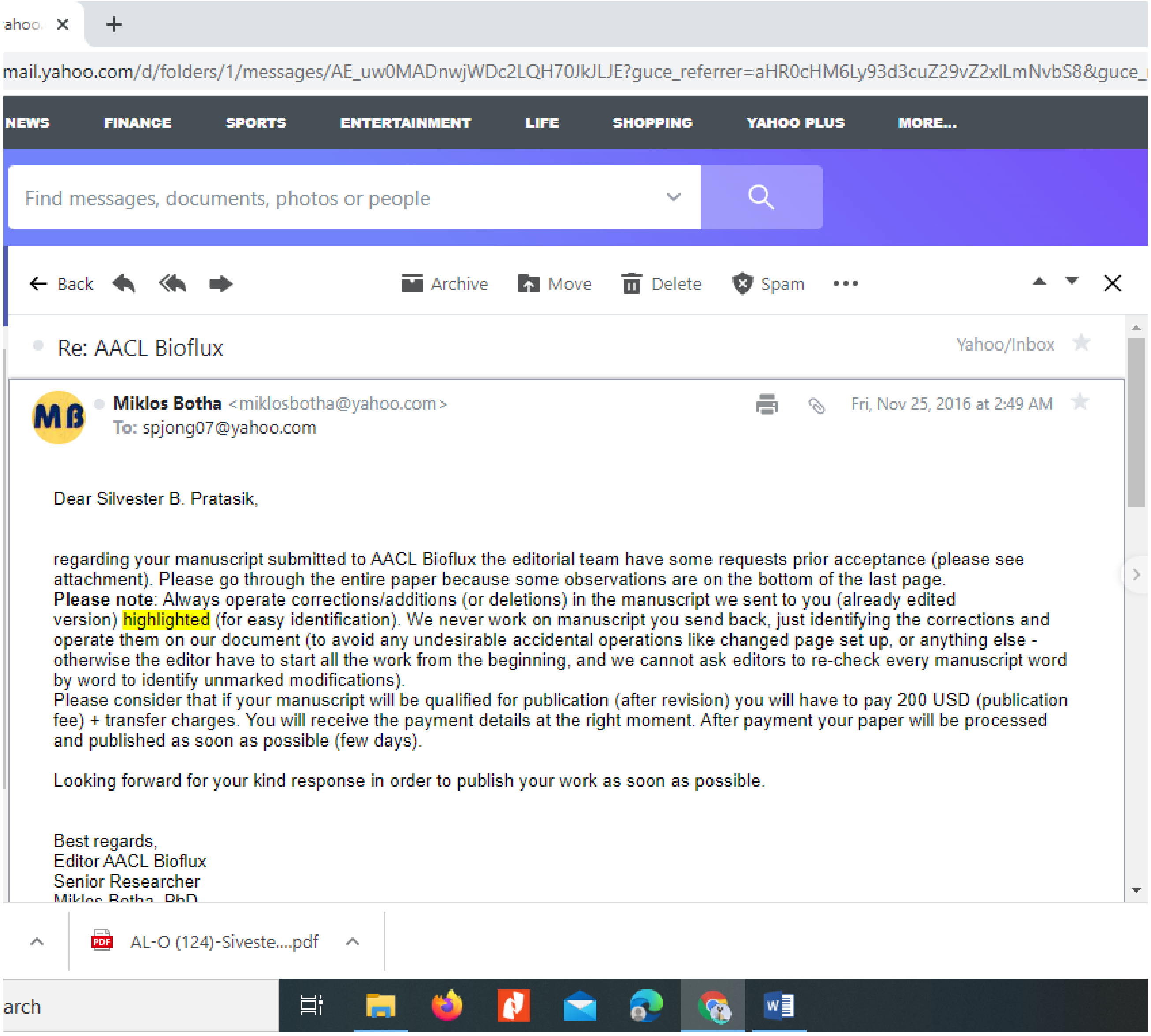

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Miklos Botha <miklosbotha@yahoo.com>
To: spjong07@yahoo.com
Fri, Nov 25, 2016 at 2:49 AM

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Miklos Botha, PhD

Korespondensi Awal Editor

Mitochondrial CO1 Genetic Marker-Based Species Diversity of Cuttlefish (Cephalopod; Mollusk) in Manado Bay and Lembeh Strait, North Sulawesi, Indonesia

