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Biological activity of sponge-associated fungi from Karimunjawa Islands, Indonesia against pathogenic *Streptococcus pneumoniae*

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Abstract. *Cristianawati O, Sabdaningsih A, Becking LE, Khoeri MM, Nuryadi H, Sabdono A, Trianto A, Radjasa OK. 2019. Biological activity of sponge-associated fungi from Karimunjawa Islands, Indonesia against pathogenic* Streptococcus pneumoniae. *Biodiversitas 20: 2143-2150.* Threats of drug-resistant *Streptococcus pneumoniae* and the urgent need for new antibiotics require prompt and sustained action for discovering bioactive compounds. This study aimed to isolate sponge-associated fungi as a candidate for sources of drugs. The sponge-associated fungi were screened against the MDR *S. pneumoniae*. The Deoxyribonucleic Acid (DNA) of fungal isolates that showed the most active was generated by using universal primers ITS1 and ITS4. While the sponge host was identified based on the molecular and by slide section. Out of twenty-nine fungal isolates from 13 marine sponges, S.06.2 isolate produced secondary metabolite that inhibiting the growth of MDR *S. pneumoniae.* Molecular identification based on the ITS region revealed that the active fungal isolate was closely related to *Curvularia lunata*, with a 99% similarity. The *C. lunata* S.06.2 was isolated from sponge *Cinachyrella australiensis.* Thus, *C. lunata* S.06.2_ LC315806 can serve as a fruitful strategy for the discovery of novel antibiotics for the treatment of MDR *S. pneumoniae.*

Keywords: *Cinachyrella australiensis, Curvularia lunata, Streptococcus pneumoniae*, marine derived-fungi

INTRODUCTION

Indonesia is the global epicenter of marine biodiversity and harbors the majority of the earth's aquatic species (von Rintelen et al. 2017). Evidence has emerged, demonstrating that many natural products extracted from marine animals and algae are the products of associated microorganisms, mostly bacteria, and fungi (Raghukumar 2008). Sponge-associated fungi have the potential to produce new chemical entities (Volgraf et al. 2008; Blunt et al. 2010; Vasanthabharathi 2012; Vicente et al. 2013; Bolanos et al. 2015; Imhoff 2016; Indraningrat et al. 2016) that actively inhibited the growths of various pathogenic microorganisms (Khoeri et al. 2011; Mayer et al. 2013; Radjasa et al. 2013; Buttachon et al. 2016; Corral et al. 2018). Our previous research also showed that sponge-associated fungi have several bioactivities such as anti- *S. aureus* and *E. coli* (Wittriansyah et al. 2016), antibacterial produced by sponge *Agelas* sp. associated fungi *Fusarium solani* (Trianto et al. 2017), and Sibero (2019) has also reported that sponge-associated fungi from a mangrove habitat were producing anti-microbial activities.

Streptococcus pneumoniae, a gram-positive bacteria (Kadioglu et al. 2008), is a facultative anaerobic pathogen (Hajaj et al. 2017) that is normally found in the human upper respiratory tract. This multidrug-resistant (MDR) bacteria may cause pneumonia, sepsis, meningitis, otitis media (O'Brien et al. 2009; Mirza et al. 2011; Hu et al. 2016; Cherazard et al. 2017), bloodstream infections and sinus infections in young children (CDC 2017). In Indonesia research on people with pneumococcal infection has already been carried out in the following areas: Jakarta (Yuliarti et al. 2012), Bandung, Central Lombok, and Padang West Sumatera (Dunne et al. 2018). The prevalence of carriers of *S. pneumoniae* among the healthy population has been sharply increasing (DKP-Jateng 2014; Farida et al. 2014; Ditjen P2P 2018) and multidrug-resistant bacteria can pose a growing threat to general health (Lestari et al. 2012).

The prevalence of *S. pneumoniae* infections in Indonesia is considerably high, especially among children and the elderly. This study focuses on sponge-associated fungi with the capability of producing compounds with anti-*S. pneumoniae* properties, collected from the Karimunjawa Islands in the North Java Sea of Indonesia.

