs. SW Sulu/Java/Flores/Banda Seas/Indian Ocean [> average no. of mutations supports FRAG; Tajima's *D* and Fu's *F* do not support population expansion for 3-1 (NW Sulu Sea)]
3-2	91.51	0.003	2-2 (T) 2-3 (I) 2-4 (T) I-T	–– –– ––	–– ++ (++) ++	1-2-11-12-13-YES	LDC/(FRAG) or FRAG/RE between SW Sulu Sea vs. E Sulu Sea vs. Java/Flores/Banda Seas/Indian Ocean [> average no. of mutations supports FRAG for 2-4 (SW Sulu Sea); Tajima's *D*, Fu's *F* and mismatch supports population expansion for 2-2 (Banda/Flores/Java Seas)]
2-2	97.42	0.000	1-4 (T) 1-5 (T) I–T	–– –– ++		1-2-3-4-NO	IBD among island chains in Java/Flores/Banda Seas/Indian Ocean [1–4: Java/Bali/Lombok; 1–5: E/W Sulawesi]
1-4	17.00	0.027	I–T		––	1-2-11-17-NO	Inconclusive outcome
1-3	99.40	0.024	B01 (I) B05 (T) I–T	–– (––)	–– ++ ––	1-2-11-12-13-YES	LDC/(FRAG) or FRAG/RE between SW Sulu Sea vs. Flores/Banda Seas/Indian Ocean [LDC is supported for B05 (SW Sulu Sea) because small no. of mutations; 1-3 (Banggi/Flores/Java Seas) shows evidence of population expansion based on Tajima's *D*, Fu's *F* and mismatch]

Table 3 *Continued*

(b) *H. kuda*			Total hierarchy: 6-steps. Total clades: 29				
Signif. clades	χ^2	sig.	Signif. subclades	D_c	D_n	Chain of inference	Inferred scenario
Total Cladogram	242.79	< 0.001	5-1 (T) 5-2 (I) I–T	–– ––	++ –– ––	1-2-11-12-13-YES	LDC/(FRAG) or FRAG/RE between Gulf of Thailand/South China/Sulu Seas vs. Sulawesi/Banda/Flores/Java Seas/Indian Ocean [> average no. of mutations supports FRAG; 5-1 does not show evidence of population expansion, but 5-2 does based on *D*, *F* and mismatch]
5-1	88.67	< 0.001	4-1 (?) 4-2 (?)	–– ––	 ++	1-2-11-12-13-YES	LDC/(FRAG) or FRAG/RE between Gulf of Thailand/South China Sea vs. South China/Sulu Seas [same inference regardless of which clade is coded as tip or interior; 4-1 shows evidence of population expansion based on *D*, *F*, and mismatch, but 4-2 does not]
4-2	43.00	< 0.001	3-3 (I) 3-4 (T)	–– ––	++	1-19-NO?	FRAG between Gulf of Thailand vs. South China Sea [> average no. of mutations supports FRAG; absence of *H. kuda* between observed samples inferred from sampling by T-S Ky & colleagues in south Vietnam]
4-1	27.03	< 0.001	3-1 (T) 3-2 (I)	–– ––	–– ++	1-2-11-12-13-YES	LDC/(FRAG) or FRAG/RE between South China/Sulu Seas vs. Sulawesi Sea [> average mutations supports FRAG; 3-2 (Sandakan) shows evidence of population expansion based on *D*, *F* and mismatch]
3-6	116.45	< 0.001	2-11 (T) 2-12 (I) 2-13 (T) I–T	–– –– ++	–– ++ ++	1-2-3-5-15 -NO (2-11) 1-2-3-5-6-7-YES (2-13)	FRAG/LDC between southern Sunda Shelf vs. Indian Ocean and IBD/LDC among populations on southern Sunda Shelf/Indian Ocean/Banda/Sulawesi Seas [population expansion supported by *D*, *F* and mismatch for 2-11 (Sunda Shelf) and 2-12 (Sunda Shelf/Indian Ocean/Flores/Sulawesi Seas), but not 2-13 (Sulawesi/Banda/Java Seas)]
3-3	10.59	0.009	2-6 (I) 2-7 (T) I–T	 –– ++	++ –– ++	1-2-3-4-9-10-NO?	FRAG or IBD between north and south South China Sea [sampling inadequate to discriminate]
3-2	13.00	0.086	2-4 (T) 2-5 (I) I–T	(––) (++)	(––) (++) (++)	1-19-20-2-3-4-9-NO	FRAG (allopatric) between northern South China Sea vs. Sulu/Sulawesi Seas [> average no. of mutation supports FRAG]
3-1	42.81	< 0.001	2-1 (T) I–T	(––) ++		1-2-3-4-9-NO	FRAG (allopatric) between northern South China Sea vs. Sulawesi Sea [> average no. of mutations supports FRAG]
2-12	271.23	< 0.001	1-18 (T) 1-19 (T) 1-22 (T) 1-23 (T) 1-24 (I) I–T	–– –– –– –– –– ++	–– (––) –– ++ ++	1-2-3-5-15-NO	FRAG/LDC between southern Sunda Shelf vs. western Indonesian islands vs. eastern Indonesian islands [*D*, *F* and mismatch support population expansion for 1–24 (eastern Indonesian islands) and partially for 1–18 and 1–22 (western Indonesian islands) and 1–19 (southern Sunda Shelf)]
1-24	111.23	0.224	C12 (T) C22 (I) C34 (T) I–T	 ––	++ –– (++) ––	1-2-11-12-NO	RE (contiguous) throughout eastern Indian Ocean/Andaman/Flores/Banda/Sulawesi Seas [accompanied by population expansion based on *D*, *F* and mismatch analysis]
1-22	18.96	0.109	C37 (T) C40 (T)		–– (++)	1-2-11-17-4-NO	IBD among populations in eastern Indian Ocean
1-19	6.55	0.323	C29 (I) I–T	++ ++	++	1-2-3-4-NO	IBD between southern Sunda Shelf and Bandar Lampung populations
1-4	36.94	0.243	A12 (I) I–T	(++) (++)	(++)	1-2-3-4-NO	IBD among populations in northern South China/Sulu/Sulawesi Seas

