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Abstract. Marine sponges are rich sources of bioactive substances with various pharmacological activities. Previous studies have shown that most bioactive compounds were originally produced by associated-microorganisms. Fungi associated with the marine sponges collected off Karimunjawa National Park were isolated and identified by morphological characteristics and molecular level analyses based on internal transcribed spacer (ITS) regions. A total of 2 isolates which were characterized, the fungi *Penicillium spinulosum* and *Trichoderma virens* have been revealed.

Key words. Associated fungi, *Penicillium spinulosum, Trichoderma virens,* sponge

1. Introduction

Our investigation on marine sponges have shown that the sponges are potential sources of anticancer compounds [1]. Many research have also proven that the sponges have pharmacological potency as antibacterial, antifungal, antimalarial, and antioxidant [2-6]. However, among the bioactive compounds, only few of them passed to the preclinical and clinical test stage, as they are source limited [7]. Extracting a large amount of a particular compound from nature will inevitably disturb the local ecology. Chemical synthetics are the preferred method for producing the bioactive compounds; however, in many cases, this method is not feasible due to the complexity and chirality of the target compounds [8].

Sponges are well known to contain a large number of microorganisms within the mesophyll that some of them permanently associated with the host sponges [9]. Some microsymbiont provide the bioactive compounds [10]. Several studies indicated that some metabolites were produced by associated microorganisms [11].

In recent years, some fungi have been shown to be sources of bioactive compounds and enzymes that important for industrial or ecological properties. Some bioactive compounds produced by the genus *Fusarium* have various biological activities, i.e. antifungal, antibacterial, and antimalarial [12-14].

In this paper, we describe the isolation and molecular identification of the sponge-associated fungi from marine sponges collected from Karimunjawa National Park, Indonesia.

2. Materials and Methods

2.1. Sponges collection

The marine sponges were collected from the Karimunjawa National Park, Karimunjawa, Indonesia by SCUBA diving at 3-15 m depth. The specimens were kept cool until the inoculation process [15].

2.2. Isolation of the associate fungus

Isolation of the fungus from the sponges was conducted using the method proposed by Safaeian *et al.* with modification of the sponge size and media. The sponges were washed with sterilized seawater before inoculation process to remove any associated microorganisms from their surface [16]. Each specimen was then cut into small piece (approx. 1 cm x 1 cm x 0.3 cm). The fungus was isolated by putting each piece of the sponge directly on the surface of malt extract agar (MEA) medium; one agar plate contains one sample to avoid contamination and confusion regarding the source of fungi. After incubation for three days, the fungal colonies were separated based on their morphological characteristics, and each colony was inoculated on a new agar plate containing MEA. This process was repeated until a pure sample of each isolate was obtained [17].

2.3. Sponge identification

The sponge host of the potential isolates was identified based on morphological and spicules characteristics according to a method proposed by Hooper [18].The features were examined for morphological characteristics including colony shape, color, texture, consistency, and surface type. For the spicules preparations, a small fragment of the sponge was placed in a small Erlenmeyer flask, then a small quantity of commercial bleach (sodium hypochlorite) was added. After the organic components dissolved leaving only the mineral skeleton, the bleach was diluted with ethanol and removed carefully. The washing process was repeated until the clean spicules were obtained. The clean spicules suspension was transferred onto a glass slide and observed and photographed at 100x and 400x magnification.

2.4. Characterization of LB-18-1 and LB-19-4 isolates

2.4.1. Phenotypic characterization. The fungal isolates were grown on MEA medium (5 days) before analysis. The photograph the morphological characteristics under a microscope. Isolate identification was conducted using a fungal taxonomical book and taxonomical guidance [19].

