

## Molecular Identification of Black Band Disease on *Pachyseris* sp in Spermonde Archipelago

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**Abstract** : This study aimed to identify species of isolated bacterial with the nitrogen base sequence analysis method on nucleotide constituents 16S rDNA genes of bacterial disease-causing Black band disease. Identification was done by isolated the bacteria, based on the appearance of the morphology of the colony, five isolates bacterias isolated from healthy coral tissue infected with BBD (C, j, L and F) and four isolates bacterias found in the coral tissue associated with BBD (N, X, Z and H). The bacterial DNA was extracted and amplified on fragments of its 16S rDNA genes using Polymerase Chain Reaction instrument. Fragments of sequences of nucleotides on the nitrogen bases constituent fragments of the gene was amplified and analyzed and to be known as its homology with nitrogen bases gene fragment sequences 16S rDNA while other origin bacteria have been originated in BankGen by using a BLAST-N program. Local alignment analysis of Samples C, j, L and F of the Gen bank data base, overall sample C had a similarity with the *Halomonas* sp, the j sample has a similarity with *Pseudoalteromonas* sp, sample L has the highest similarity with *Thiobacillus denitrifican*, samples of F has similarities to score 100% identity with *Psychromonas ingrahamii*. The coral network associated with BBD i.e. N, X, Z and h. The results of the analysis of the local alignment (local alignment) (BLASTn) shows that the sample N can be identified as *Shewanella* sp, samples of X has a similarity with *Bacillus farraquinis*, samples of Z has a similarity with *flavobacterium* and sample H has a similarity with highest *Desulfovibrio salexigen*.

**Keywords** : Black Band Disease, Bacteria, 16 S rDNA, *Pachyseris* sp.

### Introduction

Black band coral disease was reported almost 40 years ago by Anthony<sup>[2]</sup>. This disease is a kind of disease which infects coral reef network attacking malignant and spread globally at various coral species<sup>[21]</sup>, thus becoming one of coral diseases which have an important role because it can spread among colonies of corals and destroy their network and at the end will cause death in coral<sup>[18]</sup>. The microscopic observations showed the kind of diversity in the colonies of bacteria that are able to infect the network<sup>[3][19][18]</sup>, Black band disease believed to be caused by a microbial consortium<sup>[8]</sup>, but the cause of the disease agent had known BBD for sure<sup>[20]</sup>. Identification of the pathogenic bacteria cause BBD has been widely researched through the identification of molecular and microbiology<sup>[12][21][7][11][23][24][4][22]</sup> but none that can prove definitively.

Characteristics of the Black band (5-30 mm wide) that moved across the surface of the coral colonies, killed living tissue and leaving white dead network framework. Dead coral skeletons were covered by green algae. The disease can reach up to several centimeters a week (3 mm day<sup>-1</sup> cm<sup>-1</sup>) so that it can threaten coral communities<sup>[3][6][14]</sup>. Infection of this disease will be increasing in the warm water conditions but will disappear during the winter<sup>[21]</sup>.

Research on coral diseases have not been a lot of attention particularly in Indonesia. Haapkylä<sup>[13]</sup> reported the prevalence of diseases of coral in Wakatobi National Park. Some types of bacteria are also found by Massinai<sup>[16]</sup> are obtained from the results of isolation on a coral rock in the Spermonde archipelago based on phenotypic analysis methods include cell morphology, gram staining, test the oxidation and fermentation. Nevertheless, identification of bacteria based on phenotypic characters have a major weakness, namely, more over error occurred in the distinction of species and strains of bacteria. The error due to presence of phenotypic characters of unusual bacteria. Ochman<sup>[17]</sup> told that bacterial phenotypic narration suggests un static and could be changed along with the change of the organism and the environment to cause evolution.

