Phylogeography of Three Commercially Important Seahorses (Genus *Hippocampus*) in Thai Waters: An Implication from Collective Sequence Data

Thadsin Panithanarak*

ABSTRACT

All seahorse species found in Thai waters are categorized as vulnerable according to the IUCN Red List. Management and conservation plans for seahorse populations require basic knowledge of their population genetic structure and geological history. Therefore, this study aimed to investigate phylogeographical patterns of three commercially important seahorses in Thai waters, the spotted (*Hippocampus kuda*), the hedgehog (*H. spinosissimus*) and the three-spotted (*H. trimaculatus*) seahorses, based on partial sequences of the mitochondrial cytochrome *b* gene. DNA sequences of three species were derived from previous studies in phylogeny of Thai seahorses and comparative phylogeography of Southeast Asian seahorses. The analyses of partial cytochrome *b* sequences from *H. kuda* (7 haplotypes, n=40), *H. spinosissimus* (24 haplotypes, n=32) and *H. trimaculatus* (13 haplotypes, n=28) suggested a distinct phylogeographical pattern in one out of the three species studied (the Gulf of Thailand *vs*. the Andaman Sea populations in *H. kuda*). A distinct phylogeographical pattern in the shallow-water species, *H. kuda* could be explained by external factors, including geographical isolation between two oceans during the Pleistocene (2.5 million-10,000 years ago), along with ecological differences among species. The Pleistocene separation had no apparent effect on the present-day patterns of two deeper-water species, *H. spinosissimus* and *H. trimaculatus*, as their subsequent distribution could have blurred historical separation.

Keywords: Cytochrome *b*, Phylogeography, Seahorse, Thailand

INTRODUCTION

A study of phylogeography involves phylogenetic lineages and their geographical distributions, particularly within populations of the same species and among closely related species (Avise, 2000). Phylogeography of phylogenetic and population genetics maps genetic variation in order to indicate recent geographic distribution of genealogical lineages (Avise *et al*., 2016). Many studies have focused on phylogeographical patterns among marine species (reviewed in Bowen *et al*., 2016). Although marine territory seems to be featureless and without barriers to gene flow, many species that could migrate (e.g. sea turtles, pelagic fishes and some cetaceans) show phylogeographical patterns as a result of physical barriers supporting genetic differentiation and eventually allopatric speciation (Bowen *et al*., 2016).

Phylogeographical patterns in the sea, especially in highly diverse Southeast Asia, including Thai waters, are still poorly studied (reviewed in Lourie *et al*., 2005). Seahorses are among the fish species with low dispersal and accordingly they are likely to preserve their historical patterns. The use

---

Institute of Marine Science, Burapha University, Chonburi, Thailand
* Corresponding author. E-mail address: thadsin@buu.ac.th
Received 12 September 2019 / Accepted 11 December 2019

of phylogeography for a better understanding of geological history and population structure has been confirmed in many marine species, including seahorses (e.g. Lourie *et al*., 2005; reviewed in Bowen *et al*., 2016). Fourteen species of seahorses have been reported in Southeast Asia (Lourie *et al*., 2004). In this region, there are previous studies of phylogeography at a broader scale regarding four seahorse species (Lourie and Vincent, 2004; Lourie *et al*., 2005). These studies revealed three distinct phylogeographical patterns of four species observed. The first species, *Hippocampus barbouri* consisted of two separated lineages: lineage A of eastern Sulu Sea *vs*. lineage B of western Sulu/Sulawesi/Banda/Flores Seas/Indian Ocean. The second species, *H. kuda* consisted of two separated lineages: lineage A of the Pacific Ocean/South China/Sulu Seas *vs*. lineage C of the Indian Ocean/Java/Flores/Banda/Sulawesi Seas (Indian *vs*. Pacific Oceans). The third species, *H. spinosissimus* consisted of three lineages (A, B and C), but their ranges mostly overlapped, except that lineage A was geographically restricted within the Central Philippines. The fourth species, *H. trimaculatus* consisted of two separated lineages: lineage A of the Indian Ocean/Andaman/Java/South China Seas *vs*. lineage B of the Sulu/Sulawesi/Banda/Flores Seas (continental shelf *vs*. ocean populations). The phylogeographical patterns observed were a consequence of different forces that structured populations (fragmentation and/or long-distant colonization in two shallow-water species, *H. barbouri* and *H. kuda vs*. restricted dispersal with isolation by distance and range expansion in two deeper-water species, *H. spinosissimus* and *H. trimaculatus*) (Lourie *et al*., 2005). In addition, external factors, including a geographical isolation between two oceans during the Pleistocene and the Sunda Shelf flooding after glaciation, together with ecological differences among species were likely to contribute to the patterns observed (Lourie *et al*., 2005). However, specific phylogeographical patterns of seahorse species within Thai waters remains unclear. With new DNA sequence information derived from various sources of origin in Thailand (Panithanarak, 2015) coupled with previous information from Lourie and Vincent (2004) and Lourie *et al*. (2005), a better understanding of phylogeographical patterns of seahorse species within Thai waters would be possible.

Thailand is among the leading global exporters of dried seahorses (Nijman, 2010; Perry *et al*., 2010). Three species, the spotted (*Hippocampus kuda*), the hedgehog (*H. spinosissimus*) and the three-spotted (*H. trimaculatus*) seahorses were observed in studies of dried seahorse trades in both the Andaman Sea (Perry *et al*., 2010) and along the eastern coastline of the Gulf of Thailand (Laksanawimol *et al*., 2013). All three species are heavily exploited for consumption as traditional Chinese medicines (Vincent, 1995; Perry *et al*., 2010). In addition, they are listed as vulnerable on the IUCN (International Union for Conservation of Nature and Natural Resources) Red List (IUCN, 2019) and included in CITES Appendix II (CITES, 2019). Comparative information derived from phylogeographical study of three seahorse species within Thai waters could help us define evolutionarily significant units (Moritz, 1994) as well as clarify geological and ecological factors that may contribute to phylogeographical structure of the species.

This study aimed to investigate phylogeographical patterns of three commercially important seahorses in Thai waters (*Hippocampus kuda*, *H. spinosissimus* and *H. trimaculatus*) based on collective cytochrome *b* sequence data from previous studies (Lourie and Vincent, 2004; Lourie *et al*., 2005; Panithanarak, 2015) and to determine what factors facilitate their patterns.

MATERIALS AND METHODS

DNA sequences

Partial cytochrome *b* gene (cyt*b*) sequences of three commercially important seahorse species in Thai waters, i.e. *Hippocampus kuda*, *H. spinosissimus* and *H. trimaculatus* were derived from previous studies including Lourie and Vincent (2004), Lourie *et al*. (2005) and Panithanarak (2015) which were deposited in GenBank. The DNA sequences in this study are described in Tables 1, 2 and 3. The haplotype mappings of seahorse species based on approximate places of origin are shown in Figure 1.

