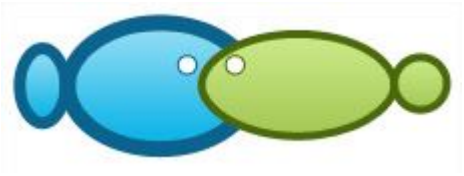

Isolation and identification of endophytic bacteria from the mangrove leaves of *Avicennia marina* and evaluation of inhibition to bacterium causing ice-ice disease

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Abstract. Endophytic bacteria are bacteria with all or part of their lives in the host tissue and mutually beneficial. This study aimed to isolate and identify endophytic bacteria from mangrove leaves of *Avicennia marina* and evaluate the inhibition to *Stenotrophomonas maltophilia* bacterium causing ice-ice disease on seaweed. The study was conducted in 2 stages, the first was isolation and identification of endophytic bacteria from mangrove leaves of *A. marina*; and the second was the inhibition test of bacterial isolates against *S. maltophilia*. Isolation and identification of endophytic bacteria from mangrove leaves of *A. marina* were obtained 18 isolates, based on sequences of 16S rRNA gene were gained 18 species and were divided into 3 groups, specifically Gammaproteobacteria, Firmicutes, and Enterobacteria. Bacteria with the best inhibition against *S. maltophilia* were *Vibrio* sp. (AMS-05), *Pseudomonas* sp. (AMS-08), and *Bacilus subtilis* (AMS-11). This study concluded that endophytic bacteria identified could inhibit *S. maltophilia* as a cause of ice-ice disease.

Key Words: 16S rRNA, bacterial combination, phylogenic tree, seaweed, *Stenotrophomonas maltophilia*.

Introduction. Seaweed is one of the marine products as export commodities with high economic value and high demand in the world market. FAO statistics (2018) release that world seaweed production is 30.4 million tons in 2015, with 29.4 million tons from the cultivation sector. The major seaweed producers are China, Indonesia, Philippines, Republic of Korea, Japan, Malaysia, Zanzibar, Madagascar, Solomon Islands, Chile, Vietnam, Tanzania, Sri Lanka, Papua New Guinea, Kiribati, India, Myanmar, Russia, South Africa and others. Demand for seaweed is increasing every year (FAO 2018). Efforts to optimize the seaweed production from cultivation experience problems that have not been resolved and cause considerable losses. One such problem is an arise of ice-ice disease and decreased seaweed production which ranges from 70 to 100% in several countries (Philippines, Indonesia, Malaysia and Tanzania) (Vairappan et al 2008).

The symptoms of ice-ice disease are appearing of white spots, waning of talus color, mucus, and covered with white flour, the epidermis peels off, and visible tissue (medulla) on the talus (Uyengco et al 1981). Ice-ice disease appears due to abiotic and biotic pressure which causes abnormal disturbances in seaweed (Andrews 1979). Bacterial infections are often a strong cause of disease in algae (Gachon et al 2010), one of the bacteria with the strongest pathogenicity is *Stenotrophomonas maltophilia* (Achmad et al 2016).

Many efforts have been made to control ice-ice disease, but the results are not optimal and affected the decline in seaweed production. Research on ice-ice disease and its control has been conducted with the method of introducing encoding genes of lysozyme enzyme (Handayani et al 2014) and superoxide dismutase enzymes (Triana et al 2016). The researches are expected to produce resistant seaweed to ice-ice disease,

but the results are not optimal. Development of seaweed production with tissue culture is expected to suppress ice-ice disease. In the last few decades, there has been a lot of research on microorganism role associated with mangrove tissue that is known an endophytic bacteria as antibacterial (Long et al 2004; Sessitsch et al 2004). Endophytic bacteria are an important source for developing new medicines for treatment in humans and other organisms (Kathiresan et al 2013).

Bacteria associated with these plants are in various plant tissues including flowers, fruit, leaves, stems, roots, and seeds (Kobayashi & Palumbo 2000). The presence of endophytic bacteria in *Avicennia marina* mangrove has been observed in roots (Janarthine et al 2011), leaves (Feliatra 2001; Ali et al 2017), and stems (Abou-Elela et al 2009), but their function is still in the research stage.

