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Revisit Study of Freshwater Sponges *Eunapius carteri* (Bowerbank, 1863) and a New Record of *Oncosclera asiatica* Manconi and Ruengsawang, 2012 (Porifera: Spongillida) in Porong River, East Java, Indonesia

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ABSTRACT

Distribution of freshwater sponges (Porifera: Spongillida) in Indonesia is currently insufficiently reported and underestimated compared to marine sponges. An inventory study on freshwater sponges in East Java after Indonesian independence in 1945 is yet to be carried out. For this reason, we reported new records of two freshwater species, *Eunapius carteri* (Bowerbank, 1863) and *Oncosclera asiatica* (Manconi and Ruengsawang, 2012) in Porong river, East Java, Indonesia. *E. carteri* species was originally described in Mumbai, India and its presence in Java was reported in 1927, 1928, and 1930. DNA barcoding and molecular phylogenetic analysis using mitochondrial COI was able to successfully identify our specimens as *E. carteri*, while analysis using the nuclear ITS markers placed our samples with other *Eunapius* species. Furthermore, in this study we report for the first time in East Java, Indonesia, the presence of *O. asiatica*. The *O. asiatica* species was originally described from Pong River located in Lower Mekong, Thailand. We concluded our specimens to be *O. asiatica* based on the morphology, skeleton, and spicule composition of the specimens that were similar to the samples recorded in Thailand. We were only successful in obtaining the COI sequence of *O. asiatica*. Furthermore, our samples did not group to the publicly available sequence of its congener, namely *Oncosclera* sp. Therefore, further molecular taxonomy and morphological analysis is needed to explore the diversity of freshwater sponges in general and to conduct species delimitation of *E. carteri* and *O. asiatica* in Java, Indonesia, and Asia

1. Introduction

Freshwater sponges (Manconi and Pronzato 2002) are classified as a minority group of sponges, in which their habitat distinguishes this order from marine sponges. In earlier centuries, biologists grouped freshwater and marine sponges (Grant 1836) with plants because they are sessile and possess a greenish-brownish color. Furthermore, the first specimen of *Spongilla lacustris* was described in 1759 by Linnaeus as "creeping, fragile, with cylindrical branches showing swellings at their ends", which the type material was preserved in the Linnean Herbarium. Sponges were not recognized as animals until 1765. Unlike marine sponges, freshwater sponges can survive in fluctuating or extreme environmental conditions, such as shortage of water, and also have the ability for long-distance dispersal because of their asexually reproduced propagules called gemmule (see detail in Manconi and Pronzato 2002). Sponges play a vital role in connecting food webs

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in freshwater ecosystems, which involve primary production between pelagic and benthic organisms in the upstream and stream. The role is displayed by Skelton and Strand 2013 that discovered a difference in carbon signature in freshwater sponges that host zoochlorella and not.

Freshwater sponges have a broad geographical distribution ranging from tropical to temperate regions and also in the Palearctic and Neotropical regions (see detail in Manconi and Pronzato 2002). However, the diversity of freshwater sponges in the Indonesian archipelago still needs to be investigated, particularly in the province of East Java. According to the World Porifera Database (WPD) (de Voogd *et al.* 2022), only five genera composed of 9 species from the Spongillidae Gray, 1867 family had been recorded on the island of Java (Gee 1930; Vorstman 1927, 1928) (see review in Manconi *et al.* 2013). We report the presence of the cosmopolitan species, namely *Eunapius carteri* (Bowerbank 1863), in the Porong river that flows through several cities in the province of East Java. This finding corroborates the last known publication dating back to 1928 by Vorstman, before the independence of Indonesia. In addition, at this watershed, we recorded for the first time the presence of *Oncosclera asiatica* Manconi and Ruengsawang 2012, a species that was initially described in Thailand.

2. Materials and Methods

2.1. Sampling Collection

Samples of *E. carteri* and *O. asiatica* (see Figure 1) were freshly collected from the Porong river at 7°28'17.4 "S, 112°31'07.3 "E on July 25th, 2020, in the city of Mojokerto. The Porong river is a straight river with a depth of less than 1 m during the dry season and can rise to 5 m during the monsoon season. Rice paddy and cornfield also exist on the riverbank (see Figure 2). Our examination included a morphological analysis covering macroscopic features, e.g., lifeform and habitat, and observations on microscopic characters' spicule composition, gemmules, and skeleton arrangement.

