Characteristics of 16S rRNA gene in seahorses from Ternate Island waters, Indonesia

[1]Aras Syazili, [2]Muhammad N. Findra, [2]Supyan, [1]Gamal M. Samadan

[1] Department of Aquaculture, Faculty of Fisheries and Marine Sciences, Khairun University, Ternate, Indonesia; [2] Department of Aquatic Resources Management, Faculty of Fisheries and Marine Sciences, Khairun University, Ternate, Indonesia. Corresponding author: M. N. Findra, muhammad.findra@gmail.com

Abstract. Seahorses (family Syngnathidae) are among the most common marine organisms in tropical and subtropical waters globally. Indonesian waters, including Ternate Island and North Maluku Province, are also habitats for seahorses. Therefore, this study aimed to assess the genetic characteristics of seahorses from the southern waters of Ternate Island based on the 16S rRNA gene. Seahorses were collected from the waters of Ternate Island for tissue sampling and DNA extraction using the 10% Chelex method. The resulting DNA was amplified with a PCR machine using Ready Mix. The pair of primers used in the amplification process include 16S-ar (5'-CGC CTG TTT ATC AAA AAC AT-3') and 16S-br (5'-CCG GTC TGA ACT CAG ATC ACG T-3'). Sequence validation results for seahorse specimens using BLASTn showed a high degree of kinship with *Hippocampus kuda* registered in Genbank, ranging from 99.16 to 99.83%. Comparing the nucleotide bases of the 16S rRNA gene sequence of the specimen with the sequences of several seahorse species from Genbank revealed specific nucleotide sites (23 sites) that could distinguish between species. Furthermore, genetic distance analysis between species resulted in varying values. The phylogenetic tree reconstructed using the Neighbor-Joining method with 1,000 bootstrap replications showed that Hippocampus species were divided into two main clades, namely Clade 1 (*H. kuda, Hippocampus reidi, Hippocampus fuscus, Hippocampus kelloggi*) and Clade 2 (*Hippocampus comes* and *Hippocampus barbouri*). In conclusion, this study successfully revealed the 16S rRNA gene sequence characteristics of seahorses and showed that partial 16S rRNA gene can be used as genetic markers in identifying seahorse species from Ternate Island as *H. kuda*.
Key Words: mitochondrial DNA, *Hippocampus kuda*, phylogenetic, specific nucleotide.

Introduction. Seahorses (family Syngnathidae) are marine biological resources that are abundantly distributed globally in both tropical and subtropical waters. This marine animal is commonly found in shallow waters of coastal ecosystems with resources such as seagrasses, coral reefs, mangroves, and seaweeds (Cohen et al 2017; Dody et al 2021; Scales 2010). Seahorses have a unique body shape, with the head resembling a horse. It is a fascinating animal due to its unique way of swimming which is not found in other marine animals. In addition, seahorses have economic value as ornamental fish and souvenirs, and are used in medicine, particularly traditional Chinese medicine. Seahorses, whether alive or dead have a high global trade value, resulting in a high economic market value, both domestically and internationally (Lourie et al 2004; Mulyawan & Saokani 2015). This marine organism is distributed in Indonesian waters, including Ternate Island and North Maluku Province. Moreover, Ternate Island is administratively included in North Maluku Province. In the waters of North Maluku, there were at least three reported species of seahorses, namely *Hippocampus kuda, Hippocampus kellogi,* and *Hippocampus spinosissimus*. In Morotai Island waters, three species were found, namely *H. kuda* (7 individuals), *H. kellogi* (17 individuals), and *H. spinosissimus* (1 individual) observed during June-July (Koroy et al 2023). In Ternate Island, only *H. kuda* was reported in the southern waters with the highest density in April for juveniles at 3.3 ind 100 m^{-2}, and the highest density for parents in October at 2.8 ind 100 m^{-2} (Dody et al 2021).

