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Population Dynamic of *Dendronephthya* sp.-Associated Bacteria in Natural and Artificial Habitats

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***Dendronephthya* sp. is a soft coral that has huge distribution starting from Indopacific, Tonga, Solomon Islands to Great Barrier Reef in Australia. However, this soft corals survive only in short period after cultivation in artificial habitat (aquarium). Recent study showed that the soft coral *Dendronephtya* sp. has an association or symbiotic relationship with several bacteria, commonly known as coral associated bacteria (CAB). In this study, we compared the population dynamic of *Dendronephthya* sp.-associated bacteria in natural and artificial habitat, resulting different bacterial community profiles using terminal restriction fragment length polymorphism (T-RFLP) analysis of bacterial community DNA. There were 15 main classes of bacterial population identified along with uncultured microorganism, uncultured organism, uncultured bacteria and unidentified organism. Members of Actinobacteria, Arthrobacteria, Chlorobia, Caldilineae, δ-proteobacteria and Proteobacteria were predicted to give contributions in the survival ability of both *Dendronephthya* sp. The cultivation of soft corals after 2 weeks in artificial habitat increases bacterial population similarity on 2 different samples by 10%. Bacterial population similarity in artificial habitat would increase along with the longer cultivation time of soft corals.**

Key words: *Dendronephthya* sp., coral associated bacteria, T-RFLP

INTRODUCTION

Indonesia is a country with the largest corals ecosystem area in the world. These coral ecosystem area has a direct association with quota or amount of corals that allowed to be traded according to *Convention International Treaty on Endangered Species* (CITES). Among hundred corals that may be traded, one of the most potential species for hobbyist is *Dendronephthya* sp. This species is a famous Alcyonia among the diver in Red Ocean and has colorful colors such as red, orange, purple, yellow, pink, and white (Fabricus & Alderslade 2001).

Dendronephthya sp. is a flower shaped soft coral with various formations. It has scratch, like thorn, along its body. These scratches or thorns are sclerites formed by silica layers and plays an important role as a supporting bundle (Fabricus & Alderslade 2001). In the natural habitat, this species grows and breeds rapidly. Recent publication reported that *Dendronephthya* sp. has green fluorescent protein (Pakhomov *et al.* 2004).

Despite of its beauty, there are only few hobbyists who are interested in cultivating this species. The reason is that these soft corals can only live about three weeks in the aquarium while others can live for months (Borneman 2001). In the marine environment, animate and inanimate surfaces are rapidly covered with colonization by bacteria, protozoa, algae, and fungi (Harder *et al.* 2003). Provided this association occurs between living organisms, this phenomenon is referred to as epibiosis. Bacterial epibiosis can be both beneficial and harmful to the host organism.

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The association between bacterial and corals plays an important role in coral health (Bourne & Munn 2005). Many studies have reported the interactions between corals and microbes (Ritchie & Smith 1995; Richardson 1998; Dobretsov & Qian 2004; Bourne & Munn 2005). The soft coral *Dendronephtya* sp. has an association or symbiosis with several bacteria (Harder *et al.* 2003), commonly known as coral associated bacteria (CAB). These bacteria play an important role on the defense mechanism against pathogenic bacteria. According to Harder *et al.* (2003), the coral tissue extracts and waterborne products of coral-associated bacteria inhibited growth and attachment of indigenous bacterial isolates, suggesting an endogenous chemical and an exogenous biological mechanism against bacterial epibiosis in this soft coral. Moreover, many of these coral associated bacteria have been served as sources of secondary metabolites including novel antibiotics (Radjasa *et al*. 2005). The association between bacteria and *Dendronephtya* sp. contribute on antifouling mechanism of other bacteria by inhibiting the attachment of macrofoulers on the surface of *Dendronephthya* sp. (Dobretsov & Qian 2004).

The association of bacteria and *Dendronephthya* sp. is also related to the survival ability of those corals. Yet, the study about the comparison of bacterial composition in *Dendronephthya* sp. in natural and artificial habitat is poorly understood. Method used for this experiment was *Terminal Restriction Fragment Length Polymorphism* (T-RFLP). T-RFLP is widely used for identifying several communities in natural ecosystem, for example: bacteria (Liu *et al.* 1997; Moeseneder *et al.* 1999), eukaryote (Marsh *et al.* 1999), and archaea (Moeseneder *et al.* 1999). When comparing the T-RFLP data generated from different

communities, variation can be found in the number and size of peaks and can be evaluated by adapting community parameters such as richness and evenness (Dunbar *et al*. 2000).

