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Evaluation of antibacterial activity and molecular characterization of bacteria from *Holothuria atra* intestine collected from anthropogenic and non-anthropogenic region in Karimunjawa, Indonesia

BAMBANG SULARDIONO♥, NINIEK WIDYORINI, DJOKO SUPRAPTO, DIAH AYUNINGRUM, ARIF RAHMAN

[1]Department of Aquatic Resources, Faculty of Fisheries and Marine Sciences, Universitas Diponegoro. Jl. Prof. H. Soedarto, S.H., Tembalang, Semarang 50275, Central Java, Indonesia. Tel.: +62-24-7474698, Fax.: +62-24-7474698, ♥email: bambangsulardiono@gmail.com

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Abstract. *Sulardiono B, Widyorini N, Suprapto D, Ayuningrum D, Rahman A. 2020. Evaluation of antibacterial activity and molecular characterization of bacteria from* Holothuria atra *intestine collected from anthropogenic and non-anthropogenic region in Karimunjawa, Indonesia. Biodiversitas 21: 3149-3155.* Evaluation of antibacterial activity and molecular characterization of bacteria from the intestine of *Holothuria atra* is needed in the anthropogenic region of Menjangan Besar, Karimunjawa. The research aims to evaluate antibacterial activity and molecular characterization of bacteria from intestine of *Holothuria atra* collected from anthropogenic and non-anthropogenic regions in Karimunjawa, Indonesia. Sea cucumber samples were collected at Menjangan Besar waters as anthropogenic region (code of HM) and Alang-alang waters as non-anthropogenics region (code of HA), Karimunjawa National Park, Indonesia. The *H. atra* sample collection was using purposive sampling method. Examination of the bacteria from isolation until molecular characterisation was done at the Tropical Marine Biotechnology Laboratory, Uiversitas Diponegoro. The isolation process was conducted using spread plate method, followed by bacterial isolates purification using streak plate method. Screening of antibacterial activity using the agar plug method, as well as molecular identification was conducted by 16S rDNA PCR amplification. The results of this study indicated that a total of 26 bacterial strains were successfully isolated from sea cucumber intestine, consisted of eleven bacterial isolates from *H. atra* in Menjangan Besar waters and the rest fifteen isolates from *H. atra* in Alang-alang waters. The preliminary assay of antibacterial test showed *H. atra* in both waters have potential bacterial isolates. Those bacterial isolates with antibacterial activity were HM1.2 and HA1.1, which based on molecular identification, showed isolates HM1.2 and HA1.1 had the closest similarity with *Bacillus paramcoides* and *Vibrio alginolyticus* consecutively, with BLAST homology 98% and 99%. The accession number for both isolates were LC550090 and LC550089, respectively.

Keywords: Antibacterial activity, *Holothuria atra*, Intestine, Karimunjawa

INTRODUCTION

Menjangan Besar waters are included in the Karimunjawa National Park area, where the waters are part of the tourism and cultivation zones (Sulisyati et al. 2018). Because there are a variety of activities that are anthropogenic, so they have an impact on the decline in the aquatic environment. Sulardiono et al. 2018 explained that the environmental status in the waters of Menjangan Besar is oligotrophic, where there is a tendency of low quality or towards eutrophication, so that it will affect the biological characteristics of the microbiological community. On the other hand, the Alang-alang waters is a rehabilitation zone. The area is imposed restrictions on activities, where the activities carried out may not cause damage to the ecosystem that is in these waters, with all its resources.

The presence of sea cucumber associated bacteria provides an opportunity to use these organisms as the main source of new bacteria and sources of bioactive compounds including antimicrobial compounds. In waters that have the potential to pollute the environment have an impact on the increase in the abundance of sedimentary bacteria, thus providing opportunities for entry into the digestive system of sea cucumbers during the feeding process as deposit-feeding. Sea cucumbers obtain food by the ingestion of marine sediment, or by filtration of seawater. Deposit-feeding holothurians are prominent members of many deep benthic communities. The association of bacteria with sea cucumbers plays an important role in various ways, including development, immunity, metabolism, and physiology (McFall-Ngai et al. 2013; Bordenstein and Theis 2015; Theis et al. 2016).