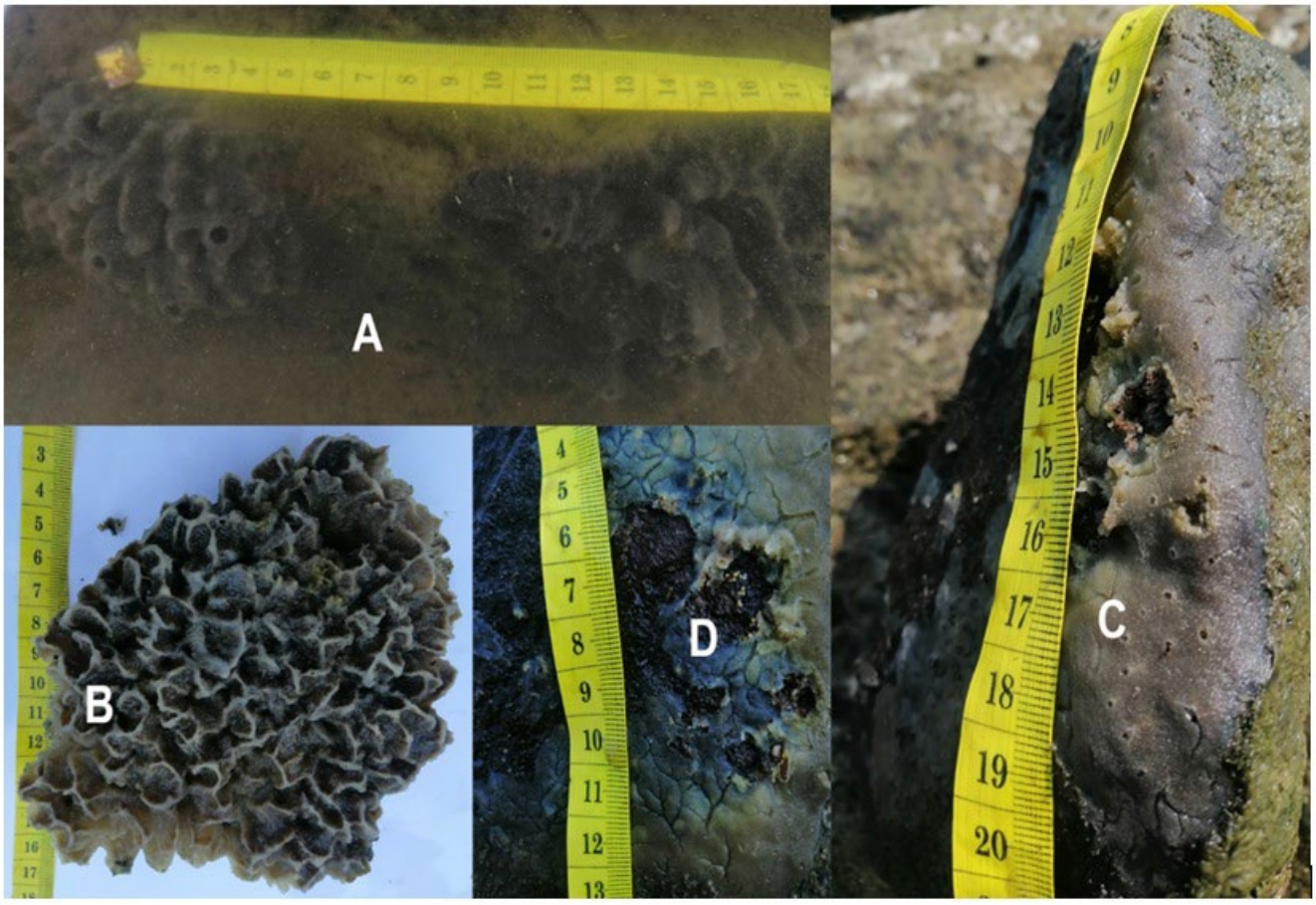

Figure 1. (A) and (B) Lifeform of *E. carteri*, (C) and (D) *O. asiatica* in Porong river. *E. carteri* was attached on hard mud substrates whereas *O. asiatica* on hard rock substrates

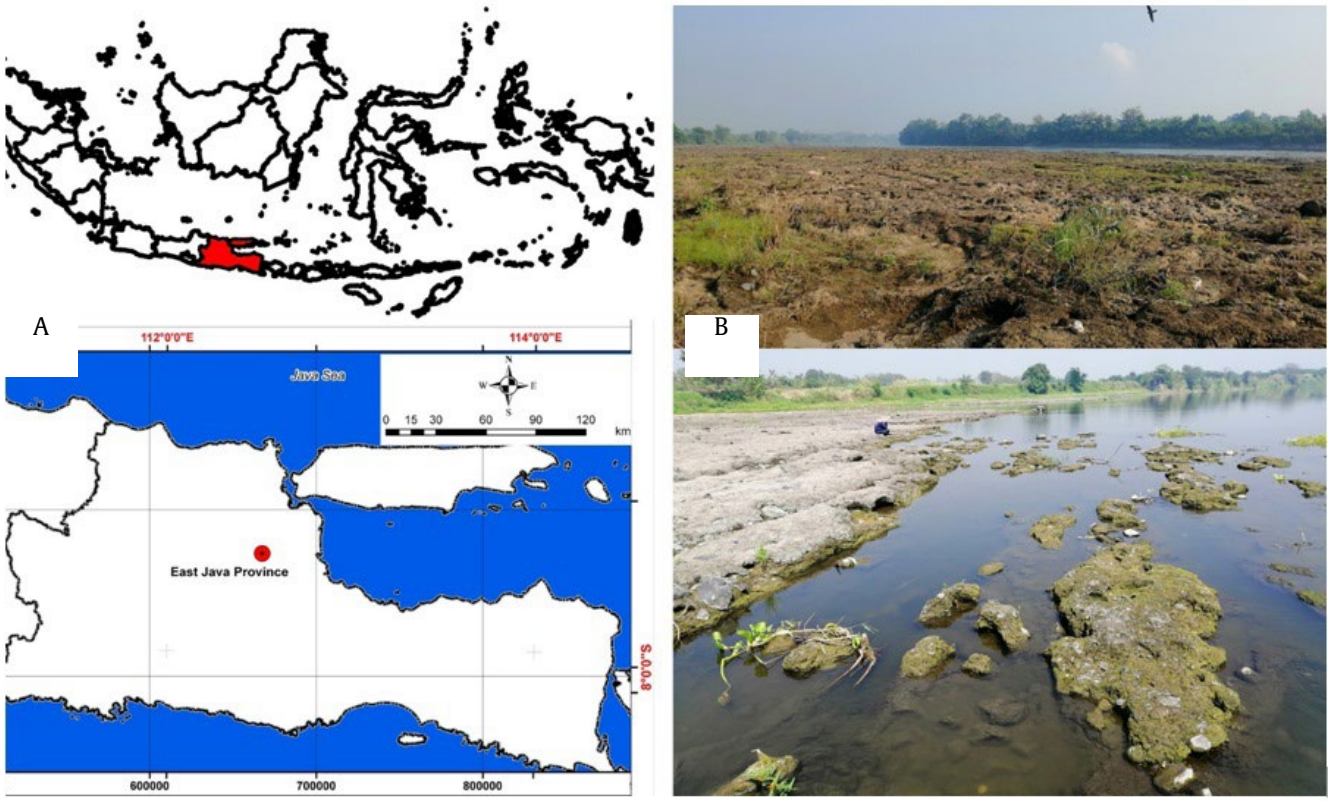

Figure 2. (A) and (B) Geographical locality of Porong river where the samples were taken in the dry season on July 25th 2020

2.2. Spicules and Skeleton Slides Preparation and Identification

Approximately ±3 cm^3 of sponge tissue was divided into three approximately equal parts. The first ±1 cm^3 tissue was immersed in bleach. After the bleach was removed entirely, the spicules were allowed to settle. Subsequently, the spicules were rinsed three times with distilled water to remove the bleach of the spicules completely and were finally preserved in 70% ethanol. The spicules were mounted on the object glass and covered with a cover slip. To observe the skeleton, the second ±1 cm^3 tissue was cross-sectioned and longitudinally cut with a hand-cutting knife. The skeleton was also mounted on the object-glass and covered with a cover slip. Entellan® | 107960-Merck Millipore was used as an adhesive substance for the spicules and skeleton slides. Finally, examinations were carried out on the type and size of the spicules, skeleton, and gemmules, which examinations followed the identification manual from Manconi and Pronzato 2002 and World Porifera Database (WPD) (de Voogd *et al.* 2022), using an Olympus CX 31 light microscope device. We compared our specimens to the description of similar species that had previously been recorded and the closest species within the same genus from Southeast Asia.

