Identification of Beche-de-mers from Indonesia by molecular approach

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Abstract. *Patantis G, Dewi AS, Fawzya YN, Nursid M. 2019. Identification of Beche-de-mers from Indonesia by molecular approach. Biodiversitas 20: 537-543.* Sea cucumbers have been commercially traded around the world due to their nutritional values and health benefits. Sea cucumbers are marketed as processed sea cucumber, also known as beche de mer, teripang, haisen or gamat. Due to the high global demand of beche-de-mers, there have been several cases of fraud due to the visual shape and color similarities of processed sea cucumbers. Additionally, the local name diversity of Indonesian sea cucumbers making the identification of beche-de-mers difficult. Therefore, this study aimed to identify commercial sea cucumbers from selected locations in Indonesia by molecular approach. The targeted Cytochrome c oxidase subunit I (COI) was used in this research. A total of 25 beche-de-mers was purchased from local markets in Indonesia, i.e. Boalemo (17 local names), Pesawaran (5), Surabaya (2) and West Lombok (1). Results showed that 22 species of beche-de-mer were successfully identified, ie. *Acaudina* sp., *Actinopyga echinites, A. lecanora, A. miliaris, Bohadschia argus, B. marmorata, B. ocellata, B. vitiensis, Holothuria* sp. *H. atra, H. coluber, H. edulis, H. excellens, H. fuscocinerea, H. fuscogilva, H. fuscopunctata, H. scabra, Pearsonothuria graeffei, Phyrella* sp., *Stichopus herrmanni, S. horrens* and *S. monotuberculatus.* The identification of Indonesian beche-de-mers using molecular method could support the identification of the local sea cucumbers to overcome mislabeled and fraud. Correct identification is important for the record of beche-de-mer trade in Indonesia and further conservation management.

Keywords: Beche-de-mer, Indonesia, molecular identification, sea cucumbers

INTRODUCTION

Sea cucumbers have been commercially used and traded as beche-de-mer, gamat, teripang or haishen for more than 1000 years (Hamel Jean-François et al. 2001) in more than 70 countries (Purcell et al. 2012). Beche-de-mers are widely consumed in Asia region as a delicacy and a traditional medicine because of their nutritional and health values (Hamel Jean-François et al. 2001). They are more than 60 sea cucumber species have been internationally marketed (Purcell et al. 2012). Indonesia, one of the biggest producer, has contributed 26 species of beche-de-mers (Purwati 2005) or even more (Choo 2008) in the international trade. However, the statistics recorded that Indonesian beche-de-mers exports only labeled as teripang, regardless the species. In addition, the local name of each teripang is varied in each region (Setyastuti and Purwati 2015) thus mislabel is common. This condition is difficult for conservation management as many of the sea cucumbers species are overfished. Therefore, it is important to identify and classify the species of the beche-de-mers from Indonesia with high-reliability result.

Identification of Indonesian sea cucumbers has been mostly performed using morphological rather than molecular method. Massin (1999) collected and morphologically identified 56 species from Spermonde archipelago; meanwhile, Purwati et al. (2010) identified 18 species from Karimunjawa Island. Other studies were conducted by Supono et al. (2014) from Lembeh Strait (22 species) and Afrely et al. (2015) from Alas Purwo National Park (8 species). All of them were identified from fresh sea cucumbers. Furthermore, Setyastuti and Purwati (2015) identified 54 Indonesian sea cucumbers in fresh, salted and dried forms. However, only 33 species were taxonomically confirmed, whereas 21 species of them could not be identified by this method. Some of the dried and salted beche-de-mers were difficult to identify due to shape similarity and ossicle damage.

Other research groups have identified Indonesian fresh sea cucumbers using a molecular method by employing the cytochrome c oxidase subunit I (COI) region. Madduppa et al. (2017) identified seven species of sea cucumbers from Kepulauan Seribu, meanwhile (Amin et al. 2016) identified one species of sea cucumber from Surabaya. The advantage of using COI over the regular method is its resistance to the degradation from heat treatment (Rasmussen and Morrissey 2008). Identification using COI region also have been applied to identify plant and animal species as ingredients in food products (Peres et al. 2007). The same technique has also been used to differentiate seafood products, i.e., fish (Di Pinto et al. 2015; Md-Zain et al. 2018), shrimp (Fernandes et al. 2017) and beche-de-mer (Uthicke et al. 2010). Based on these reasons, this study aimed to identify beche-de-mers from several regions in Indonesia using the mitochondrial COI genes.