MATERIALS AND METHODS

Sponge sample collection

The sponge samples were collected from six sites in the Karimunjawa Islands in the North Java Sea of Indonesia in March 2017 (Figure 1) through SCUBA diving. A total of 13 sponges were collected and stored in sterile plastic bags (Whirl-Pak, Nasco, USA) containing 50 ml of seawater and were immediately placed in a cooling box at temperatures below 4 ˚C (Trianto et al. 2017). Half of each sample was preserved in 70% EtOH as a voucher (Cleary et al. 2013).

Isolation of sponge-associated fungi

Sponge-associated fungi were isolated in accordance to Strobel and Daisy (2003) Using a sterile scalpel, the tissue of the sponges was cut in pieces of approximately 0.1 cm^3 in size and sprayed three times with sterilized seawater. These pieces were then rinsed for surface sterilization with sterilized seawater and 70% EtOH. MEA medium (HiMedia™, Mumbai, India) containing chloramphenicol antibiotics (100 mg/mL) was used for placing the sponge tissue in an incubator where it stayed at 28°C for seven days. Morphologically different colonies were separated and purified.

Screening for antagonistic activity against pathogenic *Streptococcus Pneumoniae*

Antimicrobial activity was determined through the agar plug method (Rahaweman et al. 2016; Sibero et al. 2017). Fresh and pure cultures of *Streptococcus pneumoniae* (RIN1) and American Type Culture Collection (ATCC), obtained from the culture collection from the EIJKMAN Institute for Molecular Biology in Jakarta, were diluted to a suspension equivalent to the 0.5 McFarland standard. The suspension was spread over the entire area of a Mueller Hinton susceptibility agar plate (Oxoid™, Basingstoke, UK) using a sterile cotton swab. Single tablets of fungi were placed on the inoculated agar plate, ensuring sufficient space between individual tablets to allow for proper measurements of inhibition zones. The plates were then incubated at 35°C for 18 hours (overnight).

Cultivation and extraction of the active fungus

One potentially active fungal isolate, namely S.06.2, was cultivated at a larger scale, using 1,000 ml sterile bottles (Schott Duran, Germany), each containing 200 ml of Malt Extract Broth (MEB) (HiMedia™) for 21 days at 27ºC. Subsequently, the fungal culture was filtered through filter paper (Advantec 7, Ø 125 mm) to separate mycelia and media. The medium was mixed with organic solvent MEB: EtOAc = 1: 2 (v/v). A separation funnel was used to separate medium and organic solvent. The organic solvent containing compounds that were secreted by the fungi was then evaporated using a rotary evaporator (Eyela® N 101, Tokyo, Japan) at 37ºC.

In vitro antibacterial assay of fungal extract

MH (Oxoid™) and MEB (HiMedia™) were used to cultivate the test bacteria and fungi, respectively. The cultures of *S. pneumoniae* were kept at 37°C overnight at under 5% CO_2 (Safari et al. 2015). The bacterium was diluted in a sterile 0.86% saline solution to obtain a cell suspension of 105 CFU/mL. The disc diffusion method was used for the assay. 0.1 mL Of diluted inoculum (105 CFU/mL) of test organism was spread on MH agar plates. Sterile paper discs (Ø 6 mm Oxoid™) impregnated with 100 µg of compounds in 15 µL ethyl acetate were used for the assay. A disc without any compound was used as a negative control. Chloramphenicol antibiotics (30 µg, Ø 6 mm Oxoid™) were used in the assay functioning as positive controls. Afterward, the plates were incubated in a CO_2 incubator for 24 hours at 37°C. Finally, the presence of a clear zone indicated antibacterial activity.

Molecular identification of the active fungal isolate S.06.2

Total DNA from mycelia was extracted using a Cheelex extraction method (Cristianawati et al. 2017, Qiu et al. 2005, Sibero et al. 2017). The internal transcribed spacer (ITS) region was amplified by using universal primer ITS 1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS 4 (5'-TCCTCCGCTTATTGATATGC-3') (Macrogen Inc., Seoul, Korea) (Sabdaningsih et al. 2017). The polymerase chain reaction (PCR) was carried out in a thermal cycler (T100™ Thermal Cycler, Bio-Rad Laboratories, California, USA). The PCR was done in volumes of 25 µL consisted of 12.5 µL of GoTaq® Green Master mix (Promega, Madison, USA), 9.5 µL of nuclease-free water, 1µL of extracted DNA template from the sponge-associated fungi and 1µL each of 10µM concentrations of the forward and reverse primer. PCR cycles were then preheated at 95°C for 3 minutes, followed by 30 cycles of initial denaturation at 95°C for 1 minute, 55°C for 1 minute and finally 72°C for 1 minute. The PCR products were checked using agarose gel 1% and visualized under UV light. PCR products with proper quality (585 bp) of band DNA were sent to 1st BASE DNA Laboratories Sdn Bhd (Malaysia) for sequencing.

