Available online at www.worldscientificnews.com

World Scientific News

An International Scientific Journal

WSN 144 (2020) 384-396 EISSN 2392-2192

Molecular diversity of the bacterial community associated with *Acropora digitifera* (Dana, 1846) corals on Rancabuaya coastline, Garut District, Indonesia

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ABSTRACT

Bacteria are one of the prokaryotic microorganisms that are symbiotic with coral reefs. The purpose of this study was to determine the diversity of bacterial communities associated with *Acropora digitifera* corals in the rancabuaya coastline of Garut district, West Java through a metagenomic and cultural approach. Stages of research include tissue isolation using waterpics, isolation of bacterial genomic DNA, sequencing using the NGS (Next Generation Sequencing) method of Hiseq using 16S rRNA V3-V4 region 341F and 806R primers for the metagenomic approach. While the culture approach, carried out inoculation, bacterial cultivation, gram staining, then proceed with the identification of molecular characteristics of DNA with 16S rRNA gene sequences. Dominantly, the results of bacterial identification were obtained as many as 77 species from 10 genera 10 families 10 orders of 10 classes and 4 phylum and unidentified reads of 6%. The results of the identification of 16S rRNA showed that the isolate ACD.P4.PH7.P had a close relationship with the Bacillus flexus strain BF strain zb strain with a similarity of 85.44%. Isolate ACD.P4.PH9.P has a close relationship with Bacillus sp. c234 with a 98.50% similarity. Isolate ACD.P4.PH9.K has an approach with the species Bacillus sp. strain 6RM1 with a similarity of 94.78%.

***Keywords*:** Acropora digitifera, Bacteria, Gen 16S rRNA, Culture, Metagenome

(Received 22 March 2020; Accepted 09 April 2020; Date of Publication 10 April 2020)

1. INTRODUCTION

Due to the inexorable rise in ocean temperature, coral bleaching has been induced among reefs around worldwide, that has already caused devastation of 19% of reef communities [1]. These detrimental effects of coral bleaching occurring worldwide demands the need for studies that integrate coral community responses (coral holobionts which includes host as well as the dynamic populations of mutualistic members) against bleaching in various understudied locations every to tentatively classify the corals of every locality according to [2] as bleaching "winners" (healthy/unaffected), "losers" (mortality after bleaching) or "survivors" (recover after bleaching). When corals experience thermal stress, expulsion of corals' alveolate endosymbiont, Symbiodinium (Family: Symbiodiniaceae) occurs by a phenomenon called coral bleaching [3]. Apart from this obligate symbiont of Scleractinian corals, there are different kinds of microbes that exist as stable, transient or sporadic members of the holobiont which resides within various microhabitats in the coral structures [4-6, 39, 40].

Rancabuaya coast is a waters area located in Garut district, West Java. There are various types of hard coral in these waters, one of which is *Acropora digitifera.* These coral reefs are symbiotic or associated with microorganisms to maintain the life of corals, which are bacteria. These microorganisms are found in the mucous layer which is used as a place for bacteria to live in association with and control the presence of pathogenic bacteria, as well as secretion of secondary metabolites as antibiotics [6].

The existence of a bacterial community in symbiosis with coral reefs can be influenced by seasonal changes, geographical differences, and pollution [7]. Corals are diverse meta-organisms that provide an essential bio-habitat for many other marine species [8], for instance, bacteria, Archaea, and microalgae (zooxanthellae) [9]. Unfortunately, recent research indicates that more than 30% of coral reefs have been destroyed due to emerging diseases [10]. Some of these diseases are attributed to coral-pathogenic micro-organisms and other factors [11-14]. These factors certainly affect the bacterial community on the coral reef, to determine the diversity of bacteria that can be used by using a metagenomic and cultural approach which of course chooses the advantages and disadvantages of each.

The purpose of this research is to find out the diversity of bacterial communities associated with *Acropora digitifera* corals on the coast of Rancabuaya, Garut Regency, West Java through a metagenomic and bioculture approach.