MATERIALS AND METHODS

Samples collection

The beche-de-mer samples were collected from various local markets in Indonesia, such as at Boalemo District, Gorontalo; Pesawaran District, Lampung; Surabaya City, East Java and West Lombok, West Nusa Tenggara between April-July 2018. A total of 47 dried-salted specimens from 25 samples with different local names were obtained.

DNA extraction and amplification

DNA of each beche-de-mer sample was isolated using the DNeasy Blood and Tissue Kit (Qiagen, Netherlands) based on the manufacturer protocol with slight modification. Approximately 30 – 50 mg of sample was cut and ground using pestle homogenizer in 180 μL lysis buffer (buffer ATL), prior to overnight incubation at 56 °C. The region of COI was amplified using polymerase chain reaction (PCR) by using CO1eF: 5'-ATAATGATAGGAGGRTTTGG-3' and COIeR: 5'-GCTCGTGTRTCTACRTCCAT-3' primers (Arndt et al. 1996). The PCR was conducted using a KOD FX Neo master mix (Toyobo, Japan) that was composed of 12.5 μL of 2x PCR buffer, 5 μL of 2 mM dNTPs, 1 μL of 10 mM each primer, 1 μL of template DNA, 0.5 μL of 1 U/μL KOD FX Neo tag and 5 μL PCR grade water. The PCR was performed in TProfessional Basic Thermocycler (Biometra, Germany) at the following conditions: initial denaturation at 98°C for 2 min, 30 cycles of denaturation at 98 °C for 30 s, primer annealing at 50°C for 30 s, extension at 72°C for 1 min, and a final extension at 72°C for 7 min. The PCR products (+ 675 base pairs) were visualized by agarose gel electrophoresis. The PCR products then were sequenced at 1st Base Laboratories (Malaysia).

Sequence data analysis and phylogenetic tree

The COI Sequences data from forward and reverse were combined using MEGA 7 (Kumar et al. 2016). The combined sequences then compared to those of references in the database by aligning the sequences using Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990) and Barcode of Life Database (BOLD) System (Ratnasingham and Hebert 2007). For the phylogenetic analysis, 40 of sequences from Gen Bank were used as references. A neighbor-joining phylogenetic tree was constructed using MEGA7 with Kimura-2 parameter models. Bootstraps of 1,000 were performed to determine the confidence levels to the nodes of the trees.

RESULTS AND DISCUSSION

A total of 25 beche-de-mers was obtained from local markets at Boalemo (17 local names), Pesawaran (5 local names), Surabaya (2 local names) and West Lombok (1 local name). All of DNA specimens (47) were successfully amplified using COI primers. The BLAST result was shown in Table 1.

Table 1. COI similarity result of Indonesian beche-de-mers generated by BLAST and BOLD System analysis

Local name	N	Collection site	Species	Similarity (%)	
				BLAST	BOLD System
Bintik (G)	2	Boalemo	*Bohadschia argus*	98	98.98
Tangkiri (G)	2	Boalemo	*Holothuria excellens*	98	99.54
Kunyit (G)	1	Boalemo	*Holothuria fuscopunctata*	99	100.00
Cera duri (G)	1	Boalemo	*Pearsonothuria graeffei*	99	99.61
Cera hitam (G)	2	Boalemo	*Holothuria atra*	99	99.85
Cera merah (G)	2	Boalemo	*Holothuria edulis*	99	99.84
Blimbing (G)	2	Boalemo	*Actinopyga echinites*	99	100.00
Polos (G)	2	Boalemo	*Bohadschia vitiensis*	99	99.85
Kapok (G)	2	Boalemo	*Actinopyga lecanora*	99	99.23
Kasur (G)	2	Boalemo	*Stichopus herrmanni/ S. vastus/ S. horrens/ S. ocellatus**	99	99.69
Ular (G)	2	Boalemo	*Holothuria coluber*	99	99.54
Lolosong (G)	2	Boalemo	*Holothuria sp./ H. fuscocinerea***	95	99.69
Duyung (G)	1	Boalemo	*Bohadschia ocellata*	98	99.39
Gosok (G)	1	Boalemo	*Holothuria scabra*	99	99.37
Susu (G)	2	Boalemo	*Holothuria fuscogilva*	99	99.54
Alolo (G)	2	Boalemo	*Bohadschia marmorata*	99	99.00
Pisang (G)	2	Boalemo	*Holothuria fuscocinerea/ H. turriscelsa***	99	99.39
Pasir (L)	2	Pesawaran	*Holothuria scabra*	99	99.00
Kelapa (L)	2	Pesawaran	*Actinopyga sp./ A. miliaris*	96	99.85
Karang (L)	2	Pesawaran	*Stichopus monotuberculatus/S. horrens*	99	99.85
Kasur (L)	2	Pesawaran	*Stichopus monotuberculatus/S. horrens*	99	100.00
Olo-olo (L)	2	Pesawaran	*Bohadschia marmorata*	99	99.85
Terung (S)	2	Surabaya	*Phyrella sp.*	88	NA
Teripang (S)	2	Surabaya	*Acaudina sp.*	87	NA
Gamat emas (LM)	3	W. Lombok	*Stichopus horrens/ S. monotuberculatus*	99	99.85