Table 1. Details of *Hippocampus kuda* sequences in Thai waters from Lourie and Vincent (2004) (1), Lourie *et al*. (2005) (2), and Panithanarak (2015) (3), including haplotypes described (H), number of individuals (N), places of origin (number of individuals from each place in brackets), accession numbers and references. *Haplotypes A22 and C12 are identical to kudaTH1 and kudaTH2 defined by Panithanarak (2015).

H	N	Place of origin	Accession no.	References
A22*	29	Bangsaen, Chonburi (19) & Koh Chang, Trad (2) & Cha-um, Phetchaburi (4) & Banpae, Rayong (4)	AY422112	2, 3
A23	1	Bangsaen, Chonburi (1)	AY422113	2
A24	1	Bangsaen, Chonburi (1)	AY422114	2
A25	2	Bangsaen, Chonburi (2)	AY422115	2
C12*	4	Aow Po, Phuket (2) & Sarasin Bridge, Thalang, Phuket (2)	AY422131	2, 3
C36	2	Sarasin Bridge, Thalang, Phuket (2)	AY422155	2
C40	1	Sarasin Bridge, Thalang, Phuket (1)	AY422159	2
Total=7	**40**	**Gulf of Thailand (33), Andaman (7)**		

Table 2. Details of *Hippocampus spinosissimus* sequences in Thai waters from Lourie and Vincent (2004) (1), Lourie *et al*. (2005) (2), and Panithanarak (2015) (3), including haplotypes described (H), number of individuals (N), places of origin (number of individuals from each place in brackets), accession numbers and references. *Haplotypes B20, C01 and C28 are identical to spinoTH6, spinoTH1 and spinoTH4, respectively defined by Panithanarak (2015).

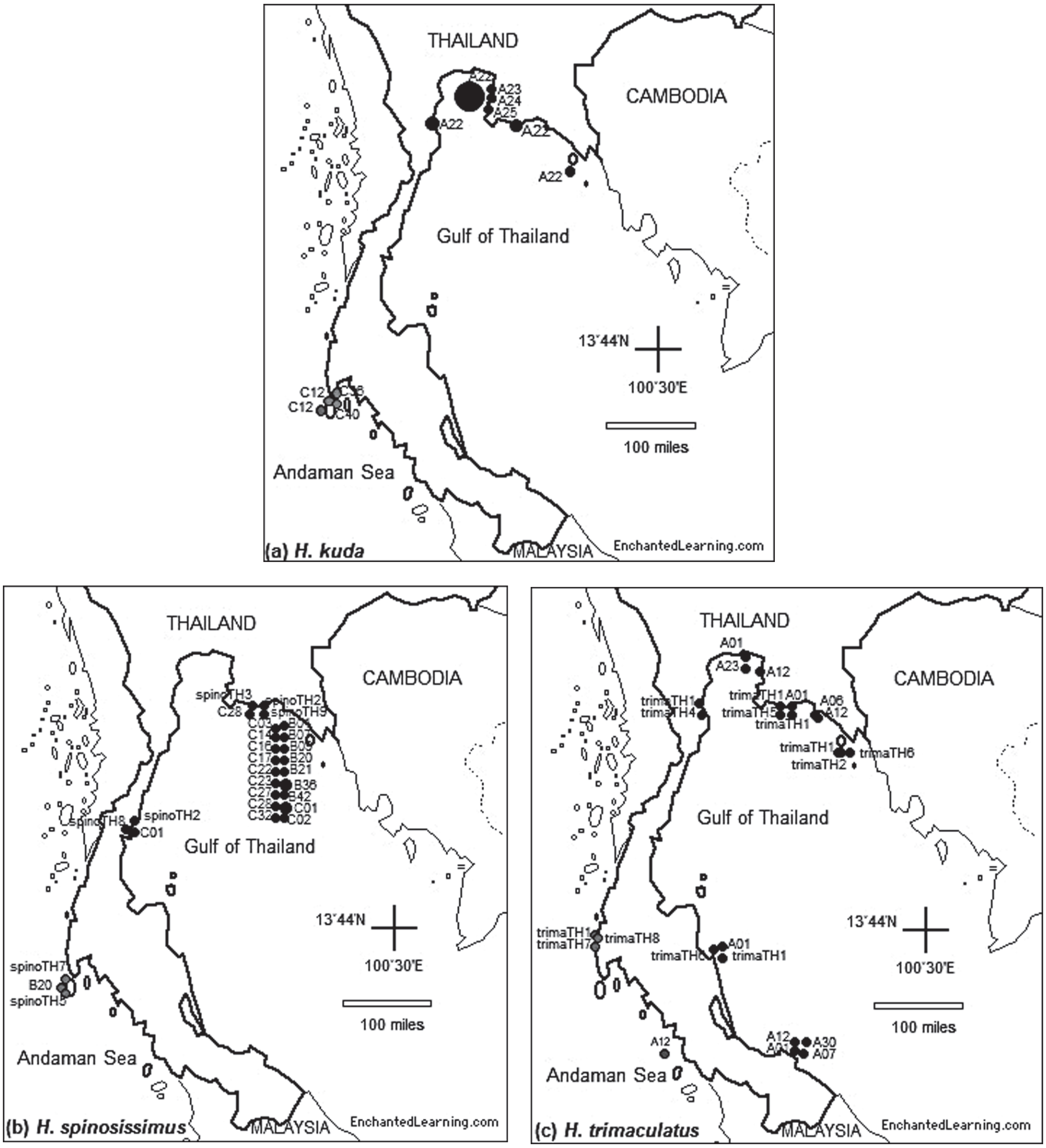
H	N	Place of origin	Accession no.	References
B01	1	Laem Sing, Chantaburi (1)	AY495742	2
B07	1	Laem Sing, Chantaburi (1)	AY495748	2
B09	1	Laem Sing, Chantaburi (1)	AY495750	2
B20*	2	Aow Po, Phuket (1) & Laem Sing, Chantaburi (1)	AY495761	2, 3
B21	1	Laem Sing, Chantaburi (1)	AY495762	2
B36	3	Laem Sing, Chantaburi (3)	AY495777	2
B42	1	Laem Sing, Chantaburi (1)	AY495783	2
C01*	4	Paknam, Chumporn (1) & Laem Sing, Chantaburi (3)	AY495786	2, 3
C02	1	Laem Sing, Chantaburi (1)	AY495787	2
C03	1	Laem Sing, Chantaburi (1)	AY495788	2
C14	1	Laem Sing, Chantaburi (1)	AY495799	2
C16	1	Laem Sing, Chantaburi (1)	AY495801	2
C17	1	Laem Sing, Chantaburi (1)	AY495802	2
C22	1	Laem Sing, Chantaburi (1)	AY495807	2
C23	1	Laem Sing, Chantaburi (1)	AY495808	2
C27	1	Laem Sing, Chantaburi (1)	AY495812	2
C28*	2	Had Mae Rampueng, Rayong (1) & Laem Sing, Chantaburi (1)	AY495813	2, 3
C32	1	Laem Sing, Chantaburi (1)	AY495817	2

Table 2. Continued.