The objective of this study is to analyze the population dynamic of *Dendronephthya* sp.-associated bacteria in natural and artificial habitat using T-RFLP.

MATERIALS AND METHODS

Sample Collection. Soft corals were collected from Selat Sunda, Kabupaten Serang, West Java, Indonesia (6° 7' 12" S; 106° 9' 1" E). Corals were carefully brought to the water surface in sealed plastic bags. Sample will be analyzed temporary; coral in the natural habitat referred as fresh sample collected from the sea and coral in artificial habitat referred as coral cultivated in the aquarium after 2 and 4 weeks.

Bacterial Community DNA. The method used in this experiment is modified method from Harder *et al.* (2003). Corals were flushed with autoclaved seawater to remove loosely attached bacteria. The surfaces of each coral colony were swabbed six times on different parts of each colony. Replicated swabs were combined in 5 ml of lysis buffer (1% SDS; 20 mM Tris-HCl pH 8; 2 mM EDTA) and 5 µl of Proteinase-K (20 mg/ml). The bacterial samples were incubated at 37 °C for 1 h then immediately frozen at -70 °C. Samples were lysed at 90 °C for 20 min. After centrifugation (9,500 x g for 20 min), supernatant was purified by phenol: chloroform: isoamyl alcohol (25:24:1) extraction. The mixture is centrifuged at 9,500 x g for 10 min and P:C:I purification were conducted twice. Then supernatant was purified by chloroform: isoamyl alcohol (24:1) and was centrifuged at 9,500 x g for 10 min. Total DNA in the supernatant was subsequently precipitated in cold isopropanol absolute and was incubated overnight at -20 °C. After centrifugation at 14,000 x g for 20 min at 4 °C, pellet DNA was washed with 500 µl cold ethanol (70%). Then the mixture was centrifuged at 16,000 x g for 3 min. The obtained DNA templates were dried in 37 °C for 10 min and resuspended in 30 µl of TE Buffer. The DNA templates were purified using Wizard® DNA Clean-Up System (Promega, Madison, WI, USA) and frozen until used.

Bacterial Community Analysis Using T-RFLP 16s rDNA Amplification for RFLP. PCR of 16S rRNA genes (rDNA) in bacterial community DNA was performed in a total volume of 25 µl containing 1 µl of DNA template, 12.5 µl GoTaq Green ® (Promega, Madison, WI, USA), 1 µl of each universal primer: 63F-FAM (5'- CAGGCCTAACACA TGCAAGTC – 3') (Moeseneder *et al.* 1999) and 1387R (5'- GGGCGGWGTGTACAAGGC – 3') (Marchesi *et al.* 1998), and ddH_2O. The 63F-FAM primer was labeled at the 5'- end with the *phosporamidite fluorochrome 5-carboxyfluorescein*. PCR was performed at 95 °C for 10 min; 30 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min; and 72 °C for 5 min. Amplified DNA was verified by electrophoresis of 2 µl of PCR mixtures in 1% agarose in 1x TAE buffer.

T-RFLP Analysis. Fluorescently labeled PCR products were purified with the QIA Quick® Gel Extraction Kit (Qiagen, The Netherlands) according to the manufacturer's protocol. The purified amplicons were single digested with endonuclease enzymes as followed *Bst*UI, *Msp*I, dan *Rsa*I (NEB Biolabs, Maryland). Digestion by *Bst*UI and *Rsa*I was performed in a total volume of 20 µl containing 2 µl restriction enzymes, 2 µl 10x restriction buffer, and 16 µl of PCR products. Digestion by *Msp*I was also performed in a total volume of 20 µl containing 1 µl restriction enzymes, 2 µl 10x restriction buffer, and 17 µl of PCR products. Digestion process using *Msp*I and *Rsa*I were incubated on 37 °C overnight, while digestion using *Bst*UI were incubated on 60 °C overnight. Digestion products were further purified by using MinElute Reaction Cleanup Kit (Qiagen, The Netherlands) according to the manufacturer's protocol.

