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Morphological and molecular identification of seahorses (*Hippocampus* spp.) from the coast of Sumatra Island, Indonesia

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Abstract. *Hanifaturahmah F, Perwitasari-Farajallah D, Butet NA, Nurilmala M, Sudrajat AO, Ochiai Y. 2020. Morphological and molecular identification of seahorses (*Hippocampus *spp.) from the coast of Sumatra Island, Indonesia. Biodiversitas 21: 4116-4123.* Seahorses (*Hippocampus* spp.) species are difficult to be identified based on their color, shape, and body size, especially for their juveniles. The purposes of this study were to identify seahorse species from Indonesian waters (Riau Islands and Lampung, Sumatra Island) based on the sequence variations in the 16S rRNA and COI genes and to analyze the kinship of these seahorses. The morphological identification was performed, followed by the validation of the identification based on a molecular approach. Both identification methods showed the one species from Riau Islands was *H. kuda*, while the others (n=2) were *H. comes*. All specimens from Lampung (n=3) were *H. comes*. The specimens of *H. comes* from Riau Islands and Lampung were the same haplotype based on the 16 S rRNA sequence. Based on the COI, *H. comes* from Riau Islands (n=2) were found to be one haplotype (haplotype 1; n=2), while those from Lampung (n=3) consisted of two haplotypes (haplotype 1; n=2 and haplotype 2; n=1). Phylogenetic analysis based on both genes revealed that *H. comes* from Riau Islands and Lampung belongs to the same clade, while *H. kuda* from Riau Islands (n=1) was included in the lineage B (Indian Ocean).

Keywords: 16S rRNA marker, COI, *Hippocampus*, morphological identification

INTRODUCTION

Seahorses (*Hippocampus* spp.), which are found all over the world, belong to Syngnathidae Family consisting of 41 species. The genus name comes from *hippos* = horse head and *campus* = sea monster (Lourie et al. 2004). They inhabit subtropical and tropical shallow coastal waters, including seagrass ecosystems, coral reefs, mangroves, and river mouths (Lourie et al. 2016). The body of the seahorses is segmented, with very small gills, a pair of pectoral fins, one dorsal fin, small anal, but without a caudal fin. Seahorses have the ability to change their body color (or camouflage) to avoid predators. It is well known that seahorses have unique reproduction properties as represented by exhibiting male pregnancy. The previous researchers reported that seahorses are monogamy (Masonjones and Lewis 2000; Kvarnemo et al. 2007) and polyandry (Masonjones and Lewis 2000; Syafiuddin et al. 2011).

Species identification of seahorses is difficult based on their color, shape, and body size. However, the cryptic and complex species cause frequent errors by the morphological identification approach. Molecular identification can improve accuracy and minimize misidentification (Bickford et al. 2007; Lourie et al. 2016). One representative technique for molecular identification is DNA barcoding, which utilizes short fragments of DNA (Hebert et al. 2003; Dasmahapatra and Mallet 2006). DNA barcoding is frequently performed using mitochondrial DNA (mtDNA), which is inherited uniparentally and does not undergo recombination. Sibling species have mtDNA with high similarity values (Urich 1990). The 16S rRNA gene is a region of mtDNA that is often used to identify species and populations, while the COI gene is used for species identification. The COI gene contains sufficient levels of variations because of its fast mutation rate and thus used for accurate identification of a wide variety of organisms, including marine species (Ward et al. 2005). In addition, the COI gene is well known to be useful as an evolutionary marker for the analysis of intra- and interspecific relationships in many marine fish and shellfish (Lakra et al. 2008; Singh et al. 2011). The COI gene was reported to be able to distinguish the variations in the nucleotide sequences in *H. kuda* from Indian waters. Eight haplotypes of *H. kuda* have been found in Indian waters based on the COI gene, while five haplotypes of *H. kuda*

were found in Indian waters based on the 16S rRNA gene (Singh et al. 2011).

Seahorses are widely distributed in the Atlantic, Indian, and Pacific Oceans. Their distribution ranges from the temperate to tropical coastal waters, with the highest diversity found in the Indo-Pacific waters (Lourie et al. 2005). In Indonesia, there are 12 species of seahorses (*Hippocampus* spp.), namely *H. barbouri, H. bargibanti, H. comes, H. denise, H. histrix, H. keloggi, H. kuda, H. spinosissimus, H. trimaculatus, H. pantohi, H. severnsi,* and *H. satomiae* (Lourie et al. 2004; Lourie and Kuiter 2008).

