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Molecular marker based on 16S rRNA gene for seahorse (*Hippocampus* spp.) from Bintan Island-Indonesia

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Abstract. Seahorse is known as the organism with different reproduction mechanism from other organisms showing male pregnancy. Phenomenon of cryptic species and complex species often lead to morphologically misidentification. DNA barcoding is commonly used as a tool for molecular identification. From 33 types of seahorses in Indonesia, 7 of them were found in Bintan waters. Thus, the objective of this study was to identify the species of seahorse by molecular marker based on 16S rRNA gene and determine the genetic relationship of seahorse from Bintan Island,Indonesia. The specimens were collected from Berakit,Bintan. DNA was extracted, then amplification of 16S rRNA gene fragment was carried out. The results showed that the sequence of amplified DNA of seahorse were *Hippocampus comes* has a high degree of kinship with *H. comes* originating from Taiwan.

Keywords: 16S rRNA gene, *Hippocampus comes*, seahorse

1. Introduction

Seahorses (*Hippocampus* spp.) comprise a genus within the Syngnathidae family that is well-known for its particular and unique body shapes [1]. They have a horse-shaped head, unique swimming manner and a different reproduction mechanism from other organisms, i.e. the female deposits eggs into the male's brood pouch [2] (figure 1). Seahorses are carnivorous and consume zooplankton such as copepods, amphipods dan mysids [3]. There are 33 known species of seahorses along the coastal areas of the world [4]. They can be found on tropical or subtropical waters, distributed worldwide along 50 ^{o}E to 50 ^{o}S, and may be found on seagrass beds, mangrove, coral reefs, estuaries or another muddy areas within less than 10 meters depth.

Unfortunately, seahorses also have a high economic value, as they are traded for traditional Chinese medicine (TCM), souvenirs, and kept as pets in aquariums, with the number of traded seahorses on global market are reaching 20 million every year [5]. Generally, seahorses are sold dried as traditional medicine raw materials, a trade that has lasted for 600 years [6]. This organism is believed to increase and balance energy flow in the body and also heal various illness, such as impotencies and infertilities, asthma, cholesterols, mumps, kidney failures and skin disease [7].

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The dramatic loss of seahorse populations has made them protected. The International Union for Conservation of Nature and Natural Resources (IUCN) Red List [8] stated that two of the species of seahorse is endangered, while 12 species are vulnerable, one species is near threatened, 12 species are least concern, and 17 other species are data deficient. All species of *Hippocampus* are included into the Appendix II of the *Convention on International Trade in Endangered Species of Wild Fauna and Flora* (CITES). In the attempts to preserve the existence of seahorses, CITES undertakes the rule that seahorses might be caught and traded with a minimum height of 10 cm, except for *Hippocampus kelloggi* as its gonads mature at >10 cm in size [9]. This rule was implemented so that seahorses had enough time to grow and reproduce before being captured and traded [10].

Fishermen in Indonesia distinguish types of seahorses visually based on color, like red, yellow, striped yellow, brown, black and striped black. Seahorse identification can not be done visually only, because it is very similar among species. Seahorse in Indonesia usually some are caught by hand, some in beach seines, and others by push-nets or scoop nets. In Indonesia, seahorses are used as traditional medicines called *jamu*. This medicine is believed for recovering all kinds of weaknesses such as impotence, loss of energy, loss of blood, pallor, loss of memory, rheumatism, and lung congestion. From 33 types of seahorses in Indonesia, 7 of them were found in Bintan waters, they are *Hippocampus barbouri*, *H. comes*, *H. histology*, *H. kellogi, H. kuda*, *H. spinosissimus* and *H. trimaculatus* [11]. *Hippocampus comes* rarely found in Berakit Cape, and is more commonly found in Bintan waters in the region of Senggarang, Sasah Bay, Sakera and Sebong Pereh.

The high level of exports of seahorses for traditional medicines causes fishermen to do various ways to catch seahorses, including using destructive methods. This event triggers a decrease in the number of seahorses in nature throughout the year. The increasing demand for seahorses will have an impact on large-scale exploitation which causes habitat degradation and even causes extinction of several species of seahorses. This means that international trade in these commodities must meet regulations that can guarantee their use will not threaten the preservation of seahorses in nature. Through DNA barcoding techniques, it is expected that taxon certainty, kinship, and tracing of species origin can be known accurately before the extinction of seahorses. Molecular markers are the right application for achieving this goal, and in order to become the basis of information in an effort to determine the management of seahorses.

