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DNA Barcoding of Dried Seahorses Illegally Sold in Metropolitan Manila, Philippines, as Traditional Chinese Medicine (TCM)

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Seahorses are a member of the Family Sygnathidae and are found in tropical and temperate coastal regions with soft, sandy bottoms, among rocks and algae, and in lagoons with strong oceanic influences. Seahorses are among the most sought-after animals, commonly marketed as traditional Chinese medicine (TCM) used to treat various diseases. The trade of these species on a global scale caused a significant decline in their population and a negative impact on the marine ecosystem. All the existing seahorse species are now recorded under Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). In the Philippines, these species are protected under the Philippine Fisheries Code (RA 8550), as amended by RA 10654, and the Philippine Wildlife Act (RA 9147). However, despite existing laws, the illegal trade of dried and live seahorses still persists. The study aims to identify seahorse species illegally traded in stores or markets in Metro Manila. A total of 22 whole-dried seahorses were purchased from different stores within Metro Manila. Nine specimens were bought from TCM stores in Binondo, Manila City, and 13 from aquatic pet shops in Cartimar Pet Center, Pasay City. Dried seahorse samples were subjected to morphological analysis and DNA barcoding. Morphological identification of all 22 samples revealed that four species of seahorses were sold in the said marketplaces. This includes *Hippocampus kuda*, *Hippocampus comes*, *Hippocampus histrix*, and *Hippocampus kellogi.* All samples underwent DNA extraction, amplification, and sequencing, with 15 sample sequences viable for BLAST and clustering analysis. Results of the clustering analysis revealed that 12 samples clustered under *Hippocampus kuda*, whereas the remaining three were under *H. comes*. Furthermore, results also showed that most of the seahorse species purchased in the three TCM stores and two aquatic pet shops were *H. kuda*, having a prevalence rate of 78% (TCM stores, Manila) and 62% (Cartimar Pet Center, Pasay), respectively. Thus, this study implies the persistent illegal selling of dried seahorses in the country, which calls for a more efficient, strict monitoring and effective policy implementation for the protection and conservation of seahorses and other marine wildlife.

Keywords: aquatic wildlife, CITES, DNA forensics, endangered species, IUCN

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INTRODUCTION

The shamanistic era of the Huang Di period marked the beginning of traditional Chinese medicine (TCM) as an alternative way of treating diseases (Catic *et al*. 2018). This practice includes various forms, including the use of plants and animals. Seahorses, species of bony fish that belong to the family Syngnathidae and the genus *Hippocampus*, with almost 50 known species worldwide, are among the most used animals in TCM due to their various medicinal purposes such as in treating impotence, infertility, renal disorders, asthma, goiter, high cholesterol, and skin and liver problems (Serite *et al.* 2021). They are also known to have anti-aging, anti-fatigue, and anti-tumor properties (Ryu *et al.* 2010). Furthermore, seahorses are also described to promote parturition and are considered as strong tonic and potent aphrodisiacs (Vincent 1996; May and Tomoda 2002).

TCM as well as its derivatives account for the largest utilization of seahorses, resulting in global trade that had reached approximately 20 million dried seahorses a year (Foster and Vincent 2005), involving more than 50 countries across the globe practicing export and imports of seahorses (Job *et al.* 2002). In the Philippines, there were several incident reports of illegal export of dried seahorses. In a study published by Sy and Melgar (2022), they recorded 19 seizure incidents from 2010–2021, wherein approximately 658.02 kg of dried seahorses were documented. Based on their report, the highest recorded number of seizure incidents was in 2019 (n = 5), whereas the highest estimated quantity of seized dried seahorses was observed in 2018, wherein approximately 211.04 kg of dried seahorses were reported. The study noted that seahorse seizure incidents peaked in 2017–2019. Additionally, they have also emphasized the large seahorse seizures that had occurred within those years, where approximately 130 kg of dried seahorses were confiscated in 2017 at Manila City, 152.94 kg were documented in Zamboanga City in 2018, and 104.54 kg of dried seahorse seizures were reported in Puerto Princesa City, Palawan, in 2019.

In addition to large-scale illegal exports, the illegal sale of dried seahorses is also prevalent in local marketplaces in the Philippines. These include TCM stores in Binondo, Manila – the country's Chinatown known for its vibrant markets offering a variety of Chinese products, including TCMs like dried seahorses. Furthermore, live seahorses have also been found to be illegally commercialized in large pet trading sites such as the Cartimar Pet Center in Pasay City (Sy and Melgar 2022). The persistent illegal trade of seahorses, whether dried or alive, poses a significant threat to the species – potentially leading to overexploitation, endangering their population and survival, as well as causing negative impacts on marine biodiversity.

