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Short Communication: DNA barcoding reveals vulnerable and not evaluated species of sea cucumbers (Holothuroidea and Stichopodidae) from Kepulauan Seribu reefs, Indonesia

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Abstract. *Madduppa H, Taurusman AA, Subhan B, Anggraini NP, Fadillah R, Tarman K. 2017. DNA barcoding reveals vulnerable and not evaluated species of sea cucumbers (Holothuroidea and Stichopodidae) from Kepulauan Seribu reefs, Indonesia. Biodiversitas 18: 893-898.* DNA Barcoding is a tool in the molecular taxonomy which allows a rapid and precise identification. This tool is needed to mitigate difficulties in identifying species of sea cucumbers in Indonesia. This study was conducted to reveal species diversity in sea cucumbers (Holothuridae and Stichopodidae), commonly harvested in the Kepulauan Seribu reefs, northern Jakarta, Indonesia, by using mitochondrial DNA. Neighbor-joining phylogenetic trees were reconstructed using the Kimura-2 parameter with 1000 of bootstrap values. The genetic distance within and between species was investigated. Conservation and trade status of the species were determined using IUCN and CITES, respectively. DNA barcoding using mitochondrial Cytochrome oxidase 1 (COI) revealed 7 species from 96 samples, of which 4 species belonged to the Stichopodidae (*Stichopus herrmanni*, *Stichopus ocellatus*, *Stichopus horrens*, *Stichopus monotuberculatus*), and 3 species to the Holothuridae (*Bohadschia bivittata*, *Actinopyga lecanora* and *Holothuria leucospilota*). Phylogenetic analysis showed that the two families were separated with a high bootstrap value. The neighbor-joining tree supported the result of identification of the sea cucumber species. The ingroup haplotypes were clustered into four main clades. *Stichopus herrmanni* and *S. ocellatus* were identified as closely related, which matches their morphological characteristics. *Bohadschia bivittata* is the most distinct species from other species due to the formation of a separate clade in the phylogenetic tree. S*tichopus. horrens* and *S. monotuberculatus* were also identified as very closely related, which might explain the frequent morphological misidentification of both types. The conservation status determined one of the investigated species was categorized as vulnerable, one as least concern, one as not evaluated, and four species in data deficient. The population trend of *S.s herrmanni* was recorded to be decreasing, whereas it remained unknown for the other 6 species. Surprisingly, all identified species in this study were not evaluated yet by CITES. This study suggests that a proper field monitoring and the establishment of a valid list of commercial and scientific names for the sea cucumbers harvested in Kepulauan Seribu is necessary. This would be valuable for keeping trade records and managing and conserving the targeted sea cucumber species in the region.

Keywords: Conservation, coral triangle, invertebrate, overfishing, taxonomy, trepan

INTRODUCTION

Sea cucumbers (Phylum Echinodermata) are common in tropical ecosystems, such as mangrove forests, seagrass meadows, and coral reefs, ranging from shallow water to over 3000 meters depth (Purcell et al. 2012). They play an important ecological role in kelp forests (Velimirov et al. 1977; Harrold and Pearse 1989) and coral reefs (Birkeland 1989). Sea cucumbers live as deposit and suspension feeders (Darsono 2002), consuming a combination of bacteria, diatoms, and detritus (Yingst 1976; Massin 1982; Moriarty 1982). They mature late, have a slow growth and low rates of recruitment (Uthicke et al. 2004; Bruckner 2005), and reproduce seasonal (Conand1981, 1993a, 1993b; Uthicke 1997).

Sea cucumbers comprise more than 350 identified species in Indonesia and are new species are still being discovered in eastern Indonesia (Purwati et al. 2008). Identification of sea cucumbers was usually done using morphological characteristics (Kamarudin and Rehan 2015). Morphological identification is conducted by macroscopic observations of the external morphology and internal organs but works only on very few species. Microscopic observations use ossicles, as their unique shapes are the most important characteristic for the morphological identification of sea cucumbers. Ossicles are small pieces of calcified material that form part of the skeleton of a sea cucumber. Identifying species by ossicle examination is a very time-consuming method that requires highly skilled experts.

