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Culturable and unculturable actinomycetes associated with the sponge *Neofibularia* from Bira Island, Indonesia

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ABSTRACT

Aims: The diversity of the actinomycete community associated with *Neofibularia* sp. from Bira Island, Indonesia, has been largely unstudied. This study was undertaken to address the paucity of information in this respect.
Methodology and results: Culturable actinomycetes were isolated and cultured on HV medium. Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) targeting the metagenomic 16S rRNA was used to analyse the structure of the actinomycete community. Five culturable actinomycetes that were isolated belonged to the genus *Streptomyces*. They showed various degrees of similarity to the reference strains *Streptomyces sampsonii* (97-99%), *Streptomyces resistomycificus* (97-99%), *Streptomyces gougerotii* (97-99%), *Streptomyces erringtonii* (97-99%), and *Streptomyces albus* (97-99%). The culturable actinomycetes isolates also showed differences in morphological characteristics as compared with the reference strains. The metagenomic analysis suggested that the actinomycete community was dominated by rare actinomycetes. Eight DGGE DNA bands that were obtained had sequences that showed similarities to *Ferrithrix thermotolerans* (88-94%), *Lamia majanohamensis* (87-92%), *Aciditerrimonas ferrireducens* (87-92%), and *Thermobispora bispora* (85-92%), while 4 bands had sequences similar to *Propionibacterium acnes* (97-100%) and another band matched sequences belonging to an uncultured bacterium clone (86-87%). The actinomycetes detected by the metagenomic approach were assigned identities that were mostly under 97.5% as compared with reference strains available in Genbank.
Conclusion, significance and impact of study: Observations from both culture and DGGE analysis give a better understanding of the diversity and community structure of actinomycetes associated with *Neofibularia* sp. The culturable actinomycetes were *Streptomyces* spp., while rare actinomycetes were dominant when the metagenomic approach was adopted. Several of these actinomycetes showed identities below 97% when matched to reference strains, indicating possible novel species associated with the sponge *Neofibularia*.

Keywords: Actinomycetes, culturable, DGGE, *Neofibularia* sp., unculturable

INTRODUCTION

Sea sponges are ancient sessile organisms that have existed since 600 million years ago and are important members in the coral reef ecosystem. Owing to its geographical location between the Indian and Pacific Oceans, Indonesia has a high diversity of sponges belonging to 830 species across various families (Hutomo and Moosa, 2005). *Neofibularia* sp. has been reported as a producer of antileishmanial, antimalarial, and antibacterial compounds (Thompson and Gallimore, 2013). Such bioactive compounds produced by sponges may have arisen from their associated microbes (Peraud, 2006). Microbe density in sponges can reach up to 10^9 cells per cm^3, covering 35% of the total biomass of sponge tissue (Hentschel *et al.*, 2012). Close association between sponges and microbes (Hentschel *et al.*, 2003) may also contribute to the high diversity of sponge-associated bacteria, including actinomycetes (Sun *et al.*, 2010).

Actinomycetes play an important role in the growth and development of sponges they are associated with, such as by providing nutrients, UV protection, defence against toxic compounds, and by stabilizing their skeletons, and decomposing residual metabolites (Shick and Dunlap, 2002). Various compounds are produced by different actinomycetes (Pathirana *et al.*, 1992), and the structures of bioactive compounds produced by marine sponge-associated actinomycetes can be different from those produced by their terrestrial counterparts (Drummond, 2006).

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The community structure of actinomycetes associated with sponge has yet to be extensively explored. Appropriate cultivation techniques are required to increase growth opportunities for the less abundant culturable actinomycetes on sponge tissue. Culturable microbes are thought to constitute less than 1%; while the other 99% are unculturable microbes (Webster *et al.*, 2001). As unculturable actinomycetes cannot be identified morphologically, the metagenomic approach is often adopted.

In this paper, the community structures of both culturable and unculturable actinomycetes were assessed using the metagenomic approach based on the 16S rRNA gene. The amplified 16S rRNA gene was subjected to denaturing gradient gel electrophoresis (DGGE) to separate genes of the similar size (Fischer and Lerman, 1983). DNA finger printing of PCR-amplified rDNA using methods such as DGGE provides information about the community structure of actinomycetes. In DGGE analysis, DNA fragments with the same length but different nucleotide sequences are separated based on the differences in mobility of PCR-amplified DNA molecules in polyacrylamide gels in a linear denaturing gradient (Muyzer *et al.*, 1993). Sequence comparisons can then be made against reference strains available in the GenBank database.

The structure of the actinomycetes community associated with *Neofibularia* sp. based on the metagenomic approach had not been reported; hence, the present study was undertaken. In combination with culture on synthetic media for culturable isolates, the two different approaches would provide better information regarding the actinomycete community associated with sponge *Neofibularia* sp. from Bira Island, Indonesia.