Note: *: unresolved species according to BLAST and BOLD System analyses; **: species identification according to BOLD System analysis; NA: result not available
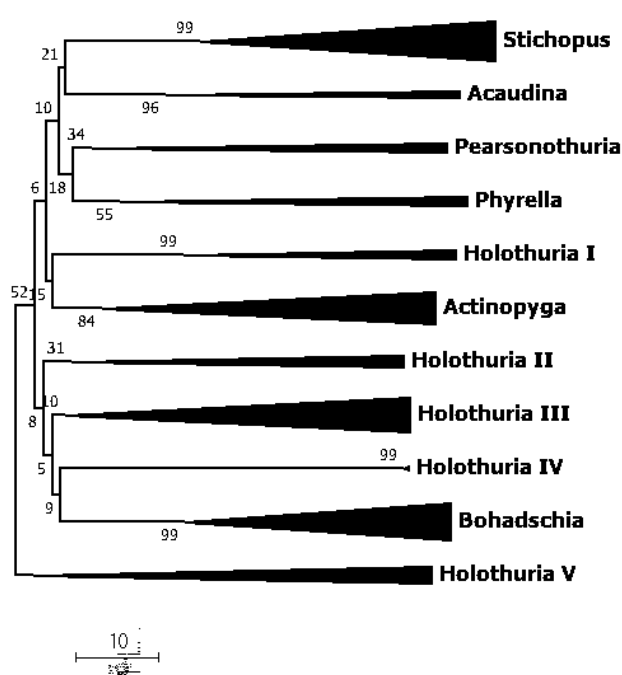
Figure 1. The phylogenetic tree of Indonesian beche-de-mers at the genus level. The tree was generated from the neighbor-joining analysis in the MEGA 7.0 software using 1000 replicates of the bootstrap test. Numbers shown next to the branches showed the bootstrap values.

The results showed that generally, the homology of beche-de-mer species in the database is between 95 to 100%, except those of samples from Surabaya (terung and teripang) that only showed 87-88% similarity. Based on the results, the beche-de-mers can be divided into seven genera, i.e., *Acaudina, Actinopyga, Bohadschia, Holothuria, Pearsonothuria, Phyrella* and *Stichopus*. BLAST and BOLD System results for "lolosong" and "pisang" gave different results. "Lolosong" was similar to *Holothuria* sp. according to BLAST, or *H. fuscocinerea* according to BOLD System. "Pisang" was identified as *Holothuria fuscocinerea* (BLAST) and *H. turriscelsa* (BOLD System). Beche-de-mers produced from sea cucumber "karang", "kasur" and "gamat emas" were identified as *Stichopus* spp. with 99% of similarity to more than one species of *Stichopus*. Since the BLAST and BOLD System analyses have not been able to determine the exact species of the origin; therefore, further analysis using the phylogenetic tree was carried out.

The phylogenetic relationship between genus is shown in Figure 1. The results showed that most of the genera formed a monophyletic clade with a high number of bootstrap, except *Holothuri*a which was formed into five clades (*Holothuria* I to V). *Holothuria* I was near *Actinopyga* clade, whereas *Holothuria* II-IV was close to *Bohadschia* clade. *Holothuria* V was distinct to the others.