H	N	Place of origin	Accession no.	References
spinoTH2	2	Paknam, Chumporn (1) & Ta Mai, Chantaburi (1)	EU179915	3
spinoTH3	1	Had Mae Rampueng, Rayong (1)	EU179917	3
spinoTH5	1	Aow Po, Phuket (1)	EU179909	3
spinoTH7	1	Aow Po, Phuket (1)	EU179911	3
spinoTH8	1	Paknam, Chumporn (1)	EU179912	3
spinoTH9	1	Ta Mai, Chantaburi (1)	EU179913	3
Total=24	**32**	**Gulf of Thailand (29), Andaman (3)**		

Table 3. Details of *Hippocampus trimaculatus* sequences in Thai waters from Lourie and Vincent (2004) (1), Lourie *et al*. (2005) (2), and Panithanarak (2015) (3), including haplotypes described (H), number of individuals (N), places of origin (number of individuals from each place in brackets), accession numbers and references. *Haplotype A01 is identical to trimaTH3 defined by Panithanarak (2015).

H	N	Place of origin	Accession no.	References
A01*	4	Banpae, Rayong (1) & Pak Panang, Nakornsrithammarach (1) & Pattani (1) & Paknam, Samut Prakan (1)	AY322436	1, 2, 3
A06	1	Laem Sing, Chantaburi (1)	AY322439	1, 2
A07	1	Pattani (1)	AY322440	1, 2
A12	5	Pattani (2) & Laem Sing, Chantaburi (1) & Bulon Island, Satun (1) & Chonburi (1)	AY322435	1, 2
A23	1	Paknam, Sumut Prakan (1)	AY322455	1, 2
A30	1	Pattani (1)	AY322462	1, 2
trimaTH1	8	Banpae, Rayong (1) & Had Mae Rampueng, Rayong (1) & Pak Panang, Nakornsrithammarach (1) & Cha-um, Phetchaburi (2) & Tub-lamu, Pangnga (2) & Koh Chang, Trad (1)	EU179925	3
trimaTH2	1	Koh Chang, Trad (1)	EU179926	3
trimaTH4	1	Cha-um, Phetchaburi (1)	EU179929	3
trimaTH5	1	Had Mae Rampueng, Rayong (1)	EU179930	3
trimaTH6	2	Pak Panang, Nakornsrithammarach (1) & Koh Chang, Trad (1)	EU179928	3
trimaTH7	1	Tub-lamu, Pangnga (1)	EU179931	3
trimaTH8	1	Tub-lamu, Pangnga (1)	EU179932	3
Total=13	**28**	**Gulf of Thailand (23), Andaman (5)**		



(a) *H. kuda*

(b) *H. spinosissimus*

(c) *H. trimaculatus*

Figure 1. Approximate geographical locations of *Hippocampus kuda* (a), *H. spinosissimus* (b) and *H. trimaculatus* (c) haplotypes. Circle size corresponds to haplotype frequency. Black circles represent Gulf of Thailand origin while grey circles represent Andaman Sea origin.

Data analyses

Genetic diversity

The DNA sequences were aligned with Clustal X 2.0 (Thompson *et al*., 1997). Genetic diversity of each species was measured in terms of number of haplotypes (H), haplotype diversity (*h*), nucleotide diversity (π), number of polymorphic or segregating sites (S) and average number of nucleotide differences (K). Genetic diversity values were calculated with DnaSP 6.12.01 (Rozas *et al*., 2017).

Phylogenetic and haplotype relationships

Phylogenetic relationships among three seahorse species in Thai waters were estimated by use of distance-based methods (other methods such as a maximum-likelihood method were not applicable due to the large data set). The best fitting models for the data set were selected by MODELTEST 3.7 (Posada and Crandall, 1998). Then, the neighbor-joining tree was constructed by PAUP* 4.0 beta 10 (Swofford, 2002). A *H. guttulatus* sequence (haplotype EX.284, accession number AF192664, Casey *et al*., 2004) was used as an outgroup. Bootstrap analysis was performed with 1000 replicates (Felsenstein, 1985) using the same settings.

A network of each seahorse species was constructed in Network 10.0.0.0 (http://www.fluxus-engineering.com) with the median-joining (Bandelt *et al*., 1999) network calculation to describe the relationship between the haplotypes.

Genetic differentiation and neutrality tests

Genetic differentiation and neutrality tests were performed considering the grouping by geography (the Gulf of Thailand *vs*. the Andaman Sea) and the grouping by the arrangement obtained from the haplotype network (as this method described the relationship between haplotypes and geographic distribution of the populations). Levels of genetic differentiation between different groups were evaluated with F_{ST} (molecular distance= pairwise difference) by Arlequin ver 3.5 (Excoffier *et al*., 2005). The neutrality tests of Tajima's D (Tajima, 1989a; 1989b) and Fu's Fs (Fu, 1997) were computed by Arlequin ver 3.5. Significance tests were performed using 1000 coalescent simulations.

Divergence times

Divergence times (the times of major separation between lineages in each species) for seahorses' cytochrome *b* gene were roughly estimated to be 1.4 % per million years based on a molecular clock hypothesis (Casey *et al*., 2004). The hypothesis relied on the separation between *Hippocampus ingens* and *H. reidi* due to the closure of the Isthmus of Panama (approximately 3 million years ago; Keigwin, 1978). According to this assumption, a pairwise genetic distance (Kimura 2-parameter, K2P) of 4.4 % between the two species was calculated as a divergence time of 1.4 % per million years. Pairwise genetic distances (K2P) within and between major lineages of *H. kuda*, *H. spinosissimus* and *H. trimaculatus* were calculated using PAUP* 4.0 beta 10 (Swofford, 2002). Then, genetic distances were converted to divergence times between major lineages of each species.

RESULTS AND DISCUSSION

Genetic diversity

Partial cytochrome *b* sequences of *Hippocampus kuda*, *H. spinosissimus* and *H. trimaculatus* used in the analyses were 666 bp in length. *Hippocampus spinosissimus* showed the highest genetic diversity among species (haplotype diversity=0.976±0.015, nucleotide diversity= 0.01072±0.00086, polymorphic sites=38 and average number of nucleotide differences=6.794, Table 4). *Hippocampus kuda* had the lowest haplotype diversity (0.469±0.094), although the species had moderate nucleotide diversity, number of polymorphic sites and average number of nucleotide differences (Table 4). This indicated high variation among *H. kuda* haplotypes. Moderate to high nucleotide diversity also suggested deep phylogenetic division in *H. kuda* and *H. spinosissimus*.