Phylogenetic analysis

The Basic Local Alignment Search Tool (BLAST) (www.ncbi.nlm.nih.gov) was used to determine the closely related fungal species with active fungal isolate S.06.2. A GTR+G+I model with 1,000 bootstrap replications was run in MEGA 7 (Kumar et al. 2016), resulting in a Maximum Likelihood phylogenetic tree of active isolates. The obtained sequences have been deposited to the DNA Data Bank of Japan (DDBJ) with the accession number at www.ddbj.nig.ac.jp.

Identification of the sponge host of the active fungus

Polyphasic identification methods were used to determine the sponge species. Specimen processing was prepared following method proposed by van Soest et al. (2014). The DNA of the sponge tissue was extracted with DNeasy blood and tissue kit (©Qiagen, Manchester, UK). Moreover, the sequences were determined with two widely molecular markers in sponge barcoding: the mitochondrial cytochrome *c* oxidase subunit I (COI) gene and the 5' end terminal part of the 28S rRNA gene (C1-D2 domains). The primers are the degenerate version of the Folmer primers as used by Erpenbeck et al. (2016) with PCR-cycles in the

following configurations: 3 minutes at 94°C, followed by 30 seconds at 94°C, 35-40 cycles of 45 seconds at 45°C, 1 minute at 70°C, and 5 minutes at 70°C. The 28S primer used were 28S-C2 (forward) and 28S-D2 (reversed) primers, also in accordance with Erpenbeck et al. (2016) with the following configurations: PCR cycles for 3 minutes at 94°C, followed by 30 seconds at 94°C, 30 cycles of 20 seconds at 51°C, 1 minute at 72°C and 5 minutes at 72°C. The PCR products were checked using agarose gel 1.7% and analyzed on Biorad Gel Documentation. The PCR products were then sent to Macrogen Laboratories (Amsterdam, The Netherlands) for sequencing. Lastly, the sponge species were determined with BLAST, and the marine Porifera database (www.marinespecies.org/porifera) was consulted for morphological identification.

RESULTS AND DISCUSSION

Collection of marine sponges

A total of 13 marine sponges were collected from six different sampling locations along the Karimunjawa Islands, Indonesia (Figure 1), with the abundant diversity of these marine sponges displayed in Table 1. Research from (Blunt et al. 2010) has explained that the oceans are home to large quantities of structurally unique natural products that are mainly accumulated in marine macrobes such as invertebrates (e.g., sponges, soft corals, tunicates) and algae. Several of these secondary metabolites have profound pharmacological properties. Sponge host *Cinachyrella* sp. was previously reported producing cytotoxic and anti-cancer compounds (Nurhayati et al. 2015). However, it is well known that a major obstacle in the ultimate development of most marine natural products is the lack of supply. Research on marine fungi has suffered neglect, despite the fungi being potent producers of secondary metabolites and bioactive substances (Kobayashi and Ishibashi 1993; Lang et al. 2007; Raghukumar 2008; Yu et al. 2008).

Screening of potential antimicrobial activity

The lists of "serious concern" threats for drug-resistant *Streptococcus pneumoniae* by the CDC (CDC 2017), and that of "medium priority" by the WHO (WHO 2017) of the urgency of the need for new antibiotics, requires prompt and sustained action to combat and contain this health concern. These lists were drawn up in a bid to guide and promote research and development of new compounds as candidates for drug treatment collected from sponge-associated fungi. Due to this urgency, further investigation of bioactive compounds from marine sponges that led to the discovery of compound anti-MDR *S. pneumoniae* is needed.

A total of 29 sponge-associated fungi were found from 13 different marine sponges species. Nine of 29 fungal isolates showed biological activity defined by the formation of inhibition zones around the fungal colonies (Table 1). Among these fungi, S.06.2 isolate was selected for further analysis as the candidate with the most potential as indicated by the clearest and largest inhibition zone (18.6±2.3 mm) against MDR *S. pneumoniae*.

