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Analysis of Bacterial Community Associated with *Aaptos* sp. from Rote and Seribu Islands

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Aaptos sp. is a marine sponge that could produce bioactive compounds such as aaptamin, aaptosin, and isoaaptamin which have activities as antitumor, antimicrobial, and antiviral. Community of bacteria associated with the sponge might correlate with production of those bioactive compounds and be affected by water environment where the sponge grow. The presence of anthropogenic stressor such as pollutans might become a burden to the waters where the biota grown and could affect the microbial biodiversity in the sponge and its active metabolite produced. The objective of this research was to analyze bacterial community associated with *Aaptos* sp. from Rote Island and Seribu Islands, using T-RFLP method. The results showed that bacterial community associated with *Aaptos* sp. from both sampling sites shared 40.81% similarity in which they were dominated by the same bacteria class of Actinobacteria, Flavobacteria, α-proteobacteria, δ-proteobacteria, and γ–proteobacteria. The bacteria collected from Rote island were more highly distributed and diverse than those from Seribu Islands. A total of 23 classes of microorganism were identified in Rote Island waters, while in Seribu Islands was 14 classes of microorganism. The presence of Actinobacteria and Proteobacteria in *Aaptos* sp., is allegedly involved in the production of secondary metabolites.

Key words: *Aaptos* sp., microbial diversity, Rote Island, Seribu Islands, T-RFLP

Aaptos sp. merupakan spons laut yang dapat memproduksi senyawa bioaktif seperti aaptamin, aaptosin, dan isoaaptamin yang memiliki aktivitas sebagai antitumor, antimikrobial, dan antiviral. Komunitas bakteri yang berasosiasi dengan spons tersebut kemungkinan berkorelasi dengan produksi komponen bioaktif dan akan dipengaruhi oleh perairan dimana spons tumbuh. Adanya stresor yang disebabkan oleh manusia seperti polutan akan menambah beban perairan ditempat biota tumbuh yang dapat mempengaruhi keragaman mikroba dalam spons dan metabolit aktif yang diproduksi. Tujuan penelitian ini adalah menganalisa komunitas bakteri yang berasosiasi dengan *Aaptos* sp. dari perairan Pulau Rote dan Kepulauan Seribu, menggunakan metoda T-RFLP. Hasil penelitian menunjukkan bahwa komunitas bakteri yang berasosiasi dengan *Aaptos* sp. dari kedua daerah sampling memiliki kesamaan 40,81%, yang didominasi oleh kelas bakteri yang sama yaitu kelas Actinobacteria, Flavobacteria, α-proteobacteria, δ-proteobacteria, dan γ-proteobacteria. Bakteri dari spons yang dikoleksi dari Pulau Rote lebih terdistribusi dan lebih beragam dibanding yang dari spons yang dikoleksi dari kepulauan Seribu. Secara total ada 23 kelas mikroorganisme sebagian besar bakteri yang diidentifikasi ada di spons dari Pulau Rote, sedangkan spons dari Kepulauan Seribu terdapat 14 kelas. Keberadaan Actinobacteria dan Proteobacteria dalam *Aaptos* sp., diduga berhubungan dengan produksi metabolit sekunder.

Kata kunci : *Aaptos* sp., Kepulauan Seribu, keragaman bakteri, Pulau Rote, T-RFLP

Aaptos sp., one of marine sponges that can be found abundantly in Indonesian waters, has been reported to produce bioactive metabolite against tumor, pathogenic microbes, and Herpes Simplex Virus type I (HSV-1) (Coutinho *et al*. 2002). It was detected that the compounds produced were alkaloids, e.g aaptamin, aaptosin and isoaaptamine; the latter compound showed potent activity against Human Immunodeficiency Virus type I (HIV-1) (Gul *et al*. 2006). *Aaptos* sp. was also been reported to produce sterol compounds (Rachmat and Muniarsih 2001), and other novel aaptamine alkaloids possesing various biological activities, including cytotoxic against murine lymphoma L5178Y cell line, antiviral, antimicrobial, antifungal, antiparasitic, α-adrenergic antagonistic, radical scavenging, and antifouling activity (Pham *et al.* 2013).