The wide use and huge market demand for seahorses necessitate management efforts such as breeding and cultivation to maintain sustainability. According to Afara et al (2023), Bahtiar et al (2023), and Pratama et al (2023), managing aquatic resources

requires information from various studies. However, limited studies have been conducted on seahorses in Ternate Island, and genetic characteristics have never been investigated. Previous studies carried out were focused on habitat characteristics and density (Dody et al 2021), as well as the growth and survival of seahorses in Juwana (Syazili et al 2023). The role of adaptation and animal development strategies is largely determined by genetic capabilities (Findra 2016). Genetic profiles can be obtained by analyzing mitochondrial DNA (Indrayani et al 2021a, Indrayani et al 2021b; Samadan et al 2024), which includes the 16S rRNA gene. This gene is a region of mitochondrial DNA often used for species and population identification (Hanifaturahmah et al 2020; Singh et al 2011). Therefore, this study aimed to assess the genetic profiles of seahorses from of Ternate Island waters.

Material and Method

Sample collection. Seahorse tissue samples were collected from the southern waters of Ternate Island (Figure 1) in February 2024. The samples collected were placed in a tube prefilled with 96% alcohol to preserve tissue samples.

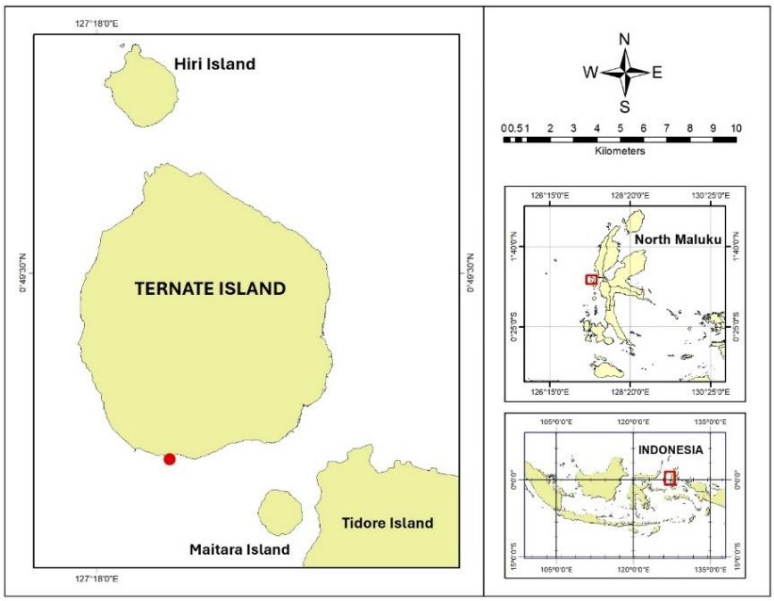

Figure 1. Sampling site (●) of seahorses at Ternate Island, Indonesia.

DNA analysis. Tissue samples measuring about 2 mm were obtained and the extraction process was carried out using the 10% Chelex method to determine total DNA. The resulting DNA was amplified with a Polymerase Chain Reaction (PCR) machine following the BIONESIA laboratory protocol, using Ready Mix. The primers used in the amplification process of seahorse samples were a pair of primers recommended by Palumbi et al (2002), namely 16S-ar (5'-CGC CTG TTT ATC AAA AAC AT-3') and 16S-br (5'-CCG GTC TGA ACT CAG ATC ACG T-3'). The total PCR reaction was 26 µL consisting of 2 µL extracted DNA template, 1.25 µL 16S-ar primer, 1.25 µL 16S-br primer, 9 µL ddH2O, and 12.5 µL Ready Mix. The reaction was amplified using an Applied Biosystems 2720 Thermal Cycler machine. In addition, the PCR temperature profile used was initial denaturation at 94°C for 5 minutes, denaturation at 94°C for 45 seconds, annealing at 53°C for 1 minute, and extension stage at 72°C for 1 minute. These stages were carried out for 35 cycles, with the final extension stage at 72°C for 2 minutes. PCR products obtained were visualized on 1% agarose gel using Nucleic Acid Gel Stain (GelRed®). Samples visualized by transmitting DNA bands were then sequenced using the services of PT. Genetika Science Jakarta.