Table 3 *Continued*

(c) *H. spinosissimus*			Total hierarchy: 5-steps. Total clades: 36				
Signif. clades	χ^2	sig.	Signif. subclades	D_c	D_n	Chain of inference	Inferred scenario
Total Cladogram	93.52	< 0.001	4-1 (T) 4-2 (T) 4-3 (I) I–T	–– (++) –– 	(++) ++ –– ––	1-2-11-12-13-YES	LDC/(FRAG) or FRAG/RE between Sulu Sea vs. rest of range and between widespread clades 4-2 and 4-3 [> average no. of mutations separating subclades supports FRAG; *D*, *F* and mismatch support population expansion in 4-2 and 4-3, but not in 4-1 (mostly Sulu Sea)]
4-2	99.28	< 0.001	3-4 (I) 3-5 (I)	 ––	++ ––	1-2-3-5-15-NO	FRAG/LDC between Andaman Sea and rest of SE Asian range sampled [population expansion supported for all subclades based on *D* and/or *F* and/or mismatch]
3-6	53.02	0.528	2-14 (I) 2-16 (T) I–T	++ (––) (++)	++ –– ++	1-2-3-4-NO	IBD among populations across entire sampled range
3-5	21.91	0.548	2-12 (T) I–T	(––) (++)	(––) ++	1-2-11-12-NO	RE (contiguous) throughout most of sampled range
3-4	5.04	0.537	2-9 (T) 2-10 (I) I–T	–– ++	(––) (++) (++)	1-2-3-4-NO	IBD among populations in the Andaman Sea
3-2	51.75	0.017	2-5 (T) I–T	 (––)	(++) 	1-2-11-12-NO	RE (contiguous) across Sunda Shelf/Sulu/South China Seas
3-1	15.00	0.062	2-1 (I) 2-2 (T) I–T	 –– 	++ –– ++	1-19-20-2-3-4-9-NO	FRAG (allopatric) between South China Sea vs. Sulu Sea
2-14	150.74	0.340	1-22 (I) 1-34 (T) 1-36 (T) I–T	(––) 	(––) (++) ++ ––	1-2-11-12-NO	RE (contiguous) across entire sampled range except extremities
2-3	15.56	0.070	1-5 (T)		(––)	1-2-11-17-4-NO	IBD among populations across Sunda Shelf/Sulu Sea
1-28	35.00	0.044	C24 (T) C25 (T)	 	(––) (++)	1-2-11-17-4-NO	IBD among populations on Sunda Shelf
1-22	150.43	0.744	C10 (T) C37 (T)	 	(++) ++	1-2-11-17-NO	Inconclusive outcome
1-19	50.63	0.925	B39 (T)	++	++	1-2-11-12-NO	RE (contiguous) across Sunda Shelf

(d) *H. trimaculatus*			Total hierarchy: 5-steps. Total clades: 9				
Signif. clades	χ^2	sig.	Signif. subclades	D_c	D_n	Chain of inference	Inferred scenario
Total cladogram	82.55	0.000	4-1 (I) 4-2 (T) I-T	–– ––	–– ++ ––	1-2-11-12-13-YES	LDC/(FRAG) or FRAG/RE between Indian Ocean/Java/South China Seas and Sulu/Banda/Flores Seas [I–T D_n is significantly large if tip status is coded the other way around, but the inferences remain the same; population expansion supported for 4-1 (west), but not for 4-2 (east) based on *D*, *F* and mismatch analysis]
4-2	26.72	0.204	3-2 (T) 3-4 (I)	–– ––		1-2-11-12-NO	RE (contiguous) among populations in the Indian Ocean/Java/Flores/Banda Sulu Seas
2-6	6.35	1.000	1-16 (I) 1-18 (I)	 (––)	(++)	1-2-3-5-6-7-YES	IBD (but with some long distance dispersal) among populations in Indian Ocean/Java/Flores/Banda/Sulu Seas

Table 3 *Continued*

Signif. clades	χ^2	sig.	Signif. subclades	D_c	D_n	Chain of inference	Inferred scenario
2-2	79.08	0.305	1-10 (T)		(++)	1-2-11-17-4-NO	IBD across Sunda Shelf
2-1	49.56	0.417	1-1 (I)	(++)		1-2-3-4-NO	IBD among populations in the Gulf of Thailand/Sunda Shelf/Indian Ocean
			1-2 (T)	(––)			
			I–T	++	++		
1-19	26.67	0.030	B10 (I)	(––)	(––)	1-2-11-12-NO	RE (contiguous) among populations eastern Indian Ocean/Java/Flores Seas
			I–T	––	(––)		
1-7	109.96	0.768	A25 (I)		(++)	1-2-11-17-4-NO	IBD between Java and rest of Sunda Shelf

Only significant clade tests are shown. Nonsignificant associations between genetics and geography are consistent with extensive intermixing but may also reflect inadequate power to detect patterns. The topological position of each clade is represented by 'I' meaning 'interior' and 'T' meaning 'tip'. Tip clades are, by definition, younger than interior clades. '?' represents situations where I–T status could not be determined. Clade distances are recorded if they are significantly large '++' or significantly small '––' ($P < 0.05$). Tests that were significant at $P < 0.1$ are shown in parentheses. Chain of inference refers to Templeton's revised inference key (2004). Summary abbreviations: FRAG, past fragmentation; LDC, long-distance colonization; RE, range expansion; IBD, restricted gene flow with isolation by distance; N/A, unable to make inference. For nesting designs see Fig. 2a–d.