Sponge such as *Aaptos* sp. is very rich in microorganism including bacteria associated within their body. About 40 % of sponge body is bacteria, and its role is very significant in the sponge metabolism (Taylor *et al*. 2007). The sponge produces similar metabolites from their associated bacteria (Radjasa *et*

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al. 2007), and there is a strong correlation between bacterial richness and secondary metabolites produced by the host (Haygood *et al*.1999). Since they are filter feeder, the sponge associated bacteria are highly affected by the water quality where the sponges grown. The presence of anthropogenic stressor such as pollutans which is more frequently occured nowadays, might become a burden to the waters and it could affect the bacterial diversity in the sponge and its active metabolite produced.

Aaptamine is a bioactive compound that was once becoming chemotaxonomic marker for sponges of the order Hadromerida where *Aaptos* sp. is one of this ordo's member. Latest report showed that there are number of species in the ordo Hadromerida producing clinically active compounds in association with microorganism including bacteria (Thomas *et al*. 2010). Our previous study on the screening of the bioctive compounds from sponges including *Aaptos* sp from Karimunjawa, Bali, and Lombok, Yogyakarta, Binuangen (Banten) waters found that the aaptamine group compound from the sponge had relatively high yield (Chasanah *et al*. 2007). Aaptamine-like compounds have also been found in sponges of other genera such as *Xestospongia*, *Suberites*, *Hymeniacidon*, and *Luffarriella* (Pham *et al*. 2013).

The objective of this research was to analyze the bacterial community associated with *Aaptos* sp. from two different locations, i.e. Rote Island and Seribu Islands.The two locations represent the less polluted and polluted waters due to human activities.

MATERIAL AND METHODS

Samples Collection. *Aaptos* sp. samples were taken by hand using scuba diving equipment from territorial waters at Rote Island (Batu Termanu) Indonesia on May 10th, 2010 (geographic location S10º40.228'; E123º05.73) and Seribu Islands (Penjaliran Barat) on April 2010, at geographic location of S05°27.720'; E106°33.603', at the same depth of 5m. Three samples of *Aaptos* sp from each location were mixed for genomic DNA isolation and analyzed to generate the bacterial community profile of the sampels from both locations.

Water (200 mL) around the *Aaptos* sp. was collected in triplicates using nansen jar and analyzed on board. Dissolved anorganic nitrogen (nitrate, nitrite, ammonia) and phosphate ions were analyzed by a colorimetric method using HACH DR-890 colorimeter. Salinities and pH were recorded using refractometer and pH meter. Dissolved oxygen (DO) and physical characteristics (water temperature, transparancy and water flow) of the waters was conducted *in situ* using DO meter, thermometer, and Secchi disk, respectively.

Samples Pre-treatment. About 25 g of *Aaptos* sp. sample was added into 25 mL of CTAB extraction buffer, blended, and centrifuged at 200 x g for 2 min. Supernatant was transferred into new microtube and centrifuged at 600 x g for 2 min, the supernatant was subsequently transferred into a microtube sterile and centrifuged at 10 000 x g for 5 min.