MATERIALS AND METHODS

Sponge sampling

Marine sponges were obtained by scuba diving in 4 to 8 meter deep water off Bira Island (5° 23' - 5°40' S, 106° 25'-106° 37' E Northern Jakarta) which belongs to the Kepulauan Seribu group of islands (5° 24' - 5° 45' S, 106° 25' - 106° 40' E), covering an area of 107.489 ha. The sponges were identified as *Neofibularia* sp.

Isolation and morphological characterisation of culturable actinomycetes

Culturable actinomycetes were isolated and cultured on Humic Acid Vitamin B Agar (HV Agar). About 1 g of sponge sample was crushed and serial dilutions up to 10^{-3} were carried out. A suspension (0.1 mL) each was taken from 10^{-1}, 10^{-2} and 10^{-3} dilutions, spread on HV Agar, and then incubated at 26-28 °C for 4 to 8 weeks. Colonies of actinomycetes were purified using Yeast Starch Agar (YSA), a selected actinomycetes medium, for 10 days at 26-28 °C. The colonies were morphologically characterized based on macroscopic (pigmentation and colour of colony) and microscopic (Gram staining, aerial hypha type, and abundance of aerial mycellia) observations using an Olympus Optilab microscope at 400× magnification. Colonies with abundance of aerial mycellia, filamentous cell and gram positive reaction were indicative of *Streptomyces* spp.

Genomic DNA isolation from culturable actinomycetes

Isolation of actinomycete genomic DNA was performed according to the Geneaid Genomic DNA Mini Kit protocol (Blood/Cultured Cell). Purity (λ 260/280 = 1.8-2) and the DNA concentration was then measured using the Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Metagenomic DNA isolation of actinomycetes associated with *Neofibularia* sp.

One gram of sponge sample was crushed into powder in liquid nitrogen, followed by the extraction of total genomic DNA using the Power Soil DNA Isolation Kit (Mobio Laboratories, Carlsbad, CA, USA). The purity and the quantity of the extract were measured as described above.

The 16S rRNA gene amplification of culturable and unculturable actinomycetes from *Neofibularia* sp.

Genomic DNA was amplified by the polymerase chain reaction (PCR) using the T1-thermocycler (Biometra, Goettingen, Germany). Genomic DNA was used as the template for amplification using two 16S rRNA gene primers specific for actinomyces, i.e. 27F (5'-AGAGTT TGATCCTGGCTCAG-3') and 16Sact1114R (5'-GAGTTGACCCCGGCRGT-3') (Martina *et al.*, 2008).

The 25 µL reaction volume consisted of 12.5 µL GoTaq Green Master Mix 2×, 0.25 µL of each primer (60 pmol), 5 µL template DNA (100 ng/µL) and 7 µL nuclease free water. Temperature gradients used for PCR were as follows: 5 min pre-denaturation at 94 °C, 1 min denaturation at 94 °C, 45 sec annealing at 65 °C (decreasing 0.5 °C for each cycle until the 20th cycle, 55 °C for the next 10 cycles), 2 min elongation at 72 °C, 7 min post elongation at 72 °C, and 15 min cooling at 4 °C (touch down amplification). PCR was performed over 30 cycles (Zhang *et al.*, 2013) and the products electrophorized on 1% agarose gel. A single DNA band per sample was observed in the G:BOX gel documentation system (Syngene, Frederick, MD, USA) to observe the 1087 bp target DNA band.

The product of first phase PCR was used as template DNA for the second phase PCR using the universal primer for bacteria, i.e. P338F-GC (5'-CGCCCGCCGCGCGCG GCGGGCGGGGCGGGGGCACGGGGGGACTCCTACGG GAGGCAGCAG-3') and P518R (5'-ATTAC CGCGGCTGCTGG-3') (Overeas *et al.*, 1997). A PCR reaction volume of 100 µL consisted of 62.5 µL GoTaq Green Master Mix 2× (Promega, Madison, WI, USA), 0.5 µL of each primer (100 pmol), 0.2 µL of the product of first phase PCR, and 36.3 µL nuclease free water. PCR for the 16S rRNA gene was conducted over 30 cycles that

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consisted of 5 min pre-denaturation at 94 °C, 1 min denaturation at 94 °C, 45 sec annealing at 55 °C, 1 min elongation at 72 °C, and 5 min post elongation at 72 °C. The PCR products (5 µL) were run on 1% agarose gel for 45 min at 80 V. The electrophoresis results were visualized after Ethidium Bromide (EtBr) staining and 180 bp bands were observed on the G:BOX gel documentation system (Syngene, Frederick, MD, USA).

