

CORRESPONDENCE

TITLE : MOLECULAR CHARACTERIZATION OF VIBRIOSIS ASSOCIATED BACTERIA FROM TRADITIONAL MUD-CRAB FARMED IN THE NORTH COAST OF CENTRAL JAVA, INDONESIA

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Sarjito Sarjito <sarjito@live.undip.ac.id>

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1 message

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<onbehalf@manuscriptcentral.com>

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Reply-To: sjst@psu.ac.th

To: sarjito@live.undip.ac.id, resti_wisnoe@yahoo.com

04-Jun-2020

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Molecular Characterization of vibriosis clinical signs-associated bacteria from traditional mud-crab farming in the North Coast of Central Java, Indonesia

Sarjito*¹, Alfabetian Harjuno Condro Haditomo¹, Restiana Wisnu Ariyati¹, Aninditia Sabdaningsih², Desrina¹, and Slamet Budi Prayitno¹

¹ Aquaculture Study Program, Aquaculture Department, Fisheries and Marine Science Faculty, Diponegoro University, Jl. Prof. Soedarto SH, Tembalang, Semarang 50275, Indonesia

² Department of Aquatic Resources, Fisheries and Marine Science Faculty, Diponegoro University, Jl. Prof. Soedarto SH, Tembalang, Semarang, Indonesia

*Email : sarjito@live.undip.ac.id; Phone +6282135870099

ABSTRACT

Mud crabs that infected by *Vibrio* bacteria can transmit diseases to the healthy cultured crustaceans such as shrimp. Moreover, the 90% mortality in mud crab life cycle stages in the traditional farming caused by *Vibrio*. Limited information on the epidemic season and the diversity of vibrio associated with mud crab in Central Java reduces options of crab farmers to prevent disease outbreaks and crop failures. This study assessed the molecular characterization of bacteria associated to clinical vibriosis in mud crab farming from the North Coast of Central Java. Twenty-five bacteria were isolated from hepatopancreas, gill and carapace of mud crab with clinical signs of vibriosis that cultured in TCBS and TSA medium. After a modified rep-PCR and further PCR amplification of 16S ribosomal RNA gene, the sequence analysis was performed. The DNA sequences were then compared with the BLAST from NCBI database, and the phylogenetic tree was constructed in accordance to the MEGA X program to identify the genus of the bacteria. The clinical signs on mud crab were associated with seven isolates, such as CJR14, CJR15, CJD23, CJK10, CJK11, CJD22, and CJR05. Based on 16S rRNA sequence analysis, these isolates were closely (92-99%) related to: *Vibrio harveyi* NCIMB1280, *Catenococcus thiocicly* TG 5-3, *Photobacterium ganghwense* FR311, *Vibrio prahaemolyticus* ATCC 1780, *Shewanella loihica* PV4, *Shewanella algae* ATCC5, and *Vibrio alginolyticus* NBRC 15630, respectively. The result of this study can be used as a preliminary design of prevention method to reduce bacterial diseases outbreak of Mud crab farming.

Keywords: bacterial diseases, mud crab, 16S rRNA, rep PCR, Vibriosis

INTRODUCTION

Mud crab, *Scylla serrata* (Forskål, 1775), is traditionally caught in and around brackish water ponds and mangrove forests along the North Coast of Central Java, Indonesia. The mangrove forests and its soils are these crab's origin habitat. Traditional brackish water pond is used for polyculture of shrimp and milk fish, Wild mud crab may intrude in these ponds, where they are caught together with the shrimp and milkfish. However, the culture of the mud crab themselves is spreading. The intrusion of wild mud crab can be a problem to crustacean's culture. Infected mud crab can carry diseases agents that may infect the healthy cultured crustaceans. To prevent damage of the crustacean culture, farmers may disinfect the pond and raise barriers that are well embedded in the bunds, but these efforts are rarely 100% effective. Bacterial diseases, especially vibriosis, was found to be a problem in fattening of the mud crab culture in Pemalang (Sarjito *et al.*, 2016) and reported to cause more than 90% mortality in all life cycle stages of mud crab growth (Jithendran *et al.*, 2010). Reported clinical signs of infected mud crab are wounds on body, decreased feed response and weakening (Jithendran *et al.*, 2010), blackening and red spots of the carapace (Sarjito *et al.*, 2014 and Sarjito *et al.*, 2016).

Vibriosis is a bacterial disease that was caused by genus vibrio bacteria (Lavilla-Pitogo *et al.*, 2004; Jithendran *et al.*, 2010). *Vibrio*'s cause frequent disease outbreaks and serious economic losses in shrimp culture (Aquilera-Rivera *et al.*, 2019) and have the potential to destroy the future of industrial culture of mud crab. Several species of *Vibrio* spp. were reported in and found to be associated with bacterial disease in mud crab: *V. fischer I*, and *V. nereis* (Wang, 2011); *V. alginolyticus* and *V. cholerae* (Najiah *et al.*, 2010 ; Wang, 2011); *Vibrio vulnificus* (Lavilla *et al.*, 2004; Shanmuga, 2008; Wang, 2011), *V. splendidus*, and *V. orientalis* (Jithendran *et al.*, 2010); *V. ordalii* (Sarjito *et al.*, 2014), *V. harrveyi* (Sarjito *et al.*, 2014; Jithendran *et al.*, 2010), *V. campbelli* (Shanmuga., 2008), and *V. parahaemolyticus* (Lavilla *et al.*, 2004; Najiah *et al.*, 2010; Shanmuga, 2008). Previous research focused on the richness of bacteria associated to vibriosis in mud crab in Central Java. The limited knowledge is available on the diversity of vibrios related to the disease outbreaks in mud crab. Local government tends to stimulate the culture of mud crab but the program lacks information on disease identification and prevention. This study was done to assess the relationship between clinical signs of vibriosis on mud crab, and the diversity of bacteria in mud crab of traditional brackish water ponds along the North Coast of Central Java, Indonesia.

MATERIALS AND METHODS

Sampling

Mud crab *S. serrata*, were collected from nine brackish water ponds of Kendal (3 ponds), Demak (3 ponds) and Rembang (3 ponds) Regency, Central Java. These three locations are the largest producers of cultivated crabs in Central Java, Indonesia (Picture 1). Based on the clinical signs for vibriosis described by Jithendran *et al.* (2010) 45 infected mud crab were selected, stored in a sterile container and carried to the Integrated Laboratory of Diponegoro University for further analysis. The average size of the caught crab, measured from 13 to 15 cm, was 14.4 ± 0.7 cm. The clinical signs, such as: red spots or brown spots on the carapace and wounds in the abdomen. Brown spot may occur due to the infection of chitinolytic bacteria that catabolize the chitin of the carapace and cause erosion and melanisation (dark brown to black pigmentation) at the site of infection (Jithendran *et al.*, 2010).



Figure 1. Research Location represented in a small red box in the left of map.

Bacterial Isolation

Forty five bacteria were isolated from gills, hemolymph, hepatopancreas, and wounds on the carapace of the infected mud crabs after culture on TCBS (Thiosulfate Citrate Bile Salts

Sucrose) and TSA (Tryptic Soy Agar) (Thomson, 2004). The colonies were purified by the streak method based on morphological appearance (Sarjito *et al.*, 2015).

Repetitive Genomic Sequences-PCR (Rep-PCR)

The isolates were analyzed using the rep-PCR of Radjasa *et al.* (2007) modified by Sarjito *et al.* (2009) in the rep-PCR, BOX AIR (5'-CTACggCAAggCgACgCTgACg-3'). The REP 1R-I and REP 2-I primers contained nucleotide inosine (I) at ambiguous position in the REP consensus. The mix PCR reagent contain of 1µL DNA template (diluted 100X), 1 µL primer, 7.5 µL Megamix Royal and 5.5 µL ddH₂O. Amplification was performed in a thermal cycler model Gene Amp PCR system 9700 with the following protocol: initial denaturation at 95°C for 5 minutes; 30 cycles of (denaturation 92°C for 1 minutes, annealing 50°C for 1,5 minutes, extension 68°C for 8 minutes) and final extension at 68°C for 10 minutes. The band DNA was visualized with injected 5 µL PCR products into 1% agarose gel and run to electrophoresis machine using 1X TBE buffer and observed in UV-transilluminator, as described by Radjasa *et al.* (2007). Grouping of isolates was conducted based on the method of Radjasa *et al.* (2007). Matrices where made from the bands position on the gel which were analyzed by the Free Tree program using the UPGMA method for constructing the phylogenetic tree. Resampling was done by bootstrapping with 1000 replications.

PCR Amplification of 16s rRNA Gene Fragments

The PCR amplification of 16S rRNA gene was performed according to Radjasa *et al.* (2007). To find out a closely complete 16S rRNA gene, the amplification was performed with two primers, GM3F as a forward primer (5'AGAGTTTGATCMTGGC-3') and GM4R as a reverse primer (5'-TACCTTGTTACGACTT-3') (Sarjito *et al.*, 2009). Genomic DNA of bacteria for PCR analysis were obtained from the destruction cell materials that taken from fresh culture bacteria, suspended in sterile water (Sigma, Germany) and subjected to five cycles of freeze (-80°C) and thaw (95°C). The PCR product was then processed to find the band DNA in the right size around 1.500 bp. The sequencing step was facilitated by PT. Genetika Science. The determined DNA sequences of samples were then compared for homology using BLAST. To find the strain the phylogenetic tree was established using the MEGA X.

RESULTS

Clinical Signs and Bacterial Isolation

The most frequent clinical signs of the infected mud crab (*S. serrata*) were wounds on the surface of the claws, the ventral, and the carapace. The isolation of bacteria using TCBS and TSA medium obtained a total of 25 pure isolates bacteria from three replicates agar plates and then stored in NA (Nutrient Agar) medium. The clinical signs of red-brown and dark spots in the carapace were found in Figure 2. Similar clinical signs were found on the abdomen.



Figure 2. The clinical signs of mud crab (*S. serrata*) infected by bacterial diseases: (a.) Red/brown spots in the carapace; (b.) Red-brown spots in the carapace wound on the abdomen.

Repetitive Genomic Sequences-PCR (Rep-PCR)

The Repetitive PCR analysis and the dendrogram related the 25 pure isolates bacteria originating from mud crabs found a seven group from total samples (Figure 3). Furthermore, the molecular identification only represented by seven isolates from each seven group (CJR14, CJR15, CJD23, CJK10, CJK11, CJD22, and CJR05).

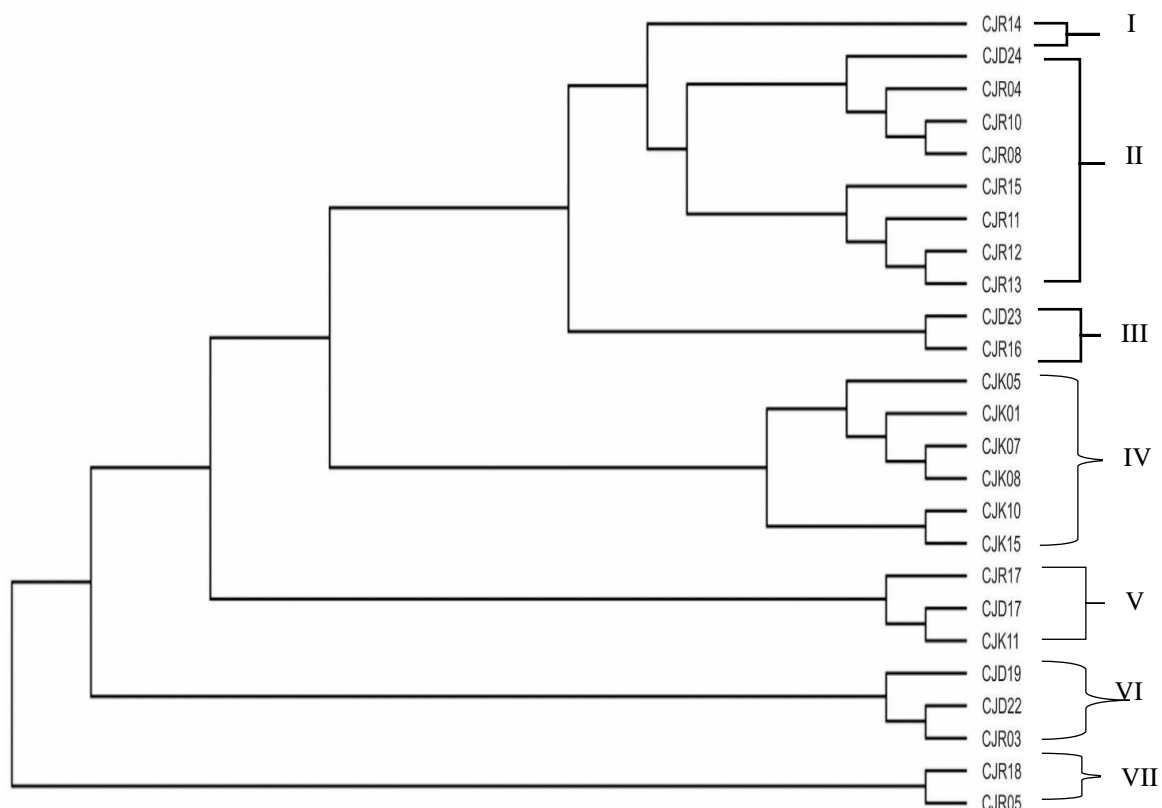


Figure 3. A dendrogram based on Rep-PCR of 25 Bacteria Isolates Associated with vibriosis clinical signs from traditional mud crab farming in the North Coastal of Central Java, Central Java, Indonesia.

Table 2. Molecular characterization of seven bacterial species associated with clinical signs of Vibriosis on mud crabs from North Coast of Central Java.

Isolates	Query Length	Close relative	Query Cover (%)	Homology (%)	Acc. Number
CJR14	1548	<i>Vibrio harveyi</i> strain NCIMB1280	98	95	NR 043165.1
CJR15	1319	<i>Catenococcus thiocicly</i> strain TG 5-3	98	98	NR 118258.1
CJD23	1387	<i>Photobacterium ganghwense</i> strain FR311	98	95	NR 043295.1

CJK10	1340	<i>Vibrio parahaemolyticus</i> ATCC 17802	99	97	NR 043689.1
CJK11	1335	<i>Shewanella loihica</i> strain PV4	97	97	NR 025794.1
CJD22	1488	<i>Shewanella algae</i> strain ATCC5192	95	92	NR 117771.1
CJR05	1362	<i>Vibrio alginolyticus</i> strain NBRC 15630	96	96	NR 122050.1

The seven vibrios associated with mud crab were closely (homology range between 95-99%) related to the *Vibrio harveyi* NCIMB1280 (CJR14), *Catenococcus thiocicly* TG 5-3 (CJR15), *Photobacterium ganghwense* FR311 (CJD23); *Vibrio prahaemolyticus* ATCC 17802 (CJK10); *Shewanella loihica* PV4 (CJK11); *Shewanella algae* ATCC5 (CJD22) and *Vibrio alginolyticus* NBRC 15630 (CJR05) (Table 2). The diversity of vibrios found in three samples locations (Table 3). We found more bacteria species in Rembang rather than in Demak and Kendal but the highest percentage of vibrio diversity compare to the total isolate in each location was in Demak (80%).

Diversity of Vibriosis Clinical Signs Associated Bacteria

The diversity of vibriosis clinical signs associated bacteria was compared from the three-sampling site to perform the percentage of species in each location (Table 3). Table 4 accommodates all of isolates that identified based on 16S rRNA gene.

Table 3. The diversity of Vibrios in Mud Crab from North Coast of Central Java per location.

Location	Code	Bacteria Species	Vibrios diversity	Isolate Number
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Rembang	CJR14;			
	CJR04;			
	CJR10;			
	CJR08;	<i>Vibrio harveyi</i> ,		
	CJR15;	<i>Catenococcus thiocicly</i> strain TG 5-3,		
	CJR11;	<i>Photobacterium ganghwense</i> strain FR311,		
	CJR12;	<i>Shewanella loihica</i> strain PV4,	6	13
	CJR13;	<i>Shewanella algae</i> strain ATCC5192,		
	CJR16;	<i>Vibrio alginolyticus</i> strain NBRC 15630,		
	CJR17;			
	CJR03;			
	CJR18;			
	CJR05			
Demak	CJD24;	<i>Catenococcus thiocicly</i> strain TG 5-3		
	CJD23;	<i>Photobacterium ganghwense</i> strain FR311		
	CJD17;	<i>Shewanella loihica</i> strain PV4	4	5
	CJD19;	<i>Shewanella algae</i> strain ATCC5192		
	CJD22			
Kendal	CJK05;			
	CJK01;			
	CJK07;	<i>Vibrio prahaemolyticus</i> ATCC 17802		
	CJK08;	<i>Shewanella loihica</i> strain PV4	2	7
	CJK10;			
	CJK15;			
	CJK11			

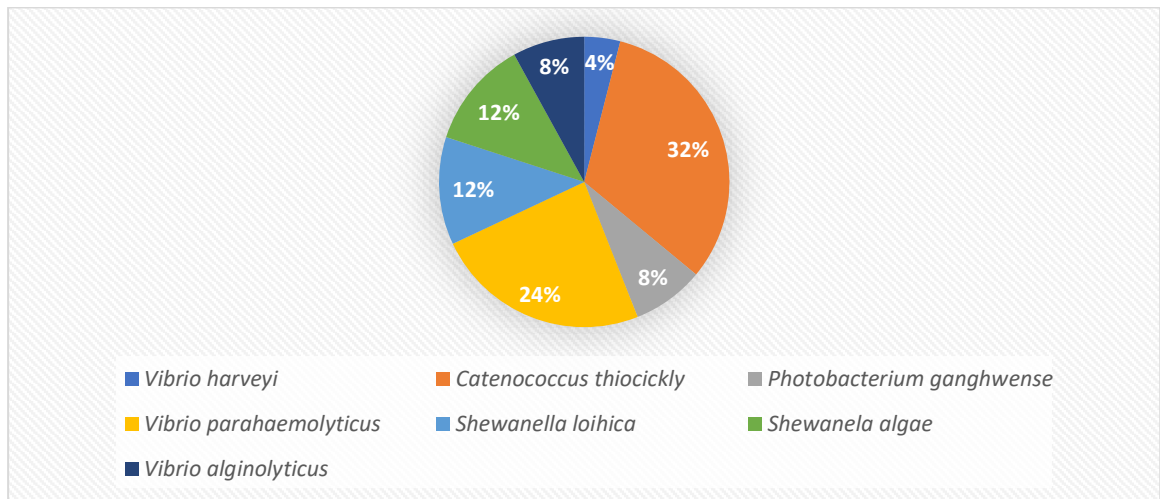


Figure 4. The bacterial diversity in Mud crab with clinical signs of Vibriosis from North Coast of Central Java.

Figure 4 represented the bacterial diversity in Mud crab with clinical signs of Vibriosis from North Coast of Central Java. The highest number species was *C. thiocicly* with the percentage 32%, while 24% was *V. parahaemolyticus*, *S. loihica* and *S. algae* in the equal percentage in 12%, as well as *P. ganghwense* and *V. alginolyticus* in 8%, and the lowest percentage was *V. harveyi* in 4%.

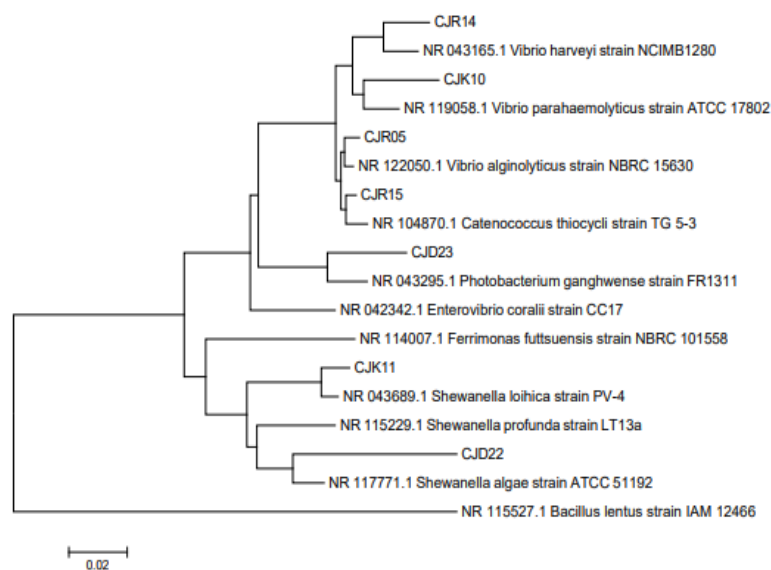


Figure 5. Phylogenetic tree of the bacteria associated with vibriosis clinical signs from traditional mud crab farming in the North Coast of Central Java, Indonesia constructed using Neighbour-Joining analysis with 1000 replicates.

In order to represent the relationship among isolates, the phylogenetic was constructed. Figure 5 shows that all of the isolates have assembled with the closely related species. The relationship between the genus performs that the bacteria genus *Vibrio* has the same clade with *Catenococcus thiocycli*. Those groups are more closely related compared to the group of *Shewanella* and *Photobacterium*.

DISCUSSION

Clinical signs

Vibriosis in mud crab was mostly known by red-brown or dark melanine spots; patches of light red spots as well as dark spots on the carapace, also wounds on the body (claws, carapace and the ventral). The identic of clinical signs have been found by Muyzer *et al.* (1995), Wang, (2011) and Lavilla *et al.* (2004). The clinical signs, such as: brown spot or red spots on the carapace and wounds in the abdomen (Figure 2) were also reported on the mud crabs that were affected by genus *vibrio* from gulf of Semarang (Sarjito *et al.*, 2014) and Pemalang (Sarjito *et al.*, 2016). 'Brown spot' may occur due to the infection of chitinolytic bacteria that catabolize the chitin of the carapace and cause erosion and melanisation (dark brown to black pigmentation) at the site of infection (Jithendran *et al.*, 2010).

The present study revealed that genus *vibrio* associated in mud crabs from traditional brackish water pond of North Coast of Central Java (Figure 1) were closely related to *V. harveyi* strain NCIMB1280 (CJR14), *C. thiocicly* strain TG 5-3 (CJR15), *P. ganghwense* strain FR311 (CJD23); *V. prahaemolyticus* ATCC 17802 (CJK10); *S. loihica* strain PV4 (CJK11); *S. algae* strain ATCC5 (CJD22) and *V. alginolyticus* strain NBRC 15630 (CJR05). This present result also revealed that diversity of vibrios associated with mud crab from brackish water pond of North Coastal of Central Java coast was lower than diversity that was found in cultured and wild crab in India (Jithendran *et al.*, 2010) and traditional brackish water surrounding of Semarang bay (Sarjito *et al.*, 2014).

In this study, four genus *Vibrionaceae* (*Vibrio*, *Shewanella*, *Photobacterium*, and *Catenococcus*) were found in mud crab. Three species of *vibrio* i.e: *V. parahaemolyticus* (CJK10), *V. harveyi* (CJR14) and *V. alginolyticus* (CJR05) also have been detected in this present study. The vibrios were frequently found as a causative agent of shell disease in *L. vannamei* shrimp and mud crab, *S. serrata*, (Estave and Herrere, 2000; Wang, 2011; soto-

Rodriguez *et al.*, 2012). *V. harveyi* has been reported as a causative agent of vibriosis in fattening mud crabs from brackish water pond in Pemalang coast (Sarjito *et al.*, 2016); extensive brackish water pond of surrounding of Gulf of Semarang (Sarjito *et al.*, 2014) and Malaysia (Najjah *et al.*, 2010). These bacteria were also reported as a causative agent of bacterial diseases in zoea stage of mud crab (Jithendran *et al.*, 2010 ; Department of Agriculture Fisheries and Forestry Fisheris Quensland 2012); adult mud crabs (Poornima *et al.*, 2012 ; Lavilla *et al.*, 2004). Moreover, Sarjito *et al.* (2014), found that this bacterium was a potential pathogen to mud crabs. The present study also revealed that *V. alginolyticus*, also found in mud crab, *S. serrata*, infected bacterial diseases in north central Java. *V. alginolyticus* was commonly reported from brackish water, estuary and marine environment. It was found as an opportunistic bacteria associated with crustacean culture and fish culture (Parenerngi *et al.*, 1993). Moreover, this bacterium was also found as an important pathogenic bacteria associated with infectious diseases in mud crab (Najjah *et al.*, 2010, Sarjito *et al.*, 2014) and shell disease of mud crab *S. serrata* grow out pond located at Mahendrapalli, Nagapattinam District, Tamil Nadu, India (Gunasekaran *et al.*, 2017), seafood, fish, shrimp, sediment and seawater (Austin and Austin, 2007).

Genus photobacterium was also found in marine water. These bacteria were reported from a sea-water sample in Ganghwa Island, South Korea. Marine bacterial strain, designated FR1311T. cells were Gram-negative, facultatively anaerobic, catalase and oxidase-positive, motile, oval or rod-shaped and halophilic (optimum sea-salt concentration for growth of 5–6 %) (Park *et al.*, 2006). This bacterium was defined as being facultatively anaerobic and weakly halophilic, and luminescent, alike most of the recognized species in the genus are luminescent. *P. ganghwense* has been described as being luminescent. Some of the luminescent species are symbionts of deep-sea fishes that hold them in specialized luminous organ (Lucena *et al.*, 2011).

The present study result showed that both *S. algae* and *S. loihica* were found associated with vibriosis in the mud crabs from traditional brackish water pond of North central java. The genus *Shewanella* is phylogenetically affiliated to the γ - Proteobacteria (Yoon *et al.*, 2004). This bacterium also closely related to genus vibrio or vibrionaceae. It has been reported as bacterial pathogen associated with massive mortality in *Scinopsis ocellata*, *Carrasius auratus* (Altun *et al.*, 2014); *Babylonia* (Shufang *et al.*, 2015); *Cynoglossus semilaevis* (Han *et al.*, 2017); and freshwater-cultured white leg shrimp, *P. vannamei* (Cao *et al.*, 2018). *Scianeops*

ocellatus (Zhang *et al.*, 2017). This bacterium causes mortalities of more than 50% of the white leg shrimps cultured in freshwater pond of Lianyuang, Jiansu province, China (Cao *et al.*, 2018).

Shewanella spp. are Gram-negative, motile bacilli. *Shewanella* spp. are found throughout the world, mainly in marine environments. The most important phenotypic characteristic of *Shewanella* spp is the production of hydrogen sulphide. They have an important role as turnover of organic material, and capable of dissimilatory reduction of various metals and other substances, such as Nitrate, Nitrite, Thiosulphate and Trimethylamine-N-oxide. In Demak the presence of *S. algae* were found only when the water temperature is more than 13°C this happend usually on July to August. This is happens because there are correlation between the salinity and the temperatur, where the isolation of *S. algae* can be performed with the salinity of 15-20%. Infection of *Shewanella* mostly happens in countries with a warm climate, or especially due to warm summers in countries with temperate climates (Holt *et al.*, 2003). *S. algae* was also found associated with milkfish gut collected from Northern Coast of Central Java (Prayitno *et al.*, 2015).

Shewanella loihica was isolated and identified from iron-rich microbial mats in the Pacific Ocean. The result shows that *S. loihica* is a Gram-negative, non-spore-forming, straight rod with a mean length of 1-8 mm and a mean width of 0-7 mm. Single flagella is used to motile. Facultative psychrotolerant anaerobe. The characteristic of the colonies are smooth, glistening, circular, flat to slightly raised, orange in color. Temperature 18 °C, pH 6.0-8.0 and Na⁺ are the optimum enviroment of *S. loihica* to grow. This bacterium was able to grow in over wide ranges of temperature and pH. The ranges of water quality that could support the growth of *S. loihica* are temperature (0–42°C), pH (4.5–10) and salt (0.5–5%). *S. loihica* plays a role as an agent of metal reduction and iron biomoneralization, based on that the existence of this bacterium can be caused by the metal content in the water. According to Newton *et al.*, (2009), the current study of *S. loihica* is focusing on the metal reduction and iron biomoneralization capabilities based on their phenotypic and phylogenetic characteristics.

Catenococcus thiocycli was identified by (Yarza *et al.*, 2013) the study found that *C. thiocycli* is part of genus Vibrionacea, order Vibrionales, class Gammaproteobacteria and

phylum Proteobacteri. This bacterium is also isolated from the Sediment in pacific ocean (David *et al.*, 2015).

Diversity of Vibriosis Clinical Signs Associated Bacteria

The diversity of vibriosis clinical signs associated bacteria was explored using molecular characterization. Rep-PCR was used to find out fingerprinting of bacterial genomes (Versalovic *et al.*, 1998). This method is an effective tool to distinguish the diversity of bacteria according to the number and size of bacterial repetitive sequences. This technique could prevent to analyse the similar isolate of bacteria based on its DNA fingerprinting. Therefore, rep-PCR is helpful to grouping *Vibrio* species. The dendrogram from Figure 3 show similarity between 25 isolates into 7 groups due to the difference of bacterial repetitive sequences. The representative isolates from each group was further examined using 16S rRNA gene for identification. A total of 7 isolates were identified and presented in Table 2. The range of homology percentage between 92-98%. The highest similarity was showed by isolate CJR15 from group II, 98% homology with *C. thiocicly* strain TG 5-3. The homology 97% was appointed to isolate CJK10 and CJK11 from group IV and V that similar to *V. parahaemolyticus* ATCC 17802 and *S. loihica* strain PV4. Followed by *V. alginolyticus* strain NBRC 15630 with 96% homology from isolate CJR05 in group VII. Moreover, isolate CJR14 and CJD23 have 95% homology with *V. harveyi* strain NCIMB1280 and *P. ganghwense* strain FR311 in group I and III, while isolate CJD22 was closely related 92% to *S. algae* strain ATCC5192 in group VI.

The isolates were continuing to be grouped based on the sampling site (Table 3). Rembang has the highest number of isolates, however from 7 group of *Vibrio*, only 6 group that were detected in this site. Demak has 4 group of *Vibrio* from 5 isolates found. Furthermore, Kendal only has 2 group of *Vibrio* from 7 isolates. The results indicated that the highest diversity of *Vibrio* was found in Rembang, rather than Demak and Kendal. It might happen since those location have a higher abrasion (Directorate of Marine and Fisheries, 2019). Miller (1989) revealed that the abrasion could decline the microbial abundances. Moreover, Figure 4 was designed to know the highest species of *Vibrionaceae* found in this study. Based on the dendrogram of rep-PCR, the highest species come from *C. thiocicly* in the group II with percentage number 32 % and the lowest was *V. harveyi* with the percentage 4%. Based on the phylogenetic tree (Figure 5), *Vibrio* and *Catenococcus* are more closely related compared to the group of *Shewanella* and *Photobacterium*.

According to the results of this study, future research should be conducted to have a deep understanding and correlation between the biotic and abiotic factors that affect the health status of Mud-crab in the traditional brackish water pond. Therefore, the metagenomic approach would be helpful to describe the bacterial structure community in the healthy and infected mud crab. Thus, the design of prevention methods to reduce bacterial disease outbreak of Mud crab farming could be effectively constructed.

CONCLUSION

This study found 25 bacteria isolates that can be associated to vibriosis clinical sign from traditional mud crab farming in the North Coast of Central Java. The isolates were closely related to *Vibrio harveyi* strain NCIMB1280 (CJR14), *Catenococcus thiocicly* strain TG 5-3 (CJR15), *Photobacterium ganghwense* strain FR311 (CJD23); *Vibrio prahaemolyticus* ATCC 17802 (CJK10); *Shewanella loihica* strain PV4 (CJK11); *Shewanella algae* strain ATCC5 (CJD22) and *Vibrio alginolyticus* strain NBRC 15630 (CJR05). Strains of these seven bacteria are well known to be pathogens for aquatic organisms. Moreover, the highest diversity of Vibrionaceae was obtained from Rembang and the highest bacterial diversity that found in three sampling sites was *C. thiocicly*.

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Original Article

Molecular Characterization of vibriosis clinical signs-associated bacteria from traditional mud-crab farming in the North Coast of Central Java, Indonesia

Sarjito^{1*}, Alfabetian Harjuno Condro Haditomo¹, Slamet Budi Prayitno¹, Aninditia Sabdaningsih², Desrina¹, and Restiana Wisnu Ariyati¹

¹Aquaculture Study Program, Aquaculture Department, Fisheries and Marine Science Faculty, Diponegoro University, Semarang, 50275, Indonesia

²Department of Aquatic Resources, Fisheries and Marine Science Faculty, Diponegoro University, Semarang, 50275, Indonesia

*Email address : sarjito@live.undip.ac.id

Abstract

This study assessed the molecular characterization of bacteria associated to clinical vibriosis in mud crab farming from the North Coast of Central Java. Twenty-five bacteria were isolated from hepatopancreas, gill and carapace of mud crab with clinical signs of vibriosis that cultured in TCBS and TSA medium. After a modified rep-PCR and further PCR amplification of 16S ribosomal RNA gene, the sequence analysis was performed. The DNA sequences were then compared with the BLAST from NCBI database, and the phylogenetic tree was constructed in accordance to the MEGA X program. The clinical signs on mud crab were associated with seven isolates: CJR14, CJR15, CJD23, CJK10, CJK11, CJD22, and CJR05. Based on 16S rRNA sequence analysis, these isolates were closely (92-99%) related to: *Vibrio harveyi* NCIMB1280, *Catenococcus thiocicly* TG 5-3, *Photobacterium ganghwense* FR311, *Vibrio*

prahaemolyticus ATCC 1780, *Shewanella loihica* PV4, *Shewanella algae* ATCC5, and *Vibrio alginolyticus* NBRC 15630.

Keywords: bacterial diseases, mud crab, 16S rRNA, rep PCR, Vibriosis

1. Introduction

Mud crab, *Scylla serrata* (Forskål, 1775), is traditionally caught in and around brackish water ponds and mangrove forests along the North Coast of Central Java, Indonesia. The mangrove forests and its soils are this crab's origin habitat. Traditional brackish water pond is used for polyculture of shrimp and milk fish, Wild mud crab may intrude in these ponds, where they are caught together with the shrimp and milkfish. However, the culture of the mud crab themselves is spreading. The intrusion of wild mud crab can be a problem to crustacean's culture. Infected mud crab can carry diseases agents that may infect the healthy cultured crustaceans. To prevent damage of the crustacean culture, farmers may disinfect the pond and raise barriers that are well embedded in the bunds, but these efforts are rarely 100% effective. Bacterial diseases, especially vibriosis, was found to be a problem in fattening of the mud crab culture in Pematang (Sarjito *et al.*, 2016) and reported to cause more than 90% mortality in all life cycle stages of mud crab growth (Jithendran, Poornima, Balasubramanian & Kulasekarapandian, 2010). Reported clinical signs of infected mud crab are wounds on body, decreased feed response and weakening (Jithendran, Poornima, Balasubramanian & Kulasekarapandian, 2010), blackening and red spots of the carapace (Sarjito, Hastuti, Samidjan, & Prayitno, 2014; and Sarjito *et al.*, 2016).

Vibriosis is a bacterial disease that was caused by genus vibrio bacteria (Lavilla-Pitogo, Celia, & Leobert, 2004; Jithendran, Poornima, Balasubramanian &

Kulasekarapandian, 2010). *Vibrio*'s cause frequent disease outbreaks and serious economic losses in shrimp culture (Aguilre-Rivera, Prieto-Davo, Rodriguez-Fuentes G, Escalante-Herrera, & Gaxiola, 2019) and have the potential to destroy the future of industrial culture of mud crab. Several species of *Vibrio* spp. were reported in and found to be associated with bacterial disease in mud crab: *V. fischeri*, and *V. nereis* (Wang, 2011); *V. alginolyticus* and *V. cholerae* (Najiah, Nadirah, Sakri & Harrison, 2010; Wang, 2011); *Vibrio vulnificus* (Lavilla-Pitogo, Celia, & Leobert, 2004; Shanmuga, 2008; Wang, 2011), *V. splendidus*, and *V. orientalis* (Jithendran, Poornima, Balasubramanian & Kulasekarapandian, 2010); *V. ordalii* (Sarjito, Hastuti, Samidjan, & Prayitno, 2014), *V. harrveyi* (Sarjito, Hastuti, Samidjan, & Prayitno, 2014; Jithendran, Poornima, Balasubramanian & Kulasekarapandian, 2010), *V. campbelli* (Shanmuga, 2008), and *V. parahaemolyticus* (Lavilla-Pitogo, Celia, & Leobert, 2004; Najiah, Nadirah, Sakri & Harrison, 2010; Shanmuga, 2008). Previous research focused on the richness of bacteria associated to vibriosis in mud crab in Central Java. The limited knowledge is available on the diversity of vibrios related to the disease outbreaks in mud crab. Local government tends to stimulate the culture of mud crab but the program lacks information on disease identification and prevention. This study was done to assess the relationship between clinical signs of vibriosis on mud crab, and the diversity of bacteria in mud crab of traditional brackish water ponds along the North Coast of Central Java, Indonesia.

2. Materials and Methods

Sampling

Mud crab *S. serrata*, were collected from nine brackish water ponds of Kendal (3 ponds), Demak (3 ponds) and Rembang (3 ponds) Regency, Central Java. These three

locations are the largest producers of cultivated crabs in Central Java, Indonesia (Picture 1). Based on the clinical signs for vibriosis described by Jithendran, Poornima, Balasubramanian & Kulasekarapandian (2010) 45 infected mud crab were selected, stored in a sterile container and carried to the Integrated Laboratory of Diponegoro University for further analysis. The average size of the caught crab, measured from 13 to 15 cm, was 14.4 ± 0.7 cm. The clinical signs, such as: red spots or brown spots on the carapace and wounds in the abdomen. Brown spot may occur due to the infection of chitinolytic bacteria that catabolize the chitin of the carapace and cause erosion and melanisation (dark brown to black pigmentation) at the site of infection (Jithendran, Poornima, Balasubramanian & Kulasekarapandian, 2010).

Figure 1. Research Location represented in a small red box in the left of map.

Bacterial Isolation

Forty five bacteria were isolated from gills, hemolymph, hepatopancreas, and wounds on the carapace of the infected mud crabs after culture on TCBS (Thiosulfate Citrate Bile Salts Sucrose) and TSA (Tryptic Soy Agar) (Thomson, 2004). The colonies were purified by the streak method based on morphological appearance (Sarjito et al, 2015).

Repetitive Genomic Sequences-PCR (Rep-PCR)

The isolates were analyzed using the rep-PCR of Radjasa *et al.* (2007) modified by Sarjito et al (2009) in the rep-PCR, BOX AIR (5'-CTACggCAAaggCgACgCTgACg-3'). The REP 1R-I and REP 2-I primers contained nucleotide inosine (I) at ambiguous position in the REP consensus. The mix PCR reagent contain of 1 μ L DNA template

(diluted 100X), 1 μ L primer, 7.5 μ L Megamix Royal and 5.5 μ L ddH₂O. Amplification was performed in a thermal cycler model Gene Amp PCR system 9700 with the following protocol: initial denaturation at 95°C for 5 minutes; 30 cycles of (denaturation 92°C for 1 minutes, annealing 50°C for 1,5 minutes, extension 68°C for 8 minutes) and final extension at 68°C for 10 minutes. The band DNA was visualized with injected 5 μ L PCR products into 1% agarose gel and run to electrophoresis machine using 1X TBE buffer and observed in UV-transilluminator, as described by Radjasa et al. (2007). Grouping of isolates was conducted based on the method of Radjasa et al. (2007). Matrices were made from the bands position on the gel which were analyzed by the Free Tree program using the UPGMA method for constructing the phylogenetic tree. Resampling was done by bootstrapping with 1000 replications.

PCR Amplification of 16s rRNA Gene Fragments

The PCR amplification of 16S rRNA gene was performed according to Radjasa et al. (2007). To find out a closely complete 16S rRNA gene, the amplification was performed with two primers, GM3F as a forward primer (5'AGAGTTTGATCMTGGC-3') and GM4R as a reverse primer (5'-TACCTTGTTACGACTT-3') (Sarjito *et al.*, 2009). Genomic DNA of bacteria for PCR analysis were obtained from the destruction cell materials that taken from fresh culture bacteria, suspended in sterile water (Sigma, Germany) and subjected to five cycles of freeze (-80°C) and thaw (95°C). The PCR product was then processed to find the band DNA in the right size around 1.500 bp. The sequencing step was facilitated by PT. Genetika Science. The determined DNA sequences of samples were then compared for homology using BLAST. To find the strain the phylogenetic tree was established using the MEGA X.

3. Results and Discussion

Clinical Signs and Bacterial Isolation

The most frequent clinical signs of the infected mud crab (*S. serrata*) were wounds on the surface of the claws, the ventral, and the carapace. The isolation of bacteria using TCBS and TSA medium obtained a total of 25 pure isolates bacteria from three replicates agar plates and then stored in NA (Nutrient Agar) medium. The clinical signs of red-brown and dark spots in the carapace were found in Figure 2. Similar clinical signs were found on the abdomen.

Figure 2. The clinical signs of mud crab (*S. serrata*) infected by bacterial diseases: (a.) Red/brown spots in the carapace; (b.) Red-brown spots in the carapace wound on the abdomen.

Vibriosis in mud crab was mostly known by red-brown or dark melanine spots; patches of light red spots as well as dark spots on the carapace, also wounds on the body (claws, carapace and the ventral). The identic of clinical signs have been found by Muyzer, Teske, Wirsén, and Jannasch (1995), Wang (2011) and Lavilla-Pitogo, Celia, & Leobert (2004). The clinical signs, such as: brown spot or red spots on the carapace and wounds in the abdomen (Figure 2) were also reported on the mud crabs that were affected by genus vibrio from gulf of Semarang (Sarjito, Hastuti, Samidjan, & Prayitno, 2014) and Pematang (Sarjito *et al.*, 2016). ‘Brown spot’ may occur due to the infection of chitinolytic bacteria that catabolize the chitin of the carapace and cause erosion and melanisation (dark brown to black pigmentation) at the site of infection (Jithendran, Poornima, Balasubramanian & Kulasekarapandian, 2010)

The present study revealed that genus vibrio associated in mud crabs from traditional brackish water pond of North Coast of Central Java (Figure 1) were closely related to *V. harveyi* strain NCIMB1280 (CJR14), *C. thiocicly* strain TG 5-3 (CJR15), *P. ganghwense* strain FR311 (CJD23); *V. prahaemolyticus* ATCC 17802 (CJK10); *S. loihica* strain PV4 (CJK11); *S. algae* strain ATCC5 (CJD22) and *V. alginolyticus* strain NBRC 15630 (CJR05). This present result also revealed that diversity of vibrios associated with mud crab from brackish water pond of North Coastal of Central Java coast was lower than diversity that was found in cultured and wild crab in India (Jithendran, Poornima, Balasubramanian & Kulasekarapandian, 2010) and traditional brackish water surrounding of Semarang bay (Sarjito, Hastuti, Samidjan, & Prayitno, 2014).

Repetitive Genomic Sequences-PCR (Rep-PCR)

The Repetitive PCR analysis and the dendrogram related the 25 pure isolates bacteria originating from mud crabs found a seven group from total samples (Figure 3). Furthermore, the molecular identification only represented by seven isolates from each seven group (CJR14, CJR15, CJD23, CJK10, CJK11, CJD22, and CJR05) (Table 1).

Figure 3. A dendrogram based on Rep-PCR of 25 Bacteria Isolates Associated with vibriosis clinical signs from traditional mud crab farming in the North Coastal of Central Java, Central Java, Indonesia.

In this study, four genus Vibrionaceae (*Vibrio*, *Shewanella*, *Photobacterium*, and *Catenococcus*) were found in mud crab. Three species of vibrio i.e: *V. parahaemolyticus* (CJK10), *V. harveyi* (CJR14) and *V. alginolyticus* (CJR05) also have been detected in this present study. The vibrios were frequently found as a causative agent of shell disease in *L. vannamei* shrimp and mud crab, *S. serrata*, (Estave and Herrere, 2000; Wang, 2011;

soto-Rodriguez *et al.*, 2012). *V. harveyi* has been reported as a causative agent of vibriosis in fattening mud crabs from brackish water pond in Pemalang coast (Sarjito *et al.*, 2016); extensive brackish water pond of surrounding of Gulf of Semarang (Sarjito, Hastuti, Samidjan, & Prayitno, 2014) and Malaysia (Najiah, Nadirah, Sakri & Harrison, 2010). These bacteria were also reported as a causative agent of bacterial diseases in zoea stage of mud crab (Jithendran, Poornima, Balasubramanian & Kulasekarapandian, 2010; Department of Agriculture Fisheries and Forestry Fisheries Queensland 2012); adult mud crabs (Poornima *et al.*, 2012 and Lavilla-Pitogo, Celia, & Leobert, 2004). Moreover, Sarjito, Hastuti, Samidjan, & Prayitno, 2014, found that this bacterium was a potential pathogen to mud crabs. The present study also revealed that *V. alginolyticus*, also found in mud crab, *S. serrata*, infected bacterial diseases in north central Java. *V. alginolyticus* was commonly reported from brackish water, estuary and marine environment. It was found as an opportunistic bacteria associated with crustacean culture and fish culture (Parenerngi *et al.*, 1993). Moreover, this bacterium was also found as an important pathogenic bacteria associated with infectious diseases in mud crab (Najiah, Nadirah, Sakri & Harrison, 2010; Sarjito, Hastuti, Samidjan, & Prayitno, 2014) and shell disease of mud crab *S. serrata* grow out pond located at Mahendrapalli, Nagapattinam District, Tamil Nadu, India (Gunasekaran, Gopalakrishnan, Deivasigamani, Muhilvannan, & Kathirkaman, 2019), seafood, fish, shrimp, sediment and seawater (Austin and Austin, 2007).

Genus photobacterium was also found in marine water. These bacteria were reported from a sea-water sample in Ganghwa Island, South Korea. Marine bacterial strain, designated FR1311T. cells were Gram-negative, facultatively anaerobic, catalase and oxidase-positive, motile, oval or rod-shaped and halophilic (optimum sea-salt

concentration for growth of 5–6 %) (Park *et al.*, 2006). This bacterium was defined as being facultatively anaerobic and weakly halophilic, and luminescent, alike most of the recognized species in the genus are luminescent. *P. ganghwense* has been described as being luminescent. Some of the luminescent species are symbionts of deep-sea fishes that hold them in specialized luminous organ (Lucena *et al.*, 2011).

The present study result showed that both *S. algae* and *S. loihica* were found associated with vibriosis in the mud crabs from traditional brackish water pond of North central java. The genus *Shewanella* is phylogenetically affiliated to the γ - Proteobacteria (Yoon, Yeo, Kim & Oh. 2004). This bacterium also closely related to genus vibrio or vibriaceae. It has been reported as bacterial pathogen associated with massive mortality in *Scinops ocellata*, *Carrasius auratus* (Altun *et al.*, 2014); *Babylonia* (Shufang, Zhang, Dequan, Shiping, & Huang, 2015); *Cynoglossus semilaevis* (Han *et al.*, 2017); and freshwater-cultured white leg shrimp, *P. vannamei* (Cao, Chen, Lu, & An, 2018). *Scianeops ocellatus* (Zhang, Zhu, & Wang, 2013). This bacterium causes mortalities of more than 50% of the white leg shrimps cultured in freshwater pond of Lianyungang, Jiansu province, China (Cao, Chen, Lu, & An, 2018).