inferences occurred even at the one-step clade level. Inferences of isolation by distance were also observed in all species, particularly at low clade levels, i.e. one- and two-step clades. Overall, inferences of fragmentation and/or long-distance colonization were more common in the two shallow-water species and range expansion and/or isolation by distance were more common in the two deeper-water species. Both *H. barbouri* and *H. kuda* showed signatures of long-distance colonization/fragmentation between marine (ocean or sea) basins at the highest phylogenetic levels (South China/Sulu Seas vs. the Sulawesi/Banda/Flores/Java Seas and the Indian Ocean), as well as (for *H. kuda*) (1) among the South China Sea, the Sulu Sea, and the Sulawesi Sea, and (2) within the same marine basin between the Gulf of Thailand vs. the South China Sea. Contiguous range expansion and/or isolation by distance were inferred among populations along the island chain of Java, Bali, and Lombok for both species. For *H. kuda*, this was also inferred between Bandar Lampung and Sungai Johor/Penyengat Island, and among populations in northern Borneo, the Philippines, and Taiwan. Both *H. spinosissimus* and *H. trimaculatus* showed clear signatures of range expansion at medium-high clade levels that may reflect postglacial recolonization of the Sunda Shelf. In most cases Tajima's *D*, Fu's *F*, and mismatch analyses supported the inferences of NCA (see Table 3a–d for summary).

As a proxy for dispersal ability, we compared the distribution of nonsingleton haplotypes across the four species. For *H. barbouri* most (9/12) nonsingleton haplotypes were restricted to single locations. For *H. kuda* 9/27 were restricted to single locations, whereas for *H. spinosissimus* and *H. trimaculatus* all but one (23/24 and 9/10, respectively) were observed in widely separated locations. The average dispersion (D_c) of nonsingleton haplotypes was significantly different across the four species (Kruskall–Wallis, d.f. = 3, $H = 29.1$, $P < 0.001$) (Fig. 3). *H. kuda* did not differ significantly from either *H. barbouri* or *H. spinosissimus* ($P = 0.204$ and $P > 0.5$, respectively), while *H. spinosissimus* and *H. trimaculatus* were marginally significantly different ($P = 0.04$) and all other comparisons were highly significant ($P \leq 0.007$).

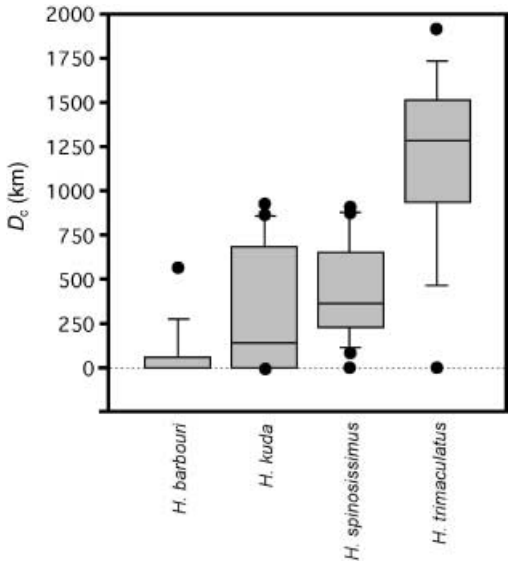

Fig. 3 Box-plot comparison of the geographical spread (clade dispersion D_c) of nonsingleton haplotypes for four different seahorse species. Horizontal bar, median D_c, vertical bars, encompass 95% of the observations, dots, extreme values.

Estimation of divergence times

The timings of the deepest splits varied across the species: from approximately 0.6 million years ago (Ma) for *H. barbouri*, 1.3 Ma for *H. kuda*, 0.9 to 1.4 Ma for the three lineages of *H. spinosissimus*, and over 2.1 Ma for *H. trimaculatus* (Fig. 2a–d, Table 1). The sublineages with restricted ranges in *H. kuda* (particularly within clade A, and the two sublineages of clade C found on the Sunda Shelf) showed minimum divergence times from closest relatives ranging from approximately 120 000–760 000 BP.

Discussion

Sharp phylogeographical breaks in all species indicate limits to dispersal over both contemporary and historical timescales. The estimated degree of population subdivision within four species of seahorse in Southeast Asia, as measured by Φ_{ST}, is high in comparison to many other marine species studied over comparable geographical scales (Rocha *et al*. 2002; Uthicke & Benzie 2003) but similar to that observed in the mantis shrimp (*Haptosquilla pulchella*) in the same area (Barber *et al*. 2002) and to the pipefish (*Urocampus carinirostris*) on the east coast of Australia (Chenoweth *et al*. 2002).

By using NCA in addition to more conventional analyses, such as AMOVA and Mantel tests, we are able to infer relative contributions to phylogeographical structure of historical events, such as vicariance of previously connected populations or long-distance colonization followed by isolation of newly founded populations, and on-going processes, such as limitation to gene flow because of restricted dispersal. To date, NCA has primarily been used in terrestrial and freshwater studies for a single species at a time but NCA is shown here to be applicable also to marine situations and comparative studies.

