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$(GTG)_5$-PCR analysis and 16S rRNA sequencing of bacteria from Sarawak aquaculture environment

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Abstract

$(GTG)_5$ PCR is a type of repetitive extragenic palindromic (rep)-PCR which amplifies the $(GTG)_5$ repetitive element that lays throughout the bacterial genome. In this study, fifty, thirty-nine and forty-nine unknown bacteria were isolated from aquaculture farms in Miri, Limbang and Lundu, respectively. $(GTG)_5$ PCR was used to screen for clonal diversity among the isolates according to sampling sites. Banding profiles obtained from electrophoresed $(GTG)_5$ PCR products were analyzed by RAPDistance Software to generate a dendrogram of neighbor joining tree (NJT) format. Based on the constructed dendrogram, representative isolates were selected for further identification. Conserved 16S rRNA region of the selected bacteria isolates were amplified and purified DNA products were sequenced. $(GTG)_5$ PCR is useful in differentiation of unknown bacterial isolates and 16S rRNA analysis species identity of the bacteria in Sarawak aquaculture environment. The high diversity of bacteria in aquaculture environment may be caused by contamination from various sources.

Introduction

Aquaculture sector is one of the important and rapidly growing sectors in Malaysia. Large number of bacteria from highly diverse bacterial species could be isolated from the aquaculture pond and its environment. Presence of a large number of bacteria in the pond indicates the presence of high nutrient level in the water. The number and species of freshwater microbiota depends on the availability of light and oxygen (Tortora *et al*., 2007). Based on previous study done by Apun *et al.* (1999), fish intestine contains the most number of bacteria from different species. Among the commonly found bacterial species in aquaculture pond and its environment were *Aeromonas*, *Escherichia*, *Enterobacter*, *Klebsiella*, *Pseudomonas*, *Vibrio*, *Bacillus*, *Listeria*, *Staphylococcus*, *Citrobacter* (Apun *et al.*, 1999) and *Edwardsiella* (Seong Wei *et al.*, 2011).

To date, repetitive element polymerase chain reaction (rep-PCR) has become one of the highly powerful molecular tools applicable for the identification of bacteria and differentiation of bacterial strains of the same species (Gomez-Gil *et al*., 2004). Although there are different methods of rep-PCR, $(GTG)_5$ has proved to show the highest discriminatory power (Mohapatra *et al.*, 2007; Gevers *et al*., 2001) and effective in screening a large amount of bacterial strains (Gevers *et al*., 2001). In addition, $(GTG)_5$-PCR is particularly useful for identification and intraspecies differentiation of bacterial genomes (Gevers *et al.*, 2001). In fact, $(GTG)_5$ PCR is a type of repetitive extragenic palindromic (rep)-PCR that amplifies the $(GTG)_5$ repetitive element that lays throughout the bacterial genomes. Apart from high discriminatory power and high-throughput of strains, this simple PCR-based method also comes with the advantages that it is a low cost and reliable tool for typing a broad range of Gram-negative bacteria and a narrow range of Gram-positive bacteria (Versalovic *et al.*, 1994; Olive & Bean, 1999; Gevers *et al.*, 2001).

Considering the need to contribute more detailed data about the bacteria identities in Sarawak aquaculture, the main intention of this study is to determine and to group the bacteria based on genetic differences through $(GTG)_5$-PCR analysis, and then identify their species based on the 16S rRNA gene sequencing.

Materials and Methods

Samples collection and processing

Sampling was carried out at aquaculture farm located at Miri, Limbang and Lundu. Three types of samples collected were sediment, water and cultured species. The sediment and water samples were taken using a sterile Polyvinyl chloride (PVC) pipe and 100 ml sterile Schott's bottle, respectively (Huys, 2003). *Litopenaeus vannamei* or Pacific White Shrimp (local name: *udang putih*) was freshly caught from the farms at Lundu and Limbang. *Pangasius pangasius* or River Catfish (local name: *ikan patin*) was freshly

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caught from Miri farm. Samples were transported to the laboratory in an ice container containing ice within 24 hours. Samples were processed immediately upon arrival in the laboratory.

