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Molecular Detection and Phylogenetic of Milky Hemolymph Disease: Case From Different Locations in Indonesia

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Abstract

Among the high significance diseases in spiny lobster farming, Milky Hemolymph Disease of Spiny Lobster (MHD-SL) is considered as the most devastating disease which caused in mortality up to 80 % and morbidity to 100%. Investigations of this disease were mostly performed for farmed lobsters, and no observation was conducted for wild lobsters. This study was carried out to identify the MHD-SL by PCR from different locations and observe the phylogenetic in comparison with several geographical regions. Adult farmed lobsters were collected from Lombok, Pangandaran, and Pegametan, while wild lobsters were obtained from Jembrana, Lombok, and Banyuwangi coastal areas. One farmed lobster from Pegametan and two farmed lobsters from Lombok were infected with the milky disease. PCR indicated the positive MHD-SL infection in a 254 bp band. Nucleotide sequence analysis demonstrated that the milky disease obtained in this study (PGMMHD-1, LOMMHD-2, and LOMMHD-3) exhibited a 99% homologous nucleotide sequence with milky disease agent from Vietnam that was uncultured Rickettsia-like Bacteria (RLB). This paper is the first report on phylogenetic analysis of milky disease from Indonesia that pointed out a closed relationship between milky disease in the present study and from Vietnam, Madagascar, Mozambique, and Tanzania.

INTRODUCTION

The most destroying disease in the farming of spiny lobster is Milky Hemolymph Disease of Spiny Lobster (MHD-SL) (Callinan and Corsin, 2009; Vu *et al*., 2014). MHD-SL is a descriptive name for the disease affecting spiny lobsters, with the syndrome is that the hemolymph of the diseased individuals become progressively turbid and milky appearance in severely affected lobsters. This appearance is caused by the enormous numbers of rickettsia-like bacteria (RLB) spreading in the hemolymph. The increase in turbidity (milkier appearance) of the hemolymph commonly relates to the rise in the moribund stage of the infected lobsters (Nunan *et al*., 2010).

Milky hemolymph disease had been recorded firstly in Vietnam, and later in Indonesia, that caused considerably high economic losses. Investigation in Vietnam in 2006 indicated that annual mortalities reached to 100%. It was reported that > 90% of farms had been devastated by this disease, and 88% of the farms suffered an ongoing outbreak in 2007. Outbreaks in a single cage were usually prolonged, with mortality of 2-5 animals per day. As a consequence, it took up only two months for the whole cage population to be lost,

when ~100 lobsters per cage were stocked (Callinan and Corsin, 2009).

Pieces of evidence imply that research is most importantly required on MHD-SL because of several reasons. It was stated that the disease could re-emerge at epidemic levels if the causative agent (RLB) become resistant to antibiotics and spread widely (Callinan and Corsin, 2009). Some literature reported that milky disease was controlled by the use of oxytetracycline (Callinan and Corsin, 2009; Thuy *et al*., 2010; DAFF, 2012; FAO, 2015). Another critical reason is that MHD-SL is to be viewed become pandemic and could affect lobster farming in other countries when the movement of the animals between countries is not regulated appropriately (Callinan and Corsin, 2009).

Milky disease in farmed lobster had been studied in Vietnam by Lightner *et al*. (2008), Hoa *et al*. (2009), Nunan *et al*. (2010), Thuy *et al*. (2010), Thuy (2011), and Vu *et al*. (2014). Nunan *et al*. (2010) demonstrated that the causative agent of milky disease in farmed *Panulirus* spp. from Vietnam was firmly related to the milky disease in other decapods crustaceans from Madagascar, Mozambique, Tanzania, and the UK. The strong relationship among these milky disease agents was indicated by the high similarity of its nucleotide sequence. In Indonesia, a study on molecular detection and cloning for milky disease agents in farmed lobster had been done in Lombok by Koesharyani *et al*. (2016).

To date, no research had been performed to investigate any occurrence of milky disease in wild lobsters. Also, there has been no available data on phylogenetic analysis of milky disease agents from Indonesia compared to those of the previous studies. It is essential to know whether milky disease from Indonesia is the same as a milky disease from other countries. Therefore, the present study was conducted to identify the MHD-SL from different locations by the molecular method and observe the phylogenetic in comparison with several geographical regions.