Lack of identification of bacteria through phenotypic analysis encouraged the identification of bacteria by other methods are more accurate. The recommended method of identification is the analysis of genotype bacteria through the reading of the base sequence of nucleotides on the framers of the nitrogen fragment of 16S rDNA genes of bacteria. Based on several considerations the method rated better than the phenotypic analysis method. The first consideration is gene 16S rDNA gene from almost all bacterial species have been determined by the sequence of nitrogen based so it can be used as a guideline if it finds new species<sup>[1]</sup>. The second consideration is the sequence of the 16SrDNA gene nitrogen based have lower intra-specific diversity in comparison with other protein gene code, as well as the nature of the fragment of 16S rDNA more sustainably.

In this research, we carried out the identification of isolated bacteria species with nitrogen bases sequence analysis method on the 16S rDNA nucleotide constituents. Identification was done by performing the isolation of bacteria, disease-causing DNA extraction of Black band disease and subsequent amplified of gene fragment of 16S rDNA using Polymerase Chain Reaction, instrument. Fragments of the gene was amplified and analyzed by sequences of bases of nitrogen to homology of nitrogen bases with sequencing the gene fragment of 16S rDNA and other bacteria that have been origin in Genbank. Thus, the necessary validation and development of tests to detect pathogenic bacteria cause disease on the reef so that it can be used as information for the control of coral health problems.

## Materials and Methods

### Sample Collection

The bacteria isolated from the infected coral disease BBD in the waters of Barrang Lompo, Badi, Bonetambung, Sarappo, Kapoposang, and the Spermonde islands, South Sulawesi starting from July 2013 to March 2014. The identification of coral diseases and signs of the disease were conducted at a depth of 1-6 m in coral reefs. All the colonies showed signs of the coral disease photographed using digital camera Nikon Coolpix P7100.

### Bacteria Isolation and Media Collection

The sample for the isolation and identification of bacteria from corals infected with black band disease were collected from the infected coral disease (n = 3). Coral samples were collected of healthy coral and coral samples infected with BBD, coral samples surface Further cleaned with sterile sea water and then inserted into the media Brain Heart Infusion Broth BHIB (Brain Heart extract 17.5 g, Pepton 10 g Glucose 2 g, Sodium chloride 5 g and Disodium hydrogen phosphate 2.5 g in 1000 ml aqua) as many as 10 ml. Samples are subsequently incorporated into the coolbox for further analyzed in the laboratory of Microbiology, Faculty of medicine and Molecular Identification in doing in the laboratory Microbiology Leibniz-Zentrum für Marine Tropical Ekologie (ZMT) Bremen, Germany. Coral bacteria-infected taken as much as 1 g then crushed in BHIB media. The grazing results of the liquid taken as much as 0.1 ml and spread into a petri dish containing agar medium, sucrosa salt agar /SSA and selective medium thiosulfate citrate bile used salt sucrose agar (TCBSA). Bacteria that grow in culture would be repeated so that it brings pure cultures of bacteria DNA extraction was performed for further.

## DNA Extraction

Bacteria grown obtained in liquid media. Cultures incubated in a shaker water bath at a temperature of 28-29 °C, 160 rpm for 24 hours. Bacterial cells were harvested by taking the suspension 1.5 ml of bacterial culture and then put in the eppendorf and centrifuged at speeds of 6000 rpm for 5 minutes, then supernatant was thrown. This step was repeated three times. DNA extraction was carried out with the method Boom's<sup>[5]</sup>, quantity and purity of DNA measured using Biophotometer plus (Cuvette µ G 1.0).