Two territorial water with the distribution of seahorses in Indonesia are Riau Islands and Lampung along Sumatra Island (Fisheries Research Center 2004). Their distribution can be influenced by geographical locations and sea currents, which allow the linkages of species in each location. The intra- and interspecies relationships can be traced based on the origin of the ancestors, one of which is based on the concept of phylogeography. Thus, this study was aimed to identify seahorse species from these locations and analyze their relationships based on 16S rRNA and COI genes from the viewpoints of preservation and utilization of Indonesian seahorses.

MATERIALS AND METHODS

Specimens sampling

The seahorses were obtained from the fishermen in the two locations, namely, the waters of Senggarang Subdistrict, Tanjung Pinang City, Riau Islands Province, and the waters of the Teluk Betung Selatan District, Bandar Lampung City, Lampung Province, Sumatra Island, Indonesia (Figure 1). The sampling was conducted in March and April 2018. The specimens used in this study were six individuals, namely, three individuals from each location with a minimum total body length of 10.0 cm. The samples were transported on ice to the laboratory, and stored in a freezer at -18 ± 2°C before use.

Morphological measurements

Morphological measurements were done using the method of Lourie et al. (2004). Some morphological parameters such as height and the average length of nose and head were measured using a ruler. In addition, the number of trunk and tail rings, cheek spines, body, and tail rings on the dorsal fins were counted manually.

DNA extraction

DNA was extracted from the tail muscle (ca. 3 g) using the Qiamp DNA Blood and Tissue kit (Qiagen, Hilden, Germany) in accordance with the company instructions with some modifications. 25 mg of samples were added 180 µL of ATL buffer and crushed manually with a plastic pestle. Then, 20 µg of Proteinase K was added to the homogenate and vortexed until thoroughly mixed. Subsequently, the mixture was incubated at 56 °C for 2 hours, and 200 µL of AL buffer was added, followed by the addition of 200 µL absolute ethanol and centrifugation at 8,000 x g for 1 min. To the supernatant was added 500 µL of AW1 buffer, centrifuged at 8,000 x g for 1 min, and the supernatant was discarded. After suspending the pellet in 500 µL of AW2 buffer, it was centrifuged at 16,000 x g, and the DNA preparation was obtained as a pellet, which was dissolved in 50 µL of buffer AW.

Amplification of 16S rRNA and COI genes

The 16S rRNA gene was amplified with a PCR apparatus (Integrated DNA Technologies, Singapore, Republic of Singapore) using a primer designed by Butet (2013, unpublished data). Amplification consisted of pre-denaturation of 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 53°C for 1 min, elongation at 72°C for 1 min, and post-elongation at 72°C for 5 min. The reaction was carried out in 25 µL of a mixture containing 3 µL (100 ng/ µL) of DNA templates, 1.5 µL of forward and reverse primers, 12.5 µL of KAPA Taq Ready Mix PCR kit (Kapa Biosystems Wilmington, MA, USA) and 6.5 µL of nuclease-free water. The COI gene was amplified using the forward primer (FishF2 5'-TCGACTAATCAATCATAAAGATATCGCGAC-3') and the reverse primer (FishR2 3'-ACTTCAGGGTGACCGAAGAATCAGAA-5') (Ward et al. 2005). Amplification steps consisted of pre-denaturation at 94°C for 3 min, 35 cycles of denaturation at 94°C for 45 sec, annealing at 58°C for 1 min and 45 sec, elongation at 72°C for 1 min, and post-elongation at 72°C for 6 min. The reaction was carried out in 25 µL of a mixture containing 3 µL (100 ng/ µL) of DNA templates, 1.25 µL of each primer, 12.5 µL of KAPATaq Ready Mix PCR kit, and 7 µL of nuclease-free water. The PCR products were subjected to agarose gel electrophoresis (1.2% gel) with 1 x TBE buffer, 1 x BlueJuice ™ Gel Loading Buffer (Thermo Fisher Scientific, Waltham, MA, USA), and SYBR® Green 1 x staining (ThermoFisher Scientific). Electrophoresis was run at 100 V for 30 min, and the DNA fragments were visualized under ultraviolet light. The nucleotide sequence was determined by a direct sequencing method presented by First BASE Laboratories (Selangor Darul Ehsan, Malaysia).