DNA barcoding is a method in molecular taxonomy using short DNA sequences to identify species. Target DNA barcoding standards for higher animals are markers of mitochondrial cytochrome oxidase subunit 1 or commonly known as CO1 markers (Hartl and Clark 1989; Amos and Hoelzel 1992). DNA barcoding has advantages in precision and accuracy in the safe identification of species compared with the morphological observations. This method has been successfully proven in different marine organisms (Jefri et al. 2015; Prehadi et al. 2015; Sembiring et al. 2015; Madduppa et al. 2016; Maulid et al. 2016; Saleky et al. 2016).

Natural populations of sea cucumbers experience pressure by a high exploitation due to the great demand from markets in Asia (Conand and Sloan 1989; Conand 1990; Conand and Byrne 1994; James and James 1994). Additionally, sea cucumbers are vulnerable to exploitation due to their slow population replenishment and their morphological complexities make them difficult to be identified to species level. Therefore, this study was conducted to identify sea cucumbers harvested in Kepulauan Seribu into species level using mitochondrial DNA.

MATERIALS AND METHODS

Sample collection and DNA extraction

A total of 96 sea cucumber specimens of various species were collected from Kepulauan Seribu, Jakarta, Indonesia (Figure 1) and put into a sea pen culture in Pramuka Island for further study. Approximately 1 cm^2 of tissue was clipped from their ventral podia or mouth mantle. The tissue was preserved in 96% ethanol until DNA extraction. The genomic DNA was isolated using a Geneaid extraction kit (Blood and Tissues) following the product's protocol.