Shewanella spp. are Gram-negative, motile bacilli. *Shewanella* spp. are found throughout the world, mainly in marine environments. The most important phenotypic characteristic of *Shewanella* spp is the production of hydrogen sulphide. They have an important role as turnover of organic material, and capable of dissimilatory reduction of various metals and other substances, such as Nitrate, Nitrite, Thiosulphate and Trimethylamine-N-oxide. In Demak the presence of *S. algae* were found only when the water temperature is more than 13°C this happend usually on July to August. This is happens because there are correlation between the salinity and the temperatur, where the

isolation of *S. algae* can be performed with the salinity of 15-20%. Infection of *Shewanella* mostly happens in countries with a warm climate, or especially due to warm summers in countries with temperate climates (Holt, Gahrn-Hansen, & Bruun, 2005). *S. algae* was also found associated with milkfish gut collected from Northern Coast of Central Java (Prayitno, Sarwan & Sarjito, 2015).

Shewanella loihica was isolated and identified from iron-rich microbial mats in the Pacific Ocean. The result shows that *S. loihica* is a Gram-negative, non-spore-forming, straight rod with a mean length of 1-8 μm and a mean width of 0-7 μm . Single flagella is used to motile. Facultative psychrotolerant anaerobe. The characteristic of the colonies are smooth, glistening, circular, flat to slightly raised, orange in color. Temperature 18 $^{\circ}\text{C}$, pH 6.0-8.0 and Na^+ are the optimum environment of *S. loihica* to grow. This bacterium was able to grow in over wide ranges of temperature and pH. The ranges of water quality that could support the growth of *S. loihica* are temperature (0–42 $^{\circ}\text{C}$), pH (4.5–10) and salt (0.5–5%). *S. loihica* plays a role as an agent of metal reduction and iron biomineralization, based on that the existence of this bacterium can be caused by the metal content in the water. According to Newton, Mori, Nakamura, Hashimoto & Watanabe (2009), the current study of *S. loihica* is focusing on the metal reduction and iron biomineralization capabilities based on their phenotypic and phylogenetic characteristics.

Catenococcus thiocycli was identified by (Yarza *et al.*, 2013) the study found that *C. thiocycli* is part of genus Vibrionaceae, order Vibrionales, class Gammaproteobacteria and phylum Proteobacteria. This bacterium is also isolated from the Sediment in Pacific Ocean (David *et al.*, 2015).

Table 1. Molecular characterization of seven bacterial species associated with clinical signs of Vibriosis on mud crabs from North Coast of Central Java.

The seven vibrios associated with mud crab were closely (homology range between 95-99%) related to the *Vibrio harveyi* NCIMB1280 (CJR14), *Catenococcus thiocicly* TG 5-3 (CJR15), *Photobacterium ganghwense* FR311 (CJD23); *Vibrio prahaemolyticus* ATCC 17802 (CJK10); *Shewanella loihica* PV4 (CJK11); *Shewanella algae* ATCC5 (CJD22) and *Vibrio alginolyticus* NBRC 15630 (CJR05) (Table 2). The diversity of vibrios found in three samples locations (Table 2). We found more bacteria species in Rembang rather than in Demak and Kendal but the highest percentage of vibrio diversity compare to the total isolate in each location was in Demak (80%).

Diversity of Vibriosis Clinical Signs Associated Bacteria

The diversity of vibriosis clinical signs associated bacteria was compared from the three-sampling site to perform the percentage of species in each location (Table 2). Table 2 accommodates all of isolates that identified based on 16S rRNA gene. The diversity of vibriosis clinical signs associated bacteria was explored using molecular characterization. Rep-PCR was used to find out fingerprinting of bacterial genomes (Versalovic, de Bruijn & Lupski, 1998). This method is an effective tool to distinguish the diversity of bacteria according to the number and size of bacterial repetitive sequences. This technique could prevent to analyse the similar isolate of bacteria based on its DNA fingerprinting. Therefore, rep-PCR is helpful to grouping *Vibrio* species.

Table 2. The diversity of *Vibrios* in Mud Crab from North Coast of Central Java per location.

The dendrogram from Figure 3 show similarity between 25 isolates into 7 groups due to the difference of bacterial repetitive sequences. The representative isolates from each group was further examined using 16S rRNA gene for identification. A total of 7 isolates were identified and presented in Table 2. The range of homology percentage between 92-98%. The highest similarity was showed by isolate CJR15 from group II, 98% homology with *C. thiocicly* strain TG 5-3. The homology 97% was appointed to isolate CJK10 and CJK11 from group IV and V that similar to *V. parahaemolyticus* ATCC 17802 and *S. loihica* strain PV4. Followed by *V. alginolyticus* strain NBRC 15630 with 96% homology from isolate CJR05 in group VII. Moreover, isolate CJR14 and CJD23 have 95% homology with *V. harveyi* strain NCIMB1280 and *P. ganghwense* strain FR311 in group I and III, while isolate CJD22 was closely related 92% to *S. algae* strain ATCC5192 in group VI

Figure 4. The bacterial diversity in Mud crab with clinical signs of Vibriosis from North Coast of Central Java.

Figure 4 represented the bacterial diversity in Mud crab with clinical signs of Vibriosis from North Coast of Central Java. The highest number species was *C. thiocicly* with the percentage 32%, while 24% was *V. parahaemolyticus*, *S. loihica* and *S. algae* in the equal percentage in 12%, as well as *P. ganghwense* and *V. alginolyticus* in 8%, and the lowest percentage was *V. harveyi* in 4%.

Figure 5. Phylogenetic tree of the bacteria associated with vibriosis clinical signs from traditional mud crab farming in the North Coast of Central Java, Indonesia constructed using Neighbour-Joining analysis with 1000 replicates.

In order to represent the relationship among isolates, the phylogenetic was constructed. Figure 5 shows that all of the isolates have assembled with the closely related species. The relationship between the genus performs that the bacteria genus *Vibrio* has the same clade with *Catenococcus thiocycli*. Those groups are more closely related compared to the group of *Shewanella* and *Photobacterium*.

The isolates were continuing to be grouped based on the sampling site (Table 2). Rembang has the highest number of isolates, however from 7 group of *Vibrio*, only 6 group that were detected in this site. Demak has 4 group of *Vibrio* from 5 isolates found. Furthermore, Kendal only has 2 group of *Vibrio* from 7 isolates. The results indicated that the highest diversity of *Vibrio* was found in Rembang, rather than Demak and Kendal. It might happen since those locations have a higher abrasion (Directorate of Marine and Fisheries, 2019). Miller (1989) revealed that the abrasion could decline the microbial abundances. Moreover, Figure 4 was designed to know the highest species of Vibrionaceae found in this study. Based on the dendogram of rep-PCR, the highest species come from *C. thiocycli* in the group II with percentage number 32 % and the lowest was *V. harveyi* with the percentage 4%. Based on the phylogenetic tree (Figure 5), *Vibrio* and *Catenococcus* are more closely related compared to the group of *Shewanella* and *Photobacterium*.

According to the results of this study, future research should be conducted to have a deep understanding and correlation between the biotic and abiotic factors that affect the

health status of Mud-crab in the traditional brackish water pond. Therefore, the metagenomic approach would be helpful to describe the bacterial structure community in the healthy and infected mud crab. Thus, the design of prevention methods to reduce bacterial disease outbreak of Mud crab farming could be effectively constructed.

4. Conclusions

This study found 25 bacteria isolates that can be associated to vibriosis clinical sign from traditional mud crab farming in the North Coast of Central Java. The isolates were closely related to *Vibrio harveyi* strain NCIMB1280 (CJR14), *Catenococcus thiocicly* strain TG 5-3 (CJR15), *Photobacterium ganghwense* strain FR311 (CJD23); *Vibrio prahaemolyticus* ATCC 17802 (CJK10); *Shewanella loihica* strain PV4 (CJK11); *Shewanella algae* strain ATCC5 (CJD22) and *Vibrio alginolyticus* strain NBRC 15630 (CJR05). Strains of these seven bacteria are well known to be pathogens for aquatic organisms. Moreover, the highest diversity of Vibrionaceae was obtained from Rembang and the highest bacterial diversity that found in three sampling sites was *C. thiocicly*.

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Figure 1. Research Location represented in a small red box in the left of map.



Figure 2. The clinical signs of mud crab (*S. serrata*) infected by bacterial diseases: (a.) Red/brown spots in the carapace; (b.) Red-brown spots in the carapace wound on the abdomen.

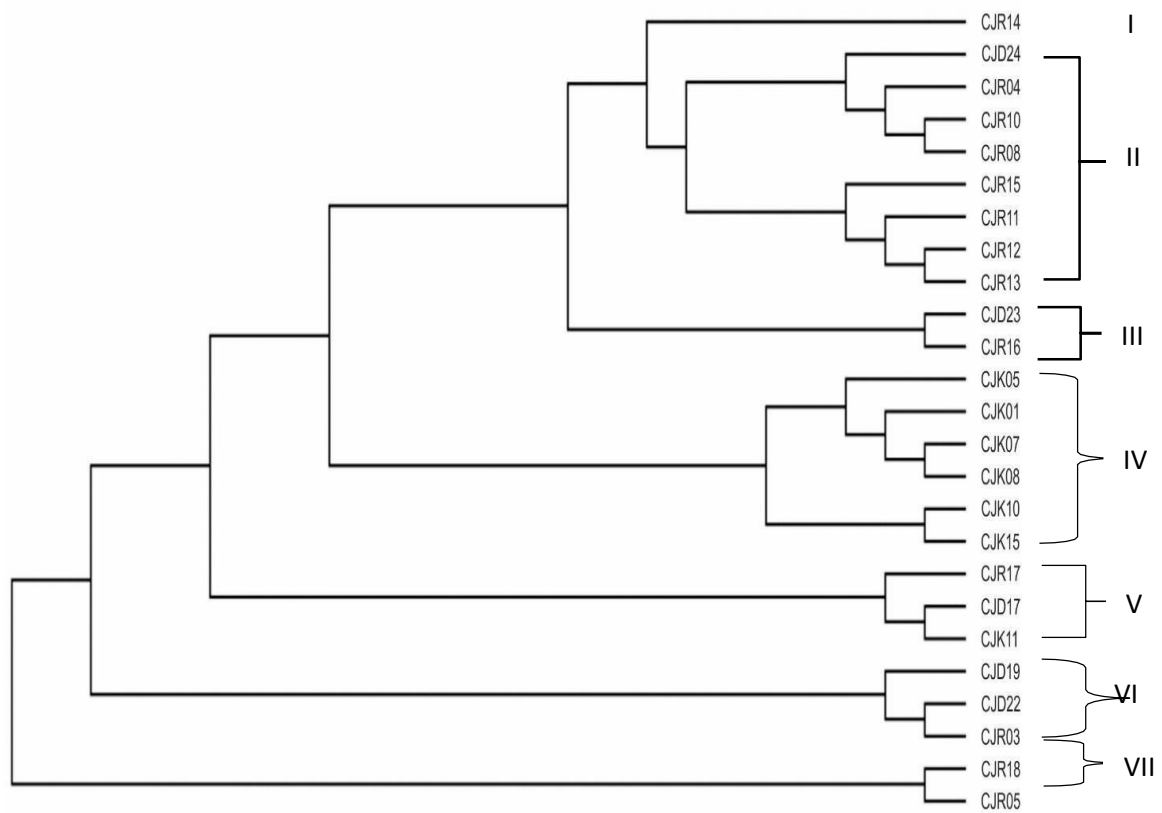


Figure 3. A dendrogram based on Rep-PCR of 25 Bacteria Isolates Associated with vibriosis clinical signs from traditional mud crab farming in the North Coastal of Central Java, Central Java, Indonesia.

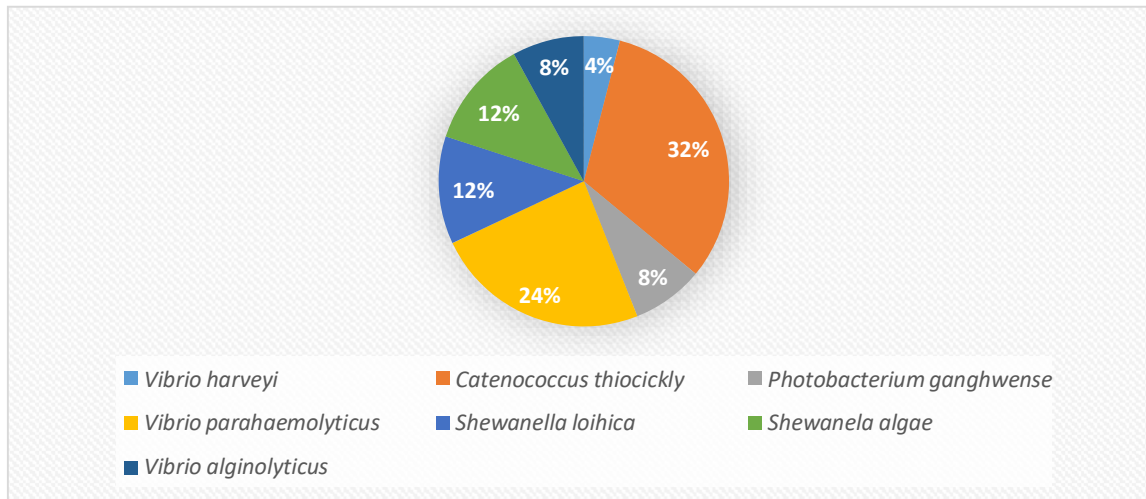


Figure 4. The bacterial diversity in Mud crab with clinical signs of Vibriosis from North Coast of Central Java.

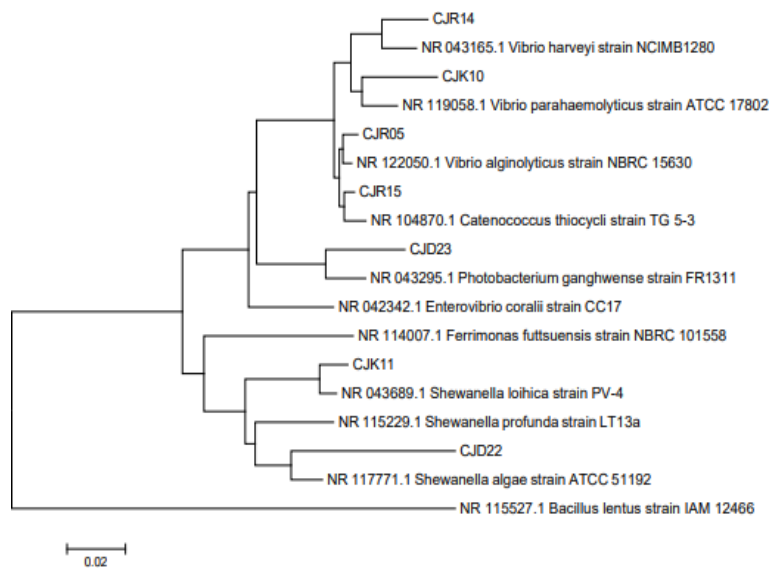


Figure 5. Phylogenetic tree of the bacteria associated with vibriosis clinical signs from traditional mud crab farming in the North Coast of Central Java, Indonesia constructed using Neighbour-Joining analysis with 1000 replicates.

Table 1. Molecular characterization of seven bacterial species associated with clinical signs of Vibriosis on mud crabs from North Coast of Central Java.

Isolates Code	Query Length	Close relative	Query Cover (%)	Homology (%)	Acc. Number
CJR14	1548	<i>Vibrio harveyi</i> strain NCIMB1280	98	95	NR 043165.1
CJR15	1319	<i>Catenococcus thiocicly</i> strain TG 5-3	98	98	NR 118258.1
CJD23	1387	<i>Photobacterium ganghwense</i> strain FR311	98	95	NR 043295.1
CJK10	1340	<i>Vibrio parahaemolyticus</i> ATCC 17802	99	97	NR 043689.1
CJK11	1335	<i>Shewanella loihica</i> strain PV4	97	97	NR 025794.1
CJD22	1488	<i>Shewanella algae</i> strain ATCC5192	95	92	NR 117771.1
CJR05	1362	<i>Vibrio alginolyticus</i> strain NBRC 15630	96	96	NR 122050.1

Table 2. The diversity of Vibrios in Mud Crab from North Coast of Central Java per location.

Location	Isolates Code	Bacteria Species	Vibrios diversity	Isolate Number	Percentage of species compare to total isolate (%)
Rembang	CJR14;				
	CJR04;				
	CJR10;				
	CJR08;				
	CJR15;	<i>Vibrio harveyi</i> strain NCIMB1280			
	CJR11;	<i>Catenococcus thiocicly</i> strain TG 5-3,			
	CJR12;	<i>Photobacterium ganghwense</i> strain FR311,			
	CJR13;	<i>Shewanella loihica</i> strain PV4,	6	13	52%
	CJR16;	<i>Shewanella algae</i> strain ATCC5192,			
	CJR17;	<i>Vibrio alginolyticus</i> strain NBRC 15630,			
	CJR03;				
	CJR18;				
	CJR05				
Demak	CJD24;	<i>Catenococcus thiocicly</i> strain TG 5-3			
	CJD23;	<i>Photobacterium ganghwense</i> strain FR311			
	CJD17;	<i>Shewanella loihica</i> strain PV4	4	5	80%
	CJD19;	<i>Shewanella algae</i> strain ATCC5192			
	CJD22				

	CJK05;			
	CJK01;			
	CJK07; <i>Vibrio prahaemolyticus</i> ATCC 17802			
Kendal	CJK08; <i>Shewanella loihica</i> strain PV4	2	7	28%
	CJK10;			
	CJK15;			
	CJK11			



Sarjito Sarjito <sarjito@live.undip.ac.id>

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1 message

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Sat, Aug 29, 2020 at 10:22

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To: sarjito@live.undip.ac.id, resti_wisnoe@yahoo.com

29-Aug-2020

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Original Article

Molecular Characterization of vibriosis clinical signs-associated bacteria from traditional mud-crab farming in the North Coast of Central Java, Indonesia

Sarjito^{1*}, Alfabetian Harjuno Condro Haditomo¹, Slamet Budi Prayitno¹, Aninditia Sabdaningsih², Desrina¹, and Restiana Wisnu Ariyati¹

¹Aquaculture Study Program, Aquaculture Department, Fisheries and Marine Science Faculty, Diponegoro University, Semarang, 50275, Indonesia

²Department of Aquatic Resources, Fisheries and Marine Science Faculty, Diponegoro University, Semarang, 50275, Indonesia

*Email address : sarjito@live.undip.ac.id

Abstract

This study assessed the molecular characterization of bacteria associated with clinical vibriosis in mud crab farming from the North Coast of Central Java. Twenty-five bacteria were isolated from hepatopancreas, gill, and carapace of mud crab that have clinical signs of vibriosis that cultured in TCBS and TSA medium. After a modified rep-PCR and further PCR amplification of the 16S ribosomal RNA gene, the sequence analysis was performed. The DNA sequences compared with the BLAST from the NCBI database. Then, the phylogenetic tree constructed by the MEGA X program. The clinical signs on mud crab were associated with seven isolates: CJR14, CJR15, CJD23, CJK10, CJK11, CJD22, and CJR05. Based on 16S rRNA sequence analysis, these isolates were firmly (92-98%) related to *Vibrio harveyi* NCIMB1280, *Catenococcus thiocicly* TG 5 – 3,

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4 *Photobacterium ganghwense* FR311, *Vibrio parahaemolyticus* ATCC 1780, *Shewanella*
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6 *loihica* PV4, *Shewanella algae* ATCC5, and *Vibrio alginolyticus* NBRC 15630.
7

8
9 **Keywords:** bacterial diseases, mud crab, 16S rRNA, rep PCR, vibriosis
10

11 12 13 **1. Introduction**

14
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16 Mud crab, *Scylla serrata* (Forskål, 1775), is traditionally caught in and around brackish
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18 water ponds and mangrove forests along the North Coast of Central Java, Indonesia. The
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20 mangrove forests and its soils are this crab's origin habitat. Traditional brackish water
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22 pond used for polyculture of shrimp and milkfish, wild mud crab may intrude in these
23
24 ponds, where they are caught together with the shrimp and milkfish. However, the culture
25
26 of the mud crab themselves is spreading. The intrusion of wild mud crab can be a problem
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28 to crustacean's culture. Infected mud crab can carry disease agents that may infect the
29
30 healthy cultured crustaceans. To prevent damage of the crustacean culture, farmers may
31
32 disinfect the pond and raise barriers that are well embedded in the bunds, but these efforts
33
34 are rarely 100% effective. Bacterial diseases, especially vibriosis, was found to be a
35
36 problem in the fattening of the mud crab culture in Pemalang (Sarjito *et al.*, 2016) and
37
38 reported to cause more than 90% mortality in all life cycle stages of mud crab growth
39
40 (Jithendran, Poornima, Balasubramanian, & Kulasekarapandian, 2010). Reported clinical
41
42 signs of infected mud crab are wounds on the body, decreased feed response and
43
44 weakening (Jithendran, Poornima, Balasubramanian, & Kulasekarapandian, 2010),
45
46 blackening and red spots of the carapace (Sarjito, Hastuti, Samidjan, & Prayitno, 2014;
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48 Sarjito *et al.*, 2016).
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55 *Vibrio's* cause various disease outbreaks and severe economic losses in shrimp
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57 culture (Aguilre-Rivera, Prieto-Davo, Rodriguez-Fuentes G, Escalante-Herrera, &
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4 Gaxiola, 2019) and have the potential to destroy the future of the industrial culture of mud
5
6 crab. Several species of *Vibrio* spp. were reported in and found to be associated with
7
8 bacterial disease in mud crab: *V. fischeri*, and *V. nereis* (Wang, 2011); *V.*
9
10 *alginolyticus* and *V. cholerae* (Najiah, Nadirah, Sakri & Harrison, 2010; Wang, 2011);
11
12 *Vibrio vulnificus* (Lavilla-Pitogo, Celia, & Leobert, 2004; Shanmuga, 2008; Wang,
13
14 2011), *V. splendidus*, and *V. orientalis* (Jithendran, Poornima, Balasubramanian, &
15
16 Kulasekarapandian, 2010); *V. ordalii* (Sarjito, Hastuti, Samidjan, & Prayitno, 2014), *V.*
17
18 *harveyi* (Sarjito, Hastuti, Samidjan, & Prayitno, 2014; Jithendran, Poornima,
19
20 Balasubramanian, & Kulasekarapandian, 2010), *V. campbelli* (Shanmuga, 2008), and *V.*
21
22 *parahaemolyticus* (Lavilla-Pitogo, Celia, & Leobert, 2004; Najiah, Nadirah, Sakri, &
23
24 Harrison, 2010; Shanmuga, 2008). Previous research focused on the richness of bacteria
25
26 associated with vibriosis in mud crab. The limited knowledge is available on the diversity
27
28 of vibrios related to the disease outbreaks in mud crab in coastal of North Central Java.
29
30 Local government tends to stimulate the culture of mud crab, but the program lacks
31
32 information on disease identification and prevention. This study was done to assess the
33
34 relationship between clinical signs of vibriosis on mud crab, and the diversity of bacteria
35
36 in mud crab of traditional brackish water ponds along the North Coast of Central Java,
37
38 Indonesia.

47 48 **2. Materials and Methods**

49 50 **Sampling**

51
52 Mud crab, *S. serrata*, were collected from nine brackish water ponds of Kendal,
53
54 Demak, and Rembang Regency, Central Java (each three ponds). These three locations
55
56 are the largest producers of cultivated crabs in Central Java, Indonesia (Picture 1). Based
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4 on the clinical signs for vibriosis described by Jithendran, Poornima, Balasubramanian,
5 & Kulasekarapandian (2010), 45 infected mud crabs were selected, stored in a sterile
6 container and carried to the Integrated Laboratory of Diponegoro University for further
7 analysis. The average size of the caught crab, measured from 13 to 15 cm, was
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14.4±0.7cm.

Figure 1. Research location represented in a small red box in the left of map.

Bacterial Isolation

Forthy-five bacteria were isolated from gills, hemolymph, hepatopancreas, and wounds on the shell of the infected mud crabs. Isolates bacteria were cultured on Thiosulfate Citrate Bile Salt Sucrose (TCBS) and Tryptic Soy Agar (TSA) (Thompson, Iida, & Swings, 2004; Sarjito *et al.*, 2016). The colonies were purified by the streak method based on morphological appearance (Sarjito *et al.*, 2016).

Repetitive Genomic Sequences-PCR (Rep-PCR)

The bacterial DNA was extracted from a 24 h-broth culture of isolate strains by using the chelex method with slight modification according to the procedure described by (de Lamballerie, Zandotti, Vignoli, Bollet, & de Micco, 1992). Then, the isolates DNA extract were analyzed using the rep-PCR of Radjasa, Nasima, Sabdono, Kita-Tsukamoto, & Ohwada (2007) was modified by Sarjito, Haditomo, Desrina, Djunaedi, & Prayitno (2018) in the rep-PCR, BOX AIR (5'-CTACggCAAaggCgACgCTgACg-3'). The REP 1R-I and REP 2-I primers contained nucleotide inosine (I) at an ambiguous position in the REP consensus. The mix PCR reagent contains one μ L DNA template (diluted 100X), one μ L primer, 7.5 μ L Megamix Royal and 5.5 μ L ddH₂O. Amplification was performed in a thermal cycler model Gene Amp PCR system 9700 with the following

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4 protocol: initial denaturation at 95°C for 5 minutes. Then 30 cycles of (denaturation 92°C
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6 for 1 minute, annealing 50°C for 1,5 minutes, extension 68°C for 8 minutes), and a final
7
8 extension at 68°C for 10 minutes. The band DNA was visualized with injected 5 µL PCR
9
10 products into 1% agarose gel and run to electrophoresis machine using 1X TBE buffer
11
12 and observed in UV-transilluminator, as described by Radjasa, Nasima, Sabdono, Kita-
13
14 Tsukamoto, & Ohwada (2007). Grouping of isolates was conducted based on the method
15
16 of Sarjito, Radjasa, Sabdono, Prayitno, & Hutabarat (2009) . Matrices were made from
17
18 the band's position on the gel, which was analyzed by the Free Tree program using the
19
20 UPGMA method for constructing the phylogenetic tree (Sarjito, Haditomo, Desrina,
21
22 Djunaedi, & Prayitno, 2018). Resampling was done by bootstrapping with 1000
23
24 replications (Prayitno, Sarwan, & Sarjito, 2015).
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29 **PCR Amplification of 16s rRNA Gene Fragments**

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31 The PCR amplification of the 16S rRNA gene was performed, according to Radjasa,
32
33 Nasima, Sabdono, Kita-Tsukamoto, & Ohwada (2007) and Sarjito, Haditomo, Desrina,
34
35 Djunaedi, & Prayitno (2018). To find out a strictly complete 16S rRNA gene, the
36
37 amplification was presented with two primers, GM3F as a forward primer
38
39 (5'AGAGTTTGATCMTGGC-3') and GM4R as a reverse primer (5'-
40
41 TACCTTGTTACGACTT-3') (Sarjito, Radjasa, Sabdono, Prayitno, & Hutabarat, 2009).
42
43 Genomic DNA of bacteria for PCR analysis was obtained from the destruction cell
44
45 materials that were taken from fresh culture bacteria, suspended in sterile water (Sigma,
46
47 Germany), and subjected to five cycles of freeze (-80°C) and thaw (95°C). The PCR
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49 product was then processed to find the band DNA in the right size around 1.500 bp. The
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51 sequencing step was facilitated by PT. Genetika Science. The determined DNA sequences
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53 of samples were then compared for homology using BLAST. The phylogenetic tree was
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4 established using MEGA X to find the closely related species (Sibero *et al.*, 2017; Sibero
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6 *et al.*, 2018; Sabdaningsih *et al.*, 2020).
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9 10 **3. Results and Discussion**

11 12 **Clinical Signs and Bacterial Isolation**

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15 The most frequent clinical signs of the infected mud crab (*S. serrata*) were wounds
16
17 on the surface of the claws, the ventral, and the carapace. The isolation of bacteria using
18
19 TCBS and TSA medium obtained 25 pure isolates of bacteria from three replicates agar
20
21 plates and then stored in NA medium. The clinical signs of red-brown and dark spots in
22
23 the carapace were found in Figure 2. Similar clinical symptoms were found on the
24
25 abdomen.
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29 **Figure 2.** The clinical signs of mud crab (*S. serrata*) infected by bacterial diseases: (a.)
30
31 Red/brown spots in the carapace; (b.) Red-brown spots in the carapace wound on the
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33 abdomen.
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37 Red-brown or dark melanin spots mostly knew as vibriosis in mud crab, patches
38
39 of light red spots as well as dark spots on the carapace, also wounds on the body (claws,
40
41 shell, and the ventral). The identic of clinical signs have been found by Muyzer, Teske,
42
43 Wirsen, and Jannasch (1995), Wang (2011), Lavilla-Pitogo, Celia, & Leobert (2004) and
44
45 Prayitno, Sarjito & Putri (2017). It was also reported on the mud crabs that were affected
46
47 by genus vibrio from the gulf of Semarang (Sarjito, Hastuti, Samidjan, & Prayitno, 2014)
48
49 and Pemalang (Sarjito *et al.*, 2016). 'Brown spot' may occur due to the infection of
50
51 chitinolytic bacteria that catabolize the chitin of the carapace and cause erosion and
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53 melanization (dark brown to black pigmentation) at the site of infection (Jithendran,
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55 Poornima, Balasubramanian & Kulasekarapandian, 2010)
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4 The vibrio's associated in mud crabs from traditional brackish water pond of
5 North Coast of Central Java (Figure 1), it was closely related to *V. harveyi* strain
6 NCIMB1280 (CJR14), *C. thiocicly* strain TG 5-3 (CJR15), *P. ganghwense* strain FR311
7 (CJD23); *V. prahaemolyticus* ATCC 17802 (CJK10); *S. loihica* strain PV4 (CJK11); *S.*
8 *algae* strain ATCC5 (CJD22) and *V. alginolyticus* strain NBRC 15630 (CJR05). The
9 result also revealed the diversity of vibrios associated with mud crab from the brackish
10 water pond. It was lower than the diversities that were found in the cultured and wild crab
11 in India (Jithendran, Poornima, Balasubramanian & Kulasekarapandian, 2010) as well as
12 traditional brackish water surrounding of Semarang bay (Sarjito, Hastuti, Samidjan, &
13 Prayitno, 2014).

24 Repetitive Genomic Sequences-PCR (Rep-PCR)

25
26
27 The Repetitive PCR analysis and the dendrogram related to the 25 pure isolates
28 bacteria originating from mud crabs found a seven group from total samples (Figure 3).
29 Furthermore, the molecular identification only represented by one isolates from each
30 seven group such as CJR14, CJR15, CJD23, CJK10, CJK11, CJD22, and CJR05 (Table
31 1).

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44 **Figure 3.** A dendrogram based on Rep-PCR of 25 bacteria isolates associated with
45 vibriosis clinical signs from traditional mud crab pond.

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49 Figure 3 shows a similarity between 25 isolates into seven groups due to the
50 difference between repetitive bacterial sequences then it was examined using the 16S
51 rRNA gene for identification. A total of seven isolates were identified and presented in
52 Table 1—the range of homology percentage between 92-98%. The highest similarity was
53 showed by isolate CJR15 from group II, 98% homology with *C. thiocicly* strain TG 5-3.
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4 The homology 97% was appointed to isolate CJK10 and CJK11 from group IV and V
5 that similar to *V. parahaemolyticus* ATCC 17802 and *S. loihica* strain PV4. Followed
6
7 by *V. alginolyticus* strain NBRC 15630 with 96% homology from isolate CJR05 in group
8
9 VII. Moreover, isolate CJR14 and CJD23 have 95% homology with *V. harveyi* strain
10
11 NCIMB1280 and *P. ganghwense* strain FR311 in group I and III, while isolate CJD22
12
13 was closely related 92% to *S. algae* strain ATCC5192 in group VI.
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19 Four genus Vibrionaceae (*Vibrio*, *Shewanella*, *Photobacterium*,
20 and *Catenococcus*) were mostly found in mud crab (Li *et al.*, 2012; Wei *et al.*, 2019).
21
22 Three species of vibrio, i.e. *V. parahaemolyticus* (CJK10), *V. harveyi* (CJR14), and *V.*
23
24 *alginolyticus* (CJR05) also have been detected in this study. The vibrios were frequently
25
26 found as a causative agent of shell disease in *L. vannamei* shrimp and mud crab, *S.*
27
28 *serrata*, (Estave, & Herrere, 2000; Wang, 2011; Soto-Rodriguez *et al.*, 2012). *V.*
29
30 *parahaemolyticus* has been found as a main pathogen in mud crab in China (Xia *et al.*,
31
32 2010) and Chakoria Coast, Bangladesh (Aftabuddin *et al.*, 2013). Additionally, *V.*
33
34 *harveyi* has been reported as a causative agent of vibriosis in fattening mud crabs from
35
36 brackish water pond in Pemalang coast (Sarjito *et al.*, 2016) and mud crab from extensive
37
38 brackish water pond of surrounding the Gulf of Semarang (Sarjito, Hastuti, Samidjan, &
39
40 Prayitno, 2014) and Malaysia (Najiah, Nadirah, Sakri, & Harrison, 2010). These bacteria
41
42 were also reported as a causative agent of bacterial diseases in zoea stage of mud crab
43
44 (Jithendran, Poornima, Balasubramanian, & Kulasekarapandian, 2010; Department of
45
46 Agriculture Fisheries and Forestry Fisheries Queensland, 2012); adult mud crabs
47
48 (Poornima *et al.*, 2012 and Lavilla-Pitogo, Celia, & Leobert, 2004). Moreover, Sarjito,
49
50 Hastuti, Samidjan, & Prayitno, 2014, found that this bacterium was a potential pathogen
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52 to mud crabs. This study also revealed that *V. alginolyticus* also found in mud crab, *S.*
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4 *serrata*, infected bacterial diseases in north-central Java. *V. alginolyticus* was commonly
5 reported from brackish water, estuary, and marine environment. It was found as an
6
7 opportunistic bacteria associated with crustacean culture and fish culture (Austin, &
8
9 Austin, 2007). Moreover, it also is seen as an important pathogenic bacteria associated
10
11 with infectious diseases in mud crab (Najiah, Nadirah, Sakri & Harrison, 2010; Sarjito,
12
13 Hastuti, Samidjan, & Prayitno, 2014) and shell disease of mud crab *S. serrata* grow out
14
15 pond located at Mahendrapalli, Nagapattinam District, Tamil Nadu, India (Gunasekaran,
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17 Gopalakrishnan, Deivasigamani, Muhilvannan, & Kathirkaman, 2019).

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24 Genus Photobacterium was found in the coastal, open-ocean and deep-sea water
25
26 (Moi *et al.*, 2017). Generally, in the seawater and sediment of marine environment, genus
27
28 Photobacterium are free-life forms, but this bacteria also colonize several animal surfaces
29
30 developing neutral or negative relationships with the host (Labella, Arahal, Castro,
31
32 Lemos, & Borrego, 2017). Surprisingly, *P. ganghwense* was detected in this study. This
33
34 bacterium, was firstly reported from a seawater in Ganghwa Island, South Korea (Park *et*
35
36 *al.*, 2006). The characteristics of this bacterium are Gram-negative, facultative anaerob,
37
38 catalase and oxidase-positive, motile, oval, rod-shaped, also halophilic (Park *et al.*, 2006).
39
40 It is even luminescent, like the common species in this genus. Some of them are symbionts
41
42 of deep-sea fishes that hold them in specialized luminous organs (Lucena *et al.*, 2011).
43
44 Moreover, Wei *et al.*, (2019) found that Photobacterium was dominant as gut microbiota
45
46 in mud crab *S. paramamosain* where collected from nine coastal areas of southern China.

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51 The result showed that both *Shewanella algae* and *Shewanella loihica* were
52
53 found associated with mud crabs that have clinical vibriosis from the traditional brackish
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55 water pond of North central java. The genus *Shewanella* is phylogenetically affiliated to
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57 the γ - Proteobacteria (Yoon, Yeo, Kim, & Oh, 2004). These bacteria were also closely
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4 related to genus vibrio or vibrionaceae (Tseng *et al.*, 2018). *Shewanella* spp., are Gram-
5
6 negative, motile bacilli. Those bacteria are found throughout the world, mainly in marine
7
8 environments. The most crucial phenotypic characteristic of *Shewanella* spp. is the
9
10 production of hydrogen sulphide. They have an essential role as a turnover of organic
11
12 material, and capable of dissimilatory reduction of various metals and other substances,
13
14 such as Nitrate, Nitrite, Thiosulphate, and Trimethylamine-N-oxide (Holt, Gahrn-
15
16 Hansen, & Bruun, 2005).
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21 *S. algae* has been reported as bacterial pathogen associated with Black Spot
22
23 Disease in Freshwater-Cultured White leg Shrimp (*Penaeus vannamei*) (Cao, Chen, Lu,
24
25 & An, 2018), causative agent of *Babylonia* (Shufang, Zhang, Dequan, Shiping, & Huang,
26
27 2015); *Cynoglossus semilaevis* (Han *et al.*, 2017), and *Scianeops ocellatus* (Zhang, Zhu,
28
29 & Wang, 2013). This bacterium causes mortalities of more than 50% of the white leg
30
31 shrimps cultured in the freshwater pond of Lianyuang, Jiansu province, China (Cao,
32
33 Chen, Lu, & An, 2018). According to Prayitno, Sarwan & Sarjito (2015) *S. algae* was
34
35 also presented in gut of milkfish from the Northern Coast of Central Java. In Demak, *S.*
36
37 *algae* was found only when the water temperature is more than 23°C. It correlates with a
38
39 wide range of the salinity and the temperature (Tseng *et al.*, 2018) where the isolation
40
41 of *S. algae* can be performed with the salinity of 15-20%. Additionally, the infection of
42
43 genus *Shewanella* mostly happens in countries with a warm climate, or mainly due to
44
45 warm summers in countries with temperate climates (Holt, Gahrn-Hansen, & Bruun,
46
47 2005). Furthermore, a literature review of the period 1999 to 2017 showed that over 64%
48
49 (9/14) of infection cases in aquatic animals were in Asia, including China, Japan,
50
51 Malaysia, and Iran (Tseng *et al.*, 2018). Although it is rare in human pathogen and
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4 symptoms of infection, the food safety of crab product should be considered since this
5
6 bacterium was already detected in mud crab farming at North Coast of Java.
7

8
9 *Shewanella loihica* was isolated and identified from iron-rich microbial mats in
10
11 the Pacific Ocean (Gao *et al*, 2006). The result shows that *S. loihica* is a gram-negative,
12
13 non-spore-forming, straight rod with a mean length of 1-8 mm and a mean width of 0-7
14
15 mm. Single flagella are used to motile-facultative psychrotolerant anaerobe. The colonies'
16
17 characteristics are smooth, glistening, circular, flat to slightly raised, orange in colour
18
19 (Gao *et al*, 2006). Temperature 18 °C, pH 6.0-8.0, and Na⁺ presence are the optimum
20
21 environmental conditions of *S. loihica* to grow. This bacterium was able to grow in over
22
23 a wide range of temperatures and pH. *S. loihica* plays a role as an agent of metal reduction
24
25 and iron bio-mineralization. Based on that, the existence of this bacterium can be caused
26
27 by the metal and nitrogen content in the water. The current study of *S. loihica* is also
28
29 focusing on metal reduction and iron bio-mineralization capabilities (Newton, Mori,
30
31 Nakamura, Hashimoto, & Watanabe, 2009; Philips *et al.*, 2018), denitrification
32
33 ($\text{NO}_3^- \rightarrow \text{N}_2$) and a respiratory ammonification ($\text{NO}_3^- \rightarrow \text{NH}_4^+$) pathway (Yoon, Sanford,
34
35 & Löffler, 2015). Therefore, this bacterium has important role on the governing the fate
36
37 of $\text{NO}_3^- / \text{NO}_2^-$ in environmental, including soils and sediments where mud crab lives
38
39 (Yoon, Cruz-García, Sanford, Ritalahti, & Löffler, 2015; Yoon, Song, Phillips, Chang,
40
41 & Song, 2019).
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48 *Catenococcus thiocyli* was identified by Yarza *et al.* (2013), the study found
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50 that *C. thiocyli* is part of genus Vibrionaceae, order Vibrionales, class
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52 Gammaproteobacteria and phylum Proteobacteria. Only few studies reported this
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54 bacterium (Zheng *et al.*, 2016; Li *et al.*, 2019; Achmad *et al.*, 2019; Guibert, Lecellier,
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56 Torda, Pochon, & Berteaux-Lecellier, 2020). This bacterium was isolated from in Sansha
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4 Yongle Blue Hole (Li *et al.*, 2019) and Pacific white shrimp (*Litopenaeus vannamei*)
5
6 larvae (Zheng *et al.*, 2016). This member of Vibrionaceae was found as a causative agent
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8 of Ice-ice diseases of the seaweed, *Kappaphycus alvarezii*, (Achmad *et al.*, 2019) and
9
10 moribund of the giant clam, *Tridacna maxima*, (Guibert, Lecellier,
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12 Torda, Pochon, & Berteaux-Lecellier, 2020).
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17 **Table 1.** Molecular characterization of seven bacterial species associated with clinical
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19 signs of Vibriosis on mud crabs from the North Coast of Central Java.
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23 The seven vibrios associated with mud crab were closely (homology range
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25 between 92-98%) related to the *Vibrio harveyi* NCIMB1280 (CJR14), *Catenococcus*
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27 *thiocicly* TG 5-3 (CJR15), *Photobacterium ganghwense* FR311 (CJD23); *Vibrio*
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29 *prahaemolyticus* ATCC 17802 (CJK10); *Shewanella loihica* PV4 (CJK11); *Shewanella*
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31 *algae* ATCC5 (CJD22) and *Vibrio alginolyticus* NBRC 15630 (CJR05) (Table 1).
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35 **Diversity of Vibriosis Clinical Signs Associated Bacteria**

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39 The diversity of vibrios in mud crab with vibriosis clinical signs were compared
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41 from the three-sampling site to perform the percentage of species in each location (Table
42
43 2). Table 2 accommodates all of the isolates that identified based on the 16S rRNA gene.
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45 The diversity of vibriosis clinical signs associated with bacteria was explored using
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47 molecular characterization by Rep-PCR. This method is a useful tool to distinguish the
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49 diversity of bacteria according to the number and size of repetitive bacterial sequences.
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51 This technique could prevent to analyse the similar isolate of bacteria based on its DNA
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53 fingerprinting. Therefore, rep-PCR is helpful in grouping *Vibrio* species. The diversity of
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55 vibrios found in three sample locations (Table 2). We found more bacteria species in
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4 Rembang rather than in Demak and Kendal. The highest percentage of vibrio diversity
5 compared to the total isolate in each location was in Demak (80%).
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10 Table 2. The diversity of Vibrios in Mud Crab from the North Coast of Central Java per
11 location.
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15 **Figure 4.** The bacterial diversity in Mud crab with clinical signs of Vibriosis from the
16 North Coast of Central Java.
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21 Figure 4 represented the bacterial diversity in Mud crab with Vibriosis's clinical signs
22 from the North Coast of Central Java. The highest number species was *C. thiocicly* with
23 the percentage 32%, while 24% was *V. parahaemolyticus*, *S. loihica* and *S. algae* in the
24 equal percentage in 12%, as well as *P. ganghwense* and *V. algynolyticus* in 8%, and the
25 lowest rate was *V. harveyi* in 4%.
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34 Figure 5. Phylogenetic tree of the bacteria associated with vibriosis clinical signs from
35 traditional mud crab farming in the North Coast of Central Java, Indonesia constructed
36 using Neighbour-Joining analysis with 1000 replicates.
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42 In order to represent the relationship among isolates, the phylogenetic was constructed.
43 Figure 5 shows that all of the strains have assembled with the closely related species. The
44 relationship between the genus performs that the bacteria genus *Vibrio* has the same clade
45 with *Catenococcus thiocycli*. Those groups are more closely related compared to the
46 group of *Shewanella* and *Photobacterium*.
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54 The isolates were continuing to be grouped based on the sampling site (Table 2).
55 Rembang has the highest number of strains. However, from seven groups of *Vibrio*, only
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4 six groups were detected in this site. Demak has four groups of *Vibrio* from five isolates
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6 found. Furthermore, Kendal only has two groups of *Vibrio* from seven strains. The results
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8 indicated that the highest diversity of *Vibrio* was found in Rembang, rather than Demak
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10 and Kendal. It might happen since those locations have a higher abrasion (Directorate of
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12 Marine and Fisheries, 2019). Miller (1989) revealed that the abrasion could decline the
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14 microbial abundances.
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19 Moreover, Figure 4 was designed to know the highest species of Vibrionaceae
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21 found in this study. Based on the dendrogram of rep-PCR, the most top species come
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23 from *C. thiocicly* in group II with a percentage number 32%, and the lowest was *V.*
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25 *harveyi* with the percentage 4%. Based on the phylogenetic tree (Figure
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27 5), *Vibrio* and *Catenococcus* are more closely related than those
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29 of *Shewanella* and *Photobacterium*.
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34 According to the results of this study, future research should be conducted to have
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36 a deep understanding and correlation between the biotic and abiotic factors that affect the
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38 health status of Mud-crab in the traditional brackish water pond. Therefore, the
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40 metagenomic approach would be helpful to describe the bacterial structure community in
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42 the healthy and infected mud crab. Thus, the design of prevention methods to reduce
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44 bacterial disease outbreak of Mud crab farming could be expertly constructed.
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49 **4. Conclusions**

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51 This study found 25 bacteria isolates that can be associated with vibriosis clinical signs
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53 from traditional mud crab farming in the North Coast of Central Java. The isolates were
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55 closely related to *Vibrio harveyi* strain NCIMB1280 (CJR14), *Catenococcus*
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57 *thiocicly* strain TG 5-3 (CJR15), *Photobacterium ganghwense* strain FR311
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4 (CJD23); *Vibrio parahaemolyticus* ATCC 17802 (CJK10); *Shewanella loihica* strain
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6 PV4 (CJK11); *Shewanella algae* strain ATCC5 (CJD22) and *Vibrio alginolyticus* strain
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8 NBRC 15630 (CJR05). Strains of these seven bacteria are well known to be pathogens
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10 for aquatic organisms. Moreover, the highest diversity of Vibrionaceae was obtained from
11
12 Rembang, and the highest bacterial diversity found in three sampling sites was *C.*
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14 *thiocicly*.
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Figure 1. Research Location represented in a small red box in the left of map.