Sea cucumbers *H. atra* live in shallow coastal waters and are widely distributed in Karimunjawa, which has an important role in the food chain system in its environment. According to Dissanayake et al. 2012, *H. atra* has a preference for seagrass habitat, where much is dominated by organic matter, gravel, and substrate, whereas according to Hartati et al. 2019 there is a relationship between habitat and species physiology ecology. Sea cucumber *H. atra* has a fairly strong susceptibility from the influence of the quality of the surrounding environment, especially the effect of infection on the body of sea cucumbers. According to Liu, et al. 2011, bacteria as microbiota in the intestine are known to play an important role in the health status of aquatic animals, which can affect the function of aquatic animals, including biological activities for the development and maintenance of the immune system and defense against pathogens. The structure of intestinal microbiome or intestinal community is strongly influenced by eating or dietary habits. Sea cucumber eating deposits increase the abundance of bacteria and the rate of remineralization of organic matter in coastal sediments, thus providing a mechanism to increase the decomposition of organic matter (MacTavish, et al. 2012). Furthermore, intestinal microbiota is considered an integral component of the host, due to its important role in modulation of the immune system, proliferation of intestinal epithelium and regulation of food energy intake. Understanding these factors, studying the microbial community is very important for applications in these aquatic animals which still require basic investigations.

To date, the study of sea cucumber gut microbiota especially bacteria from the intestine part is still limited. The latest research by Pagán-Jiménez et al. 2019 reported that the gut microbiota in sea cucumber *Holothuria glaberrima* is dominated by bacteria from the phylum Proteobacteria. However, the role of this bacteria remains unclear. Thus, this research aims to isolate the bacteria from intestine of *H. atra,* to evaluate antibacterial activity and molecular characterization of bacteria from intestine of *H. atra* collected from anthropogenic and non-anthropogenic region in Karimunjawa, Indonesia. This potential and its bacterial symbiont characteristics can be used as a database in the management of sea cucumber resources in the waters of Menjangan Besar as a tourism and cultivation zone in Karimunjawa National Park.

MATERIALS AND METHODS

Specimen collection

Holothurians of the species *H. atra* were collected from Menjangan Besar waters as a tourism and cultivation zone (code of HM) and Alang-alang waters as a rehabilitation zone (code of HA), in the Karimunjawa National Park, Indonesia, with skin diving to a depth of around 2-3 m. The total of specimens collected was ten, consists of five *specimens of H. atra* from Menjangan Besar and the rest five specimen collected from Alang-alang. During collection, sea cucumbers were put into sterile ziplock plastic bags and placed in a cool box until examined. Specimen of sea cucumber *H. atra* can be seen in Figure 1.A, while the position of taking bacteria in the intestines of sea cucumbers (Pagán-Jiménez et al. 2019), can be seen in Figure 1.B.

Environment quality observation

Observation of environment quality was conducted including physical and chemical properties; pH, temperature, visibility, current, dissolved oxygen (DO), salinity, and organic material sediment. Those parameters were measured using the proper tools including pH-meter, thermometer, Secchi disk, current ball, DO meter, refractometer, and organic material measurement in sediment test, respectively.

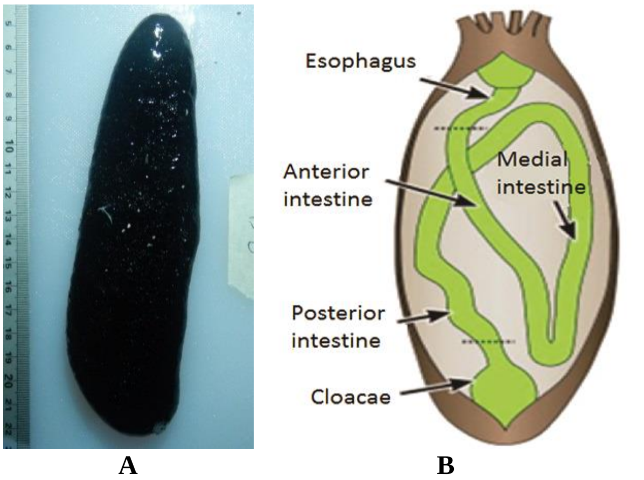

Figure 1. A. Selected *Holothuria atra* samples from Karimunjawa National Park, Indonesia. B. Position of bacterial taking in the intestine of *H. atra* (Source: Pagán-Jiménez et al. 2019)

Isolation and purification of bacteria

Isolation and purification of symbiotic bacteria were carried out at the Tropical Marine Biotechnology Laboratory, Diponegoro University. The isolation process was carried out by the serial dilution method, then the purification process is carried out by the streak plate method (Ayuningrum et al. 2019a). Before the isolation of associated bacteria, the *H. atra* specimens were dissected and the intestine pulled out under sterile condition. The intestines from each specimen were ground and serially diluted in sterile seawater, respectively. The dilution of 10^{-2} and 10^{-3} from each sample was poured on to sterile Zobell 2216E (Himedia, India) agar, incubated at 29±2°C for 3 days. The purification was conducted by picked up each different colony and streaked on to new fresh Zobell 2216E Media.