DGGE analysis of the 16S rRNA gene

The PCR product of 16S rRNA gene was run on to a 0.75 mm vertical gel containing 8% (w/v) polyacrylamide (acrylamide-bisacrylamide (37.5: 1)) in 1× Tris-acetate-EDTA (TAE). The denaturant gradient concentrations used were 30% and 70% (100% denaturant in accordance with 7M urea and 40% formamide deionisation). Electrophoresis was conducted for 7 h at 60 °C, 150 V using the D Code Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA). The electrophoresis gel was stained in the dark with EtBr for 30 min, followed by rinsing with 500 mL of 1× TAE buffer before observing the bands in the G:BOX gel documentation system (Syngene, Frederick, MD, USA). Separated bands were cut using a sterile scalpel and placed into micro tubes containing 100 µL $_{dd}H_2O$. The products of DGGE were incubated at 4 °C overnight, and then incubated again at 60 °C for 2 h, following the conditions and primers used in the second phase PCR but without the GC-clamp (Learn-Han *et al*., 2012).

16S rRNA gene sequencing for culturable and unculturable actinomycetes, bioinformatic analysis and construction of phylogenetic tree

The PCR products of the 16S rRNA gene of culturable and unculturable actinomycetes isolated from sponge were sequenced following standard protocols of DNA sequencing (ABI PRISM 3100). The sequencing results were compared to those of reference strains available in the GenBank (http://www.ncbi.nlm.nih.gov/) using the BLAST program. Phylogenetic analysis was conducted using MEGA 6 software, and the phylogenetic tree was constructed using a 1000 replication-bootstrap analysis and the neighbour-joining method (Saitou and Nei, 1987).

RESULTS

Diversity in culturable actinomycetes

A total of 20 actinomycete isolates were successfully grown in HV Agar medium and purified using YSA medium. The isolates NOAq 1.1, NRSw 4.1, NOAq 1.3, NOAq 2.1, and NOAq 1.4 showed gram (positive) staining and the type of aerial mycelia including spore chain-bearing aerial hyphae characteristic of *Streptomyces* spp. (Shirling and Gottlieb, 1966). At the same time, there were differences in colony macroscopic appearance, colony colouration and pigmentation (Figure 1, Table 1).

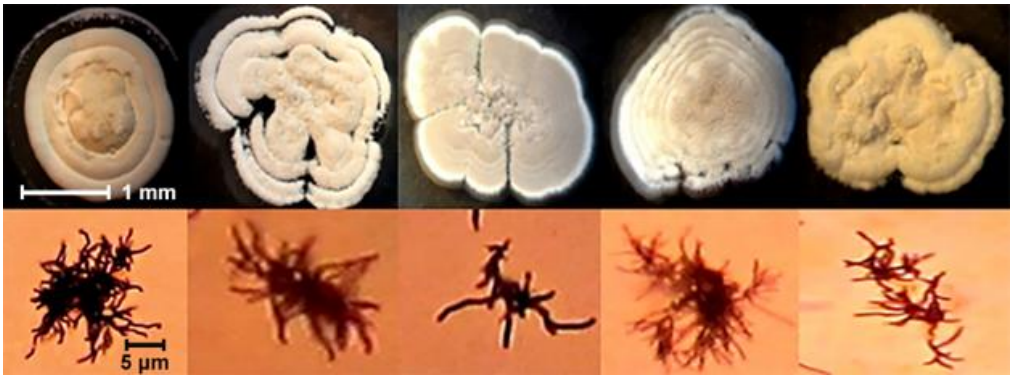

Figure 1: Macroscopic appearance of culturable actinomycetes associated with *Neofibularia* sp. showing different colony colouration (upper) and microscopic observations of actinomycetes (below). Isolates were grown on ISP 2 medium and observed after 10 days incubation at room temperature (Olympus Optilab, 400x magnification) (A = NOAq 1.1; B = NRSw 4.1; C = NOAq 1.3; D = NOAq 2.1; E = NOAq 1.4).

The 16S rRNA genes from the genomic DNA of five isolates were successfully amplified using 27F and 16Sact1114R primers with the 1087 bp DNA fragment as the target (Figure 2A). Based on the sequence data and BLAST analysis, all isolates had sequences homologous with the *Streptomyces* genus. Phylogenetic analysis indicated that the five isolates belonged to a cluster of the family *Streptomyceae* (Figure 2B). NCBI BLAST results of the culturable actinomycetes isolates NOAq1.1, NRSw 4.1, NOAq 1.3, and NOAq 2.1 showed 99% similarity with *S. sampsonii* strain NRRL B12325, *S. albus* strain J1074, and *S. resistomycificus* strain ISP 5133, while NOAq 1.4 was 97% similar to these reference strains. NOAq 1.1 and NOAq 2.1 were 99%, NRSw 4.1 and NOAq 1.3 was 98%, and NOAq 1.4 was 97% similar to *S. erringtonii* strain I36. On the other hand, NOAq 1.1 and NOAq 2.1 were 99%, NOAq 1.3 was 98%, while NRSw 4.1 and NOAq 1.4 were 97% similar to *S. gougerotii* strain NBRC 13043 (Table 2).

