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Taxonomic redescription of *Colochirus quadrangularis* (Echinodermata: Holothuroidea) from Surabaya Coastal Waters (East Java, Indonesia) with notes on new distinctive haplogroup of COI gene

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ABSTRACT

Sea cucumbers have been reported in Surabaya east coastal area for their existence and exploitation. Although *Colochirus quadrangularis* was one of species which firstly reported from Surabaya waters in 2014, this species has never been properly described. Three specimens of *C. quadrangularis* were obtained from Surabaya coastal waters. Morphological examination conducted by describing all morphological characteristic, including type and distribution of ossicle in their body. DNA-based identification was performed using cytochrome oxidase subunit I (COI) sequences analysis, including sequence comparation with BOLD database, NJ tree reconstruction, and haplotype network presentation. Result of this study showed that there are no noticeable differences of morphological features compared to previous study of *C. quadrangularis* description. Based on molecular identification, the specimens were 98.69% similar with *C. quadrangularis* from BOLD database. Conspecific divergence of sequence from this study and downloaded sequences from BOLD showed that average sequence variation within the species was 1.5%. Both morphological descriptions and DNA barcoding results confirmed existence of *C. quadrangularis* in Surabaya waters. In addition, *C. quadrangularis* of this study has unique haplotypes differentiated from previous study, collected from Singapore and Australia.

Key words : *Colochirus quadrangularis, Description, DNA barcoding, Haplotype, Surabaya waters.*

Introduction

Indonesia is a country that has high potential of marine resources. Sea cucumber has higher commercial value than other Echinoderms (Aprianto *et al.*, 2019; Tuwo and Tresnati, 2015). Asian peoples over many decades use them as nutritional delicacy and medicinal cure (Abraham *et al.*, 2002, Choo, 2008). Sea cucumber contained high value compounds such as triterpene glycosides, carotenoids, collagens, amino acids, and active compounds anticancer agent, that useful for food and biomedicine industries (Khotimchenko, 2018, Pangestuti and Arifin, 2018; Wargasetia and Permana, 2018; Purnama and Winarni, 2017; Prawitasari *et al.*, 2019). Sea cucumber is one of many major marine commodities in Indonesia, such as seaweed, grouper, spiny lobster, and winged pearl oyster (La Ode

et al., 2015). From available data, Asia and Pacific regions are the top of sea cucumber exploitation, with around 20.000 to 40.000 ton/year (Toral-Granda *et al.*, 2008). The number of exploitations for commercial and study activities were feared to lead over fishing, thus the impact could decrease the population. Indonesia is one of areas that has been overfished of sea cucumber stock worldwide (Uthicke and Conand, 2005).

Surabaya is one of the cities near the sea, like other cities in Indonesia. Some species of sea cucumber were processed to crackers and commonly consumed by local people in East Java (Suryaningrum, 2008; Putri *et al.*, 2013; Wulandari *et al.*, 2018). Existence of *C. quadrangularis* in Surabaya waters was firstly reported in 2015 with some other species (Winarni *et al.*, 2014). After that, there was not adequate taxonomical study for this species in Indonesia. DNA barcoding was successful method to identify several organisme (Lily *et al.*, 2018; Susilowati *et al.*, 2019; Hayati *et al.*, 2019) includingsea cucumber species on Indonesia. This method previously has been applied to identify Caudinidae from Surabaya (Amin *et al.*, 2016) and Holothuroidea and Stichopodidae from Kepulauan Seribu (Maduppa *et al.*, 2017). In this study, we perform morphological examinations and DNA barcoding to ensure species identity of *C. quadrangularis* specimens from Surabaya. We also construct haplotype network of COI sequences between our specimens and database sequences.

Materials and Methods

Sampling and morphological examinations

Samples of local *C. quadrangularis* sea cucumber were obtained in Surabaya's east coast (07º15'36,40" S, 112º54'17,65" E). Fresh samples were examined formorphological observations, after relaxation treatment using 7% $MgCl_2$ solution. External and internal morphology were taken by Canon EOS D600 camera, and then samples preserved in absolute ethanol to minimize tissue damages due to high water content. Ossicles were extracted from several parts of tissue (tentacle, tube feet, dorsal body and ventral body) by 10% commercial bleaching and prepared for visualisation using microscope.