Genomic DNA Isolation. Genome isolation was conducted following Zhou *et al*. (1996) with a modification. Pellets which were formed from the pre-treatment were homogenized with 750 µL CTAB extraction buffer and homogenized for 2 min at full speed. Samples were then centrifuged at 14 000 x g for 30 sec, and supernatant were transferred into sterile microtubes, frozen at -70 °C, and thawed at 65 °C (this process was done in duplicate). Afterward, samples were placed at room temperature, then, proteinase-K were added and incubated at 37 °C for 30 min, and were added with 150 µL 10% Sarkosyl detergent and incubated at 65 °C for 30 min. Samples were flipped every 10 min, centrifuged at 10 000 x g for 5 min, and the supernatants were transferred into sterile microtubes and added with equal volume of phenol:chloroform:isoamylalcohol (25:24:1), then shaked until well mixed. Samples were then centrifuged at 14 000 x g for 5 min., the liquid phase were transferred into sterile microtubes, then equal volume of chloroform were added to the samples and shaked until well mixed. Samples were centrifuged at 14 000 x g for 5 min and the liquid phase formed was transferred into sterile microtubes and added with 0.6 x volume of cold isopropanol and incubated at -20 °C for 20 min. Subsequently the samples were centrifuged at 16 000 x g for 5 min. Supernatant were discarded and the pellets formed were washed with 70% ethanol and then dried. Pellets were homogenized with 50-100 µL Tris-Cl pH 8. Genomic DNA were visualized on 1% agarose in 1 x TAE buffer, which were visualized with cyber-gold. DNA genomes were purified using DNA Wizard Genomic DNA Purification Kit® (Promega).

16S rRNA Gene Amplification and T-RFLP Analyses.16S-rRNA gene was amplified using Bio-Rad C-1000 with universal primers 27F-FAM (5'-AGA GTTTGATCCTGGCTCAG- 3') which was labeled at the 5'-end with the phosphoramiditefluorochrome 5-carboxyfluorescein (FAM) and 1387R (5'-GGGCGG WGTGTACAAGGC-3') (Marchesi *et al*. 1998). To

perform gene encoding 16S rRNA PCR, master mix was made with the following composition: 12.5 µLGoTaq (Promega, Madison, WI, USA), 1 µL primer 27F and 1387R primers (25 pmol μL^{-1}), 9.5 µL ddH_2O, and 1 µL of the DNA genome as template. PCR was performed at 95 °C for 5 min, 30 cycles of 95 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 1 min, followed by 72 °C for 10 min. PCR products were visualized on 1% agarose gel in 1x TAE buffer. The 16S rDNA was then purified using QIA quick PCR Purification Kit (Qiagen, Germany) according to the manufacturer's protocol.

T-RFLP analysis was conducted as follow: Fluorescently labeled PCR products were single digested with 3 endonuclease enzymes namely *Bsh* 1236I, *Rsa*I, and *Hpa*II for 24 h at 37 °C. Digestion was performed in a total volume of 20 µL containing 2 µL restriction enzymes, 2 µL of 10x restriction buffer, and 16 µL of PCR products. Digestion products were purified using EDTA-NaOAc precipitation method (Sambrook and Russel 2001). Pellet of the 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 Biosystem, Foster City, CA, USA) operated in GeneScan. After electrophoresis, the length of fluorescently labeled 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 (www.oardc.ohio-state.edu/trflpfragsort/index.php) with data base from Microbial Community Analysis (Shyu *et al*. 2007).

To evaluate richness and evenness, diversity statistics were calculated from each population. Population richness (S) was total number of distinct population. Diversity Index Analysis was analyzed using Shannon-Wiener Index (H), Simpson's Index (D), and Evenness (E). The Shannon-Wiener diversity index was calculated from equation $H= -\Sigma(pi)(lnpi)$ and the Simpson's Index was calculated from equation $D = 1- ((\Sigma ni (ni-1) / N (N-1))$. Evenness value was calculated from equation $E = H/H_{max}$, $H_{max} = \ln S$ (Allen *et al*. 2009).

Analysis of Aaptamine and Isoaaptamine. For analysis of aaptamine and isoaaptamine, triplicates of 5 g wet weight of frozen *Aaptos* sp. were extracted using 10 mL of methanol (pa), and centrifuged to coagulate suspended solid. The supernatan were concentrated with nitrogen and freezed dried, and redissolved in methanol providing concentration of 10 mg mL^{-1}. Concentrated supernatants (5 µL) was injected into HPLC system Shimadzu 10-AD with PDA detector and Shimpack VP ODS coloumn (2.0 mm x 150 mm). Eluent used was 20% acetonitrite/H_2O (1% TFA) at a flow rate 0.2 mL min^{-1}. Concentration of aaptamine and isoaaptamine were determined according to Dewi *et al*. (2012) as average of triplicate samples.