Bacterial isolation

The sediment and water samples were homogenized before performing 10-fold serial dilution. One gram of the fish or shrimp intestine, one ml of the homogenized water and sediment samples were diluted with 9 ml of 0.85% (w/v) saline solution. Samples were plated on duplicate Trypticase Soy Agar (TSA) and incubated at 29°C for 24 hours. Plates containing 30 to 300 colonies were selected for bacteria isolation. Five to ten colonies were randomly isolated and streaked onto TSA until pure isolate was obtained.

DNA Extraction

Bacterial DNA was extracted using the boiling-centrifugation method as described by Freschi *et al.* (2005) with modifications. Three ml of the overnight culture was centrifuged at 10,000 rpm for 5 minutes. The supernatant was discarded. The pellet was resuspended in 500 µl of sterile dH_2O and boiled for 10 minutes. Immediately, the boiled suspension was cooled in ice at 4°C for 5 minutes and centrifuged at 10,000 rpm for 10 minutes. The supernatant was used for the PCR analysis.

$(GTG)_5$ PCR analysis

$(GTG)_5$ PCR was conducted according to Matsheka *et al.* (2005) with slight modifications. A total of 25 µl PCR mixture containing 5X Taq Green Buffer (Promega, USA), 25 mM $MgCl_2$, 25 mM deoxyribonucleotide phosphate (dNTPs), 25 µM $(GTG)_5$ primer (5'- GTGGTGGTGGTGGTG-3'), sterile distilled water (dH_2O), DNA template and *Taq* DNA polymerase. The amplification begins with pre-denaturation at 95°C (7 minutes), followed by 4 cycles of denaturation, annealing and extension at 95°C (2 minutes), 36°C (2 minutes) and 72°C (2 minutes), respectively. This was followed by another 30 cycles of denaturation at 95°C (1 minute), annealing at 50°C (1 minute) and elongation at 72°C (1 minute). The final elongation was carried out at 72°C for 5 minutes. Five microliter of the amplified PCR product was electrophoresed on 1.5% (w/v) agarose gel pre-stained with 1 µl of 10 mg/ml Ethidium bromide in 1X Tris-Borate-EDTA (TBE) buffer at 100 V for 1 hour and 30 min. The gel was then visualized under UV transilluminator. Scoring was done for construction of dendrogram. Bands present were scored as "1", while absence of band was scored as "0". The data was input in RAPDistance software and phylogenetic tree was generated by the software.

Identification by 16S rRNA PCR

The genotypic identification was done using the 16S rRNA identification method (Hutter *et al*., 2003) using 519R primer (5'-GWATTACCGCGGCKGCTG-3') and 27F primer (5'-AGAGTTTGATCMTGGCTCAG-3') (Ream *et al*., 2003). The 25 µl reaction mixture consists of 5X Taq Green Buffer, 25 mM $MgCl_2$, 25 mM dNTPs, 10 µM of each primer, sterile dH_2O, DNA template and Taq DNA polymerase. The cycling conditions were as followed: pre-denaturation at 95°C (10 minutes), denaturation at 94°C (30 seconds), annealing at 55°C (1 minute), extension at 72°C (1.5 minutes) and final extension at 72°C (10 minutes) at the end of 26 cycles. The amplified PCR product was purified using QIAquick PCR purification kit (Qiagen, Germany). Four microliter of purified DNA were electrophoresed on 1.0% (w/v) agarose gel pre-stained 1 µl of 10 mg/ml EtBr in 1X TBE buffer at 80 V. The stained gel was then visualized under the UV transilluminator. The leftovers of purified DNA were sent to First BASE Laboratories Sdn. Bhd. for DNA sequencing. The DNA sequence was then compared with those in the GenBank database using the BLAST server by the National Center for Biotechnology Information (NCBI).