METHODOLOGY

Time and Place

Wild lobsters were obtained from Lombok, Jembrana, and Banyuwangi coastal areas, while farmed lobsters were collected from net cages in Lombok, Pangandaran, and Pegametan (Figure 1). A total of five lobsters were examined from each location, except for the location of Pegametan bay, ten lobsters were collected. Sample collection from Banyuwangi (mean weight = 203.18± 49.33 g), Lombok (mean weight = 211.6±34.16 g), Pangandaran (mean weight = 128.95±30.07 g), Jembrana (mean weight = 76.20±14.32 g) and Pegametan (mean weight = 123.00± 26.11 g) were performed in March, April, August, October and November 2016, respectively. All the lobsters were dry-transported to Institute for Mariculture Research and Fisheries Extension (IMRAFE), Gondol-Bali.

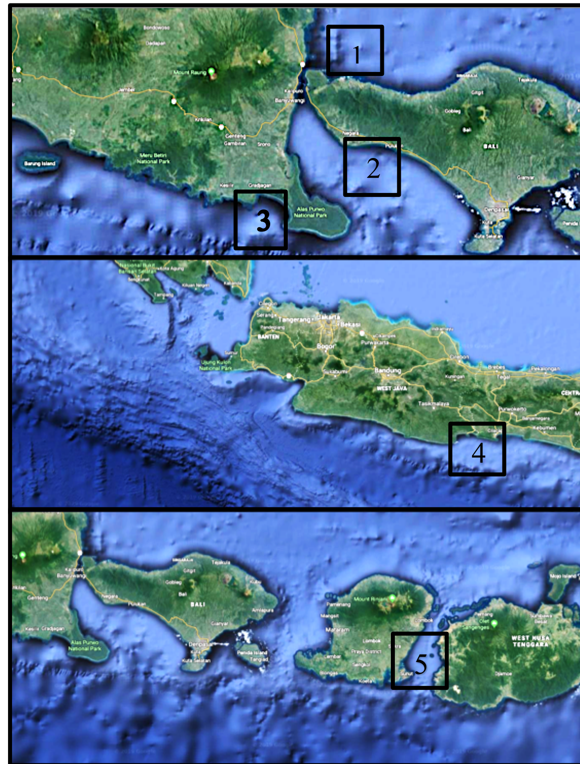

Figure 1. Sampling locations: 1) Pegametan bay, north Bali, 2) Pekutatan-Jembrana, south Bali, 3) Grajagan-Banyuwangi, 4) Pangandaran, west Java, 5) Telong Elong bay, Lombok.
Source: google map, 2019.

Research Material

Detection of milky disease agent was conducted by PCR analysis to find the presence of the causative agent of milky disease (Rickettsia-like Bacteria). Genomic DNA was extracted from muscle tissues by adding 200 – 250 μl chelex 10% in TE buffer (pH 8) in 1.5 ml microtubes, and then homogenized finely. Following that, 5– 7.5 μl pK (20 mg/ml) was administered into the microtubes. Subsequently, it was incubated first at 55°C for 2.5 h, and the second incubation was performed at 89°C for 8 min. The last step was centrifugation for 5 – 7.5 min at 13000 rpm. The supernatant was then collected and maintained at -20°C for PCR analysis. This method was modified from Martín -Platero *et al*. (2010).

Work Procedures

PCR was carried out using primer pair 254F and 254R for the targeted amplicon size 254 bp. These primers are specific for RLB. Oligonucleotide sequences of the 254F and 254R primers are 5'-CGA-GGA-CCA-GAG-ATG-GAC-CTT-3' and 5'-GCT-CAT-TGT-CAC-CGC-CAT-TGT-3', respectively (OIE, 2007; Thuy, 2011).

PCR mix was comprised of 2x KAPA 2G Fast Ready Mix, primers 254F and 254R 10μM each, DNA template, and NFW, total reaction of 10 μl. PCR was completed using Personal Thermal cycler. The thermal cycling was following Koesharyani *et al.* (2016) with modifications. Initial denaturation was done at 96°C (3 min.); then, 30 cycles of each denaturation at 96°C (15 sec.), annealing at 65°C (30 sec.), and extension at 72°C (15 sec.). The last phase was the final extension at 72°C (1 min.). The PCR products were kept at 4°C. PCR product separation was done by gel electrophoresis using 1.5% agarose gel in 1X TBE buffer with 0.05 μl/ml red safe DNA stain. A 100bp DNA ladder was used to measure the product under a UV-Transilluminator.