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ABSTRACT

This study determines cuttlefish species from Manado Bay and Lembeh Strait, North Sulawesi, based upon the mitochondrial CO1 genetic marker. Sample collection used SCUBA gear and fishermen catch. Seven cuttlefish specimens were collected in this study. DNA genomes of the samples were extracted, and CO1 gene fragments were amplified through PCR and sequenced using Big Dye© terminator chemistry (Perkin Elmer). Species identification was conducted with BLAST method. Results showed that there were four species of cuttlefish in Manado Bay and Lembeh Strait, *Sepia latimanus*, *S. pharaonis*, *Metasepia tullbergi*, and *Sepia ramani*. *S. latimanus* was represented by 4 individuals, and the rests by only one individual**.** Phylogenetic analysis revealed that all samples were separated into 4 species groups, SP1, SP3. SP4 and SP10 *(group1)*, SP5 *(group2)*, SP6 *(group3)* and SP11 *(group4)*. All species belong to important fisheries resources, but they are in insufficient data status, both fishing rate and population level.

Keywords: cuttlefish, Manado Bay, Lembeh Strait, CO1 gene.

Introduction

Manado Bay has become an income source of local communities from fishing activities and marine tourism activities, such as swimming, diving, and other water sports. It is also used for marine transport medium to reach other islands northward, such as Bunaken, Manado Tua, Siladen, Mantehage, and Naen Islands. Lembeh Strait is also a unique marine environment that has long attracted numerous underwater adventurers. Both places are holding high marine biodiversity of either economic or ecological benefit.

Cuttlefish are one of the cephalopods living in this waters and known as short-lived animals, about 1-2 years (NOVA 2007; Smith 2011). Their meat contains high amount of calcium, protein, and is low in energy, being the main meal in Mediterranian and East Asia regions (Reid et al 2005). Species diversity of cuttlefish is very high with more than a hundred species recorded. Their distribution and abundance are highly affected by the availability of suitable habitats and preys, particularly for small individuals (Neves et al 2007; Neves et al 2009). Some species live in the water deeper than 400 m, such as *Sepia australis*, *S. elegans*, *S. orbignyana* and *S. hieronis*, and some species occupy shallow waters, such as *S. latimanus*, *S. officinalis* and *S. pharaonis* (Reid et al 2005).

Cuttlefish (and cephalopods in general) are known to have diverse body patterns that can immediately change naturally controlled by its chromatophore system (Hanlon & Messenger 1996; Hanlon 2011). This cephalopod is well known as the most intelligent marine biota with the largest body-brain ratio of all invertebrates (NOVA 2007). Therefore, many studies have focused on the body pattern alterationrelated behavioral aspects to find food and avoid predation (Shohet et al 2006; Barbosa et al 2008; Kelman et al 2008; Allen et al 2009; Chiao et al 2010; Barbosa et al 2011).

According to Reid et al (2005), it is so far known that several species inhabit Indonesian waters, *Sepia bandensis, S. brevimana, S. latimanus, S. papuensis, S. pharaonis, S. sulcata, S. kiensis, S. senta, Sepiella inernus* dan *S. weberi*. For a number of years, almost no studies have been done on cuttlefish in these waters. Only one study was done on *Idiosepius pygmaeus*, small cuttlefish in Lombok waters (von Byern & Klepal 2007) that provided morphological data of *Idiosepius pygmaeus* from Indonesia and Thailand waters and their habitat conditions in order to determine the population status of the species. Low interest in cuttlefish study could result from sample collection problems as a result of no cuttlefish fisheries in Indonesia, and all cuttlefish catches are bycatch of other fisheries. The cuttlefish catch generally comes from artisanal fisheries, and the animals are exploited by fishermen in the coral reefs using handline or speargun. Species inventory from North Sulawesi waters is not also available yet, and therefore, their taxonomic work is needed**.**

Species identification is a starting point of bioecological studies, and correct species determination is fundamental to ecological studies. However, the systematics of genus *Sepia* is unclear. Morphological evidence has caused *S. officinalis* be assumed as subgenus *Sepia* sensu stricto and *S. orbignyana* Ferussac 1826 and *S. elegans* Blainville 1827 as subgenus *Rhombosepion* (Sanjuan et al 1996). Problems in species identification using morphological characteristics are also detected (Eyualem & Baxter 2003; Martinez-Arce 2009) due to failures in providing sufficient taxonomic solution and requiring good taxonomic expertise.

