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Genetic diversity of the causative agent of ice-ice disease of the seaweed *Kappaphycus alvarezii* from Karimunjawa island, Indonesia

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Abstract. An essential step in investigating the bacterial role in the occurrence of diseases in *Kappaphycus alvarezii* is the characterization of bacteria associated with this seaweed. A molecular characterization was conducted on the genetic diversity of the causative agents of ice-ice disease associated with *K. alvarezii* widely known as the main source of kappa carrageenan. *K. alvarezii* infected with ice-ice were collected from the Karimunjawa island, North Java Sea, Indonesia. Using Zobell 2216E marine agar medium, nine bacterial species were isolated from the infected seaweed. The molecular characterizations revealed that the isolated bacteria causing ice-ice disease were closely related to the genera of *Alteromonas*, *Bacillus*, *Pseudomonas*, *Pseudoalteromonas*, *Glaciecola*, *Aurantimonas*, and *Rhodococcus*. In order to identify the symptoms causative organisms, the isolated bacterial species were cultured and were evaluated for their pathogenicity. Out of 9 species, only 3 isolates were able to cause the ice-ice symptoms and consisted of *Alteromonas macleodii*, *Pseudoalteromonas issachenkonii* and *Aurantimonas corallicida*. *A. macleodii* showed the highest pathogenicity.

Keywords : Genetic diversity, causative agents, ice-ice, *Kappaphycus alvarezii*, Karimunjawa island Indonesia

1. Introduction

Genus *Kappaphycus* is farmed as raw material for the extraction of the phycocolloid, carrageenan, and as food which is considered as an important commercial commodity for many developing countries such as Indonesia, Malaysia, Philippines, and Tanzania [1]. The production of farmed seaweed in Indonesia steadily increased from 1,728,475 tonnes in 2007 to 9,298,475 tonnes in 2013 [2]. The significant increase in seaweed production consequently stimulated carrageenan industry that opens job creation, reducing unemployment and contributing to national economic growth.

Microorganisms have been identified as the colonizers of seaweeds, and bacteria have been regarded as the primary colonizer [3]. This bacterial seaweed association, however, often disturbed the seaweed production by the occurrence of disease so-called ice-ice disease. According to [4], ice-ice disease led



to a significant decrease in seaweed production and decrease in carrageenan yield compared to the healthy crop ranging from 25 to 40 %.

Bacteria are known to be the main component of seaweed epibionts with higher diversity and had implications in the development of the disease [5]. It has been further found that non-pigmented bacterium and a yellow bacterium, *Cytophaga-Flavobacterium* complex and *Vibrio-Aeromonas* complex were identified as causative microbes of the whitening of *Kappaphycus alvarezii*. [6] reported a bacterium, *Pseudoalteromonas bacteriolytica* to be the causative agent of the red spots disease of the cultured brown seaweed species, *Laminaria japonica*. In Oshoro Bay, Hokkaido, Japan, a bacterium, *Alteromonas* sp. was identified as the symptom-causative agent of *Laminaria religiosa* [7]. Further research conducted by [8] reported that *Pseudoalteromonas issachenkonii* was a bacterium that degraded algal polysaccharides, thallus of the brown alga *Fucus evanesceus*.

However, very little is known about seaweed disease and its status in Indonesia. First report on seaweed disease in Indonesia was published in early 2000, 75% of seaweed farming in Pari Island, Thousand Island was collapsed largely due to emerging diseases [9]. This phenomenon was still exists in present days. The disease was spread along with the extension of cultivation area and environmental degradation. Due to the regular outbreaks and economic loss caused by bacterial community, several strategies have been attempted to stop spreading and progressing of seaweed diseases. However, those approaches were demonstrated ineffective and there were very little success on a large spatial scale. Two alternatives approaches to identify seaweed diseases are by understanding the causative agents of particular disease and the search of anti-seaweed disease obtained from natural products [10,11].

Based on the information above, in the effort to fully understand the causative agent of *K. alvarezii* ice-ice disease in Karimunjawa island, North Java Sea, Indonesia, molecular based-approach was conducted to estimate the diversity of the causative agents and investigated the symptom causative bacteria using pathogenity test [7]. The objective of this research were to determine the diversity of the causative agents from bacterial community associated with ice-ice diseased on *K. alvarezii* and investigated the symptom causative bacteria using pathogenity test.

