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Study of Symbiont Bacteria of *Acropora digitifera* Coral From Ciletuh Bay, Sukabumi by Using Culture and Molecular Approach

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ABSTRACT

Bacteria are one of the prokaryotic microorganisms that are symbiotic with coral reefs. These microorganisms help corals in secreting mucus layers which are used as a place to live for bacteria and control the presence of pathogenic bacteria in corals. The purpose of this study was to determine the diversity of bacterial communities associated with *Acropora digitifera* corals in Ciletuh bay, West Java, by culture approach. Sampling was carried out at a depth of 4 meters. Morphology-based identification and molecular approach based on sequence of the small ribosomal unit (16S) rRNA gene were used to determine the bacterial species in the samples. A total of six pure isolates were identified based on morphological observation and the molecular DNA characteristics from three of them were identified with 16S rRNA gene sequences. The identification using 16S rRNA gene showed that the isolate ACD.P4.PH7.P had a close relationship with the BF strain and zb strain of *Bacillus flexus* (acc number MH569560.1) with a similarity of 85.44 %. While ACD.P4.PH9.P isolate has a close relationship with *Bacillus* sp. c234 (acc number FJ950647.1) with a similarity of 98.50 %. Additionally, ACD.P4.PH9.K isolates closely related to *Bacillus* sp. of strain 6RM1 (acc number MK134607.1) with a similarity of 94.78 %. This study also revealed that both microscopic identifications by morphological traits and molecular approach using 16S rRNA gene can be used in bacterial diversity assessment.

Keywords: *Acropora digitifera, bacteria, 16S rRNA gene, culture, gene sequence*

ABSTRAK

Bakteri adalah salah satu mikroorganisme prokariotik yang bersimbiosis dengan terumbu karang. Mikroorganisme ini membantu karang dalam mengeluarkan lapisan lendir yang digunakan sebagai tempat hidup bakteri dan mengendalikan keberadaan bakteri patogen di dalam karang. Tujuan dari penelitian ini adalah untuk menentukan keanekaragaman komunitas bakteri yang terkait dengan karang *Acropora digitifera* di teluk Ciletuh, Jawa Barat, dengan pendekatan kultur. Pengambilan sampel dilakukan pada kedalaman 4 meter. Identifikasi berbasis morfologi dan pendekatan molekuler berdasarkan sekuen gen rRNA unit ribosom kecil (16S) digunakan untuk menentukan spesies bakteri

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dalam sampel. Sebanyak enam isolat murni diidentifikasi berdasarkan pengamatan morfologis dan karakteristik DNA molekuler dari tiga di antaranya diidentifikasi dengan sekuens gen 16S rRNA. Identifikasi menggunakan gen 16S rRNA menunjukkan bahwa isolat ACD.P4.PH7.P memiliki hubungan yang erat dengan strain BF dan strain zb *Bacillus flexus* (nomor acc MH569560.1) dengan kesamaan 85,44%. Sedangkan isolat ACD.P4.PH9.P memiliki hubungan erat dengan *Bacillus* sp. c234 (nomor acc FJ950647.1) dengan kesamaan 98,50%. Selain itu, isolat ACD.P4.PH9.K terkait erat dengan *Bacillus* sp. dari strain 6RM1 (nomor acc MK134607.1) dengan kesamaan 94,78%. Studi ini juga mengungkapkan bahwa kedua identifikasi mikroskopis dengan ciri-ciri morfologis dan pendekatan molekuler menggunakan gen 16S rRNA dapat digunakan dalam penilaian keanekaragaman bakteri.

Kata kunci: *Acropora digitifera, bakteri, gen 16S rRNA, kultur, sekuen gen*

1. Introduction

Ciletuh Bay is located in the district of Sukabumi, West Java, Indonesia. This bay has a diverse coral reef ecosystem, both hard coral (Scleractinian) and soft corals that have holobiont with microorganisms around them, such as bacteria and zooxanthellae and other microorganisms. One of the associations between coral reefs and microorganisms is their mutual symbiosis in producing oxygen. There are various types of hard coral in these waters and one of them is *Acropora digitifera*. This species is a type of hard coral commonly found in the Indonesian Ocean which belongs to the family Acroporidae. Corals that belong to this family are commonly found in depths of 3-15 meters.