Data analysis. Data analysis was carried out using MEGA X software (Kumar et al 2018). The sequence data obtained were edited and aligned using the ClustalW method in the MEGA X program. Data was matched with the database stored at the National Center for Biotechnology Information (NCBI) through the Basic Local Alignment Search Tools

nucleotide (BLASTn) method on the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Kinship analysis was conducted using a phylogenetic tree constructed by the Neighbor-joining (NJ) method with 1000 bootstrap replications. The genetic distance was analyzed to determine its proximity to other sequence data using the p-distance method. Several seahorse sequences were used on the NCBI website as the ingroup and outgroup, namely *H. kuda* (Accession numbers AF355012, DQ288375, DQ452301), *H. comes* (Accession numbers MK017689, MK017688, MK017686), *H. reidi* (Accession numbers KY065558, DQ288368), *H. barbouri* (Accession numbers MH729375, MH729374, MH729373), *H. kelloggi* (Accession numbers FJ211369, FJ211368, AY277298), *H. fuscus* (Accession numbers DQ288371, KY065552) and *Syngnathus acus* (Accession numbers JX228167).

Results. BLASTn validation results showed that seahorse specimens from Ternate Island waters shared a high degree of kinship (ranging from 99.16 to 99.83%) with *H. kuda* registered at Genbank under accession numbers OP035079, DQ452301, FJ211365, FJ211362, MT221437, and MT221436. The proximity between seahorses from Ternate waters and other species such as *Hippocampus ingens, H. spinosissimus, H. kelloggi, H. queenslandicus, H. spinosissimus,* and *H. fuscus* ranged from 96.14 to 97.40%, respectively (Table 1).

Table 1

BLASTn results of seahorse specimen from Ternate Island based on 16S rRNA gene

Accession number	*Species name*	*Query cover (%)*	*E-value*	*Identity (%)*
OP035079	*Hippocampus kuda*	100	0.0	99.83
DQ452301	*Hippocampus kuda*	100	0.0	99.83
FJ211365	*Hippocampus kuda*	100	0.0	99.49
FJ211362	*Hippocampus kuda*	100	0.0	99.33
MT221437	*Hippocampus kuda*	100	0.0	99.16
MT221436	*Hippocampus kuda*	100	0.0	99.16
NC_024530	*Hippocampus ingens*	100	0.0	97.14
NC_029350	*Hippocampus spinosissimus*	100	0.0	96.48
KY065553	*Hippocampus ingens*	96	0.0	97.40
KF703755	*Hippocampus kelloggi*	96	0.0	96.14
NC_034319	*Hippocampus queenslandicus*	100	0.0	96.15
NC_029349	*Hippocampus kelloggi*	96	0.0	96.14

Specific nucleotides of 16S rRNA gene. A comparison of 16S rRNA gene nucleotide bases in seahorse sequences from Ternate waters and several species from Genbank showed that there were specific nucleotide sites that distinguished between species. Table 2 shows that the specific nucleotides found were at 23 sites.

Table 2

Polymorphisms of *Hippocampus* spp. specific nucleotide site based on 16S rRNA sequence

Species	*Nucleotide position*											
	106	*108*	*188*	*192*	*199*	*202*	*205*	*209*	*212*	*220*	*227*	*228*
H. kuda	C	C	T	T	A	A	C	T	C	A	A	**T**
H. comes	**T**	C	T	**C**	A	A	**T**	T	C	A	**G**	A
H. reidi	C	C	T	T	A	**T**	C	T	C	A	A	A
H. barbouri	C	**T**	**C**	T	**G**	A	C	T	C	**G**	A	A
H. kelloggi	C	C	T	T	A	A	C	**C**	**G**	A	A	A
H. fuscus	C	C	T	T	A	A	C	T	C	A	A	A
	231	*238*	*257*	*280*	*300*	*304*	*305*	*310*	*358*	*359*	*369*	
H. kuda	A	T	T	A	T	G	T	T	T	**T**	A	
H. comes	A	T	T	A	T	**A**	T	T	**C**	A	**C**	
H. reidi	A	T	T	A	**C**	G	T	T	T	A	A	

	231	238	257	280	300	304	305	310	358	359	369
H. barbouri	A	**C**	T	A	T	G	T	T	T	A	A
H. kelloggi	**T**	T	**C**	A	T	G	**C**	**C**	T	A	A
H. fuscus	A	T	T	**T**	T	G	T	T	T	A	A

Specific nucleotides are shown by red color.