2.3. DNA Barcoding Analysis

DNA extraction from the third ±1 cm^3 tissue was done by means of spin-column using InstaGene kit (Bio-Rad Laboratories, USA), in accordance with the protocol of its manufacture. Furthermore, polymerase chain reaction (PCR) using DNA templates with amplification of the Internal Transcribed Spacer (ITS) fragment by means of the protocol from Itskovich *et al.* (2015) using the ITS-Its–F1: 5′-GTAGGTGAACCTGCGGAA-3′ and ITS-Its–R1: 5′-GTTGGTTTCTTTTCCTCCGCTPCR-3′ primers, and amplification of the cytochrome oxidase-1 (CO1) fragment for standard barcoding of sponges by means of the protocols from Erpenbeck *et al.* 2016 using the dgLCO1490: 5′GGTCAACAAA TCATAAAGAYATYGG-3′ and dgHCO2198: 5′-TAAAC TTCAGGGTGACCAAARAAYCA-3′ degenerated primers designed by Meyer *et al.* (2005), were performed. Subsequently, sequencing of forward and reverse strands were performed with the ABI BigDye v3.1 chemistry (Applied Biosystems, California, USA), and primers amplification was carried out using the ABI 3730xl Genetic Analyzer, which was provided by PT Genetika Sains Indonesia (https://ptgenetika.com/about-us/), in accordance to the protocol of the manufacturer. Finally, the sequences were assembled, trimmed, and analyzed using SeaView and Bioedit

v7.2 (Hall *et al.* 2011) and subsequently checked with BLAST against GenBank (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) to ensure the obtained sequences were that of sponges and not contaminated with other organisms or sponge symbionts such as microbes.

2.4. Phylogenetic Analysis

The sequences were used for phylogenetic analysis using maximum likelihood (ML) and Bayesian inference (BI). First, the ML phylogram was generated by IQTREE v.1.6.12 (Nguyen *et al.* 2014) with an ultrafast bootstrap of 1000 replications in UFBoot2 (Hoang *et al.* 2017). Furthermore, ModelFinder (Kalyaanamoorthy *et al.* 2017) was utilized to select the optimal model of molecular evolution based on the Akaike Information (Akaike 1974), which was recognized as TVM+F+I for ITS and K3Pu+ F+I for CO1. Concurrently, the Bayesian phylogram was generated using MrBayes v.3.2.1 (Ronquist *et al.* 2012) with the GTR+G+I and GTR+G models for the ITS and CO1 sequences, respectively, because the TVM and K3Pu models are not applicable in MrBayes. Each analysis was composed of two independent runs of four Metropolis-coupled Markov chains under default temperatures with trees sampled at every 1000th generation. The analysis was automatically terminated when the chains converged, indicated by an average standard deviation of split frequencies <0.01.

3. Results

Class Demospongiae (Sollas, 1885); Order Spongillida (Manconi and Pronzato, 2002); Family Spongillidae (Gray, 1867); Genus *Eunapius* (Gray, 1867); Species *Eunapius carteri* (Bowerbank, 1863).

3.1. Diagnosis (Bowerbank, 1863)

Shape ranges from encrusting to bulbous, massive to cone-shaped, flattish to lobate, or bearing finger-like projections. The color is yellowish-brown to tan and bright green. Consistency ranges from fragile to moderately soft and compact. The surface is hispid due to tufts of emerging spicules. Oscules are sparse and conspicuous. The ectosomal skeleton was undifferentiated. Choanosomal skeleton is anisotropic. Spongin is relatively abundant. Megascleres are stout, fusiform, slightly curved, smooth oxeas (265–370 x 14–24 µm) with sharply pointed to rounded tips. Microscleres are absent. Gemmules are scattered and subspherical (440–610 µm) with gemmuloscleres (145–219 x 5–8 µm) embedded in the pneumatic layers. The foramen is tubular, simple, and without the collar. Gemmular theca is bi-layered. Habitat is standing water of freshwater ecosystems and is attached to plants, sticks, stones, or the shell of freshwater clams.

3.2. Material Examined

Six specimens 001_EC_25072020 002_EC_25072020,003_EC_25072020,004_EC_25072020, 005_EC_25072020, 006_EC_25072020, collected by Edwin Setiawan and Ahmad Yanuar on July 25, 2020, 20 cm depth, hard mud substrate, Porong river, 7°28'17.4"S, 112°31'07.3"E, Kwatu district, city of Mojokerto, East Java, Indonesia.

3.3. Description

Our specimens possessed a massive form and were attached to a hard mud substrate at a depth of approximately 20 cm. The upper surface of the sponges was hispid and had a lobate structure with a moderately soft consistency. The living specimens were yellowish light brown (Figure 3A) and turned into a light greyish black color when submerged in ethanol. The oscula were sparse and conspicuous. The skeleton was anisotropic with abundant spongin fibers (Figure 3D and E). The spicules were sharply pointed oxeas (Figure 3G). Some oxeas were modified to be curved in the middle resembling an L, T, or Y shape (Figure 3H). The gemmules (Figures 3B and C) were scattered and possessed a subspherical form. The gemmuloscleres (Figure 3F) were embedded in the pneumatic layers. The dimensions of megascleres, gemmuloscleres, and gemmules of the *E. carteri* specimens, along with the locality in which they were recorded, are shown in Table 1.

3.4. Remarks

Gee (1930) described *E. carteri* as a cosmopolitan species and a common freshwater species in Java, South China, and India. In Java, this species is frequently found in lakes and ponds, not running water. Our specimens resemble the specimen found in Java that Gee recorded. The specimen was described to possess a light brown color with a compressible but fragile structure due to a moderate amount of spongin fiber. The spicules were also slightly curved oxeas, rarely straight, smooth, and gradually sharp