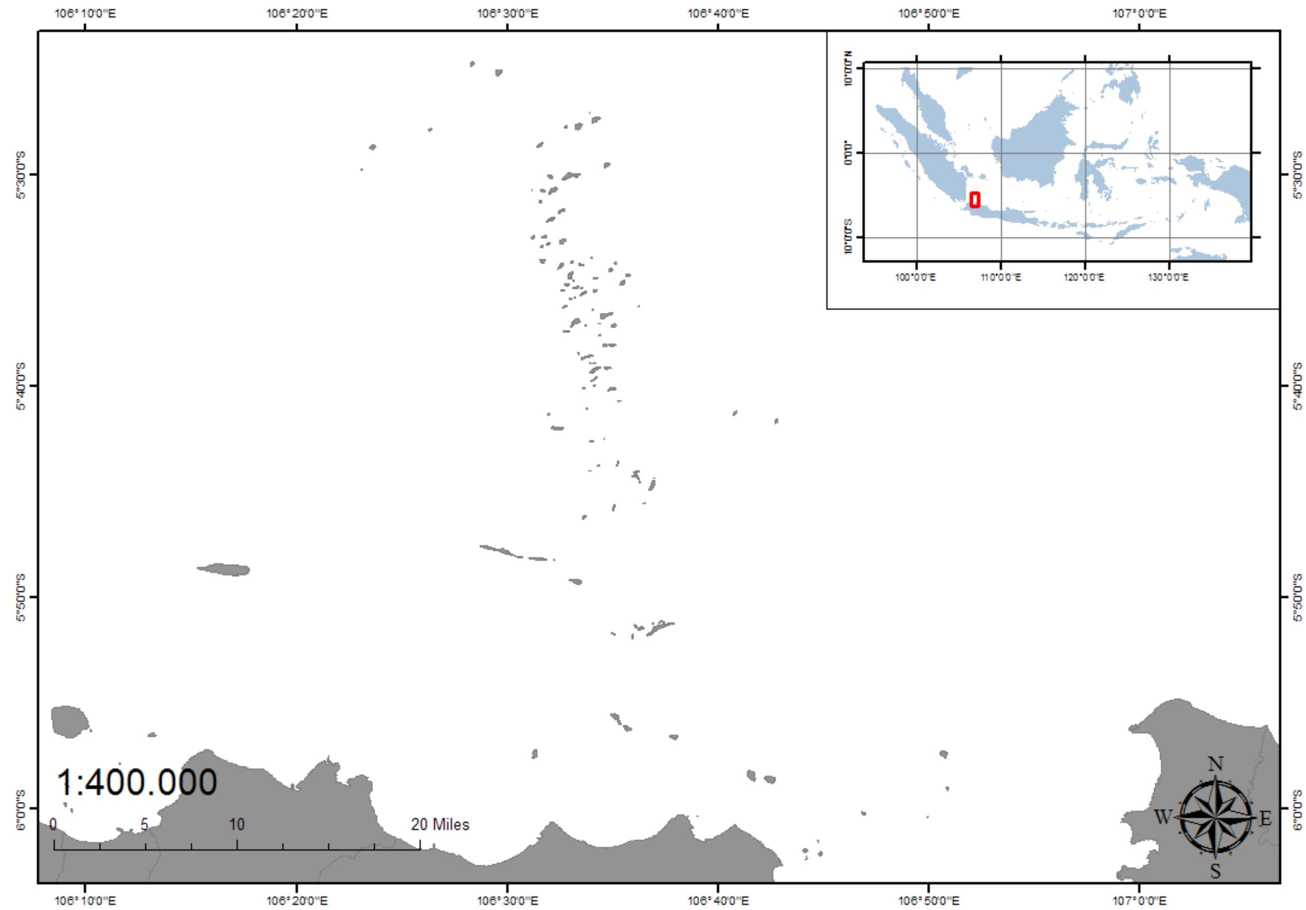

Figure 1. The map of Kepulauan Seribu, located in the north of Jakarta, Indonesia

DNA amplification and sequencing

PCR amplifications were performed in 25 µL reaction mixture containing 2 µL 25 mM $MgCl_2$, 2.5 µL 8µM dNTPs; 1.25 µL each primer pair 10 mM; 0,125 µL Taq DNA polymerase, 2.5 µL 10xPCR Buffer, 2 µL DNA template, 13.38 µL deionized water (ddH_2O). The mitochondrial cytochrome oxidase I (COI) gene was PCR-amplified using Holo_LCO: 5'-TAA TCA ACT AA (AC) CAC AAG ATT GAC GG-3 'and Holo_HCO: 5'-TAA ACT TCT GGA TG (AG) CC (AG) AA (AG) AAT CA-3 ' (Ahmed 2006). The PCR program was set as follows: an initial denaturation at 95°C for 2 min, 30 cycles of denaturation (94°C for 1 min), annealing (55 °C for 45 s), and extension (72°C for 1 min) with the final extension step at 72°C for 10 min. The PCR products were visualized to check DNA quality on 1% agarose gels (agarose 0.5 g and 50 mL TAE Buffer) with 4 µL ethidium bromide (EtBr) as a dye. The positive PCR products were sent to a sequencing facility and loaded into an ABI 3130xl automated sequencer (Applied Biosystems).

Data analysis

Sequences editing and alignment were conducted using MEGA 5 (Tamura et al. 2011). Sample identification was, performed using the Basic Local Assignment Search Tool (BLAST; Altschul et al. 1990). For phylogenetic analysis, one or more reference sequences from GenBank sequence database with the highest maximum identity to each amplicon sequence were downloaded. In the phylogenetic tree, *Antedon mediterranea* (accession number KC626509) was used as an outgroup comparison. A neighbor-joining phylogenetic tree using Kimura-2 parameter models (Kimura 1980) was reconstructed with 1000 replicates of bootstrap value. The genetic distance within and between species was investigated.

A review of the conservation status and population trend of each species was determined using IUCN (International Union for Conservation of Nature and Natural Resources) online at http://www.iucnredlist.org/. The trade status of identified species was determined using CITES (Convention on International Trade in Endangered Species) online at http://www.cites.org/.

RESULTS AND DISCUSSION

Molecular identification

All COI sequences obtained from the sea cucumber samples were aligned and sequences previously available were added to the alignment. All specimens and sampling details are shown in Table 1. A total of seven species were confirmed as sea cucumber species in this study, using mitochondrial markers cytochrome oxidase subunit 1 (CO1). From the 96 samples, BLAST results through GenBank identified *Stichopus monotuberculatus* (38 individuals) as the most frequent species, followed by *Stichopus ocellatus* (26 individuals), *Stichopus herrmanni* (22 individuals), *Holothuria leucospilota* (6 individuals), *Stichopus horrens* (2 individuals), *Bohadschia bivittata* (1 individual), and *Actinopyga lecanora* (1 individual). These seven species belong to two families, Holothuridae (*Bohadschia bivittata*, *Actinopyga lecanora* and *Holothuria leucospilota*) and Stichopodidae (*Stichopus herrmanni*, *Stichopus ocellatus*, *Stichopus horrens*, and *Stichopus monotuberculatus*).