The Convention on International Trade in Endangered Species of Wild Flora and Fauna (CITES) is a binding international arrangement that oversees global wildlife trade through permits and certificates. CITES was first established in 1975 with the primary objective of assuring the survivability of wild flora and fauna despite international trade. Seahorses are among the protected animals listed in CITES Appendix II, meaning they are subjected to strict regulation and monitoring, and international trading of the species would only be permitted if authorities grant an export permit, satisfying a condition where the trade would not cause detrimental effects on the population of these species in the future (Evanson and Foster 2011). The Philippines joined on 18 Aug 1981 and came into effect on 16 Nov 1981. Provisions of the Convention have been incorporated into Philippine laws through the Philippine Fisheries Code (RA 8550), as amended by Republic Act (RA) No. 10654, and the Philippine Wildlife Act (RA 9147) (BFAR-NFRDI 2017). These existing laws are made for the safeguarding and conservation of the country's wildlife resources, as well as their habitats. Whoever acts against these laws may face corresponding penalties, imprisonment, and cancellation of fishing permits (for fisherfolks).

However, despite existing laws protecting marine wildlife, illegal trade persists, leading to overexploitation and decreased population. At present, seahorses are IUCN red-listed, where some of the species were assessed as "vulnerable" – including *H. comes*, *H. histrix*, *H. kuda*, *H. barbouri*, and *H. kellogi* – whereas a few were assessed as endangered such as *H. capensis* and *H. whitei* (IUCN SSC 2024). This concern implies the need to implement strict regulation and monitoring of illegal wildlife trade. DNA barcoding, a molecular method that utilizes DNA sequences obtained from a tiny particular region of genes for species identification and differentiation, provides baseline information for the conservation and safeguarding of seahorses, wildlife trafficking, and resource and habitat management in the country (Wang *et al.* 2020). The mitochondrial cytochrome c oxidase subunit I (MT-COI), a highly conserved gene, is utilized as the gene marker due to its high level of genetic variation, which satisfies the criteria for animal studies (Ajmal Ali *et al.* 2014). This study offers an understanding of the effectiveness and reliability of DNA barcoding as an instrument for identifying and monitoring the illegal trade of marine wildlife (Poniente *et al.* 2022; Asis *et al.* 2014), as well as the assessment of commercial exploitation of seahorses in different marketplaces in the country for TCM use.

MATERIALS AND METHODS

Sample Collection and Processing

Twenty-two (22) whole-dried seahorse samples were randomly purchased from different TCM stores, with prices ranging from PHP 150–200. Nine samples were bought in three different TCM stores in Binondo, Manila City, Metro Manila, the Philippines (14.60192°N, 120.97565°E), whereas the remaining 13 samples were purchased in two aquatic pet shops in Cartimar Pet Center, Pasay City, Metro Manila, the Philippines (14.55064°N, 120.996995°E) (Figure 1). The purchasing of samples was permitted by the Department of Agriculture through the Bureau of Fisheries and Aquatic Resources (BFAR), in line with the memorandum of agreement (MoA) established between the concerned institution and the authors of this paper. The collected samples were morphologically examined at the Molecular Biology Laboratory of Far Eastern University (FEU), Manila City, Metro Manila, the Philippines, whereas molecular identification procedures were performed at the National Fisheries Research and Development Institute's (NFRDI) Genetic Fingerprinting Laboratory, Quezon City, Metro Manila, the Philippines. Pursuant to the MoA between the DA-BFAR and FEU, the samples were submitted to the National Museum of the Philippines for proper archival storage and preservation.

Morphological Identification

Basic morphometrics were utilized to determine the samples' eye spines, nose spines, coronet height, body spine size, cheek spines, markings or spots on the body, snout length, and more, which are considered notable characteristics that aided in distinguishing each sample from one another (Wang *et al*. 2020). Moreover, the preliminary identification of the purchased dried seahorse samples was performed using the book of Lourie *et al*. (2004) titled "A Guide to the Identification of Seahorses."

Molecular Identification

Muscle tissue samples were taken from the seahorse's tubular snout and tail (in instances when snout tissue was no longer available) using the TIANamp Marine Animals DNA Kit (Tiangen Biotech Co., Beijing, China), following the manufacturer's specification. Alongside, the protocol of cetyltrimethylammonium bromide extraction was also utilized as an alternative method for DNA extraction from ethanol-preserved tissue samples that failed to produce a positive result from the first method (using a DNA kit) employed.