According to Utama (2017), there arefifty-eight coral species from the Acroporidae family in Indonesia, including *Acropora abrolhonsensis, Acropora aspera, Acropora austrea, Acropora bifurcata, Acropora cerealis, Acropora clathrata, Acropora cytherea, Acropora digitifera, Acropora divaricata, Acropora formosa, Acropora gemmifera, Acropora humilis, Acropora hyacinthus, Acropora jacqueline, Acropora kimbeensis, Acropora listeri, Acropora loripes, Acropora microlados, Acropora millepora, Acropora monticulosa, Acropora nasuta, Acropora pulchra, Isopora palifera, Acropora retusa, Acropora robusta, Acropora samoensis, Acropora sarmentosa, Acropora secale, Acropora selago, Acropora seriata, Acropora solitaryensis, Acropora sp., Acropora spicifera, Acropora stoddarti*, *Acropora tenuis, Acropora valida, Acropora yongei, Astreopora ocellata, Astreopora sp., Isopora bruggemi, Isopora cuneata, Montipora caliculata, Montipora efflorescens, Montipora flaveolata, Montipora florida, Montipora foliosa, Montipora grissea, Montepora hispida, Montipora incrassata, Montipora informis, Montipora monasteriata, Montipora peltiformis, Montipora sp., Montipora tuberculosa, Montipora undata,* and *Montipora venosa*.

Coral reefs are symbiotic or associated with microorganisms to sustain life, one of which is associated with bacteria. In addition to sustaining coral life, bacteria can also affect coral health, nutrient intake, and coral disease (Zhang *et al*. 2015). One of the methods carried out by coral animals to initiate associations with bacteria is by secreting mucous layers containing glycoprotein.

The mucus layer has a dual role in the association process. In addition to its role of providing living space for the associated bacteria, the layer also has a role in controlling the presence of pathogenic bacteria, together with the secretion of several secondary metabolites that are antibiotics (Ritchie and Smith 1997). The existence of bacterial communities which are in symbiosis with coral reefs can be influenced by seasonal changes, geographical differences, and pollution (Zhang et al. 2015).

These factors can affect the diversity of bacterial communities in coral reefs. The presence of bacteria in the coral reef can be assessed by using culture method. This conventional method can be used for coral species identification by molecular approach. Additionally, previous study from Rinanda (2011) also demonstrated that the bacterial diversity can be conducted by isolating DNA from cultured bacteria, either from solid or liquid culture.

The purpose of this study was to determine the diversity of bacterial communities that are associated with *Acropora digitifera* corals in the water bodies of Ciletuh bay, Sukabumi, West Java, through a culture approach.

2. Materials and Methods

Acropora digitifera coral samples were taken from Ciletuh bay on coordinate

07°11'16.11" LS and 106°25'20.40" BT. Samples were then analyzed in the Laboratory of Microbiology and Molecular Biotechnology, Faculty of Fisheries and Marine Sciences, Universitas Padjadjaran.

2.1. Sampling of *Acropora digitifera* corals

Acropora digitifera coral samples were taken from the depth of 4 meters from corals with sizes of approximately 10 cm and placed into a plastic bottle containing 70% ethanol. Then they were stored in a coolbox for temporary storage and brought to the laboratory.

2.2. Bacterial screening and Morphological observations

Bacterial screening was carried out in several stages, i.e. inoculation and cultivation of bacterial colonies to determine their morphological traits and gram staining of bacterial cells to determine their shape and color. Briefly, the coral sample was crushed using a mortar. Then, a total of 1 g of sample was taken for serial dilution (7 times) in a test tube containing 9 ml of sterile seawater and then vortexed.