Genetic distances and phylogenetic trees. The genetic distances of seahorses, obtained from the alignment of 485 bp of the 16S rRNA gene sequences using the p-distance method, were 0.0000 to 0.0186 for intra-species, 0.0124 to 0.0746 for inter-species, and 0.0000 to 0.1897 for all sequences with outgroup. The summary of genetic distance analysis can be seen in Table 3. The reconstruction of phylogenetic trees using the NJ method showed that two main clades of seahorses were formed and separated from their outgroup (Figure 2).

Table 3

Genetic distance between sequences of *Hippocampus* spp. and *Syngnathus acus* based on 16S rRNA gene

	H. kuda	*H. comes*	*H. reidi*	*H. barbouri*	*H. kelloggi*	*H. fuscus*	*S. acus*
H. kuda							
H. comes	0.0746						
H. reidi	0.0155	0.0701					
H. barbouri	0.0739	0.0399	0.0646				
H. kelloggi	0.0443	0.0660	0.0412	0.0605			
H. fuscus	0.0124	0.0691	0.0052	0.0636	0.0381		
S. acus	0.1732	0.1876	0.1732	0.1869	0.1794	0.1722	

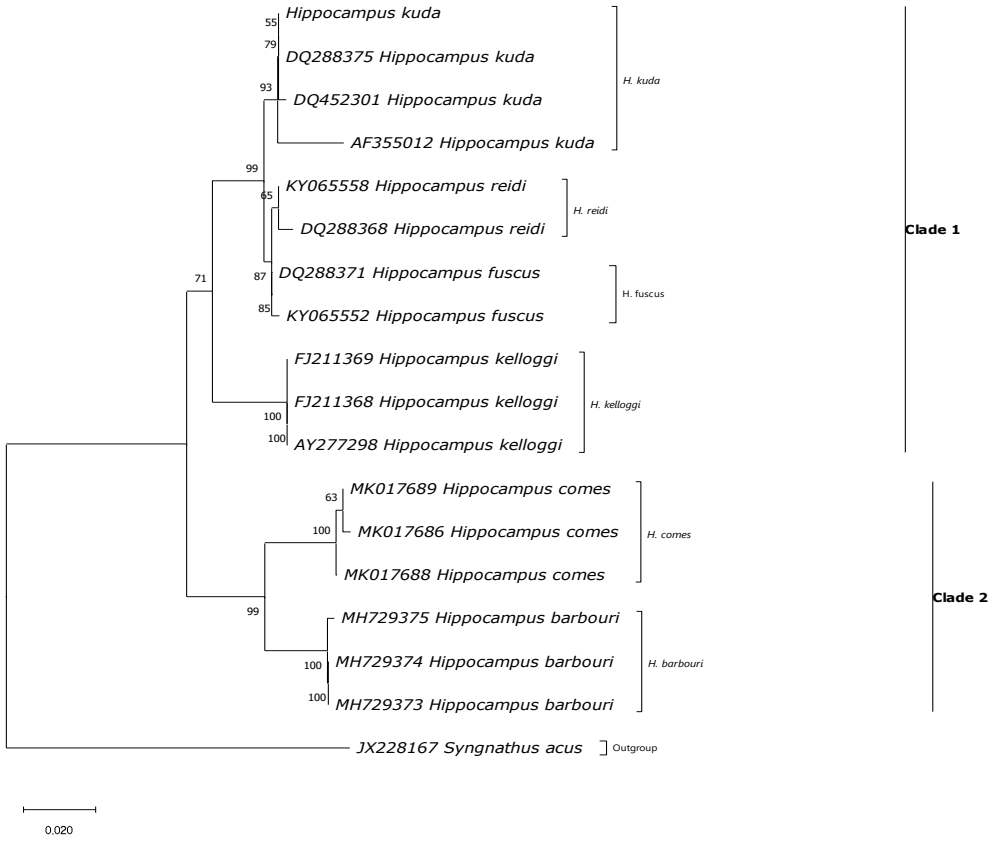

Figure 2. Phylogenetic tree of seahorse 16S sRNA sequences along 485 bp.