Table 4. Genetic diversity for three seahorse species including number of individuals (N), number of haplotypes (H), haplotype diversity (*h*), nucleotide diversity (π), polymorphic or segregating sites (S) and average number of nucleotide differences (K).

Species	**N**	**H**	***h***	**π**	**S**	**K**
Hippocampus kuda	40	7	0.469±0.094	0.00849±0.00217	22	5.651
Hippocampus spinosissimus	32	24	0.976±0.015	0.01072±0.00086	38	6.794
Hippocampus trimaculatus	28	13	0.881±0.041	0.00286±0.00041	13	1.844

The lowest nucleotide diversity (0.00286±0.00041) (in addition to the lowest number of polymorphic sites and lowest average number of nucleotide differences, Table 4) in contrast to high haplotype diversity (0.881±0.041) observed in *H. trimaculatus* indicated that there was small genetic variation among *H. trimaculatus* haplotypes (this could be due to the small number of samples). Haplotype and nucleotide diversity of the three seahorse species in this study were comparable to a previous study in Thai waters (Panithanarak, 2015), except that the diversity of *H. kuda* was slightly lower. When compared to a broader-scale study of Southeast Asian seahorses (Lourie *et al*., 2005), haplotype and nucleotide diversities of *H. spinosissimus* and *H. trimaculatus* in this study were similar (except nucleotide diversity of *H. trimaculatus* was lower). In contrast, the diversity of *H. kuda* was lower when compared to a previous study of Southeast Asian seahorses (Lourie *et al*., 2005).

Phylogenetic and haplotype relationships

The best fitting model selected by hierarchical likelihood ratio tests (hLRTs) was the HKY+G model (Hasegawa *et al*., 1985) with transition/transversion ratio set to 7.0344; nucleotide frequencies: A=0.2949, C=0.2597, G=0.1381, T=0.3073; and a gamma correction set to 0.1854. A neighbor-joining tree based on the HKY+G model is shown in Figure 2. In general, there were three major clades showing three distinct groups of species: *Hippocampus trimaculatus*, *H. kuda* and *H. spinosissimus*. Each clade consisted of a single species and was supported by high bootstrap values. Within *H. kuda* and *H. spinosissimus* clades, two subclades were identified, but there was no subclade in *H. trimaculatus*. In *H. kuda*, the separation of haplotypes into two subclades was congruent with geographical separation between the Andaman Sea and the Gulf of Thailand, whereas in *H. spinosissimus*, the haplotypes were separated by their lineages (lineages B and C) as defined in Lourie *et al*. (2005). This separation did not reveal any significant relation to geographical isolation, although the Andaman Sea haplotypes (spinoTH5, spinoTH7 and B20) were restricted within the lineage B clade.

Haplotype networks of three seahorse species, *Hippocampus kuda*, *H. spinosissimus* and *H. trimaculatus* are shown in Figures 3, 4 and 5, respectively. A haplotype network of *H. kuda* was divided into two groups (lineages A and C as defined in Lourie *et al*., 2005) and this reflected geographical separation between the Gulf of Thailand and the Andaman Sea (Figure 3). In contrast, haplotypes of *H. spinosissimus* and *H. trimaculatus* were not grouped by geography (Figures 4 and 5). The haplotypes of *H. spinosissimus* were divided into two groups (lineages B and C as defined in Lourie *et al*., 2005) with haplotype spinoTH2 separated by many mutations (32 mutational steps, Figure 4). The Andaman Sea haplotypes (spinoTH5, spinoTH7 and B20) were restricted within lineage B, while the Gulf of Thailand haplotypes were scattered between the two lineages. A star-like network of lineage C in *H. spinosissimus* indicated shallow evolutionary history (population expansion). Similarly, a haplotype network of *H. trimaculatus* was also clustered into two groups (lineage A and others). The two lineages were evenly distributed in both the Andaman Sea and the Gulf of Thailand.