Figure 2. The clinical signs of mud crab (*S. serrata*) infected by bacterial diseases: (a.) Red/brown spots in the carapace; (b.) Red-brown spots in the carapace wound on the abdomen.

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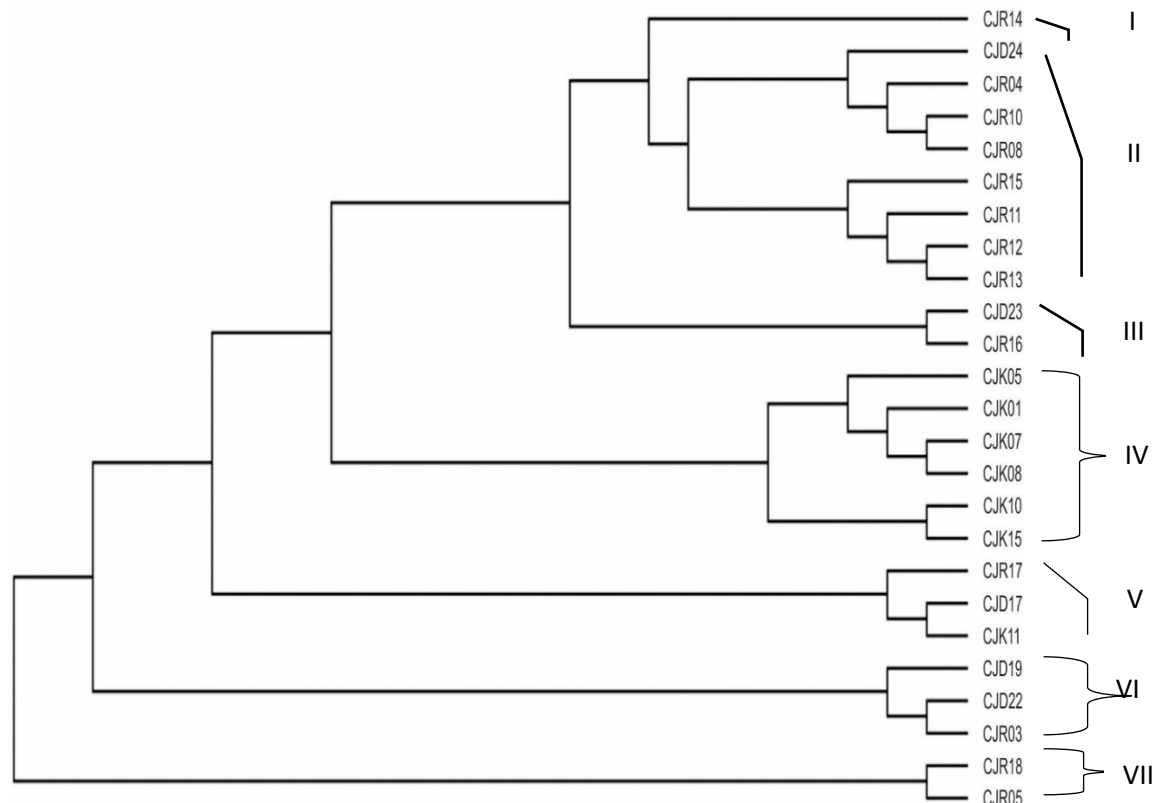


Figure 3. A dendrogram based on Rep-PCR of 25 Bacteria Isolates Associated with vibriosis clinical signs from traditional mud crab farming in the North Coastal of Central Java, Central Java, Indonesia.

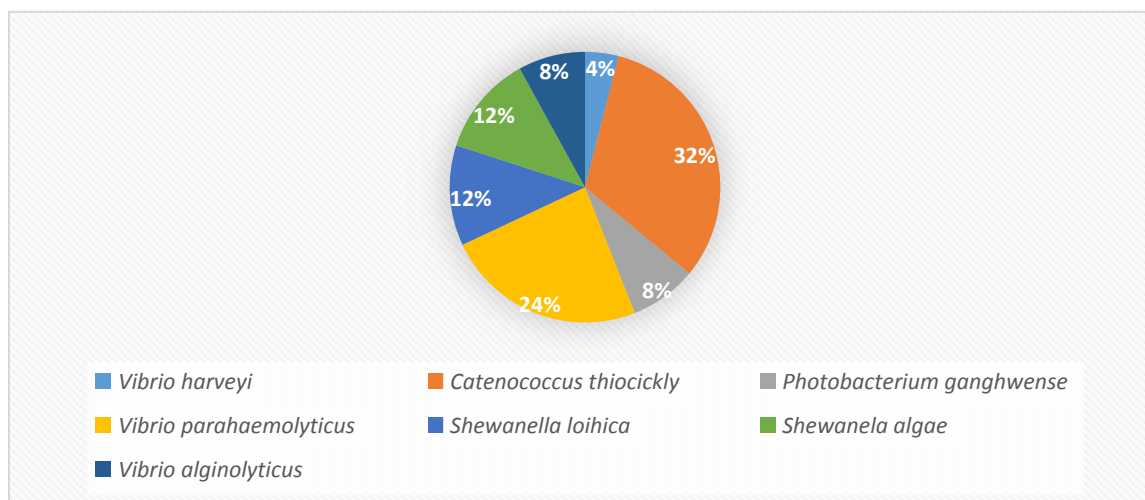


Figure 4. The bacterial diversity in Mud crab with clinical signs of Vibriosis from North Coast of Central Java.

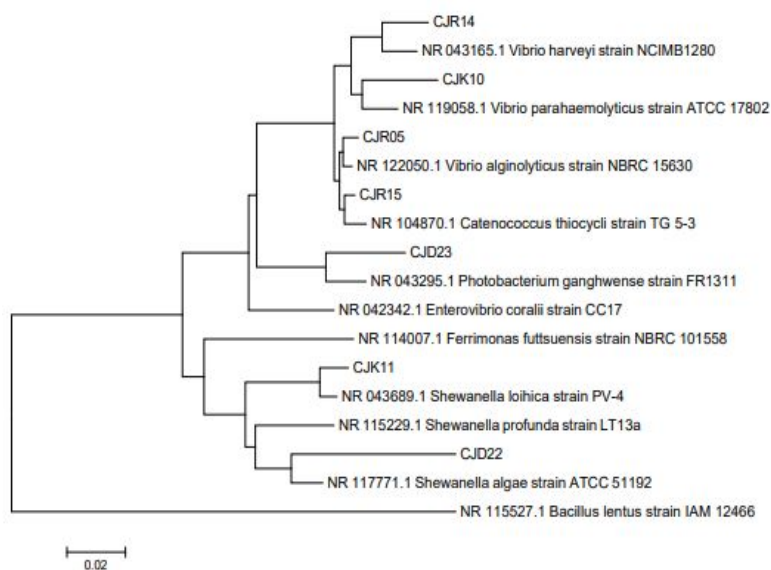


Figure 5. Phylogenetic tree of the bacteria associated with vibriosis clinical signs from traditional mud crab farming in the North Coast of Central Java, Indonesia constructed using Neighbour-Joining analysis with 1000 replicates.

Table 1. Molecular characterization of seven bacterial species associated with clinical signs of Vibriosis on mud crabs from North Coast of Central Java.

Isolates Code	Query Length	Close relative	Query Cover (%)	Homology (%)	Acc. Number
CJR14	1548	<i>Vibrio harveyi</i> strain NCIMB1280	98	95	NR 043165.1
CJR15	1319	<i>Catenococcus thiocicly</i> strain TG 5-3	98	98	NR 118258.1
CJD23	1387	<i>Photobacterium ganghwense</i> strain FR311	98	95	NR 043295.1
CJK10	1340	<i>Vibrio parahaemolyticus</i> ATCC 17802	99	97	NR 043689.1
CJK11	1335	<i>Shewanella loihica</i> strain PV4	97	97	NR 025794.1
CJD22	1488	<i>Shewanella algae</i> strain ATCC5192	95	92	NR 117771.1
CJR05	1362	<i>Vibrio alginolyticus</i> strain NBRC 15630	96	96	NR 122050.1

Table 2. The diversity of Vibrios in Mud Crab from North Coast of Central Java per location.

Location	Isolates Code	Bacteria Species	Vibrios diversity	Isolate Number	Percentage of species compare to total isolate (%)
Rembang	CJR14;				
	CJR04;				
	CJR10;				
	CJR08;				
	CJR15;	<i>Vibrio harveyi</i> strain NCIMB1280			
	CJR11;	<i>Catenococcus thiocicly</i> strain TG 5-3,			
	CJR12;	<i>Photobacterium ganghwense</i> strain FR311,	6	13	52%
	CJR13;	<i>Shewanella loihica</i> strain PV4,			
	CJR16;	<i>Shewanella algae</i> strain ATCC5192,			
	CJR17;	<i>Vibrio alginolyticus</i> strain NBRC 15630,			
	CJR03;				
	CJR18;				
	CJR05				
Demak	CJD24;	<i>Catenococcus thiocicly</i> strain TG 5-3			
	CJD23;	<i>Photobacterium ganghwense</i> strain FR311	4	5	80%
	CJD17;	<i>Shewanella loihica</i> strain PV4			

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	CJD19;	<i>Shewanella algae</i> strain ATCC5192			
	CJD22				
	CJK05;				
	CJK01;				
	CJK07;	<i>Vibrio prahaemolyticus</i> ATCC 17802			
Kendal	CJK08;	<i>Shewanella loihica</i> strain PV4	2	7	28%
	CJK10;				
	CJK15;				
	CJK11				

For Review Only



Sarjito Sarjito <sarjito@live.undip.ac.id>

**Songklanakar Journal of Science and Technology - Decision on Manuscript
IDSJST-2020-0244**

2 messages

Songklanakar Journal of Science and Technology

Tue, Nov 24, 2020 at 10:17

<onbehalfof@manuscriptcentral.com>

AM

Reply-To: proespichaya.k@psu.ac.th

To: sarjito@live.undip.ac.id, resti_wisnoe@yahoo.com

23-Nov-2020

Dear Mr. Sarjito:

Manuscript ID SJST-2020-0244 entitled "Molecular Characterization of vibriosis clinical signs-associated bacteria from traditional mud-crab farming in the North Coast of Central Java, Indonesia" which you submitted to the Songklanakar Journal of Science and Technology, **has been reviewed**. The comments from reviewer(s) are included at the bottom of this letter.

In view of the criticisms of the reviewer(s), I must decline the manuscript for publication in the Songklanakar Journal of Science and Technology at this time. However, a new manuscript may be submitted which takes into consideration these comments.

Please note that resubmitting your manuscript does not guarantee eventual acceptance, and that your resubmission will be subject to re-review by the reviewer(s) before a decision is rendered.

You will be unable to make your revisions on the originally submitted version of your manuscript. Instead, revise your manuscript using a word processing program and save it on your computer.

Once you have revised your manuscript, go to <https://mc.manuscriptcentral.com/sjst> and login to your Author Center. Click on "Manuscripts with Decisions," and then click on "Create a Resubmission" located next to the manuscript number. Then, follow the steps for resubmitting your manuscript.

You may also click the below link to start the resubmission process (or continue the process if you have already started your resubmission) for your manuscript. If you use the below link you will not be required to login to ScholarOne Manuscripts.

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Because we are trying to facilitate timely publication of manuscripts submitted to the Songklanakar Journal of Science and Technology, your resubmitted manuscript should be submitted by 22-May-2021. If you are unable to submit by this date please contact the Editorial Office for options.

I look forward to a resubmission.

Sincerely,
Assoc. Prof. Dr. Proespichaya Kanatharana
Editor in Chief, Songklanakar Journal of Science and Technology
proespichaya.k@psu.ac.th

Reviewer(s)' Comments to Author:

Reviewer: 1

Comments to the Author

Overall

This manuscript looks into the description of bacteria associated with diseased mud crabs from Indonesia. The whole experiment is nicely designed, although some additional analyses would really improve the content of this manuscript. The language of the whole manuscript needs improving.

Introduction

1. The introduction section, especially the first paragraph, needs to be re-written. The authors have the idea, but the sentences failed to bring the idea beautifully across to readers.
2. Also, the data in the second introduction paragraph needs to be updated, as there are several recent papers on *Vibrio* infection in mud crabs.

Method

3. In the method section, were the mud crabs transported live to the lab.
4. How was the 45 bacteria initially isolated?
5. Please provide the full term of rep-PCR before abbreviating it. Same as in Abstract.
6. Remove "To find out a strictly ... gene"
7. Why the authors used 16S rRNA to confirm species instead of rep-PCR? The common method should be, after rep-PCR amplification, amplicons will be selected, extracted from gel and cloned into a vector, subsequently sequenced and blasted on GenBank.

Results & Discussion

8. It would be interesting to see the link between bacteria identified at different extracted locations of mud crabs, e.g. claws, ventral, carapace.
9. The bacteria species name and the isolate codes are not aligned properly.
10. How many bands were polymorphic? What were the amplified band sizes and how many bands amplified using the REP primers?
11. In addition to the dendrogram (Fig. 3), a PCA plot is recommended to present the relationships that co-exist between and within species.

Reviewer: 2

Comments to the Author

Dear Authors,

Thank you for your hardwork to come up with this paper. Basically I think that the flow of the story is not smooth. The aim/objectives that you put in this paper is not reflecting some of the components in your paper. It seems that the Title, Abstract and your methodology is one story (molecular characterization) and your Objectives, Result and Discussion is another story (Diversity of bacteria). So, as a read, I found myself reading two different goals in your paper. Do you want to present the molecular characterization of bacteria or you want to assess the diversity of the disease associated bacteria to mud crab? I think this can be improved. Thank you.

Associate Editor

Comments to the Author:

(There are no comments.)



Comment from Reviewer.pdf

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Comment from Reviewer

1. Overall, the abstract did not reflect the content of the paper. The abstract focused on the molecular characterization, but the paper objective, result and conclusion are more focused to the diversity of the bacteria. Therefore, some changes are needed so that the paper is more focused and have one goal only.

2. Introduction:

There is little connectivity of these sentences “Traditional brackish water pond used for polyculture of shrimp and milkfish, wild mud crab may intrude in these ponds, where they are caught together with the shrimp and milkfish. However, the culture of the mud crab themselves is spreading. The intrusion of wild mud crab can be a problem to crustacean’s culture. Infected mud crab can carry disease agents that may infect the healthy cultured crustaceans. To prevent damage of the crustacean culture, farmers may disinfect the pond and raise barriers that are well embedded in the bunds, but these efforts are rarely 100% effective” to the next sentence of the first paragraph.

The sentences meant to show that the problems with crab as a carrier to infect other crustaceans and next sentence is the problem with the mud crab fattening system. There must be a sentence to connect the flow of the story in this paragraph.

3. Objective:

The two objectives in this paper are not inline with the title of this study. I would suggest that the objective is changed to something that reflecting the finding and the title of this study.

4. Materials and Methods:

Why in your abstract is 25 bacteria but in the methodology 45 bacteria?

5. Results and Discussion:

In your methodology you mentioned 45 isolates.

6. For this paragraphh,” The vibrio’s associated in mud crabs from traditional brackish water pond of the North Coast of Central Java (Figure 1), it was closely related to *V. harveyi* strain NCIMB1280 (CJR14), *C. thiocicly* strain TG 5-3 (CJR15), *P. ganghwense* strain FR311 (CJD23); *V. prahaemolyticus* ATCC 17802 (CJK10); *S. loihica* strain PV4 (CJK11); *S. algae* strain ATCC5 (CJD22) and *V. alginolyticus* strain NBRC 15630 (CJR05). The result also revealed the diversity of vibrios associated with mud crab from the brackish water pond. It was lower than the diversities that were found in the cultured and wild crab in India (Jithendran, Poornima, Balasubramanian & Kulasekarapandian, 2010) as well as traditional brackish water surrounding of Semarang Gulf (Sarjito, Hastuti, Samidjan, & Prayitno, 2014)”.

I would suggest that this paragraph is transferred to page 8, after Table 1. This is not suitable in its current location. The identification of the bacteria must come after the Rep PCR according to your Methodology.

Rebutal of Comment of Reviewers SJST-2020-0244

No.	Suggestion	Response
1.	Overall, the abstract did not reflect the content of the paper. The abstract focused on the molecular characterization, but the paper objective, result and conclusion are more focused to the diversity of the bacteria. Therefore, some changes are needed so that the paper is more focused and have one goal only	Done Please find in revised manuscript
2.	There is little connectivity of these sentences “Traditional brackish water pond used for polyculture of shrimp and milkfish, wild mud crab may intrude in these ponds, where they are caught together with the shrimp and milkfish. However, the culture of the mud crab themselves is spreading. The intrusion of wild mud crab can be a problem to crustacean’s culture. Infected mud crab can carry disease agents that may infect the healthy cultured crustaceans. To prevent damage of the crustacean culture, farmers may disinfect the pond and raise barriers that are well embedded in the bunds, but these efforts are rarely 100% effective” to the next sentence of the first paragraph. The sentences meant to show that the problems with crab as a carrier to infect other crustaceans and next sentence is the problem with the mud crab fattening system. There must be a sentence to connect the flow of the story in this paragraph	We add connecting sentence: The cause of the disease does not only occur in infected wild crabs but also in crab farming.
3.	Objective The two objectives in this paper are not in-line with the title of this study. I would suggest that the objective is changed to something that reflecting the finding and the title of this study.	This study was conducted to assessed the molecular characterization of bacteria associated with clinical symptoms and the diversity of the bacteria vibriosis infected mud crabs farming in brackish water traditional ponds along the North Coast of Central Java, Indonesia
4.	Materials and Methods: Why in your abstract is 25 bacteria but in the methodology 45 bacteria?	Thank you for your detail correction, there are 25 isolate
5.	Results and Discussion: In your methodology you mentioned 45 isolates.	

<p>6.</p>	<p>For this paragraph," The vibrio's associated in mud crabs from traditional brackish water pond of the North Coast of Central Java (Figure 1), it was closely related to <i>V. harveyi</i> strain NCIMB1280 (CJR14), <i>C. thiocicly</i> strain TG 5-3 (CJR15), <i>P. ganghwense</i> strain FR311 (CJD23); <i>V. prahaemolyticus</i> ATCC 17802 (CJK10); <i>S. loihica</i> strain PV4 (CJK11); <i>S. algae</i> strain ATCC5 (CJD22) and <i>V. alginolyticus</i> strain NBRC 15630 (CJR05). The result also revealed the diversity of vibrios associated with mud crab from the brackish water pond. It was lower than the diversities that were found in the cultured and wild crab in India (Jithendran, Poornima, Balasubramanian & Kulasekarapandian, 2010) as well as traditional brackish water surrounding of Semarang Gulf (Sarjito, Hastuti, Samidjan, & Prayitno, 2014)".</p> <p>I would suggest that this paragraph is transferred to page 8, after Table 1. This is not suitable in its current location. The identification of the bacteria must come after the Rep PCR according to your Methodology</p>	<p>Done Moved to page 8</p>
<p>7.</p>	<p>The diversity of bacteria is not clearly stated in the Methodology. In the methodology, you need to mentioned about the methodology to calculate the diversity of the bacteria so that the data you can expressed in your results and you can discuss it in your discussion.</p>	<p>Done Bacterial diversity is the percentage of the number of each species in a Dendogram group based on Rep-PCR compared to the total number of isolates. Then, the diversity of vibrios in mud crab with vibriosis clinical signs were compared from the three-sampling site to perform the percentage of species in each location</p>

Original Article

Molecular Characterization of vibriosis clinical signs-associated bacteria from traditional mud-crab farming in the North Coast of Central Java, Indonesia

Sarjito^{1*}, Alfabetian Harjuno Condro Haditomo¹, Slamet Budi Prayitno¹, Aninditia Sabdaningsih², Desrina¹, and Restiana Wisnu Ariyati¹

¹Aquaculture Study Program, Aquaculture Department, Fisheries and Marine Science Faculty, Diponegoro University, Semarang, 50275, Indonesia

²Department of Aquatic Resources, Fisheries and Marine Science Faculty, Diponegoro University, Semarang, 50275, Indonesia

*Email address : sarjito@live.undip.ac.id

Abstract

This study examines the molecular characterization of bacteria associated with clinical symptoms and the diversity of vibriosis bacteria that infected mud crab farming from three sampling locations, namely Rembang, Demak, and Kendal Districts which are the largest cultivated crab producers in the North Coast of Central Java. Twenty-five bacteria were isolated from hepatopancreas, gill, and carapace of mud crab that have clinical signs of vibriosis that cultured in TCBS and TSA medium. After a modified rep-PCR and further PCR amplification of the 16S ribosomal RNA gene, the sequence analysis was performed. The DNA sequences compared with the BLAST from the NCBI database. Then, the phylogenetic tree constructed by the MEGA X program. The clinical signs on mud crab were associated with seven isolates: CJR14, CJR15, CJD23, CJK10, CJK11, CJD22, and CJR05. Based on 16S rRNA sequence analysis, these

isolates were firmly (92-98%) related to *Vibrio harveyi* NCIMB1280, *Catenococcus thiocicly* TG 5 – 3, *Photobacterium ganghwense* FR311, *Vibrio parahaemolyticus* ATCC 1780, *Shewanella loihica* PV4, *Shewanella algae* ATCC5, and *Vibrio alginolyticus* NBRC 15630. **Bacterial Vibriosis strain *Catenococcus thiocicly* (32%) was found in each sampling location.** More type of bacteria (six out seven strains) were found in Rembang district but the highest percentage of vibrio diversity compared to the total isolate was found in Demak district (80%).

Keywords: bacterial diseases, mud crab, 16S rRNA, rep PCR, vibriosis

1. Introduction

Mud crab, *Scylla serrata* (Forskål, 1775), is traditionally caught in and around brackish water ponds and mangrove forests along the North Coast of Central Java, Indonesia. The mangrove forests and its soils are this crab's origin habitat. Traditional brackish water pond used for polyculture of shrimp and milkfish, wild mud crab may intrude in these ponds, where they are caught together with the shrimp and milkfish. However, the culture of the mud crab themselves is spreading. The intrusion of wild mud crab can be a problem to crustacean's culture. Infected mud crab can carry disease agents that may infect the healthy cultured crustaceans. To prevent damage of the crustacean culture, farmers may disinfect the pond and raise barriers that are well embedded in the bunds, but these efforts are rarely 100% effective. **The cause of the disease does not only occur in infected wild crabs but also in crab farming.** Bacterial diseases, especially vibriosis, was found to be a problem in the fattening of the mud crab culture in Pemalang (Sarjito *et al.*, 2016) and reported to cause more than 90% mortality in all life cycle

stages of mud crab growth (Jithendran, Poornima, Balasubramanian, & Kulasekarapandian, 2010). Reported clinical signs of infected mud crab are wounds on the body, decreased feed response and weakening (Jithendran, Poornima, Balasubramanian, & Kulasekarapandian, 2010), blackening and red spots of the carapace (Sarjito, Hastuti, Samidjan, & Prayitno, 2014; Sarjito *et al.*, 2016).

Vibrio's cause various disease outbreaks and severe economic losses in shrimp culture (Aguilre-Rivera, Prieto-Davo, Rodriguez-Fuentes G, Escalante-Herrera, & Gaxiola, 2019) and have the potential to destroy the future of the industrial culture of mud crab. Several species of *Vibrio* spp. were reported in and found to be associated with bacterial disease in mud crab: *V. fischeri*, and *V. nereis* (Wang, 2011); *V. alginolyticus* and *V. cholerae* (Najiah, Nadirah, Sakri & Harrison, 2010; Wang, 2011); *V. vulnificus* (Lavilla-Pitogo, Celia, & Leobert, 2004; Shanmuga, 2008; Wang, 2011), *V. splendidus*, and *V. orientalis* (Jithendran, Poornima, Balasubramanian, & Kulasekarapandian, 2010); *V. ordalii* (Sarjito, Hastuti, Samidjan, & Prayitno, 2014), *V. harveyi* (Sarjito, Hastuti, Samidjan, & Prayitno, 2014; Jithendran, Poornima, Balasubramanian, & Kulasekarapandian, 2010), *V. campbelli* (Shanmuga, 2008), and *V. parahaemolyticus* (Lavilla-Pitogo, Celia, & Leobert, 2004; Najiah, Nadirah, Sakri, & Harrison, 2010; Shanmuga, 2008). Previous research focused on the richness of bacteria associated with vibriosis in mud crab. The limited knowledge is available on the diversity of vibrios related to the disease outbreaks in mud crab in coastal of the North Central Java. Local government tends to stimulate the culture of mud crab, nevertheless, the program lacks information on disease identification and prevention.

This study was conducted to assessed the molecular characterization of bacteria associated with clinical symptoms and the diversity of the bacteria vibriosis infected

mud crabs farming in brackish water traditional ponds along the North Coast of Central Java, Indonesia.

2. Materials and Methods

Sampling

Mud crab, *S. serrata*, were collected from nine brackish water ponds of Kendal, Demak, and Rembang Regency, Central Java (each three ponds). These three locations are the largest producers of cultivated crabs in Central Java, Indonesia (Figure 1). Based on the clinical signs for vibriosis described by Jithendran, Poornima, Balasubramanian, & Kulasekarapandian (2010), 25 infected mud crabs were selected, stored in a sterile container and carried to the Integrated Laboratory of Diponegoro University for further analysis. The average size of the caught crab, measured from 13 to 15 cm, was 14.4 ± 0.7 cm.

Figure 1. Research location represented in a small red box in the left of map.

Bacterial Isolation

Forty-five bacteria were isolated from gills, hemolymph, hepatopancreas, and wounds on the shell of the infected mud crabs. Isolates bacteria were cultured on Thiosulfate Citrate Bile Salt Sucrose (TCBS) and Tryptic Soy Agar (TSA) (Thompson, Iida, & Swings, 2004; Sarjito *et al.*, 2016). The colonies were purified by the streak method based on morphological appearance (Sarjito *et al.*, 2016).

Repetitive Genomic Sequences-PCR (Rep-PCR)

The bacterial DNA was extracted from a 24 h-broth culture of isolate strains by using the chelex method with slight modification according to the procedure described

by (de Lamballerie, Zandotti, Vignoli, Bollet, & de Micco, 1992). Then, the isolates DNA extract were analyzed using the rep-PCR of Radjasa, Nasima, Sabdono, Kita-Tsukamoto, & Ohwada (2007) was modified by Sarjito, Haditomo, Desrina, Djunaedi, & Prayitno (2018) in the rep-PCR, BOX AIR (5'-CTACggCAAggCgACgCTgACg-3'). The REP 1R-I and REP 2-I primers contained nucleotide inosine (I) at an ambiguous position in the REP consensus. The mix PCR reagent contains one μL DNA template (diluted 100X), one μL primer, 7.5 μL Megamix Royal and 5.5 μL ddH₂O. Amplification was performed in a thermal cycler model Gene Amp PCR system 9700 with the following protocol: initial denaturation at 95°C for 5 minutes. Then 30 cycles of (denaturation 92°C for 1 minute, annealing 50°C for 1,5 minutes, extension 68°C for 8 minutes), and a final extension at 68°C for 10 minutes. The band DNA was visualized with injected 5 μL PCR products into 1% agarose gel and run to electrophoresis machine using 1X TBE buffer and observed in UV-transilluminator, as described by Radjasa, Nasima, Sabdono, Kita-Tsukamoto, & Ohwada (2007). Grouping of isolates was conducted based on the method of Sarjito, Radjasa, Sabdono, Prayitno, & Hutabarat (2009). This method is a useful tool for differentiating bacterial diversity according to the number and size of repeated bacterial sequences. Bacterial diversity is the percentage of the number of each species in a Dendogram group based on Rep-PCR compared to the total number of isolates. Then, the diversity of vibrios in mud crab with vibriosis clinical signs were compared from the three-sampling site to perform the percentage of species in each location.

Matrices were made from the band's position on the gel, which was analyzed by the Free Tree program using the UPGMA method for constructing the phylogenetic tree

(Sarjito, Haditomo, Desrina, Djunaedi, & Prayitno, 2018). Resampling was done by bootstrapping with 1000 replications (Prayitno, Sarwan, & Sarjito, 2015).

PCR Amplification of 16s rRNA Gene Fragments

The PCR amplification of the 16S rRNA gene was performed, according to Radjasa, Nasima, Sabdono, Kita-Tsukamoto, & Ohwada (2007) and Sarjito, Haditomo, Desrina, Djunaedi, & Prayitno (2018). To find out a strictly complete 16S rRNA gene, the amplification was presented with two primers, GM3F as a forward primer (5'AGAGTTTGATCMTGGC-3') and GM4R as a reverse primer (5'-TACCTTGTTACGACTT-3') (Sarjito, Radjasa, Sabdono, Prayitno, & Hutabarat, 2009). Genomic DNA of bacteria for PCR analysis was obtained from the destruction cell materials that were taken from fresh culture bacteria, suspended in sterile water (Sigma, Germany), and subjected to five cycles of freeze (-80°C) and thaw (95°C). The PCR product was then processed to find the band DNA in the right size around 1.500 bp. The sequencing step was facilitated by PT. Genetika Science. The determined DNA sequences of samples were then compared for homology using BLAST. The phylogenetic tree was established using MEGA X to find the closely related species (Sabdaningsih *et al.*, 2020).

3. Results and Discussion

Clinical Signs and Bacterial Isolation

The most frequent clinical signs of the infected mud crab (*S. serrata*) were wounds on the surface of the claws, the ventral, and the carapace. The isolation of bacteria using TCBS and TSA medium obtained 25 pure isolates of bacteria from three replicates agar plates and then stored in NA medium. The clinical signs of red-brown

and dark spots in the carapace were found in Figure 2. Similar clinical symptoms were found on the abdomen.

Figure 2. The clinical signs of mud crab (*S. serrata*) infected by bacterial diseases: (a.) Red/brown spots in the carapace; (b.) Red-brown spots in the carapace wound on the abdomen.

Red-brown or dark melanin spots mostly known as vibriosis in mud crab, patches of light red spots as well as dark spots on the carapace, also wounds on the body (claws, shell, and the ventral). The identical clinical signs have been found by Muyzer, Teske, Wirsen, and Jannasch (1995), Wang (2011), Lavilla-Pitogo, Celia, & Leobert (2004) and Prayitno, Sarjito & Putri (2017). It was also reported on the mud crabs that were affected by genus vibrio from the gulf of Semarang (Sarjito, Hastuti, Samidjan, & Prayitno, 2014) and Pemalang (Sarjito *et al.*, 2016). ‘Brown spot’ may occur due to the infection of chitinolytic bacteria that catabolize the chitin of the carapace and cause erosion and melanization (dark brown to black pigmentation) at the site of infection (Jithendran, Poornima, Balasubramanian & Kulasekarapandian, 2010)

Repetitive Genomic Sequences-PCR (Rep-PCR)

The Repetitive PCR analysis and the dendrogram related to the 25 pure isolates bacteria originating from mud crabs found a seven group from total samples (Figure 3). Furthermore, the molecular identification only represented by one isolates from each seven group such as CJR14, CJR15, CJD23, CJK10, CJK11, CJD22, and CJR05 (Table 1).

Figure 3. A dendrogram based on Rep-PCR of 25 bacteria isolates associated with vibriosis clinical signs from traditional mud crab pond.

Figure 3 shows a similarity between 25 isolates into seven groups due to the difference between repetitive bacterial sequences then it was examined using the 16S rRNA gene for identification. A total of seven isolates were identified and presented in Table 1—the range of homology percentage between 92-98%. The highest similarity was showed by isolate CJR15 from group II, 98% homology with *C. thiocicly* strain TG 5-3. The homology 97% was appointed to isolate CJK10 and CJK11 from group IV and V that similar to *V. parahaemolyticus* ATCC 17802 and *S. loihica* strain PV4. Followed by *V. alginolyticus* strain NBRC 15630 with 96% homology from isolate CJR05 in group VII. Moreover, isolate CJR14 and CJD23 have 95% homology with *V. harveyi* strain NCIMB1280 and *P. ganghwense* strain FR311 in group I and III, while isolate CJD22 was closely related 92% to *S. algae* strain ATCC5192 in group VI.

Table 1. Molecular characterization of seven bacterial species associated with clinical signs of Vibriosis on mud crabs from the North Coast of Central Java.

The vibrio's associated in mud crabs from traditional brackish water pond of the North Coast of Central Java (Figure 1), it was closely related to *V. harveyi* strain NCIMB1280 (CJR14), *C. thiocicly* strain TG 5-3 (CJR15), *P. ganghwense* strain FR311 (CJD23); *V. parahaemolyticus* ATCC 17802 (CJK10); *S. loihica* strain PV4 (CJK11); *S. algae* strain ATCC5 (CJD22) and *V. alginolyticus* strain NBRC 15630 (CJR05). The result also revealed the diversity of vibrios associated with mud crab from the brackish water pond. It was lower than the diversities that were found in the cultured and wild crab in India (Jithendran, Poornima, Balasubramanian & Kulasekarapandian, 2010) as well as traditional brackish water surrounding of Semarang Gulf (Sarjito, Hastuti, Samidjan, & Prayitno, 2014).

Four genus Vibrionaceae (*Vibrio*, *Shewanella*, *Photobacterium*, and *Catenococcus*) were mostly found in mud crab (Li *et al.*, 2012; Wei *et al.*, 2019). Three species of vibrio, i.e. *V. parahaemolyticus* (CJK10), *V. harveyi* (CJR14), and *V. alginolyticus* (CJR05) also have been detected in this study. The vibrios were frequently found as a causative agent of shell disease in *L. vannamei* shrimp and mud crab, *S. serrata*, (Estave, & Herrere, 2000; Wang, 2011; Soto-Rodriguez *et al.*, 2012). *V. parahaemolyticus* has been found as a main pathogen in mud crab in China (Xia *et al.*, 2010) and Chakoria Coast, Bangladesh (Aftabuddin *et al.*, 2013). Additionally, *V. harveyi* has been reported as a causative agent of vibriosis in fattening mud crabs from brackish water pond in Pemalang coast (Sarjito *et al.*, 2016) and mud crab from extensive brackish water pond of surrounding the Gulf of Semarang (Sarjito, Hastuti, Samidjan, & Prayitno, 2014) and Malaysia (Najiah, Nadirah, Sakri, & Harrison, 2010). These bacteria were also reported as a causative agent of bacterial diseases in zoea stage of mud crab (Jithendran, Poornima, Balasubramanian, & Kulasekarapandian, 2010); adult mud crabs (Poornima *et al.*, 2012 and Lavilla-Pitogo, Celia, & Leobert, 2004). Moreover, Sarjito, Hastuti, Samidjan, & Prayitno, (2014), found that this bacterium was a potential pathogen to mud crabs. *V. alginolyticus* was observed as an important pathogenic bacteria associated with infectious diseases in mud crab (Najiah, Nadirah, Sakri & Harrison, 2010; Sarjito, Hastuti, Samidjan, & Prayitno, 2014) and shell disease of mud crab *S. serrata* grow out pond located at Tamil Nadu, India (Gunasekaran, Gopalakrishnan, Deivasigamani, Muhilvannan, & Kathirkaman, 2019).

Genus *Photobacterium* was found in the coastal, open-ocean and deep-sea water (Moi *et al.*, 2017). Surprisingly, *P. ganghwense* was detected in this study. This bacterium, was firstly reported from a seawater in Ganghwa Island, South Korea (Park

et al., 2006). Furthermore, Wei *et al.*, (2019) found that Photobacterium was dominant as gut microbiota in mud crab *S. paramamosain* where collected from nine coastal areas of southern China.

The result showed that both *Shewanella algae* and *Shewanella loihica* were found in this study. They have an essential role as a turnover of organic material, and capable of dissimilatory reduction of various metals and other substances, such as Nitrate, Nitrite, Thiosulphate, and Trimethylamine-N-oxide (Holt, Gahrn-Hansen, & Bruun, 2005). *S. algae* has been reported as bacterial pathogen associated with Black Spot Disease in Freshwater-Cultured White leg Shrimp (*Penaeus vannamei*) (Cao, Chen, Lu, & An, 2018), causative agent of *Babylonia* (Shufang, Zhang, Dequan, Shiping, & Huang, 2015); *Cynoglossus semilaevis* (Han *et al.*, 2017), and *Scianeps ocellatus* (Zhang, Zhu, & Wang, 2013). According to Prayitno, Sarwan & Sarjito (2015) *S. algae* was also presented in gut of milkfish from the Northern Coast of Central Java. In Demak, *S. algae* was found only when the water temperature is more than 23°C. Additionally, the infection of genus *Shewanella* mostly occurs in countries with a warm climate (Holt, Gahrn-Hansen, & Bruun, 2005). Although it is rare in human pathogen and symptoms of infection, the food safety of crab product should be considered since this bacterium was already detected in mud crab farming at the North Coast of Central Java.

Shewanella loihica was isolated and identified from iron-rich microbial mats in the Pacific Ocean (Gao *et al.*, 2006). *S. loihica* plays a role as an agent of metal reduction and iron bio-mineralization. Based on that, the existence of this bacterium can be caused by the metal and nitrogen content in the water. Some reported study of *S. loihica* is also focusing on metal reduction and iron bio-mineralization capabilities (Newton, Mori,

Nakamura, Hashimoto, & Watanabe, 2009; Philips *et al.*, 2018), denitrification and a respiratory ammonification pathway (Yoon, Sanford, & Löffler, 2015). Therefore, this bacterium has an important role on the nutrient cycle in soils and sediments where mud crab lives.

Catenococcus thiocycli was identified by Yarza *et al.* (2013), the study found that *C. thiocycli* is part of the genus Vibrionaceae. Only few studies reported this bacterium (Zheng *et al.*, 2016; Li *et al.*, 2019; Achmad *et al.*, 2019; Guibert, Lecellier, Torda, Pochon, & Berteaux-Lecellier, 2020). This member of Vibrionaceae was found as a causative agent of Ice-ice diseases of the seaweed, *Kappaphycus alvarezii*, (Achmad *et al.*, 2019) and moribund of the giant clam, *Tridacna maxima*, (Guibert, Lecellier, Torda, Pochon, & Berteaux-Lecellier, 2020).

Diversity of Vibriosis Clinical Signs Associated Bacteria

The diversity of vibrios in mud crab with vibriosis clinical signs were compared from the three-sampling site to perform the percentage of species in each location (Table 2). Table 2 accommodates all of the isolates that identified based on the 16S rRNA gene. The diversity of vibriosis clinical signs associated with bacteria was explored using molecular characterization by Rep-PCR. This method is a useful tool to distinguish the diversity of bacteria according to the number and size of repetitive bacterial sequences. This technique could prevent to analyse the similar isolate of bacteria based on its DNA fingerprinting. Therefore, rep-PCR is helpful in grouping *Vibrio* species. The diversity of vibrios found in three sample locations (Table 2). We found more bacteria species in Rembang rather than in Demak and Kendal. The highest percentage of vibrio diversity compared to the total isolate in each location was in Demak (80%).

Table 2. The diversity of Vibrios in Mud Crab from the North Coast of Central Java per location.

Figure 4. The bacterial diversity in Mud crab with clinical signs of Vibriosis from the North Coast of Central Java.

Figure 4 represented the bacterial diversity in Mud crab with Vibriosis's clinical signs from the North Coast of Central Java. The highest number species was *C. thiocicly* with the percentage 32%, while 24% was *V. parahaemolyticus*, *S. loihica* and *S. algae* in the equal percentage in 12%, as well as *P. ganghwense* and *V. alginolyticus* in 8%, and the lowest rate was *V. harveyi* in 4%.

Figure 5. Phylogenetic tree of the bacteria associated with vibriosis clinical signs from traditional mud crab farming in the North Coast of Central Java, Indonesia constructed using Neighbour-Joining analysis with 1000 replicates.

In order to represent the relationship among isolates, the phylogenetic was constructed. Figure 5 shows that all of the strains have assembled with the closely related species. The relationship between the genus performs that the bacteria genus *Vibrio* has the same clade with *Catenococcus thiocycli*. Those groups are more closely related compared to the group of *Shewanella* and *Photobacterium*.

The isolates were continuing to be grouped based on the sampling site (Table 2). Rembang has the highest number of strains. However, from seven groups of *Vibrio*, only six groups were detected in this site. Demak has four groups of *Vibrio* from five isolates found. Furthermore, Kendal only has two groups of *Vibrio* from seven strains. The results indicated that the highest diversity of *Vibrio* was found in Rembang, rather

than Demak and Kendal. It might happen since those locations have a higher abrasion (Directorate of Marine and Fisheries, 2019). Miller (1989) revealed that the abrasion could decline the microbial abundances.

Moreover, Figure 4 was designed to know the highest species of Vibrionaceae found in this study. Based on the dendrogram of rep-PCR, the most top species come from *C. thiocicly* in group II with a percentage number 32%, and the lowest was *V. harveyi* with the percentage 4%. Based on the phylogenetic tree (Figure 5), *Vibrio* and *Catenococcus* are more closely related than those of *Shewanella* and *Photobacterium*.

According to the results, future research should be conducted to have a deep understanding and correlation between the biotic and abiotic factors that affect the health status of Mud-crab in the traditional brackish water pond. Therefore, the metagenomic approach would be helpful to describe the bacterial structure community in the healthy and infected mud crab. Thus, the design of prevention methods to reduce bacterial disease outbreak of Mud crab farming could be expertly constructed.

4. Conclusions

Strains of these seven bacteria are well known to be pathogens for aquatic organisms. Moreover, the highest diversity of Vibrionaceae was obtained from Demak, and the highest bacterial diversity found in three sampling sites was *C. thiocicly*.

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Figure 1. Research Location represented in a small red box in the left of map.



Figure 2. The clinical signs of mud crab (*S. serrata*) infected by bacterial diseases: (a.) Red/brown spots in the carapace; (b.) Red-brown spots in the carapace wound on the abdomen.

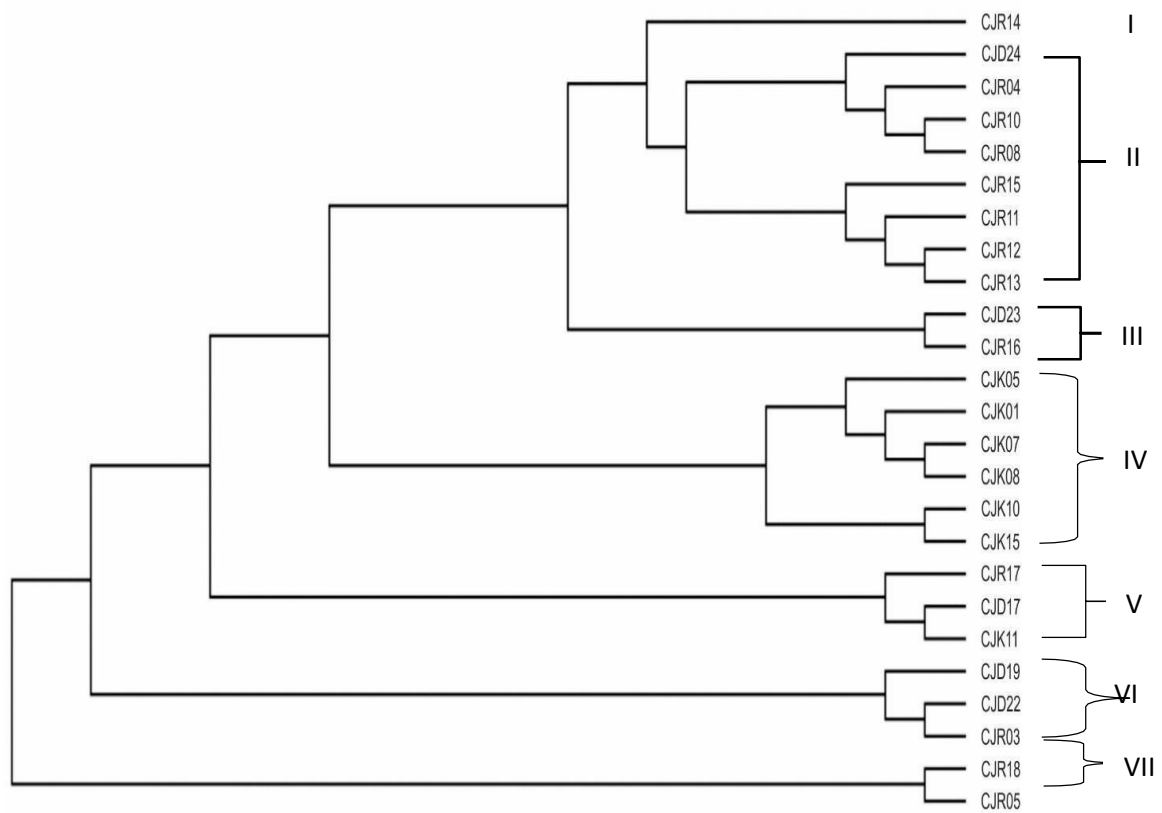


Figure 3. A dendrogram based on Rep-PCR of 25 Bacteria Isolates Associated with vibriosis clinical signs from traditional mud crab farming in the North Coastal of Central Java, Central Java, Indonesia.

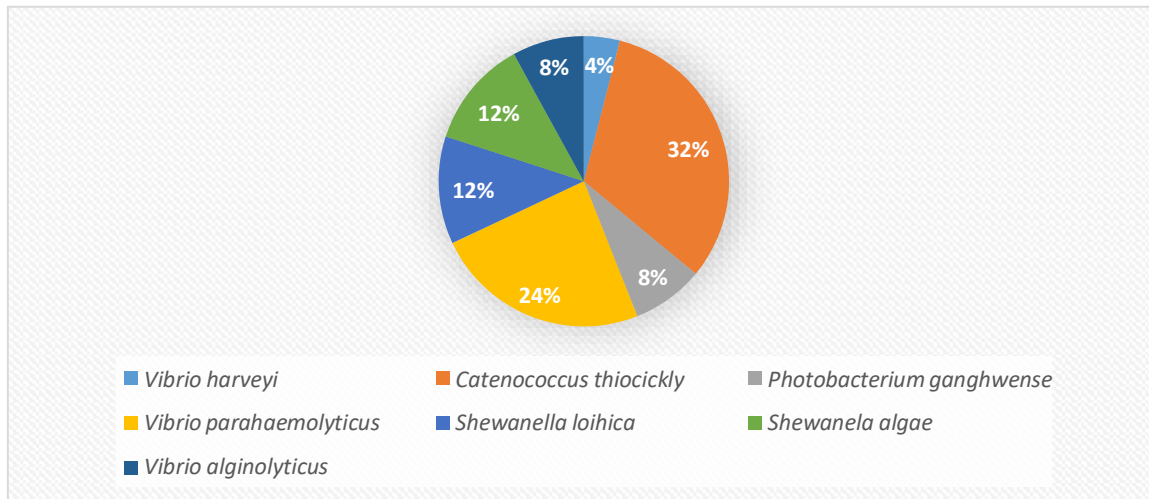


Figure 4. The bacterial diversity in Mud crab with clinical signs of Vibriosis from North Coast of Central Java.

