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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

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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 oleandoromicyn 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.

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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±0.7cm.

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 Triptic 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 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 95ºC for 5 minutes; then 30 cycles of denaturation at 92°C for 1 minute, annealing at 50ºC for 1.5 minutes, and extension at 68ºC for 8 minutes; and a final extension at 68ºC for 10 minutes. The bands of DNA were visualized from injected 5 µL 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 Dendogram 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'-TACCTTGTTACGACTT-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 (-80ºC) and thaw (95ºC). 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 disases: (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.

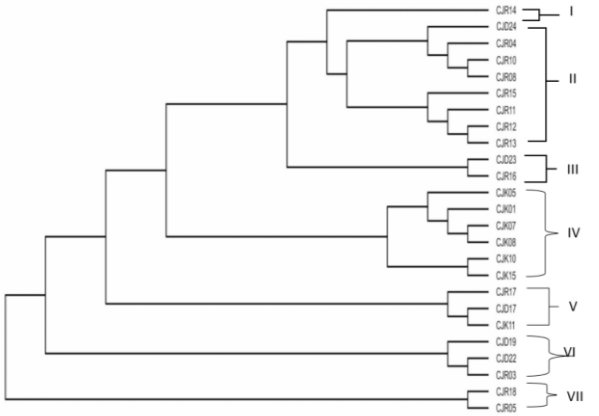

Figure 3. A dendrogram based on Rep-PCR of 25 bacteria associated with vibriosis clinical signs, isolates from mud crabs traditionally farmed in the North coast of Central Java, Indonesia.

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. parahaemolycticus* 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	*Vibrio harveyi* strain NCIMB1280	98	95	NR 043165.1
CJR15	1319	*Catenococcus thiocicly* strain TG 5-3	98	98	NR 118258.1
CJD23	1387	*Photobacterium ganghwense* strain FR311	98	95	NR 043295.1
CJK10	1340	*Vibrio parahaemolyticus* ATCC 17802	99	97	NR 043689.1
CJK11	1335	*Shewanella loihica* strain PV4	97	97	NR 025794.1
CJD22	1488	*Shewanella algae* strain ATCC5192	95	92	NR 117771.1
CJR05	1362	*Vibrio alginolyticus* 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 genuses 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 *Scianeops 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, Sanfordd, & Löfflerr, 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. algynolyticus* at 8%, and the lowest rate was for *V. harveyi* at 4%.

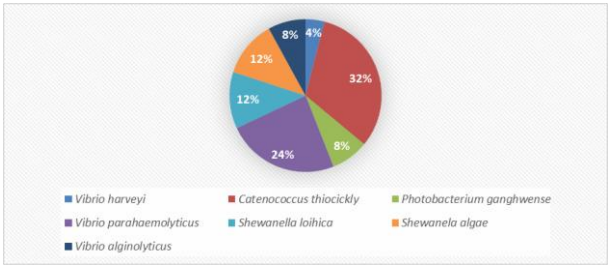

Figure 4. The bacterial diversity in mud crab with clinical signs of vibriosis from North coast of Central Java

In order to represent the relationships among isolates, the phylogenetic three was constructed. Figure 5 shows that all of the strains were associated with closely related species. The relationship between the genuses indicates that the bacterial genus Vibrio has the same clade with *Catenococcus thiocycli*. 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

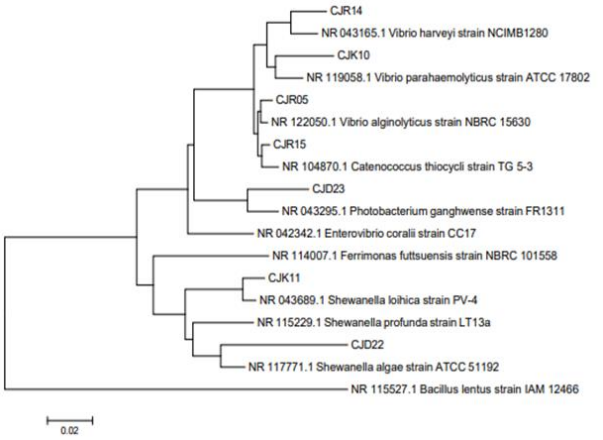

Figure 5. Phylogenetic tree of the bacteria associated with vibriosis clinical signs from mud crab traditionally farmed in the North coast of Central Java, Indonesia, constructed using neighbour-joining analysis with 1000 replicates.

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	*Vibrio harveyi* strain NCIMB1280 *Catenococcus thiocicly* strain TG 5-3, *Photobacterium ganghwense* strain FR311, *Shewanella loihica* strain PV4, *Shewanella algae* strain ATCC5192, *Vibrio alginolyticus* strain NBRC 15630,	6	13	52%
Demak	CJD24; CJD23; CJD17; CJD19; CJD22	*Catenococcus thiocicly* strain TG 5-3 *Photobacterium ganghwense* strain FR311 *Shewanella loihica* strain PV4 *Shewanella algae* strain ATCC5192	4	5	80%
Kendal	CJK05; CJK01; CJK07; CJK08; CJK10; CJK15; CJK11	*Vibrio prahaemolyticus* ATCC 17802 *Shewanella loihica* strain PV4	2	7	28%

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 Vibrionaceace 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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