2. MATERIALS AND METHODS

This research was conducted in July up to December 2019. Coral sampling was carried out in July to August 2019 in the rancabuaya waters of Garut, West Java. The process of isolation of metagenome and bacterial culture carried out at the Laboratory of Microbiology and Molecular Biotechnology, Faculty of Fisheries and Marine Sciences, Padjadjaran University. The NGS amplicon sequencing process was carried out by Novogene Co., Ltd in Singapore and Sanger sequencing was conducted by 1st Base in Singapore.

The tools used in this research are tanks, hammers, chisels, GPS, sample bottles, beaker glass, petri dishes, test tubes, ose needles, micropipets, incubators, electrophoretic vessels, thermal cyclers, UV transluminators, spectrophotometers, and microscopes.

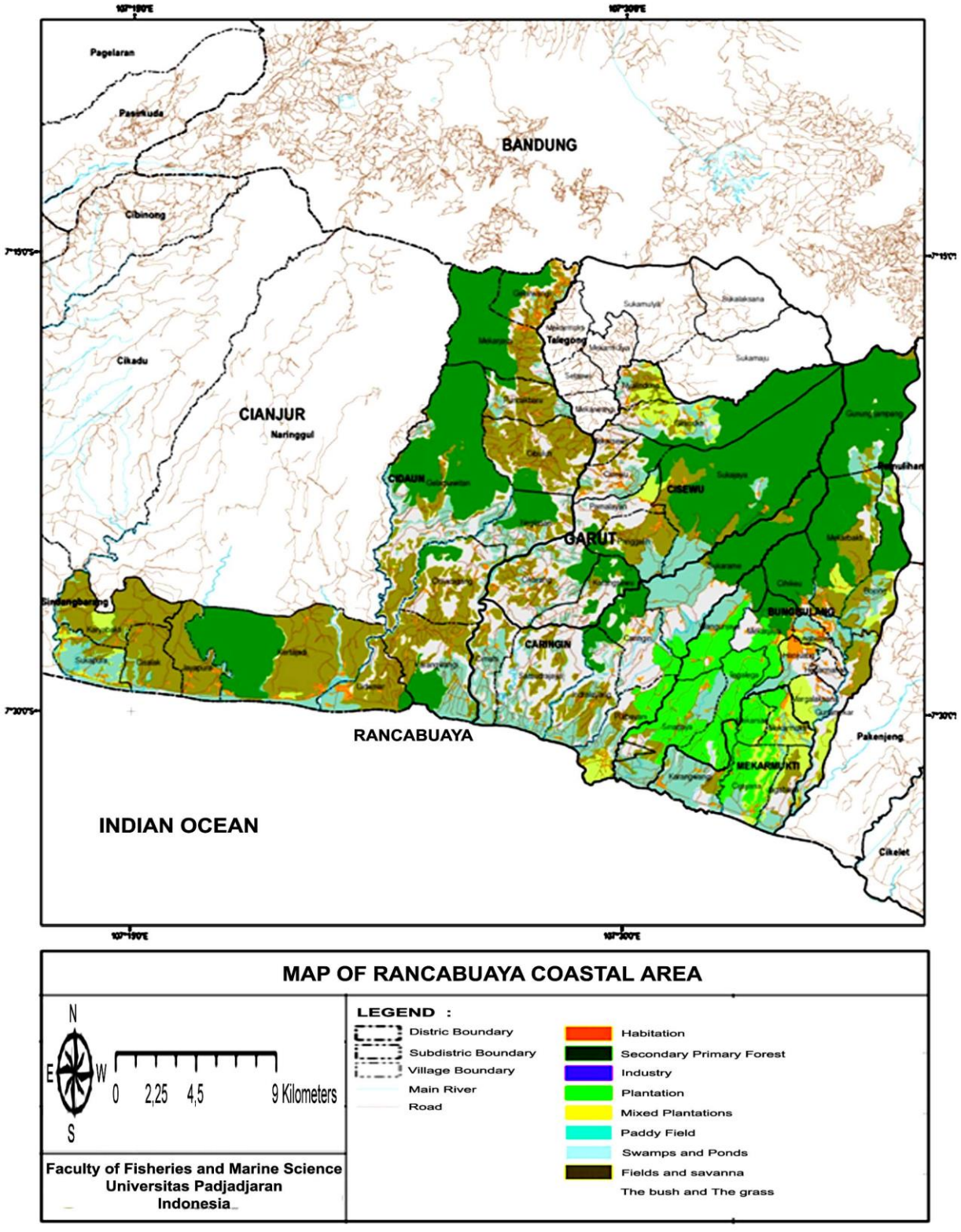

Fig. 1. Map of Rancabuaya Coastal Area

While the materials used are *Acropora digitifera* corals, seawater, ZymoBIOMICS™ DNA/RNA Miniprep Kit, Wizard Genomic DNA Purification Kit (Promega), iodine, alcohol, distilled water, Marine Agar and Sodium Broth powder, red gel, loading dye, KAPA Ladder, and agarose powder [15-20].

Acropora digitifera coral samples were taken in Rancabuaya Coastal waters using a hammer tool. *Acropora digitifera* coral samples were taken from 4 meters. Coral samples were taken of approximately 10 cm and put in plastic bottles containing 70% ethanol then stored in cool boxes for temporary storage and taken to the laboratory.

2. 1. Coral Network Isolation

The prepared samples are then sprayed using a sterile waterpipe, the result of which is fragments that descend together with a sterile seawater spray that is accommodated in a beaker glass. Then, the results of the Waterpic spray were placed in a 1.5 mL microcentrifuge tube and centrifuged using a speed of 16,000 x g within 2 minutes, the supernatant from the centrifugation results was discarded and the pellet was allowed to settle in the tube. This is done until the liquid waterpipe results run out. The centrifugation pellets were extracted using ZymoBIOMICS™ DNA/RNA Miniprep Kit.

2. 2. Isolation of Bacterial DNA Metagenome in Coral Tissues