2.4.2. Molecular identification of the fungal using 18S rRNA gene analysis. The DNA isolate was extracted with Chelex following the instruction provided by the company (Bio-Rad, US) [20]. In brief, the mycelia were added with 50 µL-100 µL aquabides (ddh2O) and 1 mL of diluted saponin (Bio-Rad, US) in PBS (Phosphate Buffer Saline). The mixture was incubated for 24 hours in 4 ^{o}C prior to centrifugation at 13,000 rpm for 1 minute for supernatant separation. A 250 µl of 10% chelex (Bio-Rad, US) was added to the DNA extract and homogenized under vortex for 20 seconds prior to centrifugation at 13,000 rpm for 2 minutes. Then, the mixture was heated at 95 ^{o}C for 45 minutes and homogenized using Vortex homogenizer. The mixture was centrifuged again at 13,000 rpm for 2 minutes. After removing the supernatant, the DNA was extracted with isopropanol (Merck, Germany) for further analyses with electrophoresis on agarose gel (Bio-Rad, US). Electrophoresis was run at 100 volts using TAE (Tris-Acetate-EDTA) (Sigma-Aldrich, US) as a running buffer. Gels were observed under UV light after staining with ethidium bromide (Bio-Rad, US). Amplification PCR was conducted using universal primer for 18s rRNA (Bio-Rad, US). Sequencing of the DNA was conducted in Genetika Science Jakarta, Indonesia.

2.4.3. Analysis of sequence alignment of 18S rRNA gene and phylogenetic analysis. Phylogenetic analysis was conducted using BLASTn software provided by Gen Bank (NCBI), followed by alignment

process using Clustal X software. The phylogenetic tree was developed using a MEGA version 5 software.

3. Results and Discussion

A total of 2 fungal symbionts from seven sponges collected off Karimunjawa National Park were isolated and identified. On the previous study, it was reported that Karimunjawa National Park provided antifungal sources from the sponge-associated fungi [21]. The fungal identification was carried out based on the morphological and molecular level.

The fungus LB-18-1 have white colony on MEA plate and the fungus LB-19-4 showed green on morphology. Based on the microscopic feature, it belongs to the genus *Trichoderma*. The occurrence of anamorphic or telomorphic phase of the fungal species [22], require the molecular level to identify the fungi. Genotypic characterization was done employing fungal 18S rRNA.

The genomic DNA from LB-18-1 isolate was amplified by PCR. This amplified gen product was used for further identification. The consensus sequence of 678bp of 18S rRNA (Figure 1) gen was generated from forward and reverse sequence data and searched through the BLAST homology data and the result showed 99% similarity with *Penicillium spinulosum* (GenBank accession no. JQ717353.1). Hence, the fungus LB-18-1 could be identified as *P. spinulosum*. The PCR product of LB-19-4 was sequenced and showed the length 623bp. After the BLAST homology searched, this fungus had 99% similarity with *Trichoderma virens* with accession no. KT803076.1.

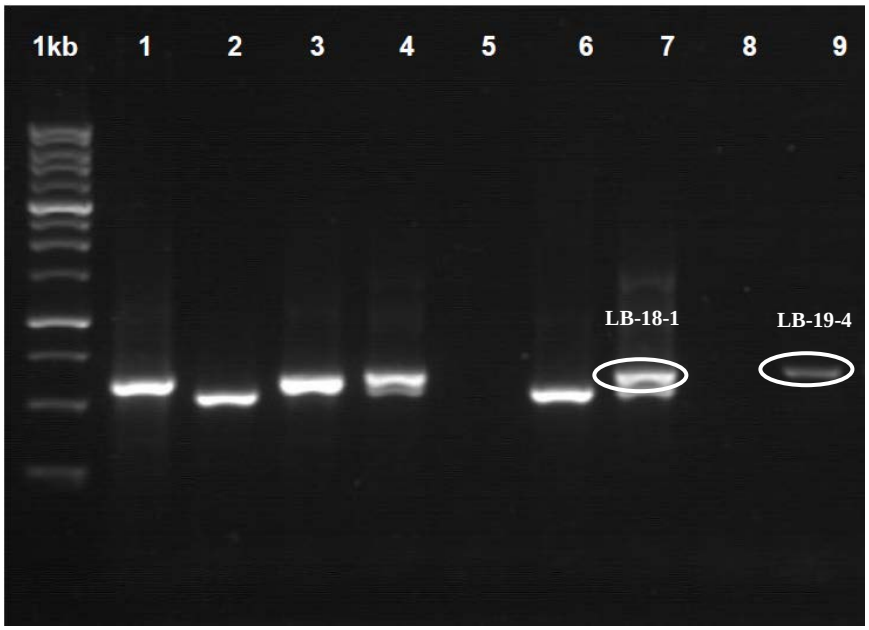

Figure 1. The fungal bands of LB-18-1 (7) and LB-19-4 (9) from PCR amplification products