Three samples of PCR products (PGMMHD-1, LOMMHD-2, and LOMMHD-3) in a total volume of 50 μl containing the amplified ~254 bp fragment was purified using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's protocol. The purified DNA was sequenced using a DNA sequencer (Macrogen, South Korea) using two oligonucleotide primers, 254F and 254R for sequence determination. The forward and reverse DNA sequences generated in this study were then aligned using Bioedit (Hall, 1999; 2011).

Data Analysis

The sequences obtained were finally subjected to BLAST analysis in the NCBI nr database. A phylogeny tree was built to demonstrate a general picture of the relationship between the nucleotide sequences of milky disease obtained in this study (PGMMHD-1, LOMMHD-2, and LOMMHD-3) and other related nucleotide sequences resulted from BLAST analysis (8 nucleotide sequences). The phylogeny tree also included 4 nucleotide sequences of milky disease agents in crab and shrimp.

The accession numbers of these milky disease agents were as follows: MOZMHD milky disease (Accession no. GU947655.1); MADMHD (Accession no. GU947656.1); TANMHD milky disease (accession no. GU947657.1); and *Carcinus maenas* milky disease (Accession no. AM749067). Alignment Explorer (CLUSTAL) in MEGA 6.0 was used to align the DNA sequences generated in this study. A dendrogram was made using the Neighbor-Joining (NJ) method (Saitou and Nei, 1987). The robustness for each branch was evaluated by bootstrapping with 1000 replications (Felsenstein, 1985). The evolutionary distances were computed using the p-distance method (Nei and Kumar, 2000). Evolutionary analyses were accomplished in MEGA6 (Tamura *et al*., 2013).

RESULTS AND DISCUSSION

The Occurence of Milky Disease

Detection of milky disease in wild and farmed lobsters was conducted by PCR analysis using 254F and 254R primers. Samples showed a DNA band of 254bp that indicated the presence of Rickettsia-Like Bacteria (the causative agent of milky disease) in muscle were stated as infected by MHD-SL (Fig. 2). Three farmed lobsters out of 35 samples were detected to be infected with the disease. The infected lobsters were one farmed lobster from Pegametan Bay, and two farmed lobsters from Lombok. PCR products of the positive infection were then labeled as PGMMHD-1, LOMMHD-2, and LOMMHD-3, and used for gene sequencing and phylogenetic analysis. PGMMHD-1 infects farmed lobster from Pegametan, while LOMMHD-2 and LOMMHD-3 infect farmed lobster from Lombok. There was no milky disease detected from the wild lobsters.

This study found that milky disease was only detected in farmed lobsters and not in wild lobsters. One farmed lobster from Pegametan (North Bali) and two samples from Lombok were infected with the milky disease. In contrast, no farmed lobster from Pangandaran was affected by the disease. Geographically, Lombok and Bali are two adjacent islands that separated by the strait between the two islands. Meanwhile, Pangandaran is located in West Java, which is separated by long distances from Bali and Lombok. It was likely that the disease could spread in between the two islands. However, it was no clarity about the route of milky disease infection in those two locations. The results suggest that biosecurity and quarantine procedures need to be actively applied to prevent milky disease introduction and translocation, particularly in Indonesia.

Finding in this study indicated that the causative agent of the milky disease emerged only in farming conditions. Some factors may likely contribute to disease introduction and transmission. A finding

by Callinan and Corsin (2009) suggested that poor hygiene could increase the risk of diseases. Lower mortalities and economic losses were experienced by farmers who dispose of waste away from the farming locations and clean their nets regularly. This indicates that the RLB is an opportunist pathogen; however, its sources in the environment are unidentified (Callinan and Corsin, 2009; Vu *et al*., 2014). A critical study in Vietnam reported that 51 fishery products used as feed for lobsters such as marine crab, goatfish, penguin wing oyster, flounder, apple snail, sardine, mantis shrimp, pony fish, anchovy were negative for RLB (Thuy, 2011). Therefore, they stated that the possibility of RLB to spread from fresh feeds to farmed lobsters is very low. However, the time and areas of their investigations were limited, so that they could not find RLB in those feeds. It was advised that the collection of fresh food samples for the detection of RLB should be conducted every month, especially at the time of milky disease outbreak using a higher number of feed samples, and in more farming areas. Therefore, more reliable results would be obtained.