Results and Discussion

One hundred and thirty-eight bacteria isolates from three different aquaculture farms were pre-screened using the $(GTG)_5$-PCR analysis. Figure 1a, 1b and 1c showed the banding profiles of the amplified bacterial $(GTG)_5$ elements in 1.5% (w/v) agarose gel from Miri, Limbang and Sampadi, respectively. The size of the bacterial isolates' $(GTG)_5$ gene from all location are within the range of 250 bp to 3,000 bp. All Limbang isolates possess different (GTG)5 pattern. Miri $(GTG)_5$ PCR profiles revealed that two isolates possess the same $(GTG)_5$ pattern. The bacterial isolates in Miri and Limbang were highly diverse. The $(GTG)_5$ pattern for Sampadi isolates were less heterogeneous compared to Miri and Limbang isolates. The (GTG)5 banding pattern for all three locations were more obvious when represented in the form of dendrograms (Figure 2a, 2b and 2c).

The objective of performing $(GTG)_5$ PCR in this study was to draw rough estimation of genetic similarity among the bacteria isolates based on the sampling locations. Screening of clonal isolates using $(GTG)_5$ PCR technique is cheap and easy. It also has

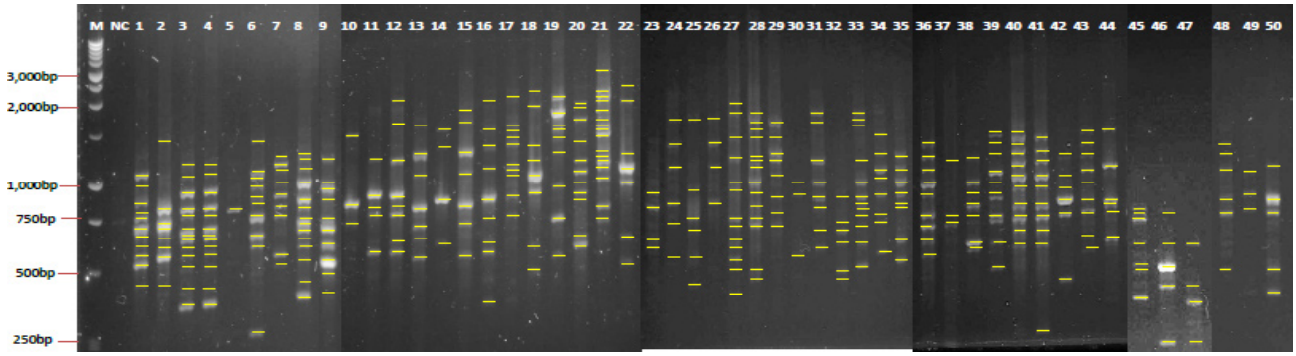

Figure 1a. Banding profiles of $(GTG)_5$ PCR for Miri isolates. Lane M: 1kb DNA Ladder (Promega, USA), NC: negative control, 1: MYY-F5, 2: MYY-F7, 3: MYY-F8, 4: MYY-F9, 5: MYY-F10, 6: MYY-W1, 7: MYY-W2, 8: MYY-W3, 9: MYY-W7, 10: MYY-2F3, 11: MYY-2F4, 12: MYY-2F5, 13: MYY-2F6, 14: MYY-2F7, 15: MYY-2F8, 16: MYY-2F9, 17: MYY-2F10, 18: MYY-2W1, 19: MYY-2W2, 20: MYY-2W3, 21: MYY-2W4, 22: MYY-2W7, 23: MYY-F4, 24: MYY-W4, 25: MYY-W5, 26: MYY-W6, 27: MYY-28, 28: MYY-W9, 29: MYY-W10, 30: MYY-2S1, 31: MYY-2S2, 32: MYY-2S3, 33: MYY-2S4, 34: MYY-2S5, 35: MYY-2S6, 36: MYY-S1, 37: MYY-S2, 38: MYY-S3, 39: MYY-S4, 40: MYY-S5, 41: MYY-S6, 42: MYY-S7, 43: MYY-F1, 44: MYY-F3, 45: MYY-2S10, 46: MYY-2F1, 47: MYY-2F2, 48: MYY-S9, 49: MYY-S10, 50: MYY-F2.