2. Material and Methods

2.1. Sampling site

Infected seaweed samples of *K. alvarezii* were collected from cultivation farms at Karimunjawa island, North Java Sea, Indonesia (Figure 1), in May 2014 (05°47'22.4"S - 110°26'57"E). After collection, the infected seaweed materials were placed onto sterile borosilicate bottles containing autoclaved seawater and brought in chilled condition, to the Tropical Marine Biotechnology Laboratory, Diponegoro University for bacterial isolation

2.2. Isolation of bacteria from infected seaweed

One gram of infected seaweed sample was grinded using mortar and diluted in 10 mL of sterilized seawater. The sample was vortexed to homogenize the solution. Afterwards, one mL of the solution was diluted into 9 mL of sterilized seawater to make 10⁻¹ seaweed dilution. The processes were repeated until 10⁻⁶ seaweed dilution. The 0,1 aliquots from 10⁻¹ to 10⁻⁷ dilution were spread onto Zobell 2216E marine agar medium. Then, they were incubated upside down at room temperature for 4x24 hours. Based on morphological features, colonies were randomly picked and purified by making streak plates [12].

2.3. Molecular Identification

Genomic DNA of each bacterial isolate from diseased seaweed was extracted by using Chelex method [13], the DNA concentrations and purity were quantified and qualified by using NanoDrop 2000 spectrophotometer. DNA extracts were amplified by PCR using universal primers 27F (5'AGAGTTTGATCMTGGCTCAG-3') and 1492 R (5'TACGGTTAACCTTGTTACGACTT-3') and temperature cycle of amplification was as follows: initial denaturation at 95° C for 3 min, and then successive denaturation (95° C for 1 min), annealing (55° C for 1 min), and extension (72° C for 1

minutes). Series of denaturation, annealing and extension were repeated until 30 cycles. A final extension was performed at 72^o C for 7 min [14]. The PCR products were analyzed by 1 % agarose gel electrophoresis and the result was observed using UVIDoc HD (UVITEC Cambridge). DNA sequencing was conducted at 1st BASE DNA Sequencing, Malaysia. Purification of PCR products and subsequent sequencing analysis were performed according to [15]. The nearly complete 16S rDNA gene sequences were used to search the genetic profile similarity in the GenBank database with the Blast algorithm to determine relative phylogenetic positions. Sequences then were aligned by using ClustalW software and the phylogenetic analyses were performed by using MEGA 6. The phylogenetic trees were determined using the neighbour-joining method with Kimura's two-parameter. The resultant tree topology was evaluated by bootstrap analyses of the neighbour-joining method based on 1.000 resampling [15].

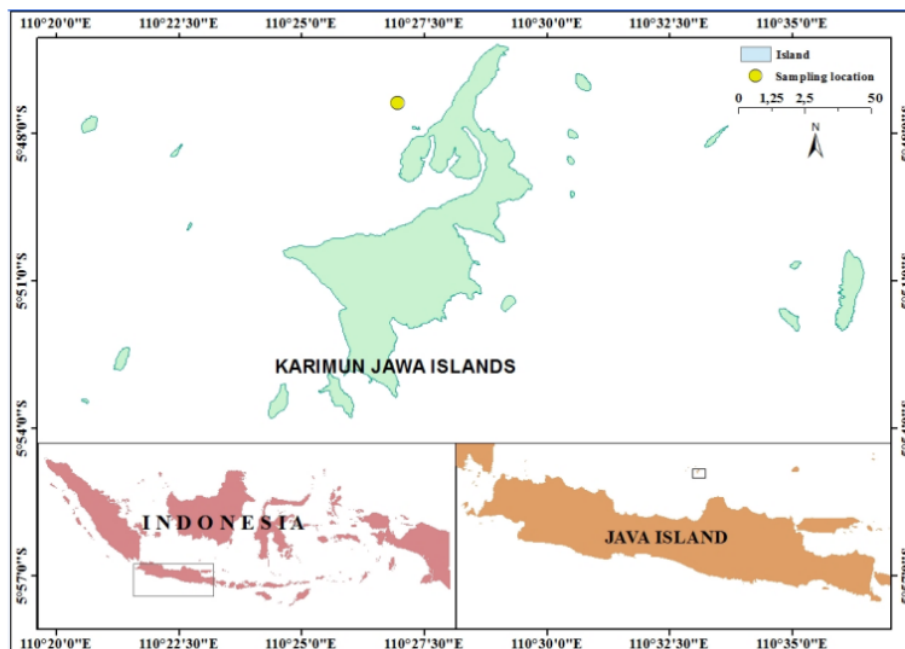


Figure 1. Sampling site for the collection of *K. alvarezii* from seaweed farm

2.4. DNA Sequences

DNA sequences of all bacterial isolates have been deposited in the DNA Database Bank of Japan (DDBJ) in the following accession numbers: LC085337-LC085345.