Pellet of digested products were mixed with 12 µl deionized formamide and 0.5 µl of internal size standard (ROX-500, Applied Biosystems, Foster City, CA, USA). This mixture was denatured for 5 min at 95 °C and immediately chilled on ice before electrophoresis on an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA) operated in GeneScan mode. After electrophoresis, the length of fluorescently labeled terminal restriction fragments (TRFs) was determined by comparison with internal standards by using GeneScan software (Applied Biosystems, Foster City, CA, USA). Fragment sizes were analyzed using FragSort ver.5.0 software with database from *Microbial Community Analysis*.

RESULTS

In this study, two *Dendronephthya* sp. were analyzed on the 0th, 2nd, and 4th week; namely D1 and D2. However, D2 did not survive on the 4th week and the bacteria population was not analyzed. The analysis of TRF patterns showed that the patterns of bacterial communities on the replicate coral colonies were fluctuating. There were several different bacterial community profiles observed on both soft corals in natural and artificial habitat (Table 1).

There were 15 main classes of bacterial population identified along with uncultured microorganism, uncultured organism, uncultured bacteria and unidentified organism. They were Actinobacteria, α-proteobacteria, Arthrobacteria, Bacilli, Bacteroidia, β-proteobacteria, Chlorobia, Cyanobacteria, Caldilineae, δ-proteobacteria, Flavobacteria, γ-proteobacteria, Mollicutes, Proteobacteria, and Sphingobacteria. Each class consisted of different bacterial populations, not only in genus and species but also in numbers.

The similarity of bacterial population on each week from D1 and D2 were tested by statistical method (PSI = $100 - 0.5 \{ \sum_{i=1}^{\infty} |ai-bi| \}$) as presented in Table 2 (Hutagalung 1998). The similarity between bacterial populations on D1 and D2 in the natural habitat is 65% and increased by 10% on the 2nd week of cultivation in artificial habitat. This might be caused by similar bacterial exposure in the artificial habitat that made the bacterial population on soft corals *Dendronephthya* sp. resulting to be more similar.

Table 1. Classes of bacterial isolates from corals surface in natural (0th week) and artificial habitat (2nd and 4th weeks). Numbers represent amount of bacterial species detected in each class

Taxonomy (class)	D1 (0th week)	D1 (2nd week)	D1 (4th week)	D2 (0th week)	D2 (2nd week)
Actinobacteria	115	ND	7	ND	ND
α-proteobacteria	90	43	23	35	32
Arthrobacteria	1	ND	1	ND	ND
Bacilli	4	ND	2	1	ND
Bacteroidia	13	1	8	10	3
β-proteobacteria	11	1	1	1	1
Chlorobia	3	ND	ND	ND	ND
Cyanobacteria	19	6	2	2	ND
Caldilineae	ND	ND	1	ND	ND
δ-proteobacteria	ND	ND	1	ND	ND
Flavobacteria	119	12	85	81	24
γ-proteobacteria	18	1	4	6	1
Mollicutes	1	ND	1	1	ND
Proteobacteria	1	ND	ND	ND	ND
Sphingobacteria	29	1	25	20	2
Uncultured microorganism	501	121	263	261	150
Uncultured organism	59	5	42	43	24
Uncultured bacteria	2054	987	633	565	308
Unidentified organism	70	19	26	29	10
Total	3108	1197	1125	1055	555

ND: not detected, D1: the 1st *Dendronephthya* sp. sample, D2: the 2nd *Dendronephthya* sp. sample.

Table 2. Similarity between bacterial population on *Dendronephthya* sp. in the natural (0th week) and artificial habitat (2nd and 4th weeks)

	D1 (0th week) (%)	D1 (2nd week) (%)	D1 (4th week) (%)	D2 (0th week) (%)	D2 (2nd week) (%)
D1 (0th week)		50	62	65	
D1 (2nd week)	50		39		75
D1 (4th week)	62	39			
D2 (0th week)	65				70
D2 (2nd week)		75		70	

D1: the 1st *Dendronephthya* sp. sample, D2: the 2nd *Dendronephthya* sp. sample.