DNA Isolation and gene amplification

Total DNA of specimens was extracted from respiratory tree using DNA Preparation Kit for Blood Animal and Plant (Jena Bioscience, Germany). Cytochrome subunit I gene amplification was partially amplified by COIce-F primer (5'-ACTGCCCA CGCCCTAGT AATGATA TTTTTTATGG TNAT GCC-3') and COIce-R primer (5'-TCGTGTGTCT ACGTCCATTCCTACTGTRA ACATRT-3') (Hoareau and Boissin, 2010). PCR was performed by mixing MyTaq Red Mix (Bioline Reagents Ltd, UK), 1 µL of the set primers (10 pmol), 2 µL DNA-template, and 8.5 µL deionized water (Himedia, India). PCR cycle consisted of a cycle (initial denaturation) at a temperature of 95 ºC for 1 minute, main cycle, and the final extension at 72 ºC for 5 minutes. Main PCR cycles performed in 40 cycles, with denaturation at 95 ºC for 30 seconds, annealing at 56 ºC for 30 seconds, extension at 72 ºC for 20 seconds. Eppendorf Personal Thermal Cycler (USA) was used to perform COI amplifications. peqGREEN DNA/RNA dye (VWR International Ltd, UK) was used to identify PCR products in 2% agarose electrophoresis system. PCR clean-up and DNA sequencing were performed in 1st Base DNA Sequencing Service, Singapore.

DNA Sequence analysis

DNA sequences of COI gene were trimmed and aligned using Geneious v.9.8 software (Kearse *et al.*, 2012) before used in further data analysis. BOLD identification system was used as online software to identify COI sequence of our samples. We constructed phylogenetic tree among our sequences and 10 downloaded sequences from BOLD system by 1000 replicates unrooted Neighbor-Joining (NJ) tree using MEGA software version 7 (Kumar *et al.*, 2016). The software also used to perform pairwise Kimura 2-parameter (K2P) sequence divergence (Kimura, 1980). To generate a haplotype network from DNA sequences, minimum spanning network was created using PopART (Population Analysis with Reticulate Trees) software (Leigh and Bryant, 2015).

Results and Discussion

Morphological Descriptions

Specimens have quadrangular or rectangular body shape, with podia spread regularly throughout the body. Body length was about 11-14 cm and diameter were 1.5-4.88 cm. Papilla were found on the

dorsal surface (bivium) and tube feet were found on the ventral surface (trivium). 10 big dendritic tentacles and ventrally 2 smaller tentacles were presented in oral part. Pointed papilla on the dorsal portion with a total of 4 rows with irregular lines and cylindrical tube feet on the ventral. Specimens have 5 anal teeth and no tail. Specimens have a simple calcareous ring without posterior and anterior elongation, as reported by Clark (1971). Internal morphology of these specimens has 5 longitudinal muscles; respiratory tree was present in left and right lateral side; gonad and intestine were present (Fig. 1).

Fresh color of the body was red in radii and papillae; white or pale in dorsal and lateral interradii, also in ventricular interradii; tip of dendritic tentacles was red, while yellowish color in tentacle base; red in ventricular tube feet. Generally, color morphology was identical with description of O'loughin *et al.* (2016) in specimen from Camden Sound in northwest Australia, but in our samples did not observed dark brown to black spotted in tentacle trunks (Fig. 1E).

Ossicles distributed almost in all body include body wall, papillae, tube feet and tentacle (Fig 2; Table 1). On dorsal and ventral wall surface have hollow ellipsoid about 102.4 – 104.5 µm long, sometimes bowl with inner bridge and knobbed with inner bridge, usually bowl with one spinous margin about 41.3 – 43.7 µm and bowl with one spinous margin with inner bridge about 49.3 – 58.6 µm long. Papillae ossicles were hollow ellipsoid and bowl with many variants shape. On tube feet, endplate about 673.5 µm diameter and almost knobbed shape

