Morphometric and genetic identification of a newly record pygmy seahorse *Hippocampus denise* in the Kepulauan Seribu reefs, Indonesia

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Abstract. The study was conducted to describe the seahorse species based on morphological and molecular characters. The pygmy seahorse in Panggang Island in Kepulauan Seribu was discovered in October 2011. The species was allegedly identified as *Hippocampus denise* (Family: Syngnathidae) described by Lourie and Randall which published in 2003. The high similarity is based on small morphometric, orange-like color and its association with sea fan *Anella* sp. Their habitat is fairly shallow at a depth between 13-24 meters compared with their sister species observed in Bali, Nusa Tenggara, and Sulawesi. The phylogenetic analysis constructed with several sequence data of *Hippocampus* sp. from Genbank shows that sample collected from Panggang Island is in the same clade with *Hippocampus denise* with 100% bootstrap value. BLAST analysis result also showed a high maximum similar identity (>99%) with the species *Hippocampus denise*. The seahorse specimen described in this study has a common typology of habitat with *Hippocampus denise*. This study shows that genetic analysis to determine the *Hippocampus denise* can be carried out to support species recognition, especially for cryptic species such as *Hippocampus* spp. There are variations in morphometric and habitat depth levels, indicating local adaptation of pygmy seahorses to the Kepulauan Seribu reefs.

Keywords: Biosystematics, Coral reefs, Reef fishes, Taxonomy

1. Introduction
The seahorse (Syngnathidae) is associated with gorgonian sea fans (*Anella reticulata*, *Muricella* sp. and *Echinogorgia* sp.). These organisms are camouflaged creatures, with their coloration and body ornamentation in the form of tubercles, they can match the stems and polyps of their gorgonian hosts. The distribution of Syngnathidae is widespread in the western Pacific, including Indonesia. These family records in Indonesia are primarily from marine, especially reef-associated in most regions [1]. The unique appearance makes the seahorse one of the marine creatures traded in the international marine ornamental trade. All species have been listed in Appendix CITES since 2002 through a licensing system (CITES II, since 5.15.04). The seahorse is generally utilized for medicine, accessories, and sometimes for religion purposes [2]. The taxonomy of seahorse is still a matter of debate because the system name is often based on the number of specimens is minimal, geographic scope insulated from the basis of naming a new species, a description of the less comprehensive
species, and lack of genetic information. On the other hand, the morphological identification conducted by macroscopic and microscopic observations of the external morphology works only on very few species. The genetic approach has often been utilized to support the identification of the type of an organism [3-4]. The identification of molecular analysis uses the concept of DNA barcoding, which refers to the sequence of nucleotide bases as an organism’s genetic code [3].

DNA barcoding is a method that uses mitochondrial DNA with one strand of DNA sufficient to compare between animal species [3-4]. DNA barcoding could identify an organism to the species level, ensuring honest trade exchanges for correct consumer information and fisheries management and conservation [4]. DNA barcoding uses primers in the PCR (polymerase chain reaction) process to amplify DNA within 600-700 bp at the mitochondrial Cytochrome Oxidase I (COI) locus [3]. According to Sanger et al. (1977) [5], PCR-sequencing was used to obtain the sequence of nucleotide bases in DNA molecules. The sequenced PCR results can determine biota species by referring to the GenBank at NCBI (National Center for Biotechnology Information). Therefore, this study was conducted to describe the specimens of pygmy seahorses (Syngnathidae) collected in Panggang Island, Kepulauan Seribu, based on genetic characteristics as a complement to morphological characteristics.

2. Materials and methods

2.1. Study site
The samples were collected in Panggang Island, in Kepulauan Seribu, in October 2011. Kepulauan Seribu Island, generally known as Thousand Islands, is a chain of islands to the north of Jakarta's coast. It forms the only regency of Jakarta, the capital of Indonesia. It consists of a string of 110 islands stretching 45 km north into the Java Sea at West Jakarta Bay. Most islands in the Kepulauan Seribu are included in The Thousand Islands Marine National Park, located in the northern part of the islands.

2.2. Morphometric and meristic measurement
A total of 3 specimens of pygmy seahorse from Panggang Island, Kepulauan Seribu, were collected and preserved using ethanol 96%. The morphology observation of collected samples was carried out using a portable digital microscope "Dinoscope". The morphometric measurements refer to the reference study [6] that described this species in 2003 based on collected samples from several locations in Indonesia (Figure 1) (Table 1).