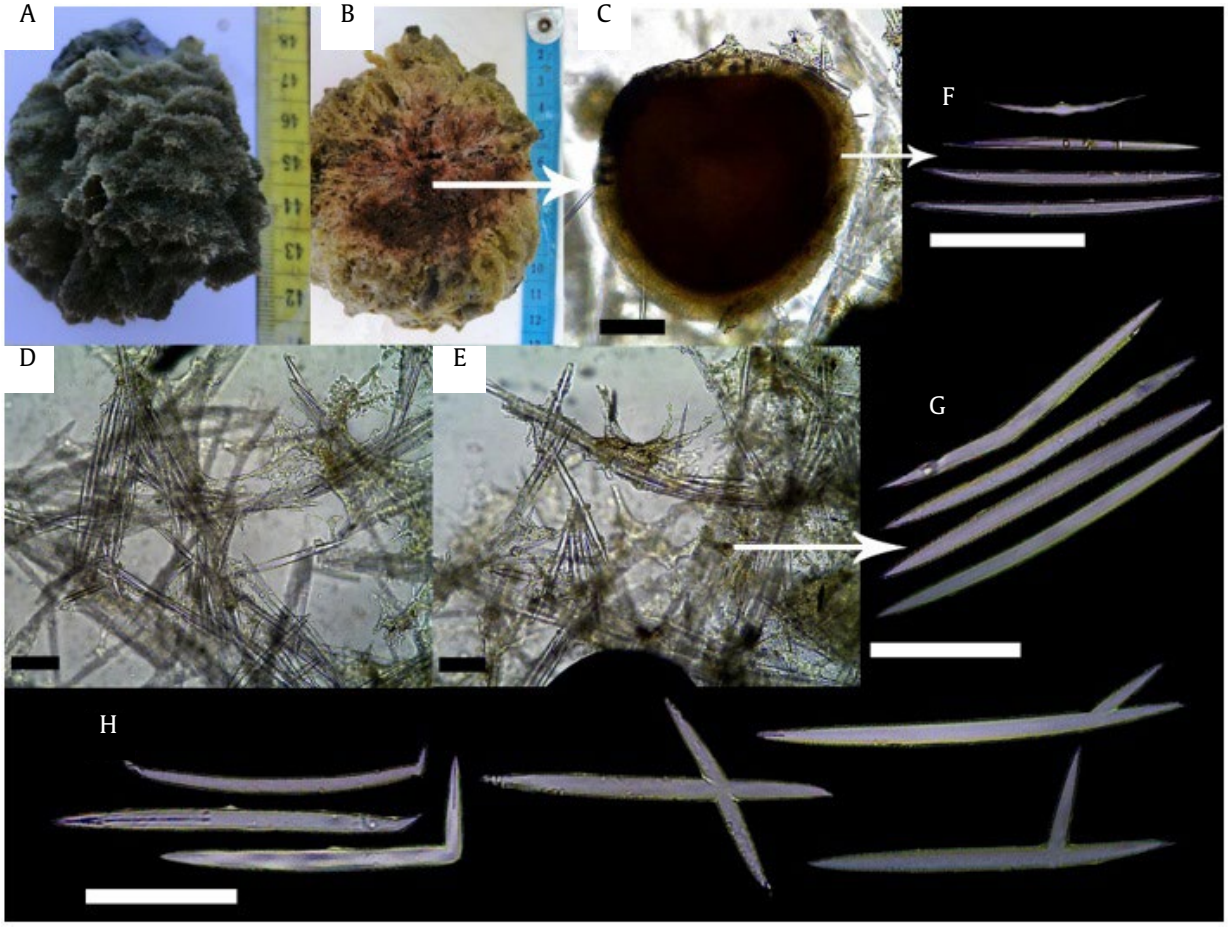

Figure 3. (A) Macroscopic outlook of the *E. carteri* specimens, apical side with osculum, (B and see arrow) basal side with gemmules, (C) microscopic outlook of gemmule, (D) tangential microscopic sections of *E. carteri*, (E) perpendicular microscopic sections of *E. carteri*, (F) oxeas gemmuloscleres, (G and H) Modified forms (abnormalities) of oxeas megascleres. The scale of (C–F) is 1 = 100 µm

Table 1. Checklist of main morpho-traits of the *E. carteri* specimens and closely related species recorded in Southeast Asia to assist in the identification of our specimens

Species/specimen number	Megascleres µm	Gemmuloscleres µm	Gemmules µm	Localities	References
E. carteri/ 001_C_25072020	Oxeas 222–**267.8**–312 × 15.9–**20.3**–30.2	Oxeas 130–**148.4**–166.7 × 7–**7.3**–7.9	Subspherical 352.2–**367**–376.8	Kaliporong East Java, Indonesia	This study
E. carteri/ 002_C_25072020	Oxeas 251.4–**288.1**–353 × 16.1–**18.6**–22.8	Oxeas 111–**128.3**–156.5 × 5.5–**6.8**–8	Subspherical 335.3–**392.6**–456.7	Kaliporong East Java, Indonesia	This study
E. carteri/ 003_C_25072020	Oxeas 201.5–**254.5**–329.4 × 15.9–**18.5**–22.8	Oxeas 147.3–**166.9**–185.8 × 7.8–**9.4**–12.2	Subspherical 299.3–**419.1**–515.3	Kaliporong East Java, Indonesia	This study
E. carteri/ 004_C_25072020	Oxeas 224.8–**258.6**–306 × 15.6–**18.7**–22.6	Oxeas 150.3–**165.7**–185.5 × 7.8–**9.4**–11.1	Subspherical 303.6–**369.8**–419.5	Kaliporong East Java, Indonesia	This study
E. carteri/ 005_C_25072020	Oxeas 239.3–**268.1**–295.9 × 12.2–**17.5**–20.7	Oxeas 158.5 –**169.5**–177.5 × 6.7–**8.07**–9.9	-	Kaliporong East Java, Indonesia	This study

Table 1. Continued

Species/specimen number	Megascleres µm	Gemmuloscleres µm	Gemmules µm	Localities	References
E. carteri/ 006_C_25072020	Oxeas 236.1–**273.3**–310.7 × 15.3–**17.7**–22.2	Oxeas 151.5–**161.7**–186.7 × 6.3–**7.8**–10.6	-	Kaliporong East Java, Indonesia	This study
E. carteri/52915	Oxeas 259–366 × 16–22	Oxeas 180–210 × 5–8	-	Java, Indonesia	(Gee 1930)
E. carteri/–	Oxeas ±300	Oxeas ±300	Subspherical ± 600	East Java	(Vorstman 1928)
E. carteri/–	Oxeas ±300	Oxeas ±300	Subspherical ± 600	West Java	(Vorstman 1927)
Eunapius crassissimus (Annandale, 1907)/–	Amphioxea 250–310 × 6–15	Amphistrongyla, Amphioxea, 80–120 3–9	Spherical 280-310	India and Malaysia	(Annandale 1907; Manconi and Pronzato 2007; Manconi *et al.* 2013; Penney and Racek 1968)
Eunapius potamolepis (Annandale, 1918)/–	Amphioxeas Amphistrongyle 240–320	Acanthostrongyles with occasionally sigmatoid & feebly curved –	Subspherical 680	Malaysia	(Annandale 1918; Manconi *et al.* 2013)
Eunapius fragilis (Leidy, 1851)/–	Amphioxea, 180–270 × 5–12	Amphioxea or amphistrongyla, with conspicuous spines 75–140 × 2–7	Subspherical, grouped into three 180-290	Malaysia	(Manconi *et al.* 2013)
Eunapius tinei (Gee, 1932)/–	Amphioxea 170–230 × 13–17 µm.	Amphioxea 70–95 × 4–7 µm	Subspherical 430–460 µm	The Philippine	(Gee 1932)
Eunapius conifer (Annandale, 1916) ZRC. POR.0274 and ZRC.POR.0275	Oxeas 210–**232.7**–255 × 7.5–**8.8**–11	Oxeas 65–**81.5**–115 × 2–**2.6**–3	Subspherical 250–**315**–350	Singapore	(Lim and Tan 2013)

pointed at the ends with a dimension similar to our specimens. In addition, some abnormalities in the end of spicules with fork ends, bulbous enlargements in the center, and other unusual characteristics that were described by Gee were also discovered in our specimens. No microscleres were detected, and gemmuloscleres possessed a similar form to megascleres. Moreover, Gee did not describe the size of gemmules, but Gee's description was similar to our specimens, in which gemmules possessed a nearly spherical shape with a flattened-out form in the basal.

Family Potamolepidae (Brien, 1967); genus *Oncosclera* (Volkmer-Ribeiro, 1970); species *Oncosclera asiatica* (Manconi and Ruengsawang, 2012).