Accurate taxonomic identification is the first step in conservation and management and can benefit from the assistance of molecular tools commonly used nowadays in identification. Morphological similarities in aquatic animals are a common phenomenon that causes misidentification. When the DNA barcode of taxonomically vouchered species are available in databases, molecular markers can assist indetermining the taxon of such species, using a faster and accessible method [12].

DNA barcoding is a method designated to assist rapid and accurate species identification using short gene regions [12]. Data generated from DNA barcode sequences might be used as a guideline in the arrangement of phylogenetic trees [13]. Nowadays, molecular-based identification is highly developed. Our previous research showed the result of DNA barcoding of sea horse from Bintan waters using COI gene markers were *Hippocampus comes* and *Hippocampus kuda* with identify value of around 98-99%. Another gene, namely 16S rRNA is reported a high degree of sensitivity, functions that do not change over time, so that random sequence changes are more accurate in an evolution [14]. The 16S rRNA gene is a highly conserved gene [15]. Thus, the objective in this study was to identify the species of seahorse based on 16S rRNA gene and determine the genetic relationship of seahorse from Bintan Island-Indonesia.

2. Materials and Methods

2.1. Sample collection

Five seahorses were obtained (Figure 1) from the genus *Hippocampus* along Bintan coastal waters, Riau Island, with the coordinate of 1°5'0"N−1°20'0"N and 104°25'0"E−104°40'0"E (figure 2), then the

samples were collected and kept inside a cool box for the transportation and stored in -20°C until used.

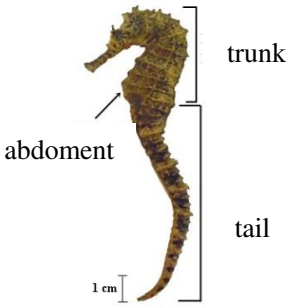

Figure 1. Seahorse (*Hippocampus* spp.).

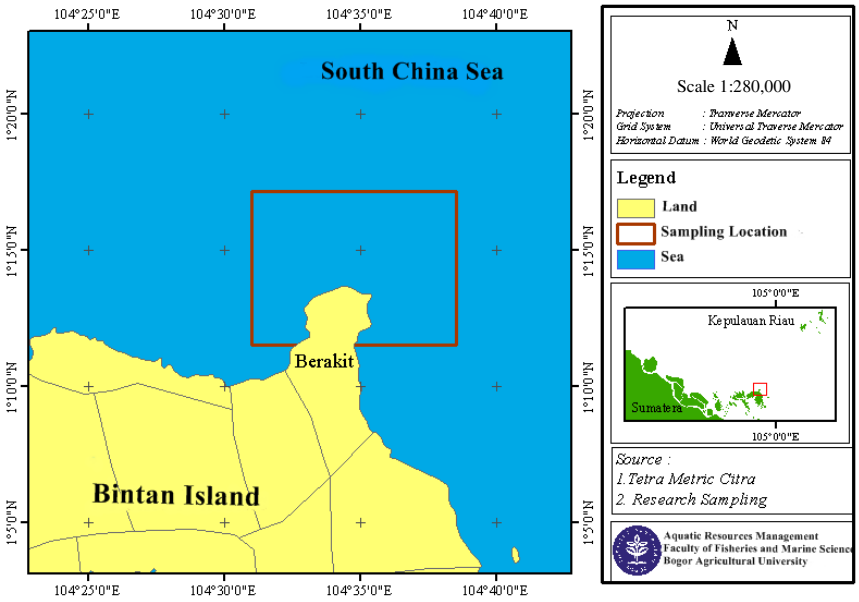

Figure 2. The location for sample collection of sea horses.

2.2. Morphological identification

The identification of five samples were done by matching the physical characteristics of seahorses to the guidelines for seahorse identification by [4]. The characteristic that was observed included meristic and morphometric characters, to get the taxonomy classification of seahorse to species level. Morphological identification was obtained from weight and length measurements, number of trunk rings, number of tail rings, eye spine, cheek spine, average of snout and head length, number of dorsal-fin rays and number of pectoral fins rays.

2.3. Molecular identification

Molecular identification of the sampels of seahorses were started from DNA extraction, DNA quality testing, DNA amplification, by PCR. Finally the PCR products were sent to a service company to obtain the sequence.