2.5. Pathogenicity test

All strains that previously isolated from diseased seaweed were cultured in 10 ml of ZoBell 2216E broth medium at 37^o C until cell density reached 10⁸ ml for 2 days. Healthy, epiphyte-free part of main branches of *K. alvarezii* were then cut into 5 cm long pieces and distributed individually into 300 mL flask containing 200 mL autoclaved seawater and were acclimated for 10 days. The cells were then

harvested by centrifugation at 5000 rpm for 10 minutes, washed twice and re-suspended with PBS solution, and then two mL subsamples of each bacterial suspension were inoculated into 200 mL SSW in 300 mL erlenmeyer flask. A set of control experiments (without bacterial inoculum) was also carried out simultaneously. All transfers of algal materials and media were done under aseptic condition in clean bench using pre-sterilized glass wares, forceps and blades. Flasks with samples were placed on a rotary shaker at 100 rpm for 7 days under approximately 50 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$, 28^o C, and 12:12 L:D cycle. Observations were conducted daily until visible sign of ice-ice by whitening of thallus was occurred up to a maximum of 1 week

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3. Results and Discussion

3.1. Isolation and Molecular Identification of Associated Bacteria from Infected Seaweed

Management of healthy seaweed aquaculture and control of ice ice disease are important component in seaweed production. The problem of ice-ice disease has not only affected the seaweed farmers but also the nation as a whole. Farmings in many producing countries suffered the drastic decline in aquaculture production with negative growth due to ice-ice disease. [4], showed that ice-ice also leads to the decrease of carrageenan yield, viscosity and gel strength of infected thalli.

To support the integrated prevention of ice ice disease, information about genetic variation of bacterial pathogens and the availability of fast and accurate detection are required. Detection method that has been developed at this time is molecular technique through analysis of genomic DNA. Here, the utilization of the 16S rDNA has been used as a systematic parameter as a marker for the identification of causative agent of ice-ice disease of seaweed *Kappaphycus alvarezii* from seaweed farming in Karimunjawa islands, North Java Sea, Indonesia.

A total of 9 bacterial strains have successfully been isolated from diseased seaweed, *K. alvarezii*. Based on the analysis of 16S rDNA sequences and phylogenetic tree, all isolates were closely related with the following bacterial genera of *Alteromonas*, *Bacillus*, *Pseudomonas*, *Pseudoalteromonas*, *Aurantimonas*, and *Rhodococcus* (Table 1 and Figure 2).

Table 1. Molecular characterization of bacterial causative agents

Isolate	Length (bp)	Closest Species	Similarity (%)	Accession Number	Group
KAKJ1	1187	<i>Alteromonas macleodii</i> 107	96%	NR_037127.1	Gammaproteobacteria
KAKJ2	1258	<i>Bacillus oceanisediminis</i> H2	91%	NR_117285.1	Bacilli
KAKJ3	1237	<i>Pseudomonas stutzeri</i> ATCC 17588	95%	NR_041715.1	Gammaproteobacteria
KAKJ4	1107	<i>Pseudoalteromonas issachenkonii</i> KMM 3549	95%	NR_025139.1	Gammaproteobacteria
KAKJ5	1060	<i>Bacillus hunanensis</i> JSM 081003	97%	NR_108948.1	Bacilli
KAKJ6	1239	<i>Bacillus megaterium</i> ATCC 14581	97%	NR_116873.1	Bacilli
KAKJ7	916	<i>Alteromonas marina</i> SW-47	96%	NR_025260.1	Gammaproteobacteria
KAKJ8	1192	<i>Aurantimonas corallicida</i> WP1	94%	NR_042319.1	Alphaproteobacteria
KAKJ9	1311	<i>Rhodococcus rhodochrous</i> DSM 43241	95%	NR_116689.1	Actinobacteria

It is predicted that one of the factors associated with high ice ice occurrence in the field is the high incidence of epiphytes. Moreover, [16] found that the combined effect of stress and biotic agents, such as opportunistic bacteria are primary factors of the ice-ice disease. Compared to previous research, [5] found that bacterial genera of *Cytophage*, *Aeromonas*, *Pseudomonas*, *Alteromonas*, *Flavobacterium*, *Arthrobacter* and *Vibrio* have been associated with the diseased seaweed *K. alvarezii*. This study showed that molecular identification of bacteria isolates have not been reported

from previous study. The difference leads to an assumption that the bacteria have site specific properties in expressing their pathogenity [1]. The 16S rRNA gene sequencing assist resolved the exact taxonomic position of seaweed bacteria, and provided more detail information on their phylogenetic position among their closest relatives (Table. 1 and Figure. 2).