Four general conclusions are discernable from the results. The first is that, in all four species, inferences of fragmentation and subsequent isolation occur at some level in the phylogeny. This inference supports the hypothesis that Pleistocene isolation of marine basins may have been important in driving diversification in Southeast Asia (McManus 1985). In none of the species, however, did the pattern reflect a simple Indian–Pacific ocean division (Benzie 1998), nor did the exact location of the phylogeographical breaks necessarily match across species. Clear signatures of smaller ocean basins are also absent (except possibly in the case of *H. kuda*). This suggests that, even if the smaller basins were important in driving diversification, subsequent dispersal has blurred the divisions, and haplotypes have spread in various directions in the different species. For example, in *H. barbouri* a divergent Sulu Sea lineage appears to be fully isolated. In *H. kuda*, the two divergent lineages in the Sulu Sea are related to South China Sea haplotypes and Sulawesi Sea haplotypes, respectively, while those in *H. spinosissimus* and *H. trimaculatus* are also found to the west (*H. spinosissimus*) and south (*H. trimaculatus*). In addition to the discordance in geographical location of the phylogenetic breaks, the absolute degree of genetic divergence also varied across the species. Despite the differences, however, all estimates fell within the Pleistocene period which is consistant with McManus' (1985) ocean basin isolation hypothesis.

The second major finding is that only three species appear to have colonized the Sunda Shelf: *H. kuda*, *H. trimaculatus*, and *H. spinosissimus*. The wide distribution of haplotypes of *H. spinosissimus* and *H. trimaculatus* across the shelf indicates that either large and diverse founding populations colonized it, or that successive colonizations occurred from large and diverse source populations. The fact that haplotypes found on the shelf are commonly identical to those found on the edge, or off the shelf *H. spinosissimus* and *H. trimaculatus* is consistent with the hypothesis that these species colonized the region since its most recent flooding approximately 14 600 BP (Hanebuth *et al*. 2000). By contrast, the shelf populations of *H. kuda* comprise almost entirely of private haplotypes. This would suggest that these populations have been isolated from those around the edge of the shelf for a significant period of time (> 120 000 BP based on a simplistic 1.4% per Myr clock). Given the even greater genetic divergence of the Gulf of Thailand populations, these populations may have been isolated for even longer (> 760 000 years), possibly in an isolated refuge. Despite our relatively intensive sampling, *H. barbouri* appears to be absent from the shelf waters. Its absence may be consistent with low levels of mobility or it may reflect ecological constraints.

The third major observation is that different forces appear to have determined the phylogeography of the different species. Fragmentation and/or long-distance colonization have primarily structured populations of the two shallow-water species (*H. barbouri* and *H. kuda*), whereas restricted dispersal with isolation by distance, and range expansion have evidently been stronger influences on the two deeper-water species (*H. spinosissimus* and *H. trimaculatus*). This is consistent with predictions based on the habitats in which the species are found. Coastal habitats are frequently more discontinuous, in that open water may be more of a barrier because of temperature, currents, predation, etc., than deeper-water habitats. Thus, dispersal in shallow habitats should be more difficult, and populations will subsequently be more isolated and subject to genetic drift. In the Red Sea, endemism is higher among species in shallow waters where ecological conditions are very unstable, than it is among deep-water groups (Goren 1986). Furthermore, oceanographic regimes in sheltered bays, which are common habitats for seahorses, may tend to retain propagules of shallow water species and promote divergence of local populations

(Scheltema *et al*. 1996). Similar patterns of retention are possibly less common in deeper open-water habitats.

Long-distance dispersal (commonly inferred as an alternative to fragmentation) is certainly biologically plausible in seahorses. Rafting of juveniles and/or adults on drifting vegetation or other holdfasts may be an important dispersal mechanism for seahorses as it is for many sessile invertebrates (Jackson 1986). Indeed, a pregnant male seahorse arriving in a new location could theoretically found an entire population, with significant consequences for the genetic composition of the new population. Rafting may play a larger role for shallow-water species than deeper-water ones. Storms primarily affect shallow water areas, causing break-up of *Sargassum* alga, which is known to be a habitat for juvenile *Hippocampus comes* (Perante *et al*. 2002), and probably also causing damage to, and transport of, seagrass and other holdfasts (Short & Echeverria 1996). The increased inference of isolation by distance, as opposed to fragmentation or long-distance colonization and isolation, in the deeper-water species could reflect the fact that deeper habitats are more stable and individuals likely disperse in a more deterministic fashion.

Finally, we see that, based on the spread of nonsingleton haplotypes, average dispersal capabilities differ across the species. *H. trimaculatus* seems to have the greatest dispersal potential and *H. barbouri* seems to have the least. In addition to effects of habitat, this difference may also relate to reproductive output: *H. trimaculatus* has the largest brood-size of the four studied species (Foster & Vincent 2004) and *H. barbouri* has the smallest (Djawad & Syafiuddin, personal communication). A simple relationship between number of offspring and successful dispersal, however, does not seem to be the case because *H. kuda* and *H. spinosissimus* differ in their reproductive output yet show similar D_c values. Furthermore, even within a single species, the sharp phylogeographical divisions observed in one area of its range contrast with widespread distributions of haplotypes in others, again suggesting that realized dispersal is more complex than can be predicted by a single factor. This is particularly the case in *H. kuda* and *H. trimaculatus*. In *H. kuda*, the narrow zone of overlap between the major lineages could be the remnant of historical Indian–Pacific ocean basin separation with limited subsequent movement of individuals. The existence of private haplotypes within *H. kuda* on the Sunda Shelf also suggests rare colonization — possibly because of lack of suitable intervening habitats and/or oceanographic factors (Morgan & Valencia 1983). In *H. trimaculatus*, the major break is between shelf and oceanic environments. It is at right angles to that expected based on major ocean basin separation. The continued maintenance of the break may reflect adaptation to different environmental conditions and/or rapid range expansion following colonization of the Sunda Shelf (Lourie & Vincent 2004). The breaks are, however, still surprising given the strong currents in the area that apparently flow at right angles to them (Wyrtki 1961).