Bioinformatic analysis

Bioinformatic analysis was performed on the obtained nucleotide sequences based on 16S rRNA and COI genes. The validation of seahorse species was done using BLASTn for each marker (16S rRNA and COI genes). Then, alignment and variation of nucleotide sequences, haplotype diversity, estimated genetic distance, and phylogenetic construction by a neighbor-joining method was conducted using the multiple sequence alignment tools ClustalW (Thompson et al. 1994) and MEGA 7 (Tamura et al. 2011). The accession numbers of nucleotides sequences for seahorse 16S rRNA genes are as follows: *H. comes* Taiwan (AF355000.1), *H. comes* Philippines (AY277289.1), *H. kuda* India (FJ541067.1), *H. kuda* South Africa (AY277289.1), *H. kuda* Thailand (DQ452301.1), *H. kuda* Philippines (AY277300.1), and *H. kuda* Japan (AP005985.1). The accession numbers of nucleotides sequences for seahorse COI genes are as follows: *H. comes*

Lampung Indonesia (GQ502134.1), *H. comes* Philippines (GQ502133.1), *H. comes* Taiwan (JX970973.1), *H. kuda* India (FJ176592.1), *H. kuda* South Africa (GU805017.1), *H. kuda* Vietnam (FJ583553.1), *H. kuda* Taiwan (GQ502153.1), and *H. kuda* Japan (AP005985.1). The species of genus *Syngnathus*, namely, *S. typhale* (NC 03279.1) and *S. schlegeli* (KJ184526.1), were used as the outgroup for both 16S rRNA and COI genes.

RESULTS AND DISCUSSION

Morphology identification

One specimen of seahorse from the Riau Islands was identified to be *H. kuda*, while the two from the same place were identified to be *H. comes* (Table 1). The three seahorse specimens from Lampung were identified to be *H. comes*. The morphological characteristics of *H. comes*, and *H. kuda* are very similar, namely, having 11 units of body rings, 34-37 units of tail rings, 1-2 units of body rings, and tail rings on the dorsal fins, and a maximum length of 18.7 cm. The only difference between *H. comes*, and *H. kuda* is the number of cheek spines. *H. comes* to have two units of cheekbones, while *H. kuda* has only one (Lourie et al. 2004). Our research group has previously reported that, based on the morphological characteristics by Lourie et al. (2004), the seahorses from the waters of Sebong Bay, Riau Islands, were identified to be *H. comes* (Nurhayati 2018). The similarity of *H. comes*, and *H. kuda* can cause errors in morphological species identification, especially in the case of juveniles, since the morphological features would generally become apparent through growth. Furthermore, the confirmation and validation using molecular techniques such as DNA barcoding are very useful to identify the species, irrespective of body size, and the part of the body.