An overall phylogenetic analysis for 96 COI gene sequences (using the neighbor-joining method and bootstrapping with 1000 replicates) indicated that nearly all species form distinct monophyletic clades in accordance with previous taxonomic work. Most samples from GenBank formed a cluster together with other specimens of the respective nominal taxon. Most genera also formed monophyletic clades, many of which were not supported by high bootstrap values. The in-group haplotypes were clustered into four main clades (I-IV) (Figure 2).

Based on data analysis, the genetic distance of the investigated specimens within species and between species shows a range of 0.0036-0.0120 and 0.0108-0.2761, respectively (Table 2). The lowest value 0.0036 is the genetic distance between individuals in *Stichopus monotuberculatus*, which means there are 3-4 different bases in 100 nucleotide sequences. While the largest genetic distance of 0.0120 for *S. horrens* means there are 12 different bases in 100 nucleotide sequences. The smallest genetic distance is between *S. herrmanni* and *S. ocellatus* with 0.0108 indicating one difference of base in 100 nucleotide base sequences, while the largest genetic distance observed between *S. ocellatus* and *Holothuria leucospilota* (27 different bases in 100 nucleotide sequences). Finally, an in-depth phylogenetic analysis of all sea cucumber families will require examination of additional, more slowly evolving, regions of the genome.

Table 1. Molecular identification of sea cucumbers in Kepulauan Seribu generated from NCBI Genbank

Species	Common name	N	Genbank max. ID (%)
Holothuridae			
Bohadschia bivittata	Two-ribboned sea cucumber	1	99
Actinopyga lecanora	Stone fish	1	99
Holothuria leucospilota	White thread fish	6	99
Stichopodidae			
Stichopus herrmanni	Curryfish	22	99
Stichopus ocellatus	Ocellated sea cucumber	26	99
Stichopus horrens	Peanutfish	2	99
Stichopus monotuberculatus	Not designated	36	100