Pellets from Waterpic coral tissue samples were extracted using ZymoBIOMICS™ DNA/RNA Miniprep Kit. The first thing to do is to put 250 mg into the ZR BashingBead™ Lysis Tube (0.1 & 0.5 mm) and add 750 µl of DNA/RNA Shield™.

The tube is then homogenized using vortex for 30 minutes, then centrifuged for 1 minute at a speed of 13,000 x g until two layers (pellets and supernatants) form on the tube. As much as 400 µl of the supernatant fluid formed was transferred into a new microcentrifuge tube. DNA/RNA Lysis Buffer was added by 1 volume (400 µl) into the microcentrifuge tube in the third step, homogeneous by means of a mix well.

The solution in step four is moved into the Spin-Away™ Filter (yellow) which is placed in the collection tube, centrifuged for 30 seconds at a speed of 13,000 x g. The Spin-Away™ Filter (yellow) is transferred into the collection tube. 400 µl of DNA/RNA Prep Buffer was added to the Spin-Away™ Filter (yellow) column and centrifuged for 30 seconds at a speed of 13,000 x g.

The liquid under the collection tube is discarded. 700 µl DNA / RNA Wash Buffer was added to the Spin-Away™ Filter (yellow) column and centrifuged for 30 seconds at a speed of 13,000 x g, the liquid under the collection tube was removed. 400 µl of DNA / RNA Wash Buffer was added to the Spin-Away™ Filter (yellow) column and centrifuged for 2 minutes at a speed of 13,000 × g. Be careful when moving the Spin-Away™ Filter (yellow) into a sterile microcentrifuge tube. 100 µl Dnase / Rnase-Free Water was added to the Spin-Away™ Filter (yellow) column, then allowed to stand for 5 minutes and centrifuged for 30 seconds at a speed of 13,000 x g to obtain DNA and RNA elution from each column.

Zymo-Spin™ III-HRC Filter is placed into a new collection tube and added 600µl ZymoBIOMICS™ HRC Prep Solution, centrifuged for 3 minutes at a speed of 8,000 x g. DNA & RNA elution (step 6) was transferred into the Zymo-SpinTM III HRC filter which was placed in a new microcentrifuge tube and centrifuged for 3 minutes at a speed of 16,000 x g. DNA & RNA elution can be used for the next step or stored in a refrigerator at -20 ºC.