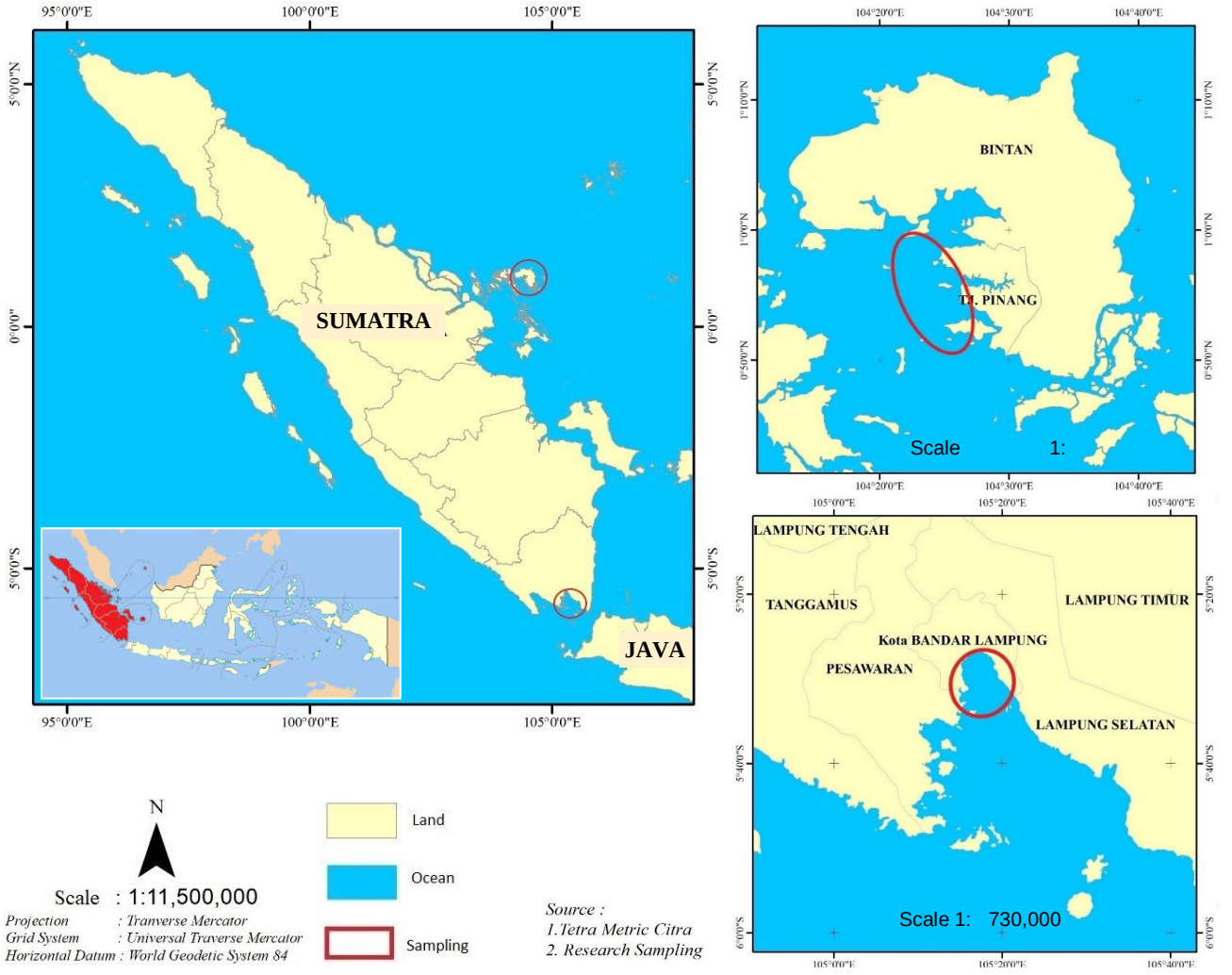

Figure 1. Sampling locations of *Hippocampus* spp. in the waters of Senggarang District, Tanjung Pinang City, Riau Islands Province (*above right*), and waters of Teluk Betung Selatan District, Bandar Lampung City, Lampung Province (*below right*), Sumatra Island, Indonesia (as encircled in the maps)

Table 1. Characteristics of morphology identification on seahorse

Description / Sample	Riau Islands			Lampung		
	HKR*	HCR1	HCR2	HCL1	HCL2	HCL3
Height (cm)	16	13	11	13	15	11
Trunk rings (unit)	11	11	11	11	11	11
Tail rings (unit)	37	36	35	36	37	34
Cheek spines (unit)	1	2	2	2	2	2
Average length of nose and head (cm)	2	2	2	2	2	2
Body rings on dorsal side (unit)	2	1	2	2	2	1
Tail rings on dorsal side (unit)	2	2	2	1	2	2
Species	*H. kuda*	*H. comes*	*H. comes*	*H. comes*	*H. comes*	*H. comes*

Note: *HKR: *Hippocampus kuda* from Riau; HCR: *H. comes* from Riau; HCL: *H. comes* from Lampung

DNA amplification

The DNA amplicons of the 16S rRNA gene consisted of a single band with a size of approximately 650 bp (Figure 2.A). The DNA amplicons of the COI gene also gave a single band with a size of approximately 700 bp (Figure 2.B).

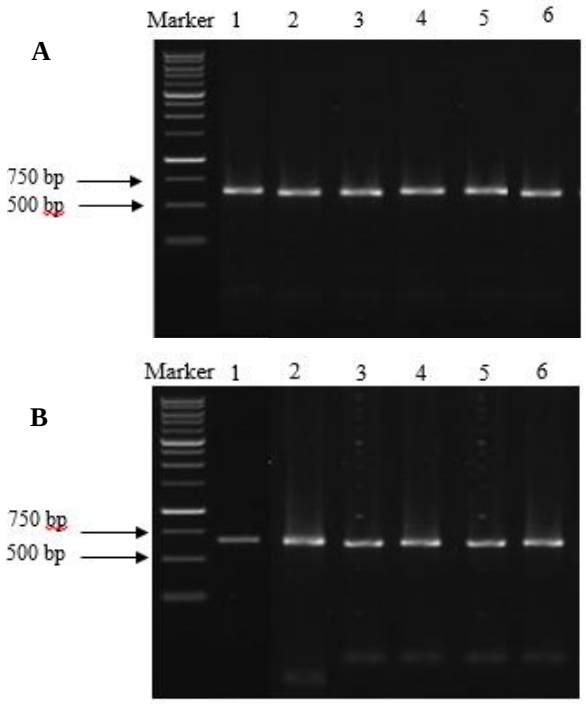

Figure 2. DNA amplification of seahorse based on (A) 16S rRNA gene and (B) COI gene. Lanes 1-3: *Hippocampus* spp. from Riau; lanes 4-6: *Hippocampus* spp. from Lampung, Sumatra, Indonesia.