Microorganism associated with mangrove leaves are very varied, so the possible function as an antibacterial also varies considering some of the test results of mangrove leaf extract can inhibit bacterial activity (Alizadeh-Behbahani et al 2012; Bakshi & Chaudhuri 2014; Aljaghthmi et al 2017). This diversity is very potential to produce novelty antibacterial. This study aimed to isolate and identify endophytic bacteria and evaluate its inhibition of ice-ice-causing bacteria. This research is expected to be used as a reference material for controlling ice-ice disease in seaweed.

Material and Method. The research was conducted from September to December 2017 at the Fish Health Laboratory, Department of Aquaculture, IPB University. The mangrove leaves of *Avicennia marina* were collected from Bojo Village, Mallusetasi Subdistrict, Barru District, South Sulawesi Province (S 4.095795° and E 119.612332°). Samples for isolation and identification of endophytic bacteria were mangrove leaves. The leaves samples were taken and put in sterile plastic bags and transported to the laboratory. Fresh leaves samples were washed using running water, then were washed with 70% ethanol for two minutes, and with sodium hypochlorite for one minute (Gayathri et al 2010). The sterile leaves were rinsed with distilled water to remove ethanol and sodium hypochlorite solutions from the leaf surface. The study was conducted in 2 stages, there were isolation and identification of endophytic bacteria from *A. marina* leaves, and inhibitory strength test of bacterial isolates against *S. maltophilia.*

Experiment 1

Isolation and identification of bacteria from mangrove leaves of A. marina. The isolation of bacterial from *A. marina* leaves was conducted by mashing 10 grams of leaves, put in a 250 mL erlenmeyer flask, then made a suspension by adding 100 mL sterile water. Dilution was conducted serially, the suspension was taken 0.1 mL, cultured in the Sea Water Complex (SWC) media to obtain pure isolates. Isolates were identified based on the Berge's Manual of Determinative Bacteriaology (Holt et al 1994), followed genotypic test by molecular.

a. DNA extraction. DNA extraction referred to protocol of the Presto^{TM} mini gDNA bacteria kit (Geneaid manufacture's Taiwan). Each isolate was cultured on SWC-broth media, incubated for 24 hours with temperature of 28^{o}C. As much as 1 mL of bacteria was centrifuged at 10,000 g for 5 minutes. Gram positive bacteria were lysed by adding 200 µL lysozyme, while Gram negative bacteria were added by 180 µL GT buffer, were vortexed and were incubated at 37^{o}C for 30 minutes.

b. Lisis. The lysis process of bacteria was carried out by adding 200 µL GB buffer, being vortexed for 10 seconds, and incubated at 70°C for 10 minutes. The DNA binding process was carried out by adding 200 µL absolute ethanol to the DNA, was centrifuged 14,000 g for 2 minutes. Washing was conducted by adding 400 µL W1 buffer to the DNA, centrifuging 14,000 g for 30 seconds, adding 600 µL wash buffer, centrifuging 14,000 g for 30 seconds. The final step was to add 50 µL elution buffer, leave it for 3 minutes, centrifuged 14,000 g for 1 minute, and stored in the temperature of -20°C.

c. Amplification. PCR amplification used universal primer of 16S rRNA with forward 63f (5-CAGGCCTAACACATGCAAGTC-3) and reverse 1387r (5-GGGCGGWGTGTACAAGGC-3) (Marchesi et al 1998) with target of 1,300 bp. Stages in PCR amplification were pre-denaturation at 94°C for 3 minutes, denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 1 minute, and final extension at 75°C for 20 minutes. The PCR product was electrophoresed using 12 µL amplicon in 1.5% agarose gel at 120 Volts and 1x Tris-acetate-electrophoresis buffer (TAE) [(0.04 mol L^{-1} Tris, 0.001 mol L^{-1} EDTA; pH 7.8)] for 15 minutes. The marker used 100 bp DNA ladder (MBI Fermentas).

d. Sequencing. Amplification products were sequenced by the services of 1st BASE DNA Sequencing in Malaysia. The sequencing results were analyzed using the basic local alignment search (BLAST) tool to compare homologies between 16S rRNA isolates and bacterial sequences at the National Center for Biotechnology Information Blast (http://www.ncbi.nlm.nih.gov). The phylogenetic tree was constructed with the Neighboring joining method using the MEGA7 program.