Screening of antibacterial activity

Screening of antibacterial activity was conducted at the Tropical Marine Biotechnology Laboratory, Diponegoro University using agar plug method (Ayuningrum et al. 2019a). Sea cucumber samples have been collected from Menjangan Besar Karimunjawa waters, and successfully filtered for their antibacterial activity against pathogenic bacteria using overlay methods (Radjasa, et al. 2013). The bacterial isolates from *H. atra* intestine were cultured for 3-4 days in the respected medium from optimal metabolite production. Agar blocks were taken from it and were placed on to NA medium which previously swapped with 0,5 Mc Farland (10^8 CFU/mL) pathogen bacteria (*Escherichia coli* and *Bacillus cereus*). Cultures then incubated at 37°C overnight prior to the incubation in fridge at 4°C for an hour. Antibacterial potential was documented and measured in millimeter (mm) and ± SD.

DNA extraction and amplification of 16S r DNA

The extraction of bacterial DNA was using chelex and saponin method (Miller et al. 1999; Ayuningrum, et al. 2017). The amplification of 16S rDNA was performed using universal primer 27F 5’-AGAGTTTGATCMTGGCTCAG-3 and 1492 R 5’-GGTTACCTTGTTACGACTT-3’ (Weisburg, et al. 1991). PCR reaction with a total of 25 μL was consist of 1μL DNA template, each primer of 2μL, ddH_2O of 10.5 μL and mix PCR (MyTaq^{TM} Red Mix-Bioline) of 12.5mL. Amplification reaction was conducted in a Thermal Cycler (BIO-RAD) T100 using optimization of consist of initial denaturation at 94 °C for 3 minutes, then followed by 30 cycles of denaturation at 94°C for 1 min each cycle, annealing at 55°C for 1 minute, extension at 72°C for 1 minute, and followed by final extension at 72°C for 7 minutes. The examination of PCR products was using gel electrophoresis with agarose 1%, and the result was visualized by using UVIDoc HD5 (UVITEC Cambridge, UK).

BLAST homology and phylogenetic tree

The PCR products from the active bacterial isolates with desired 1500 bp length were sent to 1ST Base DNA Sequencing, Malaysia through the Genetika Science provider. The resulted sequence obtained were processed to create BLAST homology and Phylogenetic analysis according to Ayuningrum et al. 2019b. The analysis of sequencing result as well as alignment and phylogenetic tree was using MEGA 6 and feature neighbor-joining. The result of phylogenetic tree construction was examined using bootstrap method.

RESULTS AND DISCUSSION

Environment and number of bacteria

Both sampling location has different chemical and physical parameter value. The result of environment quality observation was displayed in Table 1.

Based on the results of the analysis of water quality at the study site, according to Minister of the Environment Decree No. 51/2004, classified as suitable, except for organic sediment material. According to Reynold (1971) that the value of suitable organic sediment material content is 3.5-7%, so that the average measurement of organic sediment in the Menjangan Besar and Alang-alang waters (2,975% and 5,309%) is relatively low to medium. As it is known that organic material is an important factor in looking at the level of eutrophication. Eutrophication is the entry of organic matter in water bodies thereby increasing excessive fertility. This is because eutrophication causes excessive changes in chemical and biological structures in the aquatic environment, including aspects of nutrients (N and P) and bacteria.

Organic sediment content in the Menjangan Besar waters is thought to be influenced by anthropogenic activities, namely the supply of organic waste originating from settlement activities, land tourism, marine tourism, and marine aquaculture activities at the study site, then entering the aquatic environment to the bottom. The distribution of organic waste is also influenced by currents and tides. Likewise, what happens in Alang-alang waters, even though they are included in the rehabilitation zone (non-anthropogenic), but organic waste originating from settlements and hotels on the coast can also affect the quantity of waste entering the waters, which can affect the material organic in the waters, which are further influenced by currents and tides. According to the results of the study, the results of measurements of organic material content in Menjangan Besar waters are lower than Alang-alang waters. The results of analysis of variance between organic material content in Menjangan Besar waters and Alang-alang waters obtained P-value = 0.007108 < 0.05 (at 95% confidence level). This significant difference in organic material content is thought to be one of the factors that give a difference in the number of bacteria in the two study sites (Menjangan Besar as a zone that has anthropogenic effects and Alang-alang waters as a zone that has a non-anthropogenic effect).

