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Molecular characteristic of bacteria associated with healthy *Porites lutea* coral of South Malang Waters, Indonesia

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Abstract

Coral reefs are the most diverse of all marine ecosystem yet highly vulnerable to diseases and climate change impacts in which approximately 30% of corals have been affected globally. *Porites lutea* is among the most widespread coral in Indonesia, yet it is also highly impacted by the diseases. This study aimed to isolate, molecularly characterize and identify the associated bacteria that dominated the healthy *P. lutea*. The coral sampling was using snorkeling while streak method was used for bacterial isolation and purification. Molecular identification consisted of DNA extraction, 16S rRNA PCR amplification and sequencing of 16S rRNA gene flow, and BLAST homology. Results showed that the bacterium associated with healthy *P. lutea* was closely related to *Marinobacter xestospongiae, Marinobacter zheijiangensis,* and *Marinobacter mobililis* with a similarity of 96%, 96%, and 95% respectively. The bacterium can be used as a candidate for further anti-pathogenic bacterial test and may be able to inhibit the growth of pathogenic bacteria of coral diseases particularly Pink Line Syndrome that highly impact *P. lutea* in many areas.

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Introduction

Coral reefs form some of the most productive and diverse ecosystems on earth, often described as "rainforests of the sea" or as "ocean oases." The reefs are home to numerous marine species such as hard and soft corals, sponges, mollusks, crustaceans, fish, and even marine mammals (Fisher *et al*., 2015; Gross, 2013). In the marine ecosystem, coral reefs serve an important role in providing shelter, spawning and nursery grounds to a wide range of marine life (Fisher *et al*., 2015; Veron *et al*., 2009). Healthy reefs also generate income for local communities and support global economies through fisheries and coral reef tourism (Asadi and Andrimida, 2017).

Indonesia contains the highest diversity of coral reefs species. In the Bird's Head Peninsula of Indonesian Papua alone, 574 species of corals live within the area (72% of the world's total) (Veron *et al*., 2011). However, Indonesia's coral reefs are endangered due to destructive fishing practices as well as other anthropogenic threats such as sedimentation, organic pollution, and even destructive tourism activities (Putra *et al*., 2015). Moreover, the increasing sea surface temperature and the decreasing ocean pH due to the global rise of carbon dioxide elevate the damage of coral reefs ecosystem (Bruno, 2013; Orr *et al*., 2005). Those factors induce and contribute to the coral bleaching and the outbreak of coral diseases and subsequently increase death to corals over extensive areas (Séré *et al*., 2015; Weil *et al*., 2009).

The diseases of scleractinian corals were initially found in the Caribbean, and over the last 30 years, many Indo-Pacific corals have been affected with the diseases causing mortality and significant changes in coral community structures (Weil E *et al*., 2012). There are a few studies quantifying the coral diseases on Indonesian waters (Johan *et al*., 2015; Subhan *et al*., 2011). Moreover, molecular studies of the microorganisms that cause coral diseases and syndromes are even scarcer. In Karimunjawa waters, the molecular study of bacteria associated with Black Band Disease (BBD) on *Acropora* sp. coral showed that pathogenic microbial group was associated with the diseases (Sabdono and Radjasa, 2006).

Furthermore, to understand microorganisms that play a role in the White Band Disease (WBD) that infected Staghorn Coral *Acropora cervicornis*, Gignoux-Wolfsohn and Vollmer (2015) isolated and compared both the diseased and healthy-associated bacteria from the coral.

The healthy-associated bacteria may be able to produce bioactive agents with anti-pathogenic properties that could also protect against the disease-associated bacteria (Bakkiyaraj *et al*., 2013). This research aimed to isolate, molecularly characterize and identify the associated bacterium that dominated the healthy *Porites lutea* coral using 16S rRNA sequence analysis (Mignard and Flandrois, 2006). Moreover, *P. lutea* is the most abundant coral in the research area (Luthfi *et al*., 2016).