Genetic approach is an alternative method for accurate species identification. Short DNA sequence of mitochondrial genome standard area could be taken as DNA barcode for species identification (Hebert et al 2004). CO1 is the most common mitochondrial DNA gene marker used for species identification (Folmer et al 1994) due to belonging to the most conservative gene among the mitochondrial DNA protein coding genes. It has become a standard tool of molecular taxonomy and identification (Ratnasingham & Hebert 2007). Partial mitochondrial Cytochrome C Oxidase subunit 1 gene (CO1-648pb) could act as DNA barcode for animal species identification due to easily isolated and giving good resolution for animal species identification (Hebert et al 2003).

This study was aimed at discerning the cuttlefish species from Manado Bay and Lembeh Strait waters, North Sulawesi, using CO1 gene marker and providing some information on their fisheries status in North Sulawesi.

METHOD

Sample collection

Cuttlefish samples were collected in Manado Bay and Lembeh Strait waters, North Sulawesi, using SCUBA gear and fishermen catch. In this study, seven cuttlefish specimens were collected, and all samples were preserved in 95% ethanol and stored at room temperature before DNA extraction.

Extraction, PCR, and Sequencing

Genome DNA extraction of all samples used innuPREP DNA Micro Kit (Analytic Jena). The CO1 gene was amplified applying universal primer pairs

LCO1490: 5’-ggtcaacaaatcataaagatattgg-3’ and HCO2198: 5’-taaacttcagggtgaccaaaaaatca-3’ (Folmer et al 2004). Polymerase chain reaction (PCR) was carried out in 35 cycles at 95°C (30 sec.), 50°C (30 sec.), 72°C (50 sec.). The PCR product was visualized in 1% (b/v) agarosa gel electrophoresis. Bi-directional sequencing was done by First Base CO (Malaysia) using Big Dye© terminator chemistry (Perkin Elmer).

Data Analysis

The chromatogram obtained was edited using Geneious v5.6 (Drummond et al 2012). The sequences were then compared with GenBank data using BLAST (Basic Local Alignment Search Tools) method (Altschul et al 1997) and BOLDSystems (Ratnasingham & Hebert 2007). The phylogenetic tree was built using Neighbor-Joining Method (Saitou & Nei 1987). Similarity index was also calculated.

Results and Discussion

PCR outcomes were separated using 1% agarose gel electrophoresis and showed by amplification of 700 bp band (Figure 1).

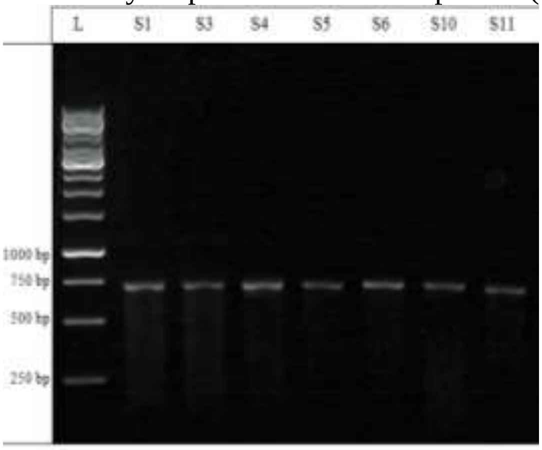

Figure 1. CO1 gene amplification of cuttlefish samples from Manado Bay and Lembeh Strait on 1% agarose gel. L is GeneRuler, Thermo Scientific as marker. S1-S11 is sample numbers.

Species Identification

The cuttlefish species recorded in this study are shown in Table 1. A total of 7 specimens were obtained through dive collection and speargun fishing. All collected samples consisted of 4 different species, *Sepia latimanus*, *Metasepia tullbergi*, *S. pharaonis*, and *S. ramani*. *S. latimanus* was represented by 4 individuals (SP1, SP3, SP4, and SP10), while *M. tullbergi*, *S. pharaonis* and *S. ramani* were only represented by one individual. Those belong to 2 genera, *Sepia* and *Metasepia*, meaning that cuttlefish in Manado Bay and Lembeh Strait are generally dominated by genus *Sepia*, family Sepiidae.