RESULT

Bacterial community profiles and its electropherograms observed from *Aaptos* sp. from two sampling areas of Rote Island and Seribu Islands can be seen at Fig 1 and Fig 2. *Aaptos* harvested from Rote island contained more diverse bacteria than those of Seribu Islands as shown in Table 1, using Shannon-Wiener index, i.e. the H value for Rote and Seribu Islands was 5.53 and 4.78, respectively. This was supported by Simpson's Index, in which the D value of the bacterial population from *Aaptos* sp. from Rote Island (0.894) was higher than that of Seribu Islands (0.884). Based on the calculation of Evenness value (E), the distribution of the bacteria population from *Aaptos* sp. In Rote Island (0.653) was more highly distributed than that of Seribu Islands (0.676). The higher D values the higher the diversity, and the bigger E values the lower in variation distribution of bacterial community.

There were 24 main classes and 1985 microorganisms of bacterial population identified along with uncultured microorganisms, uncultured organisms, uncultured bacteria, and unidentified organisms detected from *Aaptos* sp. from Seribu Islands, while *Aaptos* sp. from Rote Island which was considered richer had a total of 6128 microorganisms. Sponges from both locations shared 40.81% similarity of bacterial community, and were dominated by the same classes, i.e. Actinobacteria, Bacilli, Bacteroidia, Deinococci, α-Proteobacteria, δ-Proteobacteria, and γ-Proteobacteria. Clostridia was the only class which was found in sponge sample from Seribu Islands but was not found in those from Rote Island. On the contrary, there were 10 classes that were found in sponge from Rote Island but were not found in those from Seribu Islands.

Water quality analysis was conducted in the sampling areas at the same time of sampling (Table 2). Analysis of the bioactive compounds, i.e. aaptamine and isoaaptamine, was only carried out with samples from Seribu Islands but it could not be conducted with those from Rote Island due to the technical problem of sample storage. The *Aaptos* sp. from Seribu Islands contained 2.197% of aaptamine and 0.578% of

isoaaptamine (dried extract weight).

DISCUSSIONS

T-RFLP method has been used in this study since this method was reported to be effective in determining bacterial communities in a range of environments, highly reproducible, rapid, and amenable to field-scale experiments (Liu *et al*.1997; Dunbar *et al*. 2000). The diversity of bacterial communities associated with *Aaptos* sp. in both sampling sites showed that the bacterial community structures were different eventhough dominated by similar bacteria. Based on the water quality (Table 2), Rote Island waters showed higher water flow, indicating that the water in that location was more circulated, and this would affect the bacteria in the sponge as filter feeder organism. Stronger water flow in Rote Island waters might lead to frequent water exchange that enters the sponge body, and this might be the reason why *Aaptos* sp. from Rote Island waters contained more diverse micoorganisms compared to those of Seribu Islands. Low water flow in Seribu Islands might cause no much change of the inhabitants (bacteria) in the *Aaptos* including Clostridia from this area.

High value of phosphate in Rote Island could indicate there was domestic contaminations from human activity such as sewage containing detergent or it might be an accumulation of phosphate ion from rock decay in the marine waters that might be affected by high water flow in the waters. This contamination might be indicated by the presence of phosphate degrading bacteria such as Bacilli which was detected in more quantity in the sponge from Rote Island (Fig 1). Those bacteria could produce phophatase enzyme which plays a key role in mineralizing organic phosphate into inorganic phosphate.