The species is also vulnerable to coral diseases like Pink Line Syndrome (Ravindran *et al*., 2015). Therefore, the study of the potential bacteria that could protect against coral disease is beneficial to reduce the impact of the disease on coral reefs ecosystem.

Material and methods

Sampling and description of the site

Survey and sample collection of healthy *P. lutea* were performed in Kondang Merak waters which is part of South Malang waters and directly connected to Indian Oceans. The research station is characterized by shallow reef flat of a fringing reef with the depth of 1-5 meters during high tide and is often exposed during low tide. The sample collection was conducted at low tide with snorkelling in June 2017 ($8^0$23'50.74"S $112^0$31'6.00"E).

A fragment of healthy *P. lutea* was carefully collected using a hammer and chisel and directly placed into a polyethylene plastic bag to avoid contact with air. The sample was stored in a cooler containing ice and was directly brought to the laboratory for further experiment. The map of the study area is presented in Fig. 1.

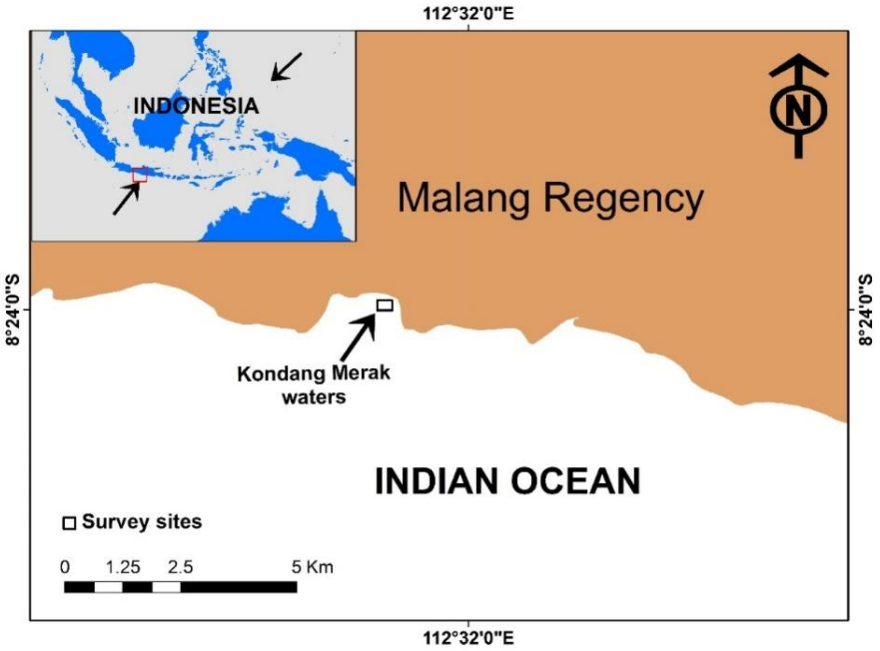

Fig. 1. The map of study area, the Kondang Merak waters.

Bacteria isolation and purification

Using an airbrush, an aerosolized jet of autoclaved seawater was sprayed at the coral fragment. The tissue of the coral was scraped using an ethanol-sterilized razor. A total amount of 1 gram of the tissue was then diluted with 9ml of autoclaved seawater and was mixed using a vortex mixer in order to suspend cells in the tissue. The resultant tissue was serially diluted until fivefold final dilution. A total amount of 50 µL of the resultant was spread on half-strength media (ZoBell 2216E marine agar) using quadrants streak-plating technique and incubated for 48 hours at room temperature. Based on the morphological feature, a colony was randomly selected and purified by using streak plate technique. The purified colony was then incubated for 24 hours, vortexed and inoculated using ZoBell 2216E marine broth for 48 hours for further molecular identification (Kawaguchi *et al.*, 2013; Sanders, 2012).