Table 1. Identification of cuttlefish in Manado Bay and Lembeh Strait using blast method.

Sample	Species Identified	Query cover (%)	Maximum Identity (%)	Access Code

SP1	*Sepia latimanus*	96	88	APO13074
SP3	*Sepia latimanus*	96	88	APO13074
SP4	*Sepia latimanus*	96	88	APO13074
SP5	*M. tullbergi*	91	86	HQ846120
SP6	*Sepia pharaonis*	97	86	HMI164538
SP10	*Sepia latimanus*	98	88	APO13074
SP11	*Sepia ramani*	97	92	HM164529

Phylogenetic analysis showed that all samples were grouped into 4 different clusters, (1) SP1, SP3, SP4 and SP10, (2) SP5, (3) SP6, and (4) SP11, respectively (Figure 2).

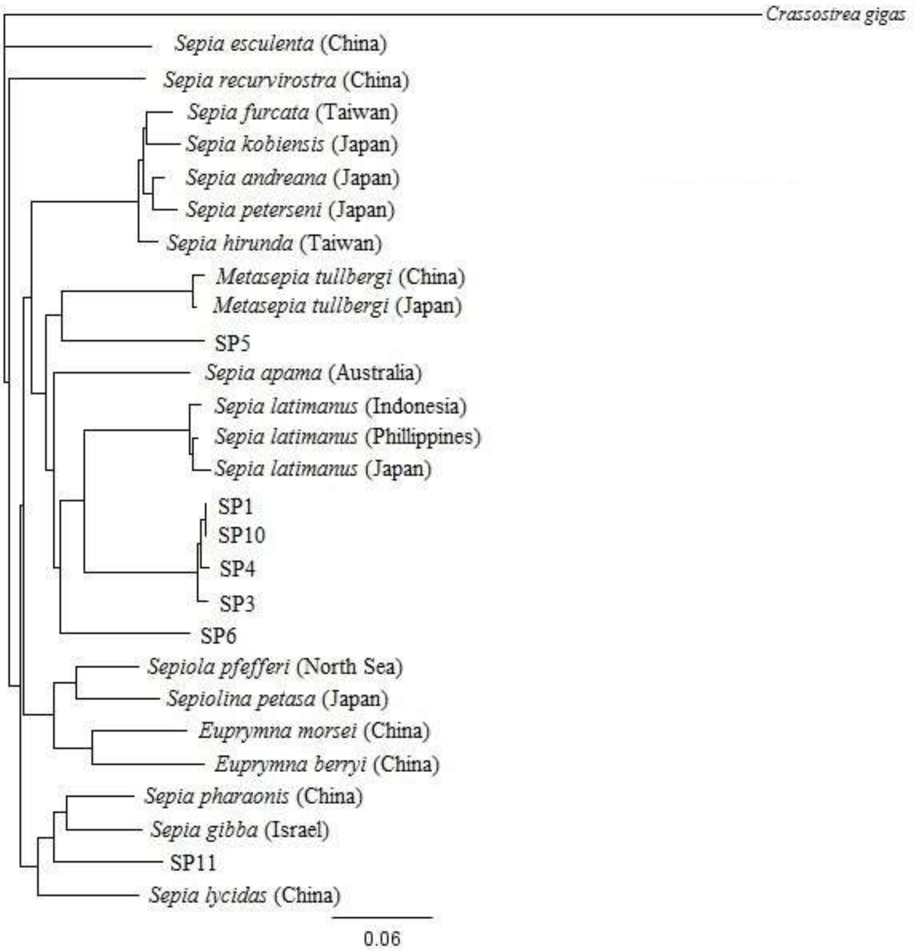

Figure 2.Phylogenetic position of the cuttlefish from Manado Bay and Lembeh Strait based on neighbor joining analysis.