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Table 1: Morphological characteristics of selected actinomycete colonies associated with *Neofibularia* sp.

Code of Isolate	Colour		Pigmentation	Aerial Mycellia	Gram Staining	Hypha Type
	Surface Colony	Colony Base				
NOAq 1.1	reddish white	reddish brown	red brick	abundant	Gram positive	Rectiflexibiles
NRSw 4.1	white	light brown	no pigmentation	abundant	Gram positive	Rectiflexibiles
NOAq 1.3	brown gray	blackish brown	light brown	abundant	Gram positive	Rectiflexibiles
NOAq 2.1	white gray	light brown	no pigmentation	abundant	Gram positive	Rectiflexibiles
NOAq 1.4	cream	reddish dark brown	reddish dark brown	abundant	Gram positive	Rectiflexibiles

After 10 days incubation at temperature room on YSA medium

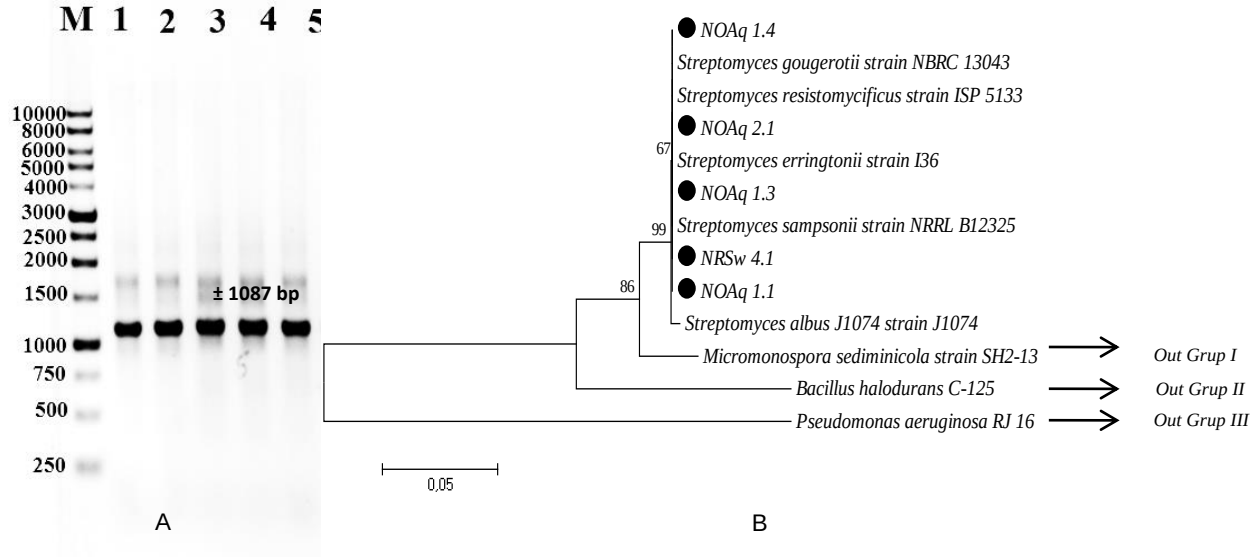

Figure 2: (A) PCR amplification of 16S rRNA (1087 bp amplicon) with primer 27F and 16Sact1114R. Lanes: 1 Kb marker (M), NOAq 1.1 (1), NRSw 4.1 (2), NOAq 1.3 (3), NOAq 2.1 (4), and NOAq 1.4 (5). (B) Phylogenetic tree of 16S rRNA gene of actinomycetes associated with *Neofibularia* sp. constructed using MEGA 6 software.

Diversity of actinomycetes based on metagenomic 16S rRNA-targeted PCR-DGGE analysis

DNA extracted from sponge tissue was successfully amplified for the 1087 bp 16S rRNA gene target. The amplicons obtained were then used as a template in the second phase of PCR to amplify the bacteria V3 region for a 180 bp gene target (Overeas *et al.*, 1997) (Figure 3A). The 16S rRNA gene amplicons were separated using DGGE. Phoretix analysis, using 1D Phoretix Software, detected 30 DGGE bands (Figure 3B), including13 dominant bands that were successfully separated on polyacrylamide gel. The DGGE bands on the gel differed in thickness and stain intensity, reflecting varying abundance of the amplicons. Bands 5, 4, and 17 were thicker than the other DNA bands (Table 3). The alignment of 13 DNA bands with reference sequences in the GenBank Database indicated that bands 13, 14, 17, and 12 were 94%, whereas band 20 was 92%, band 6 and 3 were 93%, and band 11 was 88% similar to *F. thermotolerans* strain Y005 (Figure 4). Bands 13, 6, 12, 3, 17, and 14 were 92%, band 20 was 90%, and band 11 was 87% similar to *L. majanohamensis* strain NBRC 102561. Bands 13, 12, 3, 14, and 17 were also 92%, band 20 and 6 were 91%, band 11 was 87 % similar to *A. ferrireducens* strain IC-180. Band 11 was also 85% similar to *T. bispora* strain DSM43833. DNA band 1 and 4 were 94%, band 5 was 100%, and band 9 was 95% similar to *P. acnes* strain KPA171202. Band 7 was 87%

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and 86% similar to uncultured bacterium clone nbw337c09c1 and ncm69a12c2, respectively (Table 4).