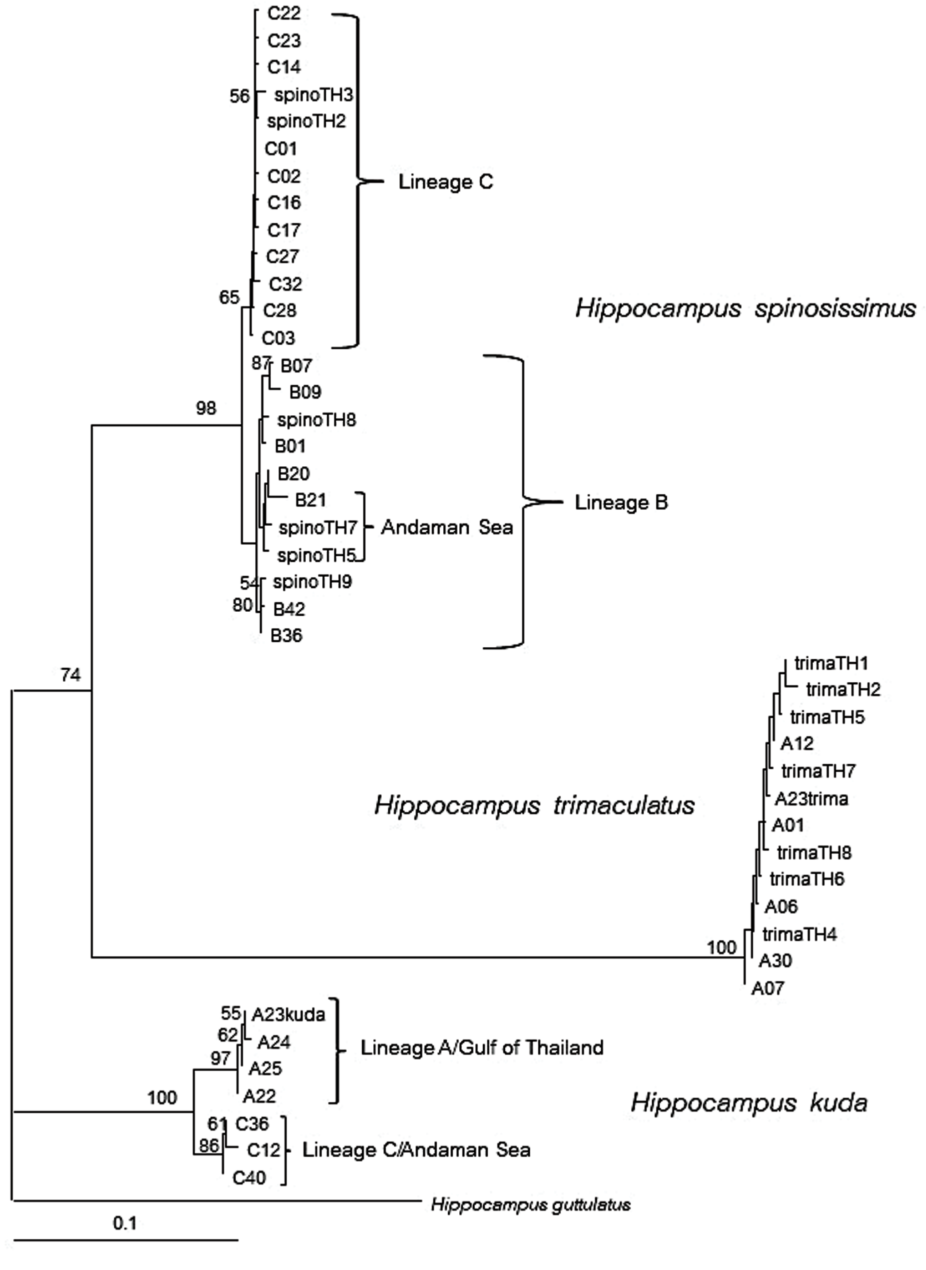


Figure 2. A neighbor-joining tree showing the relationship among *Hippocampus trimaculatus*, *H. kuda* and *H. spinosissimus* haplotypes found in Thai waters based on partial cyt*b* gene. The tree was constructed by use of the HKY+G (Hasegawa *et al*., 1985) model selected by Modeltest 3.7 (Posada and Crandall, 1998). A *H. guttulatus* sequence (accession number AF192664, Casey *et al*., 2004) was used as an outgroup. Bootstrap support values above 50 % are shown.

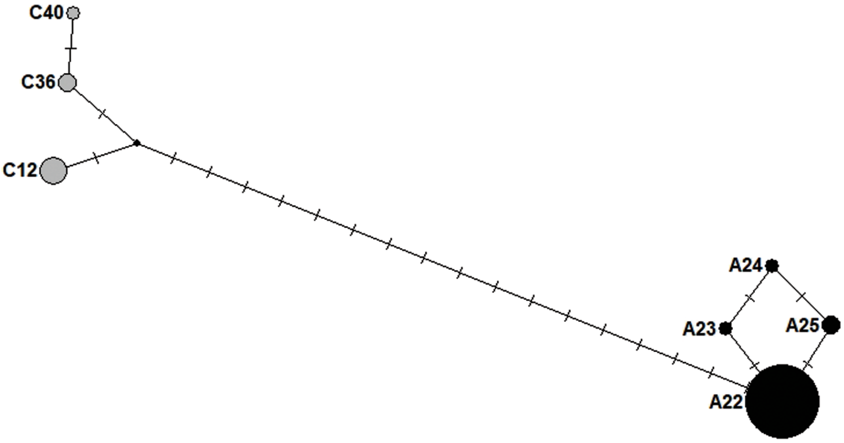


Figure 3. A haplotype network estimated by the median-joining algorithm showing relationships among *Hippocampus kuda* haplotypes inferred from partial cyt*b* gene. Circle size corresponds to haplotype frequency. Black color represents Gulf of Thailand origin while grey color represents Andaman Sea origin. Mutations between haplotypes are specified by the lines within the branches. Small black dots are non-observed haplotypes but assumed to connect observed haplotypes. To improve network drawing, lengths and positions are changed in some branches.

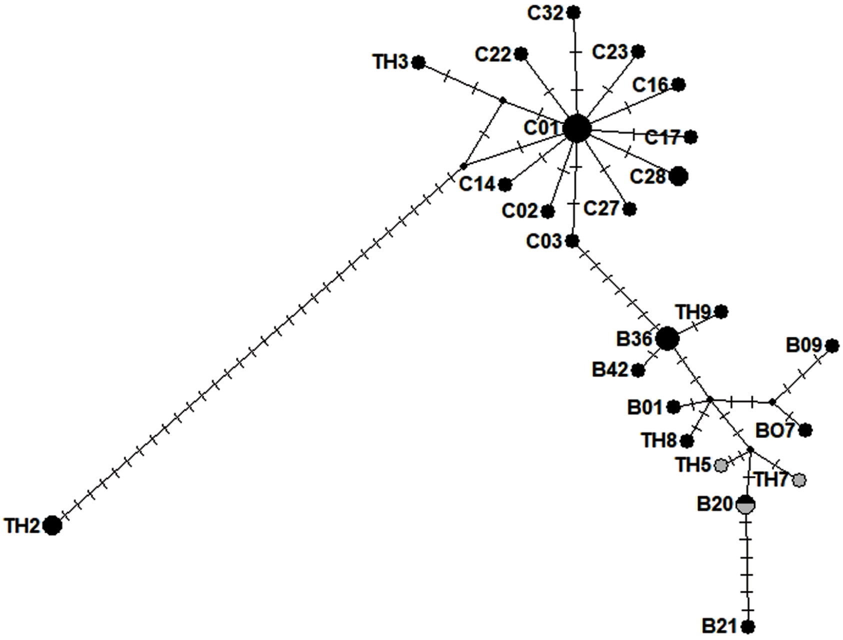


Figure 4. A haplotype network estimated by the median-joining algorithm showing relationships among *Hippocampus spinosissimus* haplotypes inferred from partial cyt*b* gene. Circle size corresponds to haplotype frequency. Black color represents Gulf of Thailand origin while grey color represents Andaman Sea origin. Mutations between haplotypes are specified by the lines within the branches. Small black dots are non-observed haplotypes but assumed to connect observed haplotypes. To improve network drawing, lengths and positions are changed in some branches.

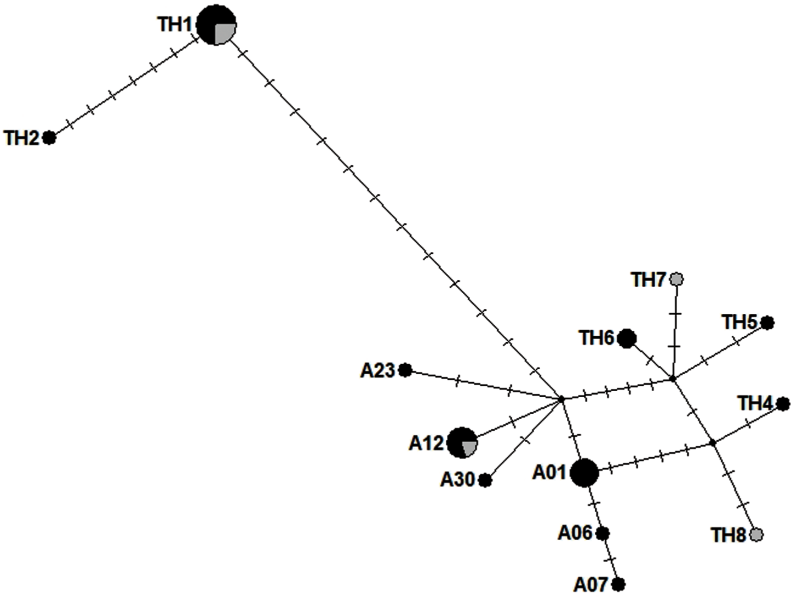


Figure 5. A haplotype network estimated by the median-joining algorithm showing relationships among *Hippocampus trimaculatus* haplotypes inferred from partial cyt*b* gene. Circle size corresponds to haplotype frequency. Black color represents Gulf of Thailand origin while grey color represents Andaman Sea origin. Mutations between haplotypes are specified by the lines within the branches. Small black dots are non-observed haplotypes but assumed to connect observed haplotypes. To improve network drawing, lengths and positions are changed in some branches.