3.5. Diagnosis (Manconi *et al.* 2012)

Thin encrusting sponges (less than 2 mm) with a hard and fragile consistency. Color in life is yellowish and turns to light brown in ethanol. A dense network of branching subdermal canals and slight hispidation are exhibited on the surface. Oscules inconspicuous. Ectosomal skeleton without special architecture, with triangular to quadrangular meshes (80–100 µm in diameter) in an irregular network of oxeas arranged in mono- to pauci-spicular tracts. Choanosomal skeleton irregularly alveolate network, extremely thin, with triangular to quadrangular meshes (93–108 µm in diameter) with scarcely developed ascending mono- to pauci-spicular fibres. Spongin was notably scanty. Megascleres are mostly acanthoxeas (140–185 × 14 µm), stout, slightly bent to rarely straight, spiny on the entire shaft with abruptly pointed tips; spines towards the tips notably curved; spines scattered on the shaft small, straight, acute, bearing microspines. Other megascleres that are oxeas and strongyloxeas are rare, and acanthostrongyles are extremely rare. Microscleres are absent. Gemmules are hemispherical, single (525–882 µm in diameter), or in groups of up to 3 gemmules partly sharing the

theca at the sponge base, strictly adhering to the basal spongin plate armed by tangential megascleres. Foramen is not evident. Gemmular theca trilayered (50 µm in thickness) with the outer layer of compact spongin covering tangentially embedded gemmuloscleres in a mosaic manner. The spongin fibres' pneumatic layer forms small, rounded meshes, with 1–4 layers of gemmuloscleres tangentially embedded—inner layer of compact spongin with sublayers. Gemmuloscleres acanthostrongyles (43–125 x 6–17 µm) elongated, from bent to rarely straight, to ovoid, with a frequently inflated shaft in the middle and large tubercles/spines particularly dense towards the tips.

3.6. Material Examined

Three specimens 001_OA_25072020 (Figure 5), 002_OA_25072020, and 003_OA_25072020, collected by Edwin Setiawan and Ahmad Yanuar, 20 cm depth, rock substrate, Porong river, 7°28'17.4"S, 112°31'07.3"E, Kwatu district, city of Mojokerto, East Java, Indonesia.

3.7. Description

The living specimens were light green to light brown in color (Figure 4A) and turned into a pale white color when submerged in ethanol. The oscula were sparse and inconspicuous. The skeleton was a loose reticulation and possessed a crumble consistency (Figure 4E). The spicules are mostly acanthoxeas (Figure 4G) with curved and sharp ending types and rare acanthostrogyles with some modification (Figure 4H) of points (Figure 4I). Microscleres are absent. Gemmules are recognized on the lower surface with a very short canal possessing a simple opening and forms into singular or grouped gemmules (Figure 4B, C and D). Gemmuloscleres are acanthostrongyles that possess various forms and lengths, i.e., short ovoid, medium bent into long straight forms with obvious spines on the tips (Figure 4F). The dimensions of megascleres, gemmuloscleres, and gemmules of the *O. asiatica* specimens, along with the locality in which they were recorded, are shown in Table 2.

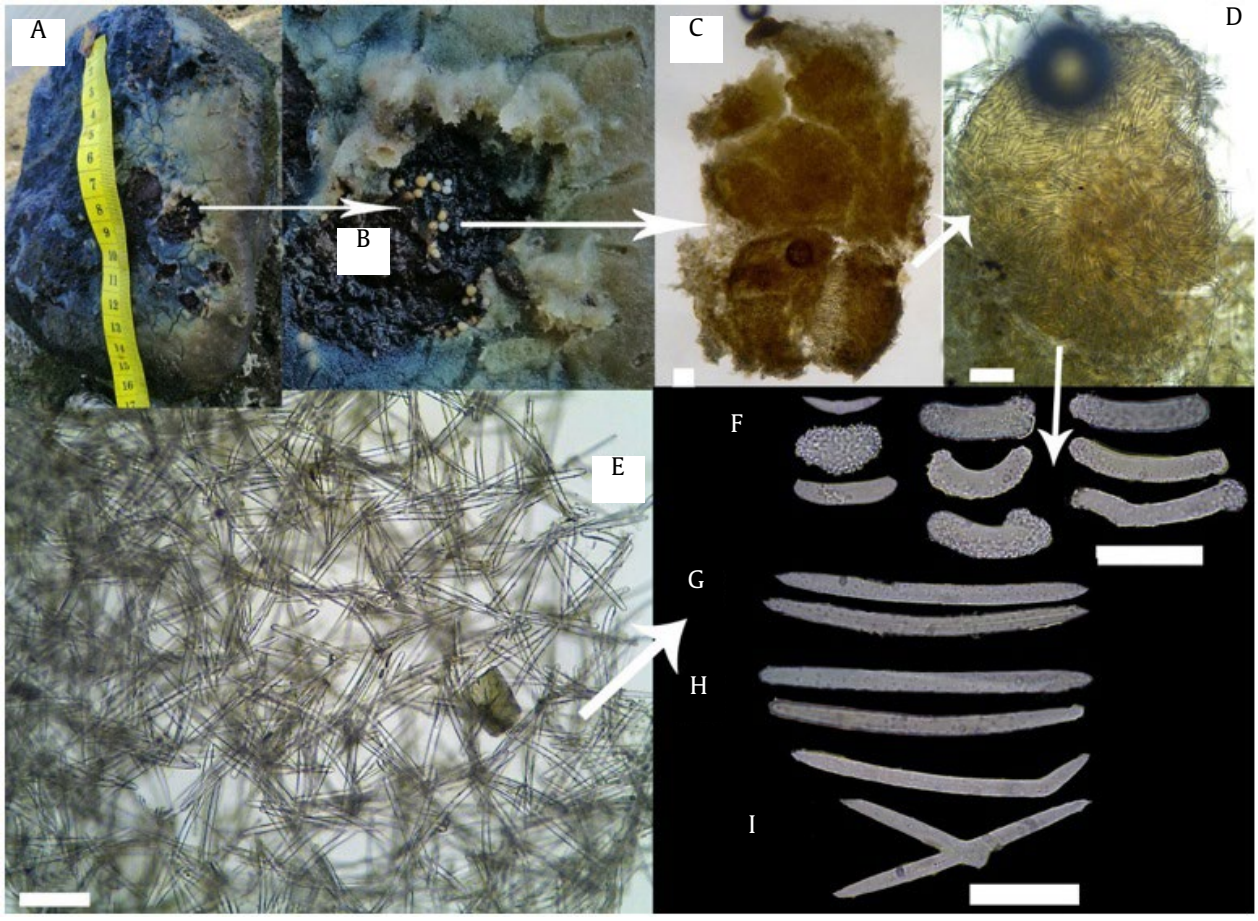

Figure 4. (A) Macroscopic outlook of *O. asiatica* specimens, (B and see arrow) Gemmules, (C and D) microscopic outlook of gemmules, (E) tangential microscopic sections of *O. asiatica*, (F) acanthostrongyles and variation of gemmuloscleres, (G) acanthooxeas, (H) acanthostrongyles, and (I) modified forms of acanthostrongyles megascleres. The scale of (C–E) is 1 = 100 µm and the scale of (F–I) is 1 = 50 µm