Actinopyga

All *Actinopyga* was grouped into one clade in the phylogenetic tree (Figure 1) and was further classified into distinct sub-clades between species with high bootstrap values (Figure 2). Both specimens of "blimbing" from Boalemo were closely related to *Actinopyga echinites*, while those of "kapok" were similar to *A. lecanora*. "Kelapa" samples from Pesawaran were close to *Actinopyga* sp. according to BLAST analysis while BOLD System suggested *A. miliaris* (Table 1). Phylogenetic analysis of "kelapa" showed that this species was close to *A. miliaris* with a high number of bootstrap (100).

Bohadschia

Five beche-de-mers were grouped into *Bohadschia* genus (Figure 3). "Alolo" and "olo-olo" from Boalemo and Pesawaran, respectively, were both identified as *Bohadschia marmorata*, "polos" was *B. vitensis*, "bintik" and "duyung" were identified as *B. argus* and *B. ocellata,* respectively.

Stichopus

There are four local sea cucumbers classified as *Stichopus* namely "kasur" and "karang" (Pesawaran), "gamat emas" (West Lombok) and "kasur" (Boalemo). Even though the BLAST analysis showed a high similarity of each beche-de-mer to more than one species (Table 1), the phylogenetic tree was able to classify each of them into separated branches with high bootstrap values that confirmed their species level (Figure 4). "Kasur" and "karang" from Pesawaran have different local names. However, the phylogenetic analysis showed that both sea cucumbers were similar to *Stichopus monotuberculatus*; whereas, the other "kasur" from Boalemo, corresponded to *S. herrmanni*. Finally, "gamat emas" from West Lombok was identified as *S. horrens*.

Holothuria

According to the phylogenetic analysis (Figure 5), ten beche-de-mers were classified into the *Holothuria* group, leading this group as the largest group among others. However, species misidentification in this group often occurs. In this research, these beche-de-mers can be distinguished unambiguously, i.e., "susu" as *H. fuscogilva*, "kunyit" as *H. fuscopunctata*, "tangkiri" as *H. excellens* and "cera hitam" as *H. atra* (Figure 5). Since COI sequence data of *H. turriscelsa* was not available in the NCBI, therefore the phylogenetic similarity of "pisang" was close to *H. fuscocinerea*. In addition, "lolosong" was similar to *H. fuscocinerea* (BOLD System) with 99.69%. However, the phylogenetic result, showed that this species was closer to *Holothuria* sp. rather than *H. fuscocinerea*. Two specimens with different local names, "pasir" (Pesawaran) and "gosok" (Boalemo), were both identified as *H. scabra*.

In this study, *Pearsonothuria, Phyrella,* and *Acaudina* were only represented by one species for each genus (Figure 6), indicating their rarity in Indonesian waters. "Cera duri" from Boalemo was similar to *Pearsonothuria graeffei*, while samples from Surabaya, "terung" and "teripang", were identified as *Phyrella* sp. and *Acaudina* sp. respectively.
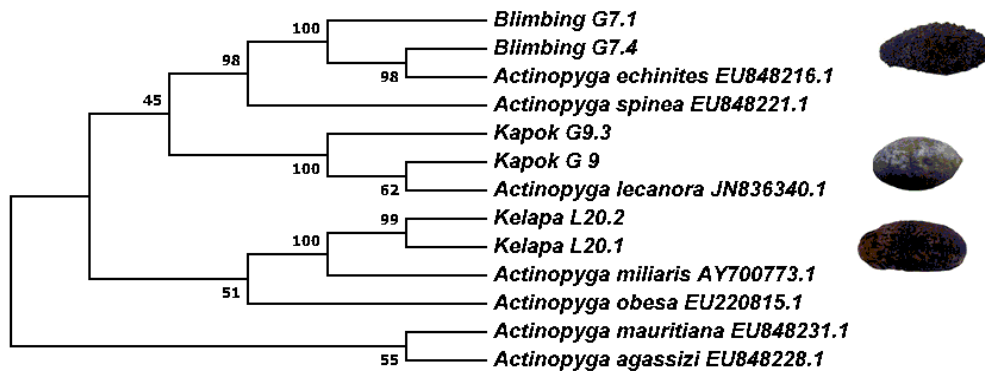
Figure 2. The phylogenetic tree of *Actinopyga* genus of Indonesian beche-de-mers. The tree was generated from the neighbor-joining analysis in the MEGA 7.0 software using 1000 replicates of the bootstrap test. Numbers shown next to the branches showed the bootstrap values. The samples were collected from Boalemo, Gorontalo (G) and Pesawaran, Lampung (L)
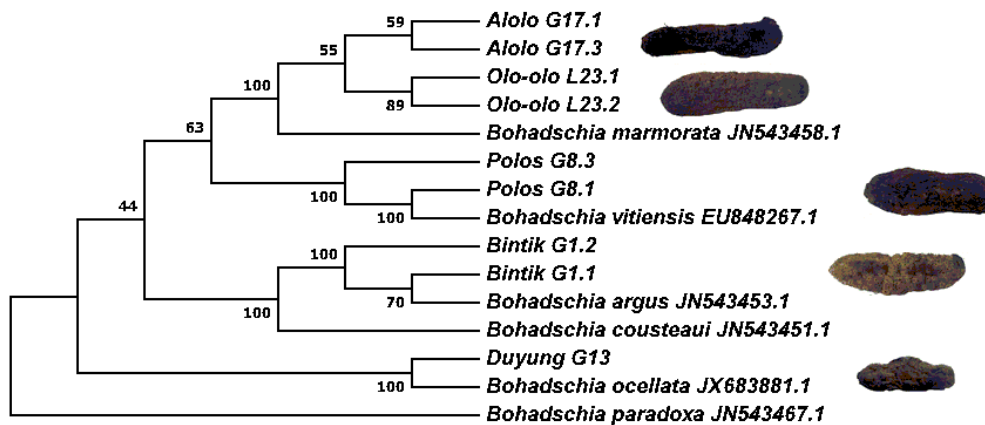