In general, phylogenetic relationships of seahorse haplotypes showed in the neighbor-joining tree (Figure 2) were consistent with the distribution of the haplotypes in the networks (Figures 3-5). However, the haplotype network of *Hippocampus trimaculatus* (Figure 5) was incongruent with the neighbor-joining tree (Figure 2). The haplotype network revealed that *H. trimaculatus* consisted of two groups (lineage A and others), while only one lineage was observed in the neighbor-joining tree. Haplotype network methods are considered more suitable for closely related haplotypes compared to traditional phylogenetic methods (Lourie *et al*., 2005). This is because the network methods can visualize alternate connections (loops) between haplotypes which are collapsed into unresolved sections of the phylogenetic tree (polytomies) (Posada and Crandall, 2001). However, the inconsistency between the phylogenetic and haplotype network approaches was possibly due to limited number of specimens from the other lineage of *H. trimaculatus*.

Genetic differentiation and neutrality tests

Genetic differentiation and neutrality tests were performed considering the grouping by geography and the grouping in the network results. For *Hippocampus kuda*, the groupings generated by geography and the networks were identical (Gulf of Thailand=lineage A, Andaman Sea=lineage C). Considering the grouping by geography (Gulf of Thailand *vs*. Andaman Sea), significant genetic differentiation was observed only in *H. kuda* populations. In *H. kuda*, the Gulf of Thailand population was significantly different from the Andaman Sea population (F_{ST}=0.65938, p<0.00001, number of permutations=1023). In contrast, for *H. spinosissimus* and *H. trimaculatus*, the Gulf of Thailand populations were not significantly different from the Andaman Sea populations (F_{ST}=0.00636 and -0.03837, respectively for *H. spinosissimus* and *H. trimaculatus*). Considering the grouping by the network results, significant genetic differentiations were observed between haplotype groups generated by the haplotype networks for all species (Figures 3-5). For *H. kuda*, the Gulf of Thailand group (lineage A) was significantly different from the Andaman Sea group (lineage C) (F_{ST}=0.65938, p<0.00001, number of permutations=1023). Lineage B of *H. spinosissimus* was significantly different from lineage C (the analysis excluded the haplotype spinoTH2) (F_{ST}=0.05123, p=0.00391, number of permutations=1023). In addition, lineage A of *H. trimaculatus* was significantly different from the other group (F_{ST}=0.37662, p<0.00001, number of permutations=1023).

Values of the neutrality tests are shown in Table 5. Considering the grouping by geography, values of Tajima's D were not significant for any of the species. Values of Fu's Fs were significant for the Gulf of Thailand group in *Hippocampus kuda* and *H. spinosissimus*. Non-significant values were observed in *H. trimaculatus* groups. Considering the grouping by haplotype networks, a value of Tajima's D was only significant for the lineage C group in *H. spinosissimus*. In addition, values of Fu's Fs were significant for more groups, including the Gulf of Thailand group in *H. kuda*, as well as the lineage B and C groups in *H. spinosissimus*. Only lineage C of *H. spinosissimus* was significant for both Tajima's D and Fu's Fs tests. Non-significant values were observed in *H. trimaculatus* groups. The results indicated population expansion in *H. kuda* populations (the Gulf of Thailand group or lineage A) and *H. spinosissimus* (the Gulf of Thailand group and both lineage B and C groups). A suggestion of population expansion in lineage C of *H. spinosissimus* was congruent with a star-like network, as seen in Figure 4.

However, genetic differentiation and neutrality tests applied in this study need to be interpreted carefully because the number of specimens from some groups (i.e. lineage C of *Hippocampus kuda*, other lineages of *H. trimaculatus*, and the Andaman Sea groups for all species) was limited, which might have had an effect on the analyses and significance levels.

Phylogeographic analyses in this study revealed a single phylogeographical pattern in one out of three heavily exploited seahorse species observed: *Hippocampus kuda* in Thai waters had a distinct phylogeographical pattern. The species consisted of two lineages (lineages A and C) confirmed by the neighbor-joining tree with high bootstrap support values (Figure 2) and the haplotype network (Figure 3). The separation of lineages A and C in *H. kuda* revealed significant relation to geographic isolation (the Gulf of Thailand *vs*. the Andaman Sea). A significant phylogeographical structure of *H. kuda* within Thai waters was congruent with previous studies (Lourie *et al*., 2005; Panithanarak *et al*., 2010). In contrast, the separation of lineages B and C in *H. spinosissimus* did not reveal any significant

Table 5. Neutrality tests for populations based on grouping by haplotype networks and geography; Gulf of Thailand (GoT) *vs*. Andaman Sea (Andaman) including Tajima's D (TD) and Fu's Fs (FF). Significant values after 1000 coalescent simulations are indicated by *p<0.05, **p<0.005 and ***p<0.0005. For *Hippocampus kuda*, the groupings generated by networks and geography are identical (lineage A=GoT, lineage C=Andaman).

Species	**Networks**					**Geography**				
	Population	**N**	**H**	**TD**	**FF**	**Population**	**N**	**H**	**TD**	**FF**
Hippocampus kuda	Lineage A	33	4	-0.83	-2.41*	GoT	33	4	-0.83	-2.41*
	Lineage C	7	3	0.77	0.67	Andaman	7	3	0.77	0.67
Hippocampus spinosissimus	Lineage B	14	11	-1.29	-3.67*	GoT	29	22	-1.12	-5.80*
	Lineage C	16	12	-2.20**	-9.75***	Andaman	3	3	0	-0.34
Hippocampus trimaculatus	Lineage A	19	11	-1.25	-2.56	GoT	23	11	-1.17	0.86
	Others	9	2	-1.10	3.10	Andaman	5	4	-1.15	1.90

relation to geographic isolation, although only lineage B had a unique population from the Andaman Sea (the neighbor-joining tree in Figure 2 and the haplotype network in Figure 4). In *H. trimaculatus*, a single lineage (lineage A) was presented in the neighbor-joining tree (Figure 2) but two lineages (lineages A and others) were shown in the haplotype network (Figure 5). Previous studies reported that in Southeast Asia, *H. trimaculatus* consisted of two major lineages, lineages A and B, although only lineage A was mainly distributed in Thai waters (Lourie and Vincent, 2004; Lourie *et al*., 2005).