The amplification of the MT-COI gene of the dried seahorses was performed using the two combinations of primers by Ward *et al*. (2005) and four of Ivanova *et al*. (2007) (Table 1), on which reaction composition and corresponding volumes of the total reaction mix was based. In every amplification, 26 µL of the total mixtures were added to the microcentrifuge tube. The first master mix following the study of Ward *et al.* (2005) was composed of 12.3-µL nuclease-free water, 2.5-µL 10x PCR buffer, 2.5-µL 2-mM diluted deoxyribonucleoside triphosphate (dNTP), 2.5 µL 25 mM $MgCl_2$, 2 µL forward primer, 2 µL reverse primer, 0.2 µL Taq DNA polymerase, and 2 µL DNA template. On the other hand, the second master mix was put together according to Ivanova *et al.* (2007) having 6.5 µL nuclease-free water, 1.5 µL 10x PCR buffer, 1.0 µL 2 mM diluted dNTP, 1.5-µL 50-mM $MgCl_2$, 0.75-µL VF2_t1 forward primer, 0.75-µL FishF2_t1 forward primer, 0.75-µL FishR2_t1 reverse primer, 0.75-µL FR1d_t1 reverse primer, 0.5-µL Taq DNA polymerase, 10-µL 5X BSA, and 2-µL DNA template. The prepared PCR cocktails were subjected to corresponding conditions based on the primers used (Table 2).

Obtained amplicons were placed into 1% agarose gel stained with ethidium bromide, positioned in an electrophoresis chamber, and run within 15 min at 100V.

Figure 1. Samples were purchased from [a] Binondo, Manila, and [b] Cartimar Pet Center.

Table 1. Primers for the amplification genetic marker, COI.

Gene marker	Name of primer	Primer 5'-3'	References
COI	FishR1	5'-TAGACTTCTGGGTGGCCAAAGAATCA-3'	Ward *et al.* (2005)
	FishF2	5'-TCGACTAATCATAAAGATATCGGCAC-3'	Ward *et al.* (2005)
COI	FishF2_t1	5'-TGTAAAACGACGGCCAGTCGACTAAT CATAAAGATATCGGCAC-3'	Ivanova *et al.* (2007)
	VF2_t1	5'-TGTAAAACGACGGCCATGCAACCAAC CACAAAGACATTGGCAC-3'	Ivanova *et al.* (2007)
	FishR2 t1	5'-CAGGAACAGCTATGACACCTCAGGGT GTCCGAARAAYCARAA-3' 5'-CAGGAAACAGCTATGACACTTCAGGG TGACCGAAGAATCAGAA-3'	Ivanova *et al.* (2007)
	FR1d_t1	5'-CAGGAAACAGCTATGACACCTCAGGG TGTCCGAARAAYCARAA-3'	Ivanova *et al.* (2007)

Table 2. The primers and their corresponding PCR parameters used to amplify the COI.

Name of primer	Initial denaturation	Denaturation	Annealing	Extension	Cycles	Final elongation
FishR1 FishF2	94°C, 5 minutes in one cycle	94 °C, 1 min	50 °C, 1 min	72 °C, 1 min	45	72 °C, 5 min
FishF2_t1 VF2_t1 FishR2 t1 FR1d_t1	94°C, 2 min in one cycle	94 °C, 30 s	52 °C,40 s	72 °C, 1 min	38	72 °C, 10 min

DNA fragments were viewed under UV using the c200 Azure Biosystems C-Series Capture documentation system. A 100-bp DNA ladder was used to identify whether the correct fragment had been successfully amplified. The positively amplified PCR products were sent to 1st Base, Malaysia, for purification and sequencing. Subsequently, completed sequence chromatograms were informed by 1st Base through email for downloading, which were eventually subjected to editing, alignment, as well as DNA barcoding analysis.

The ab1 files received from 1st Base were aligned to generate the consensus sequences of each sample with the help of the Geneious R6.1 software. *Hippocampus* COI sequences were downloaded from GenBank and were used as reference data. *Syngnathus acus*, *Cosmocampus howensis*, and *Marouba perserrata* were used as the outgroups. The Basic Local Alignment Search Tool (BLAST), notably the nucleotide BLAST algorithm or BLASTn, was utilized to match the alignment, as well as the commonalities of the sample sequences with the recognized sequences in the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). MEGA v11 was utilized to create a phylogenetic tree using the neighbor-joining method based on the Kimura two-parameter distances with bootstrapping analysis performed using 1000 replicates (Poniente *et al.* 2022; Nishimaki and Sato 2019). Additionally, pairwise distance analysis and genetic divergence were assessed to identify base differences per nucleotide site and whether DNA barcoding yielded successful results.

RESULTS AND DISCUSSION

Identification Based on Morphological Data

Table 3 presents the 22 purchased seahorse samples, their sample codes, species identification, morphometric characteristics, and actual photographs. All samples were morphologically examined and identified. The analysis of the collected data revealed that 15 seahorse samples matched with *H. kuda*. These include 22BNSEA1, 22BNSEA2, 22BNSEA4, 22BNSEA5, 22BNSEA6, 22BNSEA7, 22BNSEA9, 22CTSEA10, 22CTSEA11, 22CTSEA12, 22CTSEA13, 22CTSEA14, 22CTSEA16, 22CTSEA20, and 22CTSEA22. Sample 22BNSEA7, with a height of 22.6 cm, is considered an exceptional specimen because it has exceeded the recorded height value in literature for *H. kuda* at 17.0 cm (Lourie *et al.* 2004). Meanwhile, sample 22CTSEA22 had a missing

Table 3. Identification of morphometric characteristics as basis of morphological analysis of the collected dried seahorse samples.