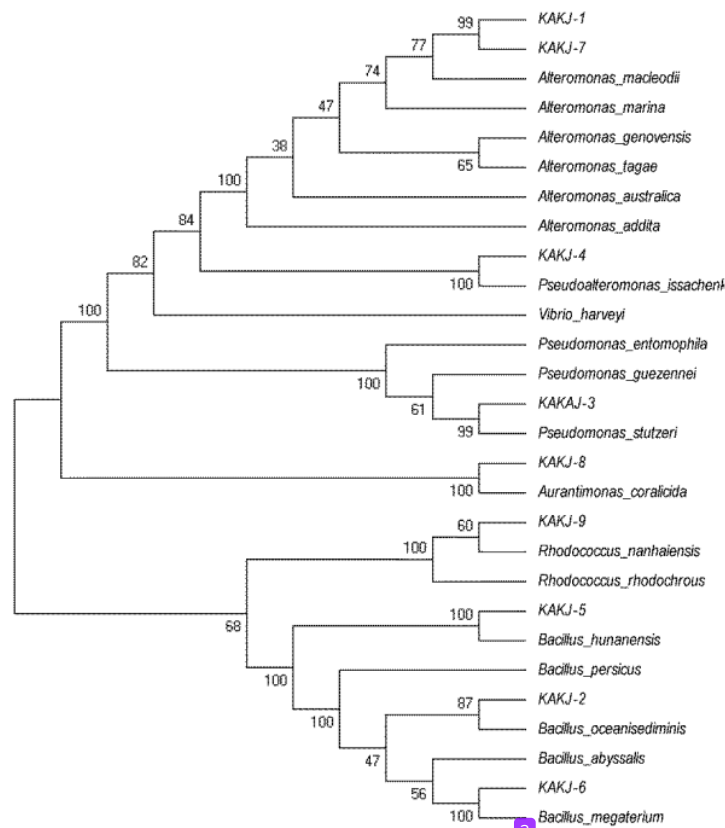


Figure 2. Phylogenetic tree of the causative agent of ice-ice disease based on the 16S rDNA sequence.

The similarity analysis showed that the isolates were between 91% and 97%. Among the isolates, strain KAKJ 5 (NR_108948.1) was similar to *Bacillus hunanensis* JSM 081003, while KAKJ 6 (NR_116873.1) similar to *Bacillus megaterium* ATCC 14581 had the higher similarity (97%) and the lowest similarity strain was found in KAKJ2, which had 91% similarity to *Bacillus oceanisediminis* H2. To estimate genetic affiliation of the pathogenic bacteria cause ice-ice disease on seaweed, a neighbor-joining tree including identified isolates and representatives marine microorganisms was constructed. A phylogenetic analysis of the 16S rDNA data for selected strains belonging to the group of the Gammaproteobacteria, Bacilli, Alphaproteobacteria, and Actinobacteria produced the dendrogram

shown in Figure 2. This comparison was made to determine the species to which the nine selected isolates are most closely related and to determine how closely the four taxa are related to each other.

3.2. Pathogenicity test

Among the nine strains screened for pathogenicity ability on healthy thallus seaweed, strains designated as KAKJ1, KAKJ4 and KAKJ8 indicated initial pathogenic activity, the thallus were distinct and showed obvious thallus deterioration as compared to the control and other bacterial species (Figure 3). The thallus seaweed inoculated with *A. macleodii* 107 (KAKJ1) showed ice-ice symptoms after 1 day post infection (1 in score), where as the thallus seaweed inoculated with *P. issachenkonii* KMM 3549 (KAKJ4) and *A. corallicida* WP1 (KAKJ8) showed symptoms after 3 days post infection (1 in score), respectively (Figure 3). After 7 days post incubation, thallus inoculated with *A. macleodii* 107 (KAKJ1) had white margins of 8,4 mm in length (3,67 in score), higher compared to other isolates (Figure 3). Bleaching and fragmentation intensified as incubation time progressed. The experimental controls remained without any symptoms for the entire duration of the experiment. These results revealed that the thallus seaweed with *A. Macleodii* 107 showed symptoms earlier as compared to other remaining pathogenic bacteria.