2. 3. NGS amplicon sequencing

The bacterial genome DNA was then sent to Novogene in Singapore for sequencing using the Next Generation Sequencing method as well as bioinformatics analysis. The data used are QCstat, statistical analysis of annotation, GraPhlAn display, and Krona Display.

2. 4. Isolation and Identification of Bacteria

The sample is pulverized using mortar, then taken as much as 1gram to be made 10 times dilution in a test tube containing 9ml of sterile seawater. Bacterial isolation was carried out using the spread plate method. Bacterial isolation took 100 µl at 5, 7 and 9 dilutions and spread on Zobell Marine Agar media. The cup contains suspense that has been flat incubated for 24 hours.

After 24 hours, the growing bacteria were observed for morphology, such as observation of the shape, color, and elevation of each bacterium to then be purified by the scratch method. The results of purification were taken 1 colony with an ose needle flattened on a glass object assisted by physiological NaCl and allowed to stand and pass on fire. 1 drop of gentian violet drops, left for 20 seconds, and washed with distilled water. 1 drop of iodine solution, let stand for 1 minute, and washed with alcohol then distilled water. 1 drop of safranin or fuchsin water, leave for 20 seconds and washed with distilled water. Dried and then observed on a microscope with a magnification of 100x objective lens.

2. 5. Molecular Identification of Bacteria

The bacterial genome DNA was isolated using the Wizard Genomic DNA Purification Kit (Promega). After bacterial DNA is obtained, then PCR amplification is performed. The primers used for PCR 16s rDNA are in accordance with Lane (1991), namely universal primers 27F and 1492R.

The PCR programs used were: initial denaturation stage (95 ºC for 3 minutes), denaturation (95 ºC for 45 seconds), annealing (52 ºC for 1 minute), elongation (72 ºC for 2 minutes) and final elongation. The PCR product mix includes GoTaq®Green Master Mix Promega (12.5 µL), primers 27 F (1.25 µL), primers 1492 R (1.25 µL), DNA templates (2 µL) and Nuclease free water (8 µL) so that the total PCR product is 25 µL. Then sanger sequencing was conducted at the 1st Base in Singapore. The FASTA obtained from the sequencing is then processed using the BioEdit™ software which is then carried out by the BLAST process on the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov). Homology BLAST results are deposited to GenBank to obtain an access number.

3. RESULTS AND DISCUSSION

Based on the NGS (Next Generation Sequencing) conducted, from the bioinformatics analysis it was found that the total data reading of 70275 with data that passed QC (Quality Control) was 65659, the sequencing results had a high effectiveness value of 93.43%. The data also obtained bases as many as 27506261 nt with an average number of 418, for the percent base value of cytosine and guanine nucleotides that is equal to 53.15. Dominated by 4 phylum 10 class 10 order 10 family 10 genus and 77 species and 6% unidentified reads. 4 phylum include phylum Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria.