Sample code	Species	Morphometric characteristics	Photograph
22BNSEA1	*Hippocampus kuda* (morphologically identified only)	Height: 17 cm Head length (HL): 3 cm Snout length (SnL): 1.2 cm HL/SnL ratio: 2.5 cm Trunk rings: 11 Tail rings: 30 Rings supporting dorsal fin: 2 trunk rings and 1 tail ring Spines: low rounded bumps; 1 cheek spine and 1 eye spine Coronet: medium height, rounded, overhanging at the back, and with broad flanges	
22BNSEA2	*Hippocampus kuda* (morphological and molecularly identified)	Height: 12 cm Head length (HL): 2.4 cm Snout length (SnL): 1.2 cm HL/SnL ratio: 2.5 cm Trunk rings: 11 Tail rings: 30 Rings supporting dorsal fin: 2 trunk rings and 1 tail ring Spines: low rounded bumps; 1 cheek spine and 1 eye spine Coronet: medium height, rounded, overhanging at the back, and with broad flanges	
22BNSEA3	*Hippocampus histrix* (morphologically identified only)	Height: 10.1 cm Head length (HL): 2.4 cm Snout length (SnL): 1.2 cm HL/SnL ratio: 2 cm Trunk rings: 11 Tail rings: 31 Rings supporting dorsal fin: 2 trunk rings and 1 tail ring Spines: well-developed, extremely long and sharp; 1 cheek spine and 1 eye spine Coronet: medium height with sharp spines in front	
22BNSEA4	*Hippocampus kuda* (morphological and molecularly identified)	Height: 17.1 cm Head length (HL): 2.2 cm Snout length (SnL): 1.1m HL/SnL ratio: 2 cm Trunk rings: 11 Tail rings: 32 Rings supporting dorsal fin: 2 trunk rings and 1 tail ring Spines: low rounded bumps; 1 cheek spine and 1 eye spine Coronet: medium height, rounded, overhanging at the back, and with broad flanges	
22BNSEA5	*Hippocampus kuda* (morphological and molecularly identified)	Height: 15.7 cm Head length (HL): 2.8 cm Snout length (SnL): 1.4 cm HL/SnL ratio: 2 cm Trunk rings: 11 Tail rings: 32 Rings supporting dorsal fin: 2 trunk rings and 1 tail ring Spines: low rounded bumps; 1 cheek spine and 1 eye spine Coronet: medium height, rounded, overhanging at the back, and with broad flanges	

Table 3. Identification of morphometric characteristics as basis of morphological analysis of the collected dried seahorse samples.

Sample code	Species	Morphometric characteristics	Photograph
22BNSEA6	*Hippocampus kuda* (morphological and molecularly identified)	Height: 15 cm Head length (HL): 2.9 cm Snout length (SnL): 1.6 cm HL/SnL ratio: 1.81 cm Trunk rings: 11 Tail rings: 28 Rings supporting dorsal fin: 2 trunk rings and 1 tail ring Spines: low rounded bumps; 1 cheek spine and 1 eye spine Coronet: medium height, rounded, overhanging at the back, and with broad flanges	
22BNSEA7	*Hippocampus kuda* (morphological and molecularly identified)	Height: 22.6 cm Head length (HL): 3.6 cm Snout length (SnL): 1.8 cm HL/SnL ratio: 2 cm Trunk rings: 11 Tail rings: 33 Rings supporting dorsal fin: 2 trunk rings and 1 tail ring Spines: low rounded bumps; 1 cheek spine and 1 eye spine Coronet: medium height, rounded, overhanging at the back, and with broad flanges	
22BNSEA8	*Hippocampus comes* (morphological and molecularly identified)	Height: 15.2 cm Head length (HL): 2.2 cm Snout length (SnL): 1 cm HL/SnL ratio: 2.2 cm Trunk rings: 11 Tail rings: 31 Rings supporting dorsal fin: 2 trunk rings and 1 tail ring Spines: well-developed and sharp; 2 cheek spines and 1 eye spine Coronet: low with distinct knobs	
22BNSEA9	*Hippocampus kuda* (morphological and molecularly identified)	Height: 15.9 cm Head length (HL): 3.3 cm Snout length (SnL): 1.9 cm HL/SnL ratio: 1.74 cm Trunk rings: 11 Tail rings: 31 Rings supporting dorsal fin: 2 trunk rings and 1 tail ring Spines: low rounded bumps; 1 cheek spine and 1 eye spine Coronet: medium height, rounded, overhanging at the back, and with broad flanges	
22CTSEA10	*Hippocampus kuda* (morphologically identified only)	Height: 12.2 cm Head length (HL): 2.7 cm Snout length (SnL): 1.2 cm HL/SnL ratio: 2.25 cm Trunk rings: 11 Tail rings: 32 Rings supporting dorsal fin: 2 trunk rings and 1 tail ring Spines: low rounded bumps; 1 cheek spine and 1 eye spine Coronet: medium height, rounded, overhanging at the back, and with broad flanges	

Table 3. Identification of morphometric characteristics as basis of morphological analysis of the collected dried seahorse samples.