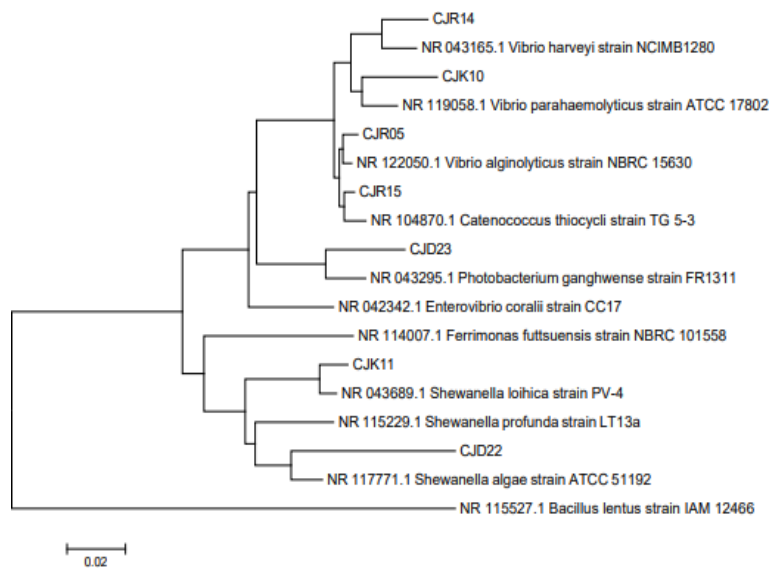


Figure 5. Phylogenetic tree of the bacteria associated with vibriosis clinical signs from traditional mud crab farming in the North Coast of Central Java, Indonesia constructed using Neighbour-Joining analysis with 1000 replicates.

Table 1. Molecular characterization of seven bacterial species associated with clinical signs of Vibriosis on mud crabs from North Coast of Central Java.

Isolates Code	Query Length	Close relative	Query Cover (%)	Homology (%)	Acc. Number
CJR14	1548	<i>Vibrio harveyi</i> strain NCIMB1280	98	95	NR 043165.1
CJR15	1319	<i>Catenococcus thiocicly</i> strain TG 5-3	98	98	NR 118258.1
CJD23	1387	<i>Photobacterium ganghwense</i> strain FR311	98	95	NR 043295.1
CJK10	1340	<i>Vibrio parahaemolyticus</i> ATCC 17802	99	97	NR 043689.1
CJK11	1335	<i>Shewanella loihica</i> strain PV4	97	97	NR 025794.1
CJD22	1488	<i>Shewanella algae</i> strain ATCC5192	95	92	NR 117771.1
CJR05	1362	<i>Vibrio alginolyticus</i> strain NBRC 15630	96	96	NR 122050.1

Table 2. The diversity of Vibrios in Mud Crab from North Coast of Central Java per location.

Location	Isolates Code	Bacteria Species	Vibrios diversity	Isolate Number	Percentage of species compare to total isolate (%)
Rembang	CJR14;				
	CJR04;				
	CJR10;				
	CJR08;				
	CJR15;	<i>Vibrio harveyi</i> strain NCIMB1280			
	CJR11;	<i>Catenococcus thiocicly</i> strain TG 5-3,			
	CJR12;	<i>Photobacterium ganghwense</i> strain FR311,			
	CJR13;	<i>Shewanella loihica</i> strain PV4,	6	13	52%
	CJR16;	<i>Shewanella algae</i> strain ATCC5192,			
	CJR17;	<i>Vibrio alginolyticus</i> strain NBRC 15630,			
	CJR03;				
	CJR18;				
	CJR05				
Demak	CJD24;	<i>Catenococcus thiocicly</i> strain TG 5-3			
	CJD23;	<i>Photobacterium ganghwense</i> strain FR311			
	CJD17;	<i>Shewanella loihica</i> strain PV4	4	5	80%
	CJD19;	<i>Shewanella algae</i> strain ATCC5192			
	CJD22				

	CJK05;			
	CJK01;			
	CJK07; <i>Vibrio prahaemolyticus</i> ATCC 17802			28%
Kendal	CJK08; <i>Shewanella loihica</i> strain PV4	2	7	
	CJK10;			
	CJK15;			
	CJK11			



Sarjito Sarjito <sarjito@live.undip.ac.id>

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Original Article

Molecular Characterization of vibriosis clinical signs-associated bacteria from traditional mud-crab farming in the North Coast of Central Java, Indonesia

Sarjito^{1*}, Alfabetian Harjuno Condro Haditomo¹, Slamet Budi Prayitno¹, Aninditia Sabdaningsih², Desrina¹, and Restiana Wisnu Ariyati¹

¹Aquaculture Study Program, Aquaculture Department, Fisheries and Marine Science Faculty, Diponegoro University, Semarang, 50275, Indonesia

²Department of Aquatic Resources, Fisheries and Marine Science Faculty, Diponegoro University, Semarang, 50275, Indonesia

*Email address : sarjito@live.undip.ac.id

Abstract

This study examines the molecular characterization of bacteria associated with clinical symptoms and the diversity of vibriosis bacteria that infected mud crab farming from three sampling locations, namely Rembang, Demak, and Kendal Districts which are the largest cultivated crab producers in the North Coast of Central Java. Twenty-five bacteria were isolated from hepatopancreas, gill, and carapace of mud crab that have clinical signs of vibriosis that cultured in TCBS and TSA medium. After a modified rep-PCR and further PCR amplification of the 16S ribosomal RNA gene, the sequence analysis was performed. The DNA sequences compared with the BLAST from the NCBI database. Then, the phylogenetic tree constructed by the MEGA X program. The clinical signs on mud crab were associated with seven isolates: CJR14, CJR15, CJD23, CJK10, CJK11, CJD22, and CJR05. Based on 16S rRNA sequence analysis, these isolates were firmly

(92-98%) related to *Vibrio harveyi* NCIMB1280, *Catenococcus thiocicly* TG 5 – 3, *Photobacterium ganghwense* FR311, *Vibrio parahaemolyticus* ATCC 1780, *Shewanella loihica* PV4, *Shewanella algae* ATCC5, and *Vibrio alginolyticus* NBRC 15630. Bacterial Vibriosis strain *Catenococcus thiocicly* (32%) was found in each sampling location. More type of bacteria (six out seven strains) were found in Rembang district but the highest percentage of vibrio diversity compared to the total isolate was found in Demak district (80%).

Keywords: bacterial diseases, mud crab, 16S rRNA, rep PCR, vibriosis

1. Introduction

Mud crab, *Scylla serrata* (Forskål, 1775), is traditionally caught in and around brackish water ponds and mangrove forests along the North Coast of Central Java, Indonesia. The mangrove forests and its soils are this crab's origin habitat. Traditional brackish water pond used for polyculture of shrimp and milkfish, wild mud crab may intrude in these ponds, where they are caught together with the shrimp and milkfish. However, the culture of the mud crab themselves is spreading. The intrusion of wild mud crab can be a problem to crustacean's culture. Infected mud crab can carry disease agents that may infect the healthy cultured crustaceans. To prevent damage of the crustacean culture, farmers may disinfect the pond and raise barriers that are well embedded in the bunds, but these efforts are rarely 100% effective. The cause of the disease does not only occur in infected wild crabs but also in crab farming. Bacterial diseases, especially vibriosis, was found to be a problem in the fattening of the mud crab culture in Pemalang (Sarjito *et al.*, 2016) and reported to cause more than 90% mortality in all life cycle stages of mud crab growth

(Jithendran, Poornima, Balasubramanian, & Kulasekarapandian, 2010). Reported clinical signs of infected mud crab are wounds on the body, decreased feed response and weakening (Jithendran, Poornima, Balasubramanian, & Kulasekarapandian, 2010), blackening and red spots of the carapace (Sarjito, Hastuti, Samidjan, & Prayitno, 2014; Sarjito *et al.*, 2016).

Vibrio's cause various disease outbreaks and severe economic losses in shrimp culture (Aguilre-Rivera, Prieto-Davo, Rodriguez-Fuentes G, Escalante-Herrera, & Gaxiola, 2019) and have the potential to destroy the future of the industrial culture of mud crab. Several species of *Vibrio* spp. were reported in and found to be associated with bacterial disease in mud crab: *V. fischeri*, and *V. nereis* (Wang, 2011); *V. alginolyticus* and *V. cholerae* (Najiah, Nadirah, Sakri & Harrison, 2010; Wang, 2011); *V. vulnificus* (Lavilla-Pitogo, Celia, & Leobert, 2004; Shanmuga, 2008; Wang, 2011), *V. splendidus*, and *V. orientalis* (Jithendran, Poornima, Balasubramanian, & Kulasekarapandian, 2010); *V. ordalii* (Sarjito, Hastuti, Samidjan, & Prayitno, 2014), *V. harveyi* (Sarjito, Hastuti, Samidjan, & Prayitno, 2014; Jithendran, Poornima, Balasubramanian, & Kulasekarapandian, 2010), *V. campbelli* (Shanmuga, 2008), and *V. parahaemolyticus* (Lavilla-Pitogo, Celia, & Leobert, 2004; Najiah, Nadirah, Sakri, & Harrison, 2010; Shanmuga, 2008). Previous research focused on the richness of bacteria associated with vibriosis in mud crab. The limited knowledge is available on the diversity of vibrios related to the disease outbreaks in mud crab in coastal of the North Central Java. Local government tends to stimulate the culture of mud crab, nevertheless, the program lacks information on disease identification and prevention. This study was conducted to assessed the molecular characterization of bacteria associated with clinical

symptoms and the diversity of the bacteria vibriosis infected mud crabs farming in brackish water traditional ponds along the North Coast of Central Java, Indonesia.

2. Materials and Methods

Sampling

Mud crab, *S. serrata*, were collected from nine brackish water ponds of Kendal, Demak, and Rembang Regency, Central Java (each three ponds). These three locations are the largest producers of cultivated crabs in Central Java, Indonesia (Figure 1). Based on the clinical signs for vibriosis described by Jithendran, Poornima, Balasubramanian, & Kulasekarapandian (2010), 25 infected mud crabs were selected, stored in a sterile container and carried to the Integrated Laboratory of Diponegoro University for further analysis. The average size of the caught crab, measured from 13 to 15 cm, was 14.4 ± 0.7 cm.

Figure 1. Research location represented in a small red box in the left of map.

Bacterial Isolation

Forty-five bacteria were isolated from gills, hemolymph, hepatopancreas, and wounds on the shell of the infected mud crabs. Isolates bacteria were cultured on Thiosulfate Citrate Bile Salt Sucrose (TCBS) and Tryptic Soy Agar (TSA) (Thompson, Iida, & Swings, 2004; Sarjito *et al.*, 2016). The colonies were purified by the streak method based on morphological appearance (Sarjito *et al.*, 2016).

Repetitive Genomic Sequences-PCR (Rep-PCR)

The bacterial DNA was extracted from a 24 h-broth culture of isolate strains by using the chelex method with slight modification according to the procedure described

by (de Lamballerie, Zandotti, Vignoli, Bollet, & de Micco, 1992). Then, the isolates DNA extract were analyzed using the rep-PCR of Radjasa, Nasima, Sabdono, Kita-Tsukamoto, & Ohwada (2007) was modified by Sarjito, Haditomo, Desrina, Djunaedi, & Prayitno (2018) in the rep-PCR, BOX AIR (5'-CTACggCAAaggCgACgCTgACg-3'). The REP 1R-I and REP 2-I primers contained nucleotide inosine (I) at an ambiguous position in the REP consensus. The mix PCR reagent contains one μL DNA template (diluted 100X), one μL primer, 7.5 μL Megamix Royal and 5.5 μL ddH₂O. Amplification was performed in a thermal cycler model Gene Amp PCR system 9700 with the following protocol: initial denaturation at 95°C for 5 minutes. Then 30 cycles of (denaturation 92°C for 1 minute, annealing 50°C for 1,5 minutes, extension 68°C for 8 minutes), and a final extension at 68°C for 10 minutes. The band DNA was visualized with injected 5 μL PCR products into 1% agarose gel and run to electrophoresis machine using 1X TBE buffer and observed in UV-transilluminator, as described by Radjasa, Nasima, Sabdono, Kita-Tsukamoto, & Ohwada (2007). Grouping of isolates was conducted based on the method of Sarjito, Radjasa, Sabdono, Prayitno, & Hutabarat (2009). This method is a useful tool for differentiating bacterial diversity according to the number and size of repeated bacterial sequences. Bacterial diversity is the percentage of the number of each species in a Dendogram group based on Rep-PCR compared to the total number of isolates. Then, the diversity of vibrios in mud crab with vibriosis clinical signs were compared from the three-sampling site to perform the percentage of species in each location.

Matrices were made from the band's position on the gel, which was analyzed by the Free Tree program using the UPGMA method for constructing the phylogenetic tree (Sarjito, Haditomo, Desrina, Djunaedi, & Prayitno, 2018). Resampling was done by bootstrapping with 1000 replications (Prayitno, Sarwan, & Sarjito, 2015).

PCR Amplification of 16s rRNA Gene Fragments

The PCR amplification of the 16S rRNA gene was performed, according to Radjasa, Nasima, Sabdono, Kita-Tsukamoto, & Ohwada (2007) and Sarjito, Haditomo, Desrina, Djunaedi, & Prayitno (2018). To find out a strictly complete 16S rRNA gene, the amplification was presented with two primers, GM3F as a forward primer (5'AGAGTTTGATCMTGGC-3') and GM4R as a reverse primer (5'-TACCTTGTTACGACTT-3') (Sarjito, Radjasa, Sabdono, Prayitno, & Hutabarat, 2009). Genomic DNA of bacteria for PCR analysis was obtained from the destruction cell materials that were taken from fresh culture bacteria, suspended in sterile water (Sigma, Germany), and subjected to five cycles of freeze (-80°C) and thaw (95°C). The PCR product was then processed to find the band DNA in the right size around 1.500 bp. The sequencing step was facilitated by PT. Genetika Science. The determined DNA sequences of samples were then compared for homology using BLAST. The phylogenetic tree was established using MEGA X to find the closely related species (Sabdaningsih *et al.*, 2020).

3. Results and Discussion

Clinical Signs and Bacterial Isolation

The most frequent clinical signs of the infected mud crab (*S. serrata*) were wounds on the surface of the claws, the ventral, and the carapace. The isolation of bacteria using TCBS and TSA medium obtained 25 pure isolates of bacteria from three replicates agar plates and then stored in NA medium. The clinical signs of red-brown and dark spots in the carapace were found in Figure 2. Similar clinical symptoms were found on the abdomen.

Figure 2. The clinical signs of mud crab (*S. serrata*) infected by bacterial diseases: (a.) Red/brown spots in the carapace; (b.) Red-brown spots in the carapace wound on the abdomen.

Red-brown or dark melanin spots mostly known as vibriosis in mud crab, patches of light red spots as well as dark spots on the carapace, also wounds on the body (claws, shell, and the ventral). The identical clinical signs have been found by Muyzer, Teske, Wirsen, and Jannasch (1995), Wang (2011), Lavilla-Pitogo, Celia, & Leobert (2004) and Prayitno, Sarjito & Putri (2017). It was also reported on the mud crabs that were affected by genus vibrio from the gulf of Semarang (Sarjito, Hastuti, Samidjan, & Prayitno, 2014) and Pemalang (Sarjito *et al.*, 2016). 'Brown spot' may occur due to the infection of chitinolytic bacteria that catabolize the chitin of the carapace and cause erosion and melanization (dark brown to black pigmentation) at the site of infection (Jithendran, Poornima, Balasubramanian & Kulasekarapandian, 2010)

Repetitive Genomic Sequences-PCR (Rep-PCR)

The Repetitive PCR analysis and the dendrogram related to the 25 pure isolates bacteria originating from mud crabs found a seven group from total samples (Figure 3). Furthermore, the molecular identification only represented by one isolates from each seven group such as CJR14, CJR15, CJD23, CJK10, CJK11, CJD22, and CJR05 (Table 1).

Figure 3. A dendrogram based on Rep-PCR of 25 bacteria isolates associated with vibriosis clinical signs from traditional mud crab pond.

Figure 3 shows a similarity between 25 isolates into seven groups due to the difference between repetitive bacterial sequences then it was examined using the 16S

rRNA gene for identification. A total of seven isolates were identified and presented in Table 1—the range of homology percentage between 92-98%. The highest similarity was showed by isolate CJR15 from group II, 98% homology with *C. thiocicly* strain TG 5-3. The homology 97% was appointed to isolate CJK10 and CJK11 from group IV and V that similar to *V. parahaemolyticus* ATCC 17802 and *S. loihica* strain PV4. Followed by *V. alginolyticus* strain NBRC 15630 with 96% homology from isolate CJR05 in group VII. Moreover, isolate CJR14 and CJD23 have 95% homology with *V. harveyi* strain NCIMB1280 and *P. ganghwense* strain FR311 in group I and III, while isolate CJD22 was closely related 92% to *S. algae* strain ATCC5192 in group VI.

Table 1. Molecular characterization of seven bacterial species associated with clinical signs of Vibriosis on mud crabs from the North Coast of Central Java.

The vibrio's associated in mud crabs from traditional brackish water pond of the North Coast of Central Java (Figure 1), it was closely related to *V. harveyi* strain NCIMB1280 (CJR14), *C. thiocicly* strain TG 5-3 (CJR15), *P. ganghwense* strain FR311 (CJD23); *V. prahaemolyticus* ATCC 17802 (CJK10); *S. loihica* strain PV4 (CJK11); *S. algae* strain ATCC5 (CJD22) and *V. alginolyticus* strain NBRC 15630 (CJR05). The result also revealed the diversity of vibrios associated with mud crab from the brackish water pond. It was lower than the diversities that were found in the cultured and wild crab in India (Jithendran, Poornima, Balasubramanian & Kulasekarapandian, 2010) as well as traditional brackish water surrounding of Semarang Gulf (Sarjito, Hastuti, Samidjan, & Prayitno, 2014).

Four genus Vibrionaceae (*Vibrio*, *Shewanella*, *Photobacterium*, and *Catenococcus*) were mostly found in mud crab (Li *et al.*, 2012; Wei *et al.*, 2019).

Three species of vibrio, i.e. *V. parahaemolyticus* (CJK10), *V. harveyi* (CJR14), and *V. alginolyticus* (CJR05) also have been detected in this study. The vibrios were frequently found as a causative agent of shell disease in *L. vannamei* shrimp and mud crab, *S. serrata*, (Estave, & Herrere, 2000; Wang, 2011; Soto-Rodriguez *et al.*, 2012). *V. parahaemolyticus* has been found as a main pathogen in mud crab in China (Xia *et al.*, 2010) and Chakoria Coast, Bangladesh (Aftabuddin *et al.*, 2013). Additionally, *V. harveyi* has been reported as a causative agent of vibriosis in fattening mud crabs from brackish water pond in Pemalang coast (Sarjito *et al.*, 2016) and mud crab from extensive brackish water pond of surrounding the Gulf of Semarang (Sarjito, Hastuti, Samidjan, & Prayitno, 2014) and Malaysia (Najiah, Nadirah, Sakri, & Harrison, 2010). These bacteria were also reported as a causative agent of bacterial diseases in zoea stage of mud crab (Jithendran, Poornima, Balasubramanian, & Kulasekarapandian, 2010); adult mud crabs (Poornima *et al.*, 2012 and Lavilla-Pitogo, Celia, & Leobert, 2004). Moreover, Sarjito, Hastuti, Samidjan, & Prayitno, (2014), found that this bacterium was a potential pathogen to mud crabs. *V. alginolyticus* was observed as an important pathogenic bacteria associated with infectious diseases in mud crab (Najiah, Nadirah, Sakri & Harrison, 2010; Sarjito, Hastuti, Samidjan, & Prayitno, 2014) and shell disease of mud crab *S. serrata* grow out pond located at Tamil Nadu, India (Gunasekaran, Gopalakrishnan, Deivasigamani, Muhilvannan, & Kathirkaman, 2019).

Genus Photobacterium was found in the coastal, open-ocean and deep-sea water (Moi *et al.*, 2017). Surprisingly, *P. ganghwense* was detected in this study. This bacterium, was firstly reported from a seawater in Ganghwa Island, South Korea (Park *et al.*, 2006). Furthermore, Wei *et al.*, (2019) found that Photobacterium was dominant as

gut microbiota in mud crab *S. paramamosain* where collected from nine coastal areas of southern China.

The result showed that both *Shewanella algae* and *Shewanella loihica* were found in this study. They have an essential role as a turnover of organic material, and capable of dissimilatory reduction of various metals and other substances, such as Nitrate, Nitrite, Thiosulphate, and Trimethylamine-N-oxide (Holt, Gahrn-Hansen, & Bruun, 2005). *S. algae* has been reported as bacterial pathogen associated with Black Spot Disease in Freshwater-Cultured White leg Shrimp (*Penaeus vannamei*) (Cao, Chen, Lu, & An, 2018), causative agent of *Babylonia* (Shufang, Zhang, Dequan, Shiping, & Huang, 2015); *Cynoglossus semilaevis* (Han *et al.*, 2017), and *Scianeps ocellatus* (Zhang, Zhu, & Wang, 2013). According to Prayitno, Sarwan & Sarjito (2015) *S. algae* was also presented in gut of milkfish from the Northern Coast of Central Java. In Demak, *S. algae* was found only when the water temperature is more than 23°C. Additionally, the infection of genus *Shewanella* mostly occurs in countries with a warm climate (Holt, Gahrn-Hansen, & Bruun, 2005). Although it is rare in human pathogen and symptoms of infection, the food safety of crab product should be considered since this bacterium was already detected in mud crab farming at the North Coast of Central Java.

Shewanella loihica was isolated and identified from iron-rich microbial mats in the Pacific Ocean (Gao *et al.*, 2006). *S. loihica* plays a role as an agent of metal reduction and iron bio-mineralization. Based on that, the existence of this bacterium can be caused by the metal and nitrogen content in the water. Some reported study of *S. loihica* is also focusing on metal reduction and iron bio-mineralization capabilities (Newton, Mori, Nakamura, Hashimoto, & Watanabe, 2009; Philips *et al.*, 2018), denitrification and a respiratory ammonification pathway (Yoon, Sanford, & Löffler, 2015). Therefore, this

bacterium has important role on the nutrient cycle in soils and sediments where mud crab lives.

Catenococcus thiocyli was identified by Yarza *et al.* (2013), the study found that *C. thiocyli* is part of genus Vibrionaceae. Only few studies reported this bacterium (Zheng *et al.*, 2016; Li *et al.*, 2019; Achmad *et al.*, 2019; Guibert, Lecellier, Torda, Pochon, & Berteaux-Lecellier, 2020). This member of Vibrionaceae was found as a causative agent of Ice-ice diseases of the seaweed, *Kappaphycus alvarezii*, (Achmad *et al.*, 2019) and moribund of the giant clam, *Tridacna maxima*, (Guibert, Lecellier, Torda, Pochon, & Berteaux-Lecellier, 2020).

Diversity of Vibriosis Clinical Signs Associated Bacteria

The diversity of vibrios in mud crab with vibriosis clinical signs were compared from the three-sampling site to perform the percentage of species in each location (Table 2). Table 2 accommodates all of the isolates that identified based on the 16S rRNA gene. The diversity of vibriosis clinical signs associated with bacteria was explored using molecular characterization by Rep-PCR. This method is a useful tool to distinguish the diversity of bacteria according to the number and size of repetitive bacterial sequences. This technique could prevent to analyse the similar isolate of bacteria based on its DNA fingerprinting. Therefore, rep-PCR is helpful in grouping *Vibrio* species. The diversity of vibrios found in three sample locations (Table 2). We found more bacteria species in Rembang rather than in Demak and Kendal. The highest percentage of vibrio diversity compared to the total isolate in each location was in Demak (80%).

Table 2. The diversity of Vibrios in Mud Crab from the North Coast of Central Java per location.

Figure 4. The bacterial diversity in Mud crab with clinical signs of Vibriosis from the North Coast of Central Java.

Figure 4 represented the bacterial diversity in Mud crab with Vibriosis's clinical signs from the North Coast of Central Java. The highest number species was *C. thiocicly* with the percentage 32%, while 24% was *V. parahaemolyticus*, *S. loihica* and *S. algae* in the equal percentage in 12%, as well as *P. ganghwense* and *V. algynolyticus* in 8%, and the lowest rate was *V. harveyi* in 4%.

Figure 5. Phylogenetic tree of the bacteria associated with vibriosis clinical signs from traditional mud crab farming in the North Coast of Central Java, Indonesia constructed using Neighbour-Joining analysis with 1000 replicates.

In order to represent the relationship among isolates, the phylogenetic was constructed. Figure 5 shows that all of the strains have assembled with the closely related species. The relationship between the genus performs that the bacteria genus *Vibrio* has the same clade with *Catenococcus thiocycli*. Those groups are more closely related compared to the group of *Shewanella* and *Photobacterium*.

The isolates were continuing to be grouped based on the sampling site (Table 2). Rembang has the highest number of strains. However, from seven groups of *Vibrio*, only six groups were detected in this site. Demak has four groups of *Vibrio* from five isolates found. Furthermore, Kendal only has two groups of *Vibrio* from seven strains. The results indicated that the highest diversity of *Vibrio* was found in Rembang, rather than Demak and Kendal. It might happen since those locations have a higher abrasion (Directorate of

Marine and Fisheries, 2019). Miller (1989) revealed that the abrasion could decline the microbial abundances.

Moreover, Figure 4 was designed to know the highest species of Vibrionaceae found in this study. Based on the dendrogram of rep-PCR, the most top species come from *C. thiocicly* in group II with a percentage number 32%, and the lowest was *V. harveyi* with the percentage 4%. Based on the phylogenetic tree (Figure 5), *Vibrio* and *Catenococcus* are more closely related than those of *Shewanella* and *Photobacterium*.

According to the results, future research should be conducted to have a deep understanding and correlation between the biotic and abiotic factors that affect the health status of Mud-crab in the traditional brackish water pond. Therefore, the metagenomic approach would be helpful to describe the bacterial structure community in the healthy and infected mud crab. Thus, the design of prevention methods to reduce bacterial disease outbreak of Mud crab farming could be expertly constructed.

4. Conclusions

Strains of these seven bacteria are well known to be pathogens for aquatic organisms. Moreover, the highest diversity of Vibrionaceae was obtained from Demak, and the highest bacterial diversity found in three sampling sites was *C. thiocicly*.

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Figure 1. Research Location represented in a small red box in the left of map.



Figure 2. The clinical signs of mud crab (*S. serrata*) infected by bacterial diseases: (a.) Red/brown spots in the carapace; (b.) Red-brown spots in the carapace wound on the abdomen.

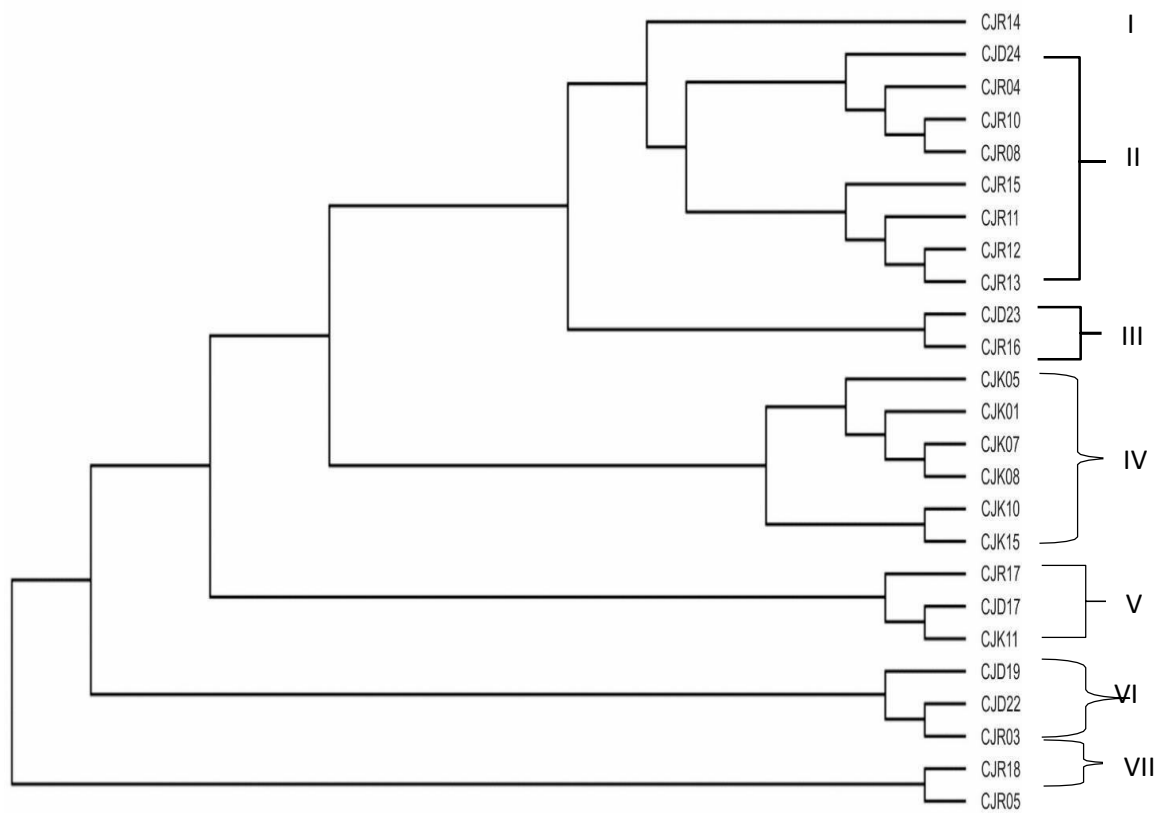


Figure 3. A dendrogram based on Rep-PCR of 25 Bacteria Isolates Associated with vibriosis clinical signs from traditional mud crab farming in the North Coastal of Central Java, Central Java, Indonesia.

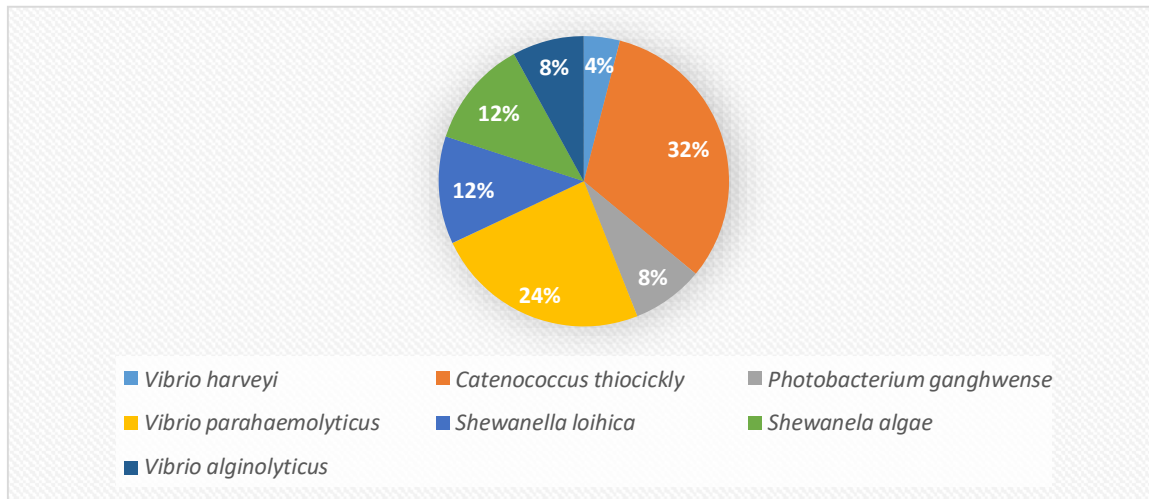


Figure 4. The bacterial diversity in Mud crab with clinical signs of Vibriosis from North Coast of Central Java.

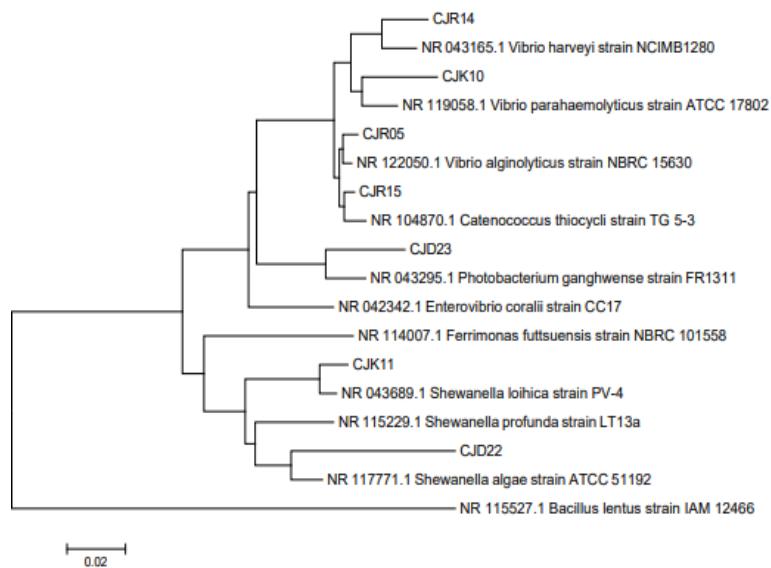


Figure 5. Phylogenetic tree of the bacteria associated with vibriosis clinical signs from traditional mud crab farming in the North Coast of Central Java, Indonesia constructed using Neighbour-Joining analysis with 1000 replicates.

Table 1. Molecular characterization of seven bacterial species associated with clinical signs of Vibriosis on mud crabs from North Coast of Central Java.

Isolates Code	Query Length	Close relative	Query Cover (%)	Homology (%)	Acc. Number
CJR14	1548	<i>Vibrio harveyi</i> strain NCIMB1280	98	95	NR 043165.1
CJR15	1319	<i>Catenococcus thiocicly</i> strain TG 5-3	98	98	NR 118258.1
CJD23	1387	<i>Photobacterium ganghwense</i> strain FR311	98	95	NR 043295.1
CJK10	1340	<i>Vibrio parahaemolyticus</i> ATCC 17802	99	97	NR 043689.1
CJK11	1335	<i>Shewanella loihica</i> strain PV4	97	97	NR 025794.1
CJD22	1488	<i>Shewanella algae</i> strain ATCC5192	95	92	NR 117771.1
CJR05	1362	<i>Vibrio alginolyticus</i> strain NBRC 15630	96	96	NR 122050.1

Table 2. The diversity of Vibrios in Mud Crab from North Coast of Central Java per location.

Location	Isolates Code	Bacteria Species	Vibrios diversity	Isolate Number	Percentage of species compare to total isolate (%)
Rembang	CJR14;				
	CJR04;				
	CJR10;				
	CJR08;				
	CJR15;	<i>Vibrio harveyi</i> strain NCIMB1280			
	CJR11;	<i>Catenococcus thiocicly</i> strain TG 5-3,			
	CJR12;	<i>Photobacterium ganghwense</i> strain FR311,			
	CJR13;	<i>Shewanella loihica</i> strain PV4,	6	13	52%
	CJR16;	<i>Shewanella algae</i> strain ATCC5192,			
	CJR17;	<i>Vibrio alginolyticus</i> strain NBRC 15630,			
	CJR03;				
	CJR18;				
	CJR05				
Demak	CJD24;	<i>Catenococcus thiocicly</i> strain TG 5-3			
	CJD23;	<i>Photobacterium ganghwense</i> strain FR311			
	CJD17;	<i>Shewanella loihica</i> strain PV4	4	5	80%
	CJD19;	<i>Shewanella algae</i> strain ATCC5192			
	CJD22				

	CJK05;			
	CJK01;			
	CJK07; <i>Vibrio prahaemolyticus</i> ATCC 17802			
Kendal	CJK08; <i>Shewanella loihica</i> strain PV4	2	7	28%
	CJK10;			
	CJK15;			
	CJK11			



Sarjito Sarjito <sarjito@live.undip.ac.id>

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Original Article

Molecular Characterization of vibriosis clinical signs-associated bacteria from traditional mud-crab farming in the North Coast of Central Java, Indonesia

Sarjito^{1*}, Alfabetian Harjuno Condro Haditomo¹, Slamet Budi Prayitno¹, Aninditia Sabdaningsih², Desrina¹, and Restiana Wisnu Ariyati¹

¹Aquaculture Study Program, Aquaculture Department, Fisheries and Marine Science Faculty, Diponegoro University, Semarang, 50275, Indonesia

²Department of Aquatic Resources, Fisheries and Marine Science Faculty, Diponegoro University, Semarang, 50275, Indonesia

*Email address : sarjito@live.undip.ac.id

Abstract

This study examines the molecular characterization of bacteria associated with clinical symptoms and the diversity of vibriosis bacteria that infected mud crab farming from three sampling locations, namely Rembang, Demak, and Kendal Districts which are the largest cultivated crab producers in the North Coast of Central Java. Twenty-five bacteria were isolated from hepatopancreas, gill, and carapace of mud crab that have clinical signs of vibriosis that cultured in TCBS and TSA medium. After a modified rep-PCR and further PCR amplification of the 16S ribosomal RNA gene, the sequence analysis was performed. The DNA sequences compared with the BLAST from the NCBI database. Then, the phylogenetic tree constructed by the MEGA X program. The clinical signs on mud crab were associated with seven isolates: CJR14, CJR15, CJD23, CJK10, CJK11, CJD22, and CJR05. Based on 16S rRNA sequence analysis, these

isolates were firmly (92-98%) related to *Vibrio harveyi* NCIMB1280, *Catenococcus thiocicly* TG 5 – 3, *Photobacterium ganghwense* FR311, *Vibrio parahaemolyticus* ATCC 1780, *Shewanella loihica* PV4, *Shewanella algae* ATCC5, and *Vibrio alginolyticus* NBRC 15630. **Bacterial Vibriosis strain *Catenococcus thiocicly* (32%) was found in each sampling location.** More type of bacteria (six out seven strains) were found in Rembang district but the highest percentage of vibrio diversity compared to the total isolate was found in Demak district (80%).

Keywords: bacterial diseases, mud crab, 16S rRNA, rep PCR, vibriosis

1. Introduction

Mud crab, *Scylla serrata* (Forskål, 1775), is traditionally caught in and around brackish water ponds and mangrove forests along the North Coast of Central Java, Indonesia. The mangrove forests and its soils are this crab's origin habitat. Traditional brackish water pond used for polyculture of shrimp and milkfish, wild mud crab may intrude in these ponds, where they are caught together with the shrimp and milkfish. However, the culture of the mud crab themselves is spreading. The intrusion of wild mud crab can be a problem to crustacean's culture. Infected mud crab can carry disease agents that may infect the healthy cultured crustaceans. To prevent damage of the crustacean culture, farmers may disinfect the pond and raise barriers that are well embedded in the bunds, but these efforts are rarely 100% effective. **The cause of the disease does not only occur in infected wild crabs but also in crab farming.** Bacterial diseases, especially vibriosis, was found to be a problem in the fattening of the mud crab culture in Pemalang (Sarjito *et al.*, 2016) and reported to cause more than 90% mortality in all life cycle

stages of mud crab growth (Jithendran, Poornima, Balasubramanian, & Kulasekarapandian, 2010). Reported clinical signs of infected mud crab are wounds on the body, decreased feed response and weakening (Jithendran, Poornima, Balasubramanian, & Kulasekarapandian, 2010), blackening and red spots of the carapace (Sarjito, Hastuti, Samidjan, & Prayitno, 2014; Sarjito *et al.*, 2016).

Vibrio's cause various disease outbreaks and severe economic losses in shrimp culture (Aguilre-Rivera, Prieto-Davo, Rodriguez-Fuentes G, Escalante-Herrera, & Gaxiola, 2019) and have the potential to destroy the future of the industrial culture of mud crab. Several species of *Vibrio* spp. were reported in and found to be associated with bacterial disease in mud crab: *V. fischeri*, and *V. nereis* (Wang, 2011); *V. alginolyticus* and *V. cholerae* (Najiah, Nadirah, Sakri & Harrison, 2010; Wang, 2011); *V. vulnificus* (Lavilla-Pitogo, Celia, & Leobert, 2004; Shanmuga, 2008; Wang, 2011), *V. splendidus*, and *V. orientalis* (Jithendran, Poornima, Balasubramanian, & Kulasekarapandian, 2010); *V. ordalii* (Sarjito, Hastuti, Samidjan, & Prayitno, 2014), *V. harveyi* (Sarjito, Hastuti, Samidjan, & Prayitno, 2014; Jithendran, Poornima, Balasubramanian, & Kulasekarapandian, 2010), *V. campbelli* (Shanmuga, 2008), and *V. parahaemolyticus* (Lavilla-Pitogo, Celia, & Leobert, 2004; Najiah, Nadirah, Sakri, & Harrison, 2010; Shanmuga, 2008). Previous research focused on the richness of bacteria associated with vibriosis in mud crab. The limited knowledge is available on the diversity of vibrios related to the disease outbreaks in mud crab in coastal of the North Central Java. Local government tends to stimulate the culture of mud crab, nevertheless, the program lacks information on disease identification and prevention.

This study was conducted to assessed the molecular characterization of bacteria associated with clinical symptoms and the diversity of the bacteria vibriosis infected

mud crabs farming in brackish water traditional ponds along the North Coast of Central Java, Indonesia.

2. Materials and Methods

Sampling

Mud crab, *S. serrata*, were collected from nine brackish water ponds of Kendal, Demak, and Rembang Regency, Central Java (each three ponds). These three locations are the largest producers of cultivated crabs in Central Java, Indonesia (Figure 1). Based on the clinical signs for vibriosis described by Jithendran, Poornima, Balasubramanian, & Kulasekarapandian (2010), 25 infected mud crabs were selected, stored in a sterile container and carried to the Integrated Laboratory of Diponegoro University for further analysis. The average size of the caught crab, measured from 13 to 15 cm, was 14.4 ± 0.7 cm.

Figure 1. Research location represented in a small red box in the left of map.

Bacterial Isolation

Forty-five bacteria were isolated from gills, hemolymph, hepatopancreas, and wounds on the shell of the infected mud crabs. Isolates bacteria were cultured on Thiosulfate Citrate Bile Salt Sucrose (TCBS) and Tryptic Soy Agar (TSA) (Thompson, Iida, & Swings, 2004; Sarjito *et al.*, 2016). The colonies were purified by the streak method based on morphological appearance (Sarjito *et al.*, 2016).

Repetitive Genomic Sequences-PCR (Rep-PCR)

The bacterial DNA was extracted from a 24 h-broth culture of isolate strains by using the chelex method with slight modification according to the procedure described

by (de Lamballerie, Zandotti, Vignoli, Bollet, & de Micco, 1992). Then, the isolates DNA extract were analyzed using the rep-PCR of Radjasa, Nasima, Sabdono, Kita-Tsukamoto, & Ohwada (2007) was modified by Sarjito, Haditomo, Desrina, Djunaedi, & Prayitno (2018) in the rep-PCR, BOX AIR (5'-CTACggCAAggCgACgCTgACg-3'). The REP 1R-I and REP 2-I primers contained nucleotide inosine (I) at an ambiguous position in the REP consensus. The mix PCR reagent contains one μL DNA template (diluted 100X), one μL primer, 7.5 μL Megamix Royal and 5.5 μL ddH₂O. Amplification was performed in a thermal cycler model Gene Amp PCR system 9700 with the following protocol: initial denaturation at 95°C for 5 minutes. Then 30 cycles of (denaturation 92°C for 1 minute, annealing 50°C for 1,5 minutes, extension 68°C for 8 minutes), and a final extension at 68°C for 10 minutes. The band DNA was visualized with injected 5 μL PCR products into 1% agarose gel and run to electrophoresis machine using 1X TBE buffer and observed in UV-transilluminator, as described by Radjasa, Nasima, Sabdono, Kita-Tsukamoto, & Ohwada (2007). Grouping of isolates was conducted based on the method of Sarjito, Radjasa, Sabdono, Prayitno, & Hutabarat (2009). This method is a useful tool for differentiating bacterial diversity according to the number and size of repeated bacterial sequences. Bacterial diversity is the percentage of the number of each species in a Dendogram group based on Rep-PCR compared to the total number of isolates. Then, the diversity of vibrios in mud crab with vibriosis clinical signs were compared from the three-sampling site to perform the percentage of species in each location.

Matrices were made from the band's position on the gel, which was analyzed by the Free Tree program using the UPGMA method for constructing the phylogenetic tree

(Sarjito, Haditomo, Desrina, Djunaedi, & Prayitno, 2018). Resampling was done by bootstrapping with 1000 replications (Prayitno, Sarwan, & Sarjito, 2015).

PCR Amplification of 16s rRNA Gene Fragments

The PCR amplification of the 16S rRNA gene was performed, according to Radjasa, Nasima, Sabdono, Kita-Tsukamoto, & Ohwada (2007) and Sarjito, Haditomo, Desrina, Djunaedi, & Prayitno (2018). To find out a strictly complete 16S rRNA gene, the amplification was presented with two primers, GM3F as a forward primer (5'AGAGTTTGATCMTGGC-3') and GM4R as a reverse primer (5'-TACCTTGTTACGACTT-3') (Sarjito, Radjasa, Sabdono, Prayitno, & Hutabarat, 2009). Genomic DNA of bacteria for PCR analysis was obtained from the destruction cell materials that were taken from fresh culture bacteria, suspended in sterile water (Sigma, Germany), and subjected to five cycles of freeze (-80°C) and thaw (95°C). The PCR product was then processed to find the band DNA in the right size around 1.500 bp. The sequencing step was facilitated by PT. Genetika Science. The determined DNA sequences of samples were then compared for homology using BLAST. The phylogenetic tree was established using MEGA X to find the closely related species (Sabdaningsih *et al.*, 2020).

3. Results and Discussion

Clinical Signs and Bacterial Isolation

The most frequent clinical signs of the infected mud crab (*S. serrata*) were wounds on the surface of the claws, the ventral, and the carapace. The isolation of bacteria using TCBS and TSA medium obtained 25 pure isolates of bacteria from three replicates agar plates and then stored in NA medium. The clinical signs of red-brown

and dark spots in the carapace were found in Figure 2. Similar clinical symptoms were found on the abdomen.

Figure 2. The clinical signs of mud crab (*S. serrata*) infected by bacterial diseases: (a.) Red/brown spots in the carapace; (b.) Red-brown spots in the carapace wound on the abdomen.

Red-brown or dark melanin spots mostly known as vibriosis in mud crab, patches of light red spots as well as dark spots on the carapace, also wounds on the body (claws, shell, and the ventral). The identical clinical signs have been found by Muyzer, Teske, Wirsen, and Jannasch (1995), Wang (2011), Lavilla-Pitogo, Celia, & Leobert (2004) and Prayitno, Sarjito & Putri (2017). It was also reported on the mud crabs that were affected by genus *Vibrio* from the gulf of Semarang (Sarjito, Hastuti, Samidjan, & Prayitno, 2014) and Pemalang (Sarjito *et al.*, 2016). ‘Brown spot’ may occur due to the infection of chitinolytic bacteria that catabolize the chitin of the carapace and cause erosion and melanization (dark brown to black pigmentation) at the site of infection (Jithendran, Poornima, Balasubramanian & Kulasekarapandian, 2010)

Repetitive Genomic Sequences-PCR (Rep-PCR)

The Repetitive PCR analysis and the dendrogram related to the 25 pure isolates bacteria originating from mud crabs found a seven group from total samples (Figure 3). Furthermore, the molecular identification only represented by one isolates from each seven group such as CJR14, CJR15, CJD23, CJK10, CJK11, CJD22, and CJR05 (Table 1).