DNA extraction was done using a commercial kit (Qiagen) according to manufacturer's protocol with small modifications. DNA quality testing was done through electrophoresis (voltage of 100 volt for 23 minutes) using a 1.2% agarose gel based on [16]. The total DNA loaded was 3 µL and the presence of total DNA confirmed using an ultraviolet machine (UV). Confirmed quality DNA was e used as a DNA template for the amplification and visualization of DNA barcode fragments. DNA amplification

was carried out by using Polymerase Chain Reaction (PCR) using a Kapa Extra Hot Start commercial kit. The primers used were designed by [17] Butet (2013, unpublished), particularly the primary 16S rRNA which was a universal primer that could be used in some aquatic biotas.

DNA amplification included 35 cycles of predenaturation at 94°C for 5 minutes, denaturation at 94°C for 45 seconds, annealing or primary attachment at 53 °C for 1 minute, elongation at 72°C for 1 minute and post-elongation at 72°C for 5 minutes. Then, the PCR products were stored for 10 minutes at 15°C. This PCR protocol was based on [18] with several temperatures and time modifications. The quality of amplified DNA was checked using a 1.2% agarose gel and visualized using an ultraviolet (UV) machine. The PCR products were sent for sequencing [19] to a sequencing service company (First Base-Malaysia).

2.4. Phlyogenetic analysis

Bioinformatic analysis in this study was performed using MEGA 5.0 software. The analysis consisted of nucleotide base sequences validations, nucleotide sequence alignments, genetic distance estimations, and phylogeny tree construction. The alignment of nucleotide sequences from sequencing results was carried out using the Clustal W method in MEGA 5.0 software [20] to analyze genetic distances and phylogeny trees. The nucleotide sequences the of 16S rRNA gene with forward and reverse primers were edited and analyzed and the genetic distance of the 16S rRNA gene sequences was analyzed using pairwise distance matrix method in MEGA 5.0 software [20]. The results were exhibited in the form of a data matrix used for analysis of interspecies kinship relationships based on the phylogeny tree formed. Phylogenetic analysis was also carried out based on a nucleotide substitution method with genetic distance and phylogeny tree construction using thebootstrapped Neighbors-Joining (NJ) method with 1000 repetitions.

3. Results

3.1. Taxonomy based on morphology

Morphologically seahorses have heads positioned at right angles to their bodies, curved trunks, pointy snouts, a long grasping tail (*prehensile*), and no tail fin [4]. Unlike fish in general, seahorses have no scales, their bodies is wrapped by bony plates or rings that function as outer skeleton. Table 1 shows the meristic and morphometric characteristics of seahorse (*Hippocampus* spp.).

Table 2. Meristic and morphometric characteristic of seahorse (*Hippocampus* spp.).

Description	Average	Unit
Weight	7.67 ± 329	gram
Length	13.4 ± 1.61	cm
Trunk rings	11 ± 0.58	unit
Tail rings	34 ± 2.16	unit
Eye spines	1 ± 0.00	unit
Cheek spines	2 ± 0.00	unit
Head and snout length	2.03 ± 0.03	cm
Dorsal fin rays	2 ± 0.00	unit
Pectoral fin rays	1 ± 0.00	unit

The results of morphometric identification on the sample used in this study shows a species of *Hippocampus comes*. These results are known by looking at the morphological features of *H. comes*, such as double cheek spines, protruding spines of the eye, slender muzzle, blunt dorsal spine, low crown, no tail spines. The color or pattern of seahorses is that there are spots or patches of pattern on the body, has fine white lines on the eyes; there is a reddish yellow on the tail and forms a line. Thus, the identification results indicate that the seahorse observed is *Hippocampus comes* morphologically.

3.2. Amplification and visualization of 16S rRNA gene fragment
Amplification of the 16S rRNA gene from DNA extracted from the samples gave positive results using an annealing temperature of 46 °C. The length of the DNA sequences obtained ranged between 500–600 bp (figure 3).