Experiment 2

Bacterial inhibition test from mangrove leaves of A. marina against ice-ice-causing bacteria. The experiment used isolate bacterium from seaweed with the symptoms of ice-ice, namely *S. maltophilia* (Achmad et al 2016). *S. maltophilia* was cultured on SWC-broth media at 28°C incubated for 24 hours. Bacteria were harvested and diluted based on the Mc Farland method (McFarland et al 2006) with a density of 10^6 cell mL^{-1}, then were cultured on SWC-gel media for challenging test activities. All isolated endophytic bacteria were cultured on SWC-broth media for 24 hours, harvested and diluted by the Mc farland method with concentration of 10^5 cell mL^{-1}. Challenging tests used the Kirby-Bauer method (Bauer et al 1966) by soaking blank disc in endophytic bacteria culture for 15 minutes, and placed on culture of *S. maltophilia* bacteria, incubated for 24 hours at 28^oC. The diameter of inhibition was measured and analyzed. Three endophytic bacteria with the best inhibitory results were taken and combined to test with *S. maltophilia* using the Kirby-Bauer method and joint culture. The results of each joint culture were cultured on a resistance medium for *S. maltophilia*, with 500 µL supertetra in 100 mL SWC-gel media. The inhibition results with the shared culture method were known by comparing the number of *S. maltophilia* colonies that grew in the resistance media of all tests.

Data analysis. Biochemical data were analyzed descriptively, while bacterial inhibition was analyzed by analysis of variance (ANOVA) using SPSS version 20. Analysis of phylogenetic trees used the MEGA7 Neighbor-joining tree program with 1000 bootstrap.

Results and Discussion

Experiment 1

Isolation and identification of endophytic bacteria from the mangrove leaves of A. marina. Biochemical testing was carried out to support the molecular identification results in bacterial isolates. Biochemical results could be seen in Table 1.

The results of the biochemical test obtained 8 genera consisting of 8 isolates from the gram negative bacteria, i.e. *Klebsiella*, *Pantoea*, 3 *Vibrio*, 2 *Enterobacter*, and *Pseudomonas* isolate; and 10 isolates from the gram positive bacteria, i.e. *Virgibacillus*, *Staphylococcus*, and 8 *Bacillus* isolates. The results of this study were different from Feliatra (2001) research. The reseach found 7 genera, namely *Neisseria, Plesiomonas, Yersinia, Corynebacterium, Bacillus, Staphylococcus* and *Acinobacter*. The other study in same species were carried out by Ali et al (2017) and established 10 genera, specifically *Paenibacillus, Bacillus, Microbacterium, Citrobacter, Lysinibacillus, Halomonas, Virgibacillus, Vibrio, Exiguobacterium,* and uncultured bacterium. There were several

bacteria species, this was alleged by the type of leaf part for isolation, aquatic environmental conditions, and season differences in sampling.

Table 1

The biochemical test results of bacterial isolates from the mangrove leaves of *A. marina*

Code of isolate	*Character*						*Genus*
	Gram	*Shape*	*SIM*	*Catalase*	*Oxidase*	*OF*	
AMS1	+	Rod	+	+	+	F	*Virgibacillus*
AMS2	-	Rod	-	+	-	F	*Klebsiella*
AMS3	-	Rod	+	+	-	F	*Pantoea*
AMS4	-	Rod	+	-	+	F	*Vibrio*
AMS5	-	Rod	+	+	+	-	*Vibrio*
AMS6	-	Rod	+	+	-	F	*Enterobacter*
AMS7	+	Sphere	-	+	-	F	*Staphylococcus*
AMS8	-	Rod	+	+	+	-	*Pseudomonas*
AMS9	-	Rod	+	-	+	F	*Vibrio*
AMS10	-	Rod	+	+	-	F	*Enterobacter*
AMS11	+	Rod	+	+	-	-	*Bacillus*
AMS12	+	Rod	+	+	+	-	*Bacillus*
AMS13	+	Rod	+	+	-	-	*Bacillus*
AMS14	+	Rod	+	+	+	-	*Bacillus*
AMS15	+	Rod	+	+	+	-	*Bacillus*
AMS16	+	Rod	+	+	-	-	*Bacillus*
AMS17	+	Rod	+	+	+	-	*Bacillus*
AMS18	+	Rod	+	+	+	-	*Bacillus*

AMS1 = isolate 1; AMS2 = isolate 2; AMS3 = isolate 3, and others; OF = oxidative fermentative .