As mentioned previously, the DGGE bands were different in thickness, indicating different isolate abundance. Band 5 which was similar to *P. acnes* strain KPA171202 had the highest abundance, with a concentration of 19.95 ng/µL, while band 17, which was similar to *F. thermotolerans* strain Y005, had a concentration of 11.75 ng/µL. Band 4 also had high abundance, at 13.45 ng/µL. However, its identity is yet to be firmly established using phylogenetic analysis and it is possibly a novel and unknown actinomycetes community from the sponge *Neofibularia*.

Table 2: Similarity of culturable actinomycetes to GeneBank references strains based on 16S rRNA sequences.

Code of Isolate	Description	Total Base (Isolate/GenBank)	Position Ordinal Base	Identity	Acession Number
NOAq 1.1 and NOAq 2.1	*S. sampsonii* strain NRRL B12325	997 / 997	45-1077	99%	NR1165081
	S. albus strain J1074	997 / 997	84-1116	99%	NR1029491
	S. resistomycificus strain ISP 5133	997 / 997	34-1066	99%	NR0421001
	S. erringtonii strain I36	997 / 997	44-1075	99%	NR1156831
	S. gougerotii strain NBRC 13043	997 / 997	62-1093	99%	NR1126101
NRSw 4.1	*S. sampsonii* strain NRRL B12325	1006 / 997	45-1077	99%	NR1165081
	S. albus strain J1074	1006 / 997	84-1116	99%	NR1029491
	S. resistomycificus strain ISP 5133	1006 / 997	34-1066	99%	NR0421001
	S. erringtonii strain I36	1006 / 997	44-1075	98%	NR1156831
	S. gougerotii strain NBRC 13043	1006 / 997	62-1093	97%	NR1126101
NOAq 1.3	*S. sampsonii* strain NRRL B12325	1007 / 997	45-1077	99%	NR1165081
	S. albus strain J1074	1007 / 997	84-1116	99%	NR1029491
	S. resistomycificus strain ISP 5133	1007 / 997	34-1066	99%	NR0421001
	S. erringtonii strain I36	1007 / 997	44-1075	98%	NR1156831
	S. gougerotii strain NBRC 13043	1007 / 997	62-1093	98%	NR1126101
NOAq 1.4	*S. sampsonii* strain NRRL B12325	1022 / 997	45-1077	97%	NR1165081
	S. albus strain J1074	1022 / 997	84-1116	97%	NR1029491
	S. resistomycificus strain ISP 5133	1022 / 997	34-1066	97%	NR0421001
	S. erringtonii strain I36	1022 / 997	44-1075	97%	NR1156831
	S. gougerotii strain NBRC 13043	1022 / 997	62-1093	97%	NR1126101

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DISCUSSION

Characterization of culturable actinomycetes

Streptomyces dominated the cultivable isolates in this study, mostly due to the isolation media and incubation temperature being suited to this genus (Vieira and Nahas, 2005). Moreover, Humic Acid Agar used in the culture is conducive to the selection for actinomycetes as it suppresses the growth of fast-growth bacteria and provides nutrition for actinomycetes,

Phylogenetic analysis based on 16S rRNA gene sequence indicated that the five culturable actinomycete isolates were most similar to *S. sampsonii* strain NRRL B12325 (NR1165081), *S. resistomycificus* strain ISP 5133 (NR0421001), *S. albus* strain J1074 (NR1029491), *S. gougerotii* strain NBRC 13043 (NR1126101), and *S. erringtonii* strain I36 (NR1156831). Although molecular identification showed high similarity with reference strains, dissimilarities in morphologycal character were also noted. This suggests that the culturable actinomycetes isolates might be different from the reference strains. The *S. sampsonii* colony is thin and yellowish white with a smooth colony surface and rectiflexibiles spore type (Jain and Jain, 2006), while the colony of the five observed isolates are differently coloured, being respectively white, reddish white, greyish brown, greyish white, and beige, with non-smooth colony surface when grown in ISP 2 media.

Table 3: DNA concentration of DGGE band from actinomycete community associated with *Neofibularia* sp.