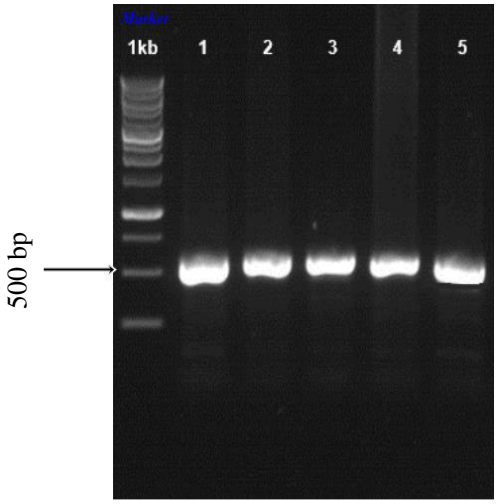

Figure 3. Electrophoresis results of 16S rRNA gene amplification (*Hippocampus* spp.) in 0.8% agarose gel. From left to right coloumn: marker, *Hippocampus* spp. sample (1-5).

3.3. DNA sequencing and alignment of the 16S rRNA gene nucleotide sequences of Hippocampus spp.
The results of validation using BLASTn showed that the five examples of seahorses taken from Bintan coastal waters, Riau Islands had a high degree of kinship (99%) with *Hippocampus comes* which had been registered in Genbank with accession number JX970974.1. Proximity between seahorses from Bintan waters with other genera such as *Hippocampus whitei*, *Hippocampus barbouri*, *Hippocampus histrix* and *Hippocampus mohnikei* each ranged between 94-97%. The nucleotide base sequence of 16S rRNA gene from the five *Hippocampus comes* was then aligned and edited using MEGA 5.0 software [20].

3.4. Genetic distance and philogeny tree 16S rRNA seahorses from Bintan coastal waters
Genetic distance describes the kinship distance between intraspecies and interspecies. The genetic distance between the genus *Hippocampus* and *Syngnathus* ranged between 0,000-0,183, the largest genetic distance value was between *H. sindonis* (NC 035827.1) and *S. typhle* (NC 030279.1) of 0.183. The value of the genetic distance between seahorse species *H. comes* with other seahorses of *Hippocampus* genera ranges from 0.000-0.093. *H. comes* from Bintan coastal waters had genetic distance around 0.000-0.004 with *H. comes* (JX970974.1) from Taiwan. The genetic distance matrix of the 16S rRNA gene fragment on *H. comes*, *Hippocampus*, *Syngnathus* spp. is presented in table 2.

Genetic distance values were used as data sources for phylogeny trees construction (figure 4). Ahylogenetic tree was used to analyze the kinship relationship of several species in the *Syngnathidae* family based on the 16S rRNA gene and produced two main clusters that separated he genus *Hippocampus* and *Syngnathus*. Based on the phylogeny tree, in the *Hippocampus* genera, the species of *Hippocampus comes* formed a subcluster that separated from other *Hippocampus* species.

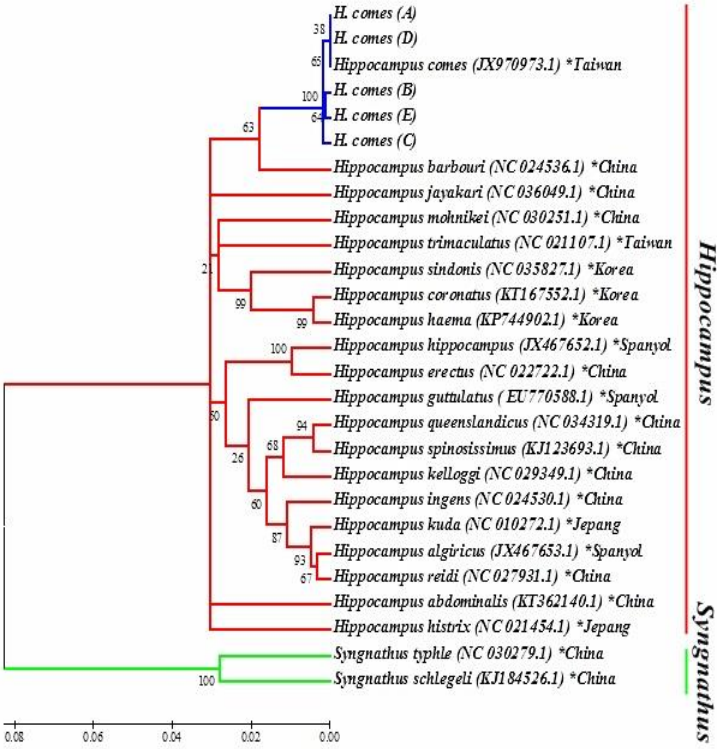

Figure 4. Phylogeny trees construction based on the 16S rRNA gene from several *Hippocampus* and *Syngnathus* species.