Bacterial inoculation was carried out by the spread plate method following the protocol from Cappuccino and Welsh (2018). Briefly, a total of 100 µl was taken from the sample at dilutions 4 and 5, then spread on the Zobell Marine Agar (Marine Agar 2216) medium produced by HiMedia Laboratories Pvt. Ltd. Mumbai-400086 India with its brand, HIMEDIA and has catalog number M384-500G in a petri dish and flattened using spreader.

The edge of the petri dish containing bacterial suspension then rotated on a bunsen which was then sealed using a plastic wrap, and incubated for 24 h. After 24 hours, the colony morphology of bacteria was observed and identified according to their shape, color and edges of each isolates. Afterwards, the bacterial colony was then purified using the streak plate technique. In brief, one ose of bacterial isolates were taken from the inoculation results, then etched into a petri dish containing Zobell Marine media.

Moreover, to divide the petri dish into four quadrants, the edge of the petri dish was rotated on a bunsen which was then sealed using plastic wrap and incubated for 24 h. After inoculation and bacterial cultivation, gram staining was performed according to the protocol from Cappuccino and Welsh (2018) to determine the shape and color of the associated bacterial cells. Briefly, one colony from the results of cultivation was taken with an ose needle, then placed on a glass object assisted by physiological NaCl and allowed to pass on fire.

Afterwards, one drop of gentian violet was added into the object glass and left for 20 s, and washed with distilled water. Then, one drop of iodine solution was dropped into the object glass and left for 1 min, then washed with alcohol and distilled water. Furthermore, one drop of safranin or fuchsin water was then added and left for 20 s, and washed with distilled water. The object glass was then dried and observed on a microscope with a magnification of 40X. Each bacterial isolates was labeled to ease identification.

2.3. Molecular Identification of Bacteria

After pure bacterial isolates were obtained from the culture, the liquid culture was then prepared using Nutrient Broth (NB) media to be produced by OXOID Limited Wade Road, Basingtoke, Hampshirw, RG24 SPW, UK with its brand, OXOID and has catalog number CM0001 which would be used for DNA extraction of bacterial genomes. The bacterial genome DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega) protocol for samples of gram positive and gram negative bacteria.

When the bacterial DNA was obtained, PCR was performed to amplify DNA. The primer used for PCR is the 16S rRNA gene in accordance to Lane (1991), which is a 27F abd 1492R universal primer. The PCR cycling parameters consisted of an initial denaturation step at 95°C for 3 min, denaturation at 95°C for 45 s, annealing step at 52°C for 1 min, elongation step at 72°C for 2 min, final elongation at 72°C for 5 min and HOLD at 4°C for 7 min.

The mixture of PCR products consist of PromTaq®Green Master Mix Promega (12.5 µL), 27 F primers (1.25 µL), 1492 R primers (1.25 µL), Nuclease free water (8 µL), and DNA template (2 µL) thus the total of PCR product is 25 µL. In this study, 1 Kb of DNA Ladder (KAPA) as a DNA size marker and DNA Loading dye as a DNA ballast were used. Red Nucleic Acid Gel Stain was used as much as 10 µL in 100mL of TAE 1X for 1% agarose gel.

Then, electrophoresis was performed to determine the presence of genomic DNA bands for 45 minutes with an 80 volt current. Afterwards, Sanger sequencing was performed

by 1st Base in Singapore. The sequencing stage was carried out at Singapore's 1st Base with results in the form of a FASTA file. The file was then processed by BioEdit and MEGA7 software for phylogenetic tree analysis.

2.4. Data Analyses

All data regarding bacterial colony morphology, shape and characteristics of bacterial cells were analyzed descriptively, whereas the FASTA files obtained from sequencing were processed using the BioEditTM software. These FASTA files were then processed further in BLAST in the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov).