Molecular identification

Molecular analysis consisted of DNA extraction, 16S rRNA *PCR* amplification and sequencing of 16S rRNA gene flow, and BLAST homology. DNA extraction was using the chelex method. 16S rRNA *PCR* amplification was using 27F universal primers (5'-AGAGTTTGATCMTGGCTCAG-3') and specific primer for eubacteria 1492R (5'-TACGGYTACCTTGTT ACGACTT-3'). BLAST homology analysis was using MEGA 5.7 (Weisburg *et al.*, 1991).

Result and discussion

Coral sample, isolation, and purification of bacteria

Massive *P. lutea* is highly distributed throughout Indonesia's seas and waters. The widespread of the coral is due to its ability to survive in a wide variety of environments like waters from low to high sedimentation and salinity. The coral is also persistent and able to adapt to various habitats such as sandy shores, rocky shores, and even reef rubbles (Zamani *et al.*, 2016). The coral is commonly found in Kondang Merak waters and often has distinct colors as responding to environmental variabilities (Luthfi *et al.*, 2016).The yellowish green color was observed on *P. lutea* in Kondang Merak waters in which the color tends to be brown at a depth more than 5 meters. The major color patterns of reef-building corals may occur due to the protein and pigment of algae that live inside the coral tissue (Dove *et al.*, 2001; Luthfi *et al.*, 2016). The sample of *P. lutea* that was used for the experiment is presented in Fig. 2.

Furthermore, the morphological observation of the bacteria isolates consisted of shape, size, elevation, and margin observation.

Each cell in the same colony in agar plate was considered a single species and could represent a pure and single isolate. The morphological appearance of the bacteria colony of healthy *P. lutea* and its morphological characteristic is presented in Fig. 3 and table 1 respectively.

Table 1. Morphological characteristic of the bacteria colony based on the quadrants streak-plating technique.

Code	Size	Shape	Elevation	Margin
P.H1	Moderate	Irregular	Flate	Lobate
P.H2	Small	Irregular	Raised	Lobate
P.H3	Small	Spindle	Raised	Lobate

Fig. 2. Healthy *P. lutea* of Kondang Merak waters. The sample was used for the study.

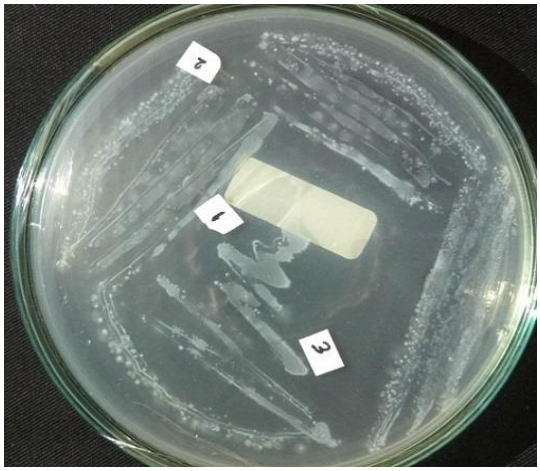

Fig. 3. Bacteria colony of healthy *P. lutea* inoculated using quadrants streak-plating technique.

Molecular identification

The purification of the bacterial colony with P.H2 code was chosen for the molecular study.

To obtain sequence data, DNA extraction had been performed in which 16S rRNA gene assisted to resolve the exact taxonomic position of the coral bacterium as the region of the gene has slow rates of evolution (Sabdono *et al.*, 2015). Several characteristics of the gene, such as evolutionary properties, ubiquity, and essential function, have allowed it to become a reliable molecular clock, and 16S rRNA sequences from distantly related bacterial lineages are also demonstrated to have similar functionalities (Case *et al.*, 2007).

16S rRNA sequence is aimed to obtain bacteria classification up to species level and to discover the genetic relationship among species (Sabdono *et al.*, 2015). The result of sequences was visualized using electropherograms. The forward and reverse sequences were then edited using MEGA 5.7 to remove the gaps and noises to eliminate the uncertainty of the sequence results. The BLAST program compared the sequences to sequence databases and calculated the statistical significance. The electropherogram of the sequence and the molecular identification are presented in Fig. 4 and table 2 respectively.