## Amplification of 16S rDNA genes with PCR

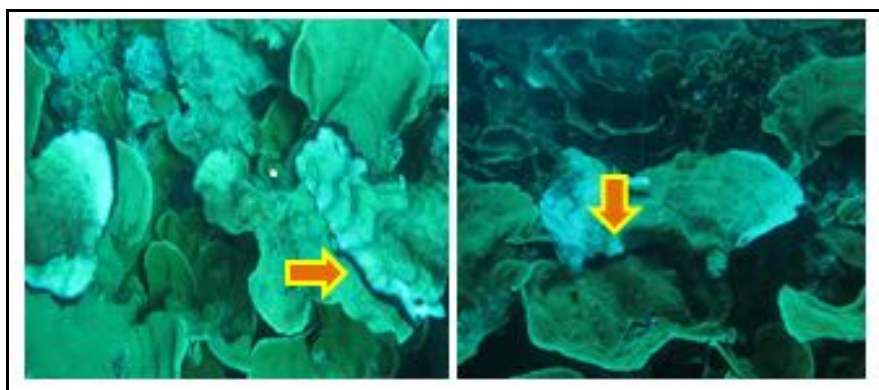
The primary use was universal primer for the domain bacteria was either a forward primer 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and reverse primer 907R (5'-CCG TCA ATT CCT TTR AGT TT-3')<sup>[15]</sup> with a target of 50 and 1000 bp. The composition of the PCR 25 µL consisted of 12.5 µL of PCR Master Mix, 1 µM Forward and Reverse Primer 0.5 µL, 5-24 ng DNA template, and the Nuclease-Free water (NFW) were added to the volume of 25 µL. All the components of the reaction were mixed into the microtube and incorporated into the PCR machine. PCR consisted of two phases, namely 1) pre-denaturation 95°C for 5 minutes; denaturation stage 95°C 30 cycles a minute; stages of annealing 55°C 1 minute, a stage extension 72°C for 3 minutes and the final extension 72°C for 10 minutes. PCR results were stored at a temperature of -20°C or electrophoresis. PCR products were analyzed through agarose gel electrophoresis using DNA Staining Serva G No. 39804.05, at the time of electrophoresis was given a marker or the marker DNA molecule (GeneRuler™ 100bp Plus DNA Ladder). Electrophoresis performed on conditions of 121 volts and 110mA for 45 minutes. Visualization of DNA using UV transilluminator.

## Sequencing and DNA Sequence Analysis

Amplified of 16S rDNA was purified using the QIAquick PCR Purification kit (Qiagen) and sequenced using an ABI PRISM 3130 XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequences were submitted to BLAST at NCBI (National Center for Biotechnology Information) to determine percentage similarity to other 16S rDNA sequences.

## Results and Discussion

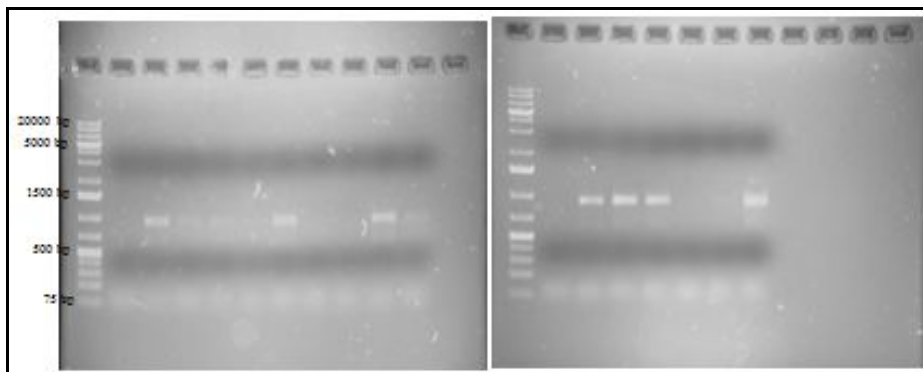
Coral bacteria associated with the disease BBD is seen in Figure 1. From the picture described that black tape showed attack disease of BBD on coral tissue on *Pachyseris* sp<sup>[19]</sup> reported that the BBD characterized by the presence of black-coloured Ribbon with a width of between 0.1-1 cm that clearly separated the living coral tissue and skeleton.



**Figure 1. *Pachyseris* sp Black Band Disease infected in the Spermonde Archipelago (source: private collection)**

Based on the appearance of the morphology of infected coral obtained, five isolated bacteria from healthy coral tissue infected with BBD (C, F, L, N and O) and four isolated bacteria found in the coral tissue associated with BBD (N, X, Z and H) performed PCR with Universal Primer.