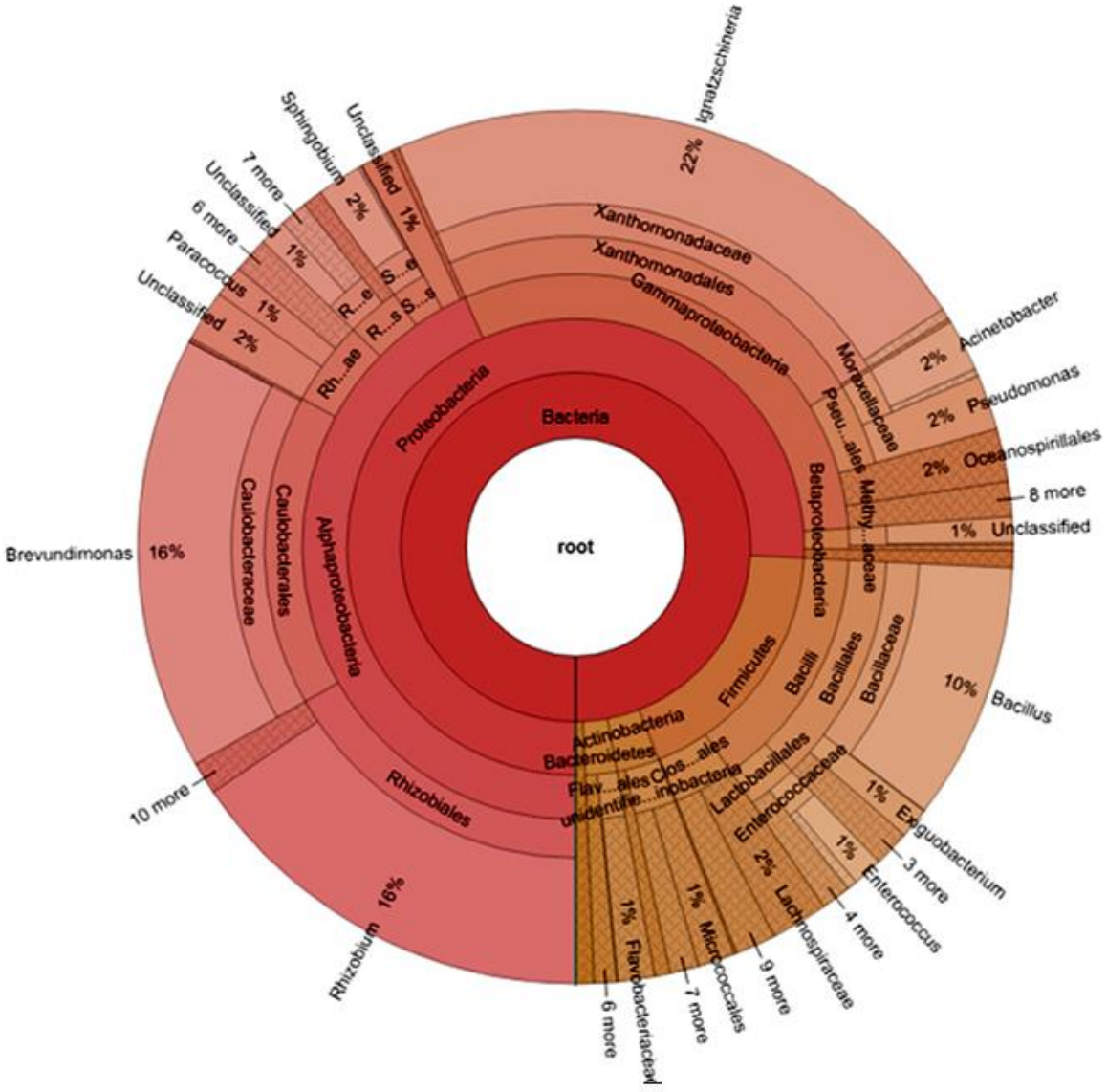

Fig. 2. Diagram of the abundance of bacteria of the *Acropora digitifera* Association

Whereas at the class level, the data obtained are dominantly found 10 classes, including Alphaproteobacteria, Gammaproteobacteria, Bacilli, Clostridia, Unidentified actinobacteria, Flavobacteriia, Betaproteobacteria, Acidimicrobiia, Deltaproteobacteria, Sphingobacteriia, and other classes. The data can be presented in the cona diagram (Fig. 1).

The results of bacterial isolation were cultured using Zobell Marine as for growth media at pH 5, 7, and 9. Bacteria grew at pH 7 and 9 only. Meanwhile, the results from isolation to the identification of bacterial cell types are presented in Table 1.

When in the same pH condition, the amount of carboxylic acid group which had no reaction with calcium ions increased for the effect of chitosan, which caused the increase of swelling ratio. In addition, the interaction between amino groups of chitosan and carboxylic acid groups of alginates is mainly electrostatic force in a lower pH while it is mainly hydrogen

bond at a higher pH. When the content of chitosan is 1.0% or 1.5%, the interaction of the NH3þ between chitosan molecules was weakened due to the increase of pH, which caused the decrease of swelling ratio.

As for the hydrogel containing no chitosan or 0.5% chitosan, the swelling ratio at pH 9 is lower than at pH 6 and pH 12. It may because a part of -COOH got a second reaction with Ca2þ contained in hydrogel, which didn't react with -COO- before. The cross-linking reaction between -COO- and Ca2þ is very fast, which may cause some -COO- cannot react with Ca2þ for the existing of β-D-mannuronic acid (M) on alginate (shown in Fig. 2). When at pH 9, a moderate swelling of hydrogel prompted the movement of Ca2þ and then the reaction between -COO- and Ca2þ decrease the swelling ratio of hydrogel [20-27].