Nucleotide Sequence and Phylogenetic Analysis of Milky Disease Agent

Nucleotide sequence and phylogenetic analysis of the three samples of milky disease (PGMMHD-1, LOMMHD-2, and LOMMHD-3) revealed that the causative agent of milky disease in the present study was similar to the milky disease agent from Vietnam that was uncultured bacterium clone RLBVN 1 (HQ130336. 1), RLBVN 2 (HQ130337.1), and VIETMHD (GU947658.1) (Fig. 3). Further, the analysis of the phylogenetic tree verified that the bacteria were closely related. The phylogenetic tree demonstrated that PGMMHD-1 was closely linked with LOMMHD-2 and LOMMHD-3.

Furthermore, the strong bootstrap value has existed between the milky disease found in this study and RLBVN 1, RLBVN 2, and VIETMHD isolates. This closed relationship was supported by the high percentage of the homologous sequences (99% similarity). Therefore, the milky disease agent obtained in this study was grouped with a milky disease agent from Vietnam. The dendrogram also demonstrated that milky disease agent in this study was closely related to milky disease agent from Mozambique (MOZMHD: GU947655. 1), Madagascar (MADMHD: GU947656.1), Tanzania (TANMHD: GU947657.1) and *Carcinus maenas* milky disease (AM749067) from the UK, which was indicated by strong bootstrap values.

The agent of milky disease in cultured lobster in Vietnam was classified in family Rickettsiaceae, which is Rickettsia-like Bacteria (Hoa *et al*., 2009). Previous studies on milky hemolymph diseases or milky hemolymph syndrome (MHS) caused by RLB recommended that the condition might be an ‘old’ disease which had been found in crab *Carcinus mediterraneous* Czerniavski (Bonami and Pappalardo, 1980) and *Penaeus monodon* (Anderson *et al*., 1987).

However, later studies showed that this disease could reemerge in other decapod species (in crab *Carcinus maenas*, shrimp *Penaeus monodon,* and *Panulirus* spp.) in new geographical areas where they were cultivated or farmed. These new geographic areas on three continents included the UK, East Africa, and Madagascar and in Asia, particularly in Vietnam (Nunan *et al*., 2010; Thuy, 2011; Vu *et al*., 2014). In Asia, milky disease had also emerged in lobster *Panulirus homarus* in Lombok, Indonesia (Koesharyani *et al*., 2016). Besides, the milky disease in farmed *P. homarus* was also occurred in Pegametan Bay, North Bali, and in Lombok, Indonesia, that had been recorded by the present paper.

Rickettsia-like Bacteria had been known as an uncultured bacterium that could not be cultured in normal agar-based nutrient mediums (Hoa *et al*., 2009). RLB strain cannot be cultivated on

tryptic soy agar, Marine Agar 2216, Brain-heart Infusion agar, Thiosulfate-citrate-bile salts-sucrose agar, and Leibovitz's L-15 Medium (Eddy *et al*., 2007; Thuy, 2011; Vu *et al*., 2014). The first report on the possibility of cultivating RLB-MHS by cell culture technology was performed using Grouper Embryonic (GE) cell line. RLB from MHS lobster could be cultured on the GE cell line that was indicated by signals of intracellular parasitism observed in GE cell culture after three days of cultivation. Unfortunately, further experiments could not be conducted due to limited equipment and facilities (Vu *et al*., 2014). As a consequence, to date, there has been no thriving culture of RLB using cell culture technology. Therefore, studies on culturing lobster RLB are needed in order to answer the questions on how to eradicate the disease without the use of antibiotics.

Although high similarities were obtained between milky disease in the present study with those of previous studies, we had a different method in performing gene sequencing. The authors of the earlier studies conducted gene sequencing from the cloned of milky disease RLB (Nunan *et al*., 2010; Thuy, 2011), whereas in our study, gene sequencing was done from the PCR products of the positive infection. This is our limitation in performing this study. However, our paper is the first report on sequencing and phylogenetic analysis of milky disease from Indonesia, which illustrates the relationship among milky disease from some countries. Considering the limitation of our study, there are opportunities for other researchers to conduct gene sequencing from the cloning of milky disease agents from Indonesia. If this research could be done, then we can compare our results with the suggested study.