Table 1. Aquatic and sediment quality in anthropogenic waters of Menjangan Besar and non-anthropogenic waters of Alang-alang

Station	Repetition	Visibility (cm)	DO (mg/L)	Depth (m)	Temp. (◦C)	Current (m/s)	pH	Salinity (ppt)	Organic material sediment
Anthropogenic waters (Menjangan Besar)	1	Not visible	4.79	4.5	30.10	0.025	7.0	32.02	3.426
	2	Not visible	4.98	3.9	29.50	0.046	6.9	31,54	2.917
	3	Not visible	5.05	3.02	30.75	0.036	6.9	31,85	2.584
	Average		4.94	3.81	30.11	0.028	6.93	31.80	2.975
Non-anthropogenic (Alang-alang)	1	Not visible	5.95	2.50	29.51	0.006	7.7	32.00	5.426
	2	38.03	5.99	2.90	30.02	0.006	7.5	32.01	5.917
	2	70.05	6.12	2.20	29.89	0.007	7.8	31,08	4.584
	Average		6.02	2.53	29.80	0.006	7.6	31.03	5.309

Isolation and characterization of bacteria

In the previous study, Sulardiono et al. (2019) reported that sea cucumber extract had ability to inhibit the growth of *E. coli* and *S. aureus* at concentration 150 mg/mL with the best activity. Sea cucumbers have unique adaptation strategy, one of which is that if sea cucumbers are in a depressed habitat environment, sea cucumbers will secrete bioactive compounds to defend against the attack of pathogenic bacteria in the water environment that enter its body. The role of bacteria for the sea cucumber, especially the producers of bioactive compound, remains unclear. Associated Actinobacteria could be an ecological advantage by providing the host with bioactive compounds for i.e., protection against infection by pathogenic bacteria or protection against predators (Seipke et al. 2012). Pringgenies et al. 2019 reported the finding that gut microbiome from Holothurians had ability to inhibit the growth of multidrug-resistant bacteria (Figure 2).

In this research, the total 26 of bacterial symbiont with different morphology (Table 2) isolated from five specimens of *H. atra* sea cucumber intestine. Those consist of 11 bacterial isolates from *H. atra* from the Menjangan Besar waters and 15 bacterial isolates from *H. atra* in Alang-alang waters as a rehabilitation zone. The comparison of total isolates from *H. atra* collected in different regions can be seen in Figure 3. These results indicate differences in the number of bacterial strains between the two locations. This difference is thought to be due to differences in the use of zones, where in the waters of Menjangan Besar there are more tourist and cultivation activities compared to Alang-alang waters as a rehabilitation zone. Evidently, the effect of the use of zones in the Menjangan Besar waters has an effect on the number of bacterial strains that are lower.

Screening of antagonistic bacteria activity

The production of antimicrobial substances by marine bacteria isolated from sea cucumber different locations in the sea has been known for long time (Sibero et al. 2019). This difference of total bacterial isolates from both regions might be related to the higher concentration of organic compounds in Menjangan Besar as anthropogenic area than in Alang-Alang as non-anthropogenic area. The environmental pressure affects biological activity in producing bioactive compounds as a form of adaptation. Microbial metabolites can play an important role in the chemical defense associated with the host and in forming the structure of microbiome communities. Metabolic and physiological changes experienced in extreme environmental conditions result in the production of structural and functional bioactive compounds (Rizzo and Giudice 2018). Thus, secondary metabolite was being prospected as antibacterial compounds for further antimicrobial development to human. The antibacterial test results showed the difference in the diameter of inhibition (DOI) zone from both locations (Figure 4).