Homogeneous BLAST results were deposited to the GenBank in order to obtain an access number that can be used to construct phylogenetic trees using MEGA 7 software. This was performed to obtain information related to the bacteria's relationship with their isolates. All data that were obtained, were analyzed comparatively and descriptively.

3. Results and Discussions

3.1. Bacterial screening and Morphological observations

A total of 6 bacterial isolates were obtained from the *Acropora digitifera* coral symbiont. These bacterial isolates were obtained from treatments of pH 7 and pH 9 with a dilution rate of 4 and 5, respectively. According to our morphological observations, from these 6 isolates, the bacterial isolate ACD.P4.PH7.P had similarity to ACD.P5.PH7.P isolate, while the isolate ACD.P4.PH9.P had similarity with ACD.P5.PH9.P isolate. Moreover, the isolate ACD.P4.PH9.K was similar to ACD.P5.PH9.K (Table 1).

3.2. DNA Isolation of Bacterial Genome

Based on the inoculation results above (Table 1), there were 6 isolates with similarities and differences. From these isolates, bacterial DNA isolation was then carried out using the Wizard Genomic DNA Purification Kit (Promega) where the results are attached in the Figure 1.

The visualization results of bacterial genome revealed that the DNA band of the bacterial genome is located above 10,000 base pairs (bp). This is due to the genome size that is greater than 10,000 bp. As demonstratedby Demerdash (2012) that bacterial DNA molecules have large sizes, which range from21,000 to 23,000 bp

The differences in DNA bands among the samples was probably caused by certain factors. Thick DNA bands show high concentrations and total DNA which were extracted intactly and have high purity too (Mulyani et al, 2011), whereas thin DNA bands could be caused by excessive physical movement during pipetting, or when flipping the solution and other factors.

Based on Figure 2, it can be deduced that the 16S rRNA gene amplification process of *Acropora digitifera* associated-bacteria using PCR was successfully performed. This also supported by the visualization results where PCR was marked by thick DNA band, and it also shows that the DNA sample band size is in accordance with the product target size (± 1465 bp). This can be observed from the produced DNA band's position that was situated between 1200 and 1600 bp. Thus, the used primers and PCR conditions amplify well in accordance to the primary target of 16S rRNA (Lauro et al. 2009).

Smears contained in the electropherogram results can be caused by the presence of contaminants in the form of either protein or the carryover of the remnants of the solution that is present when the DNA isolation process takes place (Iqbal, 2016). The same thing stated by Mulyani (2011), the smear seen during the visualization process can take the form of a solution that is still carried away during DNA isolation or it can also be degraded DNA in the DNA isolation process.

Table 1. Morphological characteristics of bacterial isolates from *Acropora digitifera*

No.	*Bacterial Colonies Morphology*					*Bacterial Cells Morphology*	
	Isolat Name	***Elevation***	***Margin***	***Whole Colony***	***Color Colony***	***Shape***	**Gram**
1.	ACD.P4.PH7.P	*Raised*	*Smooth, entire*	*Round*	*White*	Streptobacillus	*Negative*
2.	ACD.P5.PH7.P	*Raised*	*Smooth, entire*	*Round*	*White*	*Bacillus*	*Positive*
3.	ACD.P4.PH9.P	*Convex*	*Smooth, entire*	*Round*	*White*	*Bacillus*	*Positive*
4.	ACD.P4.PH9.K	*Convex*	*Smooth, entire*	*Round*	*Yellow*	*Bacillus*	*Positive*
5.	ACD.P5.PH9.P	*Convex*	*Smooth, entire*	*Round*	*White*	*Streptococcus*	*Negative*
6.	ACD.P5.PH9.K	*Convex*	*Smooth, entire*	*Round*	*Yellow*	Streptobacillus	*Negative*