4. Discussion

Based on the comparison of meristic and morphometric characteristics with identification guide [4], we identified morphologically that seahorses observed were *Hippocampus comes*. It could be seen from the body that was yellow with a black patterned tail, double cheekbones, pointy nose bone, low crown (coronet) with five spines. However, morphological identification has a weakness in classifying species. In addition, seahorses have the ability to camouflage and experience the phenomenon of cryptic and complex species, so that misidentification often occur in morphological identification. Therefore, a more accurate identification technique is needed, for instance, molecular identification.

Molecular identification of the seahorses samples from Bintan coastal waters using 16S rRNA gene provides information about the certainty of this observed seahorse species. Validation results with Basic Local Alignment Search Tool-nucleotide (BLASTn) showed that seahorses from Bintan coastal waters have a 99% similarity with *Hippocampus comes* in GenBank with accession number JX970973.1. This proved that the seahorses samples in this study came from the same genetic source and was classified as *Hippocampus comes*.

Table 2. Genetic distance based on 16S rRNA between selected species of *Hippocampus* and *Syngnathus*.

Spesies	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
S. typhle (NC 030279.1)																										
S. schlegeli (KJ184526.1)	0.056																									
H. comes (JX970973.1)	0.170	0.170																								
H. coronatus (KT167552.1)	0.175	0.162	0.075																							
H. haema (KP744902.1)	0.179	0.162	0.073	0.009																						
H. algiricus (JX467653.1)	0.164	0.157	0.063	0.093	0.088																					
H. hippocampus (JX467652.1)	0.181	0.170	0.069	0.091	0.084	0.058																				
H. abdominalis (KT362140.1)	0.170	0.155	0.073	0.075	0.071	0.075	0.065																			
H. erectus (NC 022722.1)	0.177	0.166	0.071	0.088	0.082	0.060	0.019	0.067																		
H. trimaculatus (NC 021107.1)	0.172	0.170	0.052	0.067	0.063	0.071	0.075	0.060	0.078																	
H. histrix (NC 021454.1)	0.153	0.144	0.047	0.071	0.069	0.073	0.075	0.058	0.069	0.065																
H. ingens (NC 024530.1)	0.159	0.153	0.058	0.082	0.078	0.024	0.056	0.069	0.058	0.060	0.063															
H. barbouri (NC 024536.1)	0.168	0.164	0.034	0.080	0.078	0.067	0.069	0.069	0.067	0.056	0.047	0.056														
H. reidi (NC 027931.1)	0.162	0.155	0.060	0.091	0.086	0.006	0.052	0.073	0.054	0.069	0.071	0.017	0.060													
H. kelloggi (NC 029349.1)	0.168	0.159	0.054	0.082	0.075	0.039	0.056	0.071	0.058	0.067	0.054	0.041	0.054	0.037												
H. mohnikei (NC 030251.1)	0.168	0.159	0.047	0.065	0.060	0.063	0.071	0.056	0.071	0.045	0.060	0.052	0.045	0.060	0.058											
H. queenslandicus (NC 034319.1)	0.155	0.153	0.050	0.075	0.069	0.032	0.045	0.058	0.047	0.058	0.058	0.030	0.047	0.026	0.024	0.052										
H. sindonis (NC 035827.1)	0.183	0.170	0.071	0.039	0.041	0.088	0.082	0.067	0.084	0.060	0.067	0.078	0.075	0.086	0.078	0.056	0.071									
H. jayakari (NC 036049.1)	0.164	0.164	0.060	0.073	0.069	0.067	0.071	0.078	0.069	0.060	0.065	0.050	0.058	0.060	0.058	0.052	0.047	0.067								
H. spinosissimus (KJ123693.1)	0.164	0.159	0.052	0.080	0.073	0.032	0.045	0.065	0.047	0.060	0.060	0.030	0.050	0.026	0.024	0.056	0.009	0.075	0.052							
H. kuda (NC 010272.1)	0.159	0.151	0.060	0.091	0.084	0.013	0.052	0.080	0.054	0.075	0.071	0.024	0.065	0.006	0.034	0.063	0.028	0.086	0.058	0.028						
H. guttulatus (EU770588.1)	0.168	0.162	0.043	0.091	0.086	0.043	0.050	0.065	0.056	0.063	0.069	0.047	0.058	0.041	0.041	0.060	0.034	0.082	0.069	0.034	0.047					
H. comes (A)	0.170	0.170	0.000	0.075	0.073	0.063	0.069	0.073	0.071	0.052	0.047	0.058	0.034	0.060	0.054	0.047	0.050	0.071	0.060	0.052	0.060	0.043				
H. comes (B)	0.175	0.175	0.004	0.080	0.078	0.067	0.073	0.073	0.075	0.056	0.052	0.063	0.039	0.065	0.058	0.052	0.054	0.075	0.065	0.056	0.065	0.047	0.004			
H. comes (C)	0.168	0.168	0.002	0.073	0.071	0.060	0.067	0.071	0.069	0.050	0.045	0.056	0.037	0.058	0.052	0.050	0.047	0.069	0.058	0.050	0.058	0.041	0.002	0.006		
H. comes (D)	0.170	0.170	0.000	0.075	0.073	0.063	0.069	0.073	0.071	0.052	0.047	0.058	0.034	0.060	0.054	0.047	0.050	0.071	0.060	0.052	0.060	0.043	0.000	0.004	0.002	
H. comes (E)	0.172	0.172	0.002	0.078	0.075	0.065	0.071	0.075	0.073	0.054	0.050	0.060	0.037	0.063	0.056	0.050	0.052	0.073	0.063	0.054	0.063	0.045	0.002	0.002	0.004	0.002