From the data, it can be seen that water quality of Rote Island was better than Seribu Islands waters. Ammonia content of Seribu Islands waters depicted that the waters was highly polluted with domestic waste. The pH value of Seribu Islands waters which

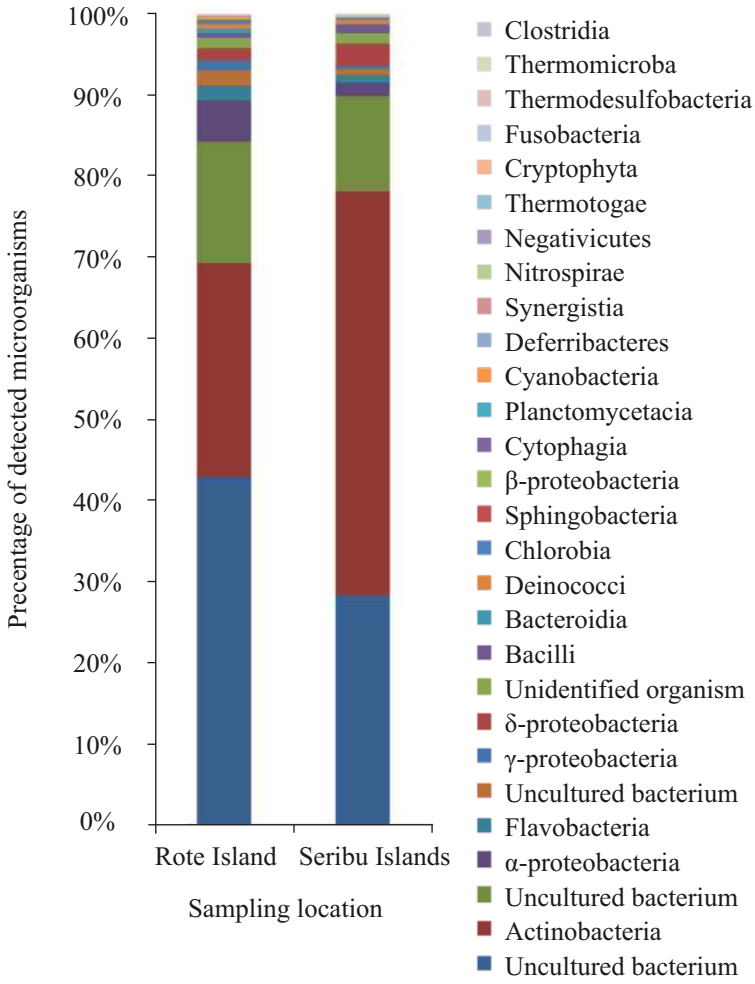

Fig 1 Percentage of detected microorganisms in *Aaptos* sp. collected from Rote Island and Seribu Islands.