Sequencing of 16S rRNA and COI gene fragments

The results of sequence analysis using BLASTn based on the 16S rRNA gene showed that two specimens from Riau Islands (HCR1 and HCR2) and three from Lampung (HCL1, HCL2, HCL3) were identical to *H. comes* (AF355000.1) with the probability of 99.6%. One individual from Riau Islands (HKR) was identical to *H. kuda* (FJ541067.1), with a probability of 99.8%.

The results based on the COI gene showed that the two individuals (HCR1 and HCR2) from the Riau Islands and three from Lampung (HCL1, HCL2, HCL3) were 99.82 - 100% identical to *H. comes*. One individual from the Riau Islands (HKR) was 99.13% identical to *H. kuda*. Identity values of 97-100% strongly suggest that the species being compared are identical (Bhattacharjee et al. 2012).

Multiple alignment and variation

The nucleotide lengths of the 16S rRNA gene in *H. comes*, *H. kuda*, and the genus *Syngnathus* as an outgroup were 484 bp from the multiple alignment results. Based on the 16S rRNA gene in *H. comes* from Riau Islands and Lampung, a 100% conservative site (484/484) was obtained (Figure 3). The nucleotide lengths of the COI gene in *H. comes*, *H. kuda*, and the genus *Syngnathus* as an outgroup was 567 bp. Based on the COI gene, *H. comes* from Riau Islands, and Lampung were 99.82% (566/567) of conservative sites, 0.18% (1/567) of variable sites, 0.18% (1/567) of substituted, and 0.18% (1/567) of a singleton. Based on the 16S rRNA gene, *H. comes* from Riau Islands, and Lampung was found to be the same haplotype, while the haplotype of *H. kuda* could not be identified due to the limited number of sample.

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HCR1 CGGCACTGCACTCAGCCTATTAATTCGAGCAGAACTAAGTCAGCCAGGAGCTTTACTAGGGGATGATCA 69
HCR2 CGGCACTGCACTCAGCCTATTAATTCGAGCAGAACTAAGTCAGCCAGGAGCTTTACTAGGGGATGATCA 69
HCL1 CGGCACTGCACTCAGCCTATTAATTCGAGCAGAACTAAGTCAGCCAGGAGCTTTACTAGGGGATGATCA 69
HCL2 CGGCACTGCACTCAGCCTATTAATTCGAGCAGAACTAAGTCAGCCAGGAGCTTTACTAGGAGATGATCA 69
HCL3 CGGCACTGCACTCAGCCTATTAATTCGAGCAGAACTAAGTCAGCCAGGAGCTTTACTAGGGGATGATCA 69
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Figure 3. The alignment of nucleotide sequences from the COI gene in *Hippocampus comes* from Riau and Lampung, Sumatra, Indonesia using ClustalW. Different types of haplotypes are shown with a highlighted base. HCR: *H. comes* from Riau; HCL: *H. comes* from Lampung