![Figure 1](image-url)

**Figure 1.** References for morphometric measurements of *Hippocampus denise* [6]. Specification: Morphometric: HL = head length; TRL = long-backs; TAL = tail length; SnL, long mouth; OD = diameter of the eye; PO = the long post-orbital (eye-to- head indentations); SnD = wide mouth; HD = head width; CH = high crown; TD4 = width between the wheel back to the backbone of the 4th and 5th; TD9 = width between the wheel back to the backbone of the 9th and 10th; PL = length of the base of the pectoral fins; DL = length of the base of the dorsal fin. SL, standard length = HL+TrL+TaL. Meristics: TRR = number of ring / ring backbone; Star = number of ring / mouth pieces; DF = number of dorsal fin spine; PF = number of bone pectoral fins.
Table 1. References for morphometric measurements in millimeter of Hippocampus denise [6]. (HL = head length; TRL = long-backs; TAL = tail length; SnL, long mouth; OD = diameter of the eye; PO = the long post-orbital (eye-to-head indentations); SnD = wide mouth; HD = head width; CH = high crown; TD4 = width between the wheel back to the backbone of the 4th and 5th; TD9 = width between the wheel back to the backbone of the 9th and 10th; PL = length of the base of the pectoral fins; DL = length of the base of the dorsal fin. SL, standard length = HL+TrL+TaL. Meristics: TRR = number of ring / ring backbone; Star = number of ring / mouth pieces; DF = number of dorsal fin spine; PF = number of bone pectoral fins).

| Morphometric parameters | SnD  | SnL   | OD   | PO   | HL   | CH   | PL   | HD   | TD4  | TD9  | TrL  | DL   | TaL | SL  |
|-------------------------|------|-------|------|------|------|------|------|------|------|------|------|------|-----|-----|
| Length (mm)             | 0.97 | 1.436 | 0.746| 1.778| 3.823| 1.995| 0.601| 2.725| 1.584| 2.073| 7.485| 1.37 | 9.751| 20.92|
| SD                      | 0.025| 0.032 | 0.037| 0.037| 0.072| 0.054| 0.02 | 0.041| 0.111| 0.152| 0.153| 0.055| 0.081| 0.321|

2.3. DNA extraction and PCR

In confirming the species of the pygmy seahorse, we process one sample collected to conduct the genetic analysis to confirm the species. Genomic DNA from Hippocampus denise sample was extracted with Qiagen extraction kits, following the manufacturer's guidelines. DNA extracts were stored at -20 °C before a polymerase chain reaction (PCR). PCRs were carried out in a total volume of 25 μl, containing 14.5 μl distilled water (ddH2O), 2.5 μl 10x PCR buffer [Applied Biosystems (AB)], 2.5 μl 8 mM dNTPs (Promega), 2 μl 25 mM MgCl2 (AB), 0.125 μl (5 unit/μl) Taq polymerase (AmpliTaq DNA Polymerase), 1.25 μl primer HCO-2198 5’ – TAA ACT TCA GGG TGA CCA AAA ATC A – 3’, 1.25 μl primer primer LCO-1490 5’– GGT CAA CAA ATC ATA AAG ATA TTG G – 3’ [7], 1 μl genomic DNA. PCRs were performed in a 2720 Thermal Cycler (Applied Biosystems) with the following thermo-profile: 94 °C for 15 seconds, followed by 38 cycles of 94 °C for 30 seconds as the denaturing step, 50 °C for 30 seconds as the annealing step, 72 °C for 45 seconds for the polymerization, and finally 72 °C for 5 minutes. PCR products were visualized in 1% agarose gels and sent to UC Berkeley DNA Sequencing Facility for clean-up and sequencing in both forward and reverse directions. Sequences were edited and aligned in MEGA 5 [8], and exploratory identification of samples was performed using the Basic Local Assignment Search Tool (BLAST) [9].

2.4. Molecular analysis

The sequence was analyzed using BLAST (Basic Local Alignment Search Tool) on Genbank NCBI (National Center for Biotechnology Information). For phylogenetic analysis, several reference sequences with the highest maximum identity to each amplicon sequence were downloaded from the GenBank NCBI sequence database. Twenty-two recognized from several seahorse species from the GenBank NCBI database were used for phylogenetic analysis. The Neighbor-joining trees was reconstructed by using MEGA 10 [8] with similar parameters to those used in the investigation of a broader phylogeny of seahorses [9-10]. The Kimura 2-parameter model of substitution was used with bootstrapping value on 1000 replicates [11].

3. Results and discussion

3.1. Morphological characteristics

A pair of pygmy seahorses were first detected in the waters of the Keplauan Seribu reefs by October 2011 (Figure 2), based upon the following facts (1) Physical measuring the performance mini <200 mm, (2) Orange-like color that is in harmony with the colors of the host rock fan/gorgonian (Annella sp.) (3) Habitat in the waters of coral reefs. This species was suspected of such species as Hippocampus denise [6]. Coral reef habitats pygmy seahorses in relatively shallow waters of the Keplauan Seribu reefs (13-24 meters), compared to his relatives that were found in the waters of Bali, Nusa Tenggara, and Sulawesi (≥ 25 m), even in Palau (=84 m). Juveniles species of pygmy seahorses measuring <2 mm with black color. The specimen’s uniqueness obtained in some field photos from the
island showed morphometric variation, particularly related to the biota camouflage efforts with the sea fans.