DNA amplification results could be seen in Figure 2. The picture showed that all isolates yielded single bands (single tape) with a size of approximately 1050 bp corresponded to a comparison using DNA marker. The magnitude of this size according to the size of the expected from the 16S rDNA genes bacterium that is 50-1500 bp.



**Figure 2. Results of Amplified DNA**

Amplification of DNA from isolated bacteria on *Pachyseris* sp single tape that have obtained indicated that the primary use was specific to primary of the amplified 16S rDNA gene in bacteria. Sequencing was one way to identify a gene. Identity of the genes that have been known to sequenced could be determined by comparing the sequence with data contained in the Gene Banks. As for results of healthy coral samples sequence C, j, L, and F aligned (alignment) with an existing sequence in the Genbank using program N-BLAST (basic local alignment search tool-nucleotide). Local alignment analysis performed against a sample C of Isolates that were in the Genbank data base. But overall the sample C has a similarity with the *Halomonas* sp, high sample identity in common C with sequence of *Halomonas* sp that is present in Genbank data base so that samples of C could be identified as *Halomonas* sp. Results of the analysis of the local alignment (local alignment) (BLASTn) showed that the sample j have in common with an identity score 99-100% with other countries. Local alignment analysis performed against the sample j of Isolates that were in the Genbank data base. But overall, the sample j has a similarity with *Pseudoalteromonas* sp. in common a high identity sample j with *Enterococcus* sp sequence which exists in the Genbank data base as well as Query cover 99-100% and the value of e-value of 0 so the sample j could be identified as *Pseudoalteromonas* sp. Analysis of local alignment (local alignment) (BLASTn) showed that the sample L have in common with the identity score 97-100% with other countries. Local alignment analysis of a sample of existing Isolates of L in the Genbank data base. From the results of analysis of samples L has similarities to the football scores and identity 97-100% with *Thiobacillus denitrificans*, however results from Alignment indicated that the results of the sample sequence similarity L has the highest with *Thiobacillus denitrificans* and has 100% similarity with *Thiobacillus denitrificans* (NR 025358.1). Analysis of local alignment (local alignment) (BLASTn) showed that the sample F have in common with the identity score 100% *Psychromonas ingrahamii* with. Local alignment analysis of a sample of existing Isolates from F in the Genbank data base. From the results of the analysis of sample F has similarities to score 100% identity with *Psychromonas ingrahamii*. So that the sample could be identified as *Psychromonas ingrahamii*.

For the infected coral BBD samples i.e. N, X, Z and h. The results of the analysis of the local alignment (local alignment) (BLASTn) showed that the sample N have in common with the identity score 90-100% with other countries. Local alignment analysis of Samples N from Isolates that were in the Gen bank data base. But overall the sample N has similarities with *Shewanella* sp. in common a high identity with a sequence of N samples *Shewanella* sp which exists in the Genbank data base Query and cover 100% and the value of e-value of 0 so that the sample can be identified as N *Shewanella* sp. The results of the analysis of the local alignment (local alignment) (BLASTn) showed that the sample X has similarities with the identity score 93-100% with other countries. Local alignment analysis performed against a sample X of Isolates that were in the Genbank data base. But overall the sample X has similarities with *Bacillus farraquinis*. High similarity identity sample sequence X with *Bacillus farraquinis* that exist in the Genbank data base as well as Query cover 91 – 100% and the value of e-value of 0 so that a sample of n could be identified as *Bacillus farraquinis*. In addition the sample X showed 100% and 100% and cover query e-value 0 with *Bacillus farraquinis* (v. 443034.1). The

results of the analysis of the local alignment (local alignment) (BLASTn) showed that the sample Z have in common with an identity score of 95-99% with other countries. Local alignment analysis of samples of Z from Isolates that were in the Genbank data base. But overall the sample Z has similarities to flavobacterium. Similarity Z samples a high identity with sequence flavobacterium that is in the Genbank data base Query and cover 85 – 100% and the value of e-value of 0 so that samples of Z can be identified as flavobacterium. Sample Z 100% has similarities, cover 100% query as well as the e-value 0 with flavobacterium (023660.1 AB). The results of the analysis of the sample H has similarities to the score 88-98% identity with the *Desulfovibrio salexigens* but from alignment results showed that the results of the sample sequence H has similarities with highest *Desulfovibrio salexigens* and has 100% similarity with *Desulfovibrio salexigens* (M 34401.1).