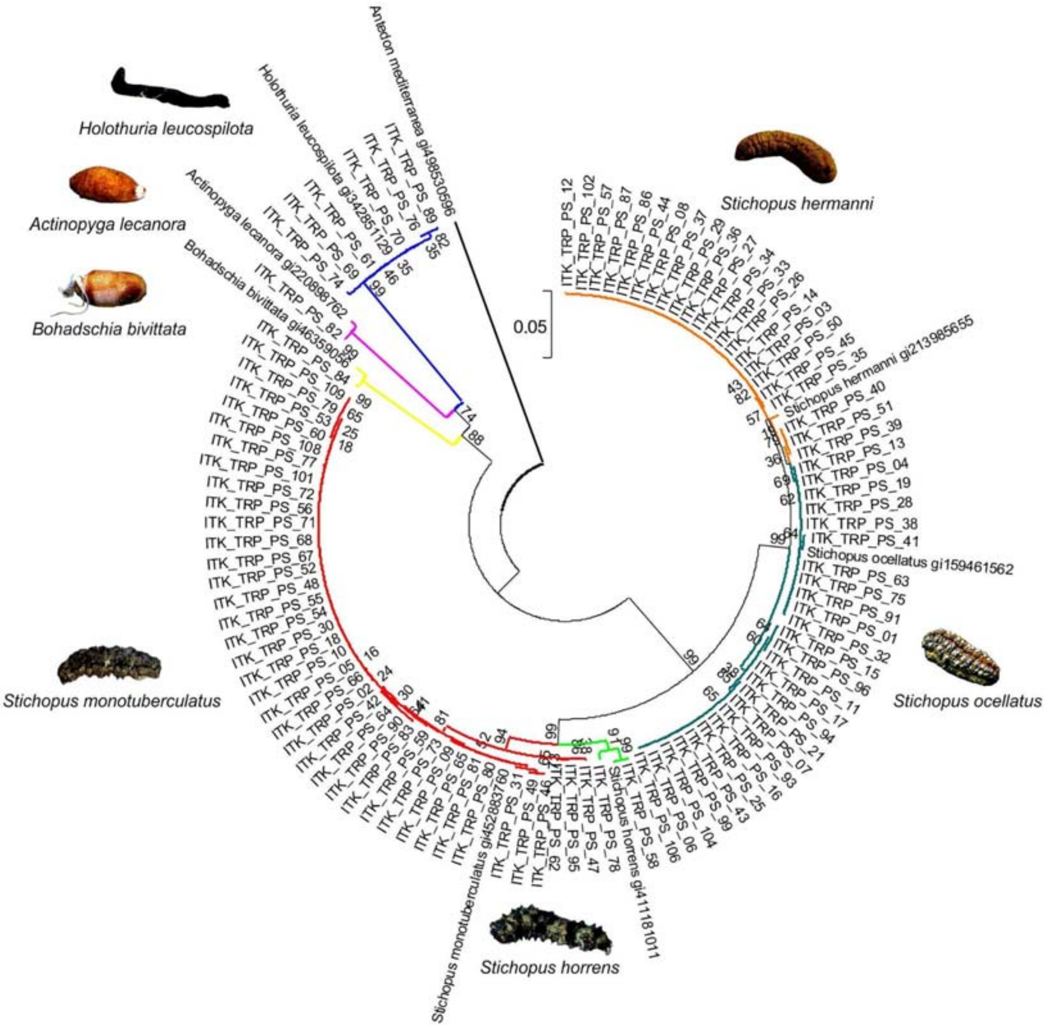

Figure 2. Neighbor-joining phylogenetic tree of 96 sequences of sea cucumber with 1000 bootstraps

Table 2. Genetic distance within and between species of sea cucumbers (Holothuridae and Stichopodidae)

Species	*Stichopus monotuberculatus*	*Holothuria leucospilota*	*Stichopus ocellatus*	*Stichopus herrmanni*	*Stichopus horrens*	*Bohadschia bivittata*	*Actinopyga lecanora*
Stichopus monotuberculatus	0.0036						
Holothuria leucospilota	0.2573	0.0066					
Stichopus ocellatus	0.0618	0.2761	0.0072				
Stichopus herrmanni	0.0619	0.2710	0.0108	0.0037			
Stichopus horrens	0.0297	0.2610	0.0605	0.0615	0.0120		
Bohadschia bivittata	0.2215	0.2006	0.2231	0.2211	0.2309	0.0108	
Actinopyga lecanora	0.2461	0.2057	0.2500	0.2491	0.2406	0.2027	0.0090

Table 3. Conservation status and population trend by IUCN (International Union for Conservation of Nature and Natural Resources) and trade status by CITES (Convention on International Trade in Endangered Species).

Species	Conservation status	Population trend	Trade status
Actinopyga lecanora	Data deficient	Unknown	Not evaluated
Bohadschia bivittata	Not evaluated	Unknown	Not evaluated
Holothuria leucospilota	Least Concern	Unknown	Not evaluated
Stichopus herrmanni	Vulnerable	Decreasing	Not evaluated
Stichopus horrens	Data deficient	Unknown	Not evaluated
Stichopus monotuberculatus	Data deficient	Unknown	Not evaluated
Stichopus ocellatus	Data deficient	Unknown	Not evaluated

Phylogenetic analysis with a neighbor-joining method for 96 COI gene sequences indicated that nearly all species form distinct monophyletic clades. A total of 7 species identified from 96 samples collected from Seribu Island in Jakarta using mitochondrial Cytochrome oxidase subunit 1 (CO1), consisted of 4 species belonging to the Stichopodidae (*Stichopus herrmanni*, *Stichopus ocellatus*, *Stichopus horrens*, *Stichopus monotuberculatus*), and 3 species belonging to the Holothuridae (*Bohadschia bivittata*, *Actinopyga lecanora* and *Holothuria leucospilota*). The genetic distance within and between species clearly showed differences between individuals within species, and between species of sea cucumbers in this study. Genetic differences are influenced by several factors, such as genetic drift and natural selection (Freeland 2005). Uthicke et al. (2010) have analyzed the relationships among many commercial species genetically.