The results of genetic distance analysis and construction of phylogeny trees with the 16S rRNA gene showed that the seahorse species had a high diversity, especially in the *Hippocampus genus*. This can be seen from the formation of two clusters, namely monophyletic clusters and paraphyletic clusters. The properties of monophyletic clusters (originating from the same ancestor) were found in the group *H. comes* from Bintan coastal waters, *H. comes* (JX970973.1) and *H. barbouri* (KF712276.1). Members of the monophyletic group are assumed to carry the same genetic and biochemical traits or patterns [22]. The nature of the paraphyletic cluster was found in other groups of species from the genus *Hippocampus*. Paraphyletic groups are groups that are parallel to each other and each group has different origins [23].

The results obtained from this study indicated that there were five polymorphisms in *H. comes* from Bintan coastal waters, which has a high degree of kinship with *H. comes* (JX970973.1) originating from Taiwan. Conserve, variable, and singleton values indicate that short fragments of the 16S rRNA gene can be used as molecular markers that accurately identify a variety of organisms to the species level [24].

Genetic distance values conceivably used to estimate intraspecies and interspecies kinships, and as a basis for phylogeny analysis. This information is useful in understanding the diversity of living things through the construction of kinship relationships. Short genetic distance values indicate a close relationship and vice versa. Genetic distance values that reached 0% showed no differences in nucleotide sequences between samples and outgroups [25]. Genetic distance of 3% or more is sufficient enough to prove the existence of species segregation [26]. This distinction can be used as a foundation for genetic distance analysis and phylogeny tree construction, as well as for the determination of interspecies relationships.

As reported in the previous study, phylogeny analysis is an instrument to infer or estimate evolutionary relationships [27]. In each branch of the phylogeny tree, there is a bootstrap value indicating the consistency or accuracy of a branch [21]. Species that are in the same branch in a phylogeny tree, have a close kinship [28].

Molecular identification studies can have a crucial role in maintaining the sustainability of *H. comes* in nature, as they are capable to provide accurate information as consideration in acts to manage seahorses. It has been known that DNA Barcoding is very effective for molecular phylogenetic studies, geographical distribution, and conservation of the biodiversity of marine ecosystems as well as to determine the authentication of species.

5. Conclusion

Seahorses from Bintan waters are morphologically identified and show that the species is similar to *Hippocampus comes*. This is supported by molecular identification through DNA barcoding techniques with a percentage similarity value of 99% with *Hippocampus comes.*