The sequencing results of 18 isolates from mangrove leaves of *A. marina* after the alignment and BLAST were showed in Table 2.

Based on Table 2, the BLAST results obtained similarity level of 16S rRNA sequences between 98-100%. The bacteria with the highest sequential similarity rate of 100% were *P. angglomerans* strain T2, *V. tritonius* strain AM2, *B. subtilis* strain BR4, *Bacillus megaterium* YC4-R4, *B. thuringiensis* strain VKK-SL-2, *B. vietnamensis* strain FJAT-46928, *Bacillus* sp. strain FJAT 47851, *B. circulans* strain MD1, while the lowest sequential similarity rate of 98% was *V. salexigens* strain JCM 30552.

The identified bacteria were endophytic bacteria and generally grow in marine waters. Gram negative bacteria found in mangrove leaves were often associated with flora, fauna, and humans. This study is similar with some previous findings, *Klebsiella* sp. TG-1 was found in starch waste water, this bacterium produced microbial flocculants (MBF-TG-1) consisting of polysaccharides (84.6%) and small amounts of protein or amino acids (11.1%) (Liu et al 2013). *Klebseilla pneumonia* was able to fixation of N_2, produced IAA hormone (Rosenblueth & Martinez-Romero 2006). Pantoea bacteria were found in plants as biocontrol of various pathogenic fungi and bacteria, producing auxin compounds, combined auxin, cytokinin, and giberelin (Chauhan et al 2015). Bacterium *V. tritonius* strain AM2 was isolated from the mangrove tissue of *A. marina*, but it was generally found from intestinal marine invertebrates and the marine environment. Matsumura et al (2014) stated that these bacteria were able to increase H_2 production as bioenergy (bioH_2) or alternative fuels by fermenting carbohydrates from seaweed and seaweed powder. *Vibrio* sp. MSSRF QS47 was isolated from *A. marina*, this bacterium produced N-acyl-Homoserine Lactone (AHL) (Viswanath et al 2015). *V. natriegens* strain ATCC 14048 was originally from *Pseudomonas natriegens*, this bacterium was commonly found in marine waters and coastal sediments. These bacteria had rapid growth, were able to utilize a variety of organic substrate as a source of carbon and energy, non-pathogenic to humans, capable of rapid biosynthesis (Wang et al 2013). *Enterobacter asburiae* was a basil bacterium belonging to the family Enterobacteriaceae, isolated from soil, water, and food products. This bacterium produced C4-HSL and C6-HSL molecules

which played a role in inhibiting the proliferation of surrounding enteric bacteria (Lau et al 2013). Then, *Enterobacter* sp. 1FTM7 could help in food fermentation of traditional Douchi mainly used to control pathogen growth (Chen et al 2015).

Table 2

Similarity percentage of nucleotide sequences from 16S rRNA gene in mangrove leaves of *A. marina*