Number of DGGE Band	DNA Concentration (ng/µL)
Blank	-1.00
1	9.30
7	9.85
14	8.75
11	10.05
3	10.25
4	13.45
5	19.95
6	11.35
9	11.10
12	10.60
13	10.65
17	11.75
20	10.75

Based on the 16S rRNA gene sequences, the five culturable actinomycetes isolates have similarities with the following species of *Streptomyces*. *Streptomyces sampsonii* was first isolated from soil and has a rectiflexible spore chain, smooth colony surface, is thin and yellowish white when grown in ISP 2 medium. In addition, *S. sampsonii* produces heptaene antifungal compounds, a polyene antibiotic with fungal inhibition activity against *Candida albicans, Aspergillus niger, Microsporum gypseum*, and *Trichophyton* (Jain and Jain, 2006).

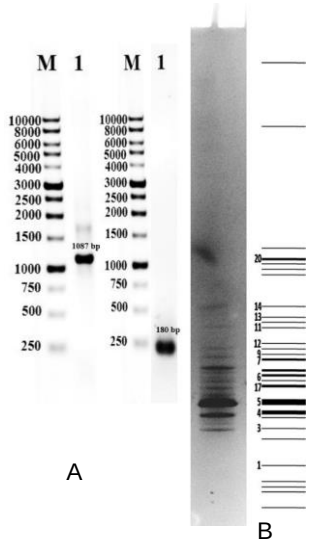

Figure 3: (A) PCR amplification of 16S rRNA (1087 bp amplicon) with primer 27F and 16Sact1114R, and second phase PCR amplification (180 bp amplicon) with primer p338F GC-*clamp* and p518R. Lanes: 1 kb marker (M); PCR amplicons (1). (B) DGGE analysis of 16S rRNA gene.

On the other hand, *S. albus* J1074 with light grey, brown, and greyish brown spores was first isolated from soil but is also found associated with marine sponges (Ian *et al.*, 2014). *Streptomyces albus* inhibits pathogen activity of *Fusarium solani* and *Helminthosporium oryzae,* and also produces xylanase that are important for rice straw pulp fermentation (Rifaat *et al.*, 2005). *Streptomyces resistomycificus* strain ISP 5133 produces resistomycin which are pentacyclic polyketides functioning in the apoptosis process of human CMK-7 leukimia megakaryoblastic cells (Shiono *et al.*, 2002). *Streptomyces erringtonii,* first isolated from the remnant of pasture hays, is aerobic, non-acid resistant, and is characterised by divided substrate mycelia with greyish white aerial spores (0.5-0.6×0.8 µm) (Santhanam *et al.*, 2013). Meanwhile, *S. gougerotii,* with rectiflexible spore chains and yellowish white spore surface when grown in ISP 2 medium, produces a gougerotin nucleoside antibiotic that has antibacterial and antivirus properties effected through the inhibition of peptide bond formation (Murao and Hayashi, 1983).

Identifications deduced from the NCBI database references that conflict with identifications based on morphological observations and other criteria are not uncommon. For example, in a study (Lisdiyanti *et al.*, 2010), the results of molecular identification of *Actinokineospora baliensis* sp. nov., *Actinokineospora cibodasensis* sp. nov., and *Actinokineospora cianjurensis* sp. nov. indicated 98.2% similarity with *Actinokineospora auranticolor* and 99.4% with *Actinokineospora terrae.* However, based on chemotaxonomic analysis, DNA hybridization and morphological characteristics (phenotype), the species were deemed different from the

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reference strains, and they were eventually identified as a novel species. In our study, the actinomycetes associated with *Neofibularia* might similarly turn out to be novel species since the isolates had different colony characteristics from the reference strains, despite having 97 to 99% similarity in 16S rRNA molecular identity with the five reference strains (Table 2).

Structure of actinomycetes community associated with *Neofibularia* sp.

The BLAST result for 13 DNA bands of actinomycetes associated with *Neofibularia* sp. indicated that the five actinomycetes groups that were identified belonged to rare actinomycetes, *viz. Ferrithrix*, *Lamia*, *Aciditerrimonas*, *Thermobispora*, and *Propionibacterium*. In addition, one DGGE band was not specifically identified using phylogenetic analysis. The number of actinomycetes isolates associated with *Neofibularia* sp. may be different for each actinomycetes community analysed, as indicated by the different thicknesses of DGGE bands. Such differences in populations of sponge-associated microbes are affected by factors such as sponge types and ages (Hentschel *et al.*, 2003). Long term adaptation process, evolution, and sponge vertical transmission are conducive to the association between many bacteria and sponges (Lee *et al*., 2001).