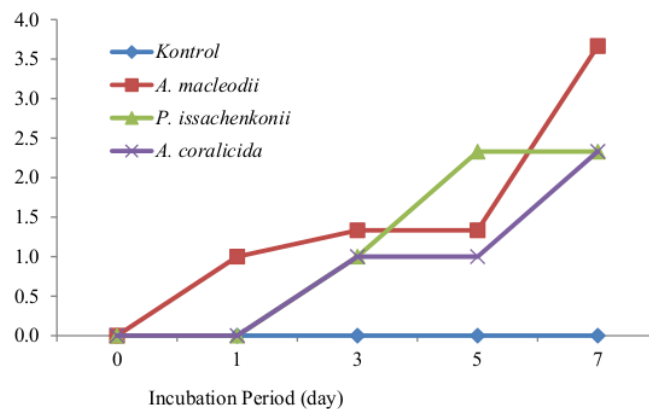


Figure 3. The average indicator of ice-ice disease symptoms on seaweed *K. alvarezii* in score during the 7 days of infection.

The pathogenicity test is the main criterion for the identification of bacteria suspected of being the aetiological agents of a plant disease. In the present pathogenicity studies, three bacterial isolates of this study were found to be pathogenic to the seaweed *K. alvarezii* as compared by inoculation method, where *A. macleodii* was found to be the worst, followed by *P. issachenkonii* KMM 3549 and *A. Corallicida* WP1. Hence, based on pathogenicity test, *A. macleodii* 107 qualifies itself as the symptom-causing bacterium. This genus was defined as comprising heterotrophic, catalase and oxidase-positive, gram-negative rods, motile by a single polar flagellum, which produce buds, grows at 15-37 °C and pH 6-9 and require NaCl for growth and positive for hydrolysis of gelatin and starch, can utilize cellobiose, D-fructose, D-glucose, D-mannose, L-lactose and maltose [17].

The ability of *A. macleodii* 107 to produce algal component-degrading enzymes and utilize various algal components may possibly be contributory factors to the development of ice-ice disease in

cultivated red seaweeds. Carrageenan and cellulose is a polysaccharide cell wall component of *Kappaphycus* species and constitutes the bulk of the cell's interstitial matrices [18]. According to [4], ice-ice disease led to a significant decrease in seaweed production and decrease in carrageenan yield and average molecular weight of carrageenan extract compared to the healthy crop ranging from 25 to 40 %. This depolymerization was attributed to the carrageenolytic activity produced and secreted by occasional bacteria. Cellulase and carrageenase isolated from bacteria are able to release epidermal and medullary protoplasm in *K. alvarezii* [19]. According to [16], these hydrolytic enzymes might be a factor in the whitening/bleaching observed in seaweed thalli during ice-ice infection. During infection the thalli at high concentration and start to utilize carrageenan, penetrate into the medulla of the seaweed thallus could cause epidermal degradation and destruction of the cell's pigment-containing plastids, resulting in the initial bleaching of the infected part [16,20].

The development of ice-ice disease in *K. alvarezii* depends on several factors to which the seaweed was exposed. [16] found that the combined effect of stress and biotic agents, such as opportunistic pathogenic bacteria, are primary factors of the ice-ice disease. The infection of the seaweed by these pathogenic bacteria may depend on their ability to successfully attach to the seaweed surface. The attachment of *A. macleodii* 107 to the seaweed thalli was required for the bacterium to promote the ice-ice disease and it is enhanced by the flagellum of the microorganism. Previous studies on the behavior of motile and non motile bacteria of *Pseudomonas fluorescens* showed that motile strains colonized the substrate more successfully than non-motile ones, due to their active flagella [21]. This may explain the possible success of higher number of *A. macleodii* 107 colonised onto the seaweed surface and start hydrolytic activity inducing the symptomatic of ice-ice disease.

Another factor shown to affect the attachment of *A. macleodii* 107 was water movement. [16] observed that continuous stirring during cultivation diminishes bacterial fixation. In the present study, water motion (continuous stirring) was not introduced during disease induction, when stirring was not used, some of the inoculated *A. macleodii* 107 cells were able to colonise the seaweed surface.

4. Conclusion

Based on the findings presented in this study, it can be concluded that among isolates which mainly appeared on ice-ice branches, three strains, designated as *A. macleodii* 107, *P. issachenkonii* KMM 3549 and *A. coralicida* WP1 were found to be pathogenic to the seaweed *K. alvarezii* as compared by inoculation period, where *A. macleodii* 107 was found to be the worst.

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