Figure 3. The phylogenetic tree of *Bohadschia* genus of Indonesian beche-de-mers. The tree was generated from the neighbor-joining analysis in the MEGA 7.0 software using 1000 replicates of the bootstrap test. Numbers shown next to the branches showed the bootstrap values. The samples collected from Boalemo, Gorontalo (G) and Pesawaran, Lampung (L)
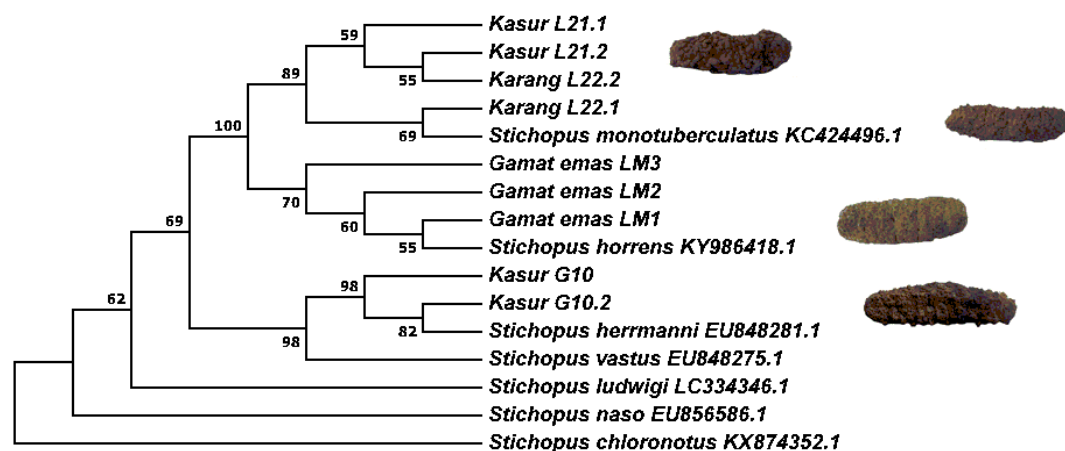