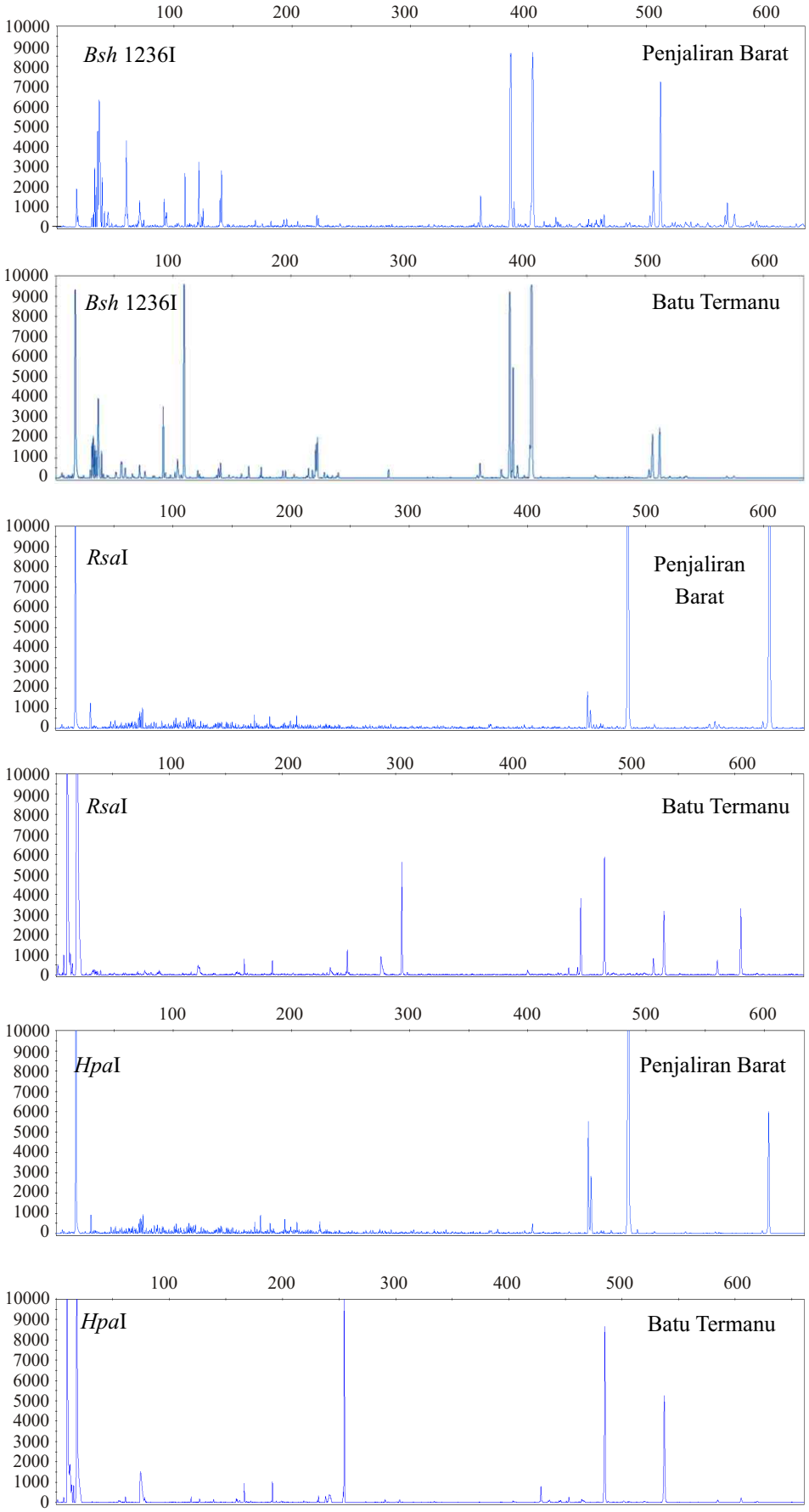

Fig 2 Electropherograms of bacterial community T-RFLPs generated from rDNAs with a labeled reverse primer and 3 endonuclease digest namely *Bsh* 1236I, *Rsa*I, and *Hpa*II obtained from representative water samples from Penjalinan Barat (Seribu Islands) and Batu Termanu (Rote Island) waters.

Table 1 The diversity index of bacterial community associated with *Aaptos* sp.

	Rote Island	Seribu Islands
H_{max}	5.533	4.787
H	3.611	3.235
D	0.894	0.884
E	0.653	0.676

D: Simpson's index; E: Evenness; H: Shannon-Wiener index; H_{max}: ln population richness

Table 2 Water quality of Rote Island and Seribu Islands

	Water flow (m/sec)	Temperature (°C)	Salinity (‰)	pH	DO	Phosphate (ppm)	Nitrate (ppm)	Nitrite (ppm)	Ammonia (ppm)
Rote Island	0.23	31	33.33	8.1	6.83	0.533	0.050	0.002	0.033
Seribu Islands	0.09	32	31.00	7.7	6.73	0.017	0.067	0.000	0.100

was below the pH required for corals growth (7.8-8.8) (Jameson and Kelty 2004) suggested that an acid contamination might be occured in the waters. Low water flow in Seribu Islands waters also causes the pollutants stay longer in the waters, therefore it affects the living microorganisms and marine biota in the waters. Previous work showed that water quality has affected the metabolite richness of the *Nephthea* spp harvested from numbers of sampling point at Alor waters (Januar *et al*. 2012).

Aaptos sp. tissue from both locations were dominated by the same bacteria, but different in quantity. The dominant bacteria from both samples were Actinobacteria, Proteobacteria, and Flavobacteria. Class Actinobacteria was very abundant in *Aaptos* sp harvested from Seribu Islands i.e. 50% compared to 26% of Rote Island waters. Seribu Island was consider more polluted than Rote Island waters.