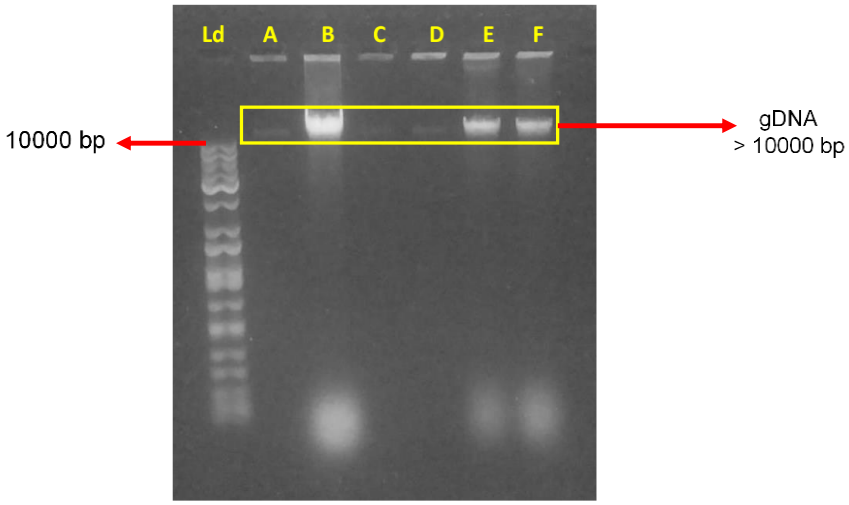

Figure 1. Genome DNA Visualization of the *Acropora Digitifera* associated-bacteria in 0.8% Agarose Gel, Ld: 1Kb DNA Ladder (KAPATM), A: Isolate ACD.P4.PH7.P, B: Isolate ACD.P5.PH7.P, C: Isolate ACD.P4.PH9.P, D: Isolate ACD.P4.PH9.K, E: Isolate ACD.P5.PH9.P, F; Isolate ACD.P5.PH9.K

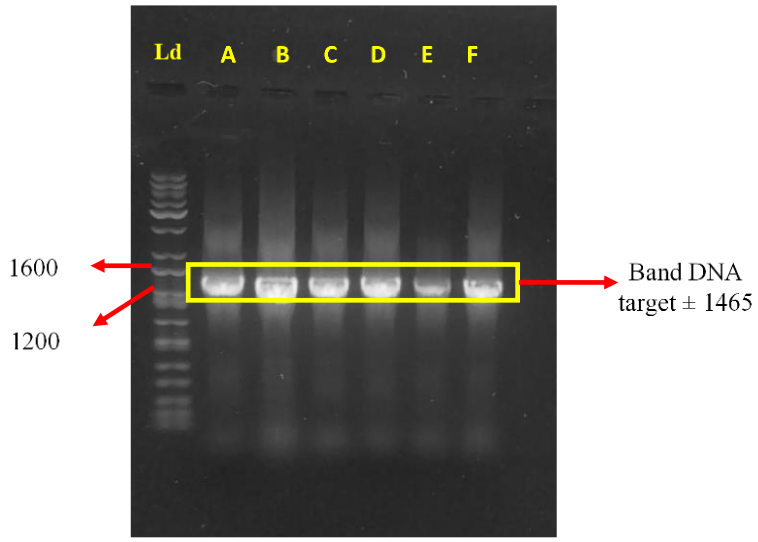

Figure 2. Gene Amplification Results of 16S rRNA with 27F and 1492R primers in 0.8% agarose gel, Ld: 1Kb DNA Ladder (KAPATM), A: Isolate ACD.P4.PH7.P, B: Isolate ACD.P5.PH7.P, C: Isolate ACD.P4.PH9.P, D: Isolate ACD.P4.PH9.K, E: Isolate ACD.P5.PH9.P, F; Isolate ACD.P5.PH9.K

With the availability of DNA band that was in accordance with the target length of its base, the amplification results were ready for sequencing.