Code of isolate	*Bacteria name*	*Level of homology (%)*	*Query/Subject*	*Access number*
AMS1	*Virgibacillus salexigens* strain JCM 30552	98	601/616 (98%)	LC016572.1
AMS2	*Klebsiella* sp. TG-1	99	947/949 (99%)	HM585430.1
AMS3	*Pantoea angglomerans* strain T2	100	1256/1256 (100%)	HQ443233.1
AMS4	*Vibrio tritonius* strain AM2	100	1772/2007 (100%)	NR_134830.1
AMS5	*Vibrio* sp. MSSRF QS47	99	829/832 (99%)	KJ877670.1
AMS6	*Entobacter asburiae* strain idli 48	99	1125/1129 (99%)	KJ830708.1
AMS7	*Staphylococcus saprophyticus* strain GRKJM1	99	449/453 (99%)	JX069805.1
AMS8	*Pseudomonas* sp. R-9	99	1240/1255 (99%)	AY914075.1
AMS9	*Vibrio natriegens* strain ATCC 14048	99	1261/1266 (99%)	CP016345.1
AMS10	*Enterobacter* sp. 1FTM7	99	1244/1258 (99%)	KC342873.1
AMS11	*Bacillus subtilis* strain BR4	100	1256/1256 (100%)	KU052617.1
AMS12	*Bacillus megaterium* YC4-R4	100	1247/1247 (100%)	CP026740.1
AMS13	*Bacillus firmus* strain PGRP4	99	1098/1099(99%)	MG229068.1
AMS14	*Bacillus thuringiensis* strain VKK-SL-2	100	1116/1116(100%)	KT714055.1
AMS15	*Bacillus subterranerus* strain FJAT-47744	99	1250/1252(99%)	MG651149.1
AMS16	*Bacillus vietnamensis* strain FJAT-46928	100	1086/1086(100%)	MG651539.1
AMS17	*Bacillus* sp. strain FJAT 47851	100	995/995(100%)	MG651253.1
AMS18	*Bacillus circulans* strain MD1	100	1253/1253(100%)	KT757520.1

AMS1 = isolate 1; AMS2 = isolate 2; AMS3 = isolate 3, and others.

Other bacteria were gram-positive and were found in marine waters, marine organisms, land, and freshwater. *V. salexigens* strain JCM 30552 was widely used in daily life. This bacterium was isolated from the sponge *Pseudoceratina purpurea* (Sinsuwan et al 2007), plants *Enhalus acoroides*, *Thalassia hemprichii* (Marhaeni et al 2011), *A. marina* (Ali et al 2017), and from shrimp paste. This bacterium had antibacterial substances, namely class II bacteriocin which was able to inhibit the growth of *Listeria monocytogenes* bacteria (Kobayashi et al 2016). *S. saprophyticus* strain GRKJM1 was found in the sea. Radjasa et al (2013) reported that this bacterium was found in coral reefs, capable to inhibit pathogenic growth of *S. aureus*, while *Pseudomonas* sp. R-9 was able to reduce hydroxychlorobiphenyls pollutants with a strong response (Lovecka et al 2004). *B. subtilis* strain BR4 were beneficial bacteria found in mangrove tissue. Boopathi et al (2017) stated that these bacteria produced biofilm inhibitors (BIC) to fight *Pseudomonas aeruginosa* strain ATCC 27853. *B. subtilis* could also be developed as a biological controlling agent for various plant diseases (Grover et al 2010). Other bacterium, *B. megaterium* YC4-R4 was found in marine waters, and it was widely used for commercial probiotics with positive effect on water quality (Hura et al 2018). The production and secretion of recombinant proteins were successfully used for important component bioconversion or biosynthesis including vitamins (Biedendieck 2016). *B. firmus* increased growth and protect against pathogens or pests (Mendis et al 2018). *B. thuringiensis*

strain VKK-SL-2 was found in marine waters and produced parasporal crystals containing one or more crystalline insecticidal proteins (Cry), which was selectively toxic to insects and was widespread in the aquatic environment (Rajashekhar et al 2017). *B. subterranerus* strain FJAT-47744 was found in waters and soil, this bacterium could dissolve phosphate to increase crop production (Tripathi & Banu 2017). *B. vietnamensis* degraded the amount of high ammonia nitrogen (TAN) and nitrite (Muthukrishnan et al 2015). *Bacillus* sp. strain FJAT 47851 and *B. circulans* strain MD1 were widely found in waters and soil and were widely used in cultivation, bioremediation, biodegradation, waste treatment, and waste production (ECCCHC 2018).