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HCR1  RHCTQPINSSRTKSARSFTRG*SNL*CYRNCPCFRNNFFYSNTNYDRRFW*LISSFNNWSA*YSLPSDKQY 69
HCR2  RHCTQPINSSRTKSARSFTRG*SNL*CYRNCPCFRNNFFYSNTNYDRRFW*LISSFNNWSA*YSLPSDKQY 69
HCL1  RHCTQPINSSRTKSARSFTRG*SNL*CYRNCPCFRNNFFYSNTNYDRRFW*LISSFNNWSA*YSLPSDKQY 69
HCL2  RHCTQPINSSRTKSARSFTRR*SNL*CYRNCPCFRNNFFYSNTNYDRRFW*LISSFNNWSA*YSLPSDKQY 69
HCL3  RHCTQPINSSRTKSARSFTRG*SNL*CYRNCPCFRNNFFYSNTNYDRRFW*LISSFNNWSA*YSLPSDKQY 69
```

Figure 4. Amino acid sequence alignment of the COI gene in *Hippocampus comes* from waters of Riau and Lampung, Sumatra Island, Indonesia using ClustalW. Amino acid residues in the different haplotypes are contained in the box. HCR: *H. comes* from Riau; HCL: *H. comes* from Lampung

Table 2. Analysis of the haplotypes in *Hippocampus comes* based on the COI gene

Samples	Haplotype
Riau	
HCR1	1
HCR2	1
Lampung	
HCL1	1
HCL2	2
HCL3	1

Note: HCR: *H. comes* Riau; HCL: *H. comes* Lampung, Sumatra Island, Indonesia

In *H. comes*, a mutation occurred at the nucleotide of the 61st position, namely, the substitution of G to A (Figure 4). This substitution seems to cause the nonsynonymous mutation of amino acid from glycine to arginine at the 21st position of the protein (Figure 5). The nonsynonymous mutation of amino acid is caused by the nucleotides changing (Page and Holmes 1998).

Based on the COI gene, *H. comes* from Riau (n=2) were found to have the haplotype 1 (n=2), while *H. comes* from Lampung (n=3) were found to have both the haplotype 1 (n=2) and haplotype 2 (n=1) (Table 2). One specimen of *H. comes* from Lampung turned out to be a specific haplotype. Therefore, this study showed that there was a difference between *H. comes* in the two localities (Riau and Lampung), and HCL2 (*H. comes* from Lampung) was different from the others. Therefore, the COI gene is useful to detect the variations at the inter- and intragenetic levels.

The two specimens of *H. comes* from Riau Islands, and two *H. comes* from Lampung shared the same haplotype (haplotype sharing). The phenomenon of haplotype sharing in the two different water can be caused by colonization. Seahorses use their tails to hook on to sea plants and other objects that are carried by the currents (rafting), so that the remote individuals can be colonized (Kuiter 2000). The phenomenon of colonization in *H. comes* thought to be due to rafting. Therefore, the dispersion of juvenile seahorses can be influenced by hydrodynamic activity. The current patterns in Indonesian waters could have caused by the West monsoon from the Asian Continent to Australia and by the East monsoon to the reverse direction (Daruwedho et al. 2016).

Genetic distance

Analysis of the COI gene showed *H. comes* population from Lampung had a genetic distance value (p) of 0.000 - 0.002 (Table 3). The population of *H. comes* from Riau Islands, and Lampung gave a value of 0.000 - 0.002. *H. comes* from Riau Islands, Lampung, Taiwan, and the Philippines showed the average value of 0.002. The COI genes of p-value less than 0.020 (2%) proved that one species is closely related (Ratnasingham and Hebert 2013). Analysis of the 16S rRNA gene showed that *H. comes* from Riau Islands, Lampung, Philippines, and Taiwan showed an average value of 0.0005 (Table 4). Based on the p values of the 16S rRNA gene less than 0.015 (1.5%), one species in the genus *Hippocampus* was closely related (Singh et al. 2011).