![Denise's pygmy seahorse](image1.png)

**Figure 2.** Denise’s pygmy seahorse *Hippocampus denise* found on gorgonian sea fans in Kepulauan Seribu (A), and a sample of Denise’s pygmy seahorse *Hippocampus denise* collected for morphological characteristics.

The Denise’s pygmy seahorse, *Hippocampus denise* [6] has a maximum length of 2.2 cm SL. The color in life is plain orange with slightly darker rings around the tail; when preserved, pale orange with tiny dark brown flecks on the nape of the neck and all over in some specimens. This species is diminutive in size. Anal fin small or absent. Rings on trunk 12; on tail 28-29. Body fleshy with inferior and ventral trunk ridges reduced to separated cross-shaped spicules embedded in the skin. Nuchal plate rounded without a raised coronet. Snout length ca. 30% in HL. Head depth ca. 50% in HL. No spines above the eye. Trunk depth (between the 9th and 10th trunk rings) ca. 7% in SL (female) and 10-15% in SL (male). The angles of certain body ridges sometimes developed into rounded tubercles (distinctly fewer and less developed compared with *H. bargibanti*).

**Table 2.** A comparison of proportional measurements as morphometric characteristics (in millimeter) of *Hippocampus denise* between reference study [6] and this study.

|               | Tr.L:SL | Ta.L:SL | HL:SL | HD:HL | Sn.HL | Sa0.5Sn.L | OD:HL | Po:HL | Ch:HL | TD9:SL | DL:SL | PL:SL |
|---------------|---------|---------|-------|-------|-------|-----------|-------|-------|-------|--------|-------|-------|
| Lourie and Randall (2003) [6] | 28.28 | 54.30 | 19.19 | 49.47 | 31.24 | 75.58 | 21.18 | 42.18 | 44.47 | 9.228 | 8.12 | 5.329 |
| This study    | 36.036 | 46.046 | 18.018 | 72.072 | 37.037 | 68.068 | 19.019 | 47.047 | 52.052 | 10.01 | 7.007 | 3.003 |

3.2. Genetic characteristics

The genetic identity of *Hippocampus denise* was estimated using a molecular marker (COI). The 698 base pairs (bp) length of the COI gene sequence was compared and analyzed. The maximum similar
identity was found at >99% from a species *Hippocampus denise* on the Genbank NCBI database (figure 3).

The resulting phylogenetic tree of 23 sequences (figure 4) shows that the genera of *Hippocampus* were constructed on the same main clade. The sample from Panggang Island (HWSQ LCO) showed the same sub-clade to *Hippocampus denise* with 100 of bootstrap value. The phylogeny tree formed has a number at the base of the branch that indicates the bootstrap value. The comparison of bootstrap values is directly proportional to the level of confidence in reconstructing the phylogeny tree. The greater the bootstrap value, the higher the level of confidence in the reconstructed phylogeny tree. There are several bootstrap values at the clade base that indicate the accuracy of branching phylogenetic trees [10-12]. The bootstrap value that appears is a measure to test the goodness of a data set model we use [10-13].

This study shows that genetic analysis to determine the *Hippocampus denise* species can be carried out to support species recognition, especially for cryptic species such as *Hippocampus* spp. However, the *Hippocampus denise* sample collected from Panggang Island showed the same position in *Hippocampus denise* sequence from the Genbank NCBI database. The specimen of seahorse described...
in this study have in common typology of habitat with species *Hippocampus denise* [6]. There are variations in morphometric and habitat depth levels, indicating local adaptation of pygmy seahorses to the Kepulauan Seribu reefs. The presence of *Hippocampus denise* is known in several countries in Indo-Pacific, such as Malaysia, Palau, Papua New Guinea, Solomon Island, and Vanuatu [6, 15]. However, the discovery of this species is related to the distribution of the type of sea fan they inhabit. It is possible that the distribution of this species is also found in other areas in Indonesia, but it has not been scientifically reported. This species of pygmy seahorse and others that may be found in the reef area is so tiny and cryptic that it is exceedingly difficult to discover. A challenge made much more difficult by its distribution far into the mesophotic zone, well beyond the range of scuba divers [16]. In this regard, it is necessary to make various efforts to reveal the potential of biodiversity in Indonesia, especially with various scientific approaches (i.e., genetic approach) [17]. By conducting this research, we also provide evidence and help disseminate genetic-related research, especially DNA barcodes in Indonesia [18-21].