Sample code	Species	Morphometric characteristics	Photograph
22CTSEA11	*Hippocampus kuda* (morphological and molecularly identified)	Height: 13.5 cm Head length (HL): 2.7 cm Snout length (SnL): 1.6 cm HL/SnL ratio: 1.69 cm Trunk rings: 11 Tail rings: 31 Rings supporting dorsal fin: 2 trunk rings and 1 tail ring Spines: low rounded bumps; 1 cheek spine and 1 eye spine Coronet: medium height, rounded, overhanging at the back, and with broad flanges	
22CTSEA12	*Hippocampus kuda* (morphologically identified only)	Height: 13.2 cm Head length (HL): 2.5 cm Snout length (SnL): 1 cm HL/SnL ratio: 2.5 cm Trunk rings: 11 Tail rings: 31 Rings supporting dorsal fin: 2 trunk rings and 1 tail ring Spines: low rounded bumps; 1 cheek spine and 1 eye spine Coronet: low, rounded, overhanging at the back, and with broad flanges	
22CTSEA13	*Hippocampus kuda* (morphological and molecularly identified)	Height: 13.5 cm Head length (HL): 2 cm Snout length (SnL): 0.8 cm HL/SnL ratio: 2.5 cm Trunk rings: 11 Tail rings: 31 Rings supporting dorsal fin: 2 trunk rings and 1 tail ring Spines: low rounded bumps; 1 cheek spine and 1 eye spine Coronet: low, rounded, overhanging at the back, and with broad flanges	
22CTSEA14	*Hippocampus kuda* (morphological and molecularly identified)	Height: 16.5 cm Head length (HL): 3 cm Snout length (SnL): 1.5 cm HL/SnL ratio: 2 cm Trunk rings: 11 Tail rings: 30 Rings supporting dorsal fin: 2 trunk rings and 1 tail ring Spines: low rounded bumps; 1 cheek spine and 1 eye spine Coronet: medium height, rounded, overhanging at the back, and with broad flanges	
22CTSEA15	*Hippocampus kellogi* (morphologically identified only)	Height: 18.5 cm Head length (HL): 3 cm Snout length (SnL): 1.5 cm HL/SnL ratio: 2 cm Trunk rings: 11 Tail rings: 28 Rings supporting dorsal fin: 2 trunk rings and 1 tail ring Spines: low and rounded, blunt-tipped; 1 cheek spine and 1 eye spine Coronet: medium height with high plate in front	

Table 3. Identification of morphometric characteristics as basis of morphological analysis of the collected dried seahorse samples.

Sample code	Species	Morphometric characteristics	Photograph
22CTSEA16	*Hippocampus kuda* (morphological and molecularly identified)	Height: 14.5 cm Head length (HL): 2.3 cm Snout length (SnL): 1.3 cm HL/SnL ratio: 1.77 cm Trunk rings: 11 Tail rings: 34 Rings supporting dorsal fin: 2 trunk rings and 1 tail ring Spines: low rounded bumps; 1 cheek spine and 1 eye spine Coronet: low, rounded overhanging at the back, and with broad flanges	
22CTSEA17	*Hippocampus comes* (morphologically identified only)	Height: 13.2 cm Head length (HL): 2.1 cm Snout length (SnL): 1.1 cm HL/SnL ratio: 1.91 cm Trunk rings: 11 Tail rings: 32 Rings supporting dorsal fin: 2 trunk rings and 1 tail ring Spines: well-developed and sharp; 2 cheek spines and 1 eye spine Coronet: low with distinct knobs	
22CTSEA18	*Hippocampus comes* (morphologically identified only)	Height: 11.1 cm Head length (HL): 1.9 cm Snout length (SnL): 1.0 cm HL/SnL ratio: 1.9 cm Trunk rings: 11 Tail rings: 31 Rings supporting dorsal fin: 2 trunk rings and 1 tail ring Spines: spines: well-developed and sharp; 2 cheek spines and 1 eye spine Coronet: low with distinct knobs	
22CTSEA19	*Hippocampus comes* (morphological and molecularly identified)	Height: 11.6 cm Head length (HL): 2.2 cm Snout length (SnL): 1.0 cm HL/SnL ratio: 2.2 cm Trunk rings: 11 Tail rings: 32 Rings supporting dorsal fin: 2 trunk rings and 1 tail ring Spines: spines: well-developed and sharp; 2 cheek spines and 1 eye spine Coronet: low with distinct knobs	
22CTSEA20	*Hippocampus kuda* (morphological and molecularly identified)	Height: 13.2 cm Head length (HL): 2.9 cm Snout length (SnL): 1.5 cm HL/SnL ratio: 1.93 cm Trunk rings: 11 Tail rings: 35 Rings supporting dorsal fin: 2 trunk rings and 1 tail ring Spines: low rounded bumps; 1 cheek spine and 1 eye spine Coronet: low, rounded overhanging at the back, and with broad flanges	

Table 3. Identification of morphometric characteristics as basis of morphological analysis of the collected dried seahorse samples.