Analysis of *H. kuda* based on the 16S rRNA gene showed that the specimens from the Riau Islands and India gave the smallest p-value of 0.002. Analysis of the COI gene showed that *H. kuda* from Riau Islands and India had the smallest value of 0.006. The smaller the p-value, the closer the kinship between species. Analysis of 16S rRNA genes showed that *H. kuda* and *H. comes* from Riau Islands, and Lampung had an average p-value of 0.064. Based on the COI gene, *H. kuda* and *H. comes* from Riau Islands and Lampung had an average value of 0.115. A p-value higher than 0.03 (3%) was sufficient to prove the occurrence of species separation (Hebert et al. 2003). The largest p-value of 0.174 was obtained for *H. kuda*, *H. comes*, and the genus *Syngnathus* based on the 16S rRNA gene (HC Taiwan with *S. typhale*). The largest p-value obtained for *H. kuda*, *H. comes*, and the genus *Syngnathus* based on the COI gene was 0.224 (among all the samples of *H. comes* and *S. typhale*).

Phylogenetic reconstruction

The phylogenetic analysis based on 16S rRNA and COI genes with the genus *Syngnathus* as outgroups showed a clear separation between *H. comes* and *H. kuda*. Based on the 16S rRNA gene, all *H. comes* specimens belonged to one large clade, supported by a bootstrap value of 100 (Figure 6). If the bootstrap value is between 95-100%, it can be concluded that the branching is highly reliable (Ubaidilah and Sutrisno 2009). Phylogenetic analysis on *H. comes* based on the 16S rRNA gene by Nurhayati (2018) gave the same results. Based on the COI gene, all *H. comes* specimens were included in one large clade, supported by a bootstrap value of 100 (Figure 6). In the one large clade of *H. comes*, there were also small clades. *H. comes* from Lampung (HCL2 and HC Lampung) were in a small clade. *H. comes* from Riau Islands (HCR1, HCR2), and Lampung (HCL1, HCL3) were in another small clade. The small clade in *H. comes* caused by the differences in genetic sequence distance.

Table 3. Genetic distances of *Hippocampus comes*, *H. kuda*, and genus *Syngnathus* based on the COI gene

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14
HCR1														
HCR2	0.000													
HCL1	0.000	0.000												
HCL2	0.002	0.002	0.002											
HCL3	0.000	0.000	0.000	0.000	0.002									
HC Taiwan (JX970973.1)	0.004	0.004	0.004	0.005	0.004									
HC Philippines (GQ502133.1)	0.002	0.002	0.002	0.004	0.002	0.005								
HKR	0.114	0.114	0.114	0.115	0.114	0.114	0.115							
HK India (FJ176592.1)	0.119	0.119	0.119	0.121	0.119	0.119	0.121	0.009						
HK Taiwan (GQ502153.1)	0.124	0.124	0.124	0.126	0.124	0.126	0.126	0.025	0.023					
HKVietnam (FJ583553.1)	0.124	0.124	0.124	0.126	0.124	0.126	0.126	0.032	0.027	0.011				
HK Japan (AP005985.1)	0.126	0.126	0.126	0.128	0.126	0.128	0.128	0.025	0.020	0.004	0.007			
HK South Africa (GU805017.1)	0.121	0.121	0.121	0.123	0.121	0.121	0.123	0.014	0.009	0.021	0.025	0.018		
Syngnathus schlegeli (KJ184526.1)	0.199	0.199	0.199	0.199	0.199	0.197	0.197	0.188	0.190	0.192	0.190	0.190	0.188	
S. typhle (NC_030279 .1)	0.224	0.224	0.224	0.224	0.224	0.224	0.222	0.218	0.215	0.222	0.220	0.220	0.217	0.135