Ferrithrix thermotolerans was first isolated from geothermal area of Yellowstone National Park, Wyoming, USA, and has small bacil cells (3-4×0.5 µm). It is a non-motile, high-temperature tolerant bacterium with a pH optimum of 1.8. It is also a heterotroph capable of oxidising iron and sulphur (Johnson *et al.*, 2009). *Propionibacterium acnes* strain KPA17202 is a non-sporic, anaerobic bacterium that favors a growth temperature of 37 °C. It is known to produce fatty acid isomerase (Liavonchanka *et al*., 2006). *Lamia majanohamensis* was first isolated from the sea cucumber *Holothuria edulis* collected from seawater off the coast of Japan (Midori *et al*., 2009). *Aciditerrimonas ferrireducens* strain IC-180 was first isolated from the solfataric zone of Hakone, Japan. It is capable of oxidising H_2, and fixing CO_2 under anaerobic conditions. While it is capable of reducing Fe^{3+}, it is not capable of oxidising Fe^{2+} (Itoh *et al*., 2011). This actinomycete is also non-filament-forming and thermoacidophilic. *Thermobispora bispora* DSM 43833 is synonymous to *Microbispora bispora* which was first isolated from compost. *Thermobispora bispora* is known to have two different transcriptionally active 16S rRNA genes, a feature not found in all microbes. The mycelia of *T. bispora* are monopodial and 0.5-0.8 µm in diameter. They have oval to round spores, and are non-motile (Wang *et al*., 1996).

The similarity of eight DNA bands with *F. thermotolerans* (with similarity up to 94%) does not necessarily confirm their identity to the reference strain. In past research, a 16S rRNA sequence homology that has <97.5% has subsequently been recognised as a distinct species (Stackebrandt and Goebel, 1994). It cannot be ruled out that in the present study, actinomycetes associated with *Neofibularia* sp. could include novel species different from *F. thermotolerans* strain Y005 despite close molecular similarities.

Again, this is true also for actinomycetes that can be classified as different species despite 16SRNA sequences that exceed 97.5% in similarity to a reference strain. For example, *Streptomyces baliensis* sp. nov., a novel actinomycetes species isolated from Indonesia, is considered a distinct species from *Streptomyces glauciniger* (98% similarity), *Streptomyces lilacinus* (97.6%), and *Streptomyces abikoensis* (97.7%) despite close sequence consensus (Otoguro *et al.*, 2009). Hence, the eight bands of unculturable actinomycetes associated with *Neofibularia* sp. which bear 94% similarity to the reference strains should be explored further for their species novelty. The novelty of actinomycetes can be fully determined through polyphasic taxonomy that is based on several characters such as morphology and physiological characters, chemotaxonomy, molecular analysis, i.e. 16S rRNA gene sequences and DNA hybridisation.

Comparison of culturable and unculturable actinomycete diversity

The culture and DGGE approaches adopted in the present study are used to assess the diversity of culturable an unculturable actinomycetes in parallel with the work of Zhang *et al.* (2006) and Xin *et al.* (2008). With the DGGE method, four rare actinomycetes were identified, while the cultivation technique recovered isolates came from a single genus, i.e. *Streptomyces*. It appears that the community of actinomycetes associated with *Neofibularia* are dominated by rare actinomycetes from the non-*Streptomyces* group. These bacteria that show similarity with *Ferrithrix*, *Lamia*, *Aciditerrimonas*, *Thermobispora*, and *Propionibacterium* cannot as yet be cultured for further characterization. On the other hand, the cultured isolates were not detected in the DGGE approach. This could be indicative of a very low abundance of *Streptomyces* spp. that failed to be amplified using DGGE primers (Sun *et al.*, 2010). Possibly also, their presence could have been represented by very thin bands on the DGGE polyacrylamide gel, and they were not selected for sequencing. In our study, only 13 dominant bands out of 30 bands that appeared by phoretix analysis were sequenced. Therefore, the *Streptomyces* spp. might have been represented among the remaining 17 unsequenced bands. The present work can provide a foundation towards better understanding of the diversity of actinomycetes associated with sponge *Neofibularia* off Bira Island in the Indonesian archipelago.

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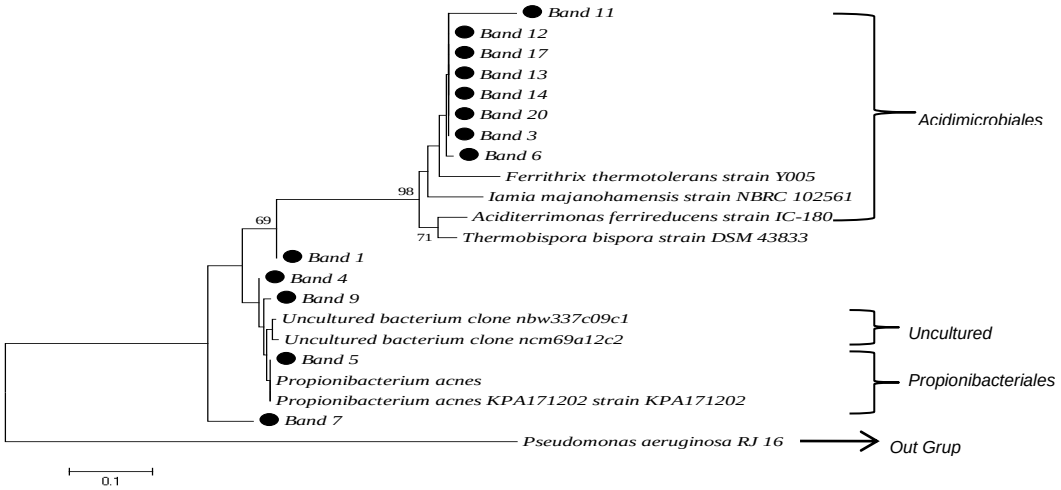