Discussion. Amplification of 16S rRNA gene fragments in seahorse specimens from Ternate Island showed good results at 53°C annealing temperature, with 593 bp obtained as the length of the sequence. This signifies that the 16S-ar and 16S-br primer pairs used were suitable for the seahorse samples. Another study reported that samples from Riau Islands, Indonesia, were amplified at the same annealing temperature with sequence lengths ranging from 650 to 700 bp (Hanifaturahmah et al 2020). The difference in sequence length was due to distinct primers used in this study. However, the DNA sequence could not be compared with this study because it was not found in Genbank. The seahorse specimen from Ternate Island showed a very high degree of similarity with *H. kuda* already registered in GenBank. This implies that the specimen from Ternate might be *H. horse* or very genetically similar to this species. Table 1 shows that the level of identity ranged from 96.14% to 97.40% when compared with other species such as *H. ingens, H. spinosissimus, H. kelloggi,* and *H. queenslandicus*. Although the similarity was significant, this identity was lower than that of *H. kuda*. This confirmed that the specimens from Ternate were more closely related to *H. kuda* than the other species analyzed. According to Nurilmala et al (2019), the molecular identification of seahorse samples using the 16S rRNA gene can provide information on species' identity. Comparison of 16S rRNA gene nucleotide bases in several seahorse species showed specific nucleotide sites that can distinguish between species (Table 2). *H. kuda* had 2 specific sites (228 and 359), *H. comes* had 7 specific sites (106, 192, 205, 227, 304, 358, and 369), *H. reidi* had 2 specific sites (202 and 300), *H. barbouri* had 5 specific sites (108, 188, 199, 220, and 238), *H. kelloggi* had 5 specific sites (108, 188, 199, 220, and 238), and *H. fuscus* had a single specific site (280). According to Findra et al (2020), specific nucleotides can differentiate between species. In addition, nucleotide polymorphisms can distinguish between populations and individuals (Butet et al 2019; Findra et al 2017).

The results of this study showed that the genetic distance between species had varying values. This distance represented the difference in nucleotide proportions between pairs of species. The smaller the values, the closer the kinship between the species. Table 3 showed that the genetic distance between *H. kuda* and *H. fuscus* was 0.0124, which implied very high genetic closeness. This might signify that both had a relatively recent ancestor in their evolutionary history. The genetic distance between *H. horse* and *H. reidi* was 0.0155, showing a fairly high closeness, was slightly larger than the distance between *H. fuscus* and *H. reidi*. The genetic distance between *H. reidi* and *H. fuscus* (0.0052) was very small, suggesting two evolutionarily close species. Meanwhile, a larger value of 0.0746 was observed between *H. kuda* and *H. comes*. This suggested that the species were more genetically distinct than some other pairs of the genus *Hippocampus*. All *Hippocampus* species showed a considerable genetic distance to *S. acus* (between 0.1722 and 0.1876), suggesting that the species is quite distant evolutionarily from all *Hippocampus* species analyzed. The inclusion of *S. acus* as an outgroup clarified the phylogenetic position of seahorses within the family Syngnathidae. This allowed the genetic relationship between two different genera within the same family to be determined, as represented in the phylogenetic tree that was formed in Figure 2. *S. acus* had a significant genetic distance from *Hippocampus* and was used as a positive control in the analysis. This confirmed that the genetic distance analysis method worked correctly and that the small genetic distance between *Hippocampus* species reflected close kinship. In this context, outgroups were used in establishing kinship relationships by functioning as controls and comparisons (Butet et al 2019; Subositi & Widodo 2010).

A phylogenetic tree of seahorse 16S rRNA sequences reconstructed using the NJ method with 1000 bootstrap replicates showed the relationships between seahorse species and one outgroup species (Figure 2). It also showed that *Hippocampus* species were divided into two main clades. Clade 1 included *H. horse, H. reidi, H. fuscus,* and *H. kelloggi*, and Clade 2 included *H. comes* and *H. barbouri. S. acus* was used as an outgroup to provide evolutionary context and help root the phylogenetic tree. Specimens of *H. kuda* from Ternate and Genbank (DQ288375, DQ452301, AF355012) were in the same group with very high bootstrap values, suggesting that these samples had very strong genetic similarities. This was the case for several other species in Clade 1 and 2 (*H. reidi* and *H. fuscus*), which also exhibited a strong clustering with each other, with high bootstrap values

showing a close relationship. The phylogenetic reconstruction by Nurilmala et al (2019) also showed a similar tree structure, with *H. horse, H. reidi, H. kellogi,* and several other species in the same branching indicating a close kinship. Meanwhile, *H. comes* and *H. barbouri* were in the same branching but separated from *H. horse, H. reidi,* and *H. kellogi*. This phylogenetic tree provided insight on how Hippocampus species might have evolved from a common ancestor. Grouping species based on genetic proximity helped in understanding the evolutionary history and divergence patterns among species.