Figure 4. The phylogenetic tree of *Stichopus* genus of Indonesian beche-de-mers. The tree was generated from the neighbor-joining analysis in the MEGA 7.0 software using 1000 replicates of the bootstrap test. Numbers shown next to the branches showed the bootstrap values. The samples collected from Boalemo, Gorontalo (G), Pesawaran, Lampung (L)and West Lombok (LM)
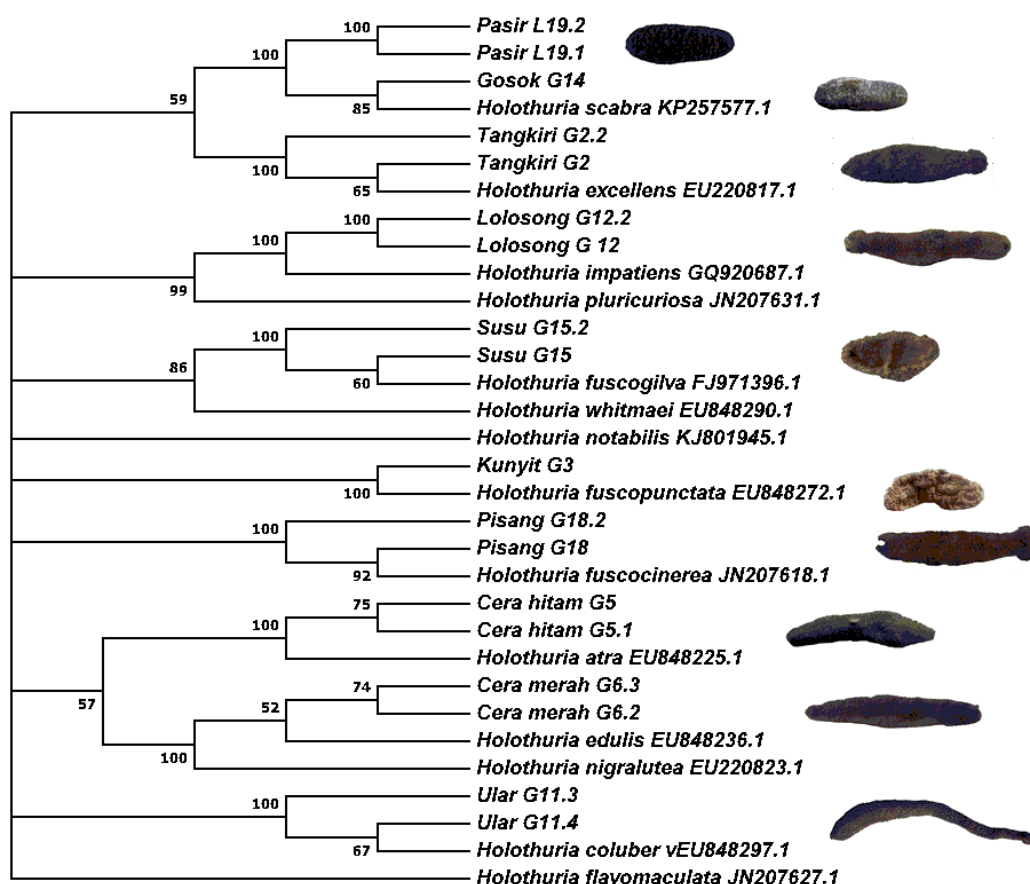
Figure 5. The phylogenetic tree of *Holothuria* genus of Indonesian beche-de-mers. The tree was generated from the neighbor-joining analysis in the MEGA 7.0 software using 1000 replicates of the bootstrap test. Numbers shown next to the branches showed the bootstrap values. The samples collected from Boalemo, Gorontalo (G) and Pesawaran, Lampung (L)
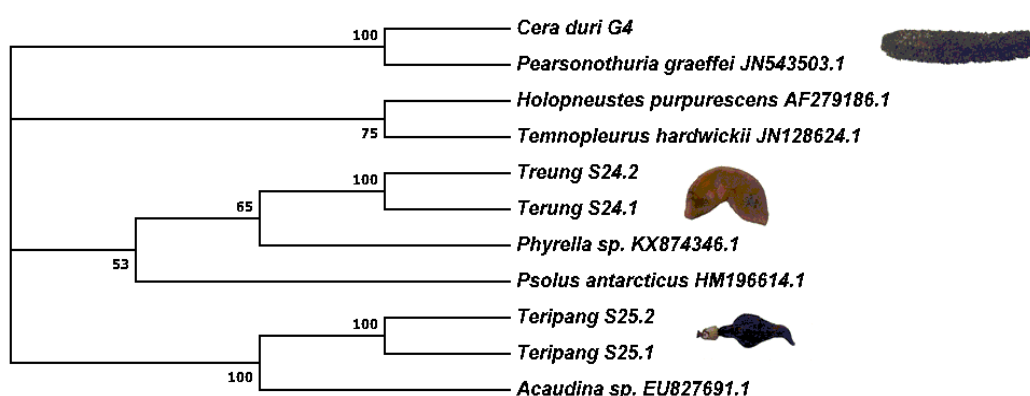

Figure 6. The phylogenetic tree of *Pearsonothuria, Phyrella* and *Acaudina* genera. The tree was generated from the neighbor-joining analysis in the MEGA 7.0 software using 1000 replicates of the bootstrap test. Numbers shown next to the branches showed the bootstrap values. The samples collected from Boalemo, Gorontalo (G) and Surabaya, East Java (S)