Sample code	Species	Morphometric characteristics	Photograph
22CTSEA21	*Hippocampus comes* (morphological and molecularly identified)	Height: 13.9 cm Head length (HL): 2.4 cm Snout length (SnL): 1.3 cm HL/SnL ratio: 1.85 cm Trunk rings: 11 Tail rings: 34 Rings supporting dorsal fin: 2 trunk rings and 1 tail ring Spines: spines: well-developed and sharp; 2 cheek spines and 2 eye spines Coronet: low with distinct knobs	
22CTSEA22	*Hippocampus kuda* (morphological and molecularly identified)	Height: 14.2 cm Head length (HL): Unavailable data Snout length (SnL): Unavailable data HL/SnL ratio: Unavailable data Trunk rings: 10 Tail rings: 33 Rings supporting dorsal fin: 2 trunk rings and 1 tail ring Spines: low rounded bumps; 1 cheek spine and 1 eye spine Coronet: low, rounded overhanging at the back, and with broad flanges	

snout when obtained. Even though it is an incomplete specimen, the other remaining morphometric traits and molecular analysis confirmed its identity as *H. kuda*.

On the other hand, samples 22BNSEA3 and 22CTSEA15 exhibit morphological characteristics of *H. histrix* and *H. kellogi*, respectively. 22BNSEA3 has well-developed, extremely long, sharp spines prominent in the eye, cheek, nose, and front of the coronet, whereas 22CTSEA15 exhibits low and rounded spines and has a distinct coronet. Remarkably, both species possess one cheek and one eye spine. Furthermore, the remaining five seahorse samples – namely 22BNSEA8, 22CTSEA17, 22CTSEA18, 22CTSEA19, and 22CTSEA21 – were identified as *H. comes*, which possess distinctive two cheek spines, low coronets, and narrow heads, making them easily recognized from the other seahorse species.

Identification Based on Molecular Data

Successful PCR amplification and sequencing are among the metrics used to assess DNA barcoding (Hou *et al.* 2015). Approximately 90.91% of the seahorse samples collected (20 out of 22) showed positive results in PCR amplification. This high success rate suggests that the DNA extracted from these samples was of good quality and sufficient quantity for the PCR reaction, even though the seahorse samples were dried and subjected to manufacturing procedures. Samples 22BNSEA1 and 22CTSEA18 – with 0.015 and 0.016 µg/µL concentration values, respectively, at lid factor 10 nano spectrophotometry – did not produce positive results, likely because these samples were relatively the most degraded among the collected samples. However, out of 20 positive amplicons, five of them failed to produce good-quality sequences – including samples 22BNSEA3, 22CTSEA10, 22CTSEA12, 22CTSEA15, and 22CTSEA17. Therefore, only 15 dried seahorse samples have viable molecular data that were utilized for further analysis.

Dried seahorse samples may yield unfavorable results due to DNA deterioration brought on by several circumstances – including storage conditions, manufacturing procedures, and preservation techniques (Hou *et al.* 2015; Wang *et al.* 2020). Additionally, dried seahorses sold in TCM markets frequently undergo various production processes – including washing, boiling, and drying – which can adversely affect the quality and amount of DNA extracted from the samples. Furthermore, preservation techniques and storage conditions may also influence DNA degradation (Hou *et al.* 2015).

The constructed phylogenetic tree showed five *Hippocampus* species groups: *H. kuda*, *H. reidi*, *H. fuscus*, *H. whitei*, and *H. comes* (Figure 2). Specifically, the tree revealed that samples examined belonged to two groups *H. kuda* and *H. comes*, supported with 92 and 100% bootstrap values, respectively. Twelve (12) samples were identified as *H. kuda* – namely 22BNSEA2,