Conclusions. The 16S rRNA gene sequence of seahorses from Ternate Island was successfully characterized. *H. kuda* was in the same clade as *H. reidi, H. fuscus,* and *H. kelloggi*, showing close kinship. This study also proved that partial 16S rRNA genes can be used as genetic markers in species identification of Ternate Island seahorses as *H. kuda*.

Acknowledgements. The authors are grateful to Khairun University for funding this study through the grant research 2024 (PKUPT scheme) with the contract number: 01/PEN-PKUPT/PG.12/2024. The authors are also grateful to Ms. Ela for assisting in seahorse sample collection in Ternate waters.

Conflict of interest. The authors declare no conflict of interest.

References

Afara M. Y., Sara L., Halili, Findra M. N., 2023 [Growth patterns and condition factors of red shrimp (*Parhippolyte uveae*) in swamp waters at Koguna Beach Area, Buton Regency, Southeast Sulawesi]. Juvenil: Jurnal Ilmiah Kelautan dan Perikanan 4(1): 43-50. [In Indonesian].

Bahtiar, Permatahati Y. I., Findra M. N., Fekri L., 2023 Production, biomass, and turnover of exploited mangrove clams (*Geloina expansa*, Mousson 1849) in Kendari Bay mangrove forest, Southeast Sulawesi Indonesia. BIO Web of Conferences 74:03009.

Butet N. A., Dewi I. A. B. P., Zairion Z., Hakim A. A., 2019 Species validation of mole crabs based on molecular marker of 16s rRNA from Bantul and Purworejo waters. Journal of Tropical Fisheries Management 3(2):28–35.

Cohen F. P. A., Valenti W. C., Planas M., Calado R., 2017 Seahorse aquaculture, biology and conservation: knowledge gaps and research opportunities. Reviews in Fisheries Science & Aquaculture 25(1):100–111.

Dody S., Manuputty G. D., Limmon G. V., 2021 Habitat characteristcs and density of spotted seahorse *Hippocampus kuda* at waters of Ternate Island and surroundings, Maluku Utara, Indonesia. IOP Conference Series: Earth and Environmental Science 805(1):012006.

Findra M. N., 2016 [Population and genetic profile study of giant clams (Bivalvia, Cardiidae, Tridacninae) in the Wakatobi National Park waters]. Master Thesis. Bogor Agricultural University, 46 p. [In Indonesian].

Findra M. N., Setyobudiandi I., Butet N. A., Solihin D. D., 2017 Genetic profile assessment of giant clam genus *Tridacna* as a basis for resource management at Wakatobi National Park waters. ILMU KELAUTAN: Indonesian Journal of Marine Sciences 22(2):67-74.

Findra M. N., Setyobudiandi I., Solihin D. D., Butet N. A., 2020 Characteristics of cytochrome C oxidase subunit I gene in giant clam from Wakatobi National Park Waters, Indonesia. IOP Conference Series: Earth and Environmental Science 584(1):012009.

Hanifaturahmah F., Perwitasari-Farajallah D., Butet N. A., Nurilmala M., Sudrajat A. O., Ochiai Y., 2020 Morphological and molecular identification of seahorses (*Hippocampus* spp.) from the coast of Sumatra Island, Indonesia. Biodiversitas Journal of Biological Diversity 21(9):4116–4123.

Indrayani I., Findra M. N., Jufri A., Hidayat H., Pariakan A., 2021a Genetic variations of *Cheilopogon nigricans* in the Makassar Strait, Indonesia. Indo Pacific Journal of Ocean Life 5(1):22-28.