Stichopus herrmanni and *S. ocellatus* were identified as closely related. Based on morphological characteristics, both species have similarities, e.g. yellow to orange in color and freckles scattered across the body, or only spots. Whereas *S. ocellatus* can be gray with white fringes, *S. ocellatus* rather has dark brown or black freckles (Purcell et al. 2012). The relationship between *S. horrens* and *S. monotuberculatus* is very close, which might also explain the frequent morphologic misidentification of both types. Morphologically, both have colors that vary from gray, beige, dark red to dark brown or black with patches of different color on the dorsal part. In addition, *S. horrens* has huge 'thumb-tack' shaped table ossicles of the dorsal papillae, while *S. monotuberculatus* has ossicles of different shapes (Purcell et al. 2012). In phylogeny, *Bohadschia bivittata* has the most different base sequences so that this species forms a separate clade to the six other species. *Bohadschia bivittata* displays a light brown color with two brown bands across the dorsal part. This species is usually buried in the sand during the day. *Actinopyga lecanora* and *Holothuria leucospilota* have a close relationship, but differ morphologically. *Actinopyga lecanora* has a cream-colored to brown body with bright spots or dark spots on the body with a white anus (Purcell et al. 2012). *A. lecanora* will harden like a stone when threatened (Purwati et al. 2008). The entire body of *H. leucospilota* is black and has an elongated shape with half of the posterior portion being slightly widened.

Conservation and trade status

Table 3 shown the Conservation status and population trend by IUCN (International Union for Conservation of Nature and Natural Resources) and trade status by CITES (Convention on International Trade in Endangered Species). Based on IUCN categories, one of the investigated species is categorized as vulnerable, one as least concern, one as not evaluated, and four species as data deficient. Populations of *Stichopus herrmanni* are in a decreasing trend, while this remains unknown for all other six species. Surprisingly, all identified species in this study were not evaluated yet by CITES.

The exploitation of sea cucumbers in the world began in the 1980s (Purcell et al. 2012). From 2001 until 2011, 3000 to 7000 tons of sea cucumbers were traded per year in Indonesia (MMAF 2011). Due to their little capacity for natural recovery and replenishment, some populations of some species have been reduced to such low levels that it lead to their economic and ecological extinction (Purcell et al. 2012).

On the markets, the variety of forms and products of sea cucumbers makes them difficult to identify to species level. The trepan is mainly boiled or fumigated and will appear very different. In addition, even in a complete individual, most sea cucumbers are morphologically complex and difficult to identify. As a result, most Indonesian trade records are only recorded as Teripang for all species traded. This will, in turn, give impact on the management of targeted species and also for the statistical trade records. This study showed that all identified sea cucumber species were closely related, which shows the importance of using genetic identification for effective conservation and management. Other studies have shown the importance of molecular identification for certain fisheries (e.g. Uthicke et al. 2010; Prehadi et al. 2015; Sembiring et al. 2015; Madduppa et al. 2016).

Worldwide, a total of 58 sea cucumber species that are economically important belong to two main families, Holothuridae and Stichopodidae (Purcell et al. 2012), and about 25 species of them are economically important in Indonesian waters (Darsono 2002). Some populations of sea cucumbers are over-harvested (e.g. Uthicke and Conand 2005). However, few efforts for mitigation such as developing efficient aquaculture and stock enhancement programs are undertaken Indonesia. The Kepulauan Seribu once was an important are of sea cucumber diversity and abundance in Indonesia. However, because of exploitation and habitat destruction, the density of sea cucumbers in the Kepulauan Seribu has declined to about 0.016-1.1089 ind./m^2 (Hana 2011). Some other coral reef communities have also shown a gradient from Jakarta Bay to the northern part of Kepulauan Seribu (Madduppa et al. 2013). Six of identified sea cucumber species in this study belong to commercially important sea cucumbers of the world by FAO (2012), and are exploited for food in Southeast Asia (Choo 2008). Unfortunately, the trade status of the seven species identified in this study has not been evaluated by CITES. Recently, the national strategic plan for the period 2016 until 2020 for sea cucumber conservation has been issued by the Ministry of Marine Affairs and Fisheries of Indonesia (Sadili et al. 2015). The strategic plan includes providing data and information of sea cucumber fisheries, establishing conservation areas for important habitats, increasing population numbers of sea cucumbers, and developing a national regulation and a standard operational procedure of sea cucumber production (Sadili et al. 2015). The fast identification of species, proper trade records, and stock assessments in exploited areas are important to protect these species from local extinction. Therefore, this study suggests a proper field monitoring at important sea cucumber sites and establishing of a valid list of commercial and scientific names for the sea cucumbers harvested in Kepulauan Seribu.

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