3.3. Nucleotide Sequences Analysis from Sanger Sequencing and Phylogenetic Trees Reconstruction

The amplified sample was then sent for sanger sequencing. Furthermore, the obtained results were in the form of nucleotide bases from three sequenced bacterial samples in which the data consist of forward sequences and reverse sequences in .ab1 and .seq formats. The nucleotide sequence was then processed using BioEdit™ software in which the results were consensus of forward and reverse sequences.

The consensus results were further processed to match the available data at GenBank in NCBI (National Center of Biotechnology Information) by using the Nucleotide BLAST program in the Nucleotide collection (nr / nt) database with its bacterial organisms (taxid: 2) online. The identification results of the three sequenced bacterial samples are presented in Table 2.

The percentage of query cover from the

Table 2. Identification results of Bacterial 16S rRNA gene with BLAST Program

No.	Isolate Code	Species	*Query Cover*	E value	*Identity* (%)	*Accesion Number*
1.	ACD.P4.PH7.P	*Bacillus flexus* strain Bf strain zb	99 %	0,0	85,44	MH569560.1
2.	ACD.P4.PH9.P	*Bacillus* sp. c234	100 %	0,0	98,50	FJ950647.1
3.	ACD.P4.PH9.K	*Bacillus* sp. strain 6RM1	100 %	0,0	94,78	MK134607.1

three samples BLAST ranged from 99–100% (BLAST). This results are in coherence with the Claverie and Notredame (2003) where the query coverage value of above 80% is moderately high. Therefore, the high percentage of query cover from our samples indicating a high similarity of the base length of the data sequence from samples with those in the Genbank (Miller et al. 1990).

The identity values of the three samples ranged from 85 – 98% where the ACD.P4.PH7.P sample had the lowest percentage (85.44%). In contrast, the ACD.P4.PH9.P sample had the highest percentage (98.50%). Identity values above 97% indicate that the species found in the sample had higher similarity to those in the Genbank and is not a new species (Pangastuti 2006) as shown in the ACD.P4.PH9.P sample which has an identity value of 98.50% with the species *Bacillus* sp.

While for ACD.P4.PH9.K sample, the identity value is lower than 97% indicating that the species obtained in the sample is considered as a new species within the same genus, i.e. Bacillus. Our result is supported by Drancourt et al. (2000) which stated that 16S rRNA gene sequence data that has an identity value of ≤97% indicates that the species are in the same genus where as the identity value between 89 – 93% shows that the species are in the same family but different genus. On the other hand, the sample ACD.P4.PH7.P has an identity value of 85.44%, suggesting that the species are situated in the different family.

The results of phylogenetic tree reconstruction in the sequence ACD.P4.PH7.P showed a close relationship with the species *Bacillus flexus* of strain Bf and strain zb (Acc. No. MH569560.1, Figure 3). Moreover, the obtained phylogenetic tree construction revealed that the sequence ACD.P4.PH7.P has a bootstrap value of 30% and an identity of 85.44%. This means that from the phylogeny tree reconstruction of a thousand times, the sequence ACD.P4.PH7.P has a 30% relationship with *Bacillus flexus* of strain Bf and strain zb (Acc. No. MH569560.1).

Bacillus flexus bacteria, which is classified from the genus of Bacillus, is a gram-positive, rod-shaped, motile bacteria. The genus Bacillus has various roles especially in the fields of bioremediation. This genus has the abilities to degrade organic compounds such as protein, starch, cellulose, and hydrocarbons. Additionally, this genus also capable of producing antibiotics, plays animportant role in nitrification and dentrification, nitrogen fixing, chemolitotrophic and thermophilic (Claus and Berkeley, 1986). As demonstrated by Jebeli *et al*. 2017, that *Bacillus flexus* has the ability to remediate arsenic from water [As (V) (28%) and As (III) (45%)], especially in batch conditions.