A literature survey covering more than 23,000 bioactive microbial products, i.e., antifungal, antibacterial, antiviral, cytotoxic and immunosuppressive agents, shows that the producing organisms are mainly from the fungal kingdom. Hence, fungi represent one of the most promising sources of bioactive compounds (Brakhage et al. 2004). Fungi derived from marine sources are considered as constituting a vast reservoir of secondary metabolites (Saleem et al. 2007). The fungus S.06.2 is considerable of great interest as a new, promising source of biologically active products for producing chemical diversity of the secondary metabolites.

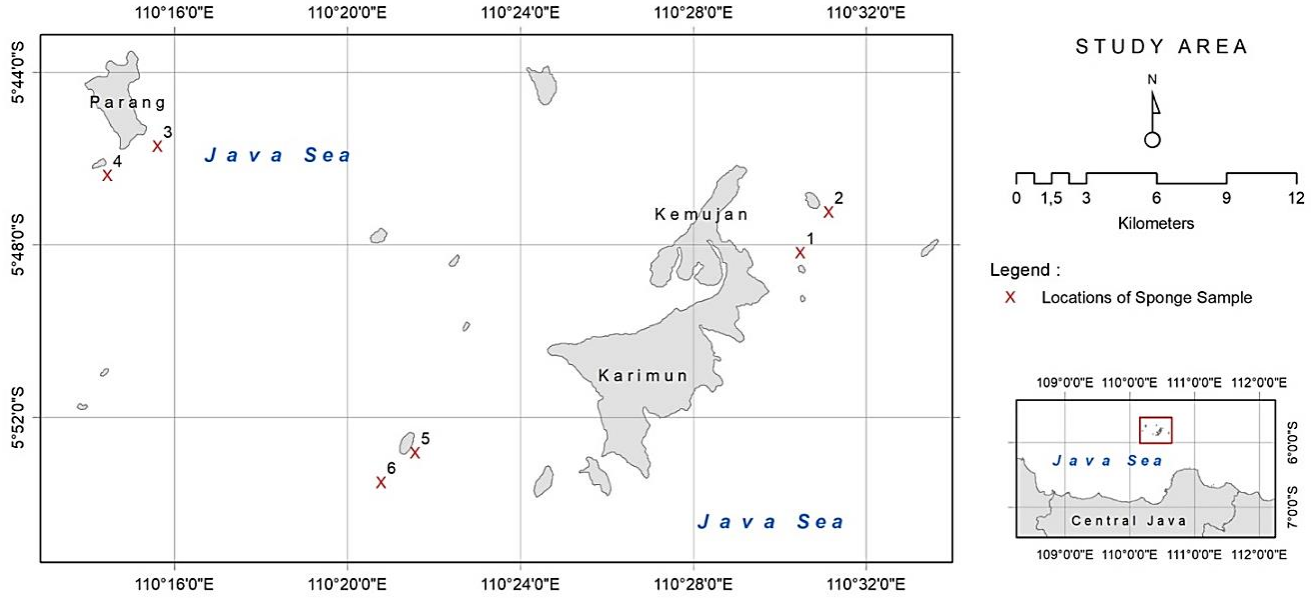


Figure 1. A map of Karimunjawa Islands, Indonesia, showing the location of the sample sites. The image in the lower-right shows the location of the Karimunjawa Islands system in the North Java Sea. Source: GeoEye-1 satellite data from 2014

Table 1. Screening of sponge-associated fungi against MDR and ATCC *S. pneumoniae*

Location	Sponge sources	Fungal isolates	*S. Pneumoniae* ATCC	MDR
1	S.16	S.16.1	-	-
	S.19	S.19.1	-	-
		S.19.2	-	-
		S.19.3	-	-
		S.19.4	-	-
		S.19.5	-	-
		S.19.6	-	-
		S.19.7	-	-
		S.19.8	-	+
		S.19.9	-	-
		S.19.10	-	-
		S.19.12	+	+
		S.19.13	-	-
	S.03	S.03.1	-	-
		S.03.2	-	-
		S.03.3	-	-
		S.03.4	-	-
2	T.14	T.14.3	+	+
3	C.06.B	C.06.B	-	-
	S.06	**S.06.2***	-	+
	C.10.B	C.10.B (2)	+	+
	S.23	S.23.1	-	-
	S.28	S.28.1	+	-
		S.28.3	+	-
4	C.05.B	C.05.B.1	+	-
	C.08.B	C.08.B	-	-
	S.103	S.103.1	-	-
5	S.55	S.55.1	+	-
		S.55.2	-	-