The results of this study are strong, but there are limitations to the conclusions that can be drawn. The unequal sample sizes across species (n = 91–264) reflect the somewhat opportunistic nature of the collections yet had little effect on the estimation of genetic parameters (results not shown) or on our conclusions. The unequal geographical sampling represents a potentially greater problem. In particular, *H. spinosissimus* and *H. trimaculatus* were sampled most extensively on the Sunda Shelf, where the greatest range expansion might be expected, and only a single *H. spinosissimus* individual was found in eastern Indonesia, an area that showed fragmentation in *H. trimaculatus*. While we are relatively confident that the absence of *H. barbouri* on the Sunda Shelf is real, the lack of *H. spinosissimus* in eastern Indonesia is more likely to be the result of insufficient sampling. A related issue is that samples of the two deeper-water species (*H. spinosissimus* and *H. trimaculatus*) were mostly collected by small trawl boats, whereas the two shallow-water species (*H. barbouri* and *H. kuda*) were generally collected by hand. This means that the 'populations' of the former are likely to originate from wider geographical areas than 'populations' of the latter, with the potential that higher diversities in the deep-water species may reflect their wider source of origin. Despite this potential bias, the broad patterns remain the same: distributions of individual haplotypes are far wider in the two deeper-water species than in the shallow-water ones. Further samples and analyses of other molecular markers, including nuclear ones, will address the extent of these potential sampling artefacts.

Further research, both on seahorses and on other marine species, will be valuable in determining the generality of our results. We delimited the bounds of this study geographically, restricting our sampling to Southeast Asia. To put the results of this study into a wider context, samples from further afield would be needed. *H. barbouri* is the only species whose entire distribution lies within Southeast Asia. Its sister taxa (*Hippocampus whitei*, and *Hippocampus subelongatus*/*Hippocampus angustus*/*Hippocampus comes*) are distributed in eastern, western, and northwestern Australia and Southeast Asia, respectively (Lourie *et al*. 1999b) and are separated from *H. barbouri* by approximately 6.9% (K2P distance, cytochrome *b* sequence data) (Casey *et al*. 2004). Two samples, initially identified as *H. barbouri*, from Irian Jaya (West Papua) clustered with *H. angustus* from northwestern Australia (unpublished data – S. Lourie). Specimens of putative *H. kuda* from India and East Africa were genetically different from those in Southeast Asia, and a third lineage, restricted to New Guinea was also revealed (Lourie 2004). Samples from Queensland, Australia, identified as *Hippocampus queenslandicus* by Peter Southgate (Horne 2001) fell out within both lineage A and B of *H. spinosissimus*

(Teske *et al.* 2005). In *H. trimaculatus*, more extensive geographical sampling indicated that samples from the Sunda Shelf area of Southeast Asia are genetically identical or extremely close to specimens from India and Japan (Lourie & Vincent 2004).

Conservation implications

Significant conservation concerns arise from overexploitation for domestic and international trade of all four of the species of Southeast Asian seahorses studied here (Vincent 1996; CITES 2003). Comparative phylogeography has the potential to provide insights into the patterns and processes that determine and maintain species' distributions, and hence can help inform conservation decisions. Four major implications for conservation can be drawn from our results. First, at the scale of Southeast Asia, all species are restricted in their dispersal capabilities to some degree. *H. barbouri* appears most restricted and *H. trimaculatus* least, particularly on the Sunda Shelf. Thus, for each species, sufficient viable and geographically close populations, within phylogeographical regions, need to be maintained to enable recolonization if necessary. Second, *H. barbouri* and *H. kuda* are characterized by relatively isolated populations with locally monophyletic lineages. These need to be managed as separate units since the probability that they will be recolonized from elsewhere is likely to be low over ecological timeframes. Third, the maintenance of abrupt phylogeographical divisions in *H. barbouri*, *H. kuda* and *H. trimaculatus* could also imply ecological adaptation and thus any introductions or reintroductions should logically derive from the same genetic lineage. Finally, the extremely high diversity in *H. spinosissimus* could imply historically large populations (Roman & Palumbi 2003). Further analysis of genetic data will be valuable in estimating demographic parameters for these seahorse species.

In conclusion, we see here that historical events, ongoing gene flow, and ecological differences combine in complex ways to determine present-day phylogeographical patterns across species. Although we can not be absolutely certain of the particular causes of observed patterns, comparative analyses can elucidate common threads. Here we show phylogeographical patterns that reflect Pleistocene isolation of marine basins in Southeast Asia, postglacial recolonization of the Sunda Shelf, and differential patterns between deep- and shallow-water species. The implications of these patterns for conservation include identification of evolutionarily significant units and distinct phylogeographical breaks, indications of different dispersal capabilities across the species, and new ecological hypotheses regarding the mechanisms by which they may have achieved, and possibly maintain, their current distributions.

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Supplementary material

Supplementary material is available from http://www.blackwellpublishing.com/products/journals/suppmat/MEC/MEC2464/MEC2464sm.htm

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Appendix

Locations, sources, and haplotypes observed among samples of (a) *Hippocampus barbouri* (b) *Hippocampus kuda* (c) *Hippompus spinosissimus* and (d) *Hippocampus trimaculatus*. A more detailed list including individual catalogue and GenBank numbers is available online (see supplementary material)