Figure 3. A dendrogram based on Rep-PCR of 25 bacteria isolates associated with vibriosis clinical signs from traditional mud crab pond.

Figure 3 shows a similarity between 25 isolates into seven groups due to the difference between repetitive bacterial sequences then it was examined using the 16S rRNA gene for identification. A total of seven isolates were identified and presented in Table 1—the range of homology percentage between 92-98%. The highest similarity was showed by isolate CJR15 from group II, 98% homology with *C. thiocicly* strain TG 5-3. The homology 97% was appointed to isolate CJK10 and CJK11 from group IV and V that similar to *V. parahaemolyticus* ATCC 17802 and *S. loihica* strain PV4. Followed by *V. alginolyticus* strain NBRC 15630 with 96% homology from isolate CJR05 in group VII. Moreover, isolate CJR14 and CJD23 have 95% homology with *V. harveyi* strain NCIMB1280 and *P. ganghwense* strain FR311 in group I and III, while isolate CJD22 was closely related 92% to *S. algae* strain ATCC5192 in group VI.

Table 1. Molecular characterization of seven bacterial species associated with clinical signs of Vibriosis on mud crabs from the North Coast of Central Java.

The vibrio's associated in mud crabs from traditional brackish water pond of the North Coast of Central Java (Figure 1), it was closely related to *V. harveyi* strain NCIMB1280 (CJR14), *C. thiocicly* strain TG 5-3 (CJR15), *P. ganghwense* strain FR311 (CJD23); *V. parahaemolyticus* ATCC 17802 (CJK10); *S. loihica* strain PV4 (CJK11); *S. algae* strain ATCC5 (CJD22) and *V. alginolyticus* strain NBRC 15630 (CJR05). The result also revealed the diversity of vibrios associated with mud crab from the brackish water pond. It was lower than the diversities that were found in the cultured and wild crab in India (Jithendran, Poornima, Balasubramanian & Kulasekarapandian, 2010) as well as traditional brackish water surrounding of Semarang Gulf (Sarjito, Hastuti, Samidjan, & Prayitno, 2014).

Four genus Vibrionaceae (*Vibrio*, *Shewanella*, *Photobacterium*, and *Catenococcus*) were mostly found in mud crab (Li *et al.*, 2012; Wei *et al.*, 2019). Three species of vibrio, i.e. *V. parahaemolyticus* (CJK10), *V. harveyi* (CJR14), and *V. alginolyticus* (CJR05) also have been detected in this study. The vibrios were frequently found as a causative agent of shell disease in *L. vannamei* shrimp and mud crab, *S. serrata*, (Estave, & Herrere, 2000; Wang, 2011; Soto-Rodriguez *et al.*, 2012). *V. parahaemolyticus* has been found as a main pathogen in mud crab in China (Xia *et al.*, 2010) and Chakoria Coast, Bangladesh (Aftabuddin *et al.*, 2013). Additionally, *V. harveyi* has been reported as a causative agent of vibriosis in fattening mud crabs from brackish water pond in Pemalang coast (Sarjito *et al.*, 2016) and mud crab from extensive brackish water pond of surrounding the Gulf of Semarang (Sarjito, Hastuti, Samidjan, & Prayitno, 2014) and Malaysia (Najiah, Nadirah, Sakri, & Harrison, 2010). These bacteria were also reported as a causative agent of bacterial diseases in zoea stage of mud crab (Jithendran, Poornima, Balasubramanian, & Kulasekarapandian, 2010); adult mud crabs (Poornima *et al.*, 2012 and Lavilla-Pitogo, Celia, & Leobert, 2004). Moreover, Sarjito, Hastuti, Samidjan, & Prayitno, (2014), found that this bacterium was a potential pathogen to mud crabs. *V. alginolyticus* was observed as an important pathogenic bacteria associated with infectious diseases in mud crab (Najiah, Nadirah, Sakri & Harrison, 2010; Sarjito, Hastuti, Samidjan, & Prayitno, 2014) and shell disease of mud crab *S. serrata* grow out pond located at Tamil Nadu, India (Gunasekaran, Gopalakrishnan, Deivasigamani, Muhilvannan, & Kathirkaman, 2019).

Genus *Photobacterium* was found in the coastal, open-ocean and deep-sea water (Moi *et al.*, 2017). Surprisingly, *P. ganghwense* was detected in this study. This bacterium, was firstly reported from a seawater in Ganghwa Island, South Korea (Park

et al., 2006). Furthermore, Wei *et al.*, (2019) found that Photobacterium was dominant as gut microbiota in mud crab *S. paramamosain* where collected from nine coastal areas of southern China.

The result showed that both *Shewanella algae* and *Shewanella loihica* were found in this study. They have an essential role as a turnover of organic material, and capable of dissimilatory reduction of various metals and other substances, such as Nitrate, Nitrite, Thiosulphate, and Trimethylamine-N-oxide (Holt, Gahrn-Hansen, & Bruun, 2005). *S. algae* has been reported as bacterial pathogen associated with Black Spot Disease in Freshwater-Cultured White leg Shrimp (*Penaeus vannamei*) (Cao, Chen, Lu, & An, 2018), causative agent of *Babylonia* (Shufang, Zhang, Dequan, Shiping, & Huang, 2015); *Cynoglossus semilaevis* (Han *et al.*, 2017), and *Scianeps ocellatus* (Zhang, Zhu, & Wang, 2013). According to Prayitno, Sarwan & Sarjito (2015) *S. algae* was also presented in gut of milkfish from the Northern Coast of Central Java. In Demak, *S. algae* was found only when the water temperature is more than 23°C. Additionally, the infection of genus *Shewanella* mostly occurs in countries with a warm climate (Holt, Gahrn-Hansen, & Bruun, 2005). Although it is rare in human pathogen and symptoms of infection, the food safety of crab product should be considered since this bacterium was already detected in mud crab farming at the North Coast of Central Java.

Shewanella loihica was isolated and identified from iron-rich microbial mats in the Pacific Ocean (Gao *et al.*, 2006). *S. loihica* plays a role as an agent of metal reduction and iron bio-mineralization. Based on that, the existence of this bacterium can be caused by the metal and nitrogen content in the water. Some reported study of *S. loihica* is also focusing on metal reduction and iron bio-mineralization capabilities (Newton, Mori,

Nakamura, Hashimoto, & Watanabe, 2009; Philips *et al.*, 2018), denitrification and a respiratory ammonification pathway (Yoon, Sanford, & Löffler, 2015). Therefore, this bacterium has an important role on the nutrient cycle in soils and sediments where mud crab lives.

Catenococcus thiocyli was identified by Yarza *et al.* (2013), the study found that *C. thiocyli* is part of the genus Vibrionaceae. Only few studies reported this bacterium (Zheng *et al.*, 2016; Li *et al.*, 2019; Achmad *et al.*, 2019; Guibert, Lecellier, Torda, Pochon, & Berteaux-Lecellier, 2020). This member of Vibrionaceae was found as a causative agent of Ice-ice diseases of the seaweed, *Kappaphycus alvarezii*, (Achmad *et al.*, 2019) and moribund of the giant clam, *Tridacna maxima*, (Guibert, Lecellier, Torda, Pochon, & Berteaux-Lecellier, 2020).

Diversity of Vibriosis Clinical Signs Associated Bacteria

The diversity of vibrios in mud crab with vibriosis clinical signs were compared from the three-sampling site to perform the percentage of species in each location (Table 2). Table 2 accommodates all of the isolates that identified based on the 16S rRNA gene. The diversity of vibriosis clinical signs associated with bacteria was explored using molecular characterization by Rep-PCR. This method is a useful tool to distinguish the diversity of bacteria according to the number and size of repetitive bacterial sequences. This technique could prevent to analyse the similar isolate of bacteria based on its DNA fingerprinting. Therefore, rep-PCR is helpful in grouping *Vibrio* species. The diversity of vibrios found in three sample locations (Table 2). We found more bacteria species in Rembang rather than in Demak and Kendal. The highest percentage of vibrio diversity compared to the total isolate in each location was in Demak (80%).

Table 2. The diversity of Vibrios in Mud Crab from the North Coast of Central Java per location.

Figure 4. The bacterial diversity in Mud crab with clinical signs of Vibriosis from the North Coast of Central Java.

Figure 4 represented the bacterial diversity in Mud crab with Vibriosis's clinical signs from the North Coast of Central Java. The highest number species was *C. thiocicly* with the percentage 32%, while 24% was *V. parahaemolyticus*, *S. loihica* and *S. algae* in the equal percentage in 12%, as well as *P. ganghwense* and *V. alginolyticus* in 8%, and the lowest rate was *V. harveyi* in 4%.

Figure 5. Phylogenetic tree of the bacteria associated with vibriosis clinical signs from traditional mud crab farming in the North Coast of Central Java, Indonesia constructed using Neighbour-Joining analysis with 1000 replicates.

In order to represent the relationship among isolates, the phylogenetic was constructed. Figure 5 shows that all of the strains have assembled with the closely related species. The relationship between the genus performs that the bacteria genus *Vibrio* has the same clade with *Catenococcus thiocycli*. Those groups are more closely related compared to the group of *Shewanella* and *Photobacterium*.

The isolates were continuing to be grouped based on the sampling site (Table 2). Rembang has the highest number of strains. However, from seven groups of *Vibrio*, only six groups were detected in this site. Demak has four groups of *Vibrio* from five isolates found. Furthermore, Kendal only has two groups of *Vibrio* from seven strains. The results indicated that the highest diversity of *Vibrio* was found in Rembang, rather

than Demak and Kendal. It might happen since those locations have a higher abrasion (Directorate of Marine and Fisheries, 2019). Miller (1989) revealed that the abrasion could decline the microbial abundances.

Moreover, Figure 4 was designed to know the highest species of Vibrionaceae found in this study. Based on the dendrogram of rep-PCR, the most top species come from *C. thiocicly* in group II with a percentage number 32%, and the lowest was *V. harveyi* with the percentage 4%. Based on the phylogenetic tree (Figure 5), *Vibrio* and *Catenococcus* are more closely related than those of *Shewanella* and *Photobacterium*.

According to the results, future research should be conducted to have a deep understanding and correlation between the biotic and abiotic factors that affect the health status of Mud-crab in the traditional brackish water pond. Therefore, the metagenomic approach would be helpful to describe the bacterial structure community in the healthy and infected mud crab. Thus, the design of prevention methods to reduce bacterial disease outbreak of Mud crab farming could be expertly constructed.

4. Conclusions

Strains of these seven bacteria are well known to be pathogens for aquatic organisms. Moreover, the highest diversity of Vibrionaceae was obtained from Demak, and the highest bacterial diversity found in three sampling sites was *C. thiocicly*.

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Figure 1. Research Location represented in a small red box in the left of map.



Figure 2. The clinical signs of mud crab (*S. serrata*) infected by bacterial diseases: (a.) Red/brown spots in the carapace; (b.) Red-brown spots in the carapace wound on the abdomen.

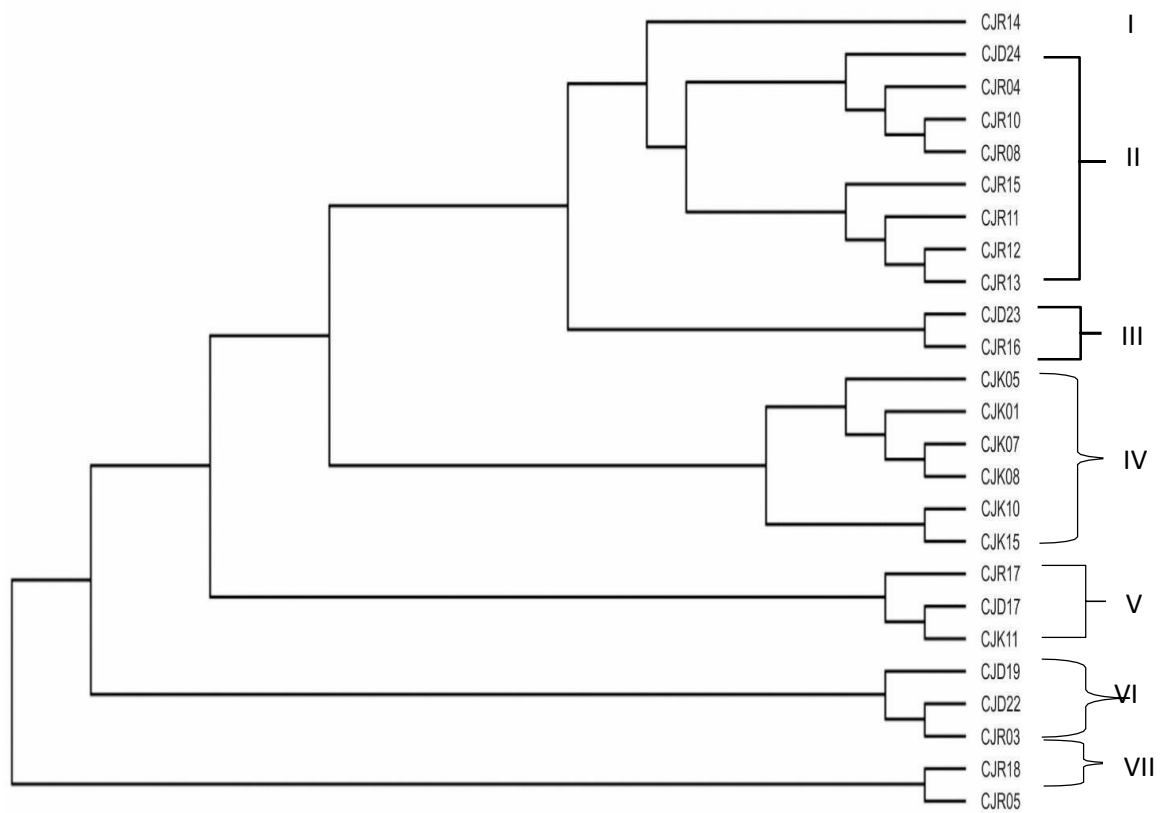


Figure 3. A dendrogram based on Rep-PCR of 25 Bacteria Isolates Associated with vibriosis clinical signs from traditional mud crab farming in the North Coastal of Central Java, Central Java, Indonesia.

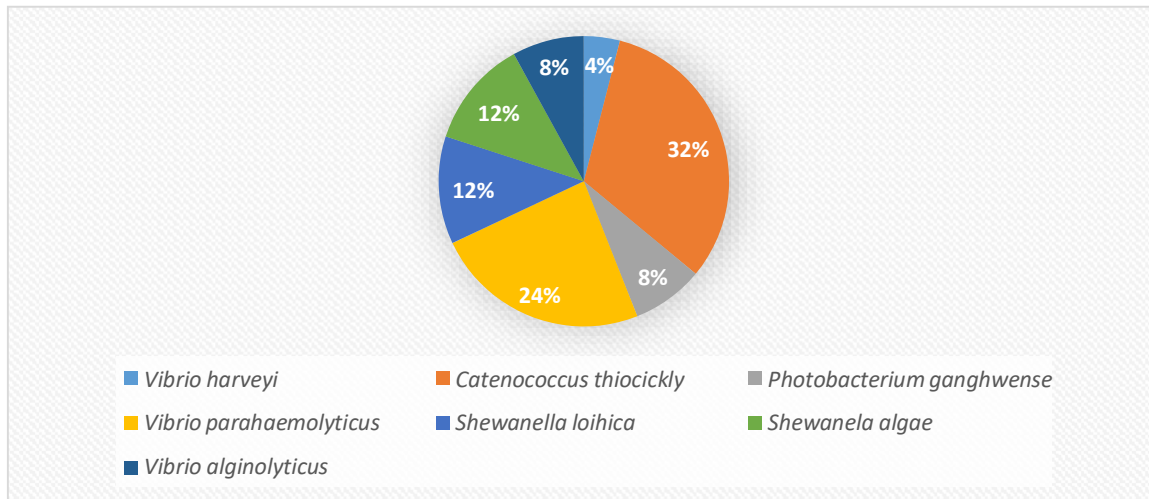


Figure 4. The bacterial diversity in Mud crab with clinical signs of Vibriosis from North Coast of Central Java.

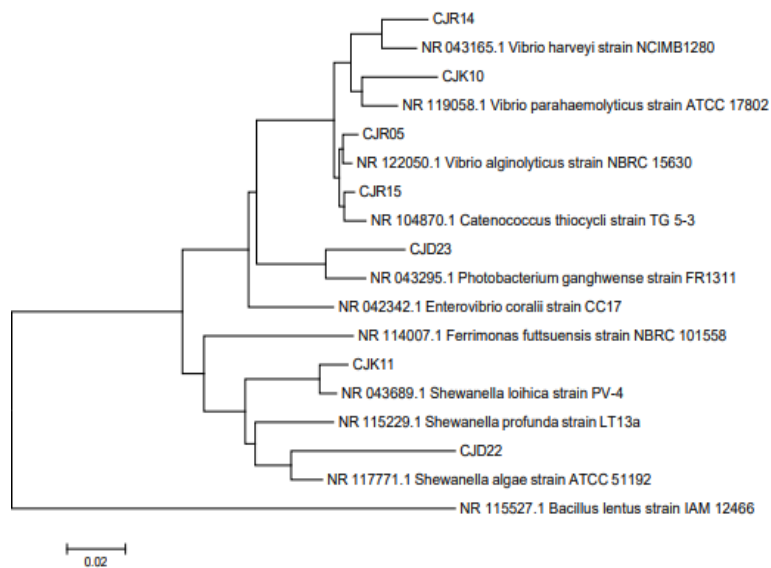


Figure 5. Phylogenetic tree of the bacteria associated with vibriosis clinical signs from traditional mud crab farming in the North Coast of Central Java, Indonesia constructed using Neighbour-Joining analysis with 1000 replicates.

Table 1. Molecular characterization of seven bacterial species associated with clinical signs of Vibriosis on mud crabs from North Coast of Central Java.

Isolates Code	Query Length	Close relative	Query Cover (%)	Homology (%)	Acc. Number
CJR14	1548	<i>Vibrio harveyi</i> strain NCIMB1280	98	95	NR 043165.1
CJR15	1319	<i>Catenococcus thiocicly</i> strain TG 5-3	98	98	NR 118258.1
CJD23	1387	<i>Photobacterium ganghwense</i> strain FR311	98	95	NR 043295.1
CJK10	1340	<i>Vibrio parahaemolyticus</i> ATCC 17802	99	97	NR 043689.1
CJK11	1335	<i>Shewanella loihica</i> strain PV4	97	97	NR 025794.1
CJD22	1488	<i>Shewanella algae</i> strain ATCC5192	95	92	NR 117771.1
CJR05	1362	<i>Vibrio alginolyticus</i> strain NBRC 15630	96	96	NR 122050.1

Table 2. The diversity of Vibrios in Mud Crab from North Coast of Central Java per location.

Location	Isolates Code	Bacteria Species	Vibrios diversity	Isolate Number	Percentage of species compare to total isolate (%)
Rembang	CJR14;				
	CJR04;				
	CJR10;				
	CJR08;				
	CJR15;	<i>Vibrio harveyi</i> strain NCIMB1280			
	CJR11;	<i>Catenococcus thiocicly</i> strain TG 5-3,			
	CJR12;	<i>Photobacterium ganghwense</i> strain FR311,			
	CJR13;	<i>Shewanella loihica</i> strain PV4,	6	13	52%
	CJR16;	<i>Shewanella algae</i> strain ATCC5192,			
	CJR17;	<i>Vibrio alginolyticus</i> strain NBRC 15630,			
	CJR03;				
	CJR18;				
	CJR05				
Demak	CJD24;	<i>Catenococcus thiocicly</i> strain TG 5-3			
	CJD23;	<i>Photobacterium ganghwense</i> strain FR311			
	CJD17;	<i>Shewanella loihica</i> strain PV4	4	5	80%
	CJD19;	<i>Shewanella algae</i> strain ATCC5192			
	CJD22				

	CJK05;			
	CJK01;			
	CJK07; <i>Vibrio prahaemolyticus</i> ATCC 17802			
Kendal	CJK08; <i>Shewanella loihica</i> strain PV4	2	7	28%
	CJK10;			
	CJK15;			
	CJK11			



Sarjito Sarjito <sarjito@live.undip.ac.id>

Submission Confirmation for Molecular Characterization of vibriosis clinical signs-associated bacteria from traditional mud-crab farming in the North Coast of Central Java, Indonesia - [EMID:96d755c898127dc1]

1 message

SJST <em@editorialmanager.com>
Reply-To: SJST <sjst@psu.ac.th>
To: Sarjito Sarjito <sarjito@live.undip.ac.id>

Sat, May 29, 2021 at 12:18 AM

Dear Mr Sarjito,

Your submission entitled "Molecular Characterization of vibriosis clinical signs-associated bacteria from traditional mud-crab farming in the North Coast of Central Java, Indonesia" has been received by journal Songklanakarin Journal of Science and Technology

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1 message

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Reply-To: SJST <sjst@psu.ac.th>
To: Sarjito Sarjito <sarjito@live.undip.ac.id>

Wed, Jun 2, 2021 at 3:19 PM

CC: "Alfabetian Harjuno Condro Haditomo" condrohaditomo@gmail.com, "Slamet Budi Prayitno" sbudiprayitno@gmail.com, "Aninditia Sabdaningsih" aninditia@gmail.com, "Restiana Wisnu Ariyati" restianawisnu@lecturer.undip.ac.id

Dear Mr Sarjito,

Your submission entitled "Molecular Characterization of vibriosis clinical signs-associated bacteria from traditional mud-crab farming in the North Coast of Central Java, Indonesia" has been assigned the following manuscript number: SJST-D-21-00164.

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To: Sarjito Sarjito <sarjito@live.undip.ac.id>

Wed, Aug 11, 2021 at 4:04 PM

CC: yeong@umt.edu.my

Ref.: Ms. No. SJST-D-21-00164

Article Title: "**Molecular Characterization of vibriosis clinical signs-associated bacteria from traditional mud-crab farming in the North Coast of Central Java, Indonesia**"

Songklanakarin Journal of Science and Technology

Dear *Mr Sarjito*,

Reviewers have now commented on your paper. You will see that they are advising that you **revise your manuscript**. If you are prepared to undertake the work required, I would be pleased to reconsider my decision.

For your guidance, reviewers' comments are appended below.

If you decide to revise the work, please submit a list of changes or a rebuttal against each point which is being raised when you submit the revised manuscript.

Your revision is due by **2021-09-10 23:59:59**.

To submit a revision, go to <https://www.editorialmanager.com/sjst/> and log in as an Author. You will see a menu item call Submission Needing Revision. You will find your submission record there.

Yours sincerely

Proespichaya Kanatharana
Chief Editor
Songklanakarin Journal of Science and Technology

Comments from the Editor and Reviewers :

Reviewer #2: Please fix it according to the review suggestion. The abstract should be reflected the contents.

There is additional documentation related to this decision letter. To access the file(s), please click the link below. You may also login to the system and click the 'View Attachments' link in the Action column.

[View Attachments](#)

Reviewer's Responses to Questions

Does the title of this paper clearly and sufficiently reflect its contents?

Reviewer #1: Yes

Reviewer #2: **No**: The title doesn't reflect the content

Are the keywords and abstracts/summary informative?

Reviewer #1: Yes

Reviewer #2: Yes

Are the references relevant and up-to-date?

Reviewer #1: Yes

Reviewer #2: Yes

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2 attachments



SJST-D-21-00164 revised.pdf
1446K



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1323K

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Molecular Characterization of vibriosis clinical signs-associated bacteria from traditional mud-crab farming in the North Coast of Central Java, Indonesia
--Manuscript Draft--

Manuscript Number:	SJST-D-21-00164
Full Title:	Molecular Characterization of vibriosis clinical signs-associated bacteria from traditional mud-crab farming in the North Coast of Central Java, Indonesia
Article Type:	Original Article
Section/Category:	Agriculture
Keywords:	bacterial diseases; mud crab, 16S rRNA; rep PCR; vibriosis
Manuscript Region of Origin:	INDONESIA
Abstract:	<p>This study examines the molecular characterization of bacteria associated with clinical symptoms and the diversity of vibriosis bacteria that infected mud crab farming from three sampling locations, namely Rembang, Demak, and Kendal Districts. Twenty-five bacteria were isolated from hepatopancreas, gill, and carapace of mud crab that have clinical signs of vibriosis that cultured in TCBS and TSA medium. After a modified rep-PCR and further PCR amplification of the 16S-rRNA gene, the sequence analysis was performed. The DNA sequences compared with the BLAST from the NCBI database. Then, the phylogenetic tree constructed by the MEGA X program. The clinical signs on mud crab were associated with seven isolates: CJR14, CJR15, CJD23, CJK10, CJK11, CJD22, and CJR05. Based on 16S-rRNA sequence analysis, these isolates were firmly (92-98%) related to <i>Vibrio harveyi</i> NCIMB-1280, <i>Catenococcus thiocicly</i> TG5-3, <i>Photobacterium ganghwense</i> FR311, <i>Vibrio parahaemolyticus</i> ATCC-1780, <i>Shewanella loihica</i> PV4, <i>Shewanella algae</i> ATCC5, and <i>Vibrio alginolyticus</i> NBRC-15630.</p>

COMMENTS OF REVIEWER 1
SJST-D-21-00164_REVISÉD

Rebutal of Comment of Reviewers SJST-2020-0244

No.	Suggestion	Response
1.	<p>Overall, the abstract did not reflect the content of the paper. The abstract focused on the molecular characterization, but the paper objective, result and conclusion are more focused to the diversity of the bacteria. Therefore, some changes are needed so that the paper is more focused and have one goal only</p> <p>The abstract does not describe how many samples were taken from each station location. Abstract does not show the results as stated in the title. Such as clinical signs of crabs infected with Vibrio. The bacterial isolates obtained were not the same between those in the abstract and those in the text.</p>	<p>Done Please find in revised manuscript</p>
2.	<p>There is little connectivity of these sentences “Traditional brackish water pond used for polyculture of shrimp and milkfish, wild mud crab may intrude in these ponds, where they are caught together with the shrimp and milkfish. However, the culture of the mud crab themselves is spreading. The intrusion of wild mud crab can be a problem to crustacean’s culture. Infected mud crab can carry disease agents that may infect the healthy cultured crustaceans. To prevent damage of the crustacean culture, farmers may disinfect the pond and raise barriers that are well embedded in the bunds, but these efforts are rarely 100% effective” to the next sentence of the first paragraph. The sentences meant to show that the problems with crab as a carrier to infect other crustaceans and next sentence is the problem with the mud crab fattening system. There must be a sentence to connect the flow of the story in this paragraph</p> <p>The background is a little confusing with wild mud crabs and mud crabs farming. Wild mud crab can infect shrimp culture, but in this study samples were taken from mud crab farming, what is the relationship? Why not take wild mud crabs according to the waters raised in this study?</p>	<p>We add connecting sentence: The cause of the disease does not only occur in infected wild crabs but also in crab farming.</p>

3.	<p>Objective</p> <p>The two objectives in this paper are not in line with the title of this study. I would suggest that the objective is changed to something that reflecting the finding and the title of this study.</p> <p>The purpose of this study was to characterize mud crabs that have Vibrio clinical signs of infection, but there are not described in the abstract.</p>	<p>This study was conducted to assessed the molecular characterization of bacteria associated with clinical symptoms and the diversity of the bacteria vibriosis infected mud crabs farming in brackish water traditional ponds along the North Coast of Central Java, Indonesia</p>
4.	<p>Materials and Methods:</p> <p>Why in your abstract is 25 bacteria but in the methodology 45 bacteria?</p> <p>Bacterial isolates obtained from mud crab are not the same as presented in the abstract. There seems to be a number of bacteria isolated from the samples and pure isolates obtained.</p> <p>There are 25 infected mud crabs selected.</p>	<p>Thank you for your detail correction, there are 25 isolate</p>
5.	<p>Results and Discussion:</p> <p>In your methodology you mentioned 45 isolates.</p>	

6.	<p>For this paragraph," The vibrio's associated in mud crabs from traditional brackish water pond of the North Coast of Central Java (Figure 1), it was closely related to V. harveyi strain NCIMB1280 (CJR14), C. thioicly strain TG 5-3 (CJR15), P. ganghwense strain FR311 (CJD23); V. prahaemolyticus ATCC 17802 (CJK10); S. loihica strain PV4 (CJK11); S. algae strain ATCC5 (CJD22) and V. alginolyticus strain NBRC 15630 (CJR05). The result also revealed the diversity of vibrios associated with mud crab from the brackish water pond. It was lower than the diversities that were found in the cultured and wild crab in India (Jithendran, Poornima, Balasubramanian & Kulasekarapandian, 2010) as well as traditional brackish water surrounding of Semarang Gulf (Sarjito, Hastuti, Samidjan, & Prayitno, 2014)".</p> <p>I would suggest that this paragraph is transferred to page 8, after Table 1. This is not suitable in its current location. The identification of the bacteria must come after the Rep PCR according to your Methodology</p>	<p>Done Moved to page 8</p>
7.	<p>The diversity of bacteria is not clearly stated in the Methodology. In the methodology, you need to mentioned about the methodology to calculate the diversity of the bacteria so that the data you can expressed in your results and you can discuss it in your discussion.</p>	<p>Done Bacterial diversity is the percentage of the number of each species in a Dendogram group based on Rep-PCR compared to the total number of isolates. Then, the diversity of vibrios in mud crab with vibriosis clinical signs were compared from the three-sampling site to perform the percentage of species in each location</p>
8.	<p>Red-brown or dark melanin spots mostly knew as vibriosis in mud crab does not appear in abstract. Abstract does not describe the research title.</p>	
9.	<p>The results indicated that the highest diversity of Vibrio was found in Rembang, rather than Demak and Kendal. But, in Conclusions : according to molecular characterization, the highest diversity of Vibrionaceae was obtained from Demak,??</p>	

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Original Article

Molecular Characterization of vibriosis clinical signs-associated bacteria from traditional mud-crab farming in the North Coast of Central Java, Indonesia

Sarjito^{1*}, Alfabetian Harjuno Condro Haditomo¹, Slamet Budi Prayitno¹, Aninditia Sabdaningsih², Desrina¹, and Restiana Wisnu Ariyati¹

¹Aquaculture Study Program, Aquaculture Department, Fisheries and Marine Science Faculty, Diponegoro University, Semarang, 50275, Indonesia

²Department of Aquatic Resources, Fisheries and Marine Science Faculty, Diponegoro University, Semarang, 50275, Indonesia

*Email address : sarjito@live.undip.ac.id

Abstract

This study examines the molecular characterization of bacteria associated with clinical symptoms and the diversity of vibriosis bacteria that infected mud crab farming from three sampling locations, namely Rembang, Demak, and Kendal Districts. Twenty-five bacteria were isolated from hepatopancreas, gill, and carapace of mud crab that have clinical signs of vibriosis that cultured in TCBS and TSA medium. After a modified rep-PCR and further PCR amplification of the 16S-rRNA gene, the sequence analysis was performed. The DNA sequences compared with the BLAST from the NCBI database. Then, the phylogenetic tree constructed by the MEGA X program. The clinical signs on mud crab were associated with seven isolates: CJR14, CJR15, CJD23, CJK10, CJK11, CJD22, and CJR05. Based on 16S-rRNA sequence analysis, these isolates were firmly (92-98%) related to *Vibrio harveyi* NCIMB-1280, *Catenococcus thiocicly* TG5-3,

1 *Photobacterium ganghwense* FR311, *Vibrio parahaemolyticus* ATCC-1780, *Shewanella*
2
3 *loihica* PV4, *Shewanella algae* ATCC5, and *Vibrio alginolyticus* NBRC-15630.
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8 **Keywords:** bacterial diseases, mud crab, 16S rRNA, rep PCR, vibriosis
9

10 **1. Introduction**

11
12 Mud crab, *Scylla serrata* (Forskål, 1775), is traditionally caught in and around brackish
13
14 water ponds and mangrove forests along the North Coast of Central Java, Indonesia. The
15
16 mangrove forests and its soils are this crab's origin habitat. Traditional brackish water
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18 pond used for polyculture of shrimp and milkfish, wild mud crab may intrude in these
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20 ponds, where they are caught together with the shrimp and milkfish. However, the culture
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22 of the mud crab themselves is spreading. The intrusion of wild mud crab can be a problem
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24 to crustacean's culture. Infected mud crab can carry disease agents that may infect the
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26 healthy cultured crustaceans. To prevent damage of the crustacean culture, farmers may
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28 disinfect the pond and raise barriers that are well embedded in the bunds, but these efforts
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30 are rarely 100% effective. The cause of the disease does not only occur in infected wild
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32 crabs but also in crab farming. Bacterial diseases, especially vibriosis, was found to be a
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34 problem in the fattening of the mud crab culture in Pemalang (Sarjito *et al.*, 2016) and
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36 reported to cause more than 90% mortality in all life cycle stages of mud crab growth
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38 (Jithendran, Poornima, Balasubramanian, & Kulasekarapandian, 2010). Reported clinical
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40 signs of infected mud crab are wounds on the body, decreased feed response and
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42 weakening (Jithendran *et al.*, 2010), blackening and red spots of the carapace (Sarjito,
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44 Hastuti, Samidjan, & Prayitno, 2014; Sarjito *et al.*, 2016).
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1 Vibrio's cause various disease outbreaks and severe economic losses in shrimp
2 culture (Aguilre-Rivera, Prieto-Davo, Rodriguez-Fuentes G, Escalante-Herrera, &
3 Gaxiola, 2019) and have the potential to destroy the future of the industrial culture of mud
4 crab. Several species of *Vibrio* spp. were reported in and found to be associated with
5 bacterial disease in mud crab: *V. fischeri*, and *V. nereis* (Wang, 2011); *V.*
6 *alginolyticus* and *V. cholerae* (Najiah, Nadirah, Sakri & Harrison, 2010; Wang, 2011); *V.*
7 *vulnificus* (Lavilla-Pitogo, Celia, & Leobert, 2004; Shanmuga, 2008; Wang, 2011), *V.*
8 *splendidus*, and *V. orientalis* (Jithendran et al., 2010); *V. ordalii* and *V. harveyi* (Sarjito
9 et al., 2014; Jithendran et al., 2010), *V. campbelli* (Shanmuga, 2008), and *V.*
10 *parahaemolyticus* (Lavilla-Pitogo et al., 2004; Najiah et al., 2010; Shanmuga, 2008).
11 Previous research focused on the richness of bacteria associated with vibriosis in mud
12 crab. The limited knowledge is available on the diversity of vibrios related to the disease
13 outbreaks in mud crab in coastal of the North Central Java. Local government tends to
14 stimulate the culture of mud crab, nevertheless, the program lacks information on disease
15 identification and prevention. This study was conducted to assessed the molecular
16 characterization of bacteria associated with clinical symptoms and the diversity of the
17 bacteria vibriosis infected mud crabs farming in brackish water traditional ponds along
18 the North Coast of Central Java, Indonesia.

2. Materials and Methods

Sampling

Mud crab, *S. serrata*, were collected from nine brackish water ponds of Kendal,
Demak, and Rembang Regency, Central Java (each three ponds). These three locations
are the largest producers of cultivated crabs in Central Java, Indonesia (Figure 1). Based

1 on the clinical signs for vibriosis described by Jithendran et al. (2010), 25 infected mud
2 crabs were selected, stored in a sterile container and carried to the Integrated Laboratory
3
4 of Diponegoro University for further analysis. The average size of the caught crab,
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6 measured from 13 to 15 cm, was 14.4 ± 0.7 cm.
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9
10 **Figure 1.** Research location
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15 **Bacterial Isolation**

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18 Forty-five bacteria were isolated from gills, haemolymph, hepatopancreas, and wounds
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20 on the shell of the infected mud crabs. Isolates bacteria were cultured on Thiosulfate
21
22 Citrate Bile Salt Sucrose (TCBS) and Tryptic Soy Agar (TSA) (Thompson, Iida, &
23
24 Swings, 2004; Sarjito *et al.*, 2016). The colonies were purified by the streak method based
25
26 on morphological appearance (Sarjito *et al.*, 2016).
27
28

29 **Repetitive Sequences-based PCR (Rep-PCR)**

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31
32 The bacterial DNA was extracted from a 24 h-broth culture of isolate strains by
33
34 using the chelex method with slight modification according to the procedure described
35
36 by (de Lamballerie, Zandotti, Vignoli, Bollet, & de Micco, 1992). Then, the isolates
37
38 DNA extract were analyzed using the rep-PCR of Radjasa, Nasima, Sabdono, Kita-
39
40 Tsukamoto, & Ohwada (2007) was modified by Sarjito, Haditomo, Desrina, Djunaedi, &
41
42 Prayitno (2018) in the rep-PCR, BOX A1R (5'-CTACggCAAaggCgACgCTgACg-3').
43
44 The REP 1R-I and REP 2-I primers contained nucleotide inosine (I) at an ambiguous
45
46 position in the REP consensus. The mix PCR reagent contains one μ L DNA template
47
48 (diluted 100X), one μ L primer, 7.5 μ L Megamix Royal and 5.5 μ L ddH₂O. Amplification
49
50 was performed in a thermal cycler model Gene Amp PCR system 9700 with the following
51
52 protocol: initial denaturation at 95°C for 5 minutes. Then 30 cycles of (denaturation 92°C
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1 for 1 minute, annealing 50°C for 1,5 minutes, extension 68°C for 8 minutes), and a final
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3 extension at 68°C for 10 minutes. The band DNA was visualized with injected 5 µL PCR
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5 products into 1% agarose gel and run to electrophoresis machine using 1X TBE buffer
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7 and observed in UV-transilluminator, as described by Radjasa et al, (2007). Grouping of
8
9 isolates was conducted based on the method of Sarjito, Radjasa, Sabdono, Prayitno, &
10
11 Hutabarat (2009). The method is a useful tool to distinguish the bacterial strain from the
12
13 three-sampling site into group based on the fingerprinting of interspersed repetitive DN
14
15 A sequences of BOX element using BOX A1R primer, therefore each group could be
16
17 then identified using 16S-rRNA gene. Bacterial diversity is the percentage of the number
18
19 of each species in a Dendogram group based on Rep-PCR compared to the total number
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21 of isolates. Then, the diversity of vibrios in mud crab with vibriosis clinical signs were
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23 compared from the three-sampling site to perform the percentage of species in each
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25 location.

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Matrices were made from the band's position on the gel, which was analysed by the Free Tree program using the UPGMA method for constructing the phylogenetic tree (Sarjito et al., 2018). Resampling was done by bootstrapping with 1000 replications (Prayitno, Sarwan, & Sarjito, 2015).

PCR Amplification of 16S-rRNA Gene Fragments

The PCR amplification of the 16S-rRNA gene was performed, according to Radjasa et al. (2007) and Sarjito et al. (2018). To find out a 16S-rRNA gene, the amplification was presented with two primers, GM3F as a forward primer (5'AGAGTTTGATCMTGGC-3') and GM4R as a reverse primer (5'-TACCTTGTTACGACTT-3') (Sarjito et al., 2009). Genomic DNA of bacteria for PCR analysis was obtained from the destruction cell

1 materials that were taken from fresh culture bacteria, suspended in sterile water (Sigma,
2 Germany), and subjected to five cycles of freeze (-80°C) and thaw (95°C). The PCR
3 product was then processed to find the band DNA in the right size around 1.500 bp. The
4 sequencing step was facilitated by PT. Genetika Science. The determined DNA sequences
5 of samples were then compared for homology using BLAST. The phylogenetic tree was
6 established using MEGA X to find the closely related species (Sabdaningsih *et al.*, 2020).
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9 10 11 12 13 14 15 16 17 **3. Results and Discussion**

18 19 **Clinical Signs and Bacterial Isolation**

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22 The most frequent clinical signs of the infected mud crab (*S. serrata*) were wounds
23 on the surface of the claws, the ventral, and the carapace. The isolation of bacteria using
24 TCBS and TSA medium obtained 25 pure isolates of bacteria from three replicates agar
25 plates and then stored in NA medium. The clinical signs of red-brown and dark spots in
26 the carapace were found in Figure 2. Similar clinical symptoms were found on the
27 abdomen.
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30 31 32 **Figure 2.** The clinical signs of mud crab

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35 Red-brown or dark melanin spots mostly knew as vibriosis in mud crab, patches
36 of light red spots as well as dark spots on the carapace, also wounds on the body (claws,
37 shell, and the ventral). The identic of clinical signs have been found by Muyzer, Teske,
38 Wirsén, and Jannasch (1995), Wang (2011), Lavilla-Pitogo *et al.* (2004) and Prayitno,
39 Sarjito & Putri (2017). It was also reported on the mud crabs that were affected by genus
40 vibrio from the gulf of Semarang (Sarjito *et al.*, 2014) and Pematang (Sarjito *et al.*, 2016).
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1 chitin of the carapace and cause erosion and melanisation (dark brown to black
2
3 pigmentation) at the site of infection (Jithendran et al., 2010)
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5 6 **Repetitive Sequences-based PCR (Rep-PCR)**

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8 The Repetitive PCR analysis and the dendrogram related to the 25 pure isolates
9
10 bacteria originating from mud crabs found a seven group from total samples (Figure 3).
11
12 Furthermore, the molecular identification only represented by one isolate from each seven
13
14 group such as CJR14, CJR15, CJD23, CJK10, CJK11, CJD22, and CJR05 (Table 1) and
15
16 the remaining isolates were assumed as the same species within the group according to
17
18 the similar pattern of DNA size from rep-PCR result.
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26 **Figure 3.** A dendrogram

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29 Figure 3 shows a similarity between 25 isolates into seven groups due to the
30
31 difference between repetitive bacterial sequences then it was examined using the 16S-
32
33 rRNA gene for identification. A total of seven isolates were identified and presented in
34
35 Table 1—the range of homology percentage between 92-98%. The highest similarity was
36
37 showed by isolate CJR15 from group II, 98% homology with *C. thiocicly* strain TG5-3.
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39 The homology 97% was appointed to isolate CJK10 and CJK11 from group IV and V
40
41 that similar to *V. parahaemolyticus* ATCC-17802 and *S. loihica* strain PV4. Followed
42
43 by *V. alginolyticus* strain NBRC-15630 with 96% homology from isolate CJR05 in group
44
45 VII. Moreover, isolate CJR14 and CJD23 have 95% homology with *V. harveyi* strain
46
47 NCIMB-1280 and *P. ganghwense* strain FR311 in group I and III, while isolate CJD22
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49 was closely related 92% to *S. algae* strain ATCC-5192 in group VI.
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57 **Table 1.** Molecular characterization

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1 The vibrio's associated in mud crabs from traditional brackish water pond of the
2
3 North Coast of Central Java (Figure 1), it was closely related to *V. harveyi* strain NCIMB-
4
5 1280 (CJR14), *C. thiocicly* strain TG 5-3 (CJR15), *P. ganghwense* strain FR311
6
7 (CJD23); *V. prahaemolyticus* ATCC 17802 (CJK10); *S. loihica* strain PV4 (CJK11); *S.*
8
9 *algae* strain ATCC5 (CJD22) and *V. alginolyticus* strain NBRC-15630 (CJR05). The
10
11 result also revealed the diversity of vibrios associated with mud crab from the brackish
12
13 water pond. It was lower than the diversities that were found in the cultured and wild crab
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15 in India (Jithendran et al., 2010) as well as traditional brackish water surrounding of
16
17 Semarang Gulf (Sarjito et al., 2014).