Table 2. The pure isolated bacteria from *Holothuria atra* intestine

Isolate code	Morphological characteristics				
	Color	**Size**	**Shape**	**Margin**	**Elevation**
M1	Clear white	Small	Round	lobate	Convex
M2	Clear white	Pin point	Round	Entire	Convex
M3	Turbid white	Big	Round	Entire	Convex
M4	White	Big	Filament	Filament	Flat
M5	White	Small	Round	Entire	Convex
M6	Yellowish white	Small	Round	Entire	Convex
M7	Turbid white	Big	Filament	Filament	Flat
M8	Clear white	Small	Round	Entire	Convex
M9	White	Small	Round	Entire	Convex
M10	Turbid white	Medium	Round	Entire	Convex
M11	Clear white	Big	Round	Lobate	Convex
A1	Yellowish white	Small	Round	Entire	raised
A2	White	Medium	Round	Entire	Convex
A3	Turbid white	Medium	Round	Entire	Raised
A4	White	Medium	Round	Entire	Raised
A5	Translucent white	Small	Round	Entire	Convex
A6	Yellow	Pint point	Round	Entire	Convex
A7	Yellowish white	Small	Round	Entire	Convex
A8	Turbid white	Big	Round	Entire	Raised
A9	White	Small	Round	Entire	Convex
A10	Turbid White	Medium	Round	Entire	Convex
A11	Translucent White	Big	Round	Entire	Raised
A12	Yellowish White	Pin point	Round	Entire	Convex
A13	White	Medium	Round	Entire	Convex
A14	Clear white	Medium	Round	Entire	Convex
A15	Yellowish white	Small	Irregular	Entire	Raised

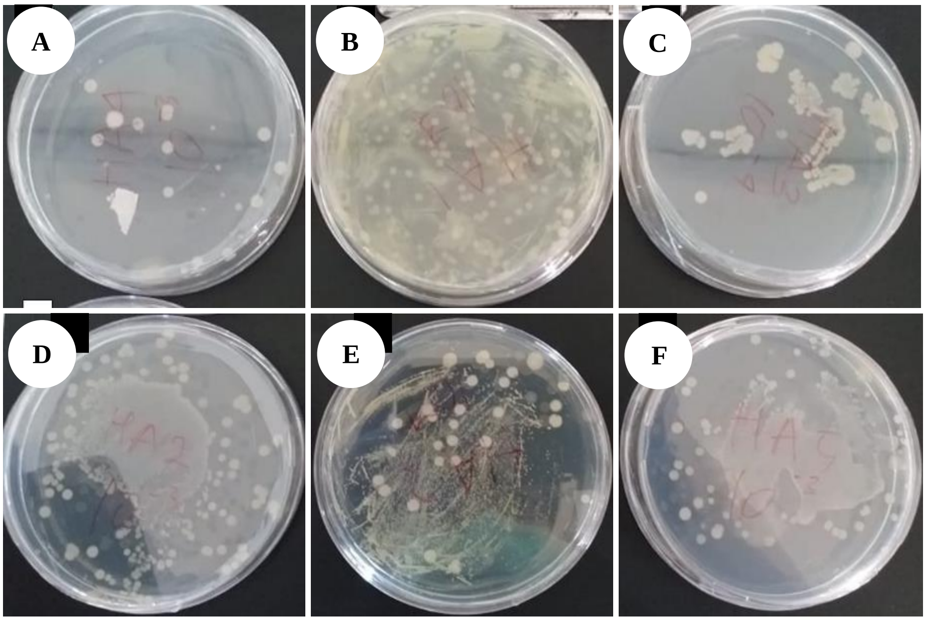

Figure 2. Some of the isolation results of the bacteria from *Holothuria atra* intestine. A-F refer to bacterial isolate A4, A1, A3, A2, A6 and A5 before purification

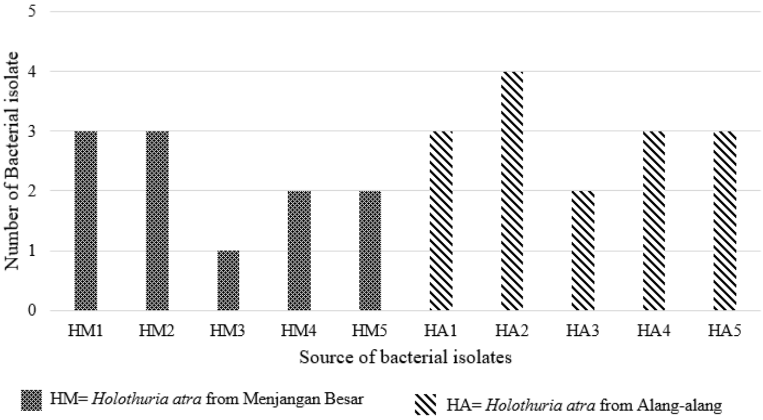

Figure 3. Number of Isolated Bacterial Strain from *Holothuria atra* collected at different regions. HM (*Holothurians* from Menjangan), and HA (Holothurians from Alang-alang)