The phylogenetic tree of 18 endophytic bacteria using the MEGA7 program were divided into 3 major clades, i.e. clade 1 consisting of AMS2, AMS3, AMS6, AMS10 isolates which were compared with bacteria *K. pneumonia* strain HSL4 (KJ401316.1), *P. dispersa* strain DSM 30073 (NR116797.1), *E. hormaechei* strain OCC21 (KU525319.1) in the Enterobacteria group; clade 2 consisting of isolates AMS4, AMS5, AMS8, AMS9 which were compared with *V. alginolyticus* strain ATCC 17749 (NR 117895.1), *V. proteolyticus* strain MSSRF QS49 (KP640643.1), *Pseudomonas* sp. strain 3 MSSRF QS31 (KP640641.1), *V. furnissii* strain MSSRF QS57 in the Gammaproteobacteria group; clade 3 consisting of AMS1, AMS7, AMS11, AMS12, AMS13, AMS14, AMS15, AMS16, AMS17, and AMS18 isolates which were compared with *V. necropolis* LMG 19488 (NR118486.1), *S. sciuri* subsp. *sciuri* strain DSM 20345T (AJ421446.1), *B. subtilis spizizenii* strain ATCC 6633 (NR 118486.1), *B. toyonensis* strain Z013 (MG255974.1). The phylogenetic tree was shown in Figure 1.

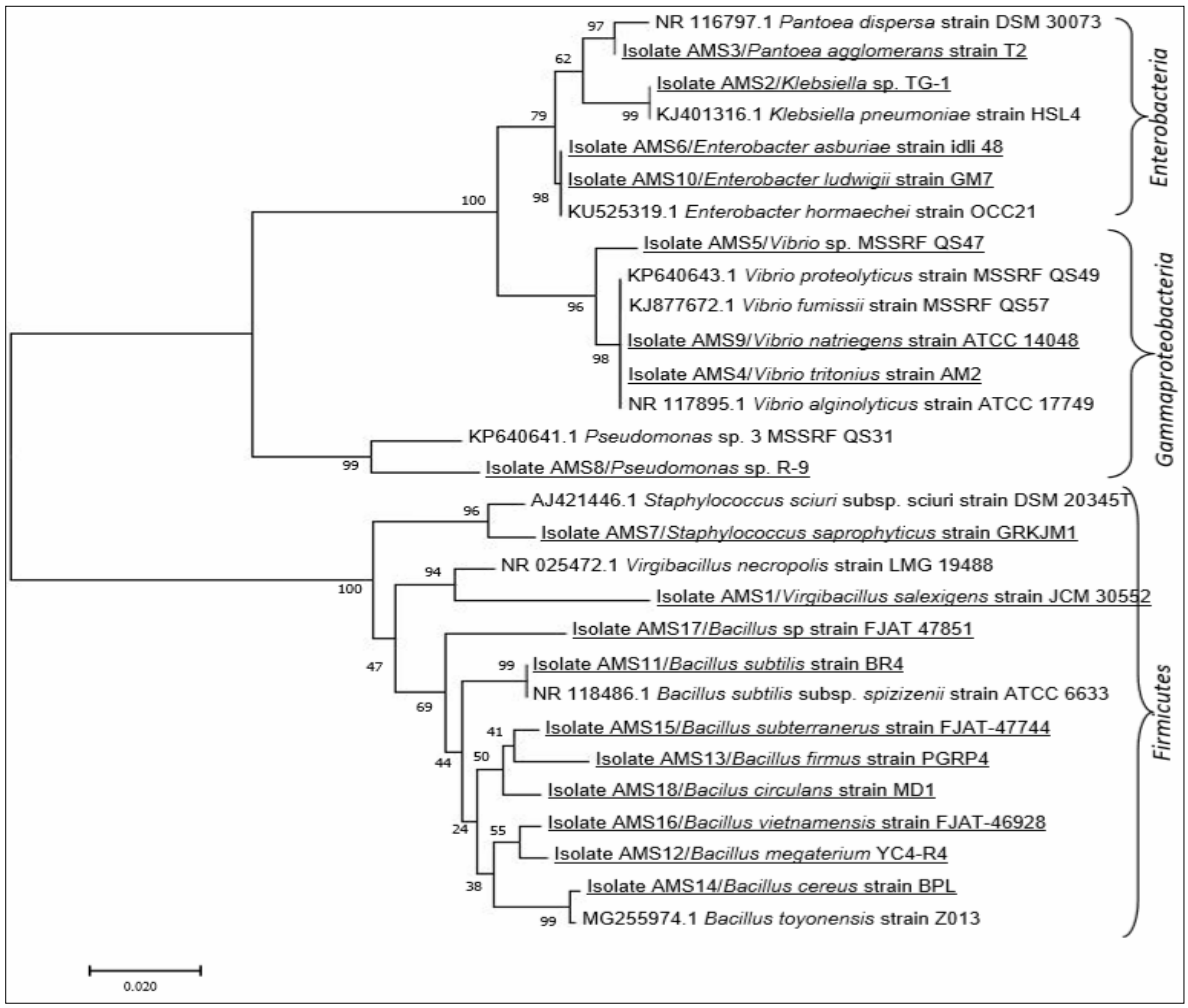

Figure 1. Phylogenic tree based on the nucleotide sequence of the 16S rRNA gene of endophytic bacteria isolates from mangrove leaves of *A. marina*.