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24 Four genus Vibrionaceae (*Vibrio*, *Shewanella*, *Photobacterium*,
25
26 and *Catenococcus*) were mostly found in mud crab (Li et al., 2012; Wei et al., 2019).
27
28 Three species of vibrio, i.e. *V. parahaemolyticus* (CJK10), *V. harveyi* (CJR14), and *V.*
29
30 *alginolyticus* (CJR05) also have been detected in this study. The vibrios were frequently
31
32 found as a causative agent of shell disease in *L. vannamei* shrimp and mud crab, *S.*
33
34 *serrata*, (Estave, & Herrere, 2000; Wang, 2011; Soto-Rodriguez et al., 2012). *V.*
35
36 *parahaemolyticus* has been found as a main pathogen in mud crab in China (Xia et al.,
37
38 2010) and Chakoria Coast, Bangladesh (Aftabuddin et al., 2013). Additionally, *V.*
39
40 *harveyi* has been reported as a causative agent of vibriosis in fattening mud crabs from
41
42 brackish water pond in Pemalang coast (Sarjito et al., 2016) and mud crab from extensive
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44 brackish water pond of surrounding the Gulf of Semarang (Sarjito et al., 2014) and
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46 Malaysia (Najiah et al., 2010). These bacteria were also reported as a causative agent of
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48 bacterial diseases in zoea stage of mud crab (Jithendran et al., 2010); adult mud crabs
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50 (Poornima et al., 2012 and Lavilla-Pitogo et al., 2004). Moreover, Sarjito et al., (2014),
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52 found that this bacterium was a potential pathogen to mud crabs. *V. alginolyticus* was
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1 observed as an important pathogenic bacterium associated with infectious diseases in mud
2 crab (Najiah et al., 2010) and shell disease of mud crab *S. serrata* grow out pond located
3 at Tamil Nadu, India (Gunasekaran, Gopalakrishnan, Deivasigamani, Muhilvannan, &
4 Kathirkaman, 2019).
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11 Genus Photobacterium was found in the coastal, open-ocean and deep-sea water
12 (Moi et al., 2017). Surprisingly, *P. ganghwense* was detected in this study. This
13 bacterium, was firstly reported from a seawater in Ganghwa Island, South Korea (Park et
14 al., 2006). Furthermore, Wei et al., (2019) found that Photobacterium was dominant as
15 gut microbiota in mud crab *S. paramamosain* were collected from nine coastal areas of
16 southern China.
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26 The result showed that both *Shewanella algae* and *Shewanella loihica* were
27 found in this study. They have an essential role as a turnover of organic material, and
28 capable of dissimilatory reduction of various metals and other substances, such as Nitrate,
29 Nitrite, Thiosulphate, and Trimethylamine-N-oxide (Holt, Gahrn-Hansen, & Bruun,
30 2005). *S. algae* has been reported as bacterial pathogen associated with Black Spot
31 Disease in Freshwater-Cultured White leg Shrimp (*Penaeus vannamei*) (Cao, Chen, Lu,
32 & An, 2018), causative agent of *Babylonia* (Shufang, Zhang, Dequan, Shiping, & Huang,
33 2015); *Cynoglossus semilaevis* (Han et al., 2018), and *Scianeops ocellatus* (Zhang, Zhu,
34 & Wang, 2013). According to Prayitno et al. (2015) *S. algae* was also presented in gut of
35 milkfish from the Northern Coast of Central Java. In Demak, *S. algae* was found only
36 when the water temperature is more than 23°C. Additionally, the infection of genus
37 *Shewanella* mostly occurs in countries with a warm climate (Holt et al., 2005). Although
38 it is rare in human pathogen and symptoms of infection, the food safety of crab product
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1 should be considered since this bacterium was already detected in mud crab farming at
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3 the North Coast of Central Java.
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6 *Shewanella loihica* was isolated and identified from iron-rich microbial mats in
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8 the Pacific Ocean (Gao *et al.*, 2006). *S. loihica* plays a role as an agent of metal reduction
9
10 and iron bio-mineralization. Based on that, the existence of this bacterium can be caused
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12 by the metal and nitrogen content in the water. Some reported study of *S. loihica* is also
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14 focusing on metal reduction and iron bio-mineralization capabilities (Newton, Mori,
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16 Nakamura, Hashimoto, & Watanabe, 2009; Philips *et al.*, 2018), denitrification and a
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18 respiratory ammonification pathway (Yoon, Sanford, & Löffler, 2015). Therefore, this
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20 bacterium has important role on the nutrient cycle in soils and sediments where mud crab
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22 lives.
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28 *Catenococcus thiocycli* was identified by Yarza *et al.* (2013), the study found
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30 that *C. thiocycli* is part of genus Vibrionaceae. Only few studies reported this bacterium
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32 (Zheng *et al.*, 2016; Li *et al.*, 2019; Achmad *et al.*, 2019; Guibert, Lecellier,
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34 Torda, Pochon, & Berteaux-Lecellier, 2020). This member of Vibrionaceae was found
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36 as a causative agent of Ice-ice diseases of the seaweed, *Kappaphycus alvarezii*, (Achmad
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38 *et al.*, 2019) and moribund of the giant clam, *Tridacna maxima*, (Guibert *et al.*, 2020).
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43 **Diversity of Vibriosis Clinical Signs Associated Bacteria**

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47 The diversity of vibrios in mud crab with vibriosis clinical signs were compared
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49 from the three-sampling site to perform the percentage of species in each location (Table
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51 2). Table 2 accommodates all of the isolates that identified based on the 16S-rRNA gene.
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53 The diversity of vibriosis clinical signs associated with bacteria was explored using
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55 molecular characterization by rep-PCR. This method is a useful tool to distinguish the
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1 diversity of bacteria according to the number and size of repetitive bacterial sequences.
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3 This technique could prevent to analyse the similar isolate of bacteria based on its DNA
4 fingerprinting. Therefore, rep-PCR is helpful in grouping *Vibrio* species. The diversity of
5 vibrios found in three sample locations (Table 2). We found more bacteria species in
6 Rembang rather than in Demak and Kendal. The highest percentage of vibrio diversity
7 compared to the total isolate in each location was in Demak (80%).
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17 **Table 2.** The diversity of Vibrios

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20 **Figure 4.** The bacterial diversity in Mud crab

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24 Figure 4 represented the bacterial diversity in Mud crab with Vibriosis's clinical signs
25 from the North Coast of Central Java. The highest number species was *C. thiocicly* with
26 the percentage 32%, while 24% was *V. parahaemolyticus*, *S. loihica* and *S. algae* in the
27 equal percentage in 12%, as well as *P. ganghwense* and *V. algynolyticus* in 8%, and the
28 lowest rate was *V. harveyi* in 4%.
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38 **Figure 5.** Phylogenetic tree

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41 In order to represent the relationship among isolates, the phylogenetic was constructed.
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43 Figure 5 shows that all of the strains have assembled with the closely related species. The
44 relationship between the genus performs that the bacteria genus *Vibrio* has the same clade
45 with *Catenococcus thiocycli*. Those groups are more closely related compared to the
46 group of *Shewanella* and *Photobacterium*.
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55 The isolates were continuing to be grouped based on the sampling site (Table 2).
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57 Rembang has the highest number of strains. However, from seven groups of *Vibrio*, only
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1 six groups were detected in this site. Demak has four groups of *Vibrio* from five isolates
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3 found. Furthermore, Kendal only has two groups of *Vibrio* from seven strains. The results
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5 indicated that the highest diversity of *Vibrio* was found in Rembang, rather than Demak
6
7 and Kendal. It might happen since those locations have a higher abrasion (Directorate of
8
9 Marine and Fisheries, 2019). Miller (1989) revealed that the abrasion could decline the
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11 microbial abundances.
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17 Moreover, Figure 4 was designed to know the highest species of Vibrionaceae
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19 found in this study. Based on the dendrogram of rep-PCR, the most top species come
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21 from *C. thiocicly* in group II with a percentage number 32%, and the lowest was *V.*
22
23 *harveyi* with the percentage 4%. Based on the phylogenetic tree (Figure
24
25 5), *Vibrio* and *Catenococcus* are more closely related than those
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27 of *Shewanella* and *Photobacterium*.
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33 According to the results, future research should be conducted to have a deep
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35 understanding and correlation between the biotic and abiotic factors that affect the health
36
37 status of Mud-crab in the traditional brackish water pond. Therefore, the metagenomic
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39 approach would be helpful to describe the bacterial structure community in the healthy
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41 and infected mud crab. Thus, the design of prevention methods to reduce bacterial disease
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43 outbreak of Mud crab farming could be expertly constructed.
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47 48 49 **4. Conclusions**

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51 This study revealed that strains of these seven groups of bacteria are well known to be
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53 pathogens for aquatic organisms. Moreover, according to molecular characterization, the
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55 highest diversity of Vibrionaceae was obtained from Demak, and the highest bacterium
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57 found in all three sampling sites was *C. thiocicly*.
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Figure 1. Research Location represented in a small red box in the left of map.



Figure 2. The clinical signs of mud crab (*S. serrata*) infected by bacterial diseases: (a.) Red/brown spots in the carapace; (b.) Red-brown spots in the carapace wound on the abdomen.

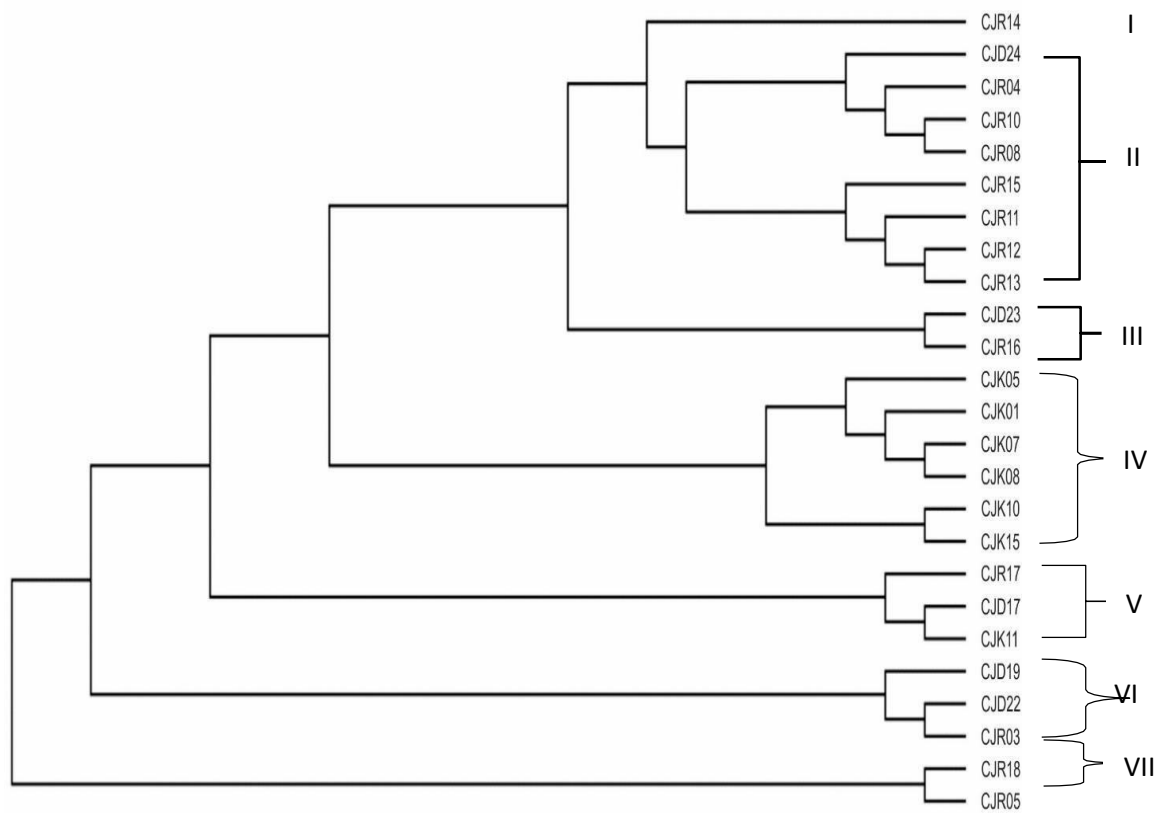


Figure 3. A dendrogram based on Rep-PCR of 25 Bacteria Isolates Associated with vibriosis clinical signs from traditional mud crab farming in the North Coastal of Central Java, Central Java, Indonesia.

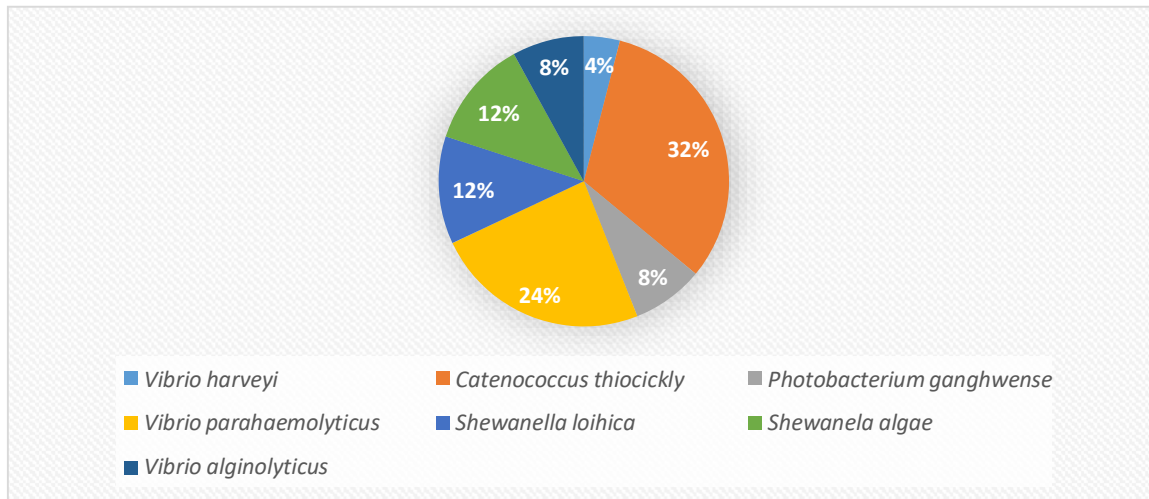


Figure 4. The bacterial diversity in Mud crab with clinical signs of Vibriosis from North Coast of Central Java.

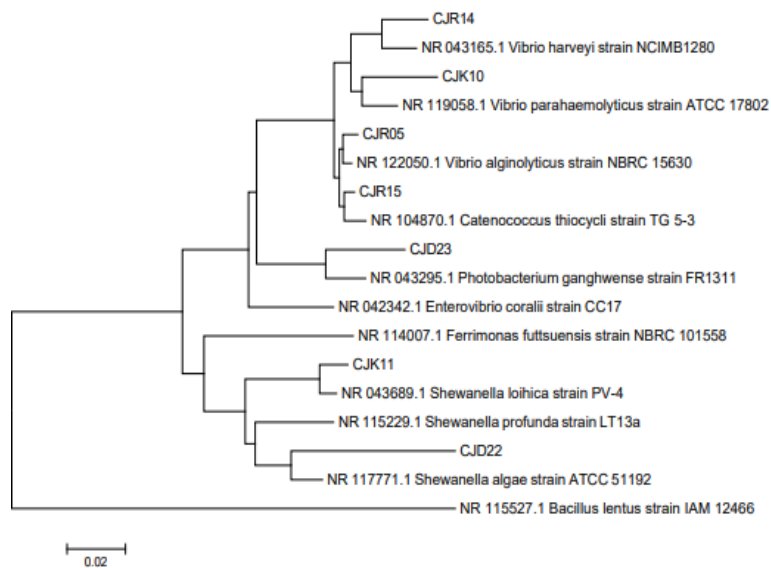


Figure 5. Phylogenetic tree of the bacteria associated with vibriosis clinical signs from traditional mud crab farming in the North Coast of Central Java, Indonesia constructed using Neighbour-Joining analysis with 1000 replicates.

Table 1. Molecular characterization of seven bacterial species associated with clinical signs of Vibriosis on mud crabs from North Coast of Central Java.

Isolates Code	Query Length	Close relative	Query Cover (%)	Homology (%)	Acc. Number
CJR14	1548	<i>Vibrio harveyi</i> strain NCIMB1280	98	95	NR 043165.1
CJR15	1319	<i>Catenococcus thiocicly</i> strain TG 5-3	98	98	NR 118258.1
CJD23	1387	<i>Photobacterium ganghwense</i> strain FR311	98	95	NR 043295.1
CJK10	1340	<i>Vibrio parahaemolyticus</i> ATCC 17802	99	97	NR 043689.1
CJK11	1335	<i>Shewanella loihica</i> strain PV4	97	97	NR 025794.1
CJD22	1488	<i>Shewanella algae</i> strain ATCC5192	95	92	NR 117771.1
CJR05	1362	<i>Vibrio alginolyticus</i> strain NBRC 15630	96	96	NR 122050.1

Table 2. The diversity of Vibrios in Mud Crab from North Coast of Central Java per location.

Location	Isolates Code	Bacteria Species	Vibrios diversity	Isolate Number	Percentage of species compare to total isolate (%)
Rembang	CJR14;				
	CJR04;				
	CJR10;				
	CJR08;				
	CJR15;	<i>Vibrio harveyi</i> strain NCIMB1280			
	CJR11;	<i>Catenococcus thiocicly</i> strain TG 5-3,			
	CJR12;	<i>Photobacterium ganghwense</i> strain FR311,			
	CJR13;	<i>Shewanella loihica</i> strain PV4,	6	13	52%
	CJR16;	<i>Shewanella algae</i> strain ATCC5192,			
	CJR17;	<i>Vibrio alginolyticus</i> strain NBRC 15630,			
	CJR03;				
	CJR18;				
	CJR05				
Demak	CJD24;	<i>Catenococcus thiocicly</i> strain TG 5-3			
	CJD23;	<i>Photobacterium ganghwense</i> strain FR311			
	CJD17;	<i>Shewanella loihica</i> strain PV4	4	5	80%
	CJD19;	<i>Shewanella algae</i> strain ATCC5192			
	CJD22				

	CJK05;			
	CJK01;			
	CJK07; <i>Vibrio prahaemolyticus</i> ATCC 17802			
Kendal	CJK08; <i>Shewanella loihica</i> strain PV4	2	7	28%
	CJK10;			
	CJK15;			
	CJK11			

	CJK05;			
	CJK01;			
	CJK07; <i>Vibrio prahaemolyticus</i> ATCC 17802			
Kendal	CJK08; <i>Shewanella loihica</i> strain PV4	2	7	28%
	CJK10;			
	CJK15;			
	CJK11			

Molecular Characterization of vibriosis ~~clinical signs~~-associated bacteria from traditional mud-crab farming in the North Coast of Central Java, Indonesia --Manuscript Draft--

Manuscript Number:	SJST-D-21-00164
Full Title:	Molecular Characterization of vibriosis clinical signs -associated bacteria from traditional mud-crab farming in the North Coast of Central Java, Indonesia
Article Type:	Original Article
Section/Category:	Agriculture
Keywords:	bacterial diseases; mud crab, 16S rRNA; rep PCR; vibriosis
Manuscript Region of Origin:	INDONESIA
Abstract:	<p>This study examines the molecular characterization of bacteria associated with clinical symptoms and the diversity of vibriosis bacteria that infected mud crab farming from three sampling locations, namely Rembang, Demak, and Kendal Districts. Twenty-five bacteria were isolated from hepatopancreas, gill, and carapace of mud crab that have clinical signs of vibriosis that cultured in TCBS and TSA medium. After a modified rep-PCR and further PCR amplification of the 16S-rRNA gene, the sequence analysis was performed. The DNA sequences compared with the BLAST from the NCBI database. Then, the phylogenetic tree constructed by the MEGA X program. The clinical signs on mud crab were associated with seven isolates: CJR14, CJR15, CJD23, CJK10, CJK11, CJD22, and CJR05. Based on 16S-rRNA sequence analysis, these isolates were firmly (92-98%) related to <i>Vibrio harveyi</i> NCIMB-1280, <i>Catenococcus thioicly</i> TG5-3, <i>Photobacterium ganghwense</i> FR311, <i>Vibrio parahaemolyticus</i> ATCC-1780, <i>Shewanella loihica</i> PV4, <i>Shewanella algae</i> ATCC5, and <i>Vibrio alginolyticus</i> NBRC-15630.</p>

COMMENTS OF REVIEWER 2
SJST-D-21-00164_REVIEWER (2)

Rebutal of Comment of Reviewers SJST-2020-0244

No.	Suggestion	Response
1.	Overall, the abstract did not reflect the content of the paper. The abstract focused on the molecular characterization, but the paper objective, result and conclusion are more focused to the diversity of the bacteria. Therefore, some changes are needed so that the paper is more focused and have one goal only	Done Please find in revised manuscript
2.	There is little connectivity of these sentences “Traditional brackish water pond used for polyculture of shrimp and milkfish, wild mud crab may intrude in these ponds, where they are caught together with the shrimp and milkfish. However, the culture of the mud crab themselves is spreading. The intrusion of wild mud crab can be a problem to crustacean’s culture. Infected mud crab can carry disease agents that may infect the healthy cultured crustaceans. To prevent damage of the crustacean culture, farmers may disinfect the pond and raise barriers that are well embedded in the bunds, but these efforts are rarely 100% effective” to the next sentence of the first paragraph. The sentences meant to show that the problems with crab as a carrier to infect other crustaceans and next sentence is the problem with the mud crab fattening system. There must be a sentence to connect the flow of the story in this paragraph	We add connecting sentence: The cause of the disease does not only occur in infected wild crabs but also in crab farming.
3.	Objective The two objectives in this paper are not in-line with the title of this study. I would suggest that the objective is changed to something that reflecting the finding and the title of this study.	This study was conducted to assessed the molecular characterization of bacteria associated with clinical symptoms and the diversity of the bacteria vibriosis infected mud crabs farming in brackish water traditional ponds along the North Coast of Central Java, Indonesia
4.	Materials and Methods: Why in your abstract is 25 bacteria but in the methodology 45 bacteria?	Thank you for your detail correction, there are 25 isolate
5.	Results and Discussion: In your methodology you mentioned 45 isolates.	

<p>6.</p>	<p>For this paragraph," The vibrio's associated in mud crabs from traditional brackish water pond of the North Coast of Central Java (Figure 1), it was closely related to <i>V. harveyi</i> strain NCIMB1280 (CJR14), <i>C. thiocicly</i> strain TG 5-3 (CJR15), <i>P. ganghwense</i> strain FR311 (CJD23); <i>V. prahaemolyticus</i> ATCC 17802 (CJK10); <i>S. loihica</i> strain PV4 (CJK11); <i>S. algae</i> strain ATCC5 (CJD22) and <i>V. alginolyticus</i> strain NBRC 15630 (CJR05). The result also revealed the diversity of vibrios associated with mud crab from the brackish water pond. It was lower than the diversities that were found in the cultured and wild crab in India (Jithendran, Poornima, Balasubramanian & Kulasekarapandian, 2010) as well as traditional brackish water surrounding of Semarang Gulf (Sarjito, Hastuti, Samidjan, & Prayitno, 2014)".</p> <p>I would suggest that this paragraph is transferred to page 8, after Table 1. This is not suitable in its current location. The identification of the bacteria must come after the Rep PCR according to your Methodology</p>	<p>Done Moved to page 8</p>
<p>7.</p>	<p>The diversity of bacteria is not clearly stated in the Methodology. In the methodology, you need to mention about the methodology to calculate the diversity of the bacteria so that the data you can express in your results and you can discuss it in your discussion.</p>	<p>Done Bacterial diversity is the percentage of the number of each species in a Dendogram group based on Rep-PCR compared to the total number of isolates. Then, the diversity of vibrios in mud crab with vibriosis clinical signs were compared from the three-sampling site to perform the percentage of species in each location</p>

Original Article

Molecular Characterization of vibriosis ~~clinical signs~~-associated bacteria from traditional mud-crab farming in the North Coast of Central Java, Indonesia

Sarjito^{1*}, Alfabetian Harjuno Condro Haditomo¹, Slamet Budi Prayitno¹, Aninditia Sabdaningsih², Desrina¹, and Restiana Wisnu Ariyati¹

¹Aquaculture Study Program, Aquaculture Department, Fisheries and Marine Science Faculty, Diponegoro University, Semarang, 50275, Indonesia

²Department of Aquatic Resources, Fisheries and Marine Science Faculty, Diponegoro University, Semarang, 50275, Indonesia

*Email address : sarjito@live.undip.ac.id

Abstract

This study examines the molecular characterization of bacteria associated with clinical symptoms and the diversity of vibriosis bacteria that ~~infected~~ mud crab farming from three sampling locations, namely Rembang, Demak, and Kendal Districts. Twenty-five



bacteria were isolated from hepatopancreas, gill, and carapace of mud crab that have clinical signs of vibriosis that cultured in TCBS and TSA medium. After a modified rep-PCR and further PCR amplification of the 16S-rRNA gene, the sequence analysis was performed. The DNA sequences compared with the BLAST from the NCBI database. Then, the phylogenetic tree constructed by the MEGA X program. The clinical signs on mud crab were associated with seven isolates: CJR14, CJR15, CJD23, CJK10, CJK11, CJD22, and CJR05. Based on 16S-rRNA sequence analysis, these isolates were firmly (92-98%) related to *Vibrio harveyi* NCIMB-1280, *Catenococcus thiocicly* TG5-3,

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1 *Photobacterium ganghwense* FR311, *Vibrio parahaemolyticus* ATCC-1780, *Shewanella*
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3 *loihica* PV4, *Shewanella algae* ATCC5, and *Vibrio alginolyticus* NBRC-15630.
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8 **Keywords:** ~~bacterial diseases~~, mud crab, 16S rRNA, rep PCR, vibriosis
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10 11 12 **1. Introduction**

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14 Mud crab, *Scylla serrata* (Forskål, 1775), is traditionally caught in and around brackish
15 water ponds and mangrove forests along the North Coast of Central Java, Indonesia. The
16 mangrove forests and its soils are this crab's origin habitat. Traditional brackish water
17 pond used for polyculture of shrimp and milkfish, wild mud crab may intrude in these
18 ponds, where they are caught together with the shrimp and milkfish. However, the culture
19 of the mud crab themselves is spreading. The intrusion of wild mud crab can be a problem
20 to crustacean's culture. Infected mud crab can carry disease agents that may infect the
21 healthy cultured crustaceans. To prevent damage of the crustacean culture, farmers may
22 disinfect the pond and raise barriers that are well embedded in the bunds, but these efforts
23 are rarely 100% effective. The cause of the disease does not only occur in infected wild
24 crabs but also in crab farming. Bacterial diseases, especially vibriosis, was found to be a
25 problem in the fattening of the mud crab culture in Pemalang (Sarjito *et al.*, 2016) and
26 reported to cause more than 90% mortality in all life cycle stages of mud crab growth
27 (Jithendran, Poornima, Balasubramanian, & Kulasekarapandian, 2010). Reported clinical
28 signs of infected mud crab are wounds on the body, decreased feed response and
29 weakening (Jithendran *et al.*, 2010), blackening and red spots of the carapace (Sarjito,
30 Hastuti, Samidjan, & Prayitno, 2014; Sarjito *et al.*, 2016).
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1 Vibrio's cause various disease outbreaks and severe economic losses in shrimp
2 culture (Aguilre-Rivera, Prieto-Davo, Rodriguez-Fuentes G, Escalante-Herrera, &
3 Gaxiola, 2019) and have the potential to destroy the future of the industrial culture of mud
4 crab. Several species of *Vibrio* spp. were reported in and found to be associated with
5 bacterial disease in mud crab: *V. fischeri*, and *V. nereis* (Wang, 2011); *V.*
6 *alginolyticus* and *V. cholerae* (Najiah, Nadirah, Sakri & Harrison, 2010; Wang, 2011); *V.*
7 *vulnificus* (Lavilla-Pitogo, Celia, & Leobert, 2004; Shanmuga, 2008; Wang, 2011), *V.*
8 *splendidus*, and *V. orientalis* (Jithendran et al., 2010); *V. ordalii* and *V. harveyi* (Sarjito
9 et al., 2014; Jithendran et al., 2010), *V. campbelli* (Shanmuga, 2008), and *V.*
10 *parahaemolyticus* (Lavilla-Pitogo et al., 2004; Najiah et al., 2010; Shanmuga, 2008).
11 Previous research focused on the richness of bacteria associated with vibriosis in mud
12 crab. The limited knowledge is available on the diversity of vibrios related to the disease
13 outbreaks in mud crab in coastal of the North Central Java. Local government tends to
14 stimulate the culture of mud crab, nevertheless, the program lacks information on disease
15 identification and prevention. This study was conducted to assessed the molecular
16 characterization of bacteria associated with clinical symptoms and the diversity of the
17 bacteria vibriosis infected mud crabs farming in brackish water traditional ponds along
18 the North Coast of Central Java, Indonesia.

2. Materials and Methods

Sampling

Mud crab, *S. serrata*, were collected from nine brackish water ponds of Kendal,
Demak, and Rembang Regency, Central Java (each three ponds). These three locations
are the largest producers of cultivated crabs in Central Java, Indonesia (Figure 1). Based

1 on the clinical signs for vibriosis described by Jithendran et al. (2010), 25 infected mud
2 crabs were selected, stored in a sterile container and carried to the Integrated Laboratory
3 of Diponegoro University for further analysis. The average size of the caught crab,
4 measured from 13 to 15 cm, was 14.4±0.7cm.
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10 **Figure 1.** Research location
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14 **Bacterial Isolation**

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18 Forty-five bacteria were isolated from gills, haemolymph, hepatopancreas, and wounds
19 on the shell of the infected mud crabs. Isolates bacteria were cultured on Thiosulfate
20 Citrate Bile Salt Sucrose (TCBS) and Tryptic Soy Agar (TSA) (Thompson, Iida, &
21 Swings, 2004; Sarjito *et al.*, 2016). The colonies were purified by the streak method based
22 on morphological appearance (Sarjito *et al.*, 2016).
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30 **Repetitive Sequences-based PCR (Rep-PCR)**

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32 The bacterial DNA was extracted from a 24 h-broth culture of isolate strains by
33 using the chelex method with slight modification according to the procedure described
34 by (de Lamballerie, Zandotti, Vignoli, Bollet, & de Micco, 1992). Then, the isolates
35 DNA extract were analyzed using the rep-PCR of Radjasa, Nasima, Sabdon, Kita-
36 Tsukamoto, & Ohwada (2007) was modified by Sarjito, Haditomo, Desrina, Djunaedi, &
37 Prayitno (2018) in the rep-PCR, BOX A1R (5'-CTACggCAAaggCgACgCTgACg-3').
38 The REP 1R-I and REP 2-I primers contained nucleotide inosine (I) at an ambiguous
39 position in the REP consensus. The mix PCR reagent contains one µL DNA template
40 (diluted 100X), one µL primer, 7.5 µL Megamix Royal and 5.5 µL ddH₂O. Amplification
41 was performed in a thermal cycler model Gene Amp PCR system 9700 with the following
42 protocol: initial denaturation at 95°C for 5 minutes. Then 30 cycles of (denaturation 92°C
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1 for 1 minute, annealing 50°C for 1,5 minutes, extension 68°C for 8 minutes), and a final
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3 extension at 68°C for 10 minutes. The band DNA was visualized with injected 5 µL PCR
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5 products into 1% agarose gel and run to electrophoresis machine using 1X TBE buffer
6
7 and observed in UV-transilluminator, as described by Radjasa et al, (2007). Grouping of
8
9 isolates was conducted based on the method of Sarjito, Radjasa, Sabdono, Prayitno, &
10
11 Hutabarat (2009). The method is a useful tool to distinguish the bacterial strain from the
12
13 three-sampling site into group based on the fingerprinting of interspersed repetitive DN
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15 A sequences of BOX element using BOX A1R primer, therefore each group could be
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17 then identified using 16S-rRNA gene. Bacterial diversity is the percentage of the number
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19 of each species in a Dendogram group based on Rep-PCR compared to the total number
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21 of isolates. Then, the diversity of vibrios in mud crab with vibriosis clinical signs were
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23 compared from the three-sampling site to perform the percentage of species in each
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25 location.
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33 Matrices were made from the band's position on the gel, which was analysed by
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35 the Free Tree program using the UPGMA method for constructing the phylogenetic tree
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37 (Sarjito et al., 2018). Resampling was done by bootstrapping with 1000 replications
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39 (Prayitno, Sarwan, & Sarjito, 2015).
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45 **PCR Amplification of 16S-rRNA Gene Fragments**

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47 The PCR amplification of the 16S-rRNA gene was performed, according to Radjasa et al.
48
49 (2007) and Sarjito et al. (2018). To find out a 16S-rRNA gene, the amplification was
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51 presented with two primers, GM3F as a forward primer (5'AGAGTTTGATCMTGGC-
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53 3') and GM4R as a reverse primer (5'-TACCTTGTTACGACTT-3') (Sarjito et al., 2009).
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55 Genomic DNA of bacteria for PCR analysis was obtained from the destruction cell
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1 materials that were taken from fresh culture bacteria, suspended in sterile water (Sigma,
2 Germany), and subjected to five cycles of freeze (-80°C) and thaw (95°C). The PCR
3 product was then processed to find the band DNA in the right size around 1.500 bp. The
4 sequencing step was facilitated by PT. Genetika Science. The determined DNA sequences
5 of samples were then compared for homology using BLAST. The phylogenetic tree was
6 established using MEGA X to find the closely related species (Sabdaningsih *et al.*, 2020).
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16 3. Results and Discussion

19 Clinical Signs and Bacterial Isolation

22 The most frequent clinical signs of the infected mud crab (*S. serrata*) were wounds
23 on the surface of the claws, the ventral, and the carapace. The isolation of bacteria using
24 TCBS and TSA medium obtained 25 pure isolates of bacteria from three replicates agar
25 plates and then stored in NA medium. The clinical signs of red-brown and dark spots in
26 the carapace were found in Figure 2. Similar clinical symptoms were found on the
27 abdomen.
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37 **Figure 2.** The clinical signs of mud crab

39 Red-brown or dark melanin spots mostly knew as vibriosis in mud crab, patches
40 of light red spots as well as dark spots on the carapace, also wounds on the body (claws,
41 shell, and the ventral). The identic of clinical signs have been found by Muyzer, Teske,
42 Wirsen, and Jannasch (1995), Wang (2011), Lavilla-Pitogo *et al.* (2004) and Prayitno,
43 Sarjito & Putri (2017). It was also reported on the mud crabs that were affected by genus
44 vibrio from the gulf of Semarang (Sarjito *et al.*, 2014) and Pemalang (Sarjito *et al.*, 2016).
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1 chitin of the carapace and cause erosion and melanisation (dark brown to black
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3 pigmentation) at the site of infection (Jithendran et al., 2010)
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5 6 **Repetitive Sequences-based PCR (Rep-PCR)**

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8 The Repetitive PCR analysis and the dendrogram related to the 25 pure isolates
9
10 bacteria originating from mud crabs found a seven group from total samples (Figure 3).
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12 Furthermore, the molecular identification only represented by one isolate from each seven
13
14 group such as CJR14, CJR15, CJD23, CJK10, CJK11, CJD22, and CJR05 (Table 1) and
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16 the remaining isolates were assumed as the same species within the group according to
17
18 the similar pattern of DNA size from rep-PCR result.
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25 **Figure 3.** A dendrogram

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29 Figure 3 shows a similarity between 25 isolates into seven groups due to the
30
31 difference between repetitive bacterial sequences then it was examined using the 16S-
32
33 rRNA gene for identification. A total of seven isolates were identified and presented in
34
35 Table 1—the range of homology percentage between 92-98%. The highest similarity was
36
37 showed by isolate CJR15 from group II, 98% homology with *C. thiocicly* strain TG5-3.
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39 The homology 97% was appointed to isolate CJK10 and CJK11 from group IV and V
40
41 that similar to *V. parahaemolyticus* ATCC-17802 and *S. loihica* strain PV4. Followed
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43 by *V. alginolyticus* strain NBRC-15630 with 96% homology from isolate CJR05 in group
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45 VII. Moreover, isolate CJR14 and CJD23 have 95% homology with *V. harveyi* strain
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47 NCIMB-1280 and *P. ganghwense* strain FR311 in group I and III, while isolate CJD22
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49 was closely related 92% to *S. algae* strain ATCC-5192 in group VI.
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57 **Table 1.** Molecular characterization

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1 The vibrio's associated in mud crabs from traditional brackish water pond of the
2
3 North Coast of Central Java (Figure 1), it was closely related to *V. harveyi* strain NCIMB-
4
5 1280 (CJR14), *C. thiocicly* strain TG 5-3 (CJR15), *P. ganghwense* strain FR311
6
7 (CJD23); *V. prahaemolyticus* ATCC 17802 (CJK10); *S. loihica* strain PV4 (CJK11); *S.*
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9 *algae* strain ATCC5 (CJD22) and *V. alginolyticus* strain NBRC-15630 (CJR05). The
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11 result also revealed the diversity of vibrios associated with mud crab from the brackish
12
13 water pond. It was lower than the diversities that were found in the cultured and wild crab
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15 in India (Jithendran et al., 2010) as well as traditional brackish water surrounding of
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17 Semarang Gulf (Sarjito et al., 2014).
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24 Four genus Vibrionaceae (*Vibrio*, *Shewanella*, *Photobacterium*,
25 and *Catenococcus*) were mostly found in mud crab (Li et al., 2012; Wei et al., 2019).
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27 Three species of vibrio, i.e. *V. parahaemolyticus* (CJK10), *V. harveyi* (CJR14), and *V.*
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29 *alginolyticus* (CJR05) also have been detected in this study. The vibrios were frequently
30
31 found as a causative agent of shell disease in *L. vannamei* shrimp and mud crab, *S.*
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33 *serrata*, (Estave, & Herrere, 2000; Wang, 2011; Soto-Rodriguez et al., 2012). *V.*
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35 *parahaemolyticus* has been found as a main pathogen in mud crab in China (Xia et al.,
36
37 2010) and Chakoria Coast, Bangladesh (Aftabuddin et al., 2013). Additionally, *V.*
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39 *harveyi* has been reported as a causative agent of vibriosis in fattening mud crabs from
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41 brackish water pond in Pemalang coast (Sarjito et al., 2016) and mud crab from extensive
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43 brackish water pond of surrounding the Gulf of Semarang (Sarjito et al., 2014) and
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45 Malaysia (Najiah et al., 2010). These bacteria were also reported as a causative agent of
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47 bacterial diseases in zoea stage of mud crab (Jithendran et al., 2010); adult mud crabs
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49 (Poornima et al., 2012 and Lavilla-Pitogo et al., 2004). Moreover, Sarjito et al., (2014),
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51 found that this bacterium was a potential pathogen to mud crabs. *V. alginolyticus* was
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1 observed as an important pathogenic bacterium associated with infectious diseases in mud
2 crab (Najiah et al., 2010) and shell disease of mud crab *S. serrata* grow out pond located
3 at Tamil Nadu, India (Gunasekaran, Gopalakrishnan, Deivasigamani, Muhilvannan, &
4 Kathirkaman, 2019).
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11 Genus Photobacterium was found in the coastal, open-ocean and deep-sea water
12 (Moi et al., 2017). Surprisingly, *P. ganghwense* was detected in this study. This
13 bacterium, was firstly reported from a seawater in Ganghwa Island, South Korea (Park et
14 al., 2006). Furthermore, Wei et al., (2019) found that Photobacterium was dominant as
15 gut microbiota in mud crab *S. paramamosain* were collected from nine coastal areas of
16 southern China.
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26 The result showed that both *Shewanella algae* and *Shewanella loihica* were
27 found in this study. They have an essential role as a turnover of organic material, and
28 capable of dissimilatory reduction of various metals and other substances, such as Nitrate,
29 Nitrite, Thiosulphate, and Trimethylamine-N-oxide (Holt, Gahrn-Hansen, & Bruun,
30 2005). *S. algae* has been reported as bacterial pathogen associated with Black Spot
31 Disease in Freshwater-Cultured White leg Shrimp (*Penaeus vannamei*) (Cao, Chen, Lu,
32 & An, 2018), causative agent of *Babylonia* (Shufang, Zhang, Dequan, Shiping, & Huang,
33 2015); *Cynoglossus semilaevis* (Han et al., 2018), and *Scianeops ocellatus* (Zhang, Zhu,
34 & Wang, 2013). According to Prayitno et al. (2015) *S. algae* was also presented in gut of
35 milkfish from the Northern Coast of Central Java. In Demak, *S. algae* was found only
36 when the water temperature is more than 23°C. Additionally, the infection of genus
37 *Shewanella* mostly occurs in countries with a warm climate (Holt et al., 2005). Although
38 it is rare in human pathogen and symptoms of infection, the food safety of crab product
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1 should be considered since this bacterium was already detected in mud crab farming at
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3 the North Coast of Central Java.
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6 *Shewanella loihica* was isolated and identified from iron-rich microbial mats in
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8 the Pacific Ocean (Gao *et al.*, 2006). *S. loihica* plays a role as an agent of metal reduction
9
10 and iron bio-mineralization. Based on that, the existence of this bacterium can be caused
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12 by the metal and nitrogen content in the water. Some reported study of *S. loihica* is also
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14 focusing on metal reduction and iron bio-mineralization capabilities (Newton, Mori,
15
16 Nakamura, Hashimoto, & Watanabe, 2009; Philips *et al.*, 2018), denitrification and a
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18 respiratory ammonification pathway (Yoon, Sanfordd, & Löfflerr, 2015). Therefore, this
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20 bacterium has important role on the nutrient cycle in soils and sediments where mud crab
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22 lives.
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28 *Catenococcus thiocycli* was identified by Yarza *et al.* (2013), the study found
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30 that *C. thiocycli* is part of genus Vibrionaceae. Only few studies reported this bacterium
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32 (Zheng *et al.*, 2016; Li *et al.*, 2019; Achmad *et al.*, 2019; Guibert, Lecellier,
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34 Torda, Pochon, & Berteaux-Lecellier, 2020). This member of Vibrionaceae was found
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36 as a causative agent of Ice-ice diseases of the seaweed, *Kappaphycus alvarezii*, (Achmad
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38 *et al.*, 2019) and moribund of the giant clam, *Tridacna maxima*, (Guibert *et al.*, 2020).
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43 **Diversity of Vibriosis Clinical Signs Associated Bacteria**

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47 The diversity of vibrios in mud crab with vibriosis clinical signs were compared
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49 from the three-sampling site to perform the percentage of species in each location (Table
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51 2). Table 2 accommodates all of the isolates that identified based on the 16S-rRNA gene.
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53 The diversity of vibriosis clinical signs associated with bacteria was explored using
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55 molecular characterization by rep-PCR. This method is a useful tool to distinguish the
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1 diversity of bacteria according to the number and size of repetitive bacterial sequences.
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3 This technique could prevent to analyse the similar isolate of bacteria based on its DNA
4 fingerprinting. Therefore, rep-PCR is helpful in grouping *Vibrio* species. The diversity of
5 vibrios found in three sample locations (Table 2). We found more bacteria species in
6 Rembang rather than in Demak and Kendal. The highest percentage of vibrio diversity
7 compared to the total isolate in each location was in Demak (80%).
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17 **Table 2.** The diversity of Vibrios

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20 **Figure 4.** The bacterial diversity in Mud crab

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24 Figure 4 represented the bacterial diversity in Mud crab with Vibriosis's clinical signs
25 from the North Coast of Central Java. The highest number species was *C. thiocicly* with
26 the percentage 32%, while 24% was *V. parahaemolyticus*, *S. loihica* and *S. algae* in the
27 equal percentage in 12%, as well as *P. ganghwense* and *V. algynolyticus* in 8%, and the
28 lowest rate was *V. harveyi* in 4%.
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38 **Figure 5.** Phylogenetic tree

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41 In order to represent the relationship among isolates, the phylogenetic was constructed.
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43 Figure 5 shows that all of the strains have assembled with the closely related species. The
44 relationship between the genus performs that the bacteria genus *Vibrio* has the same clade
45 with *Catenococcus thiocycli*. Those groups are more closely related compared to the
46 group of *Shewanella* and *Photobacterium*.
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55 The isolates were continuing to be grouped based on the sampling site (Table 2).
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57 Rembang has the highest number of strains. However, from seven groups of *Vibrio*, only
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1 six groups were detected in this site. Demak has four groups of *Vibrio* from five isolates
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3 found. Furthermore, Kendal only has two groups of *Vibrio* from seven strains. The results
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5 indicated that the highest diversity of *Vibrio* was found in Rembang, rather than Demak
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7 and Kendal. It might happen since those locations have a higher abrasion (Directorate of
8
9 Marine and Fisheries, 2019). Miller (1989) revealed that the abrasion could decline the
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11 microbial abundances.
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16 Moreover, Figure 4 was designed to know the highest species of Vibrionaceae
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18 found in this study. Based on the dendrogram of rep-PCR, the most top species come
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20 from *C. thiocicly* in group II with a percentage number 32%, and the lowest was *V.*
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22 *harveyi* with the percentage 4%. Based on the phylogenetic tree (Figure
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24 5), *Vibrio* and *Catenococcus* are more closely related than those
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26 of *Shewanella* and *Photobacterium*.
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33 According to the results, future research should be conducted to have a deep
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35 understanding and correlation between the biotic and abiotic factors that affect the health
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37 status of Mud-crab in the traditional brackish water pond. Therefore, the metagenomic
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39 approach would be helpful to describe the bacterial structure community in the healthy
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41 and infected mud crab. Thus, the design of prevention methods to reduce bacterial disease
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43 outbreak of Mud crab farming could be expertly constructed.
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48 **4. Conclusions**

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51 This study revealed that strains of these seven groups of bacteria are well known to be
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53 pathogens for aquatic organisms. Moreover, according to molecular characterization, the
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55 highest diversity of Vibrionaceae was obtained from Demak, and the highest bacterium
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57 found in all three sampling sites was *C. thiocicly*.
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Figure 1. Research Location represented in a small red box in the left of map.



Figure 2. The clinical signs of mud crab (*S. serrata*) infected by bacterial diseases: (a.) Red/brown spots in the carapace; (b.) Red-brown spots in the carapace wound on the abdomen.

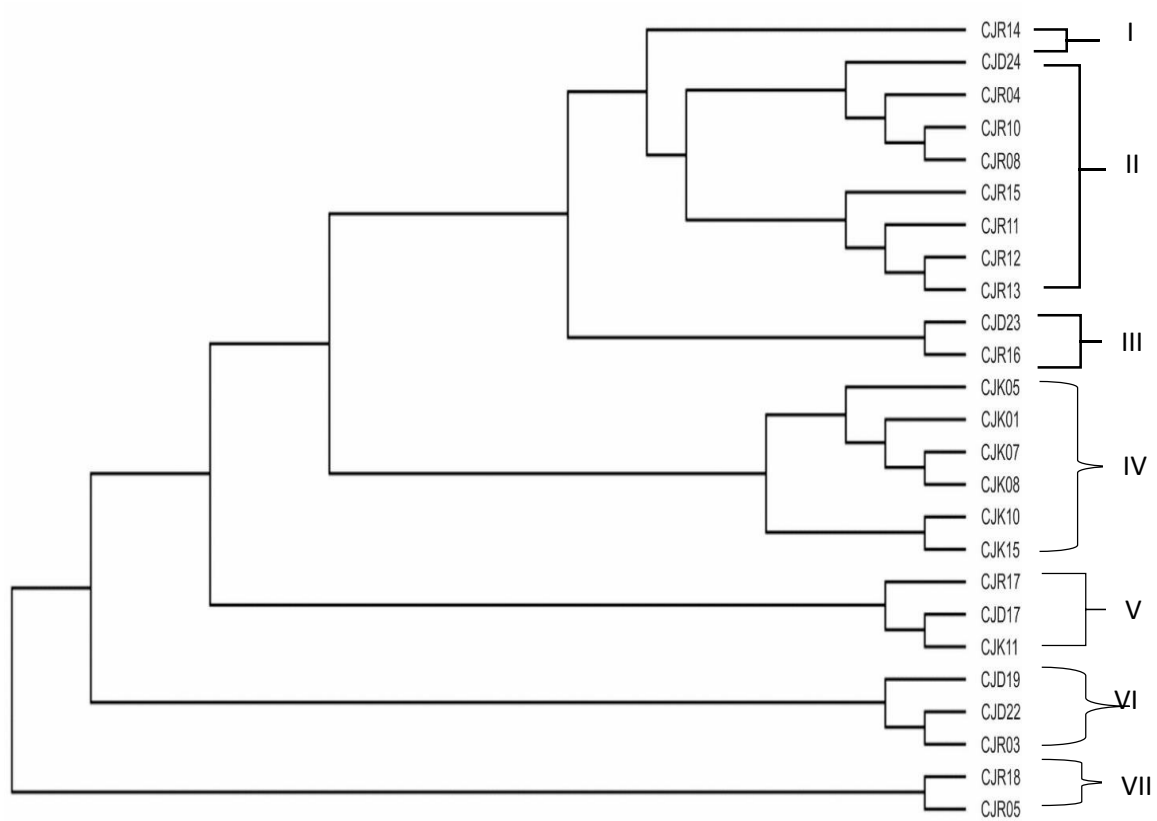


Figure 3. A dendrogram based on Rep-PCR of 25 Bacteria Isolates Associated with vibriosis clinical signs from traditional mud crab farming in the North Coastal of Central Java, Central Java, Indonesia.