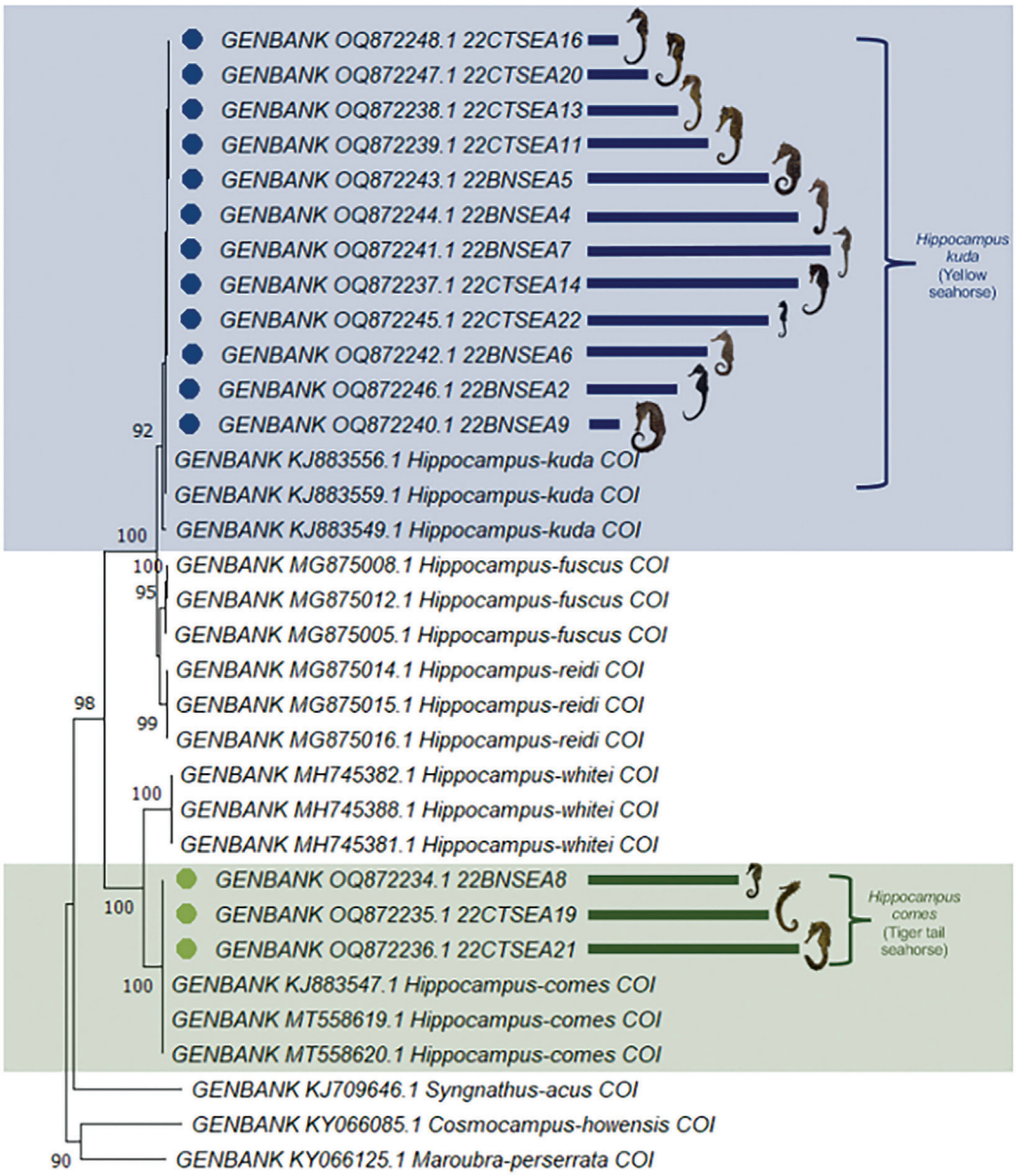

Figure 2. Neighbor-joining tree of seahorse samples deduced from COI sequences with 1000 bootstrap replicates.

22BNSEA4, 22BNSEA5, 22BNSEA6 22BNSEA7, 22BNSEA9, 22CTSEA11, 22CTSEA13, 22CTSEA14, 22CTSEA16, 22CTSEA20, and 22CTSEA22 – whereas the remaining three samples were clustered under *H. comes*, which are 22BNSEA8, 222CTSEA19, and 22CTSEA21. Moreover, the pairwise analysis revealed that samples 22BNSEA2, 22BNSEA4, 22BNSEA5, 22BNSEA6 22BNSEA7, 22BNSEA9, 22CTSEA11, 22CTSEA13, 22CTSEA14, 22CTSEA16, 22CTSEA20, and 22CTSEA22 have genetic distance ranging from 0.000–0.012 to *H. kuda*, whereas samples 2BNSEA8, 22CTSEA19, and 22CTSEA21 have genetic distances of 0.000 to *H. comes*. Hebert *et al.* (2003) noted that a genetic difference value of greater than three percent is the threshold for species recognition on whether it belongs to the same species or not.