Based on the phylogeny tree, there were 3 groups, namely Gammaproteobacteria, Firmicutes, and Enterobacteria. Gammaproteobacteria group contained 4 bacteria, i.e. *V. tritonius* strain AM2, *Vibrio* sp. MSSRF QS47, *Pseudomonas* sp. R-9 and *V. natriegens* strains of ATCC 14048. Firmicutes group have 10 bacteria, i.e. *S. saprophyticus* strain GRKJM1, *V. salexigens* strain JCM 30552, *B. subtilis* strain BR4, *B. megaterium* strain YC4-R4, *B. firmus* strain PGRP4, *B. thuringiensis* strain VKK-SL-2, *B. subterranerus* strain FJAT-47744, *B. vietnamensis* strain FJAT-46928, *Bacillus* sp. strain FJAT 47851, and *B. circulans* strain MD1. *Enterobacteria* groups have 4 bacteria, i.e. *Klebsiella* sp. TG-1, *P. angglomerans* strain T2, *E. asburiae* strain idli 48, and *Enterobacter* sp. 1FTM7.

The result of bacteria identification was obtained from mangroves, mostly from Gammaproteobacteria and Firmicutes. Gammaproteobacteria were widespread in the tropic and subtropic oceans (Jing et al 2015), and the dominant community of mangroves with 86.66% of the total community (Zhang et al 2017), epiphytic bacterial communities in seaweed and core microbiota in seagrass roots (Egan et al 2013), this group was widely isolated from rhizosphere sediments of mangroves (Gomes et al 2014). The Firmicutes group was bacteria that were often found in plants. This bacteria group dominated in mangrove sediments (Vincent et al 2013; Alzubaidy et al 2016), and was commonly found in plants, helped to increase productivity by producing metabolites and compounds needed for plant growth (Bibi et al 2017).

Experiment 2. The results of the inhibition test from endophytic bacteria of mangrove leaves on ice-ice-causing bacteria in seaweed could be observed in Table 3. The analysis showed that the average inhibition of endophytic bacteria had significant effect ($p < 0.05$) on *S. maltophilia*.

Table 3

Inhibitory activity of bacteria against *S. maltophilia*

Bacteria	*Inhibitory activity (mm) (X±SD)*
V. salexigens AMS-01	8.05 ± 0.11^{abc}
Klebsiella sp. AMS-02	8.00 ± 0.00^{abc}
P. angglomerans AMS-03	8.40 ± 0.38^{abcde}
V. tritonius AMS-04	8.75 ± 0.18^{cdef}
Vibrio sp. AMS-05	9.80 ± 0.76^{h}
E. asburiae AMS-06	8.00 ± 0.00^{abc}
S. saprophyticus AMS-07	8.00 ± 0.00^{abc}
Pseudomonas sp. AMS-08	9.55 ± 0.87^{gh}
V. natriegens AMS-09	9.05 ± 0.41^{defgh}
Enterobacter sp. AMS-10	8.00 ± 0.00^{abc}
B. subtilis AMS-11	9.20 ± 0.45^{fgh}
B. megaterium AMS-12	8.30 ± 0.11^{abcd}
B. firmus AMS-13	7.85 ± 0.14^{ab}
B. thuringiensis AMS-14	7.70 ± 0.11^{a}
B. subterranerus AMS-15	8.60 ± 0.22^{bcdef}
B. vietnamensis AMS-16	8.85 ± 0.22^{defg}
Bacillus sp. AMS-17	9.15 ± 0.22^{efgh}
B. circulans AMS-18	8.75 ± 0.18^{cdef}

Different superscripts in the same column show real-time results ($p < 0.05$).