This result contrasted with the results of research conducted by<sup>[11]</sup> that found healthy corals surface community (brain-massive coral) was dominated by green sulfur bacteria,  $\alpha$ -proteobacter, planctomycetales and firmicutes. This discrepancy was suspected because of the difference in a host of different species of coral. Further reported by Frias-Lopez<sup>[11]</sup> that every species of coral have different microbial diversity. Based on the progress of the molecular identification techniques, which are the Molecular characterization of the conduct on the Community Association-causing bacteria which infects coral BBD <sup>[7][11][23]</sup>. Microbial communities found BBD who dominated the heterotrophic bacteria<sup>[12][7]</sup>, sulfide-oxidizing bacteria of the genus *Beggiatoa*<sup>[9]</sup> and the sulfate-reducing bacteria are corected from some of the genus *Desulfovibrio*<sup>[24]</sup>.

## Conclusion

The *Halomonas* sp bacterium strain C, *Pseudoalteromonas* sp strain j, *Thiobacillus denitrificans* strain L and *Psychromonas ingrahamii* strain F were bacteria associated with healthy corals in the Spermonde Archipelago on *Pachyseris* sp. As for samples of the infected coral BBD applied anxiety N could be identified as *Shewanella* sp, strain X with *Bacillus farraquinis*, bacteria *Flavobacterium* strain Z and *Desulfovibrio salexigens* strain H. The creation of validation would be the development of a test to detect bacterial pathogens cause disease on the reef so that it can be informed as an important factor to the control of health problems.

## References

1. Acinas, S. G., Marcelino. L. A, Klepac-Ceraj. V and M. F. Polz 2004. Divergence and Redundancy of 16S rRNA Sequences in Genomes with Multiple rrn Operons. *Journal of Bacteriology* 186(9): 2629-2635.
2. Antonius, A. 1973. New observations on coral destruction in reefs. In Tenth Meeting of the Association of Island Marine Laboratories of the Caribbean. University of Puerto Rico (Mayaguez): 3.
3. Antonius, A. 1981. the 'band' disease in coral reefs. *Proc 6th Int Coral reef Symp* 2: 7-14.
4. Barneah, O., E. Ben-Dov, E. Kramarsky-Winter and A. Kushmaro 2007. Characterization of black band disease in Red Sea stony corals. *Environmental microbiology*. 98: 1995-2006.
5. Boom, R., C. J. Sol, M. M. Salimans, C. L. Jansen, P. M. Wertheim-van Dillen and J. van der Noordaa 1990. Rapid and simple method for purification of nucleic acids. *Journal of Clinical Microbiology*. 28(3): 495-503.
6. Carlton, R. G. and L. L. Richardson 1995. Oxygen and sulfide dynamics in a horizontally migrating cyanobacterial mat: Black band disease of corals. *FEMS Microbiology Ecology*. 182: 155-162.
7. Cooney, R. P., O. Pantos, M. D. Le Tissier, M. R. Barer, A. G. O'Donnell and J. C. Bythell 2002. Characterization of the bacterial consortium associated with black band disease in coral using molecular microbiological techniques. *Environ Microbiol*. 47: 401-413.
8. Dinsdale, E. A. 2000. Abundance of Black-Band Disease on Coral from on Location on the Great Barrier Reef: a Comparison with Abundance in the Caribbean Region, *Proc 9th Int Coral Reef Symp*, Bali, Indonesia 2 :1239-1243.
9. Ducklow, H. W. and R. Mitchell 1979. Composition of mucus released by coral reef coelentrates. *Limnology Oceanography*. 24(4): 706-714.
10. Frias-Lopez, J., J. S. Klaus, G. T. Bonheyo and B. W. Fouke 2004. Bacterial Community Associated with Black Band Disease in Corals. *Applied and Environmental Microbiology*. 70(10): 5955-5962.
11. Frias-Lopez, J., A. L. Zerkle, G. T. Bonheyo and B. W. Fouke 2002. Partitioning of Bacterial Communities between Seawater and Healthy, Black Band Diseased, and Dead Coral Surfaces. *Applied and Environmental Microbiology*. 68(5): 2214-2228.
12. Garrett, P. and H. Ducklow 1975. Coral Disease in Bermuda. *Nature*. 253: 349-350.