The similarity of bacterial populations between 0th week and 2nd week of cultivation for D1 and D2 was 50 and 70% respectively. After 4th week of cultivating D1, the similarity of bacterial population was increased by 12% compared to the 2nd week of cultivation.

DISCUSSION

The numbers of each bacterial population on D1 were different with organisms on D2. Each week of cultivation period in artificial habitat also showed several different bacterial populations. This might happen because every living organism is unique and not similar, and so the amount of bioactive compound produced by each organisms that may inhibit the growth of other organism. The bacterial population in the artificial habitat was also decreasing by time.

The bacterial communities associated corals were found to be largely coral specific (Ritchie & Smith 2004). Shifts in bacterial community could affect the health of the coral (Kapley *et al.* 2007). Several studies showed also that the distinct of both composition and function of microbiota were associated with healthy and diseased corals (Cooney *et al.* 2002; Frias-Lopez *et al.* 2002; Pantos *et al.* 2003; Bourne 2005; Reis *et al.* 2009). It has been demonstrated that the bacterial community of the whole coral colony was affected even when a very small part of the colony showed signs of disease (Pantos *et al.* 2003). Sekar *et al.* (2008) proposed that degrading water quality, such as by increasing nutrients could thrive certain virulent proteobacteria.

The class of Caldilineae and δ-proteobacteria were not detected in the natural habitat but after 4 week of cultivation, species from these classes were detected. The presence of these species in the 4th week of cultivation might come from the artificial habitat and due to the change of bioactive compounds produced by other bacterial attachment on the soft corals that facilitate these species to attach.

The population dynamic of *Dendronephthya* sp.-associated bacteria might play big role in the survival ability of this soft corals. Members of Actinobacteria, Arthrobacteria, Chlorobia, Caldilineae, δ-proteobacteria and Proteobacteria were predicted to give contributions in the survival ability of D1 and D2. The presence of those classes of bacteria might help D1 to live 2 weeks longer than D2. In order to attain coral survivability, Rohwer and Kelley (2004) hypothesized that coral associated bacteria play a role in resistance to disease through competition for nutrients, space, or production of antibiotics. According to Harder *et al.* (2003), there were 3 main class of cultured bacteria identified on the body of soft corals *Dendronephthya* sp. in the natural habitat. They were γ-proteobacteria, α-proteobacteria, and Cytophaga-Flexibacter-Bacteroides. Compared to result from Harder *et al.* (2003), there was a similarity of predicted classes. The class of proteobacteria were detected using cultured dependent and cultured independent method (T-RFLP).

Proteobacteria were the largest and most diverse group of eubacteria. Proteobacteria are commonly found in marine environment and might be used as a biomarker for marine bacteria (Taylor *et al.* 2007). Moreover,

Proteobacteria could produce low molecular-weight biological active compounds with antimicrobial, cytotoxic agent, antibiotics, and bioactive extract (Thomas *et al.* 2010). Although the 16S rRNA studies showed that they were phylogenetically related, proteobacteria vary markedly in many respects. Most members are facultatively or obligately anaerobic, chemoautotrophs, and heterotrophic, but there are numerous exceptions. Comparison of 16S rRNA sequences has shown that the proteobacteria are composed of five classes, they are α-proteobacteria, β-proteobacteria, γ-proteobacteria, δ-proteobacteria, and ε-proteobacteria.

The difference of bacterial population similarity on D1 and D2 might be caused by the difference of bioactive compounds produced by each samples. Longer time of soft corals cultivation in the artificial habitat would increase the bacterial population similarity which might be caused by the mortality, birth rate, or migration of certain bacteria that might contribute in the survival ability of this coral.

Coral associated bacteria (CAB) that played big roles in the survival ability of soft corals *Dendronephthya* sp., especially in the artificial habitat, was provided from class of proteobacteria. In this research, the dynamic population of CAB can be studied by T-RFLP method. The result showed that the cultivation of soft corals after 2 weeks in artificial habitat increased bacterial population similarity on 2 different species by 10%. Longer time of soft corals cultivation in the artificial habitat would increase the bacterial population similarity.

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