Morphology-based identification of dried marine samples can be challenging due to the limitations of morphological characters. In many cases, these samples may have undergone changes in shape, color, and texture during the drying process, making it difficult to identify them based on morphological features alone (Hou *et al.* 2015). DNA barcoding offers an alternative approach that relies on the analysis of DNA sequences that are less subject to changes caused by environmental factors. The COI gene is widely

used in DNA barcoding because it has several advantages, including conserved regions and sufficient sequence variation among closely related species (Wang *et al*. 2020). COI has been successfully used to identify various animal species, including marine invertebrates. However, while DNA barcoding has proven to be a valuable tool for species identification, it also has some limitations.

The success of DNA barcoding relies on the availability of reference sequences for comparison, and in some cases, the reference sequences may not be available (Hou *et al.* 2015). In the case of seahorses, the availability of GenBank sequences has been steadily increasing, making DNA barcoding a viable option for their identification. Several studies have used DNA barcoding to identify seahorses, including *H. trimaculatus*, *H. spinosissimus*, and *H. kelloggi* (Zeng *et al.* 2019), as well as *H. kuda* and *H. comes* (Nurilmala *et al*. 2020). These studies utilized COI sequences from GenBank as reference sequences for species identification. However, Hebert *et al.* (2003) stated that DNA barcoding can be limited by intraspecific variation, which can result in the misidentification of individuals within the same species. Thus, they acclaimed that morphological data and molecular forensics obtained through DNA barcoding are complementary and can be used together to improve species identification. Morphological data provides valuable information on the external features of the organism. In contrast, molecular data can be used to identify cryptic species or to confirm the identity of a morphologically ambiguous specimen (Hou *et al*. 2015).

Prevalence of Seahorse as TCM in Metro Manila

TCM has played a vital role in the Philippines' healthcare system because of its diverse therapeutic capabilities and affordability. However, it is also evident that at the expense of the high demand for TCM products, a decrease in the population of living organisms used for TCM would also decline (Sy and Melgar 2022; Law 2021; Rosa *et al.* 2013). Among these living organisms are seahorses that are popularly used as an agent for curing several diseases, resulting in an excessive decrease in their population due to overexploitation and smuggling. The illegal trade of CITES-listed species of seahorses in the Philippines remains rampant despite the laws to protect it, including RA 9147 (Wildlife Resources Conservation and Protection Act) and RA 8550 (The Philippine Fisheries Code of 1998), as amended by RA 10654 (Foster *et al*. 2019). It was also mentioned by Sy and Melgar (2022) that, despite the governing laws inclined toward conservation for the mentioned species, the problem in the Philippines is that although there are penalties on the stated national laws, implementation of the penalty for violators is either not observed or are penalized less severely, resulting into a lower monetary fine and a shorter jail sentence. This circumstance implies that conservation efforts for the endangered, threatened, and protected marine species remain ineffective and disregarded due to poor management and policy implementation, specifically in marine protected areas, as well as no-take reserves (Zhang and Vincent 2019).

Moreover, the findings of this study revealed the prevalence rate of illegally traded dried seahorses from different TCM stores in Binondo, Manila, and aquatic pet shops in Cartimar Pet Center, Pasay City. Based on the assessment, most of the seahorse species purchased in different TCM stores of Binondo were *H. kuda* (seven out of nine), having a prevalence rate of 78%, whereas both *H. comes* (one out of nine) and *H. histrix* (one out of nine) had a prevalence rate of 11%. On the other hand, in the aquatic pet shops of Cartimar Pet Center, *H. kuda* (eight out of 13) had the highest prevalence of 62%, followed by *H. comes* (four out of 13), which had a 31% prevalence rate, then *H. kellogi* (one out of 13), having the lowest prevalence rate of 7%. Considering this as solid evidence, the illegal trade of marine wildlife persists in the country. Additionally, the identified seahorse species illegally sold in said local markets were identified as vulnerable, which undoubtedly poses a huge concern to their population. Thus, the Philippines should take efficient action toward conserving seahorses by paying more attention to fisheries, trade, and conservation. Furthermore, the country should also focus on providing better policy interventions and sustainable management frameworks in the promotion of the conservation and protection of marine wildlife.

CONCLUSION

DNA barcoding provides solid experimental and theoretical foundations for species identification. This tool is vital to promote the conservation, management, and formulation of laws concerning seahorses in the Philippines. The study has also noted the persistent illegal trading of dried seahorses across Metropolitan Manila, causing the risk of species overexploitation and having detrimental effects on marine biodiversity. In this regard, the study suggests conducting further investigation into the trading patterns and sites where dried seahorses are commercially sold, including but not limited to TCM stores and black markets in the Philippines. It is also important to identify the primary sources for the collection of live or fresh seahorses. Moreover, future studies may focus on examining other threatened species used as TCM, like sea dragons and manta and devil rays, to provide baseline information that could support the conservation and protection of the country's marine wildlife. Furthermore,

the study also recommends a more in-depth review of existing policies to promote strict implementation of regulation and monitoring of wildlife trade.

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NOTES ON APPENDICES

The complete appendices section of the study is accessible at https://philjournal.dost.gov.ph

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