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References

[1] Wilson A and Orr J 2011 The evolutionary origins of Syngnathidae: pipefishes and seahorses *J. Fish Biol.* **6** 1603–1623
[2] Mulyawan AE and Soekani J 2015 Karakteristik habitat dan kelimpahan kuda laut (*Hippocampus barbouri*) yang terangkap di Kepulauan Tanakeke Kabupaten Takalar *J. Balik Diwa*. **2** 13–19
[3] Foster SJ and Vincent ACJ 2004 Life history and ecology of seahorses: implications for conservation and management *J. Fish. Biol*. **65** 1-61
[4] Lourie SA, Foster SJ, Cooper EW, and Vincent AC 2004 *A Guide to the Identification of Seahorses Project Seahorse and TRAFFIC North America*. University of British Columbia and World Wildlife Fund, Washington DC
[5] Vincent ACJ, Foster SJ and Koldewey HJ. 2011. Conservation and management of seahorses and other Syngnathidae *J. Fish Biol*. **78** 1681–1724
[6] Vincent AC 1996 The international trade in seahorses TRAFFIC International Cambridge, UK. 163 pp.
[7] Sreepada R, Desai U and Naik S 2002 The plight of Indian sea horses : Need for conservation and management *Current Science* **8** 377–378
[8] [IUCN] International Union for Conservation of Nature. Red list of threatened species http://www. Iucnredlist.org. [Retrieved on March 2018].
[9] Foster SJ and Vincent ACJ 2005 Enhancing sustainability of the international trade in seahorses with a single minimum size limit *Conserv. Biol*. **4** 1044–1050
[10] [CITES] 2004. Convention on the International Trade in Endangered Species of Wild Flora and Fauna Appendise I, II, and III (http://www.cites.org/index.html) [Retrieved on March 2018].
[11] Fianda C, Pratomo A and Idris F 2015 Identifikasi dan inventarisasi jenis kuda laut (*Hippocampus* sp.) yang hidup di perairan Pulau Bintan Provinsi Kepulauan Riau Kepulauan Riau (ID): Universitas Maritim Raja Ali Haji
[12] Hebert PDN and Gregory T 2005 The promise of DNA barcoding for taxonomy *Systematic Biology*. **5** 852–859
[13] Hajibabaei M and Steinke D 2012 DNA Barcode Standarts : New comunity standars adopted for fungi and protists *Barcode Bulletin*. **3** 1–17
[14] Patel JB 2001 16S rRNA gene sequencing for bacterial pathogen identification in the clinical laboratory *J. Mol. Diagnostics* **4** 313–321
[15] Rinanda T 2011 Analisis sekuensing 16S rRNA di bidang mikrobiologi *J. Kedokteran Syiah Kuala*. **3** 172–177
[16] Westermeier R and Gronau S 2005 Electrophoresis in practice: a guide to methods and applications of DNA and protein separations. Weinheim (GE): Wiley-VCH
[18] Yuwono T 2006 *Teori dan amplifikasi Polymerase Chain Reaction* Yogyakarta (ID): ANDI
[19] Sanger F, Micklen S and Coulson A. 1977 DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America*. 12 5463–5467
[20] Tamura K, Peterson D, Peterson N, Stecher G, Nei M and Kumar S 2011 MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods *Mol. Biol. Evol*. **10** 2731–2739
[21] Tamura K, Dudley J, Nei M and Kumar S 2007 MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0. *Mol. Biol. Evol*. **8**: 1596–1599

[22] Topik H 2005 Systematic study of subtribe Aeridinae (*Orchidaeceae*) [Disertasi]. Japan: University of Tokyo
[23] Rahayu S. 2011. Kekerabatan *Hoya* (Asclepiadaseae) Sumatera berdasarkan karakter morfologi. *Bulletin Kebun Raya*. **1** 19–28
[24] Zhou J, Bruns MA and Tiedje JM 1996 DNA recovery from soils of diverse composition. *Appl. Environ. Microbiol*. (2): 316–322.
[25] Sahara A, Prastowo J, Widayanti R and Nurcahyo W 2015 Kekerabatan genetik caplak *Rhiphicephalus* (*Boophilus*) *microplus* asal Indonesia berdasarkan sekuen *internal transcribed spacer-2 J. Veteriner* **3** 310–319
[26] Hebert PDN, Cywinska A and Ball SL 2003 Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London*. 270 313-322
27] Brinkman FSL and Leipe DD 2001 Phylogenetic analysis In: Bioinformatics: A Practical Guide to the Analysis of Genes and Proteins. Baxevanis AD dan Ouellette BFF (Eds). John Willey and Sons. 323–358
[28] Li S, Pearl DK, Doss H 1999 Phylogenetic tree construction using Markov chain Monte Carlo. *J. Am. Stat. Assoc.* **95** 493–508