Crassostrea gigas was a representative of bivalve used as comparison from outgroups to better clarify the genetic kinship between the cuttlefish specimen and the genbank data. All samples, but SP6, were in the same group as each species group indicating that they belong to the same reference species of the genbank data. This finding also shows that the same species inhabiting Manado Bay and Lembeh Strait waters reflects very strong kinship. SP1, 3, 4 and 10 are in the group of *Sepia latimanus*, but only SP1 and SP10 have 100% similarity compared with SP3 and SP4, while SP3 and SP4 have similarity level range of 98.9% to 99.2%. It reflects that there are genetic variations among the same species inhabiting Manado Bay and Lembeh Strait waters. Nevertheless, SP1, 3, 4 and 10 possess lower similarity to *S. latimanus* from other Indonesia waters, the Philippines and Japan listed in the genbank, 86.9-88%, indicating that the kinship could be affected by geographic distance and aquatic environmental conditions of different locations worldwide. *S. latimanus* is the second largest cuttlefish species after *Sepia apama* (Reid et al 2005). This species can grow to 50 cm mantle length and reach 10 kg body weight, even though this maximum individual size was not found in this study, indicating that their population is declining. Recent status of *S. latimanus* is in the category of data insufficient (IUCN 2014). In Indonesia, particularly North Sulawesi, its fisheries status is also unclear due to no cuttlefish fisheries in this area. All cuttlefish catches were obtained from speargun fishing in the coral reefs by taking advantages of their spawning migration, and mostly unreported. This species is widely distributed in the Indo-Pacific from Andaman Sea, the east to Fiji, and the south to the north of Australia (Reid et al 2005). The species is common in the coral reef up to 30 m deep (Norman 2000). In Manado Bay and Lembeh Strait, *S. latimanus* goes to shallow waters around 3-4 m deep and use the branching corals to lay eggs.

SP5 identified as *Metasepia tullbergi* in blast method occurred at the same group as *M. tullbergi* from China and Japan recorded in the genbank even though they had only 86% similarity level (Appendix 1). This comparison, however, has confirmed that SP5 collected in this study is *M. tullbergi*. This species is a small-sized cuttlefish species inhabiting neritic demersal zone. This species was usually found in sandy, muddy and stony substrate waters in continental shelf about 100 m (Norman 2003; Reid et al 2005). Present observations in Lembeh Strait and Manado Bay waters demonstrated that they could be found on sandy bottom at 18-20 m deep. This species is widely distributed in the Indo-Pacific (Reid et al 2005). Beside Indonesian waters, the species was recorded in Brunei Darussalam, Camboja, China, Japan, North and South Korea, Malaysia, Phillippine, Singapore, Taiwan, Thailand, and Vietnam, respectively (Barratt & Allcock 2012).

M. tullbergi has been used as part of public fisheries, and because of its bright colors, the species could be taken for aquarium fish trade (Reid et al 2005). This species belongs to insufficient data category as well (Barratt & Allcock 2012). Although there is high fishing pressure in some areas, no fishing rate data is available. This species occurrence in North Sulawesi waters, such as Lembeh Strait and Manado Bay, has highly supported the underwater tourism activities (personal observations). In Lembeh Strait, they were found in about 12-20 m deep, while in Manado Bay, this species was recorded at 18 m deep.

SP6 has the identity of *Sepia pharaonis* through blast method (Table 1). It, however, is widely separated from *S. pharaonis* of China and sufficiently close to *S. latimanus* (Figure 2). SP6 has also lower similarity level to *S. pharaonis* of China (85%) than *S. latimanus* of Japan, Philippines and Indonesia (86%-87%) (Appendix 1).

Based on similarity level, SP6 has only 85% similarity to *S. pharaonis* from China and *S. kobiensis* from Japan, but lower than other genus members compared in this study, except that *S. recurvirostra* and *S. esculenta* (83%) from China (Appendix 1). It could result from low availability of comparison data in the genbank. Nevertheless, its phylogenetic position is far from *S. pharaonis* of the genbank, indicating that SP6 could be different species. Its closeness to *S. latimanus* in the phylogenetic tree could also occur since SP6 is probably part of complex species of *S. latimanus*, and therefore, this study suggested further taxonomic test on SP6.

Sepia pharaonis has maximum dorsal mantle length (DML) of 420 mm and maximum weight of 5 kg (Carpenter & Neim 1998). This species is distributed in south and east China Sea, Indonesia, Malaysia, Philippines, north Australia, Suez Bay, Zanzibar, Madagascar to Arabian Sea. In Manado Bay, our study found that this species, especially juvenile, about 5 cm DML, was active at night. They were found between coral branches, reflecting that coral physical structure plays important role in providing shelter to young cuttlefish. This habit is related with their strategy to avoid predators and search for food. The same behavior was also recorded by FAO (Reid et al 2005).