Discussion

The molecular methods have been widely used to identify various marine organisms and their processed products, including sea cucumbers, because of their specificity. In this study, we successfully identified 25 beche-de-mers from Boalemo, Pesawaran, Surabaya, and West Lombok with this particular technique. This molecular method compliments the morphological identification of commercial sea cucumbers from Indonesia that has been reported previously (Setyastuti and Purwati 2015). At the genus level, *Holothuria* group has more than one clades. This result was similar to the phylogenetic tree of commercial beche-de-mers by Uthicke et al. (2010), in which *H. atra* and *H. edulis* clades were next to

Bohadschia, whereas *H. coluber*, *H. fuscogilva,* and *H. fuscopunctata* were grouped into different clades (Uthicke et al. 2010). They suggested that these results might indicate high variability of COI in the *Holothuria* genus. In addition, *Holothuria* consists of 18 subgenera that further divided into 160 species, while *Actinopyga, Bohadschia,* and *Pearsonothuria* have not been divided into subgenera (Honey-Escandón et al. 2012). The genetic distance among genus and species already discussed by Uthicke et al. (2010), therefore in this result we focused on the compatibility of the reported species identification from previous studies (Purwati 2005, 2006; Purwati et al. 2010; Setyastuti and Purwati 2015).

The identification of local beche-de-mers from Indonesia in *Actinopyga* genus was difficult. Purwati (2006) and Purwati et al. (2010) inconsistently reported that "kapuk" from West Lombok and "kapuk" from Karimunjawa were similar to *A. lecanora*, whereas in other papers, "kapuk" and "kapok" were similar to *A. miliaris* (Purwati 2005) or *A. miliaris* and *A. echinites*, respectively (Setyastuti and Purwati 2015). These ambiguities may be due to the different location of sample collection or visual similarity of this genus after being processed. Generally, the *Actinopyga* beche de mer has an oval shape, *A. miliaris* and *A. lecanora* have black and brown-black color with a size between 10-12 cm, while *A. echinites* have a lighter color with a size between 8-15 cm (Purcell et al. 2012). Our results suggested that beche-de-mer with the local name of "kapok" from Boalemo was *A. lecanora*. Other local names of *Actinopyga* based on the morphological identification are shown in Table 2.

In the current study, slightly different local names in the *Bohadschia* group, "alolo" (Boalemo) and "olo-olo" (Pesawaran), showed similarity to *B. marmorata*. Additionaly, Purwati (2005) reported another local name of *B. marmorata* as "olok-olok". "Polos" and "bintik" from Boalemo were respectively identified as *B. vitiensis* and *B.*

argus. These results were in agreement with those of previous studies (Setyastuti and Purwati 2015). However, the identification of "duyung" was different. "Duyung" was identified as *Thelenota anax* (amberfish) (Purwati 2005, 2006), while in this study the same species was identified as *B. ocellata* (= *Holothuria ocellata*). Our result was based on one specimen of "duyung" that were collected from different locations to that of the previous report. Therefore more studies need to be performed to secure the species identity of "duyung". According to the IUCN Red List of Threatened Species, generally, the conservation status of *Bohadschia* members is secure. *B. argus* has a stable population and has been categorized as least concern (Conand et al. 2013) while *B. marmorata* (Conand and Purcell 2013) has a decreasing population and has been classified as data deficient. The conservation status of *B. vitiensis* and *B. ocellata* have not been listed yet.

In some Indonesian regions, all *Stichopus* labeled as "gamat" (Setyastuti and Purwati 2015), regardless of species. Identification of *Stichopus* is often mistaken between *S. horrens, S. monotuberculatus, S. quadrifasciatus* and *S. naso* (Purcell et al. 2012) due to their similar appearances. A thorough and careful morphological identification is crucial to differentiate these four species (Massin 1999). In this study, the BLAST and BOLD System analysis for *Stichopus* group showed that there were more than one species that have high similarities (Table 1). Further analysis using a phylogenetic tree (Figure 4) separated *S. horrens*, *S. herrmanni,* and *S. monotuberculatus*. This is in agreement with the previous study by Uthicke et al. (2010) that found that the phylogenetic tree of COI sequences could be used to differentiate *Stichopus* because of their distinct clades and high bootstrap values among species. The conservation status of *S. herrmanni* is already vulnerable, while *S. monotuberculatus* and *S. horrens* are data deficient (Conand et al. 2013).