13. Haapkylä, J., A. Seymour, J. Trebilco and D. Smith 2007. Coral disease prevalence and coral health in the Wakatobi Marine Park, south-east Sulawesi, Indonesia. *Journal of the Marine Biological Association of the United Kingdom*. 87(2): 403-414.
14. Kuta, K. G. and L. L. Richardson 1997. Black band disease and the fate of diseased coral colonies in the Florida Keys. . *Proc 8th Int Coral Reef Sym 1* : 575-578.
15. Lane, D. J., Pace, B, Olsen, G .J, Stahl, D. A., Sogin. M .L. and Pace. N. R. 1985. Rapid Determination of 16S ribosomal RNA sequences for Phylogenetic Analyses. *P Natl Acad Sci USA*. 82: 6955-6959.
16. Massinai, A. 2012. Kondisi dan Sebaran Penyakit pada Karang Batu (Stony coral) Di Kepulauan Spermonde. Disertasi. Program Pascasarjana Universitas Hasanuddin. 190hal
17. Ochman, H. 2005. Genomes on The Shrink. *Proc Natl Acad Sci, USA*. 102: 11959-11960.
18. Richardson, L. L. 1998. Coral diseases: what is really known? *Trends in Ecology & Evolution*. 13(11): 438-443.
19. Richardson, L. L., Kuta KG., Schnell S. and C. RG. 1997. Ecology of the black band disease microbial consortium. *Proc. 8th. Intl. Coral Reef Symp*. 1:597-600.
20. Richardson, L. L. and K. G. Kuta 2003. Ecological physiology of the black band disease cyanobacterium *Phormidium corallyticum*. *FEMS microbiology ecology*. 433: 287-298.
21. Rutzler, K. and D. L. Santavy 1983a. The black band disease of Atlantic reef corals. I. Description of the cyanophyte pathogen. *P.S.Z.N.I Mar Ecol*. 4: 301-319.
22. Sato, Y., D. G. Bourne and B. L. Willis 2009. Dynamics of seasonal outbreaks of black band disease in an assemblage of *Montipora* species at Pelorus Island (Great Barrier Reef, Australia). *Proceedings of the Royal Society B: Biological Sciences*. 276(1668): 2795-2803.
23. Sekar R., Mills K, Remily ER, Voss JD and Richardson L. L. 2006. Microbial communities in the surface mucopolysaccharide layer and the black band microbial mat of black band-diseased *Sidestrea siderea*. *Applied and Environmental Microbiology*. 72(9): 5963-5973.
24. Viehman S., Mills DK., Meichel GW. and Richardson. LL. 2006. Culture and identification of *Desulfivibrio* spp from corals infected by black band disease on Dominican and Florida Keys reefs. *Dis Aquat Org*. 69: 119-127.
25. Ici Piter Kulu , Abdul Latief Abadi , Aminudin Afandhi and Nooraidawati; Morphological and Molecular Identification of *Beauveria bassiana* as Entomopathogen Agent from Central Kalimantan Peatland, Indonesia, *International Journal of ChemTech Research*, 2014, Vol.8, No.4, pp 2079-2084,

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