Table 2. Checklist of main morpho-traits of the *O. asiatica* specimens and the *Oncosclera* recorded in Southeast Asia to assist the identification of our specimens

Species/specimen number	Megascleres µm	Gemmuloscleres µm	Gemmules µm	Localities	References
O. asiatica/ 001_OA_25072020	Acanthoxeas 128.4–**150**–173.8 × 8.8–**11.2**–14.3 Acanthostrogyles 124.2–**142**–159.5 × 9.4–**14.4**–17.7	Acanthostrogyles 43–**60.2**–83.8 × 13.8–**17.3**–20	471–**492**–516	Kaliporong East Java, Indonesia	This study
O. asiatica/ 002_OA_25072020	Acanthoxeas 134.4–**137**–176 × 6.3–**12.7**–12.9 Acanthostrogyles 144–**155.7**–157.3 × 10.6–**12**–13.5	Acanthostrogyles 38.4–**59.2**–87.2 × 14.3–**17.3**–24.4	442–**473**–502	Kaliporong East Java, Indonesia	This study
O. asiatica/ 003_OA_25072020	Acanthoxeas 127.4–**150.1**–177.3 × 10–**11.5**–16 Acanthostrogyles 126.5–**146.7**–158.8 × 10–**11.4**–14.4	Acanthostrogyles 37.2–**64.2**–80 × 6.6–**14.2**–18.3	455–**472**–501	Kaliporong East Java, Indonesia	This study
O. asiatica/ MSNG 56534a	Acanthoxeas 140–185 × 14 Acanthostrongyles 133–159 × 9–12	Acanthostrongyles 43–125 × 6–17	525– 882	Mekong, Thailand	(Manconi *et al.* 2012)

3.8. Remarks

To date, *O. asiatica* is the sole species of the Oncosclera genus that has been recorded and described, originating from Southeast Asia. Our specimens from the Porong river possess similar characteristics to that described by Manconi *et al.* (2012) regarding morphological character, i.e., life color, consistency, skeleton structure, and spicule composition, including gemmules and gemmuloscleres type.

3.9. Molecular Analysis

We were able to obtain four ITS sequences from the E. carteri specimens 001_EC_25072020, 002_EC_25072020, 003_EC_25072020, 004_EC_25072020 (accession number MZ496391, MZ496392, MZ496393, and MZ496394 respectively) whereas, attempts to obtain ITS sequences from the *E. carteri* specimens 005_EC_25072020, 006_EC_25072020 and the *O. asiatica* 001_OA_25072020, 002_OA_25072020, and 003_OA_25072020 failed due to symbiont amplification. The length of the *E. carteri* ITS sequences was 713 bp, exhibiting a 97-98 % similarity to ITS sequences of EF151948 and *Eunapius conifer* EF151939 (Figure 5). Furthermore, we were able to sequence CO1 fragment of three *E. carteri* specimens 001_EC_25072020, 002_EC_25072020, and 003_EC_25072020 (accession number MZ502317, MZ502318, and MZ502319 respectively) and two O. asiatica 001_OA_25072020, 002_OA_25072020 specimens (accession number MZ502320 and MZ502321 respectively). While attempts to obtain sequences from the other specimens failed due to symbiont contamination. The length of the *E. carteri* and *O. asiatica* sequences were 673-686 bp. The *E. carteri* sequences obtained were 99-100% identical to MH319717, whereas the *O. asiatica* sequences obtained were 99 % identical to *Spongilla lacustris* KU759841 (Figure 6).

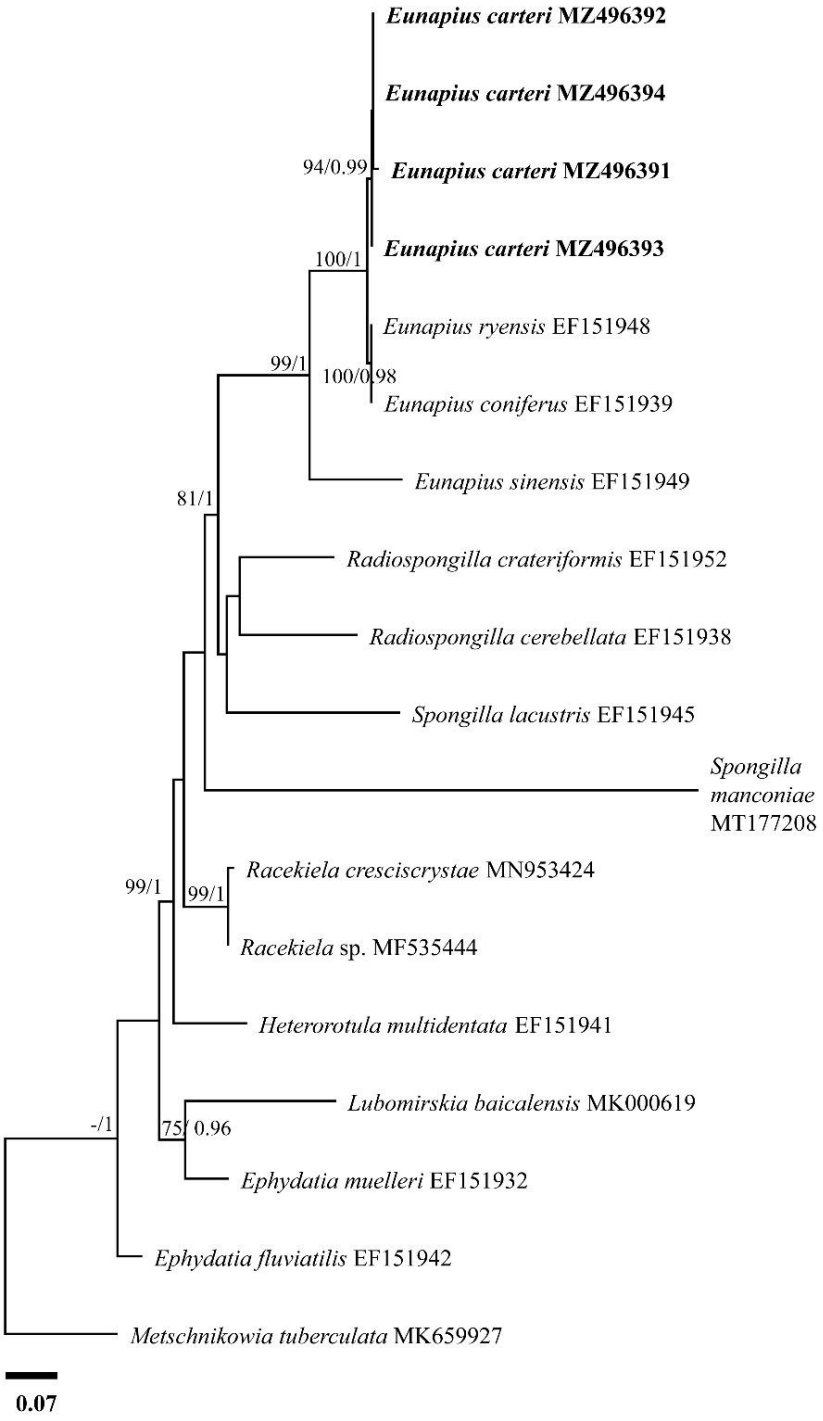

Figure 5. Maximum likelihood ITS phylogram of *E. carteri* specimens rooted with freshwater sponges from Baikal Lake Metschikowia tuberculate MK6599927. The number on the branches represent bootstrap proportions (BP)/ posterior probabilities (PP) of Bayesian inference. The scale bars indicate the number of substitutions per sites. The obtained sequences from this study are in bold