Indrayani I., Sambah A. B., Kurniawan A., Pariakan A., Jufri A., Findra M. N., Wiadnya D. G. R., 2021b First genetic record and phylogenetic relationships *Cheilopogon spilonotopterus* (Bleker, 1865) originates from the Makassar Strait, Indonesia. Ecology, Environment and Conservation Paper 27:399-402.
Koroy K., Nurafni, Alwi D., 2023 Distribution patterns of sea horses based on sex caught in the waters of Morotai Island. East Asian Journal of Multidisciplinary Research 2(10): 4089–4096.
Kumar S., Stecher G., Li M., Knyaz C., Tamura K., 2018 MEGA X: Molecular evolutionary genetics analysis across computing platforms. Molecular Biology and Evolution 35(6): 1547–1549.
Lourie S. A., Foster S. J., Cooper E. W. T., Vincent, A. C. J., 2004 A guide to the identification of seahorses. University of British Columbia and World Wildlife Fund, 114 p.
Mulyawan A. E., Saokani J., 2015 [Habitat characteristics and abundance of seahorses (*Hippocampus barbouri*) caught in Tanakeke Islands, Takalar Regency]. Jurnal Balik Diwa 6(2):13–19. [In Indonesian].
Nurilmala M., Nurhayati K. H., Butet N. A., Sudrajat A. O., 2019 Molecular marker based on 16S rRNA gene for seahorse (*Hippocampus* spp.) from Bintan Island-Indonesia. IOP Conference Series: Earth and Environmental Science 278(1):012054.
Palumbi S., Martin A., Romano S., McMillan W. O., Stice L., Grabowski G., 2002 The simple fool's guide to PCR (Version 2.0). Department of Zoology and Kewalo Marine Laboratory, University of Hawaii, 45 p.
Pratama M. F., Sara L., Halili, Findra M. N., 2023 [Habitat characteristics of red shrimp (*Parhippolyte uveae*) in swamp waters around the Koguna Beach area, Buton Regency, Southeast Sulawesi]. Habitus Aquatica 4(1):8-16. [In Indonesian].
Samadan G. M., Supyan, Findra M. N., 2024 Genetic characteristics of *Macrobrachium lar* from Gane Timur, Halmahera Island, Indonesia, based on mitochondrial COI gene. AACL Bioflux 17(4):1543–1550.
Scales H., 2010 Advances in the ecology, biogeography and conservation of seahorses (genus *Hippocampus*). Progress in Physical Geography: Earth and Environment 34(4):443–458.
Singh K., Lakra W. S., Gopalakrishnan A., Modayil M. J., Malakar A. K., Sobti R. C., 2011 Molecular identification and phylogenetic relationship of seahorse, *Hippocampus kuda* (Bleeker 1852) using mitochondrial 16S rRNA and COI gene sequences from east and west coasts of India. Indian Journal of Animal Sciences 81(1):97–101.
Subositi D., Widodo H., 2010 [Interspecific phylogenetic relationship of genus *Stevia* members based on Maturase K (matK) gene]. Jurnal Tumbuhan Obat Indonesia 3(1): 33–41. [In Indonesian].
Syazili A., Samadan G. M., Ali Y. S. L., Yuliana, Muchdar F., Pakidi C. S., 2023 [Growth and survival of seahorses (*Hippocampus kuda*) juwana treated with brown algae *Padina australis* extract through immersion]. Musamus Fisheries and Marine Journal 6(1):1-8. [In Indonesian].
*** https://blast.ncbi.nlm.nih.gov/Blast.cgi

Received: 04 July 2024. Accepted: 02 October 2024. Published online: 19 October 2024.
Authors:
Aras Syazili, Department of Aquaculture, Faculty of Fisheries and Marine Sciences, Khairun University, 97719, Ternate, Indonesia, e-mail: arassyasili@unkhair.ac.id
Muhammad Nur Findra, Department of Aquatic Resources Management, Faculty of Fisheries and Marine Sciences, Khairun University, 97719, Ternate, Indonesia, e-mail: muhammad.findra@gmail.com
Supyan, Department of Aquatic Resources Management, Faculty of Fisheries and Marine Sciences, Khairun University, 97719, Ternate, Indonesia, e-mail: supyan77@ymail.com
Gamal Mustik Samadan, Department of Aquaculture, Faculty of Fisheries and Marine Sciences, Khairun University, 97719, Ternate, Indonesia, e-mail: gmsamadan@unkhair.ac.id

How to cite this article:
Syazili A., Findra M. N., Supyan, Samadan G. M., 2024 Characteristics of 16S rRNA gene in seahorses from Ternate Island waters, Indonesia. AACL Bioflux 17(5):2122-2128.