Table 4. Genetic distances of *Hippocampus comes*, *H. kuda*, and genus *Syngnathus* based on the 16S rRNA gene

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14
HCR1														
HCR2	0.000													
HCL1	0.000	0.000												
HCL2	0.000	0.000	0.000											
HCL3	0.000	0.000	0.000	0.000										
HC Philippines (AY277289.1)	0.000	0.000	0.000	0.000	0.000									
HC Taiwan (AF355000.1)	0.002	0.002	0.002	0.002	0.002	0.002								
HKR	0.064	0.064	0.064	0.064	0.064	0.064	0.066							
HK India (FJ541067.1)	0.062	0.062	0.062	0.062	0.062	0.062	0.064	0.002						
HK South Africa (AY277299.1)	0.064	0.064	0.064	0.064	0.064	0.064	0.066	0.004	0.002					
HK Philippines (AY277300.1)	0.062	0.062	0.062	0.062	0.062	0.062	0.064	0.006	0.004	0.006				
HK Thailand (DQ452301.1)	0.064	0.064	0.064	0.064	0.064	0.064	0.066	0.009	0.006	0.009	0.002			
HK Japan (AP005985.1)	0.062	0.062	0.062	0.062	0.062	0.062	0.064	0.006	0.004	0.006	0.000	0.002		
Syngnathus typhle (NC03279.1)	0.172	0.172	0.172	0.172	0.172	0.172	0.174	0.170	0.168	0.170	0.166	0.168	0.166	
Syngnathus schlegeli (KJ184526.1)	0.164	0.164	0.164	0.164	0.164	0.164	0.166	0.155	0.153	0.155	0.151	0.153	0.151	0.057

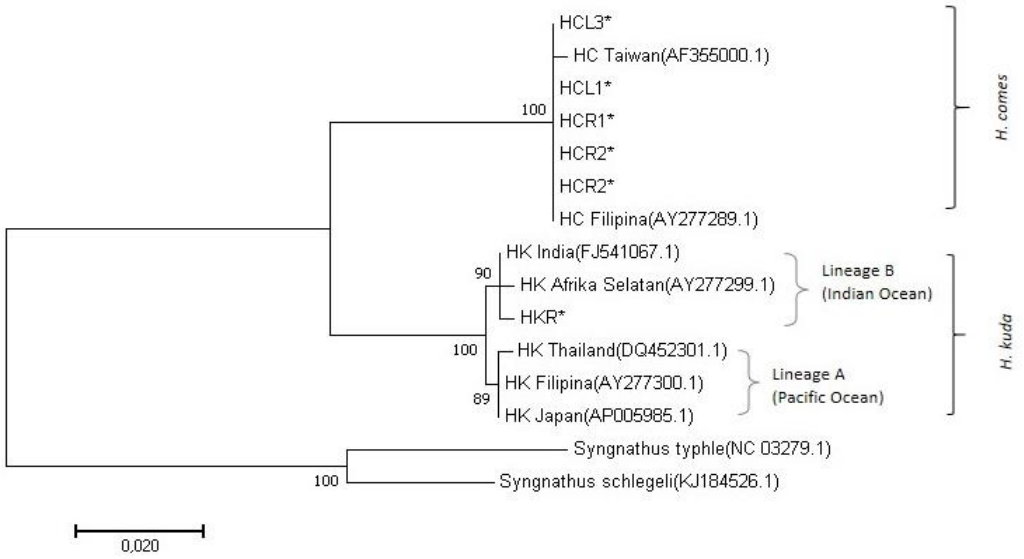

Figure 5. The phylogenic tree of *Hippocampus comes*, *H. kuda* and the genus *Syngnathus* based on 16S rRNA gene made by the neighbor-joining method with 1000 times bootstrap. Lineages A and B were determined, according to Teske et al. (2005).

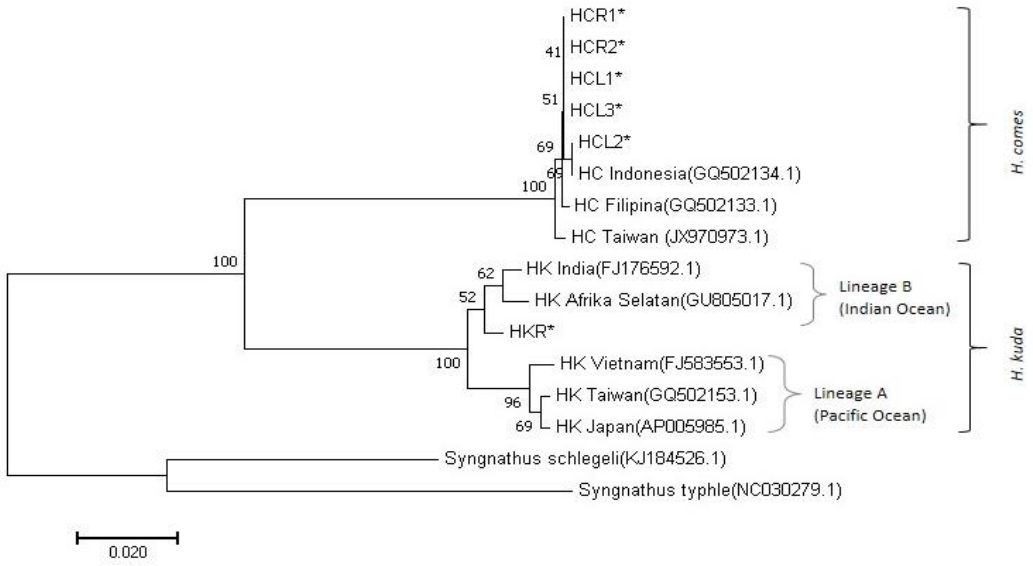

Figure 6. The phylogenic tree of *Hippocampus comes*, *H. kuda* and the genus *Syngnathus* based on the COI gene made by the neighbor-joining method with 1000 times bootstrap. Lineages A and B were determined according to Teske et al. (2005)

Phylogenetic analysis based on 16S rRNA and COI genes in *H. kuda* resulted in the separation into two groups, supported by a bootstrap value of 100. Analysis of the mtDNA control region from *H. kuda* by Teske et al. (2005) showed the same result. *H. kuda* was found to be divided into the lineage A (Pacific Ocean) and the lineage B (Indian Ocean). Based on the 16S rRNA gene, *H. kuda* from Riau Islands was in a clade with *H. kuda* from India and South Africa. Based on the COI gene, *H. kuda* from Riau Islands belonged to one clade with *H. kuda* from India and South Africa. Based on the mtDNA control region, *H. kuda* from Indonesian waters were found to be in the clade with *H. kuda* from India and South Africa (lineage B) (Teske et al. 2005). This suggests that, based on the 16S rRNA and COI genes, *H. kuda* from Riau Islands belong to lineage B (Indian Ocean).

In conclusion, both morphological and molecular analyses using 16S rRNA and COI genes showed that the seahorses from Riau Island were identified to be *H. comes* (n=2) and *H. kuda* (n=1) and those from Lampung were *H. comes* (n=3). Molecular analysis is essential to explore the haplotype diversity and their relationships. The COI gene could provide distinguished haplotypes of *H. comes* from both water. Phylogenetic analyses of *H. comes* from both water showed that all the specimens belonged to the same clade. These findings would contribute to the preservation and utilization of Indonesian seahorse.

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