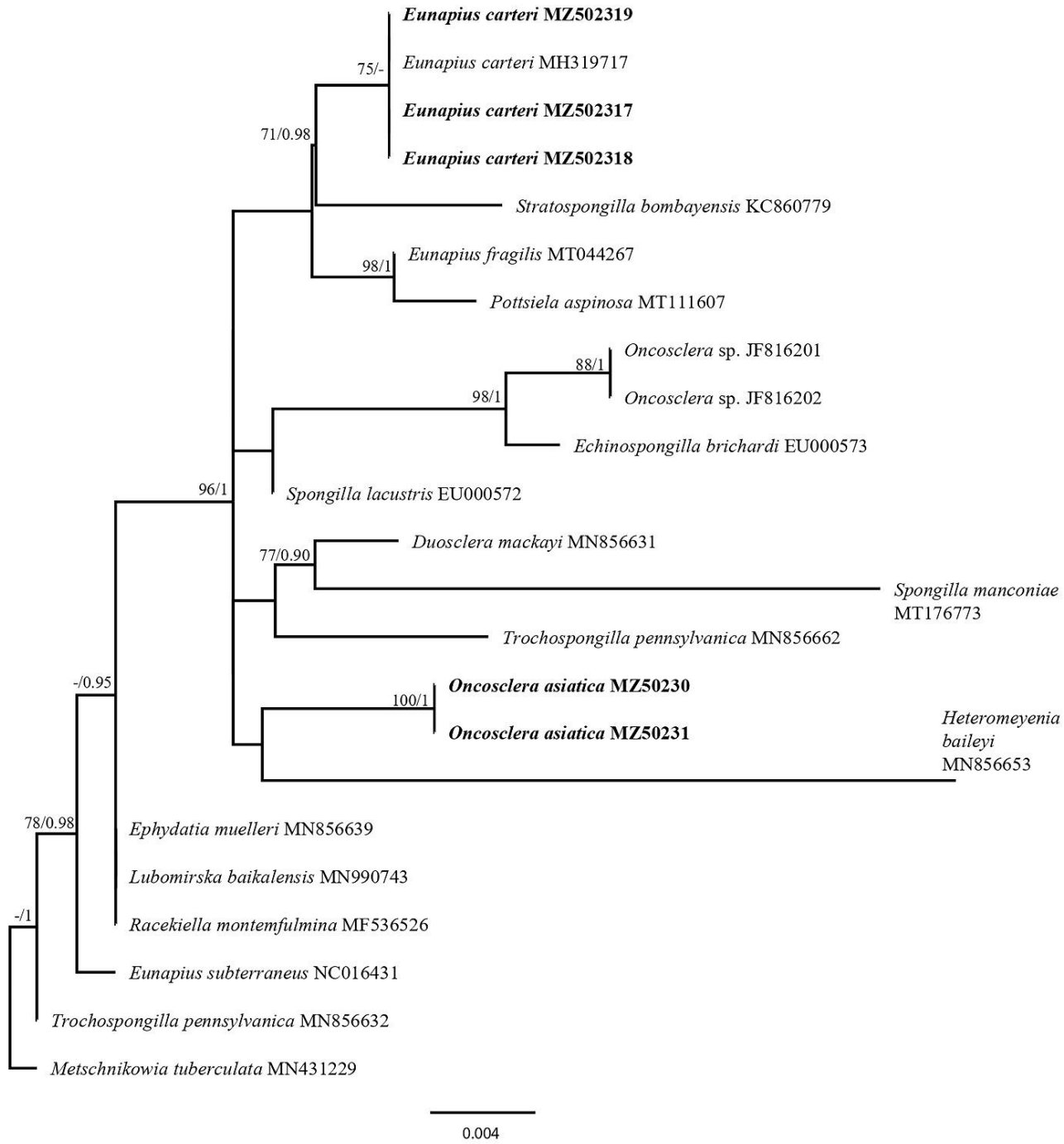

Figure 6. Maximum likelihood CO1 phylogram of *E. carteri* and *O. asiatica* specimens rooted with freshwater sponges from Baikal Lake Metschikowia tuberculate MN431229. The number on the branches represent bootstrap proportions (BP)/posterior probabilities (PP) of Bayesian inference. The scale bars indicate the number of substitutions per sites. The obtained sequences from this study are in bold

4. Discussion

In the classification of the zoogeographic zone, Indonesia is divided into two regions, namely the Oriental and Australasian regions. Sumatra, Java, and Borneo are grouped into Oriental areas, including Southeast Asia, the Indian subcontinent, and southwestern China. While Celebes, Molucca, Lesser Sunda, and Papua are grouped into the Australasian region. The diversity of freshwater sponges between these two zoogeographic regions is similar, possessing around thirty species, which is comparable to the Nearctic region and fewer than the Afrotropical (49 species), Palearctic (59 species), and Neotropical (63 species) regions (see detail in Manconi and Pronzato 2007). The diversity of freshwater sponges in Indonesia is insufficiently investigated. Only a few manuscripts were published after Indonesia's independence, solely from Celebes (Meixner *et al.* 2007; von Rintelen *et al.* 2007). This condition is in contrast to other Southeast Asian countries (see Calcinai *et al.* 2020; Lim and Tan 2013; Manconi *et al.* 2013).

Nine species out of five genera were described from the island of Java (see review in Manconi *et al.* 2013; Vorstman 1927, 1928), namely *Ephydatia fortis* Weltner 1895, *Ephydatia ramsayi* (Haswell 1883 (1882)), *Radiospongilla cerebellata* (Bowerbank 1863), *Radiospongilla crateriformis* (Potts 1882), *Radiospongilla indica* (Annandale 1907), *Trochospongilla latouchiana* Annandale 1907, *Trochospongilla philottiana* Annandale 1907, *Umborotula bogorensis* (Weber 1890), and *Eunapius carteri* (Bowerbank 1863). *E. carteri* is a cosmopolitan species that inhabits a broad geographical area, including Europe (Carballeira 2018; Pronzato and Manconi 2001), Africa (Manconi and Pronzato 2009), and the Indian Subcontinent (Jakhalekar and Ghate 2013). Our findings confirmed the existence of *E. carteri* on the island of Java (Gee 1930; Vorstman 1927, 1928). We did not discover the other four species of *E. carteri* known to be found in East Java within the area of the scope of our study, but it may possibly be found with further exploration of other river basins and lakes described by Vorstman 1928.