Figure 4: Phylogenetic tree based on DDGE separation of 16S rRNA gene of actinomycetes associated with *Neofibularia* sp. The phylogenetic tree was constructed using MEGA 6 software.

Table 4: Similarity of actinomycetes associated with *Neofibularia* sp. with GeneBank references strains, based on BLAST analysis of 16S rRNA sequence.

Band	Description	Query Cover	Total Base (GenBank/DGGE Band)	Position Ordinal Base	Identity	Accession Number
1, 4	*P. acnes* strain KPA171202	100%	130 / 128	375-503	94%	NR0746751
	P. acnes	100%	130 / 128	375-503	94%	NR0408471
5	*P. acnes* strain KPA171202	97%	130 / 128	375-503	100%	NR0746751
	P. acnes	97%	130 / 128	375-503	100%	NR0408471
9	*P. acnes* strain KPA171202	97%	131 / 128	375-503	95%	NR0746751
	P. acnes	97%	131 / 128	375-503	95%	NR0408471
7	Uncultured bacterium clone nbw337c09c1	77%	127 / 128	357-482	87%	GQ0907041
	Uncultured bacterium clone ncm69a12c2	77%	127 / 128	357-482	86%	KF1082841
13, 12,	*F. thermotolerans* strain Y005	99%	125 / 125	381-508	94%	NR0427511
14, 17	*L. majanohamensis* strain NBRC 102561	99%	125 / 125	359-486	92%	NR0416341
	A. ferrireducen strain IC-180	100%	125 / 126	366-494	92%	NR1129721
	T. bispora strain DSM 43833	100%	125 / 126	390-518	92%	NR0746801
20	*F. thermotolerans* strain Y005	99%	123 / 125	381-508	92%	NR0427511
	L. majanohamensis strain NBRC 102561	99%	123 / 125	359-486	90%	NR0416341
	A. ferrireducen strain IC-180	100%	123 / 126	366-494	91%	NR1129721
	T. bispora strain DSM 43833	100%	123 / 126	390-518	91%	NR0746801
6	*F. thermotolerans* strain Y005	99%	125 / 125	381-508	93%	NR0427511
	L. majanohamensis strain NBRC 102561	99%	125 / 125	359-486	92%	NR0416341
	A. ferrireducen strain IC-180	100%	125 / 126	366-494	91%	NR1129721
	T. bispora strain DSM 43833	100%	125 / 126	390-518	91%	NR0746801
3	*F. thermotolerans* strain Y005	97%	125 / 125	381-508	93%	NR0427511
	L. majanohamensis strain NBRC 102561	97%	125 / 125	359-486	92%	NR0416341
	A. ferrireducen strain IC-180	98%	125 / 126	366-494	92%	NR1129721
	T. bispora strain DSM 43833	98%	125 / 126	390-518	92%	NR0746801
11	*F. thermotolerans* strain Y005	100%	128 / 125	381-508	88%	NR0427511
	L. majanohamensis strain NBRC 102561	100%	128 / 125	359-486	87%	NR0416341
	A. ferrireducen strain IC-180	100%	128 / 126	366-494	87%	NR1129721
	T. bispora strain DSM 43833	100%	128 / 126	390-518	85%	NR0746801

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CONCLUSION

This is the first study of the community structure of actinomycetes associated with *Neofibularia* sp. The community of actinomycetes comprises both culturable and unculturable actinomycetes. The combined culture and DGGE approaches provide an informative appraisal of the actinomycete diversity that includes rare actinomycetes identified through the latter procedure. Of this group, eight DGGE bands had the highest similarity of 94% with *F. thermotolerans* strain Y005, four bands were most similar (97-100%) to *P. acnes* strain KPA171202, and one band had the highest similarity of 77% with uncultured bacterium clone nbw337c09c1. The culturable actinomycetes isolated in this study were all from the *Streptomyces* group, and were most similar (97-99%) to *S. sampsonii* strain NRRL B12325, *S. resistomycificus* strain ISP 5133, and *S. albus* strain J1074.

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