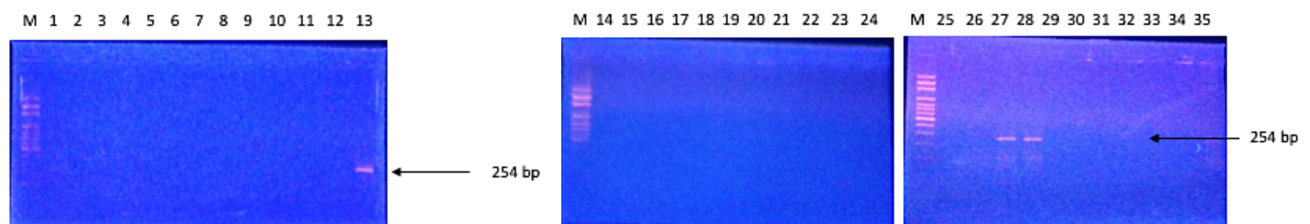

Figure 2. PCR image amplification for the detection of milky disease in wild and farmed lobster *P. homarus* using primer 254F and 254R. M = marker DNA ladder 100 bp, 1-5 = Banyuwangi wild lobster, 6–10 = Lombok wild lobster, 11-15 = Pegametan farmed lobster, 16-20 = Pangandaran farmed lobster, 21-25 = Jembrana wild lobster, 26-30 = Lombok farmed lobster, 31-35 = Pegametan farmed lobster. The positive milky disease infection was expressed in a 254 bp band. Only 3/35 lobsters were infected with the milky disease; 2 samples from farmed lobster in Lombok, and 1 sample from farmed lobster in Pegametan bay. The milky disease was not detected in wild lobsters.

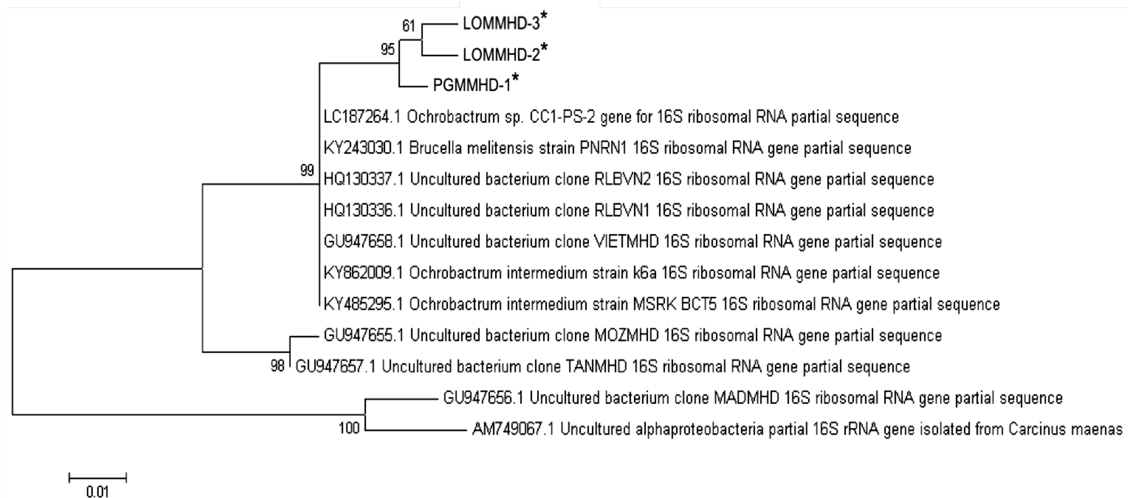

Figure 3. Phylogenetic tree constructed based on the neighbor-joining (NJ) method of the milky hemolymph disease of spiny lobster (MHD-SL) nucleotide sequences from farmed spiny lobster *P. homarus*. Numbers at the branch points show bootstrap confidence values (1000 replicates). The milky disease found in this study is indicated with * symbols (PGMMHD-1, LOMMHD-2, and LOMMHD-3). All the three milky diseases obtained in this study were clustered with the causative agents of milky diseases in Vietnam that are uncultured bacterium clone RLBVN 1 (HQ130336.1) (99% similarity), RLBVN 2 (HQ130337.1) (99% similarity), and VIETMHD (GU947658.1) (99% similarity). Milky disease in this study was closely related with MOZMHD (GU947655.1) (98% similarity), MADMHD (GU947656.1) (100% similarity), TANMHD (GU947657.1) (98% similarity) and *Carcinus maenas* milky disease (AM749067) (100% similarity).

CONCLUSION

This study found that milky disease was only detected in farmed lobster *P. homarus*, and no milky disease was observed in wild lobsters. Therefore, it is required to investigate the source of infection in the farmed lobsters. The causative agent of milky disease obtained in this study was similar (99%) to milky disease agents in Vietnam, which was classified as a group of uncultured bacteria that cannot be cultured using various types of bacterial media culture. As a consequence, further study is needed, especially on how to culture the rickettsia-like bacteria through cell culture technology.

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