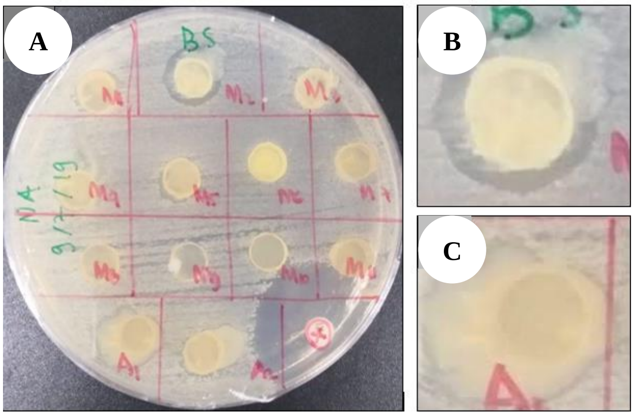

Figure 4. Screening of antagonistic activity from bacterial strain isolated from *H. atra* intestine against pathogenic bacteria. Code M is for bacterial isolates isolated from *H. atra* in Menjangan water, meanwhile, A is for bacterial strains from *H. atra* in Alang-alang water. The zone of inhibition is from HM1.2 with diameter of inhibition (DOI) 8.2±1.5 mm and HA1.1. with DOI 6.3±1.5mm.

The ability of intestine bacteria from *H. atra* to produce antibacterial compounds indicate the presence of probiotic bacteria inside its intestine. That probiotic might be beneficial to protect the life of *H. atra* against harmful bacteria in water environment. Authors opinion that sea cucumber is benthic deposit feeder, so that the presence of waste organic material in their food might contain harmful bacteria that activate the probiotic inside its intestine. Probiotics can produce bacteriocin to fight pathogens that are selective only against some strains of pathogens (Yuniastuti 2014). The size of DOI was slightly different from both zone, indicated the different kinds of bioactive compounds or different amounts of bioactive compounds. However, this still needs further study.

Amplification of partial 16S rDNA and the phylogenetic tree

PCR-based protocols mark the ribosomal RNA gene subunit (rDNA) for study purposes in microbial taxonomy, phylogenetics, evolution, and ecology (dos Santos, et al. 2019). 16S rDNA was used as the marker for molecular identification bacteria. The PCR product was visualized and resulted in the band with approximately 1500 bp (Figure 5). The bacteria performed antibacterial activity (HM1.2 and HA1.1) in the prior step were successfully identified using molecular approach as *Bacillus paramycoides* and *Vibrio alginolyticus* with BLAST homology 98% and 99%.

The results obtained 11 strains of bacterial isolates from Menjangan Besar waters as anthropogenic areas and 15 strains of bacterial isolates from Alang-Alang waters as non-anthropogenic areas, The antibacterial test results showed the difference in the diameter of inhibition (DOI) zone from both locations. Molecular identification showed isolates HM1.2 and HA1.1 had the closest similarity with *Bacillus paramycoides* and *Vibrio alginolyticus* with homology Blast 98% and 99%.

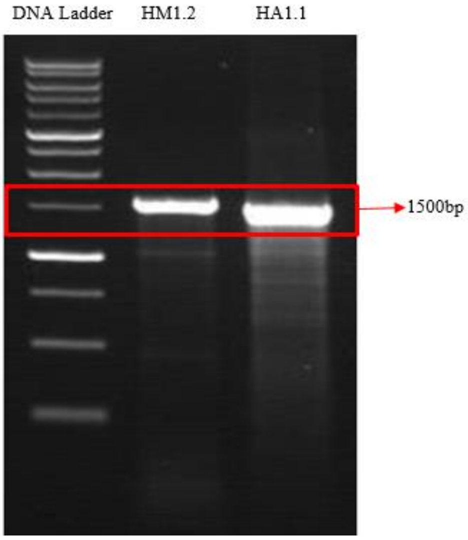

Figure 5. Visualization of electrophoresis result of bacterial strain HM1.2 and HA1.1 showing bands with a proximal length 1500 bp.