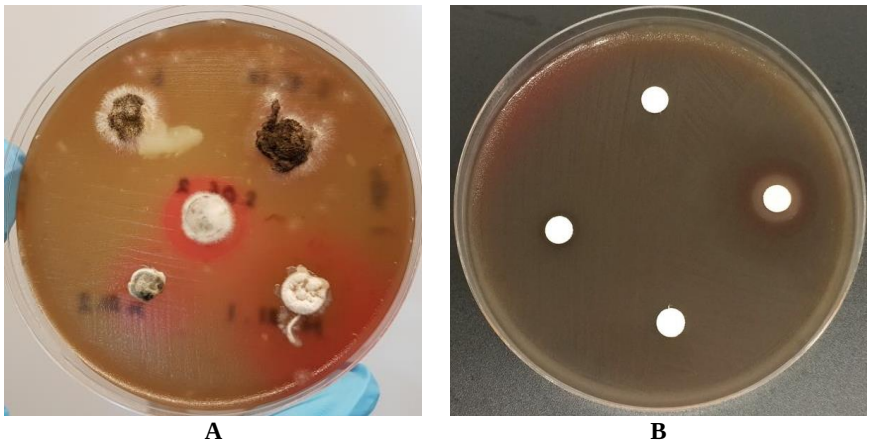
Note: (+) Exhibited antibacterial activity (-) Did not exhibit antibacterial activity (*) Exhibited significant antibacterial activity. No sponge was collected from location 6.

Phylogenetic analysis of active fungal isolate

Through identification based on the classical taxonomy, including micro and macromorphological features, the isolated fungus S.06.2 was identified as *Curvularia lunata*. Phylogenetic inference based on the ITS region revealed that isolate S.06.2 was a member of the genus *Curvularia.* It clustered together with several species from the genus *Curvularia* (Figure 3). The DNA sequence of the ITS region of fungal isolate S.06.2 showed 99% homology. Therefore, *C. lunata* WJCY01 (KY404177) is the species closest related to isolate S.06.2. The sequence data of isolate S.06.2 has been submitted to GenBank under accession number LC315806.

The isolate S.06.2 and several species of genus *Curvularia* formed an independent clade with an intrageneric sequence divergence of 2.7%. The presence of generic types of its genus, *C. lunata,* showed that the clade is an authentically of the genus *Curvularia.* This genus is a sister group of *Cochliobulus* and is separated by an intergeneric sequence divergence of 7.3%. Furthermore, similarity scores between isolate S.06.2 and *Curvularia lunata* using BLAST reached as high as 99%. Therefore, fungal isolate S.06.2 can be identified as *C. lunata*.

In this study, the crude extract EtOAc of fungus *Curvularia* sp. showed biological activity against human pathogen MDR *S. pneumoniae*. Marine-derived fungi *Curvularia* sp. produces anti-inflammatory (Ding et al. 2019), antibacterial (Han et al. 2014, Liu et al. 2019) and anti-cytotoxic (Liu et al. 2019) compounds. *Curvularia* sp. has also been reported as phytotoxic and as an antifungal metabolite (Yin et al. 2018). Compounds isolated from the EtOAc extract of *Curvularia* sp. produced new antioxidant and antibacterial compounds (Venkatachalam et al. 2011). New compounds isolated from *Curvularia* sp., strain M12, were isolated from a leaf (Mondol et al. 2017). *Curvularia* sp. from red algae *Acanthophora spicifera* produces a cytotoxic (Greve et al. 2008) which could be a unique resource for the development of new pharmaceuticals. The appearance of an inhibition zone with S.06.2 isolate against MDR *S. pneumoniae* has opened up the possibility for finding an alternative solution to *S. pneumoniae* infections, as the priority list of pathogens will help the case as reported by the CDC to reduce drug-resistant infection per year, excess hospitalizations, deaths and excess medical cost per year around the world (CDC 2017).