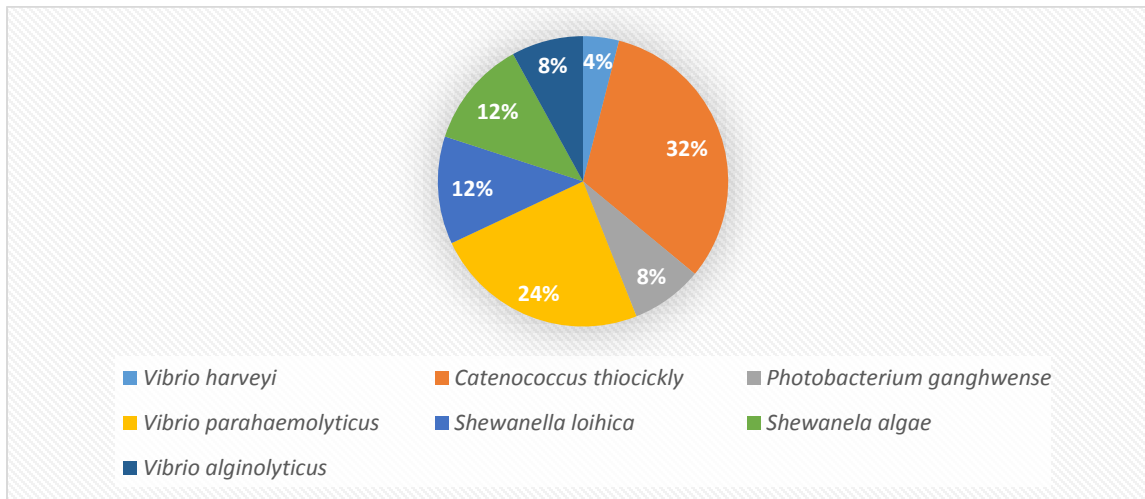


Figure 4. The bacterial diversity in Mud crab with clinical signs of Vibriosis from North Coast of Central Java.

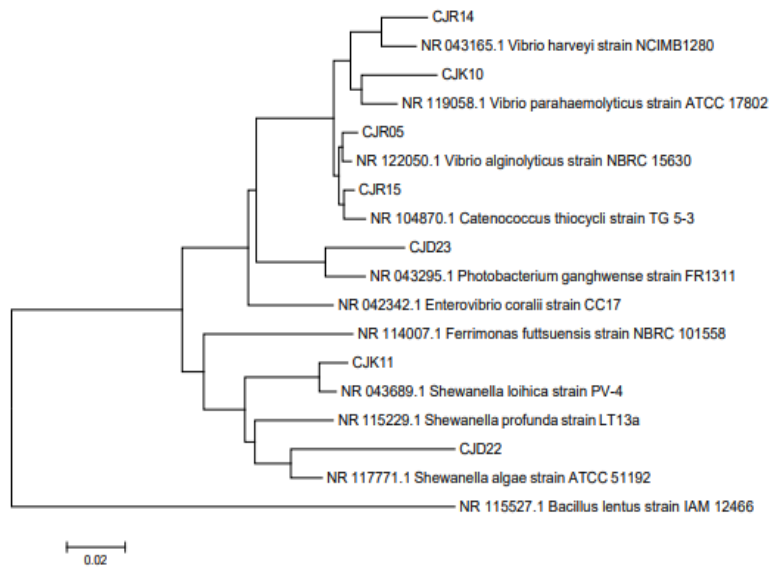


Figure 5. Phylogenetic tree of the bacteria associated with vibriosis clinical signs from traditional mud crab farming in the North Coast of Central Java, Indonesia constructed using Neighbour-Joining analysis with 1000 replicates.

Table 1. Molecular characterization of seven bacterial species associated with clinical signs of Vibriosis on mud crabs from North Coast of Central Java.

Isolates Code	Query Length	Close relative	Query Cover (%)	Homology (%)	Acc. Number
CJR14	1548	<i>Vibrio harveyi</i> strain NCIMB1280	98	95	NR 043165.1
CJR15	1319	<i>Catenococcus thiocicly</i> strain TG 5-3	98	98	NR 118258.1
CJD23	1387	<i>Photobacterium ganghwense</i> strain FR311	98	95	NR 043295.1
CJK10	1340	<i>Vibrio parahaemolyticus</i> ATCC 17802	99	97	NR 043689.1
CJK11	1335	<i>Shewanella loihica</i> strain PV4	97	97	NR 025794.1
CJD22	1488	<i>Shewanella algae</i> strain ATCC5192	95	92	NR 117771.1
CJR05	1362	<i>Vibrio alginolyticus</i> strain NBRC 15630	96	96	NR 122050.1

Table 2. The diversity of Vibrios in Mud Crab from North Coast of Central Java per location.

Location	Isolates Code	Bacteria Species	Vibrios diversity	Isolate Number	Percentage of species compare to total isolate (%)
Rembang	CJR14;				
	CJR04;				
	CJR10;				
	CJR08;	<i>Vibrio harveyi</i> strain NCIMB1280			
	CJR15;	<i>Catenococcus thiocicly</i> strain TG 5-3,			
	CJR11;	<i>Photobacterium ganghwense</i> strain FR311,			
	CJR12;	<i>Shewanella loihica</i> strain PV4,	6	13	52%
	CJR13;	<i>Shewanella algae</i> strain ATCC5192,			
	CJR16;	<i>Vibrio alginolyticus</i> strain NBRC 15630,			
	CJR17;				
	CJR03;				
	CJR18;				
	CJR05				
Demak	CJD24;	<i>Catenococcus thiocicly</i> strain TG 5-3			
	CJD23;	<i>Photobacterium ganghwense</i> strain FR311			
	CJD17;	<i>Shewanella loihica</i> strain PV4	4	5	80%
	CJD19;	<i>Shewanella algae</i> strain ATCC5192			
	CJD22				

	CJK05;			
	CJK01;			
	CJK07; <i>Vibrio prahaemolyticus</i> ATCC 17802			
Kendal	CJK08; <i>Shewanella loihica</i> strain PV4	2	7	28%
	CJK10;			
	CJK15;			
	CJK11			



Sarjito Sarjito <sarjito@live.undip.ac.id>

Submission Confirmation for SJST-D-21-00164R1 - [EMID:5dec3c4a36b52a00]

1 message

SJST <em@editorialmanager.com>
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To: Sarjito Sarjito <sarjito@live.undip.ac.id>

Wed, Sep 8, 2021 at 6:16 PM

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Molecular Characterization of vibriosis clinical signs-associated bacteria from traditional mud-crab farming in the North Coast of Central Java, Indonesia

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Rebutal of Comment of Reviewers SJST-D-2100164

No.	Suggestion	Response
1.	The abstract does not describe how many samples were taken from each station location. Abstract does not show the results as stated in the title. Such as clinical signs of crabs infected with Vibrio. The bacterial isolates obtained were not the same between those in the abstract and those in the text.namely Rembang (13 isolates), Demak (5 isolates), and Kendal (7 isolates) Districts. In total, twenty-five bacteria were isolated from.....
2.	The background is a little confusing with wild mud crabs and mud crabs farming. Wild mud crab can infect shrimp culture, but in this study, samples were taken from mud crab farming, what is the relationship? Why not take wild mud crabs according to the waters raised in this study?	It Has been change accordingly
3.	The purpose of this study was to characterize mud crabs that have Vibrio clinical signs of infection, but there are not described in the abstract	We add sentence: The clinical signs i.e. red-brown spots that mostly detected in the infected mud crabs carapace as well as wounds on their body of mud crabs associated with seven isolates
4.	Bacterial isolates obtained from mud crab are not the same as presented in the abstract. There seems to be a number of bacteria isolated from the samples and pure isolates obtained. There are 25 infected mud crabs selected.	<i>Has been adopted accordingly.</i> 9 moribund mud crabs was isolated from 3 study sites, 25 isolates were obtained and after characterization break down into 7 groups

Rebutal of Comment of Reviewers SJST-D-2100164

No.	Suggestion	Response
1	In your method you mentioned 45 bacteria were isolated? Please check again	Thank you for detail Already check, twenty-five isolates
2	The cause of the disease does not only occur in infected wild crabs but also in crab farming. Need to explain properly	We change the sentence: <i>Has been re arranged accordingly</i> Disease caused by infiltration of infected wild crabs into brackish water ponds reduces shrimp culture production as well as mud crab farming. So, we collect sample from crab farming

3	<p>The wounds could be due to physical wound, not necessarily due to bacterial infection, these wounds were not shown in figure 2</p> <p>Did all the samples had similar clinical signs? If not, state percentage</p> <p>The result should also include the different bacterial species found from different organs....</p>	<p>The wounds shown in figure two, left hand figure shown clearer (yellow circle)</p> <p>Almost all the samples have similar sights, a certain part soft broken carapace</p>
4	<p>Which vibrio specifically? Please relate the clinical signs to the bacteria found</p>	<p>Add detail</p>

Original Article

Molecular Characterization of vibriosis associated bacteria from traditional mud-crab farming in the North Coast of Central Java, Indonesia

Sarjito^{1*}, Alfabetian Harjuno Condro Haditomo¹, Slamet Budi Prayitno¹, Aninditia Sabdaningsih², Desrina¹, and Restiana Wisnu Ariyati¹

¹Aquaculture Study Program, Aquaculture Department, Fisheries and Marine Science Faculty, Diponegoro University, Semarang, 50275, Indonesia

²Department of Aquatic Resources, Fisheries and Marine Science Faculty, Diponegoro University, Semarang, 50275, Indonesia

*Email address : sarjito@live.undip.ac.id

Abstract

This study examines the molecular characterization of bacteria associated with clinical symptoms and the diversity of vibriosis that infected mud crab farming from three sampling locations, namely **Rembang, Demak, and Kendal Districts**. Their clinical signs were red-brown spots on carapace and wounds. Twenty five bacterial isolates were gained from hepatopancreas, gills and carapace of nine infected mud crabs that cultured in TCBS and TSA medium. Molecular characterization was carried out through modified rep-PCR then followed by the 16S-rRNA gene amplification. The results indicated that Seven out of 25 isolates, namely CJR14, CJR15, CJD23, CJK10, CJK11, CJD22 and CJR05 were 92-98% firmly related to that *Vibrio harveyi* NCIMB-1280, *Catenococcus thiocicly* TG5-3, *Photobacterium ganghwense* FR311, *Vibrio parahaemolyticus* ATCC-1780, *Shewanella loihica* PV4, *Shewanella algae* ATCC5, and *Vibrio alginolyticus* NBRC-15630.

This study revealed that 25 isolates collected from infected mud crabs were grouped into seven groups of bacteria. These seven groups were well known to be pathogens for aquatic organisms. Moreover, according to molecular characterization, the highest diversity of Vibrionaceae was obtained from Demak, and the highest bacterium found in all three sampling sites was *C. thiocicly*.

Keywords: mud crab, 16S rRNA, rep PCR, vibriosis

1. Introduction

Mud crab, *Scylla serrata* (Forskål, 1775), is traditionally caught in and around brackish water ponds and mangrove forests along the North Coast of Central Java, Indonesia. The mangrove forests and its soils are this crab's origin habitat. Since around twenty years, mud crabs are traditionally farmed in North coast of Central Java to ensure production and size. But, the seeds are still collected from the wild. Najiah, Nadirah, Sakri and Harrison (2010) reported that wild crabs in Setiu Wetland, Malaysia were infected by 12 species of bacteria that resistance to antibiotics linomycine, ampicillin, amocillin and oleandromycin, 94.5%, 90.1%, 86.8% and 78.0% respectively. These indicated the safety of wild mud crabs for human consumption and antibiotic resistance. Sarjito et al (2016) reported that bacterial disease has caused mud crabs fattening obstacle in Pemalang District. Moreover Jithendran, Poornima, Balasubramanian, and Kulasekarapandian (2010) stated that bacterial disease in mud crabs caused mortality over 90% in all life cycle stages with clinical signs such as wounds on the body, decreased feed response and weakening. Sarjito, Hastuti, Samidjan, and Prayitno (2014); Sarjito et al. (2016) added blackening and red spots of carapace.

Several species of *Vibrio* spp. were reported associated with bacterial disease in mud crabs, such as : *V. fischeri* (Sarjito, Hastuti, Samidjan, & Prayitno, 2014; Sarjito *et al.*, 2016; Wang, 2011), *V. nereis* (Wang, 2011); *V. alginolyticus* and *V. cholerae* (Najiah, Nadirah, Sakri & Harrison, 2010; Sarjito *et al.*, 2016; Wang, 2011); *V. vulnificus* (Lavilla-Pitogo, Celia & Leobert, 2004; Shanmuga, 2008; Wang, 2011), *V. splendidus*, and *V. orientalis* (Jithendran, Poornima, Balasubramanian, & Kulasekarapandian, 2010); *V. ordalii*, and *V. harveyi* (Sarjito, Hastuti, Samidjan, & Prayitno, 2014; Jithendran, Poornima, Balasubramanian, & Kulasekarapandian, 2010), *V. campbelli* (Shanmuga, 2008), and *V. parahaemolyticus* (Lavilla-Pitogo, Celia, & Leobert, 2004; Shanmuga, 2008, Najiah, Nadirah, Sakri & Harrison, 2010; Wang, 2011, Sarjito *et al.*, 2016), *V. harveyi*, *V. cholerae*, *V. parahaemolyticus*, *V. alginolyticus* and *V. fischeri* (Sarjito *et al.*, 2016), *V. alginolyticus* and *V. harveyi* (Sarjito, Desrina, Haditomo, & Prayitno, 2018). The limited knowledge is available on the diversity of vibrios related to the disease outbreaks in mud crab in coastal of the North Central Java. However, local government tends to stimulate the culture of mud crabs, although the information on disease identification and prevention is lacking. Wild mud crabs actually a threat for the traditional polyculture shrimp and milkfish, and semi intensive shrimp culture system. It is because wild mud crabs intrude earthen brackish water ponds, causes fish and shrimp escape and disease spread. Disease caused by infiltration of infected wild crabs into brackish water ponds reduces fish and shrimp production.

Based on some background above, this study was conducted to assessed the molecular characterization of bacteria associated with clinical symptoms and the diversity of the bacterial vibriosis infected mud crabs farming in brackish water traditional ponds along the North Coast of Central Java, Indonesia.

2. Materials and Methods

Sampling

Nine mud crabs, *S. serrata*, were collected from nine brackish water ponds of Kendal, Demak, and Rembang Regency, Central Java (each three ponds). These three locations are the largest producers of cultivated crabs in Central Java, Indonesia (Figure 1). Based on the clinical signs for vibriosis described by Jithendran, Poornima, Balasubramanian, and Kulasekarapandian (2010), those infected mud crabs were stored in a sterile container and carried to the Integrated Laboratory of Diponegoro University for further analysis. The average size of the caught crab, measured from 13 to 15 cm, was 14.4 ± 0.7 cm.

Figure 1. Research location

Bacterial Isolation

Twenty-five bacteria were isolated from gills, haemolymph, hepatopancreas, and wounds on the shell of the infected mud crabs. Isolates bacteria were cultured on Thiosulfate Citrate Bile Salt Sucrose (TCBS) and Tryptic Soy Agar (TSA) (Thompson, Iida, & Swings, 2004; Sarjito *et al.*, 2016). The colonies were purified by the streak method based on morphological appearance (Sarjito *et al.*, 2016).

Repetitive Sequences-based PCR (Rep-PCR)

The bacterial DNA was extracted from a 24 h-broth culture of isolate strains by using the chelex method with slight modification according to the procedure described

by (de Lamballerie, Zandotti, Vignoli, Bollet, & de Micco, 1992). Then, the isolates DNA extract were analyzed using the rep-PCR of Radjasa, Nasima, Sabdono, Kita-Tsukamoto, & Ohwada (2007) was modified by Sarjito, Haditomo, Desrina, Djunaedi, & Prayitno (2018) in the rep-PCR, BOX A1R (5'-CTACggCAAaggCgACgCTgACg-3'). The REP 1R-I and REP 2-I primers contained nucleotide inosine (I) at an ambiguous position in the REP consensus. The mix PCR reagent contains one μL DNA template (diluted 100X), one μL primer, 7.5 μL Megamix Royal and 5.5 μL ddH₂O. Amplification was performed in a thermal cycler model Gene Amp PCR system 9700 with the following protocol: initial denaturation at 95°C for 5 minutes. Then 30 cycles of denaturation 92°C for 1 minute, annealing 50°C for 1,5 minutes, extension 68°C for 8 minutes, and a final extension at 68°C for 10 minutes. The band DNA was visualized with injected 5 μL PCR products into 1% agarose gel and run to electrophoresis machine using 1X TBE buffer and observed in UV-transilluminator, as described by Radjasa, Nasima, Sabdono, Kita-Tsukamoto, and Ohwada (2007). Grouping of isolates was conducted based on the method of Sarjito, Radjasa, Sabdono, Prayitno, and Hutabarat (2009). The method is a useful tool to distinguish the bacterial strain from the three-sampling site into group based on the fingerprinting of interspersed repetitive DN A sequences of BOX element using BOX A1R primer, therefore each group could be then identified using 16S-rRNA gene. Bacterial diversity is the percentage of the number of each species in a Dendogram group based on Rep-PCR compared to the total number of isolates. Then, the diversity of vibrios in mud crab with vibriosis clinical signs were compared from the three-sampling site to perform the percentage of species in each location.

Matrices were made from the band's position on the gel, which was analysed by the Free Tree program using the UPGMA method for constructing the phylogenetic tree

(Sarjito, Haditomo, Desrina, Djunaedi, & Prayitno, 2018). Resampling was done by bootstrapping with 1000 replications (Prayitno, Sarwan, & Sarjito, 2015).

PCR Amplification of 16S-rRNA Gene Fragments

The PCR amplification of the 16S-rRNA gene was performed, according to Radjasa, Nasima, Sabdono, Kita-Tsukamoto, & Ohwada. (2007) and Sarjito, Haditomo, Desrina, Djunaedi, & Prayitno (2018). To find out a 16S-rRNA gene, the amplification was presented with two primers, GM3F as a forward primer (5'AGAGTTTGATCMTGGC-3') and GM4R as a reverse primer (5'-TACCTTGTTACGACTT-3') (Sarjito, Radjasa, Sabdono, Prayitno, & Hutabarat, 2009). Genomic DNA of bacteria for PCR analysis was obtained from the destruction cell materials that were taken from fresh culture bacteria, suspended in sterile water (Sigma, Germany), and subjected to five cycles of freeze (-80°C) and thaw (95°C). The PCR product was then processed to find the band DNA in the right size around 1.500 bp. The sequencing step was facilitated by PT. Genetika Science. The determined DNA sequences of samples were then compared for homology using BLAST. The phylogenetic tree was established using MEGA X to find the closely related species (Sabdaningsih *et al.*, 2020).

3. Results and Discussion

Clinical Signs and Bacterial Isolation

The clinical signs of the infected mud crab (*S. serrata*) collected from three studied locations were wounds on the surface of the claws, the ventral, the carapace, and abdomen. Red-brown and dark spots in the carapace were found in Figure 2. Isolation of bacteria on TCBS medium in tri replicate obtained 25 pure isolates.

Figure 2. The clinical signs of mud crab

Red-brown or dark melanin spots on body surface, patches of light red spots as well as dark spots on the carapace, also wounds on the body (claws, shell, and the ventral) were known as vibriosis in mud crabs. Similar clinical signs have been described by Muyzer, Teske, Wirsén, and Jannasch (1995), Wang (2011), Lavilla-Pitogo et al. (2004) and Prayitno, Sarjito & Putri (2017). Those clinical signs also reported on the mud crabs infected with *V. harveyi*, *V. fischeri* and *V. ordalii* from the gulf of Semarang (Sarjito, Hastuti, Samidjan, and Prayitno, 2014) and *V. harveyi*, *V. cholerae*, *V. parahaemolyticus*, *V. alginolyticus* and *V. fischeri* from Pemalang (Sarjito et al., 2016). ‘Brown spot’ may occur due to the infection of chitinolytic bacteria that catabolize the chitin of the carapace and cause erosion and melanisation (dark brown to black pigmentation) at the site of infection (Jithendran, Poornima, Balasubramanian, & Kulasekarapandian, 2010).

Repetitive Sequences-based PCR (Rep-PCR)

The Repetitive PCR analysis and the dendrogram related to the 25 pure isolates bacteria originating from mud crabs revealed to seven groups (Figure 3). Furthermore, for molecular identification was represented by one isolate each group namely CJR14, CJR15, CJD23, CJK10, CJK11, CJD22, and CJR05 (Table 1). The remaining isolates were assumed as the same species within the group according to the similar pattern of DNA size from rep-PCR result.

Figure 3. A dendrogram

Figure 3 shows similarity of 25 isolates and divided into seven groups according to the differences between repetitive bacterial sequences. Then they were examined using 16S-rRNA gene for identification. A total of seven isolates were identified and presented

in Table 1, the range of homology percentage between 92-98%. The highest similarity was showed by isolate CJR15 from group II with 98% homology to *C.thiocicly* strain TG5-3. The homology 97% was appointed to isolate CJK10 and CJK11 from group IV and V that similar to *V. parahaemolyticus* ATCC-17802 and *S. loihica* strain PV4. Followed by CJR05 from group VII with homology 96% and similar to *V. alginolyticus* strain NBRC-15630. Moreover, isolate CJR14 and CJD23 in group I and III have 95% homology to *V. harveyi* strain NCIMB-1280 and *P. ganghwense* strain FR311, while isolate CJD22 was 92% closely related to *S. algae* strain ATCC-5192 in group VI.

Table 1. Molecular characterization

The vibrio's in mud crabs from traditional brackish water pond of the North Coast of Central Java (Figure 1 were closely related to *V.harveyi* strain NCIMB-1280 (CJR14), *C.thiocicly* strain TG 5-3 (CJR15), *P. ganghwense* strain FR311 (CJD23); *V.parahaemolyticus* ATCC 17802 (CJK10); *S. loihica* strain PV4 (CJK11); *S. algae* strain ATCC5 (CJD22) and *V. alginolyticus* strain NBRC-15630 (CJR05). The result revealed the diversity of vibrios associated with mud crab from traditional mud crabs farming that distrust production and result in significant losses. These bacterial diversity results were lower than the diversities found in the cultured and wild crab in India (Jithendran, Poornima, Balasubramanian, & Kulasekarapandian, 2010) as well as extensive brackish water ponds surrounding Semarang Gulf (Sarjito, Hastuti, Samidjan, & Prayitno, 2014).

Four genus Vibrionaceae (*Vibrio*, *Shewanella*, *Photobacterium*, and *Catenococcus*) were mostly found in mud crab (Li *et al.*, 2012; Wei *et al.*, 2019). Three species of vibrio,

namely *V. parahaemolyticus* (CJK10), *V. harveyi* (CJR14), and *V. alginolyticus* (CJR05) also have been detected in this study. The vibrios were frequently found as a causative agent of shell disease in mud crab (*S. serrata*), and shrimp (*L. vannamei*) (Estave, & Herrere, 2000; Wang, 2011; Soto-Rodriguez et al., 2012). *V. parahaemolyticus* has been found as a main pathogen in mud crab in China (Xia *et al.*, 2010) and Chakoria Coast, Bangladesh (Aftabuddin et al., 2013). Additionally, *V. harveyi* has been reported as a causative agent of vibriosis in fattening mud crabs from brackish water pond in Pemalang coast (Sarjito *et al.*, 2016,) and mud crab from extensive brackish water pond of surrounding the Gulf of Semarang (Sarjito, Hastuti, Samidjan, & Prayitno, 2014) and Malaysia (Najiah, Nadirah, Sakri, & Harrison., 2010). These bacteria were also reported as a causative agent of bacterial diseases in zoea stage of mud crab (Jithendran, Poornima, Balasubramanian, & Kulasekarapandian, 2010); adult mud crabs (Poornima et al., 2012 and Lavilla-Pitogo et al., 2004). Moreover, Sarjito, Hastuti, Samidjan and Prayitno (2014), found that this bacterium was a potential pathogen to mud crabs. *V. alginolyticus* was observed as an important pathogenic bacterium associated with infectious diseases in mud crab (Najiah, Nadirah, Sakri, & Harrison, 2010) and shell disease of mud crab *S. serrata* grow out pond located at Tamil Nadu, India (Gunasekaran, Gopalakrishnan, Deivasigamani, Muhilvannan, & Kathirkaman, 2019).

Genus *Photobacterium* was found in the coastal, open-ocean and deep-sea water (Moi *et al.*, 2017). Wei *et al.*, (2019) found that *Photobacterium* was dominant as gut microbiota in mud crab *S. paramamosain* were collected from nine coastal areas of southern China. Surprisingly, *P. ganghwense* was detected in this study. This bacterium, was firstly reported from a seawater in Ganghwa Island, South Korea (Park *et al.*, 2006).

Shewanella algae and *Shewanella loihica* were found in this study. They both have an essential role as a turnover of organic material, and capable of dissimilatory reduction of various metals and other substances, such as Nitrate, Nitrite, Thiosulphate, and Trimethylamine-N-oxide (Holt, Gahrn-Hansen, & Bruun, 2005). *S. algae* has been reported as bacterial pathogen associated with Black Spot Disease in Freshwater-Cultured White leg Shrimp, *L. vannamei*, (Cao, Chen, Lu, & An, 2018), causative agent of *Babylonia* (Shufang, Zhang, Dequan, Shiping, & Huang, 2015); *Cynoglossus semilaevis* (Han *et al.*, 2018), and *Sciameops ocellatus* (Zhang, Zhu, & Wang, 2013). According to Prayitno *et al.* (2015) *S. algae* was also presented in gut of milkfish from the Northern Coast of Central Java. In Demak, *S. algae* was found only when the water temperature is more than 23°C. Additionally, the infection of genus *Shewanella* mostly occurs in countries with a warm climate (Holt, Gahrn-Hansen, & Bruun, 2005). Although, it is rarely found in human pathogen and exhibited symptoms of infection since this bacterium was already detected in mud crab farming at the North Coast of Central Java, the present of this bacterium in the mud crabs products might be considered.

Shewanella loihica was isolated and identified from iron-rich microbial mats in the Pacific Ocean (Gao *et al.*, 2006). *S. loihica* plays a role as an agent of metal reduction and iron bio-mineralization. Based on that, the existence of this bacterium can be associated with metal and nitrogen content in the water. Some reported study of *S. loihica* was also focusing on metal reduction and iron bio-mineralization capabilities (Newton, Mori, Nakamura, Hashimoto, & Watanabe, 2009; Philips *et al.*, 2018), denitrification and a respiratory ammonification pathway (Yoon, Sanford, & Löffler, 2015). Therefore, this bacterium has important role on the nutrient cycle in soils and sediments where mud crab lives.

Catenococcus thiocyli was identified by Yarza *et al.* (2013), the study found that *C. thiocyli* is part of genus Vibrionaceae. Only few studies reported this bacterium (Zheng *et al.*, 2016; Li *et al.*, 2019; Achmad *et al.*, 2019; Guibert, Lecellier, Torda, Pochon, & Berteaux-Lecellier, 2020). This member of Vibrionaceae was found as a causative agent of Ice-ice diseases of the seaweed, *Kappaphycus alvarezii*, (Achmad *et al.*, 2019) and moribund of the giant clam, *Tridacna maxima*, (Guibert, Lecellier, Torda, Pochon, & Berteaux-Lecellier, 2020).

Diversity of Vibriosis Clinical Signs Associated Bacteria

The diversity of vibrios in mud crab with vibriosis clinical signs were compared from the three-sampling site to perform the percentage of species in each location (Table 2). Table 2 accommodates all of the isolates that identified based on the 16S-rRNA gene. The diversity of vibriosis clinical signs associated with bacteria was explored using molecular characterization by rep-PCR. This method is a useful tool to distinguish the diversity of bacteria according to the number and size of repetitive bacterial sequences. This technique could prevent to analyse the similar isolate of bacteria based on its DNA fingerprinting. Therefore, rep-PCR is helpful in grouping *Vibrio* species. The diversity of vibrios found in three sample locations (Table 2). We found more bacteria species in **Rembang (13 isolates) rather than Kendal (7 isolates) and Demak (5 isolates)**. The highest percentage of vibrio diversity compared to the total isolate in each location was in Demak (80%).

Table 2. The diversity of Vibrios

Figure 4. The bacterial diversity in Mud crab

Figure 4 represented the bacterial diversity in Mud crab with Vibriosis's clinical signs from the North Coast of Central Java. The highest number species was *C. thiocicly* with the percentage of 32%, while 24% was *V. parahaemolyticus*, *S. loihica* and *S. algae* in the equal percentage which is in 12%, as well as *P. ganghwense* and *V. alginolyticus* in 8%, and the lowest rate was *V. harveyi* in 4%.

Figure 5. Phylogenetic tree

In order to represent the relationship among isolates, the phylogenetic tree was constructed. Figure 5 shows that all of the strains have assembled with the closely related species. The relationship between the genus performs that the bacteria genus *Vibrio* has the same clade with *Catenococcus thiocycli*. Those groups are more closely related compared to the group of *Shewanella* and *Photobacterium*.

The isolates were continuing to be grouped based on the sampling site (Table 2). Rembang has the highest number of strains. However, from seven groups of *Vibrio*, only six groups were detected in this site. Demak has four groups of *Vibrio* from five isolates found. Furthermore, Kendal only has two groups of *Vibrio* from seven strains. The results indicated that the highest diversity of *Vibrio* was found in Rembang, rather than Demak and Kendal. It might happen since those locations have a higher abrasion (Directorate of Marine and Fisheries, 2019). Miller (1989) revealed that the abrasion could decline the microbial abundances.

Moreover, Figure 4 was designed to know the highest species of *Vibrionaceae* found in this study. Based on the dendrogram of rep-PCR, the predominant species was

from *C. thiocicly* in group II with a percentage number 32%, and the lowest was *V. harveyi* with the percentage of 4%. Based on the phylogenetic tree (Figure 5), *Vibrio* and *Catenococcus* are more closely related each other than those of *Shewanella* and *Photobacterium*.

According to the results above, future research should be conducted to have a deep understanding and correlation between the biotic and abiotic factors that affect the health status of Mud-crab in the traditional mud crab farming. Therefore, the metagenomic approach would be helpful to describe the bacterial structure community in the healthy and infected mud crab. Thus, the design of prevention methods to reduce bacterial disease outbreak of Mud crab farming could be expertly constructed.

4. Conclusions

This study revealed that 25 isolates collected from infected mud crabs were grouped into seven groups of bacteria are well known to be pathogens for aquatic organisms. Moreover, according to molecular characterization, the highest diversity of Vibrionaceae was obtained from Demak, and the highest bacterium found in all three sampling sites was *C. thiocicly*.

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Figure 1. Research Location represented in a small red box in the left of map.



Figure 2. The clinical signs of mud crab (*S. serrata*) infected by bacterial diseases: (a.) Red/brown spots in the carapace; (b.) Red-brown spots in the carapace wound on the abdomen.

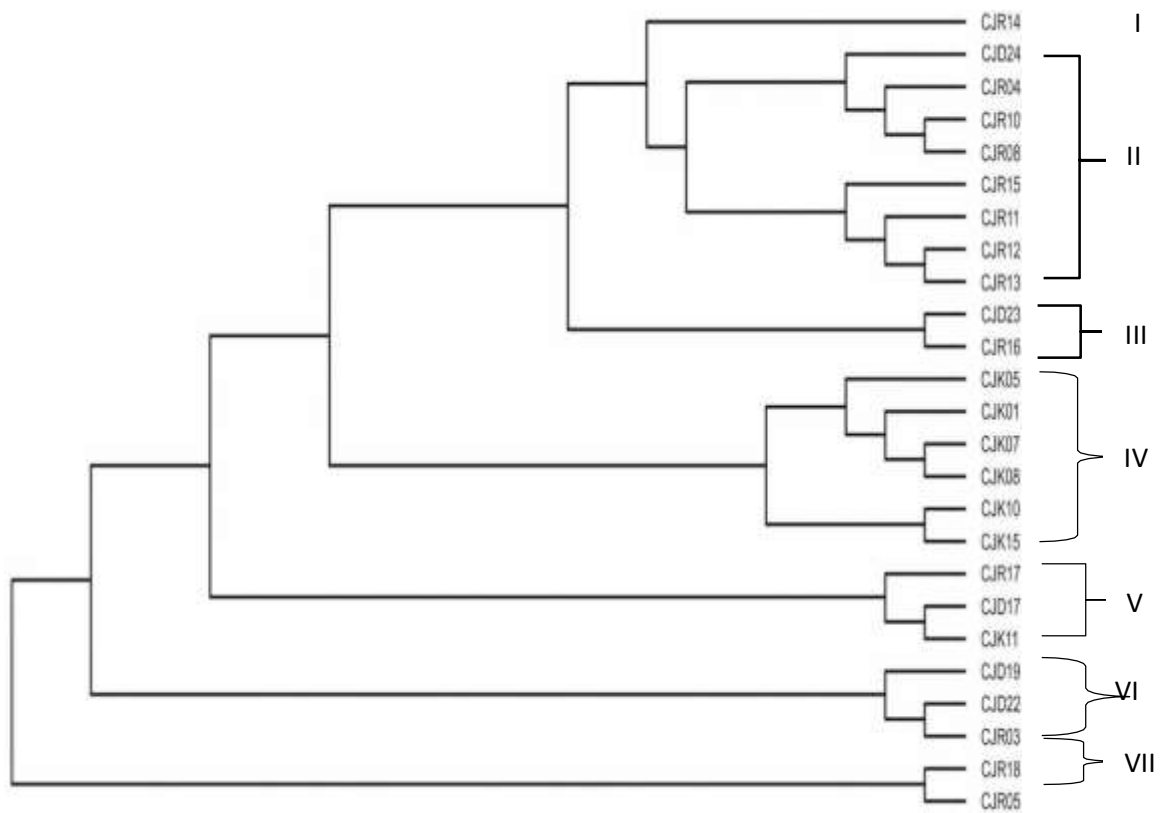


Figure 3. A dendrogram based on Rep-PCR of 25 Bacteria Isolates Associated with vibriosis clinical signs from traditional mud crab farming in the North Coastal of Central Java, Central Java, Indonesia.

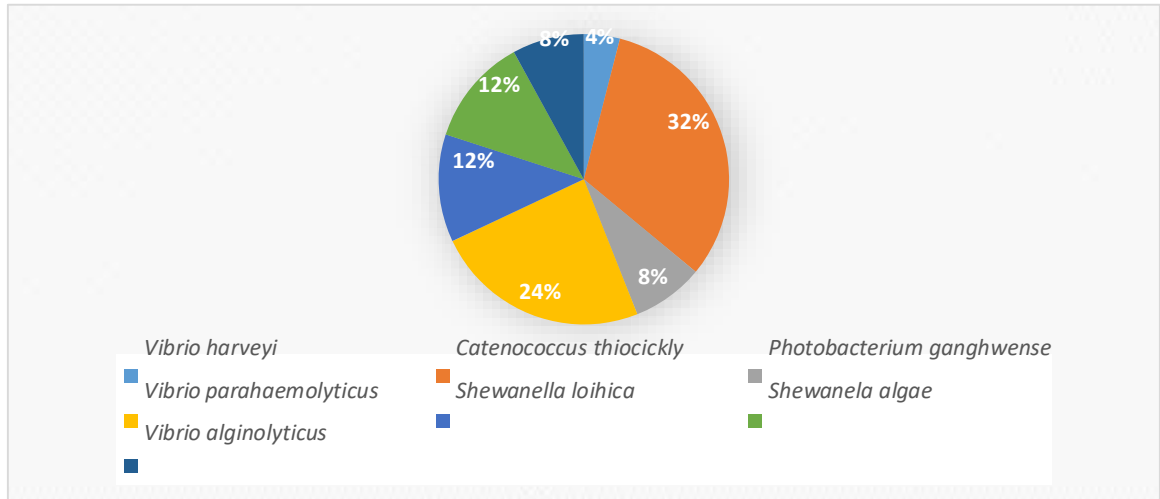


Figure 4. The bacterial diversity in Mud crab with clinical signs of Vibriosis from North Coast of Central Java.

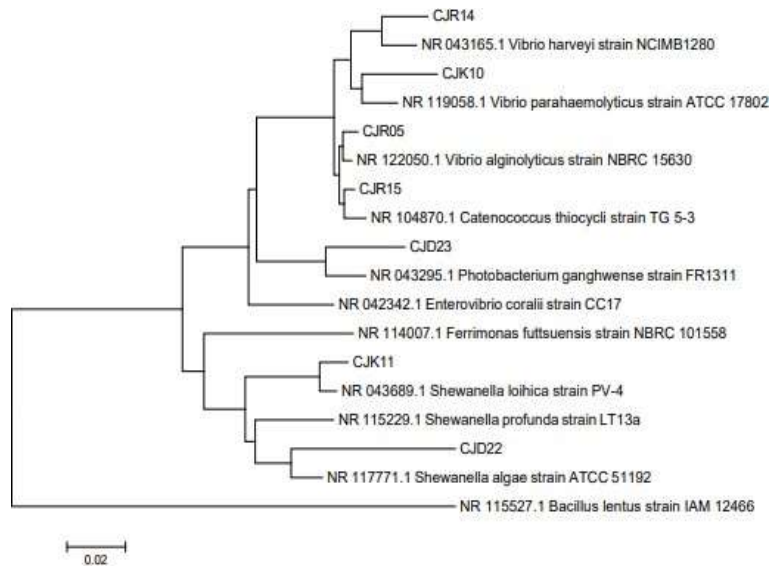


Figure 5. Phylogenetic tree of the bacteria associated with vibriosis clinical signs from traditional mud crab farming in the North Coast of Central Java, Indonesia constructed using Neighbour-Joining analysis with 1000 replicates.

Table 1. Molecular characterization of seven bacterial species associated with clinical signs of Vibriosis on mud crabs from North Coast of Central Java.

Isolates Code	Query Length	Close relative	Query Cover (%)	Homology (%)	Acc. Number
CJR14	1548	<i>Vibrio harveyi</i> strain NCIMB1280	98	95	NR 043165.1
CJR15	1319	<i>Catenococcus thiocicly</i> strain TG 5-3	98	98	NR 118258.1
CJD23	1387	<i>Photobacterium ganghwense</i> strain FR311	98	95	NR 043295.1
CJK10	1340	<i>Vibrio parahaemolyticus</i> ATCC 17802	99	97	NR 043689.1
CJK11	1335	<i>Shewanella loihica</i> strain PV4	97	97	NR 025794.1
CJD22	1488	<i>Shewanella algae</i> strain ATCC5192	95	92	NR 117771.1
CJR05	1362	<i>Vibrio alginolyticus</i> strain NBRC 15630	96	96	NR 122050.1

Table 2. The diversity of Vibrios in Mud Crab from North Coast of Central Java per location.

Location	Isolates Code	Bacteria Species	Vibrios diversity	Isolate Number	Percentage of species compare to total isolate (%)
Rembang	CJR14;				
	CJR04;				
	CJR10;				
	CJR08;				
	CJR15;	<i>Vibrio harveyi</i> strain NCIMB1280			
	CJR11;	<i>Catenococcus thiocicly</i> strain TG 5-3,			
	CJR12;	<i>Photobacterium ganghwense</i> strain FR311,			
	CJR13;	<i>Shewanella loihica</i> strain PV4,	6	13	52%
	CJR16;	<i>Shewanella algae</i> strain ATCC5192,			
	CJR17;	<i>Vibrio alginolyticus</i> strain NBRC 15630,			
	CJR03;				
	CJR18;				
	CJR05				
Demak	CJD24;	<i>Catenococcus thiocicly</i> strain TG 5-3			
	CJD23;	<i>Photobacterium ganghwense</i> strain FR311			
	CJD17;	<i>Shewanella loihica</i> strain PV4	4	5	80%
	CJD19;	<i>Shewanella algae</i> strain ATCC5192			
	CJD22				

	CJK05;			
	CJK01;			
	CJK07; <i>Vibrio prahaemolyticus</i> ATCC 17802			
Kendal	CJK08; <i>Shewanella loihica</i> strain PV4	2	7	28%
	CJK10;			
	CJK15;			
	CJK11			



Sarjito Sarjito <sarjito@live.undip.ac.id>

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Original Article

Molecular characterization of vibriosis associated bacteria
from traditional mud-crab farmed
in the North Coast of Central Java, IndonesiaSarjito^{1*}, Alfabetian Harjuno Condro Haditomo¹, Slamet Budi Prayitno¹,
Aninditia Sabdaningsih², Desrina¹, and Restiana Wisnu Ariyati¹¹ Aquaculture Study Program, Department of Aquaculture, Faculty of Fisheries and Marine Science,
Diponegoro University, Semarang, 50275 Indonesia² Department of Aquatic Resources, Faculty of Fisheries and Marine Science,
Diponegoro University, Semarang, 50275 Indonesia

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Abstract

This study examines the molecular characterization of bacteria associated with clinical symptoms and the diversity of vibriosis infecting farmed mud crab from three sampling locations, namely Rembang, Demak, and Kendal Districts. The clinical symptom was red-brown spots on carapace and wounds. Twenty-five bacterial isolates were gained from hepatopancreas, gills and carapace of nine infected mud crabs, by culturing in TCBS and TSA medium. Molecular characterization was carried out through modified rep-PCR followed by 16S-rRNA gene amplification. The results indicate that seven out of the 25 isolates, namely CJR14, CJR15, CJD23, CJK10, CJK11, CJD22 and CJR05 were 92-98% firmly related to *Vibrio harveyi* NCIMB-1280, *Catenococcus thiocicly* TG5-3, *Photobacterium ganghwense* FR311, *Vibrio parahaemolyticus* ATCC-1780, *Shewanella loihica* PV4, *Shewanella algae* ATCC5, and *Vibrio alginolyticus* NBRC-15630. This study revealed that the 25 isolates found from infected mud crabs fell into seven groups of bacteria. These seven groups were well-known as pathogens for aquatic organisms. Moreover, according to molecular characterization, the highest diversity of Vibrionaceae was obtained from Rembang, and the bacterium most found in all three sampling sites was *C. thiocicly*.

Keywords: mud crab, 16S rRNA, rep PCR, vibriosis

1. Introduction

Mud crab, *Scylla serrata* (Forskål, 1775), is traditionally caught in and around brackish water ponds and mangrove forests along the North coast of Central Java, Indonesia. Since around twenty years, mud crabs have been farmed in the North coast of Central Java to ensure production quantity and crab size. However, the seeds are still collected from the wild. Najiah, Nadirah, Sakri and Harrison (2010) reported that wild crabs in Setiu Wetland, Malaysia, were

infected by 12 species of bacteria resistant to the antibiotics linomycine, ampicillin, amocillin and oleandomycin at 94.5%, 90.1%, 86.8% and 78.0% respectively. These indicated safety issues in human consumption of wild mud crabs due to the antibiotic resistance. Sarjito *et al.* (2016) reported that bacterial disease has become an obstacle for fattening mud crabs in Pemalang District. Moreover, Jithendran, Poornima, Balasubramanian, and Kulasekara pandian (2010) stated that bacterial disease in mud crabs caused mortality of over 90% in all life cycle stages, with clinical signs including wounds on the body, decreased feed response and weakening. Sarjito, Hastuti, Samidjan, and Prayitno (2014); and Sarjito *et al.* (2016) added blackening and red spots of carapace to the symptoms.

*Corresponding author

Email address: sarjito@live.undip.ac.id

Several species of *Vibrio* spp. have been reported as associated with bacterial diseases in mud crabs, such as *V. fischeri* (Sarjito, Hastuti, Samidjan, & Prayitno, 2014; Sarjito *et al.*, 2016; Wang, 2011), *V. nereis* (Wang, 2011); *V. alginolyticus* and *V. cholerae* (Najiah, Nadirah, Sakri & Harrison, 2010; Sarjito *et al.*, 2016; Wang, 2011); *V. vulnificus* (Lavilla-Pitogo, Celia & Leobert, 2004; Shanmuga, 2008; Wang, 2011), *V. splendidus* and *V. orientalis* (Jithendran, Poornima, Balasubramanian, & Kulasekara pandian, 2010); *V. ordalii* and *V. harveyi* (Sarjito, Hastuti, Samidjan, & Prayitno, 2014; Jithendran, Poornima, Bala subramanian, & Kulasekarapandian, 2010), *V. campbelli* (Shanmuga, 2008) and *V. parahaemolyticus* (Lavilla-Pitogo, Celia, & Leobert, 2004; Shanmuga, 2008, Najiah, Nadirah, Sakri & Harrison, 2010; Wang, 2011, Sarjito *et al.*, 2016), *V. harveyi*, *V. cholerae*, *V. parahaemolyticus*, *V. alginolyticus* and *V. fischeri* (Sarjito *et al.*, 2016), *V. alginolyticus* and *V. harveyi* (Sarjito, Desrina, Haditomo, & Prayitno, 2018). Only limited knowledge is available on the diversity of vibrios related to the disease outbreaks in mud crab in coastal North Central Java. However, local government tends to stimulate the culturing of mud crabs, although information on disease identification and prevention is lacking. Wild mud crabs are actually a threat to the traditional polyculture shrimp and milkfish, and semi-intensive shrimp culture system. This is because wild mud crabs intrude earthen brackish water ponds, causing fish and shrimp to escape and diseases to spread. Diseases caused by the infiltration of infected wild crabs into brackish water ponds reduces fish and shrimp production. Based on the background above, this study was conducted to assess the molecular characterization of bacteria associated with clinical symptoms, and the diversity of the bacterial vibriosis infecting mud crabs farmed in brackish water traditional ponds along the North coast of Central Java, Indonesia.

2. Materials and Methods

2.1 Sampling

Nine mud crabs, *S. serrata*, were sampled from nine brackish water ponds of Kendal, Demak, and Rembang Regency, Central Java (each for three ponds). These three locations are the largest producers of cultivated crabs in Central Java, Indonesia (Figure 1). Based on the clinical signs for vibriosis described by Jithendran, Poornima, Balasubramanian, and Kulasekarapandian (2010), these infected mud crabs were stored in a sterile container and carried to the Integrated Laboratory of Diponegoro University for further analysis. The average size of the caught crab, in the range from 13 to 15 cm, was 14.4 ± 0.7 cm.

2.2 Bacterial isolation

Twenty-five bacteria were isolated from gills, haemolymph, hepatopancreas, and wounds on the shell of the infected mud crabs. Isolates of the bacteria were cultured on Thiosulfate Citrate Bile Salt Sucrose (TCBS) and on Tryptic Soy Agar (TSA) (Thompson, Iida, & Swings, 2004; Sarjito *et al.*, 2016). The colonies were purified by the streak method, based on morphological appearance (Sarjito *et al.*, 2016).



Figure 1. Research locations are represented by the small red boxes in the left-hand-side map.