4. Conclusion
This study shows that genetic analysis to determine the *Hippocampus denise* can be carried out to support species recognition, especially for cryptic species such as *Hippocampus spp*. There are variations in morphometric and habitat depth levels, indicating local adaptation of pygmy seahorses to the Kepulauan Seribu reefs.

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References

[1] Nelson J S 1994 *Fishes of the world* 3rd edn (New York: John Wiley & Sons, Inc.) p 600
[2] Rosa I M, Alves R R, Bonifácio K M, Mourão J S, Osório F M, Oliveira T P and Nottingham M C 2005 Fishers’ knowledge and seahorse conservation in Brazil J. Ethnobiol. Ethnomed 1(1) 1-5
[3] Hebert P D, Cywinska A, Ball S L and DeWaard J R 2003 Biological identifications through DNA barcodes Proc. R. Soc. B: Biol. Sci. 270(1512) 313-21
[4] Madduppa H, Ayuningtyas R U, Subhan B and Arafat D 2016 Exploited but unevaluated: DNA barcoding reveals skates and stingrays (Chordata, Chondrichthyes) species landed in the Indonesian fish market *IJMS* 21 77-84
[5] Sanger F, Nicklen S and Coulson A R 1977 DNA sequencing with chain-terminating inhibitors *Proc. Natl. Acad. Sci.* 74(12) 5463-7
[6] Lourie S A and Randall J E 2003 A new pygmy seahorse, *Hippocampus denise* (Teleostei Syngnathidae) from the Indo-Pacific *Zool. Stud.* 42(2) 284-91
[7] Vrijenhoek R 1994 DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates *Mol. Mar. Biol. Biotechnol.* 3(5) 294-9
[8] Kumar S, Stecher G, Li M, Knyaz C and Tamura K 2018 MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms *Mol. Biol. Evol.* 35 1547-9
[9] Tamura K, Nei M, Kumar S 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method *Proc. Natl. Acad. Sci.* 101 11030-35.
[10] Saitou N and Nei M 1987 The neighbor-joining method: A new method for reconstructing phylogenetic trees *Mol. Biol. Evol.* 4:406-25
[11] Felsenstein J 1985 Confidence limits on phylogenies: An approach using the bootstrap *Evolution* 39 783-91
[12] Altschul S F, Gish W, Miller W, Myers E W and Lipman D J 1990 Basic local alignment search
[13] Horiike T, Miyata D, Hamada K, Saruhashi S, Shinozawa T, Kumar S, Chakraborty R, Komiyama T and Tateno Y 2009 Phylogenetic construction of 17 bacterial phyla by new method and carefully selected orthologs *Gene* 429(1-2) 59-64

[14] Dharmayanti N L 2011 Filogenetika molekuler: metode taksonomi organisme berdasarkan sejarah evolusi. *Wartazoa* 21(1) 1-0.

[15] Allen GR, Erdmann MV 2012 *Reef fishes of the East Indies* (Perth: Universitity of Hawai’i Press)

[16] Foster R, Bridge T C and Bongaerts P 2012 The first record of *Hippocampus denise* (Syngnathidae) from Australia *Aqua, Int J Ichthyol* 18 55-7

[17] Madduppa H, Cahyani N K, Anggoro A W, Subhan B, Jefri E, Sani L M, Arafat D, Akbar N and Bengen D G 2021 eDNA metabarcoding illuminates species diversity and composition of three phyla (chordata, mollusca and echinodermata) across Indonesian coral reefs *Biodivers. Conserv.* 30(11) 3087-114

[18] Madduppa H, Martaulina R, Zairion Z, Renjani R M, Kawaroe M, Anggraini N P, Subhan B, Verawati I and Sani LM 2021 Genetic population subdivision of the blue swimming crab (Portunus pelagicus) across Indonesia inferred from mitochondrial DNA: Implication to sustainable fishery *Plos one* 16(2) e0240951

[19] Madduppa H, Putri A S, Wicaksono R Z, Subhan B, Akbar N, Ismail F, Arafat D, Prabuning D, Sani L M I, Srimariana E S and Baksir A 2020 Morphometric and DNA Barcoding of endemic Halmaheran walking shark (*Hemiscyllium halmahera*, Allen, 2013) in North Maluku, Indonesia: Morphogenetic of endemic Halmaheran walking shark *Biodiversitas* 21(7) 3331-43

[20] Toha A H, Widodo N, Subhan B, Himawan M R, Tania C, Noor B A, Stewart B S, and Madduppa H H 2016 Close genetic relatedness of whale sharks, *Rhincodon typus* in the Indo-Pacific region *AACL Bioflux* 9(3) 458-65

[21] Bramandito A, Subhan B, Prartono T, Anggraini NP, Januar HiI and Madduppa H 2018 Genetic diversity and population structure of *Siganus fuscescens* across urban reefs of Seribu Islands, Northern of Jakarta, Indonesia *Biodiversitas* 19(6) 1993-2002