A **B**

Figure 2. Inhibition zone (Fungi S.06.2 against *S. pneumoniae*) with the (A) Agar plug method and the (B) Disc-diffusion method. (Photos: O. Cristianawati)

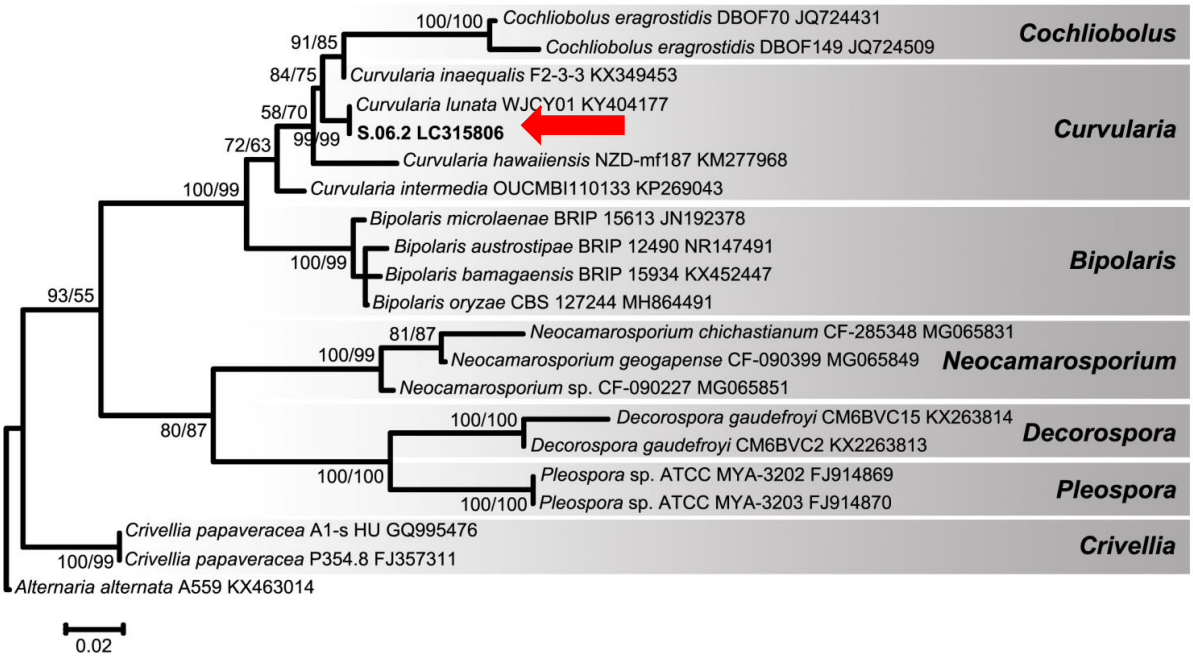
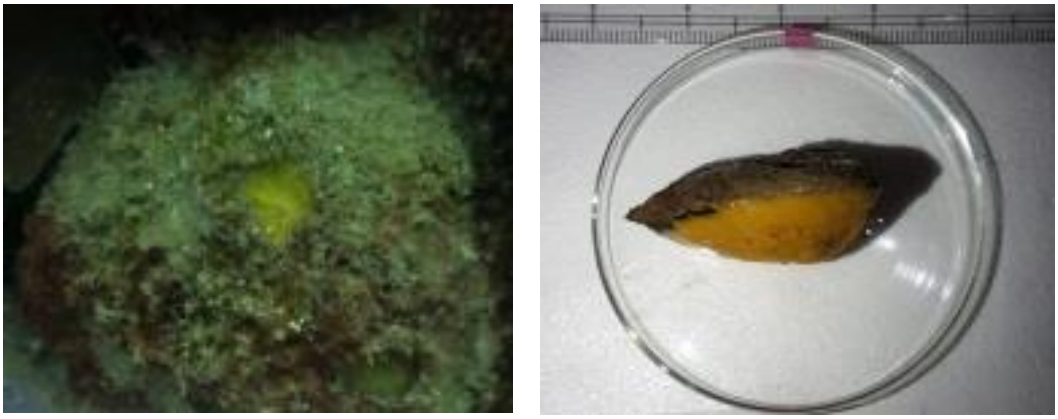
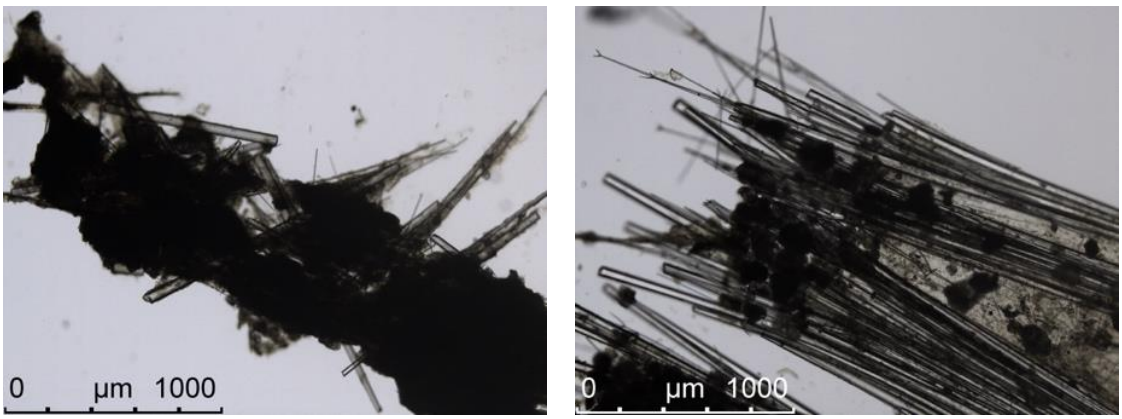