SP11 possesses the identity of *Sepia ramani*, and the blast method has positioned it in the same group as *S. pharaonis* (China), *S. gibba* (Israel), and *S. lycidas* (China) (Figure 2). It is supported by intra- and interspecific similarity level, in which the similarity ranges from 83.1% to 90%, with the highest shown by *S. pharaonis* (China), followed by *S. gibba* (Israel), and *S. lycidas* (China). Strong kinship between *S. pharaonis* and SP11 supports the FAO morphological identification (Carpenter & Neim 1998; Reid et al 2005) that *S. ramani* is a complex species of *S. pharaonis.* With other comparison species of genus Sepia, the similarity level ranges from 88.3% to 89.2% (Appendix 1). Nevertheless, the phylogenetic analysis on 50 specimens collected from all geographic distribution of *S. pharaonis* (Anderson et al 2010) also found that the species was a complex one of 5 or 6 sub-groups of *S. ramani*, depending upon the taxonomic status of *S. ramani* and suggested more morphological and morphometric studies to support the genetic data.

Sepia ramani was described as new species from southeast Indian waters (Neethiselvan 2001). The difference between both species is that *S. ramani* has a longer club with 15-24 subequal large suckers, while *S. pharaonis* has 6 large medial suckers, 3 or 4 of them are much bigger than the rests. *S. ramani* has also 14-16 transverse rows of normal-sized suckers at the proximal edge of hectochotylized arm, while *S. pharaonis* has only 10–12 rows. Other characters of both species are very similar. *S. ramani* was reported from Indian Ocean along south Indian coast and around Sri Lanka at the depth of about 100 m and could grow up to 375 m DML (Reid et al 2005). *Sepia ramani* and *S. Prabahari* are very similar to *S. pharaonis*, but these 3 species have been numerously exploited in the southeast (Reid et al 2005; Anderson et al 2010). Our study revealed that in Manado Bay, small *S. ramani* was found at around 6 m depth on sandy bottom, and this species is firstly reported from Indonesia waters. It reflects that there are possibly more cuttlefish species living in this waters, so that more research activities are needed in order to reach more habitat types, and therefore, more sufficient equipment are required. This species did not become fishing target of the speargunners since bigger individuals were not encountered in the shallow waters.

Variations in interspecific similarity level reflect that there are different genetic characteristics within *S. latimanus,* collected along Arakan to Lembeh Strait waters, and these variations show wider genetic difference with geographic distance. Similar situation is also exhibited in different cuttlefish species collected in this study. Based on the phylogenetic analysis, SP6 identified as *S. pharaonis*, is, in fact, separated from the same species of China and closer to *S. latimanus*. It reveals that genetic characteristics held by SP6 are different from that of *S. latimanus* or *S.*

pharaonis (Appendix 1). Thus, a detail study on SP6 is needed to gain better explanation through combination of supporting taxonomic methods. Nevertheless, the use of morphological and molecular methods in this study has revealed supporting direction in recognizing the cuttlefish species, particularly from North Sulawesi waters.

Furthermore, new record of the cuttlefish reported from Indonesia waters, such as *S. ramani* (SP11), and difference shown by SP6 and *S. pharaonis* (genbank) between blast method and neighbor-joining analysis have revealed that Indonesian Sea, North Sulawesi in particular, with high habitat diversity, still holds a lot of undescribed cuttlefish species. This study limited our observations to the shallow water habitats reachable by scuba dives, while the cuttlefish distribution can reach 200 m deep. Thus, the use of better equipment to maximize the number of cuttlefish specimens will be able to enrich the information, especially in relation with more species collections. All the cuttlefish species recorded in this study belong to important fisheries, but they are in the status of insufficient data, both fishing rate and population level, despite high fishing pressures, so that their population is easily threatened to extinct. In Indonesia, particularly North Sulawesi, where there is no cuttlefish fisheries, the catch data would also not be possible to obtain. In addition, habitat destructions in the coastal areas, such as coral reefs and seagrass beds, supporting their early stage of life could also accelerate their extinction.

Conclusions.