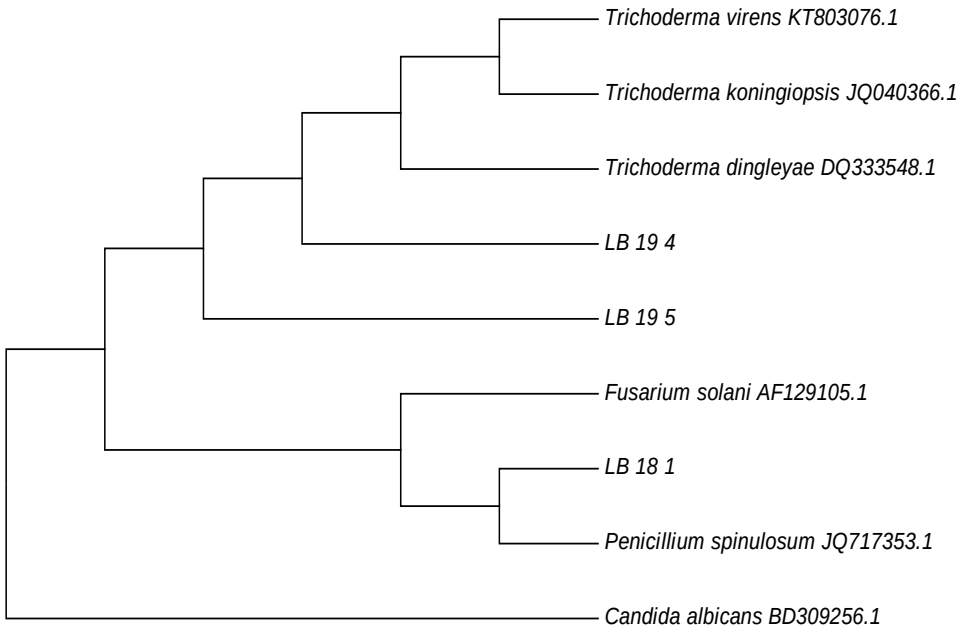

Figure 2. The phylogenetic tree of LB-18-1 and LB-19-4

Penicillium belongs to the sponge-generalist genera as it can be found in more than one sponge and dominantly isolated from sponges [23]. *Penicillium* is particularly known for their ability to produce important bioactive compounds. Alkaloids from *P. aurantiogriseum* have been reported to have cytotoxic activity against several cell lines [24].

The genus of *Trichoderma* has frequently been reported as the marine-derived fungus. This genus also widely discussed as terrestrial fungi mostly isolated from soil [25]. The serious observation and identification of marine-derived fungi have been done in a significant progress [26]. The marine isolates have been studied to have taxonomically close relation to species that are from terrestrial environments [27]. On the other hand, it produces different and unique secondary metabolites compare to the terrestrial one [28]. In the marine area, *Trichoderma* species were found ranging from the tidal zone and wetland [29]. The members of *Trichoderma* produce numerous metabolites and have been evaluated for its bioactivity on a various study as biocatalisator [30], antioxidant [31], biological control agent [32], anticancer [33], and antibiotic [34]. The recent report provides the information that the fungi from Karimunjawa National Park could be utilized as the source of the bioactive substance.

4. Conclusion

A total of 2 isolates which were characterized, the fungi *Penicillium spinulosum* and *Trichoderma virens* have been identified from sponges collected off Karimunjawa National Park. The further study about its bioactivities needs to be done to reveal the potential of Karimunjawa National Park biodiversity.

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