Based on Figure 4, the sequence ACD.P4.PH9.P has a close relationship with the species *Bacillus* sp. c234 (Acc. No. FJ950647.1). The bootstrap value between the two sequences is higher than the ACD.P4.PH7.P sequence, which is 55% with

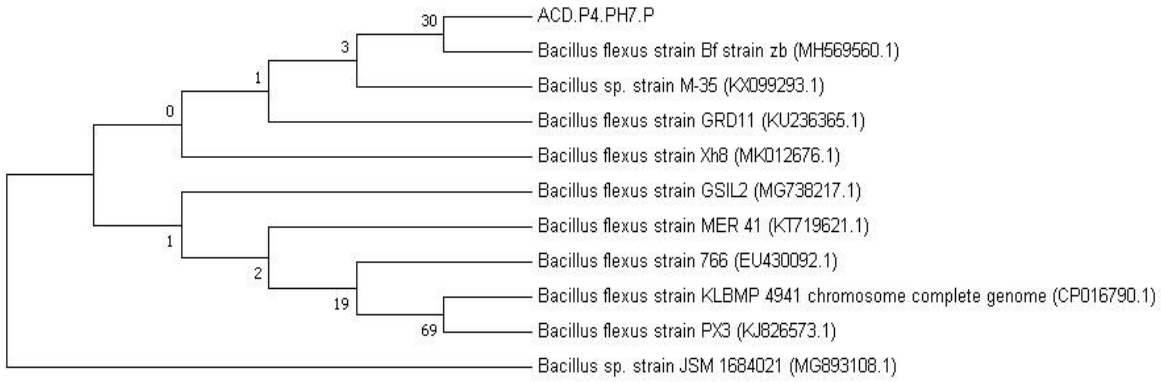

Figure 3. Phylogeny Tree of ACD.P4.PH7.P Bacteria Isolate Based on 16S rRNA Sequence Neighbor – Joining Method, Bootstrap 1000x

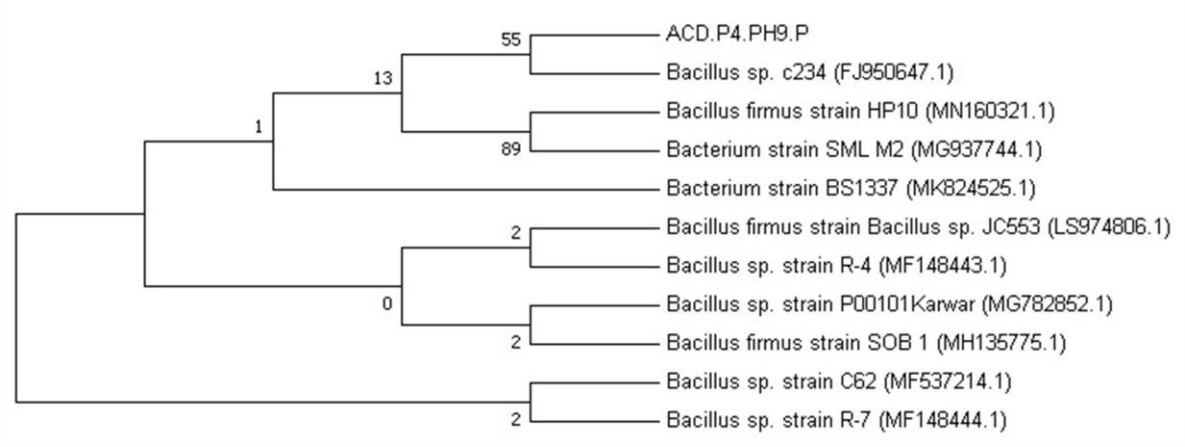

Figure 4. Phylogeny Tree of ACD.P4.PH9.P Bacteria Isolate Based on 16S rRNA Sequence Neighbor – Joining Method, Bootstrap 1000x