Table 1. Morphology of Bacterial Isolates.

No.	Isolat Name	Elevation	Margin	Whole Colony	Color Colony	Cell Shape	Gram Bacteria
1.	ACD.P4.PH7.P	Raised	Smooth, entire	Round	White	*Streptobasil*	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	*Streptobasil*	Negative

Table 2. Results of Identification of Bacterial 16S rRNA Genes with the BLAST Program.

No.	Isolates Code	Species	*Query Cover*	E value	*Identity* (%)	*Accession 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

The cover query value in the three samples ranges from 99-100%. The query coverage value is a value that indicates the percentage of nucleotide lengths that are aligned with the database in BLAST. According to (Miller et al. 1990) the higher the value of query coverage, the sequence of the data has the same base length as the data in Genbank so that this makes it easier when analyzed.

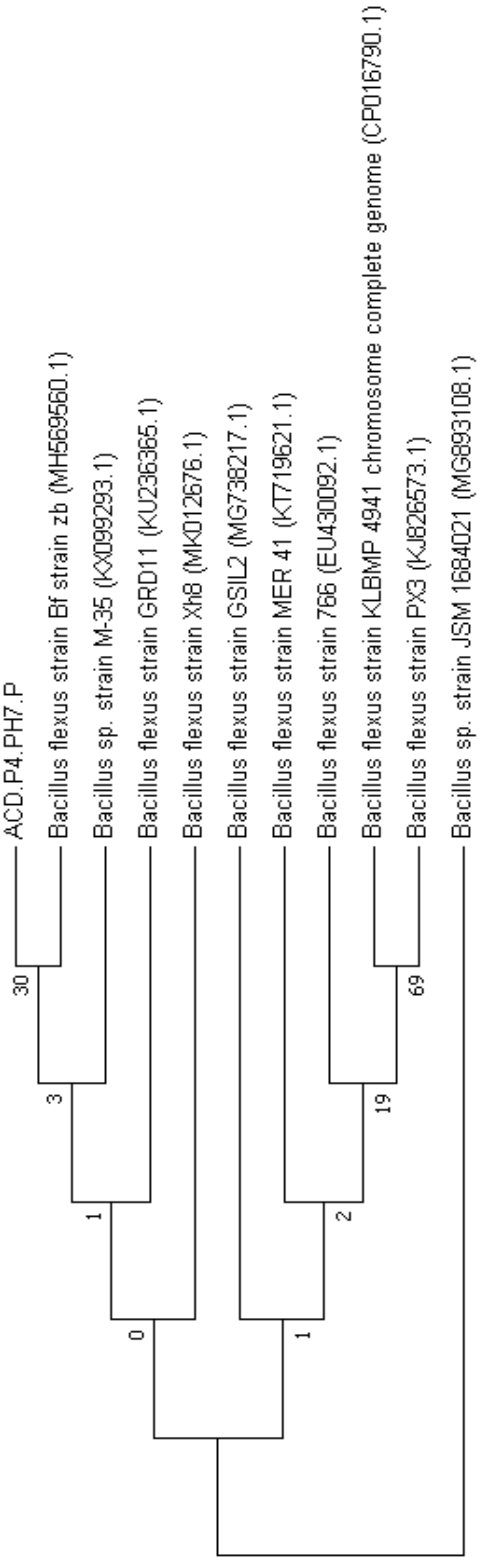

Fig. 3. Phylogeny Trees of ACD.P4.PH7.P Bacteria Isolate Based on 16S rRNA Sequence Neighbor - Joining Method, Bootstrap 1000 times