(a) *H. barbouri*

Pop	Locality	*n*	Latitude	Longitude	Haplotypes	Source
1	Busuanga (Coron), Palawan, Philippines	13	approx. 11°54′N	approx. 120°12′E	A01(11), A04(2)	primary buyer
	Busuanga (Salvacion)	8	approx. 11°55′N	approx. 119°23′E	A01(7), A02	primary buyer
2	Dumaran, Palawan, Philippines	4	approx. 10°32′N	approx. 119°48′E	A01(2), A02, A03	fisher
3	Pulau Balembangen, Sabah, Malaysia	5	approx. 07°12′N	approx. 117°00′E	B01, B05(3), B19(1)	fisher
4	Jolo, Sulu Arch., Philippines	5	approx. 05°58′N	approx. 121°06′E	B16(2), B17(2), B18	fisher?
5	Tawi-Tawi, Sulu Arch., Philippines	1	approx. 05°10′N	approx. 120°10′E	B16	fisher?
6	P. Tanakeke (Butung), Sulawesi, Indonesia	8	05°28.470′S	119°18.670′E	B01(6), B02, B04	SL
	P. Tanakeke (Kampea)	8	05°28.021′S	119°17.135′E	B01(7), B02	SL
	P. Tanakeke (Labbol Lamber)	4	05°27.893′S	119°18.380′E	B01(2), B08, B14	SL
7	Bone, Sulawesi, Indonesia	1	approx. 04°33′S	approx. 120°24′E	B01	fisher
8	Bau Bau, Sulawesi, Indonesia	9	approx. 05°27′S	approx. 122°36′E	B01, B13(4), B15(4)	fisher
9	Cilacap, Java, Indonesia	6	approx. 07°44′S	approx. 109°00′E	B01(5), B10	primary buyer
10	Bali, Indonesia	8	possibly approx. 08°30′S	possibly approx. 115°00′E	B03, B09(4), B12(3)	aquarium exporter
11	Lombok (Batu Nampar), Indonesia	9	approx. 08°52′S	approx. 116°24′E	B01(7), B06, B07	fisher
	Lombok (P. Petagan and P. Lampu)	12	approx. 08°25′S	approx. 116°45′E	B01(10), B06, B11	fisher

(b) *H. kuda*

Pop	Locality	*n*	Latitude	Longitude	Haplotypes	Source
1	Bang Saen, Chonburi, Thailand	21	approx.13°16′N	approx. 100°56′E	A22(17), A23, A24, A25(2)	fisher
2	Sungai Johor, Johor, Malaysia	25	approx. 01°05′N	approx. 104°00′E	C14, C15(2), C16, C17, C18(8), C19, C29(11)	researcher
3	Penyengat Island, Riau, Indonesia	17	approx. 00°56′N	approx. 104°25′E	C13, C17, C18(8), C29(4), C30(2), C31	fisher
4	Sarasin Bridge, Thalang, Phuket, Thailand	5	approx. 08°00′N	approx. 098°21′E	C12(2), C36(2), C40	fisher
5	Pasumpahan, Padang, Sumatra, Indonesia	20	approx. 01°07′S	approx. 100°22′E	C25, C26(3), C27, C36(11), C37(3), C39	SL
6	Bandar Lampung, Sumatra, Indonesia	22	approx. 05°32′S	approx. 105°17′E	C26(7), C29, C35, C36(9), C37(4)	fisher
7	Pangandaran, Java, Indonesia	12	approx. 07°41′S	approx. 108°40′E	C26, C36(9), C34, C41	fisher/SL
8	Gilimanuk, Bali, Indonesia	6	approx. 08°10′S	approx. 114°26′E	C22(2), C26(2), C36, C44	SL
9	Sandakan, Sabah, Malaysia	21	approx. 05°48′N	approx. 118°06′E	A01, A02, A03(2), A12(4), A05, A04, A06(6), A07, A08, C22(3)	fisher
10	Nha Trang, Khan Hoa, Vietnam	17	approx. 12°15′N	approx. 109°10′E	A18(2), A19(12), A20, A21(2)	fisher
11	Bau Bau, Sulawesi, Indonesia	8	approx. 05°27′S	approx. 122°36′E	C09, C22(5), C33, C43	fisher
12	Lembeh Strait, Sulawesi, Indonesia	19	approx. 01°29′N	approx. 125°14′E	A06(2), C07, C10, C21, C22(7), C24, C28, C32, C42, C45, C46, C47	SL
13	Padre Burgos, Luzon, Philippines	4	approx. 13°57′N	approx. 120°56′E	A02, A09, A12(2)	fisher
14	Ta Pong Bay, southern Taiwan	20	approx. 22°29′N	approx. 120°35′E	A09, A12(8), A15(8), A17(3)	researcher
15	Batu Nampar, Lombok, Indonesia	4	approx. 08°52′S	approx. 116°24′E	C21, C22(2), C44	fisher
16	Labuan Bajo, Flores, Indonesia	1	approx. 08°29′S	approx. 119°53′E	C22	fisher
	Pulau Komodo, Flores, Indonesia	2	approx. 08°35′S	approx. 119°30′E	C11, C22	fisher
17	Kendari, Sulawesi, Indonesia	1	approx. 03°59′S	approx. 122°33′E	C44	fisher
18	Daram Island, Samar, Philippines	3	approx. 11°38′N	approx. 124°47′E	A11, A12, A15	fisher
19	Jandayan Island, Bohol, Philippines	3	approx. 10°10′N	approx. 124°10′E	A15, A16, C22	fisher/researcher
20	Tagkawayan, Quezon, Philippines	6	approx. 14°00′N	approx. 122°37′E	A12(4), A13, A14	fisher/researcher

Appendix *Continued*

(b) *H. kuda continued*

Pop	Locality	*n*	Latitude	Longitude	Haplotypes	Source
21	Busuanga, Palawan, Philippines	2	approx. 11°55′N	approx. 119°23′E	A10, A15	fisher
22	Kampong Som, Cambodia	3	approx. 10°30′N	approx. 104°12′E	A22(3)	fisher
23	Pulau Tambelan, Riau, Indonesia	1	approx. 01°00′S	approx. 107°30′E	C18	fisher
24	Tanjung Kelayan, Belitung, Indonesia	1	approx. 02°34′S	approx. 107°42′E	C29	fisher
25	Pulau Laut, Kalimantan, Indonesia	1	approx. 03°15′S	approx. 116°11′E	C22	fisher
26	Manado, Sulawesi, Indonesia	1	approx. 01°30′N	approx. 124°55′E	C22	fisher
	Likupang, Sulawesi, Indonesia	1	approx. 01°40′N	approx. 125°04′E	C47	SL
27	Tampo, Sulawesi, Indonesia	13	approx. 04°37′S	approx. 122°53′E	C08(2), C20, C22(7), C23, C42, C46	1st level buyer
28	Magellanes, Sorsogon, Luzon, Philippines	4	approx. 12°49′N	approx. 123°52′E	A02(2), A03, A15	fisher