On the other hand, class Proteobacteria was found more likely higher in Rote Island waters compared to that of Seribu Islands waters. Actinobacteria was reported to be the most morphologically diverse prokaryotes, and are widely distributed in both terrestrial and aquatic ecosystems (Servin *et al.* 2008). One of the well studied Actinobacteria species is *Streptomyces* sp. Metabolite compounds produced by *Streptomyces* sp. was reported could exhibit cytotoxic activity against cancer cell lines (Chasanah *et al.* 2009; Khan *et al.* 2010). Study conducted by Suthindhiran and Kanabiran (2010) also reported that marine Actinomycetes was very potential in producing the active metabolites. *Streptomyces* has been used for commercial production of different compounds for various therapeutic agents such as tetracycline as antibacteria, amphotericin as antifungal, and tacrolimusas immunosuppressant (Suthindhiran and Kannabiran 2010).

Another dominating class was Proteobacteria (α, β, δ, and γ-proteobacteria) which was found more abundant in *Aaptos* sp. from Rote Island, i.e. about 7% compared to 4% in *Aaptos* sp. from Seribu Islands waters. Li *et al*. (2006) reported that Proteobacteria are common in marine environment and might be used as a biomarker on sponge host. Proteobacteria have been suggested to have varied effects on sponge hosts such as nitrogen fixation. Proteobacteria especially γ-proteobacteria has been reported to produce the most chemically diverse bioactive natural products such as agrochelin, a cytotoxic thiazole alkaloid from *Agrobacterium* and B-90063, a dimericoxazolepyridone analog from *Blastobacter*, which indicates the biomedical potential of this class of Proteobacteria. In addition, an early investigation of the α-proteobacterium *Thalassospira* sp. strain CNJ328, resulted in the discovery of unique immunosuppressive peptides, thalassospiramides A and B (Oh *et al*. 2007).

Kalinovskaya *et al*. (2004) reported that Proteobacteria could produce low molecular-weight biological active compounds with antimicrobial and surface-active properties. Another research by Radjasa *et al*. (2007) reported that metabolites of Proteobacteria which was isolated from *Aaptos* sp. from Panjang Island, Jepara, were active against multi drugs resistant strains of microorganism. All of the above studies indicated that there is correlation of sponge bioactive compounds with Proteobacteria and/or Actinobacteria, which were dominant microorganism in *Aaptos* sp. used in this study. Sponge from ordo Suberitidae where *Aaptos* sp. is one of the member was reported to produce therapeutic important bioactive compounds of microbial origin. Alpha, β, γ, δ-Proteobacteria, and Actinobacteria has been reported to be possible contributors of pharmacologically relevant secondary metabolites of sponges, in addition to Firmicutes, Cyanobacteria, and Fungi (Thomas *et al*. 2010).

In this study, the bioactive compounds, i.e. aaptamine and isoaaptamine have been extracted from the *Aaptos* sp. tissue from Seribu Islands, but we failed to extract those from sample from Rote island waters due to the failure of the sample storage. Aaptamine in the *Aaptos* sp. tissue harvested from Seribu Islands was 2.197% and isoaaptamine was 0.578% (dried extract weight). Since the same compounds from Rote samples could not be analyzed, we could not compare both samples.

To conclude, the structure of bacterial community in the *Aaptos* sp. harvested from Seribu Islands and Rote Island waters was affected by water quality, but in this study, we can not yet correlate the bacterial community with the bioactive content of the sponge, i.e. aaptamine and isoaaptamine. Bacterial community associated with *Aaptos* sp. from Rote Island which was less polluted and having higher water flow, was more diverse and highly distributed than that of Seribu Islands. However, they both shared 40.81% similarity of microorganism, from which they had the same dominant bacteria, i.e. Actinobacteria and Proteobacteria that are well known as bioactive producer.

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