Figure 3. A Maximum Likelihood (ML) phylogenetic tree based on ITS region using GTR+G+I model with 1,000 bootstrap replications. The number of each node indicates bootstrap values (NJ/ML). Bootstrap values below 50% are not shown. The isolate of sponge-associated fungi is marked in bold letters with a red arrow



A **B**

Figure 4. The sponge of species *Cinachyrella australiensis* with (A) an underwater picture and (B) a surface water picture. (Photos: O. Cristianawati)



A **B**

Figure 5. Sections of the *Cinachyrella australiensis*, namely the (A) Ectosome and the (B) Choanosome consisting of spicules. (Photos: L. E. Becking.)

Table 2. Level of nucleotide similarity of selected sponge for primer cytochrome *c* oxidase subunit I and primer 28S

Sample Code	Sequence Length	Identical Species (bp)	Homology (%)
S.06	*Cinachyrella australiensis* voucher QMG 320216	819	97.32

Identification of sponge hosts

Combining molecular and morphological data seems to be the most promising approach to a better understanding of phylogenetic relationships (Lafay et al. 1995; Smith et al. 2011). Nucleotide Similarity of Sponge S.06 for CO1 and 28S is presented in Table 2. The sponge S.06 was identified as *Cinachyrella australiensis.* Morphological identification showed that this is globular sponges of max with yellow buds, massive and hispid. It has a distinctly radial form with a pronounced radial orientation of megascleres (Figure 5), as a small basal attachment and almost solid spicule core at the center and the surface part of the sponge S.06 is coated with dark sediment and algae (Figure 4). Ectosome has fewer sigmas than the choanosome and a whitish ectosome layer which is visible with the naked eye (Carter 1886).

The *C. lunata* S.06.2 isolate was obtained from sponge *C. australiensis* from location 3 at the Karimunjawa Islands in Indonesia (Figure 1). *C. australiensis* can usually be found on firm surfaces such as rocks, but some sponges can attach themselves to soft sediment through a root-like base (Van Soest 2019). The morphology of *C. australiensis* showed one obvious oscule at the top of the sponge.

To the best of our knowledge, the presence of *C. lunata* derived from *C. australiensis* against pathogenic or MDR gram-positive bacteria *S. pneumoniae* in Indonesia has not yet been studied. The *C. australiensis* sponge-associated *C. lunata* fungus produces bioactive compounds that can be used in the treatment of multi-drug resistant respiratory human pathogens *S. pneumoniae.* However, further studies, particularly on bioassay-guided purification and structure elucidation, are needed to determine the anti-MDR *S. pneumoniae* compound.

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