Several *E. carteri* congeners that are cosmopolitan were recorded to inhabit Southeast Asia, namely *Eunapius crassissimus* (Annandale 1907), *Eunapius fragilis* (Leidy 1851), and *Eunapius conifer* (Annandale 1916) (see Table 1). Furthermore, two other species recorded in Southeast Asia, namely *Eunapius potamolepis* (Annandale 1918) from Malaysia and *Eunapius tinei* (Gee 1932) from the Philippines, were described as endemic species. Two other species from different genera were recorded in Thailand: *Corvospongilla siamensis* Manconi and Ruengsawang 2012, and *Oncosclera asiatica* Manconi and Ruengsawang 2012, in which the latter was discovered for the first time in Indonesia in our study. Several descriptions of *E. carteri* recorded in Java (Gee 1930; Vorstman 1927, 1928) were in accordance with our species identification, namely oxeas form and dimensions of megascleres, gemuloscleres, and gemmules that are subspherical (see Table 1). We classify our specimens as *E. carteri* due to several distinguishing characteristics, namely the megascleres form, which was an amphioxea form with a thinner size (6–15 µm), and the similar dimension of gemmuloscleres (80–120 x 3–9 µm). Specimens of *E. crassisimus* from Malaysia possess gemmules of spherical form, which is in contrast to our specimens that possess gemmules of subspherical form, which is a characteristic of E carteri (Annandale 1907; Manconi and Pronzato 2007; Manconi *et al.* 2013; Penney and Racek 1968). We did not classify our specimens as *E. potamolepis* due to the different forms of megascleres of *E. potamolepis*, which are amphistrongyle and amphioxeas, and also, this species possesses much bigger gemmules (±680 µm) compared to our specimens (Annandale 1918; Manconi *et al.* 2013). Another Malaysian species, namely *E. fragilis*, possesses amphioxea megascleres and amphioxea or amphistrongyla gemmuloscleres (Manconi *et al.* 2013), which differs from our specimens. The *E. tinei* endemic species from the Philippines possesses megascleres that are amphioxea and gemmuloscleres that are much smaller (70–95 x 4–7 µm) compared to our specimens, which distinguishes our specimens from this species (Gee 1932). We also did not classify our specimens to the recently recorded species from Singapore, namely *E. conifer*, due to its gemmuloscleres that are much smaller (65–81.5–115 µm x 2–2.6–3 µm) compared to our specimens and also its conical gemmules with a flattened subspherical form (250–315–350 µm) which differs to that of our specimens, despite the similar oxeas form of gemmuloscleres (Lim and Tan 2013).

The *O. asiatica* samples documented in this study are important for further exploration and future distribution studies because this species had never been reported to exist in Indonesia. To date, seventeen Oncosclera species have been validly recognized. The *O. asiatica* species have been recorded in Southeast Asia (Manconi *et al.* 2012). A different Asian species, namely *Oncosclera kaniensis* Matsuoka and Masuda 2000 from the Palaearctic region (Japan), has been declared extinct (†). The other *Oncosclera* species were described from the Neotropical region or Latin America (see detail in Manconi *et al.* 2013). Volkmer-Ribeiro 1970 separated the *Oncosclera* genus from the *Stratospongilla* genus. The *Stratospongilla* genus and the *Eunapius* genus belong to the same family, namely *Spongillidae*. *Oncosclera* differs from *Stratospongilla* due to the absence of free microscleres from the inner symplasm, the total absence of a gemmule pneumatic layer, and the great variability of the gemmuloscleres form. Furthermore, the *O. asiatica* species shares similar microtraits of megascleres and gemmuloscleres with two other congeners, namely *Oncosclera jewelli* (Volkmer 1963) from the Neotropical region and *Oncosclera gilsoni* (Topsent 1912) from the Autralasian region. However, the gemmular morph of *O. jewelli* and *O. gilsoni* are different from that of *O. asiatica*, in which the gemmular morph of *O. jewelli* and *O. gilsoni* are free in the skeleton, while the gemmular morph of *O. asiatica* is strictly adhering to the basal spongin plate. Gemmuloscleres of *O. asiatica* are extremely larger than that of *O. gilsoni* and foramina are present in the gemmules of *O. gilsoni*. Furthermore, *O. asiatica* possess spicules of acanthoxeas and acanthostrongyles (megascleres) and elongated acanthostrongyles (gemmuloscleres) that are similar to the Neotropical species *Oncosclera intermedia* Bonetto and Ezcurra de Drago 1973 but ovoid gemmuloscleres are absent in the *Oncosclera intermedia* Bonetto and Ezcurra de Drago 1973 species (see detail Manconi *et al.* 2013).

The utilization of molecular markers supports the identification of both the *E. carteri* and *O. asiatica* species since barcoding has been frequently advocated for species delimitation due to limited morphological characteristics and phenotypic plasticity (Woerheide and Erpenbeck 2007; Woerheide *et al.* 2007). CO1 has been utilized and recognized as the most suitable marker for the barcoding of almost all metazoans (Hebert *et al.* 2003a, 2003b). However, it has limitations on taxa that possess a slow evolution rate (Huang *et al.* 2008; Shearer *et al.* 2002). For this reason, additional markers have been suggested, including the I3-M11extension region (Erpenbeck *et al.* 2006), which is an additional fragment of CO1, 28S rDNA (see www.spongesbarcoding.com), and ITS that was proposed by Itskovich *et al.* 2015 for better suitability in assessing intra and interspecific variations in freshwater sponges. In this study, we exhibited that the ITS marker can separate Eunapius from other clades or groups. This result can be seen in Figure 5, where Eunapius ryuensis (Sasaki 1970), a cosmopolitan species originally described in South Korea, and *E. conifer*, a cosmopolitan species originally described in China, were shown to be the closest taxa of *E. carteri*. However, the ITS marker failed to obtain sequences from *O. asiatica*. Furthermore, the CO1 marker was unable to group both the *Eunapius* clade and the *Oncosclera* clade and could not separate between the two clades. This result can be seen in Figure 6, where our *E. carteri* specimens were closest to *Stratospongilla bombayens*, and our *O. asiatica* specimens could not be assigned to the *Oncosclera* clade, in addition to the polyphyly of the other *Eunapius* taxa.

In conclusion, the exploration of freshwater sponges in Indonesia is challenging but provides an opportunity to enrich data on sponge biodiversity, especially on the island of Java, in which the last known publication dates back to the colonial era (Gee 1930; Vorstman 1927, 1928) making the data outdated. Furthermore, the exploration of freshwater sponges is advantageous since microbial symbionts in freshwater sponges are widely explored for novel potentials (e.g., Costa *et al.* 2013; Gaikwad *et al.* 2016; Laport *et al.* 2019).

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