Table 2. The local name comparison of Indonesian beche-de-mers

Species	Present study	Setyastuti and Purwati (2015)	Common name
Acaudina sp.	Teripang	-	-
Actinopyga echinites	Blimbing	Kunyit, ladu-ladu, kapok, kapuk, billala, bilado, kassi	Deepwater redfish
Actinopyga lecanora	Kapok	Batu, balibi, hitam	Stonefish
Actinopyga miliaris	Kelapa	Kapok, kapuk, lotong, gamet, sepatu, hitam	Blackfish
Bohadschia argus	Bintik	Ular mata, gamat bati, bintik, cempedak, patola	Leopardfish
Bohadschia marmorata	Alolo, olo-olo	Kawasa, olok-olok, getah putih, pulut, benang, krido polos	Chalky cucumber
Bohadschia vitiensis	Polos	Olok-olok, gatta, gama, polos	Brown sandfish
Bohadschia ocellata	Duyung	Kacang goreng	-
Holothuria atra	Cera hitam	Lakling hitam, coklat, hitam, dara, keling, cera	Lollyfish
Holothuria coluber	Ular	Taikokong, talengko	Snakefish
Holothuria edulis	Cera merah	Dada, cera, perut, lakling merah, takling, batu keling	Pinkfish
Holothuria excellens	Tangkiri	Hitam	-
Holothuria fuscocinerea	Pisang	Coklat, lakling coklat	-
Holothuria fuscogilva	Susu	Susu putih, bissawa	White teatfish
Holothuria fuscopunctata	Kunyit	Susu putih, kuning, kunyit	Elephant trunkfish
Holothuria sp.	Lolosong	Pulut	Bottleneck sea cucumber
Holothuria scabra	Gosok, pasir	Gosok, pasir, buang kulit, putih, kamboa	Sandfish
Pearsonothuria graeffei	Cera duri	Bintik merah, gombyok, sutra, cera duri, gemuk, bati, donga	Blackspotted sea cucumber
Phyrella sp.	Terung	-	-
Stichopus herrmanni	Kasur	Gamet emas, gamet kacang, taikongkong	Curryfish
Stichopus horrens	Gamat emas	Kacang goreng, taikongkong, kacang, susu, rengget	Dragonfish
Stichopus monotuberculatus	Karang, kasur	Gamet pace	Dragonfish

Misidentification of *Holothuria* group is also common in Indonesia such as for *H. fuscogilva*, *H. fuscopunctata*, *H. atra* and *H. excellens* (Setyastuti and Purwati 2015). In this study, these beche-de-mers can be distinguished clearly. *H. scabra* was easily identified, despite its various local names. This sea cucumber is popular in Indonesia regions and is known as "pasir", "gosok", "buang kulit", "putih" and "kamboa" (Setyastuti and Purwati 2015). The lack of sequence of *H. turriscelsa* in the NCBI resulted "pisang" was identified as *H. fuscocinerea* in this phylogenetic analysis. However, the results may be different if both *H. fuscocinerea* and *H. turriscelsa* sequences were compared. The conservation status of most of the *Holothuria* members has not been evaluated. Some other *Holothuria* is data deficient and least concern. The status of *H. scabra* and *H. fuscogilva,* is endangered and vulnerable, respectively (Conand et al. 2013; Hamel et al. 2013).

"Teripang" and "terung" from Surabaya are traded locally, either for immediate consumption or as raw material for crackers. The identification of "teripang" in this study showed similarity to *Acaudina* sp. These results were similar to those of Amin and co-workers (2016) who identified the species using morphology and molecular identifications (Amin et al. 2016). In addition, the identification of *P. graeffei* is consistent with local name reported by Setyastuti and Purwati (2015). Others region called *P. graeffei* as "bantun", "donga", "karang" or "jepun" (West Lombok) and "gamat gombyok" (Karimunjawa) (Purwati 2006; Purwati et al. 2010).

To conclude, the identification of Indonesian beche-de-mers using molecular method could provide additional data and support for the identification of the local sea cucumbers to avoid mislabeling. Correct and reliable identification is important for the record of beche-de-mers trade in Indonesia and further conservation management as some of them are vulnerable and endangered. Collection of more samples from extended regions of Indonesia will be necessary as different location have different names of beche-de-mers.

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