The molecular identification using BLAST program showed that the bacterium associated with healthy *P. lutea* was closely related to *Marinobacter xestospongiae, Marinobacter zheijiangensis, Marinobacter mobililis,* and *Marinobacter nitratireducens* with a similarity of 96%, 96%, 95%, and 95% respectively. *Marinobacter* spp. is Proteobacteria that also could be found in seawater. Some of the strains and species of *Marinobacter* have been demonstrated to degrade hydrocarbons. Meanwhile, *M. xestospongiae* is a gram-negative bacteria that usually lives inside the tissue of marine sponge *Xestospongia testudinaria* (Lee *et al.*, 2012); therefore, this finding gives a new insight that the bacteria also live in healthy *P. lutea* tissues. The bacteria could be expected to play a role as anti-pathogenic bacterial coral symbiont if it shows antagonistic activities against PLS associated bacteria that are highly impacted *P. lutea* tissues.

Table 2. The molecular identification of bacterium associated with healthy *P. lutea* coral.

Kode NCBI	Nama Spesies	Max score	Total score	Query cover (%)	E-value	Similarity (%)
NR 109066.1	*Marinobacter xestospongiae*	1244	1244	82%	0.0	96%
NR 044457.1	*Marinobacter zheijiangensis*	1227	1227	82%	0.0	96%
NR 044456.1	*Marinobacter mobilis*	1206	1206	82%	0.0	95%
NR 136469.1	*Marinobacter nitratireducens*	1204	1204	82%	0.0	95%
NR 042807.1	*Mrinobacter algicola*	1192	1192	79%	0.0	96%
NR 025671.1	*Marinobacter lipolyticus*	1186	1186	82%	0.0	95%
NR 043718.1	*Marinobacter koorensis*	1181	1181	82%	0.0	95%
NR 044340.1	*Marinobacter gooseongensis*	1177	1177	82%	0.0	95%
NR 145917.1	*Marinobacter confluentis*	1175	1210	79%	0.0	96%
NR 074765.1	*Marinobacter adhaerens*	1175	1175	82%	0.0	95%

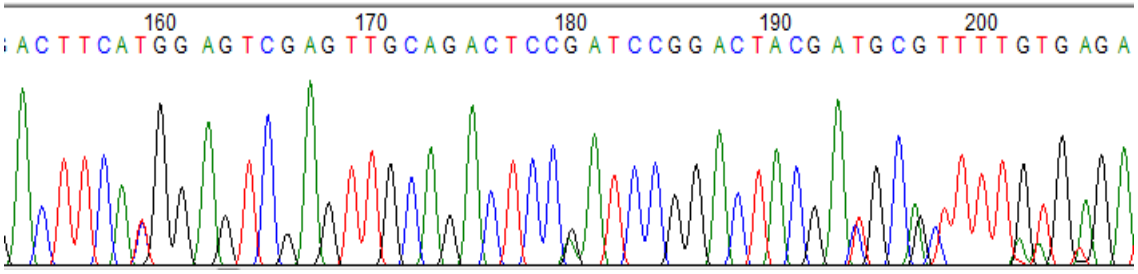

Fig. 4. The electropherogram of the sequence data of bacteria associated with healthy *P. lutea* coral.

Conclusions

Based on the molecular study, it was revealed that the bacterium associated with healthy *P. lutea* of Kondang Merak waters was closely related to *Marinobacter xestospongiae, Marinobacter zheijiangensis,* and *Marinobacter mobililis* with a similarity of 96%, 96%, and 95% respectively. *M. xestospongiae* as the closest relative of the bacterium sample has never been reported to be associated with coral; therefore, this finding gives a better understanding of the ecology of the bacteria. The bacterium could be a potential agent to inhibit the growth of PLS bacteria if it could demonstrate inhibition zone in antagonistic activity test.

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