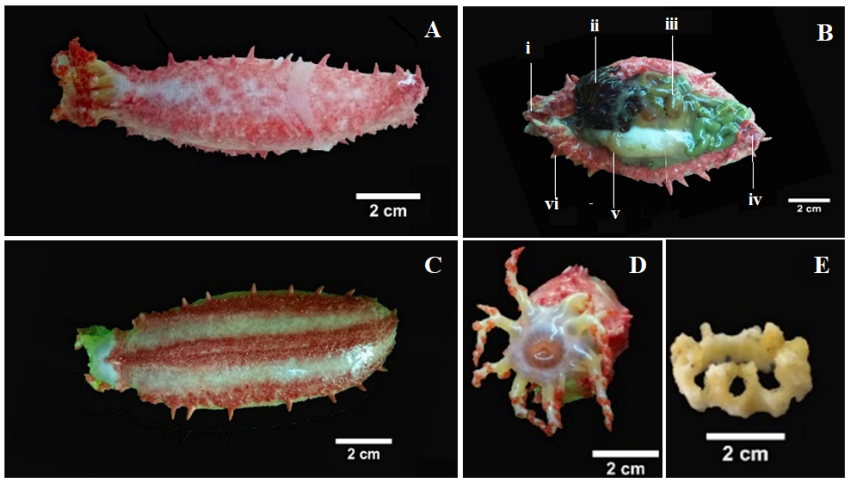

Fig. 1. Photo of fresh specimen of *C. quadrangularis*from Surabaya waters. (A) dorsal side (*bivium*); (B) ventral side (*trivium*); (C) internal anatomy; (D) shape and number of tentacles; (E) calcareous ring. Parts of internal organ:anterior part or tentacle (i), gonad (ii), intestine (iii), anus (iv), respiratory tree (v) dorsal papillae (vi).

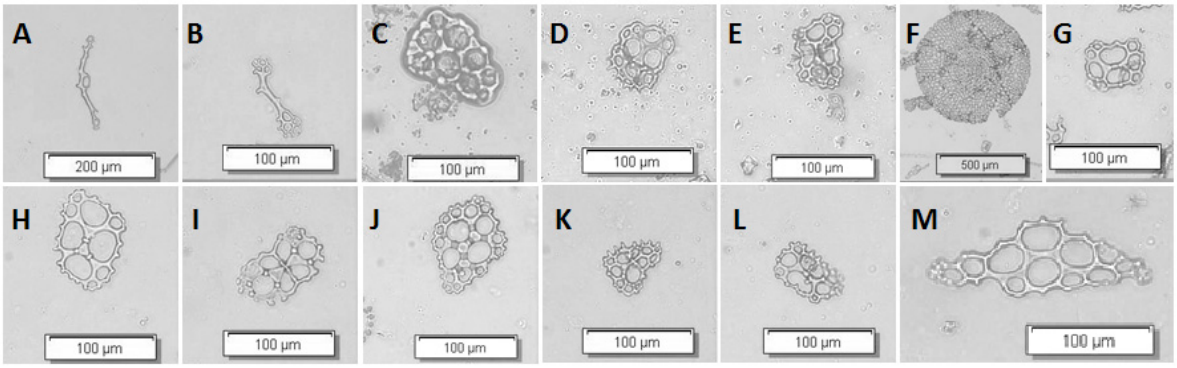

Fig. 2. Ossicles types of *C. quadrangularis* from Surabaya coastal waters. A: *tentacle rods*; B: *rossettes*; C: *hollow ellipsoid*; D: *bowl with one spinosus margin*; E: *bowl with inner bridge*; F: *endplate*; G: *bowl*; H: *spinosus edge*; I: *ellipsoid with inner bridge*; J: *knobbed with inner bridge*; K: *knobbed*; L: *bowl with one spinosus margin and inner bridge*; M: *support rod plates*

in range 62.7 – 84.7 bowl µm long. On tentacle, tentacle rods up to 177 µm and rosettes about 81 - 82 µm long. Tube feet was highest diversity of ossicles compared to other part of body, while tentacle was lowest diversity of ossicles.

Molecular Study

In this study, mitochondrial cytochrome oxidase subunit I gene was used to identify the specimens. 660-687 bp sequences of COI gene were obtained from 3 sea cucumber specimens, without presence any stop codons, insertion and deletion sites. BOLD identification system resulted highest similarity to *C. quadrangularis*, with 98,69% sequence identity to BOLD database. It indicated that specimens in this study confirmed as *C. quadrangularis*, because sequence divergence did not exceed 2%. Ward *et al.* (2008) reported that intraspecies divergence of COI in Echinodermata vary from 0-3.04%.