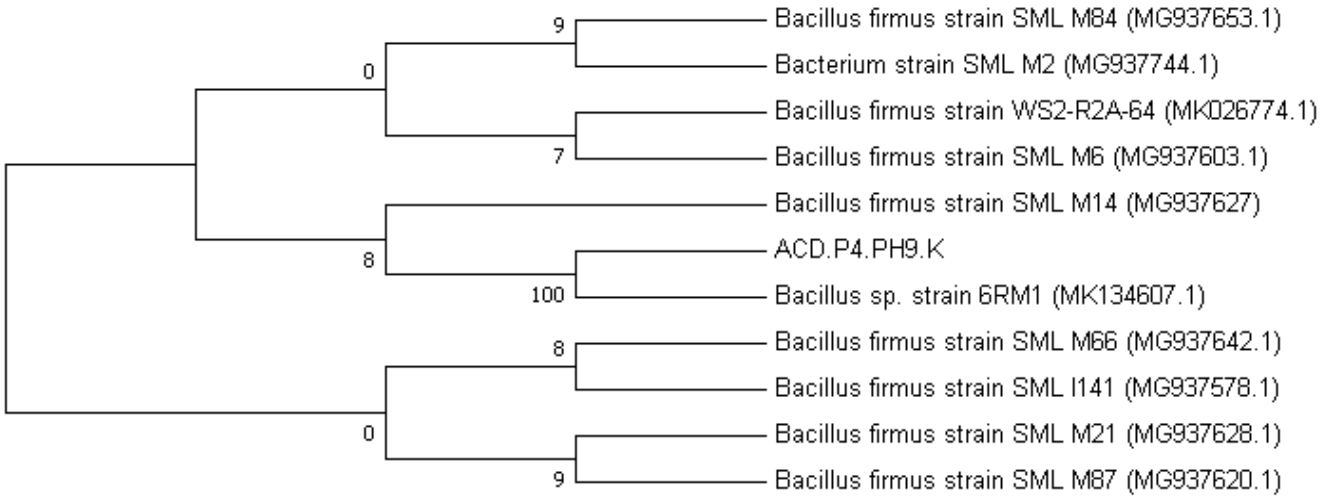

Fig. 4. Phylogeny Trees of ACD.P4.PH9.P Bacteria Isolate Based on 16S rRNA Sequence Neighbor - Joining Method, Bootstrap 1000 times

This is complemented by the statement of [28-33] that the value of query coverage above 80% is quite high. With a query cover value obtained of 99-100%, this shows that the sample has the same base length as the one in GenBank. The identity values of the three samples ranged from 85 - 98% with samples that found that the identity values were found in the ACD.P4.PH7.P sample with the acquisition of 85.44% and the highest identity value was in the ACD.P4.PH9.P sample ie amounted to 98.50%.

Identity values above 97% indicate that the species is the same species as those in Genbank and is not a new species [34-38] as shown in the ACD.P4.PH9.P sample which found an identity value of 98.50% with the species Bacillus sp.

As for the ACD.P4.PH9.K sample, the identity value is below 97%, which shows that the species obtained in the sample are new species but are in the same genus, Bacillus. This is supported by [35-38] that 16S rRNA gene sequence data that has an identity value ≤ 97% can be stated that the isolates are in the same genus. While the identity value between 89 - 93% shows a different family. Whereas in the ACD.P4.PH7.P sample with an identity value of 85.44%.

The results of phylogenetic tree reconstruction in the ACD.P4.PH7.P sequence showed a kinship with the species of Bacillus flexus strain Bf strain zb (Acc. No. MH569560.1). From the phylogenetic tree construction obtained, it can be seen that the ACD.P4.PH7.P sequence has a bootstrap value of 30% and an identity of 85.44%. This means that from 1000 times the phylogeny tree reconstruction, the sequence of ACD.P4.PH7.P has a relationship of 30% with Bacillus flexus strain Bf strain zb (Acc. No. MH569560.1).

Based on Figure 3, the sequence ACD.P4.PH9.P has a close kinship 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 an identity value of 98.50. This means that from 1000 times the reconstruction of phylogeny trees, the sequence ACD.P4.PH9.P has a kinship of 55% with Bacillus sp. c234 (Acc. No. FJ950647.1).