This isolate of endophytic bacteria had a different inhibitory activity against *S. maltophilia*. This could be seen in Table 3, where the inhibition of 3 endophytic bacteria was the strongest, namely *Vibrio* sp. AMS-05, then *Pseudomonas* sp. AMS-08, and *B. subtilis* AMS-11. These bacteria were potential bacterial candidates to inhibit ice-ice-causing bacteria on seaweed. Pelczar & Chan (2005) explained that a weak inhibition indicated low antibacterial activity, whereas a strong inhibition indicated greater antibacterial activity. Furthermore, Davis & Stout (1971) stated that inhibition was categorized to very strong (inhibition 20 mm or more), strong (inhibition 10-20 mm),

medium (inhibition 5-10 mm) and weak (inhibition < 5 mm). Measurement of bacterial inhibition against *S. maltophilia* showed inhibitory ability at a moderate level.

The inhibition of each bacterium was thought in the quorum condition and the inhibitory spectrum of each bacterium worked more specifically to control *S. maltophilia*. In addition, the phase of cell adaptation and multiplication determined the inhibitory performance of bacteria. Interaction between bacteria helped to recognize, inhibit, and produce secondary metabolites. Defoirdt et al (2004) stated that quorum sensing was the coordination of bacterial gene expression in response to population density by producing, releasing, and recognizing small signal molecules (autoinducers), the bacteria pathogenicity was controlled by quorum or anti quorum sensing microorganisms with potential quenchers in regulating quorum sensing function of pathogenic bacteria.

The results of the inhibition test of *Pseudomonas* sp. AMS-08, *Vibrio* sp. AMS-05, and *B. subtilis* AMS-11 combinations against *S. maltophilia* (Table 4) with the Kirby-Bauer method showed that all combination treatments had inhibitory activity against pathogens and were able to reduce pathogen growth through combined culture. The bacterial combination was statistically significant ($p < 0.05$) on *S. maltophilia* with the Kirby-Bauer test and culture method.

Table 4

Inhibitory activity of bacteria combination against *S. maltophilia*

Bacteria	*Inhibitory activity*	
	Kirby-Bauer method (mm)	*Mix culture (%)*
Pseudomonas sp. AMS-08 + *Vibrio* sp. AMS-05	8.00 ± 0.01^{a}	41 ± 1.15^{a}
Pseudomonas sp. AMS-08 + *B. subtilis* AMS-11	8.00 ± 0.03^{a}	40 ± 0.58^{a}
Vibrio sp. AMS-05 + *B. subtilis* AMS-11	8.00 ± 0.00^{a}	41 ± 1.00^{a}
Pseudomonas sp. AMS-08 + *Vibrio* sp. AMS-05 + *B. subtilis* AMS-11	7.70 ± 0.02^{b}	40 ± 0.58^{a}
control (without endophytic bacteria)	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}

Different superscripts in the same column show real-time results ($p < 0.05$).

The combination of the three bacteria was less synergistic than a single bacterium. It was suspected that a combination of bacteria was antagonistic to fellow bacteria. It produced secondary metabolites to inhibit each other and could not work together to inhibit pathogens. Secondary metabolites played a role in defense, competition, and act as signals for interaction or communication (Brader et al 2014). Bacteria did not recognize or communicate through quorum sensing with other bacteria and would change their metabolism according to their individual needs. They would inhibit each other and decrease the inhibition of pathogens. Molina et al (2003) stated that degradation of acyl homoserine lactones could be a biological control mechanism of plant diseases by potential microbes, but could inhibit controlling mechanism of other strains. The potential bacteria were able to compete in space and food with pathogenic bacteria and had a much faster growth than pathogenic bacteria so that they could maintain populations optimally (Chrisnawati et al 2009).

Conclusions. Isolation and identification of endophytic bacteria from mangrove leaves of *A. marina* were obtained 18 species and were divided into 3 groups, specifically Gammaproteobacteria, Firmicutes, and Enterobacteria. Endophytic bacteria identified could inhibit *S. maltophilia* as a cause of ice-ice disease. The best inhibitions were *Vibrio* sp. (AMS-05), *Pseudomonas* sp. (AMS-08), and *Bacilus subtilis* (AMS-11).

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