Identical BOLD identification results also represented in NJ tree reconstruction (Figure 3A), showed that genetic distance was below 3%. Using 617 bp homologous sequences from our study and downloaded sequences from two different geographicalsites (Singapore and Australia), pairwise Kimura 2-parameter (K2P) sequence diver-

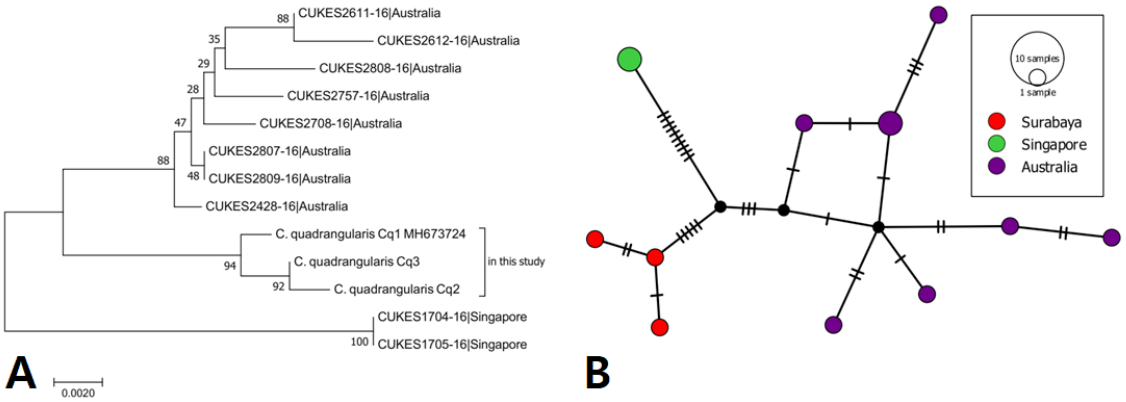

Fig. 3. COI sequence analysis of *C. quadrangularis* obtained from this sudy and previous database. (A) Unrooted Neighbor-Joining (NJ) tree; (B) Minimum spanning network of *C. quadrangularis* from this study and BOLD database (specimens collected from Singapore and Australia). A total 617 bases in the final dataset were analyzed.The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The circular diameter in network is proportional to frequency of the haplotype, the color is marked by sampling area, mutations showed as hatch marks, black dots indicate hypothetical ancestral haplotypes.

Table 1. Ossicle types and distribution in body of *C. quadrangularis* specimen from Surabaya waters

Ossicles type	Tentacles	*Tube feet*	Dorsal papillae	Ventral body wall	Dorsal body wall
Bowl		√	√		
Bowl with inner bridge			√	√	
Bowl with one spinous margin			√	√	√
Bowl with one spinous margin and inner bridge		√		√	√
Ellipsoid with inner bridge		√			
Endplate		√			
Hollow ellipsoid			√	√	√
Knobbed		√			
Knobbed with inner bridge		√			√
Rods	√				
Rossettes	√				
Spinous edge		√			
Support rod plates		√			

gence ranged from 0.2%-2.8%, with average 1.5%. This results was higher than result of Ward *et al.* (2008), who found intraspecific variation to average 0.48%, and range between 0% to 0.85% in Holothuroidea. It was possibly caused by different geographical range of analyzed COI sequences. Due to isolation by distance and phylogeographic structure, the intraspecific variation may increases with larger geographical scale of sampling (Bergsten *et al.*, 2012; Amin *et al.*, 2019). NJ tree reconstruction of our result also indicated that same geographical site will be clustered in a group. Specimens from Surabaya were closely related to Australian specimens, compared to specimens from Singapore. This pattern also be obtained in haplotype network (Figure 3B). Minimum spanning network of COI haplotypes showed that lowest mutation between Surabaya and hypothetical ancestral of Australian haplotype was 8 nucleotides, compared 13 nucleotides mutation between Singapore and Australian hypothetical ancestral. In our study, we confirmed three different haplotype of *C. quadrangularis* from Surabaya and they were distinctive haplogroup, differentiated from Singapore and Australian haplogroup. This study was a fundamentally beginning point for further studies to manage the fisheries policies of this species, especially in Indonesia.

Conclusion

In present study, we examine *C. quadrangular is* specimens from Surabaya coastal waters and confirmed as *C. quadrangularis* based on morphology and molecular approach. Three unique haplotypes of cytochrome oxidase subunit I gene also be detected from Surabaya, mismatched from Singapore and Australia haplogroups.

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