(c) *H. spinosissimus*

Pop	Locality	*n*	Latitude	Longitude	Haplotypes	Source
1	Thai/Myanmar border	9	exact location unknown		B22, B23, B24(4), B26(2), B30	buyer
2	Pulau Pangkor?, Perak, Malaysia	7	approx. 04°15′N	approx. 100°34′E	B24(2), B26, B27, B31, B39, C21	TCM shop
3	Bandar Lampung, Sumatra, Indonesia	9	approx. 05°32′S	approx. 105°17′E	B12, B15, B36(2), C01(2), C22(2), C34	fisher
4	Tanjung Tinggi, Belitung, Indonesia	1	approx. 02°33′S	approx. 107°43′E	C01	fisher
5	Jepara, Java, Indonesia	15	approx. 07°00′S	approx. 110°30′E	B03, B04, B12, B25, B36(2), B40, C01(4), C11, C27, C33, C35	fisher
6	Kalimantan Selatan (Pulau Laut, Pulau Kunyit), Indonesia	3	approx. 04°00′S	approx. 116°00′E	C25, C36, B40	fisher
	Kalimantan Selatan (Pulau Laut, Lontar)	1	approx. 03°58′S	approx. 116°02′E	B39	fisher
	Kalimantan Selatan (Pulau Laut, Teluk Tamiang)	4	approx. 04°03′S	approx. 116°02′E	B06, B16, B36, B40	fisher
	Kalimantan Selatan (Pulau Laut, Gemuruh)	1	approx. 03°57′S	approx. 116°03′E	C40	fisher
	Kalimantan Selatan (Pagatan)	1	approx. 03°36′S	approx. 115°58′E	B19	fisher
7	Pulau Pejantan, Tambelan, Indonesia	1	approx. 00°10′N	approx. 107°15′E	C17	fisher
8	Mersing, Johor, Malaysia	19	approx. 02°25′N	approx. 103°50′E	A02, B05, B13, B34, B35, B36(2), B38, B43, C01(3), C05, C06, C07, C13(2), C19, C38	primary buyer
9	Kapas to Pulau Tenggol, Terengganu, Malaysia	5	approx. 05°30′N	approx. 103°30′E	B10, B28, B36, C18, C26	fisher
10	Laem Sing, Chanthaburi, Thailand	22	approx. 12°10′N	approx. 102°10′E	B01, B07, B09, B20, B21, B36(3), B42, C01(3), C02, C03, C14, C16, C17, C22, C23, C27, C28, C32	fisher
11	Cambodia (near Kampot)	4	approx. 10.50°N	approx. 104.20°E	B17, B37, C04, C27	fisher
	Cambodia (near Sihanoukville)	1	approx. 10°38′N	approx. 103°30′E	B36	fisher
	Cambodia (Kampong Som)	3	10°38.615′N	103°29.770′E	B36, B41, C13	fisher
	Cambodia (Kampong Som)	5	10°36.212′N	103°29.183′E	B11, B18, B36, C01, C27	fisher
12	near Rach Gia?, Kien Giang, Vietnam	2	approx. 10°00′N	approx. 105°00′E	B36, C24	?
13	Nha Trang, Khan Hoa, Vietnam	5	approx. 12°15′N	approx. 109°10′E	A01, C27(3), C29	?
14	near Santubong, Sarawak, Malaysia	4	approx. 01°43′N	approx. 110°18′E	B08, C01, C02, C20	fisher
15	Kota Kinabalu, Sabah, Malaysia	5	approx. 06°30′N	approx. 116°06′E	B36, C13, C18, C27(2)	fisher
	Kota Kinabalu (near P. Mentanani)	2	approx. 06°43′N	approx. 116°20′E	B05, C08	fisher
near	Kota Kinabalu (Pulau Tiga)	6	approx. 05°42′N	approx. 115°38′E	B04, B05, B33, C09, C31, C32	fisher
near	Kota Kinabalu (outside Labuan)	1	approx. 05°15′N	approx. 115°10′E	B44	primary buyer

Appendix *Continued*

(c) *H. spinosissimus continued*

Pop	Locality	*n*	Latitude	Longitude	Haplotypes	Source
16	Pulau Banggi, Sabah, Malaysia	3	approx. 07°05′N	approx. 117°00′E	B32, B36, C15	fisher
17	Pulau Malawali, Sabah, Malaysia	4	approx. 07°00′N	approx. 117°20′E	A03, B07, B20, C01	fisher
18	Dumaran, Palawan, Philippines	4	approx. 10°32′N	approx. 119°48′E	B36, C10, C35, C37	fisher
19	Cavite, Luzon, Philippines	5	approx. 14°29′N	approx. 120°54′E	B14, B20, B39, C12, C37	?
20	Cawangan, Masbate, Philippines	4	approx. 11°50′N	approx. 123°45′E	A03, B02(2), B26	fisher
21	Cebu, Philippines (Suwangan, Bantayan Island)	8	approx. 11°12′N	approx. 123°45′E	A03(8)	fisher
	Cebu (Panitugan, Santafe)	1	approx. 11°12′N	approx. 123°45′E	A03	fisher
	Bohol (Handumon)	2	approx. 10°10′N	approx. 124°10′E	A03, B02	fisher
	Bohol (Bienunido, Malinguin Island)	3	approx. 10°07′N	approx. 124°22′E	A03, C39 (2)	fisher
22	I-Lan, Taiwan	1	approx. 24°46′N	approx. 121°45′E	B10	primary buyer
23	Lembeh Strait, Sulawesi, Indonesia	1	01°29.422′N	125°14.215′E	B29	SL