2.3 Repetitive sequences-based PCR (Rep-PCR)

The bacterial DNA was extracted from a 24 h broth culture of isolate strains by using the chelex method with slight modification to the procedure described by (de Lamballerie, Zandotti, Vignoli, Bollet, & de Micco, 1992). Then, the isolated DNA extracts were analyzed using the rep-PCR of Radjasa, Nasima, Sabdono, Kita-Tsukamoto, & Ohwada (2007) as modified by Sarjito, Haditomo, Desrina, Djunaedi, & Prayitno (2018) in the rep-PCR, BOX A1R (5'-CTACggCAAggCgACgCTgACg-3'). The REP 1R-I and REP 2-I primers contained nucleotide inosine (I) at an ambiguous position in the REP consensus. The mix PCR reagent contains one μ L DNA template (diluted 100X), one μ L primer, 7.5 μ L Megamix Royal and 5.5 μ L ddH₂O. Amplification was performed in a thermal cycler model Gene Amp PCR system 9700 with the following protocol: initial denaturation at 95°C for 5 minutes; then 30 cycles of denaturation at 92°C for 1 minute, annealing at 50°C for 1.5 minutes, and extension at 68°C for 8 minutes; and a final extension at 68°C for 10 minutes. The bands of DNA were visualized from injected 5 μ L of PCR products into 1% agarose gel that was run in an electrophoresis machine using 1X TBE buffer and observed under UV-transilluminator, as described by Radjasa, Nasima, Sabdono, Kita-Tsukamoto, and Ohwada (2007). Grouping of the isolates was conducted based on the method of Sarjito, Radjasa, Sabdono, Prayitno, and Hutabarat (2009). The method is a useful tool for placing the bacterial strains from the three sampling sites into groups, based on the fingerprinting of interspersed repetitive DNA sequences of BOX element using BOX A1R primer. Each group could be then identified using 16S-rRNA gene. Bacterial diversity is the percentage of the number of each species in a Dendrogram group based on Rep-PCR compared to the total number of isolates. Then, the diversity of vibrios in mud crab with clinical signs of vibriosis were compared from the three-sampling sites, to assess the percentages of species by location.

Matrices were made from the band positions on the gel, which were analysed by the Free Tree program using the UPGMA method for constructing the phylogenetic tree (Sarjito, Haditomo, Desrina, Djunaedi, & Prayitno, 2018).

Resampling was done by bootstrapping with 1000 replications (Prayitno, Sarwan, & Sarjito, 2015).

2.4 PCR amplification of 16S-rRNA gene fragments

The PCR amplification of the 16S-rRNA gene was performed, according to Radjasa, Nasima, Sabdono, Kita-Tsukamoto, & Ohwada (2007) and Sarjito, Haditomo, Desrina, Djunaedi, & Prayitno (2018). To target the 16S-rRNA gene the amplification used two primers, GM3F as a forward primer (5'-AGAGTTTGATCMTGGC-3') and GM4R as a reverse primer (5'-TACCTTGTACGACTT-3') (Sarjito, Radjasa, Sabdono, Prayitno, & Hutabarat, 2009). Genomic DNA of bacteria for PCR analysis was obtained from the lysing of cell materials that were taken from freshly cultured bacteria, suspended in sterile water (Sigma, Germany), by subjecting to five cycles of freeze (-80°C) and thaw (95°C). The PCR product was then processed to find the DNA band of the right size around 1.500 bp. The sequencing step was facilitated by PT. Genetika Science. The determined DNA sequences of samples were then compared for homology using BLAST. The phylogenetic tree was established using MEGA X to find closely related species (Sabdaningsih *et al.*, 2020).

3. Results and Discussion

3.1 Clinical Signs and Bacterial Isolation

The clinical signs of the infected mud crab (*S. serrata*) collected from the three studied locations were wounds on the surface of the claws, the ventral, the carapace, and abdomen. Red-brown and dark spots in the carapace were found, as seen in Figure 2. Isolation of bacteria on TCBS medium in three replicates obtained 25 pure isolates.



Figure 2. The clinical signs of mud crab (*S. serrata*) infected by bacterial diseases: (a) red/brown spots in the carapace; and (b) red-brown spots in the carapace wound on the abdomen.

Red-brown or dark melanin spots on body surface, patches of light red spots as well as dark spots on the carapace, and also wounds on the body (claws, shell, and the ventral) are known as indications of vibriosis in mud crabs. Similar clinical signs have been described by Muyzer, Teske, Wirsén, and Jannasch (1995), Wang (2011), Lavilla-Pitogo *et al.* (2004) and Prayitno, Sarjito & Putri (2017). Similar clinical signs are also reported in mud crabs infected with *V. harveyi*, *V. fischeri* and *V. ordalii* from the gulf of Semarang (Sarjito, Hastuti, Samidjan, and Prayitno, 2014) and *V. harveyi*, *V. cholerae*, *V. parahaemolyticus*, *V. alginolyticus* and *V. fischeri* from Pemalang (Sarjito *et al.*, 2016). 'Brown spot' may occur due to an infection by chitinolytic bacteria that catabolize the chitin of the carapace and cause erosion

and melanisation (dark brown to black pigmentation) at the site of infection (Jithendran, Poornima, Balasubramanian, & Kulasekarapandian, 2010).

3.2 Repetitive sequences-based PCR (Rep-PCR)

The Repetitive PCR analysis and the dendrogram related to the 25 pure isolates bacteria originating from mud crabs revealed seven groups (Figure 3). Furthermore, for molecular identification each group was represented by one isolate, namely CJR14, CJR15, CJD23, CJK10, CJK11, CJD22, and CJR05 (Table 1). The remaining isolates were assumed to be of the same species within a group, according to their similar patterns of DNA size from the rep-PCR results.

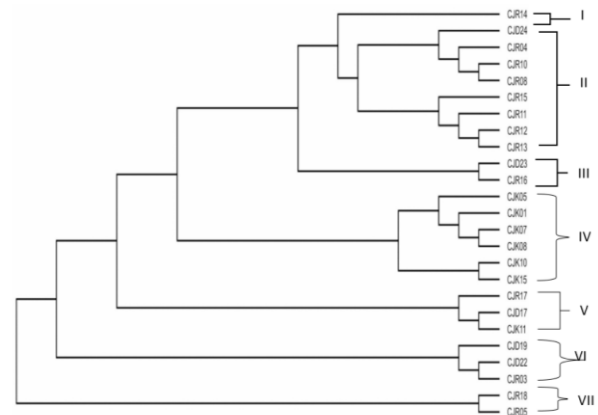


Figure 3. A dendrogram based on Rep-PCR of 25 bacteria associated with vibriosis clinical signs, isolates from mud crabs traditionally farmed in the North coast of Central Java, Indonesia.

Figure 3 shows similarity of the 25 isolates divided into seven groups according to the differences between repetitive bacterial sequences. Then these were examined using 16S-rRNA gene for identification from a total of seven isolates, identified as presented in Table 1, with the range of homology percentage being 92-98%. The highest similarity was by isolate CJR15 from group II having 98% homology to *C. thiocicly* strain TG5-3. The homology level 97% was appointed to isolates CJK10 and CJK11 from group IV and V that were similar to *V. parahaemolyticus* ATCC-17802 and *S. loihica* strain PV4. In rank order this was followed by CJR05 from group VII with 96% homology to *V. alginolyticus* strain NBRC-15630. Moreover, isolates CJR14 and CJD23 in groups I and III had 95% homology to *V. harveyi* strain NCIMB-1280 and *P. ganghwense* strain FR311, while isolate CJD22 was 92% closely related to *S. algae* strain ATCC-5192 in group VI.

The vibrios in mud crabs from traditional brackish water ponds of the North coast of Central Java (Figure 1) were closely related to *V. harveyi* strain NCIMB-1280 (CJR14), *C. thiocicly* strain TG 5-3 (CJR15), *P. ganghwense* strain FR311 (CJD23); *V. parahaemolyticus* ATCC 17802 (CJK10); *S. loihica* strain PV4 (CJK11); *S. algae* strain ATCC5 (CJD22) and *V. alginolyticus* strain NBRC-15630 (CJR05). The results revealed the diversity of vibrios associated with mud crabs

Table 1. Molecular characterization of seven bacterial species associated with clinical signs of vibriosis in mud crabs from North coast of Central Java

Isolate code	Query length	Close relative	Query cover (%)	Homology (%)	Acc. Number
CJR14	1548	<i>Vibrio harveyi</i> strain NCIMB1280	98	95	NR 043165.1
CJR15	1319	<i>Catenococcus thiocicly</i> strain TG 5-3	98	98	NR 118258.1
CJD23	1387	<i>Photobacterium ganghwense</i> strain FR311	98	95	NR 043295.1
CJK10	1340	<i>Vibrio parahaemolyticus</i> ATCC 17802	99	97	NR 043689.1
CJK11	1335	<i>Shewanella loihica</i> strain PV4	97	97	NR 025794.1
CJD22	1488	<i>Shewanella algae</i> strain ATCC5192	95	92	NR 117771.1
CJR05	1362	<i>Vibrio alginolyticus</i> strain NBRC 15630	96	96	NR 122050.1

from traditional farming that impact production negatively and result in significant losses. These bacterial diversity results were lower than the diversities found in the cultured and wild crab in India (Jithendran, Poornima, Bala subramanian, & Kulasekarapandian, 2010) as well as in extensive brackish water ponds surrounding Semarang Gulf (Sarjito, Hastuti, Samidjan, & Prayitno, 2014).

Four genera of Vibrionaceae (*Vibrio*, *Shewanella*, *Photobacterium*, and *Catenococcus*) were mostly found in mud crab (Li *et al.*, 2012; Wei *et al.*, 2019). Three species of vibrio, namely *V. parahaemolyticus* (CJK10), *V. harveyi* (CJR14), and *V. alginolyticus* (CJR05) also have been detected in this study. The vibrios have been frequently found as causative agents of shell disease in mud crab (*S. serrata*), and in shrimp (*L. vannamei*) (Estave, & Herrere, 2000; Wang, 2011; Soto-Rodriguez *et al.*, 2012). *V. parahaemolyticus* has been found as a main pathogen in mud crab in China (Xia *et al.*, 2010) and in Chakoria coast, Bangladesh (Aftabuddin *et al.*, 2013). Additionally, *V. harveyi* has been reported as a causative agent of vibriosis in fattening mud crabs from brackish water pond in Pemalang coast (Sarjito *et al.*, 2016,) and in mud crab from extensive brackish water ponds surrounding the Gulf of Semarang (Sarjito, Hastuti, Samidjan, & Prayitno, 2014) and in Malaysia (Najiah, Nadirah, Sakri, & Harrison, 2010). These bacteria were also reported as causative agents of bacterial diseases in zoea stage of mud crab (Jithendran, Poornima, Balasubramanian, & Kulasekarapandian, 2010); and in adult mud crabs (Poornima *et al.*, 2012 and Lavilla-Pitogo *et al.*, 2004). Moreover, Sarjito, Hastuti, Samidjan and Prayitno (2014), found that this bacterium was a potential pathogen to mud crabs. *V. alginolyticus* was observed as an important pathogenic bacterium associated with infectious diseases in mud crab (Najiah, Nadirah, Sakri, & Harrison, 2010) and in shell disease of mud crab *S. serrata* grown in pond located at Tamil Nadu, India (Gunasekaran, Gopalakrishnan,

Deivasigamani, Muhilvannan, & Kathirkaman, 2019). Genus *Photobacterium* was found in the coastal, open-ocean and deep-sea water (Moi *et al.*, 2017). Wei *et al.*, (2019) found that *Photobacterium* was dominant as gut microbiota in mud crab *S. paramamosain* collected from nine coastal areas of southern China. Surprisingly, *P. Ganghwense* was detected in this study. This bacterium, was firstly reported from seawater in Ganghwa Island, South Korea (Park *et al.*, 2006).

Shewanella algae and *Shewanella loihica* were found in this study. They both have an essential role in the turnover of organic material, and are capable of dissimilatory reduction of various metals and other substances, such as

Nitrate, Nitrite, Thiosulphate, and Trimethylamine-N-oxide (Holt, Gahrn-Hansen, & Bruun, 2005). *S. algae* has been reported as bacterial pathogen associated with Black Spot Disease in Freshwater-Cultured White leg Shrimp, *L. vannamei* (Cao, Chen, Lu, & An, 2018), as causative agent in *Babylonia* (Shufang, Zhang, Dequan, Shiping, & Huang, 2015); *Cynoglossus semilaevis* (Han *et al.*, 2018), and *Sciaenops ocellatus* (Zhang, Zhu, & Wang, 2013). According to Prayitno *et al.* (2015) *S. algae* was also present in gut of milkfish from the Northern coast of Central Java. In Demak, *S. algae* was found only when the water temperature exceeded 23°C. Additionally, the infection of genus *Shewanella* mostly occurs in countries with a warm climate (Holt, Gahrn-Hansen, & Bruun, 2005). Although it is rarely found as a human pathogen and exhibited symptoms of infection since this bacterium was already detected in mud crab farming at the North coast of Central Java, the presence of this bacterium in the mud crab products might be considered.

Shewanella loihica was isolated and identified from iron-rich microbial mats in the Pacific Ocean (Gao *et al.*, 2006). *S. loihica* plays a role as an agent of metal reduction and iron bio-mineralization. Based on that, the existence of this bacterium can be associated with metal and nitrogen content in the water. Some reported studies of *S. loihica* have also focused on metal reduction and iron bio-mineralization capabilities (Newton, Mori, Nakamura, Hashimoto, & Watanabe, 2009; Philips *et al.*, 2018), and on denitrification and a respiratory ammonification pathway (Yoon, Sanford, & Löffler, 2015). Therefore, this bacterium has an important role in the nutrient cycle in soils and sediments where the mud crab lives.

Catenococcus thiocycli was identified by Yarza *et al.*, (2013), and the study found that *C. thiocycli* is part of genus Vibrionaceae. Only few studies have reported this bacterium (Zheng *et al.*, 2016; Li *et al.*, 2019; Achmad *et al.*, 2019; Guibert, Lecellier, Torda, Pochon, & Berteaux-Lecellier, 2020). This member of Vibrionaceae was found as a causative agent of Ice-ice diseases of the seaweed, *Kappaphycus alvarezii* (Achmad *et al.*, 2019) and moribund of the giant clam, *Tridacna maxima* (Guibert, Lecellier, Torda, Pochon, & Berteaux-Lecellier, 2020).

3.3 Diversity of bacteria associated with vibriosis clinical signs

The diversity of vibrios in mud crab with vibriosis clinical signs were compared between the three sampling sites, determining the percentages of species by location

(Table 2). Table 2 accommodates all of the isolates that were identified based on the 16S-rRNA gene. The diversity of vibriosis clinical signs associated bacteria was explored using molecular characterization by rep-PCR. This method is a useful tool to assessing the diversity of bacteria according to the number and size of repetitive bacterial sequences. This technique could prevent analysing similar isolates of bacteria based on its DNA fingerprinting, and was therefore helpful in grouping the *Vibrio* species. The diversity of vibrios found in the three sample locations is seen in Table 2. We found more bacteria species in Rembang (6 vibrios) than in Demak (4 vibrios) and Kendal (2 vibrios) The highest percentage of vibrio diversity compared to the total isolates was in Demak (80%).

Figure 4 presents the bacterial diversity in Mud crab with Vibriosis's clinical signs from the North coast of Central Java. The highest number species was for *C. thiocicly* with the percentage of 32%, while 24% was *V. parahaemolyticus*, and *S. loihica* and *S. algae* had equal percentages at 12%, as well as *P. ganghwense* and *V. alginolyticus* at 8%, and the lowest rate was for *V. harveyi* at 4%.

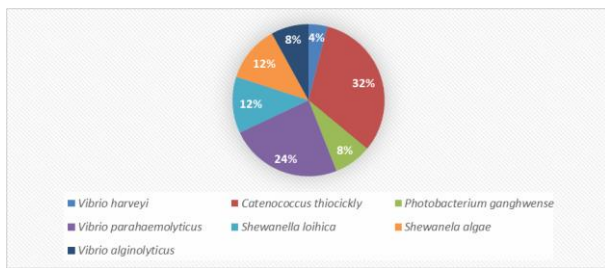


Figure 4. The bacterial diversity in mud crab with clinical signs of vibriosis from North coast of Central Java

Table 2. The diversity of vibrios in mud crab from North coast of Central Java by location

Location	Isolates	Bacteria Species	Vibrios diversity	Isolate Number	Percentage of species compare to total isolate (%)
Rembang	CJR14; CJR04; CJR10; CJR08; CJR15; CJR11; CJR12; CJR13; CJR16; CJR17; CJR03; CJR18; CJR05	<i>Vibrio harveyi</i> strain NCIMB1280 <i>Catenococcus thiocicly</i> strain TG 5-3, <i>Photobacterium ganghwense</i> strain FR311, <i>Shewanella loihica</i> strain PV4, <i>Shewanella algae</i> strain ATCC5192, <i>Vibrio alginolyticus</i> strain NBRC 15630,	6	13	52%
Demak	CJD24; CJD23; CJD17; CJD19; CJD22	<i>Catenococcus thiocicly</i> strain TG 5-3 <i>Photobacterium ganghwense</i> strain FR311 <i>Shewanella loihica</i> strain PV4 <i>Shewanella algae</i> strain ATCC5192	4	5	80%
Kendal	CJK05; CJK01; CJK07; CJK08; CJK10; CJK15; CJK11	<i>Vibrio prahaemolyticus</i> ATCC 17802 <i>Shewanella loihica</i> strain PV4	2	7	28%

In order to represent the relationships among isolates, the phylogenetic tree was constructed. Figure 5 shows that all of the strains were associated with closely related species. The relationship between the genera indicates that the bacterial genus *Vibrio* has the same clade with *Catenococcus thiocicly*. Those groups are more closely related than the groups of *Shewanella* and *Photobacterium*.

The isolates were also grouped based on the sampling site (Table 2). Rembang has the highest number of strains. However, from the seven groups of *Vibrio*, only six groups were detected in this site. Demak has four groups of *Vibrio* from five isolates found. Furthermore, Kendal only has two groups of *Vibrio* from seven strains. The results indicate

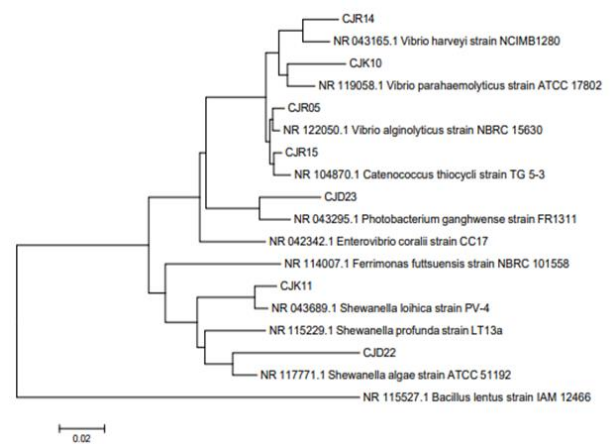


Figure 5. Phylogenetic tree of the bacteria associated with vibriosis clinical signs from mud crab traditionally farmed in the North coast of Central Java, Indonesia, constructed using neighbour-joining analysis with 1000 replicates.

that the highest diversity of *Vibrio* was found in Rembang, superior to those of Demak and Kendal. This might be because those locations have a higher abrasion (Directorate of Marine and Fisheries, 2019); Miller (1989) revealed that the abrasion could decrease microbial abundances.

Moreover, Figure 4 was designed to show the highest species of Vibrionaceae found in this study. Based on the dendrogram of rep-PCR, the dominant species was from *C. thiocicly* in group II at 32%, and the lowest was *V. harveyi* at 4%. Based on the phylogenetic tree (Figure 5), *Vibrio* and *Catenococcus* are more closely related to each other than to *Shewanella* and *Photobacterium*.

According to the results above, future research should be conducted for a deep understanding of correlations between biotic and abiotic factors that affect the health status of Mud-crab in traditional mud crab farming. Therefore, the metagenomic approach could be helpful for describing the bacterial community structures in the healthy and infected mud crabs. Then, the design of prevention methods to reduce bacterial disease outbreaks impacting mud crab farming could be pursued based on such knowledge.

4. Conclusions

This study revealed that 25 isolates collected from infected mud crabs fell into seven groups of bacteria well-known as pathogens for aquatic organisms. Moreover, according to molecular characterizations, the highest diversity of Vibrionaceae was obtained from Rembang, and the bacterium most found at all three sampling sites was *C. thiocicly*.

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Original Article

Molecular characterization of vibriosis associated bacteria from traditional mud-crab farmed in the North Coast of Central Java, Indonesia

Sarjito Sarjito^{1*}, Alfabetian Harjuno Condro Haditomo¹, Slamet Budi Prayitno¹,
Aninditia Sabdaningsih², Desrina¹, and Restiana Wisnu Ariyati¹

¹ Aquaculture Study Program, Department of Aquaculture, Faculty of Fisheries and Marine Science,
Diponegoro University, Semarang, 50275 Indonesia

² Department of Aquatic Resources, Faculty of Fisheries and Marine Science,
Diponegoro University, Semarang, 50275 Indonesia

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Abstract

This study examines the molecular characterization of bacteria associated with clinical symptoms and the diversity of vibriosis infecting farmed mud crab from three sampling locations, namely Rembang, Demak, and Kendal Districts. The clinical symptom was red-brown spots on carapace and wounds. Twenty-five bacterial isolates were gained from hepatopancreas, gills and carapace of nine infected mud crabs, by culturing in TCBS and TSA medium. Molecular characterization was carried out through modified rep-PCR followed by 16S-rRNA gene amplification. The results indicate that seven out of the 25 isolates, namely CJR14, CJR15, CJD23, CJK10, CJK11, CJD22 and CJR05 were 92-98% firmly related to *Vibrio harveyi* NCIMB-1280, *Catenococcus thiocicly* TG5-3, *Photobacterium ganghwense* FR311, *Vibrio parahaemolyticus* ATCC-1780, *Shewanella loihica* PV4, *Shewanella algae* ATCC5, and *Vibrio alginolyticus* NBRC-15630. This study revealed that the 25 isolates found from infected mud crabs fell into seven groups of bacteria. These seven groups were well-known as pathogens for aquatic organisms. Moreover, according to molecular characterization, the highest diversity of Vibrionaceae was obtained from Rembang, and the bacterium most found in all three sampling sites was *C. thiocicly*.

Keywords: mud crab, 16S rRNA, rep PCR, vibriosis

1. Introduction

Mud crab, *Scylla serrata* (Forskål, 1775), is traditionally caught in and around brackish water ponds and mangrove forests along the North coast of Central Java, Indonesia. Since around twenty years, mud crabs have been farmed in the North coast of Central Java to ensure production quantity and crab size. However, the seeds are still collected from the wild. Najiah, Nadirah, Sakri and Harrison (2010) reported that wild crabs in Setiu Wetland, Malaysia, were

infected by 12 species of bacteria resistant to the antibiotics linomycine, ampicillin, amocillin and oleandomycin at 94.5%, 90.1%, 86.8% and 78.0% respectively. These indicated safety issues in human consumption of wild mud crabs due to the antibiotic resistance. Sarjito *et al.* (2016) reported that bacterial disease has become an obstacle for fattening mud crabs in Pemalang District. Moreover, Jithendran, Poornima, Balasubramanian, and Kulasekara pandian (2010) stated that bacterial disease in mud crabs caused mortality of over 90% in all life cycle stages, with clinical signs including wounds on the body, decreased feed response and weakening. Sarjito, Hastuti, Samidjan, and Prayitno (2014); and Sarjito *et al.* (2016) added blackening and red spots of carapace to the symptoms.

*Corresponding author

Email address: sarjito@live.undip.ac.id

Several species of *Vibrio* spp. have been reported as associated with bacterial diseases in mud crabs, such as *V. fischeri* (Sarjito, Hastuti, Samidjan, & Prayitno, 2014; Sarjito *et al.*, 2016; Wang, 2011), *V. nereis* (Wang, 2011); *V. alginolyticus* and *V. cholerae* (Najiah, Nadirah, Sakri & Harrison, 2010; Sarjito *et al.*, 2016; Wang, 2011); *V. vulnificus* (Lavilla-Pitogo, Celia & Leobert, 2004; Shanmuga, 2008; Wang, 2011), *V. splendidus* and *V. orientalis* (Jithendran, Poornima, Balasubramanian, & Kulasekara pandian, 2010); *V. ordalii* and *V. harveyi* (Sarjito, Hastuti, Samidjan, & Prayitno, 2014; Jithendran, Poornima, Bala subramanian, & Kulasekarapandian, 2010), *V. campbelli* (Shanmuga, 2008) and *V. parahaemolyticus* (Lavilla-Pitogo, Celia, & Leobert, 2004; Shanmuga, 2008, Najiah, Nadirah, Sakri & Harrison, 2010; Wang, 2011, Sarjito *et al.*, 2016), *V. harveyi*, *V. cholerae*, *V. parahaemolyticus*, *V. alginolyticus* and *V. fischeri* (Sarjito *et al.*, 2016), *V. alginolyticus* and *V. harveyi* (Sarjito, Desrina, Haditomo, & Prayitno, 2018). Only limited knowledge is available on the diversity of vibrios related to the disease outbreaks in mud crab in coastal North Central Java. However, local government tends to stimulate the culturing of mud crabs, although information on disease identification and prevention is lacking. Wild mud crabs are actually a threat to the traditional polyculture shrimp and milkfish, and semi-intensive shrimp culture system. This is because wild mud crabs intrude earthen brackish water ponds, causing fish and shrimp to escape and diseases to spread. Diseases caused by the infiltration of infected wild crabs into brackish water ponds reduces fish and shrimp production. Based on the background above, this study was conducted to assess the molecular characterization of bacteria associated with clinical symptoms, and the diversity of the bacterial vibriosis infecting mud crabs farmed in brackish water traditional ponds along the North coast of Central Java, Indonesia.

2. Materials and Methods

2.1 Sampling

Nine mud crabs, *S. serrata*, were sampled from nine brackish water ponds of Kendal, Demak, and Rembang Regency, Central Java (each for three ponds). These three locations are the largest producers of cultivated crabs in Central Java, Indonesia (Figure 1). Based on the clinical signs for vibriosis described by Jithendran, Poornima, Balasubramanian, and Kulasekarapandian (2010), these infected mud crabs were stored in a sterile container and carried to the Integrated Laboratory of Diponegoro University for further analysis. The average size of the caught crab, in the range from 13 to 15 cm, was 14.4 ± 0.7 cm.

2.2 Bacterial isolation

Twenty-five bacteria were isolated from gills, haemolymph, hepatopancreas, and wounds on the shell of the infected mud crabs. Isolates of the bacteria were cultured on Thiosulfate Citrate Bile Salt Sucrose (TCBS) and on Tryptic Soy Agar (TSA) (Thompson, Iida, & Swings, 2004; Sarjito *et al.*, 2016). The colonies were purified by the streak method, based on morphological appearance (Sarjito *et al.*, 2016).



Figure 1. Research locations are represented by the small red boxes in the left-hand-side map.

2.3 Repetitive sequences-based PCR (Rep-PCR)

The bacterial DNA was extracted from a 24 h broth culture of isolate strains by using the chelex method with slight modification to the procedure described by (de Lamballerie, Zandotti, Vignoli, Bollet, & de Micco, 1992). Then, the isolated DNA extracts were analyzed using the rep-PCR of Radjasa, Nasima, Sabdono, Kita-Tsukamoto, & Ohwada (2007) as modified by Sarjito, Haditomo, Desrina, Djunaedi, & Prayitno (2018) in the rep-PCR, BOX A1R (5'-CTACggCAAggCgACgCTgACg-3'). The REP 1R-I and REP 2-I primers contained nucleotide inosine (I) at an ambiguous position in the REP consensus. The mix PCR reagent contains one μ L DNA template (diluted 100X), one μ L primer, 7.5 μ L Megamix Royal and 5.5 μ L ddH₂O. Amplification was performed in a thermal cycler model Gene Amp PCR system 9700 with the following protocol: initial denaturation at 95°C for 5 minutes; then 30 cycles of denaturation at 92°C for 1 minute, annealing at 50°C for 1.5 minutes, and extension at 68°C for 8 minutes; and a final extension at 68°C for 10 minutes. The bands of DNA were visualized from injected 5 μ L of PCR products into 1% agarose gel that was run in an electrophoresis machine using 1X TBE buffer and observed under UV-transilluminator, as described by Radjasa, Nasima, Sabdono, Kita-Tsukamoto, and Ohwada (2007). Grouping of the isolates was conducted based on the method of Sarjito, Radjasa, Sabdono, Prayitno, and Hutabarat (2009). The method is a useful tool for placing the bacterial strains from the three sampling sites into groups, based on the fingerprinting of interspersed repetitive DNA sequences of BOX element using BOX A1R primer. Each group could be then identified using 16S-rRNA gene. Bacterial diversity is the percentage of the number of each species in a Dendrogram group based on Rep-PCR compared to the total number of isolates. Then, the diversity of vibrios in mud crab with clinical signs of vibriosis were compared from the three-sampling sites, to assess the percentages of species by location.

Matrices were made from the band positions on the gel, which were analysed by the Free Tree program using the UPGMA method for constructing the phylogenetic tree (Sarjito, Haditomo, Desrina, Djunaedi, & Prayitno, 2018).

Resampling was done by bootstrapping with 1000 replications (Prayitno, Sarwan, & Sarjito, 2015).

2.4 PCR amplification of 16S-rRNA gene fragments

The PCR amplification of the 16S-rRNA gene was performed, according to Radjasa, Nasima, Sabdono, Kita-Tsukamoto, & Ohwada (2007) and Sarjito, Haditomo, Desrina, Djunaedi, & Prayitno (2018). To target the 16S-rRNA gene the amplification used two primers, GM3F as a forward primer (5'-AGAGTTTGATCMTGGC-3') and GM4R as a reverse primer (5'-TACCTTGTACGACTT-3') (Sarjito, Radjasa, Sabdono, Prayitno, & Hutabarat, 2009). Genomic DNA of bacteria for PCR analysis was obtained from the lysing of cell materials that were taken from freshly cultured bacteria, suspended in sterile water (Sigma, Germany), by subjecting to five cycles of freeze (-80°C) and thaw (95°C). The PCR product was then processed to find the DNA band of the right size around 1.500 bp. The sequencing step was facilitated by PT. Genetika Science. The determined DNA sequences of samples were then compared for homology using BLAST. The phylogenetic tree was established using MEGA X to find closely related species (Sabdaningsih *et al.*, 2020).

3. Results and Discussion

3.1 Clinical Signs and Bacterial Isolation

The clinical signs of the infected mud crab (*S. serrata*) collected from the three studied locations were wounds on the surface of the claws, the ventral, the carapace, and abdomen. Red-brown and dark spots in the carapace were found, as seen in Figure 2. Isolation of bacteria on TCBS medium in three replicates obtained 25 pure isolates.



Figure 2. The clinical signs of mud crab (*S. serrata*) infected by bacterial diseases: (a) red/brown spots in the carapace; and (b) red-brown spots in the carapace wound on the abdomen.

Red-brown or dark melanin spots on body surface, patches of light red spots as well as dark spots on the carapace, and also wounds on the body (claws, shell, and the ventral) are known as indications of vibriosis in mud crabs. Similar clinical signs have been described by Muyzer, Teske, Wirsen, and Jannasch (1995), Wang (2011), Lavilla-Pitogo *et al.* (2004) and Prayitno, Sarjito & Putri (2017). Similar clinical signs are also reported in mud crabs infected with *V. harveyi*, *V. fischeri* and *V. ordalii* from the gulf of Semarang (Sarjito, Hastuti, Samidjan, and Prayitno, 2014) and *V. harveyi*, *V. cholerae*, *V. parahaemolyticus*, *V. alginolyticus* and *V. fischeri* from Pemalang (Sarjito *et al.*, 2016). 'Brown spot' may occur due to an infection by chitinolytic bacteria that catabolize the chitin of the carapace and cause erosion

and melanisation (dark brown to black pigmentation) at the site of infection (Jithendran, Poornima, Balasubramanian, & Kulasekarapandian, 2010).

3.2 Repetitive sequences-based PCR (Rep-PCR)

The Repetitive PCR analysis and the dendrogram related to the 25 pure isolates bacteria originating from mud crabs revealed seven groups (Figure 3). Furthermore, for molecular identification each group was represented by one isolate, namely CJR14, CJR15, CJD23, CJK10, CJK11, CJD22, and CJR05 (Table 1). The remaining isolates were assumed to be of the same species within a group, according to their similar patterns of DNA size from the rep-PCR results.

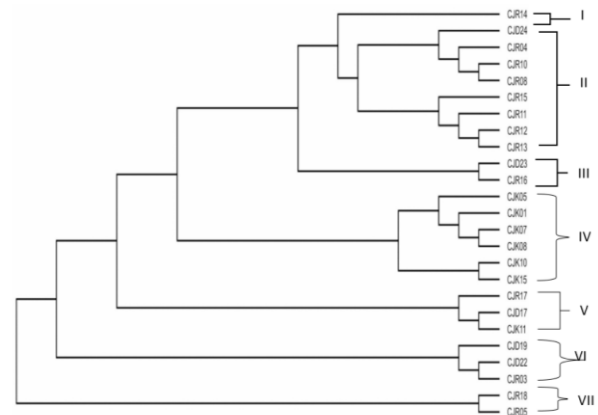


Figure 3. A dendrogram based on Rep-PCR of 25 bacteria associated with vibriosis clinical signs, isolates from mud crabs traditionally farmed in the North coast of Central Java, Indonesia.

Figure 3 shows similarity of the 25 isolates divided into seven groups according to the differences between repetitive bacterial sequences. Then these were examined using 16S-rRNA gene for identification from a total of seven isolates, identified as presented in Table 1, with the range of homology percentage being 92-98%. The highest similarity was by isolate CJR15 from group II having 98% homology to *C. thiocicly* strain TG5-3. The homology level 97% was appointed to isolates CJK10 and CJK11 from group IV and V that were similar to *V. parahaemolyticus* ATCC-17802 and *S. loihica* strain PV4. In rank order this was followed by CJR05 from group VII with 96% homology to *V. alginolyticus* strain NBRC-15630. Moreover, isolates CJR14 and CJD23 in groups I and III had 95% homology to *V. harveyi* strain NCIMB-1280 and *P. ganghwense* strain FR311, while isolate CJD22 was 92% closely related to *S. algae* strain ATCC-5192 in group VI.

The vibrios in mud crabs from traditional brackish water ponds of the North coast of Central Java (Figure 1) were closely related to *V. harveyi* strain NCIMB-1280 (CJR14), *C. thiocicly* strain TG 5-3 (CJR15), *P. ganghwense* strain FR311 (CJD23); *V. parahaemolyticus* ATCC 17802 (CJK10); *S. loihica* strain PV4 (CJK11); *S. algae* strain ATCC5 (CJD22) and *V. alginolyticus* strain NBRC-15630 (CJR05). The results revealed the diversity of vibrios associated with mud crabs

Table 1. Molecular characterization of seven bacterial species associated with clinical signs of vibriosis in mud crabs from North coast of Central Java

Isolate code	Query length	Close relative	Query cover (%)	Homology (%)	Acc. Number
CJR14	1548	<i>Vibrio harveyi</i> strain NCIMB1280	98	95	NR 043165.1
CJR15	1319	<i>Catenococcus thiocicly</i> strain TG 5-3	98	98	NR 118258.1
CJD23	1387	<i>Photobacterium ganghwense</i> strain FR311	98	95	NR 043295.1
CJK10	1340	<i>Vibrio parahaemolyticus</i> ATCC 17802	99	97	NR 043689.1
CJK11	1335	<i>Shewanella loihica</i> strain PV4	97	97	NR 025794.1
CJD22	1488	<i>Shewanella algae</i> strain ATCC5192	95	92	NR 117771.1
CJR05	1362	<i>Vibrio alginolyticus</i> strain NBRC 15630	96	96	NR 122050.1

from traditional farming that impact production negatively and result in significant losses. These bacterial diversity results were lower than the diversities found in the cultured and wild crab in India (Jithendran, Poornima, Bala subramanian, & Kulasekarapandian, 2010) as well as in extensive brackish water ponds surrounding Semarang Gulf (Sarjito, Hastuti, Samidjan, & Prayitno, 2014).

Four genera of Vibrionaceae (*Vibrio*, *Shewanella*, *Photobacterium*, and *Catenococcus*) were mostly found in mud crab (Li *et al.*, 2012; Wei *et al.*, 2019). Three species of vibrio, namely *V. parahaemolyticus* (CJK10), *V. harveyi* (CJR14), and *V. alginolyticus* (CJR05) also have been detected in this study. The vibrios have been frequently found as causative agents of shell disease in mud crab (*S. serrata*), and in shrimp (*L. vannamei*) (Estave, & Herrere, 2000; Wang, 2011; Soto-Rodriguez *et al.*, 2012). *V. parahaemolyticus* has been found as a main pathogen in mud crab in China (Xia *et al.*, 2010) and in Chakoria coast, Bangladesh (Aftabuddin *et al.*, 2013). Additionally, *V. harveyi* has been reported as a causative agent of vibriosis in fattening mud crabs from brackish water pond in Pemalang coast (Sarjito *et al.*, 2016,) and in mud crab from extensive brackish water ponds surrounding the Gulf of Semarang (Sarjito, Hastuti, Samidjan, & Prayitno, 2014) and in Malaysia (Najiah, Nadirah, Sakri, & Harrison, 2010). These bacteria were also reported as causative agents of bacterial diseases in zoea stage of mud crab (Jithendran, Poornima, Balasubramanian, & Kulasekarapandian, 2010); and in adult mud crabs (Poornima *et al.*, 2012 and Lavilla-Pitogo *et al.*, 2004). Moreover, Sarjito, Hastuti, Samidjan and Prayitno (2014), found that this bacterium was a potential pathogen to mud crabs. *V. alginolyticus* was observed as an important pathogenic bacterium associated with infectious diseases in mud crab (Najiah, Nadirah, Sakri, & Harrison, 2010) and in shell disease of mud crab *S. serrata* grown in pond located at Tamil Nadu, India (Gunasekaran, Gopalakrishnan,

Deivasigamani, Muhilvannan, & Kathirkaman, 2019). Genus *Photobacterium* was found in the coastal, open-ocean and deep-sea water (Moi *et al.*, 2017). Wei *et al.*, (2019) found that *Photobacterium* was dominant as gut microbiota in mud crab *S. paramamosain* collected from nine coastal areas of southern China. Surprisingly, *P. Ganghwense* was detected in this study. This bacterium, was firstly reported from seawater in Ganghwa Island, South Korea (Park *et al.*, 2006).

Shewanella algae and *Shewanella loihica* were found in this study. They both have an essential role in the turnover of organic material, and are capable of dissimilatory reduction of various metals and other substances, such as

Nitrate, Nitrite, Thiosulphate, and Trimethylamine-N-oxide (Holt, Gahrn-Hansen, & Bruun, 2005). *S. algae* has been reported as bacterial pathogen associated with Black Spot Disease in Freshwater-Cultured White leg Shrimp, *L. vannamei* (Cao, Chen, Lu, & An, 2018), as causative agent in *Babylonia* (Shufang, Zhang, Dequan, Shiping, & Huang, 2015); *Cynoglossus semilaevis* (Han *et al.*, 2018), and *Sciaenops ocellatus* (Zhang, Zhu, & Wang, 2013). According to Prayitno *et al.* (2015) *S. algae* was also present in gut of milkfish from the Northern coast of Central Java. In Demak, *S. algae* was found only when the water temperature exceeded 23°C. Additionally, the infection of genus *Shewanella* mostly occurs in countries with a warm climate (Holt, Gahrn-Hansen, & Bruun, 2005). Although it is rarely found as a human pathogen and exhibited symptoms of infection since this bacterium was already detected in mud crab farming at the North coast of Central Java, the presence of this bacterium in the mud crab products might be considered.

Shewanella loihica was isolated and identified from iron-rich microbial mats in the Pacific Ocean (Gao *et al.*, 2006). *S. loihica* plays a role as an agent of metal reduction and iron bio-mineralization. Based on that, the existence of this bacterium can be associated with metal and nitrogen content in the water. Some reported studies of *S. loihica* have also focused on metal reduction and iron bio-mineralization capabilities (Newton, Mori, Nakamura, Hashimoto, & Watanabe, 2009; Philips *et al.*, 2018), and on denitrification and a respiratory ammonification pathway (Yoon, Sanford, & Löffler, 2015). Therefore, this bacterium has an important role in the nutrient cycle in soils and sediments where the mud crab lives.

Catenococcus thiocycli was identified by Yarza *et al.*, (2013), and the study found that *C. thiocycli* is part of genus Vibrionaceae. Only few studies have reported this bacterium (Zheng *et al.*, 2016; Li *et al.*, 2019; Achmad *et al.*, 2019; Guibert, Lecellier, Torda, Pochon, & Berteaux-Lecellier, 2020). This member of Vibrionaceae was found as a causative agent of Ice-ice diseases of the seaweed, *Kappaphycus alvarezii* (Achmad *et al.*, 2019) and moribund of the giant clam, *Tridacna maxima* (Guibert, Lecellier, Torda, Pochon, & Berteaux-Lecellier, 2020).

3.3 Diversity of bacteria associated with vibriosis clinical signs

The diversity of vibrios in mud crab with vibriosis clinical signs were compared between the three sampling sites, determining the percentages of species by location

(Table 2). Table 2 accommodates all of the isolates that were identified based on the 16S-rRNA gene. The diversity of vibriosis clinical signs associated bacteria was explored using molecular characterization by rep-PCR. This method is a useful tool to assessing the diversity of bacteria according to the number and size of repetitive bacterial sequences. This technique could prevent analysing similar isolates of bacteria based on its DNA fingerprinting, and was therefore helpful in grouping the *Vibrio* species. The diversity of vibrios found in the three sample locations is seen in Table 2. We found more bacteria species in Rembang (6 vibrios) than in Demak (4 vibrios) and Kendal (2 vibrios) The highest percentage of vibrio diversity compared to the total isolates was in Demak (80%).

Figure 4 presents the bacterial diversity in Mud crab with Vibriosis's clinical signs from the North coast of Central Java. The highest number species was for *C. thioicly* with the percentage of 32%, while 24% was *V. parahaemolyticus*, and *S. loihica* and *S. algae* had equal percentages at 12%, as well as *P. ganghwense* and *V. alginolyticus* at 8%, and the lowest rate was for *V. harveyi* at 4%.

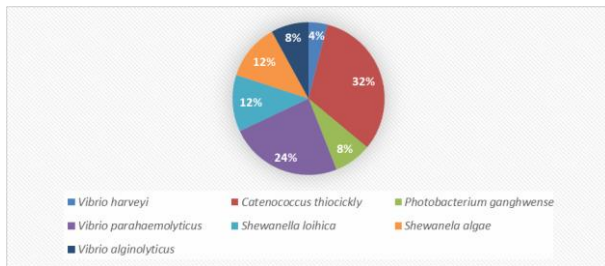


Figure 4. The bacterial diversity in mud crab with clinical signs of vibriosis from North coast of Central Java

Table 2. The diversity of vibrios in mud crab from North coast of Central Java by location

Location	Isolates	Bacteria Species	Vibrios diversity	Isolate Number	Percentage of species compare to total isolate (%)
Rembang	CJR14;	<i>Vibrio harveyi</i> strain NCIMB1280	6	13	52%
	CJR04;	<i>Catenococcus thioicly</i> strain TG 5-3,			
	CJR10;	<i>Photobacterium ganghwense</i> strain FR311,			
	CJR08;	<i>Shewanella loihica</i> strain PV4,			
	CJR15;	<i>Shewanella algae</i> strain ATCC5192,			
	CJR11;	<i>Vibrio alginolyticus</i> strain NBRC 15630,			
	CJR12;				
	CJR13;				
	CJR16;				
	CJR17;				
	CJR03;				
	CJR18;				
	CJR05				
Demak	CJD24;	<i>Catenococcus thioicly</i> strain TG 5-3	4	5	80%
	CJD23;	<i>Photobacterium ganghwense</i> strain FR311			
	CJD17;	<i>Shewanella loihica</i> strain PV4			
	CJD19;	<i>Shewanella algae</i> strain ATCC5192			
	CJD22				
Kendal	CJK05;	<i>Vibrio prahaemolyticus</i> ATCC 17802	2	7	28%
	CJK01;	<i>Shewanella loihica</i> strain PV4			
	CJK07;				
	CJK08;				
	CJK10;				
	CJK15;				
	CJK11				

In order to represent the relationships among isolates, the phylogenetic tree was constructed. Figure 5 shows that all of the strains were associated with closely related species. The relationship between the genera indicates that the bacterial genus *Vibrio* has the same clade with *Catenococcus thioicly*. Those groups are more closely related than the groups of *Shewanella* and *Photobacterium*.

The isolates were also grouped based on the sampling site (Table 2). Rembang has the highest number of strains. However, from the seven groups of *Vibrio*, only six groups were detected in this site. Demak has four groups of *Vibrio* from five isolates found. Furthermore, Kendal only has two groups of *Vibrio* from seven strains. The results indicate

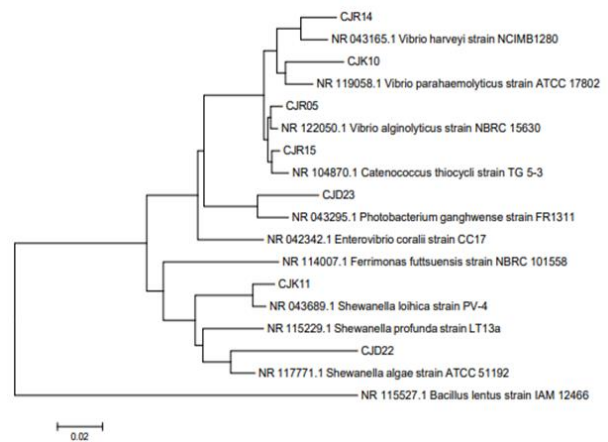


Figure 5. Phylogenetic tree of the bacteria associated with vibriosis clinical signs from mud crab traditionally farmed in the North coast of Central Java, Indonesia, constructed using neighbour-joining analysis with 1000 replicates.

that the highest diversity of *Vibrio* was found in Rembang, superior to those of Demak and Kendal. This might be because those locations have a higher abrasion (Directorate of Marine and Fisheries, 2019); Miller (1989) revealed that the abrasion could decrease microbial abundances.

Moreover, Figure 4 was designed to show the highest species of Vibrionaceae found in this study. Based on the dendrogram of rep-PCR, the dominant species was from *C. thiocicly* in group II at 32%, and the lowest was *V. harveyi* at 4%. Based on the phylogenetic tree (Figure 5), *Vibrio* and *Catenococcus* are more closely related to each other than to *Shewanella* and *Photobacterium*.

According to the results above, future research should be conducted for a deep understanding of correlations between biotic and abiotic factors that affect the health status of Mud-crab in traditional mud crab farming. Therefore, the metagenomic approach could be helpful for describing the bacterial community structures in the healthy and infected mud crabs. Then, the design of prevention methods to reduce bacterial disease outbreaks impacting mud crab farming could be pursued based on such knowledge.

4. Conclusions

This study revealed that 25 isolates collected from infected mud crabs fell into seven groups of bacteria well-known as pathogens for aquatic organisms. Moreover, according to molecular characterizations, the highest diversity of Vibrionaceae was obtained from Rembang, and the bacterium most found at all three sampling sites was *C. thiocicly*.

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