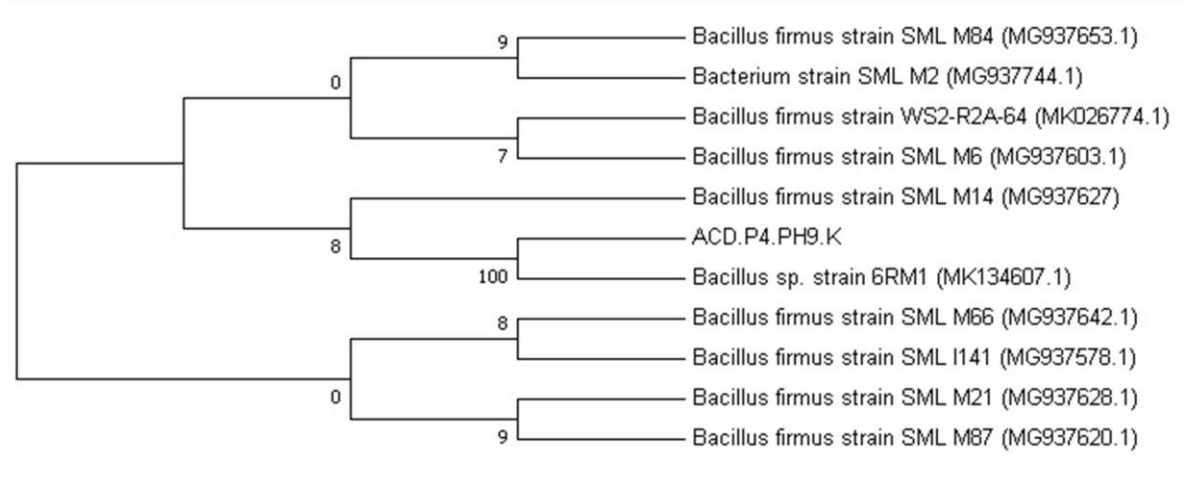

Figure 5. Phylogenic Tree of ACD.P4.PH9.K Bacteria Isolate Based on 16S rRNA Sequence Neighbor – Joining Method, Bootstrap 1000x

an identity value of 98.50 %. This means that from the phylogeny trees' reconstruction of a thousand times, the sequence ACD.P4.PH9.P has a 55% relationship with *Bacillus* sp. c234 (Acc. No. FJ950647.1). On the other hand, the ACD.P4.PH9.K sequence is closely related to the species *Bacillus* sp. of strain 6RM1 (Acc. No. MK134607.1, Figure 5) The obtained bootstrap value is 100% with an identity value of 94.78%. This means that from the phylogeny trees' reconstruction of a thousand times, the sequence ACD.P4.PH9.K has a 100% relationship with *Bacillus* sp. of strain 6RM1 (Acc. No. MK134607.1).

Between the picture (Figure 4 and Figure 5), both have the same kinship, i.e. with the species *Bacillus* sp. which belongs to the Bacillus genus. Bacillus is a rod-shaped bacteria that is often found in land or water, including the sea. These bacteria are classified into aerobic gram-positive bacteria or facultative aerobic with several types of Bacillus producing extracellular enzymes that can hydrolyze complex proteins and polysaccharides (Hatmanti, 2000).

In addition there are several strains. A strain is a collection of several types of cells. Strains are considered as small differences found in the same bacterial species. Strain differences in bacterial species can be caused by environmental factors and evolutionary processes.

4. Conclusions

Based on the present study, from the isolation processes of the *Acropora digitifera* symbiotic bacteria, 6 isolates of pure bacteria were obtained, where 3 of them were sequenced. It was discovered that the bacteria from the isolate ACD.P4.PH7.P has a close

relationship with *Bacillus flexus* of strain BF and zb (Acc. No. MH56956060 .1) with 85.44% similarity.

Isolate ACD.P4.PH9.P has a close relationship with *Bacillus* sp. c234 (Acc. No. FJ950647.1) with a similarity of 98.50%. Isolate ACD.P4.PH9.K has a close relationship with the species *Bacillus* sp. of strain 6RM1 (Acc. No. MK134607.1) with a 94.78% similarity. Further research should be done concerning the bacterial association with the sea water and the sediments around these coral reefs.

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