(d) *H. trimaculatus*

Pop	Locality	*n*	Latitude	Longitude	Haplotypes	Source
1	Thai/Myanmar border	2	exact location unknown		A12, A26	buyer
2	Bulon Island, Thailand	1	approx. 06°50′N	approx. 099°30′E	A12	buyer
3	near Pulau Pangkor, Perak, Malaysia	3	approx. 04°15′N	approx. 100°34′E	A12, A18, A20	fisher
4	Anyer (Pulau Sangiang or P. Panaitan), Java, Indonesia	2	approx. 06°00′ or 06°30′S	approx. 105°50′ or 105°15′E	A01, A29	fisher
5	Bandar Lampung, Sumatra, Indonesia	2	approx. 05°32′S	approx. 105°17′E	A05, A12	fisher
6	Pangandaran, Java, Indonesia	3	approx. 07°41′S	approx. 108°40′E	A25, B01, B15	fisher
7	Batu Nampar, Lombok, Indonesia	8	approx. 08°52′S	approx. 116°24′E	B01, B06, B10(4), B12, B13	fisher
8	Labuan Bajo, Flores, Indonesia	2	approx. 08°29′S	approx. 119°53′E	B01, B14	fisher
9	Kendari, Sulawesi, Indonesia	1	approx. 04°00′S	approx. 123°00′E	B01	boy in market
10	Karimunjawa, Java, Indonesia	1	approx. 05°53′S	approx. 110°26′E	A20	fisher
11	Jepara, Central Java, Indonesia	1	approx. 06°30′S	approx. 110°30′E	A25	fisher
12	Kalimantan Selatan (Pulau Laut, Rampa), Indonesia	1	approx. 03°14′S	approx. 116°12′E	B09	fisher
	Kalimantan Selatan (Tanjung Dewa)	2	approx. 03°10′S	approx. 116°20′E	A10, B01	fisher
	Kalimantan Selatan (Pagatan)	2	approx. 03°36′S	approx. 115°58′E	A12, A20	fisher
13	Indramayu, West Java, Indonesia	2	approx. 06°15′S	approx. 108°30′E	B11, B12	fisher
14	Mersing, Johor, Malaysia	12	approx. 02°25′N	approx. 103°50′E	A04, A06, A09, A12(3), A14, A15, A17, A20, A22, A30	TCM shop
15	near Pulau Kapas and P. Tenggol, Terengganu, Malaysia	2	approx. 04°45′N	approx. 103°40′E	A12, A24	fisher
16	Pattani, Thailand	5	approx. 07°N	approx. 101°E	A01, A07, A12(2), A30	fisher
17	Gulf of Thailand (Ban Koh Prerd, Laem Sing, Chanthaburi) Thailand	2	approx. 12°10′N	approx. 102°10′E	A06, A12	fisher
	Gulf of Thailand (Paknam, Samut Prakan)	2	approx. 13°25′N	approx. 100°36′E	A01, A23	fisher
	Gulf of Thailand (Chonburi)	1	approx. 13°15′N	approx. 100°40′E	A12	researcher
18	Cambodia (Kampong Som)	4	10.60355°N	103.48604°E	A12, A16, A20, A27	fisher
	Cambodia (Kep)	1	10.48060°N	104.32182°E	A21	fisher
	Cambodia (Lob)	1	10.43218°N	104.43185°E	A22	fisher
19	Song-Doc, Thang, Minh Hai, Vietnam	1	approx. 09°00′N	approx. 104°45′E	A13	fisher?
20	Nha Trang, Khanh Hoa, Vietnam	6	approx. 12°15′N	approx. 109°10′E	A11, A12, A18, A30, A31, A20	fisher?
21	Thuan An, Vietnam	1	exact location unknown		A03	fisher?
22	Santubong, Kuching, Sarawak, Malaysia	5	approx. 01°50′N	approx. 110°15′E	A08, A12(2), A20(2)	fisher
23	Pulau Tiga, Sabah, Malaysia	1	approx. 05°42′N	approx. 115°38′E	B01	fisher
24	Pulau Tigabu, Sabah, Malaysia	4	approx. 07°00′N	approx. 117°20′E	B01, B03, B04, B08	fisher
25	Dumaran, Palawan, Philippines	1	approx. 10°32′N	approx. 119°48′E	B01	fisher
26	Iloilo, Philippines	2	exact location unknown		B01, B02	fisher

Appendix *Continued*

(d) *H. trimaculatus continued*

Pop	Locality	*n*	Latitude	Longitude	Haplotypes	Source
27	Daram Island, West Samar, Philippines	1	approx. 11°38′N	approx. 124°47′E	B01	fisher
28	Cebu (Suwangan, Bantayan Island)	2	approx. 11°12′N	approx. 123°45′E	B01, B07	fisher
	Bohol (Maumauan Island)	1	approx. 10°08′N	approx. 124°08′E	B05	fisher
	Bohol (Nasingin Island)	1	approx. 10°08′N	approx. 124°08′E	B01	fisher
29	Taiwan (I-Lan)	1	approx. 24°46′N	approx. 121°45′E	A01	TCM shop
	Taiwan (Keelung Island)	1	approx. 25°15′N	approx. 121°40′E	A30	fisher

Samples that were < approx. 50 km apart were combined and given single population numbers. Particularly for *H. spinosissimus* and *H. trimaculatus*, the uncertainty associated with sampling precluded finer resolution of populations. A more detailed list including individual catalogue and GenBank numbers is available online (see supplementary material). [TCM, traditional Chinese medicine; SL, collected from the wild by Sara Lourie]