Possible factors that could influence a phylogeographical pattern in one out of three seahorse species were geological history and ecological differences. Geological events that may impact present-day genetic distribution of marine species include a geographical isolation between the Indian and the Pacific Oceans (McManus, 1985) and the Sunda Shelf flooding after glaciation (Voris, 2000). A significant phylogeographical structure of *Hippocampus kuda* may possibly be explained by a geographical isolation of two oceans hypothesis (McManus, 1985). It is possible that the separation of lineages A and C in *H. kuda* is associated with lowered sea levels during the Pleistocene (2.5 million-10,000 years ago). This is congruent with the estimated divergence time between the split of two lineages (1.98-2.31 million years ago, Table 6). During the Pleistocene, when repeated glacial cycles occurred, coupled with dramatically falling of global sea levels, many areas of land in Southeast Asia were exposed, such as the Sunda Shelf, and existing islands were linked by land bridges (Voris, 2000). Consequently, geographical isolation of the Indian and Pacific Ocean populations would be possible. Previous study also showed significant phylogeographical structure among *H. kuda* populations in Thai waters based on other mitochondrial markers (mitochondrial control region) following the Pleistocene isolation of ocean basins hypothesis (Panithanarak *et al*., 2010). The separation of lineages B and C in *H. spinosissimus* would have also occurred during the Pleistocene, as estimated divergence time (0.76-1.87 million years ago, Table 6) fell within this period. However, the diversification between the two lineages did not reflect the simple Pleistocene isolation. Other factors, such as dispersal ability assumed by biological and/or ecological differences could have had more influence on the species diversification. The Last Glacial Maximum occurred about 19,000 years BP, when sea levels were at the lowest (about 130 m lower than existing levels) (Yokoyama *et al*., 2000). After glacial melting, gradual and then rapid rise of sea levels followed and led to the Sunda Shelf flooding (about 14,600 BP) (Hanebuth *et al*., 2000). The possibility that *H. spinosissimus* recolonized the Sunda Shelf after the postglacial flooding was suggested by recent population expansion signs (a star-like network and significant values of Tajima's D and Fu's Fs observed in lineage C of *H. spinosissimus*). The Andaman Sea

Table 6. Pairwise genetic distances (distance measure: Kimura 2-Parameter) within and between each species lineage. Genetic distances are shown as percentage (%). Divergence times (million years, mya) were estimated from genetic distances between the species lineages.

Species lineage	**Pairwise genetic distance (%)**		**Divergence time (mya)**
	within lineage	**between lineages**	
Hippocampus kuda			
-Lineage A (Gulf of Thailand)	0.15-0.30	2.77-3.24	1.98-2.31
-Lineage C (Andaman Sea)	0.15-0.45		
Hippocampus spinosissimus			
-Lineage B	0.15-1.84	1.06-2.62	0.76-1.87
-Lineage C	0.15-0.76		
Hippocampus trimaculatus	0.15-0.76	-	-

population might be retained with greater structure within lineage B than in lineage C. In *H. trimaculatus*, only one lineage was observed in the neighbor-joining tree; therefore, divergence time estimation was not applicable.

Ecological differences among seahorse species could define their dispersal capabilities. *Hippocampus kuda*, *H. spinosissimus* and *H. trimaculatus* are supposed to be different in dispersal rate, according to their dissimilar habitats. Generally, *H. kuda* is a species found in shallow water inhabiting seagrass, mangrove, estuarine and mud-covered areas at depths below 10 m (Lourie *et al*., 1999; Choo and Liew, 2003). These habitats are likely to be fragmented along the coast, and suitable habitats are rare and may be separated from one another. In contrast, *H. spinosissimus* and *H. trimaculatus* are deeper-water species occupying areas at a minimum of 10-15 m depth and inhabiting open-water substrates, e.g. sand, pebbles and non-living bivalves (Lourie *et al*., 1999) or living with sponges, octocorals and sea-whips (Choo and Liew, 2003). These habitats would involve fewer barriers and thus *H. spinosissimus* and *H. trimaculatus* populations may be more highly connected. Ecological differences could play a major role in shaping the phylogeographical structure of these three seahorse species. *Hippocampus kuda* dispersal is thought to be low. Consequently, population structure of *H. kuda* reflects historical arrangement of land and sea, i.e. Pleistocene separation of ocean basins. *Hippocampus spinosissimus* and *H. trimaculatus* dispersal capabilities are assumed to be higher than *H. kuda*. Subsequent distribution of the two deeper-water species could blur historical separations (except in the case of the Andaman Sea population within lineage B of *H. spinosissimus*). Thereby, present-day population structure of *H. spinosissimus* and *H. trimaculatus* does not follow a simple separation of ocean basins hypothesis. In *H. spinosissimus*, recolonization of Thai waters may occur from two sources of populations (lineages B and C), while it is unclear whether *H. trimaculatus* has recolonized from a single population or more. Further studies focused especially on *H. trimaculatus* from various localities within Thai waters are needed to verify both the species pattern and structure. In general, further sampling and analyses based on other potential molecular markers, including in the nuclear genome will confirm and address some artefacts that may originate from these collective data analyses.

CONCLUSION

In conclusion, a distinct phylogeographical pattern was observed in one (*Hippocampus kuda*) out of three commercially important seahorse species in Thai waters. Two external factors that could facilitate the phylogeographical pattern were geological history and ecological differences between species. An event in geological history that could influence the pattern in *H. kuda* was the isolation of Indian and Pacific Oceans during the Pleistocene. The historical ocean basin separation had no effect on the present-day patterns in *H. spinosissimus* and *H. trimaculatus*, as subsequent distribution of the two species could have blurred historical separation. Ecological differences between a shallow-water species, *H. kuda* and two deeper-water species, *H. spinosissimus* and *H. trimaculatus* also contribute to the distinct phylogeographical pattern observed.

LITERATURE CITED

Avise, J.C. 2000. **Phylogeography: The History and Formation of Species.** Harvard University Press. Cambridge. 464 pp.

Avise, J.C., B.W. Bowen and F.J. Ayala. 2016. In the light of evolution X: Comparative phylogeography. **Proceedings of the National Academy of Sciences of the United States of America** 113: 7957–7961.