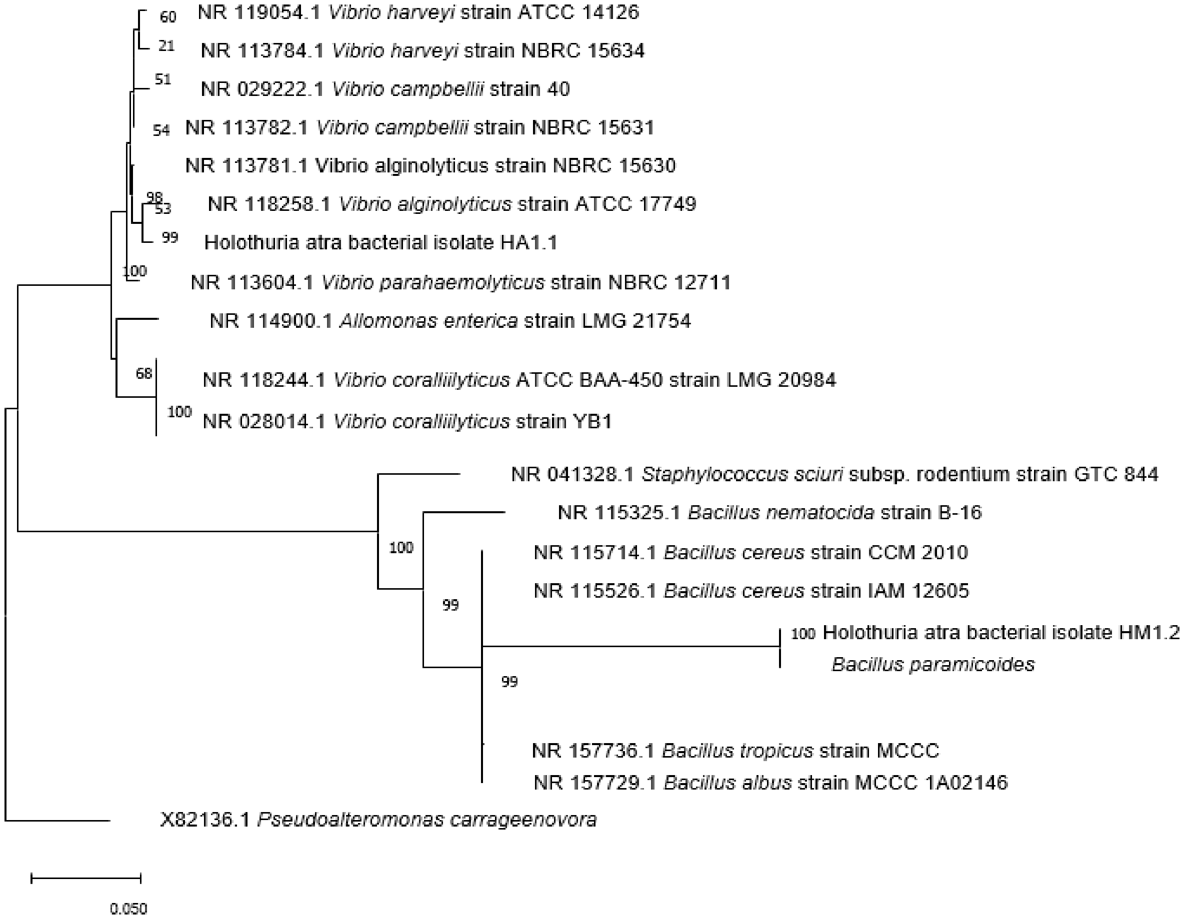

Figure 6. Phylogenetic tree of bacterial strain HM1.2 and HA1.1

In conclusion, based on the environment quality observation, both locations have a difference in visibility and organic material sediment, with Menjangan Besar considered more turbid than Alang-alang. This turbidity is due to the stronger water current based on this study. The hypothesis of pathogenic bacteria commonly found in anthropogenic water was not proven with this approach, because in Alang-alang also found *Vibrio algynolyticus* inside the *H. atra'*s body. *V. algynolyticus* is considered as pathogenic bacteria causing vibriosis in shrimp culture. Thus, to prove that hypothesis is still needed further research with another approach such as metagenomics. But, along with that, we found an interesting fact that the antibacterial properties from *H. atra* is not only from itself but also from associated bacteria living in the body, which not being reported before.

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