Meanwhile, the ACD.P4.PH9.K sequence has a very close kinship with the species Bacillus sp. strain 6RM1 (Acc. No. MK134607.1) The bootstrap value obtained is 100% with an identity value of 94.78%. This means that from 1000 times the phylogeny tree reconstruction, the sequence ACD.P4.PH9.K has a 100% kinship with Bacillus sp. strain 6RM1 (Acc. No. MK134607.1).

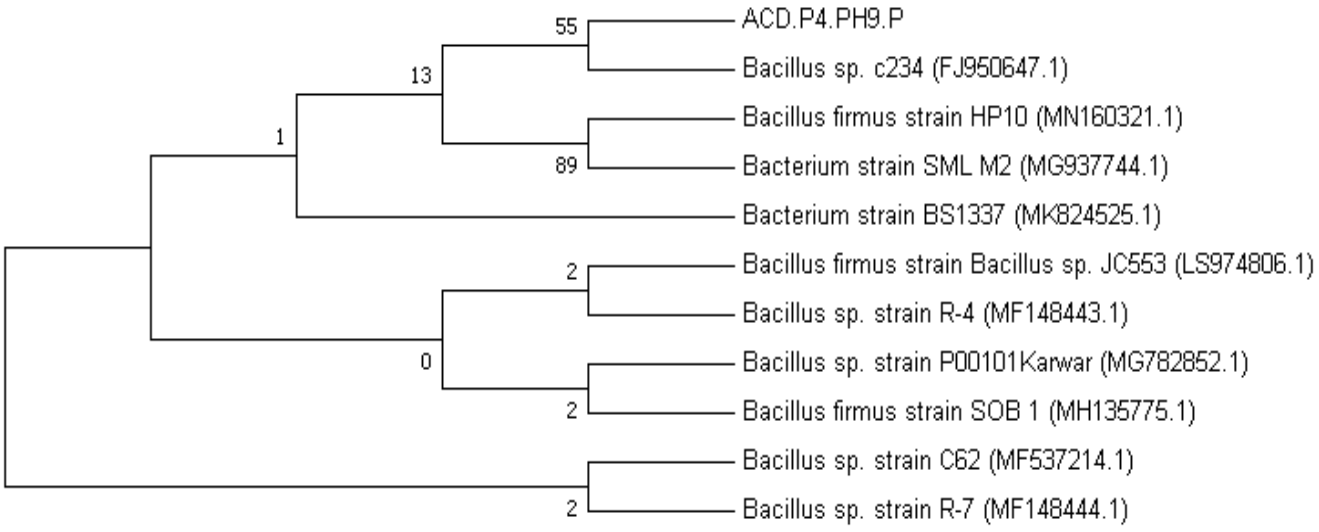

Fig. 5. Bacterial Trees of ACD.P4.PH9.K 16S rRNA-Based Bacteria Isolates for the Neighboring - Joining Method, Bootstrap 1000times

With this study, the preliminary bacterial profiling of different coral genera each showing resistance and susceptibility has been made out. This study lays the foundation for the bacterial profile of Rancabuaya corals. Assumed that the phylogenetic evolution of bacteria showing the phylogenies of bacterial taxa evolved out as the taxonomic level segmentation phylum to species.

4. CONCLUSIONS

Based on the metagenomic approach, dominance obtained 4 phylum 10 class 10 order 10 family 10 genus and 77 species and unidentified reads of 6%. Bacteria from the phylum level that found the highest abundance value were Phylobacteria bacterium at 76%, while from the class level which had the highest abundance value, Alphaproteobacteria with a value of 43.35%.

Based on the culture approach, the ACD.P4.PH7.P isolate was found to be close to the Bacillus flexus strain BF strain zb strain (acc number MH569560.1) with a similarity of 85.44%. Isolate ACD.P4.PH9.P has a close relationship with Bacillus sp. c234 (acc number FJ950647.1) with a similarity of 98.50%. Isolate ACD.P4.PH9.K has an approach with the species Bacillus sp. strain 6RM1 (acc number MK134607.1) with a 94.78% similarity. The bacteria produced in the culture method are also found in the metagenome approach, that is, of the genus Bacillus.

Acknowledgment

The authors wish to thank Faculty of Fisheries and Marine Science, Universitas Padjadjaran, which funded the study through Research Grant program.

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