Bandelt, H.J., P. Forster and A. Röhl. 1999. Median-joining networks for inferring intraspecific phylogenies. **Molecular Biology and Evolutions** 16: 37–48.

Bowen, B.W., M.R. Gaither, J.D. DiBattista, M. Iacchei, K.R. Andrews, W.S. Grant, R.J. Toonen and J.C. Briggs. 2016. Comparative phylogeography of the ocean planet. **Proceedings of the National Academy of Sciences of the United States of America** 113: 7962–7969.

Casey, S.P., H.J. Hall, H.F. Stanley and A.C.J. Vincent. 2004. The origin and evolution of seahorses (genus *Hippocampus*): a phylogenetic study using the cytochrome *b* gene of mitochondrial DNA. **Molecular Phylogenetics and Evolution** 30: 261–272.

Choo, C.K. and H.C. Liew. 2003. Spacial distribution, substrate assemblages and size composition of seahorses (Family Syngnathidae) in the coastal waters of Peninsular Malaysia. **Journal of the Marine Biological Association of the United Kingdom** 83: 271–276.

CITES. 2019. **Proposals for amendment of Appendices I and II.** Results of the 12th meeting of the conference of the parties 2002. Convention on international trade in endangered species of wild fauna and flora secretariat, Geneva, Switzerland. http://www.cites.org. Cited 9 Sep 2019.

Excoffier, L., G. Laval and S. Schneider. 2005. Arlequin ver. 3.0: An integrated software package for population genetics data analysis. **Evolutionary Bioinformatics Online** 1: 47–50.

Felsenstein, J.W. 1985. Confidence limits on phylogenies: an approach using the bootstrap. **Evolution** 39: 783–791.

Fu, Y. 1997. Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. **Genetics** 147: 915–925.

Hanebuth, T., K. Stattegger and P.M. Grootes. 2000. Rapid flooding of the Sunda Shelf: a late-glacial sea level record. **Science** 288: 1033–1035.

Hasegawa, M., K. Kishino and T. Yano. 1985. Dating the human-ape splitting by a molecular clock of mitochondrial DNA. **Journal of Molecular Evolution** 22: 160–174.

IUCN. 2019. **The IUCN red list of threatened species.** IUCN (The World Conservation Union) Species Survival Commission, Gland, Switzerland. https://www.iucnredlist.org. Cited 9 Sep 2019.

Keigwin, L.D. 1978. Pliocene closing of the Isthmus of Panama, based on biostratigraphic evidence from nearby Pacific Ocean and Caribbean Sea cores. **Geology** 6: 630–634.

Laksanawimol, P., S. Petpiroon and P. Damrongphol. 2013. Trade of seahorses, *Hippocampus* spp. (Actinopertygii: Syngnathiformes: Syngnathidae), on the East Coast of the Gulf of Thailand. **Acta Ichthyologica et Piscatoria** 43: 229–235.

Lourie, S.A., A.C.J. Vincent and H.J. Hall. 1999. **Seahorse: An identification guide to the world's species and their conservation.** Project Seahorse. London. 213 pp.

Lourie, S.A. and A.C.J. Vincent. 2004. A marine fish follows Wallace's Line: the phylogeography of the three-spotted seahorse (*Hippocampus trimaculatus*, Syngnathidae, Teleostei) in Southeast Asia. **Journal of Biogeography** 31: 1975–1985.

Lourie, S.A., S.J. Foster, E.W.T. Cooper and A.C.J. Vincent. 2004. **A Guide to the Identification of Seahorses.** Project Seahorse and TRAFFIC North America. University of British Columbia and World Wildlife Fund. Washington D.C. 114 pp.

Lourie, S.A., D.M. Green and A.C.J. Vincent, 2005. Dispersal, habitat differences, and comparative phylogeography of Southeast Asian seahorses (Syngnathidae: *Hippocampus*). **Molecular Ecology** 14: 1073–1094.

McManus, J.W. 1985. **Marine speciation, tectonics and sea level changes in Southeast Asia.** Proceedings of the Fifth International Coral Reef Congress, Tahiti 1985: 133–138.

Moritz, C. 1994. Defining 'evolutionarily significant units' for conservation. **Trends in Ecology and Evolution** 9: 373–375.

Nijman, V. 2010. An overview of international wildlife trade from Southeast Asia. **Biodiversity and Conservation** 19(4): 1101–1114.

Panithanarak, T. 2015. **Phylogeny of Thai seahorses inferred from mitochondrial DNA cytochrome *b* gene.** Proceedings of the Burapha University International Conference 2015: 1010–1023.

Panithanarak, T., R. Karuwancharoen, U. Na-Nakorn and T.T.T. Nguyen. 2010. Population genetics of the spotted seahorse (*Hippocampus kuda*) in Thai waters: implications for conservation. **Zoological Studies** 49(4): 564–576.

Perry, A.L., K.E. Lunn and A.C.J. Vincent. 2010. Fisheries, large-scale trade, and conservation of seahorses in Malaysia and Thailand. **Aquatic Conservation: Marine and Freshwater Ecosystems** 20(4): 464–475.

Posada, D. and K.A. Crandall. 1998. Modeltest: testing the model of DNA substitution. **Bioinformatics** 14: 817–818.

Posada, D. and K.A. Crandall. 2001. Intraspecific gene genealogies: trees grafting into networks. **Trends in Ecology and Evolution** 16: 37–45.

Rozas, J., A. Ferrer-Mata, J.C. Sanchez-DelBarrio, S. Guirao-Rico, P. Librado, S. Ramos-Onsins and A. Sanchez-Gracia. 2017. DnaSP v6: DNA sequence polymorphism analysis of large datasets. **Molecular Biology and Evolutions** 34: 3299–3302.

Swofford, D.L. 2002. **PAUP*-phylogenetic analysis using parsimony (*and other methods), Version 4.0b10.** Sinauer Associates. Sunderland, Massachusetts. 142 pp.

Tajima, F. 1989a. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. **Genetics** 123: 585–595.

Tajima, F. 1989b. The effect of change in population size on DNA polymorphism. **Genetics** 123: 597–601.

Thompson, J.D., T.J. Gibson, F. Plewniak, F. Jeanmougin and D.G. Higgins. 1997. The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. **Nucleic Acids Research** 24: 4876–4882.

Vincent, A.C.J. 1995. Trade in seahorse for traditional Chinese medicines, aquarium fishes and curios. **TRAFFIC Bulletin** 15(3): 125–128.

Voris, H.K. 2000. Maps of Pleistocene sea levels in Southeast Asia: shorelines, river systems, time durations. **Journal of Biogeography** 27: 1153–1167.

Yokoyama, Y., K. Lambeck., P.D. Deckker, P. Johnston and L.K. Fifield . 2000. Timing of the last glacial maximum from observed sea-level minima. **Nature** 406: 713–716.