Acknowledgement

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Appendix 1. Similarity Index

No.	Sample Name	Similarity Level (%)																											
		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	27	28
1	SP1	100																											
2	SP3	98.8	100																										
3	SP4	99.2	98.9	100																									
4	SP5	83.4	83.1	83.1	100																								
5	SP6	85.1	84.9	84.9	85	100																							
6	SP10	100	98.8	99.2	83	85	100																						
7	SP11	83.2	83.2	83.4	82	84	83	100																					
8	*Sepia furcata* (Taiwan)	83.7	83.5	83.5	84	85	84	85.7	100																				
9	*Metasepia tullbergi* (China)	83.6	82.2	81.4	86	85	82	84	83.5	100																			
10	*Sepia apama* (Australia)	85.1	84.9	84.6	84	86	85	83.1	84.9	85	100																		
11	*S. hirunda* (Taiwan)	83.8	83.7	83.7	85	86	84	86.3	96.8	85	85.4	100																	
12	*Metasepia tullbergi* (Japan)	81.6	82.2	81.4	86	85	82	84.6	83.8	99	85.4	85	100																
13	*S. latimanus* (Indonesia)	87.5	87.7	87.7	85	87	88	84.9	83.4	84	85.4	84	84	100															
14	*S. esculenta* (China)	83.4	83.7	83.5	80	83	83	85.5	84.5	83	83.2	85	83	81.9	100														
15	*S. andreana* (Japan)	83.4	83.2	83.2	85	86	83	85.4	97.6	84	85.4	98	84	83.5	84.5	100													
16	*Sepiola pfefferi* (North Sea)	86	86.1	86	85	85	86	86.4	85.7	84	85.1	86	85	84.9	84.9	85.4	100												
17	*Sepia latimanus* (Philippine)	87.8	88	88	85	87	88	84.9	83.4	84	85.5	84	84	99.1	82.5	83.5	84.9	100											
18	*Sepiolina petasa* (Japan)	82.9	83.1	82.6	85	84	83	86	86.3	84	84.1	87	85	83.1	82.9	86.4	91.9	83	100										
19	*Sepia latimanus* (Japan)	86.7	86.9	86.9	84	86	87	84.9	82.6	84	85.2	84	84	98	82	82.8	84.5	99	83	100									
20	*S. pharaonis* (China)	85.2	84.9	85.1	84	85	85	90.2	86.4	85	85.8	87	85	85.4	86.3	8.72	88	85	86	85	100								
21	*Crassostrea gigas*	60.9	60.6	60.9	60	59	61	59.1	59.7	59	60.4	60	59	58.8	60	59.7	61	59	61	58	60	100							
22	*Sepia peterseni* (Japan)	82.8	82.6	82.6	85	86	83	85.4	96.2	84	84.9	97	84	82.9	84.1	98	85.2	83	86	82	86.9	59.2	100						
23	*S. gibba* (Israel)	85.1	84.8	84.6	85	86	85	89.2	86.3	84	83.8	87	85	84.5	86.1	87.2	87.5	85	87	84	92.4	58.9	86	100					
24	*S. lycidas* (China)	82.9	82.8	82.5	85	86	83	88.3	85.1	85	84.9	86	86	84.9	85.7	85.7	87.3	85	87	85	89	59.2	85	89.3	100				
25	*S. kobiensis* (Japan)	83.7	83.2	83.5	84	85	84	84.8	96.6	83	85.1	96	83	82.5	84.1	96.8	85.2	83	86	82	86.4	59.5	96	86.7	85.1	100			
26	*Euprymna morsei* (China)	84.1	83.6	84.1	83	82	84	84.3	83.4	82	84.1	84	83	84	84.3	83.5	88.7	84	87	84	86.4	58.8	83	85.5	84.9	82.6	100		
27	*E. berryi* (China)	82.9	83.4	82.9	84	82	83	83.2	83.5	82	81.7	85	82	82.9	82.2	83.2	88.9	83	86	83	83.7	58.8	83	84.8	84.6	83.5	89.3	100	
28	*Sepia recurvirostra* (China)	82.5	82.5	82.5	84	83	83	86	84.3	83	82.8	86	84	81.4	85.1	85.2	86	82	86	82	85.5	60.1	84	86.6	87.3	84.1	84	82.9	100