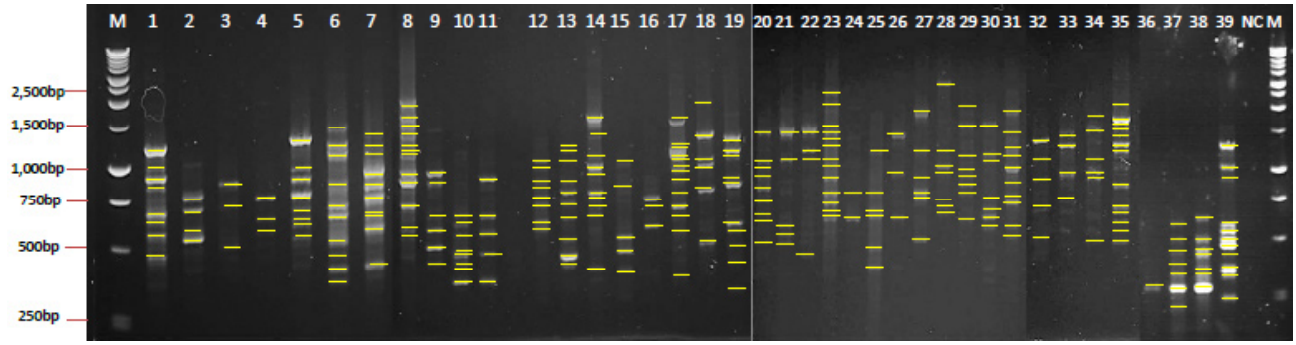

Figure 1b. Banding profiles of $(GTG)_5$ PCR for Limbang isolates. Lane M: 1kb DNA Ladder (Promega, USA), 1: LBG-S4, 2: LBG-S6, 3: LBG-S7, 4: LBG-P5, 5: LBG-P7, 6: LBG-P8, 7: LBG-W2, 8: LBG-W6, 9: LBG-W8, 10: LBG-P2, 11: LBG-P10, 12: LBG-2S1, 13: LBG-2S2, 14: LBG-2S3, 15: LBG-2S5, 16: LBG-2S7, 17: LBG-2S8, 18: LBG-2S9, 19: LBG-2S10, 20: LBG-2P1, 21: LBG-2P2, 22: LBG-2P3, 23: LBG-2P4, 24: LBG-2P5, 25: LBG-2P6, 26: LBG-2P7, 27: LBG-2P8, 28: LBG-2P9, 29: LBG-2P10, 30: LBG-2W2, 31: LBG-2W3, 32: LBG-S2, 33: LBG-P1, 34: LBG-P6, 35: LBG-W3, 36: LBG-P4, 37: LBG-2W4, 38: LBG-2W5, 39: LBG-2W6, NC: negative control.

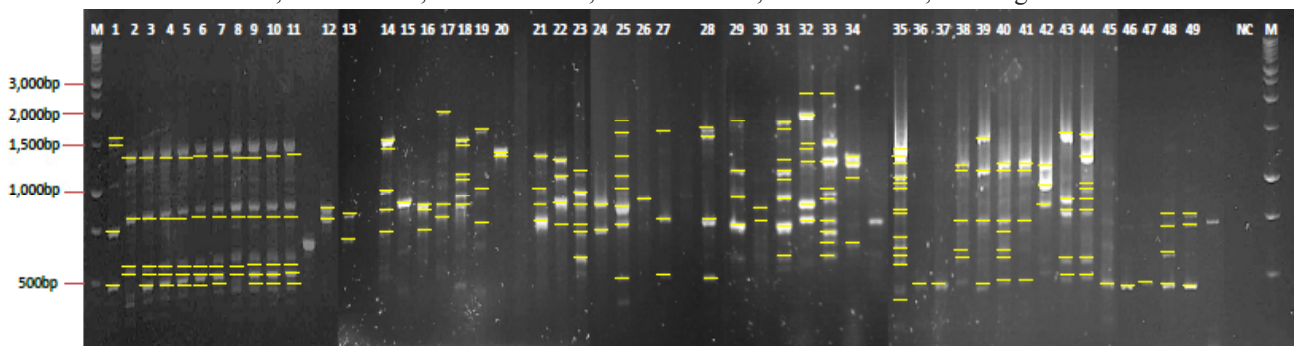

Figure 1c. Banding profiles of $(GTG)_5$ PCR for Sampadi isolates. Lane M: 1kb DNA ladder (Promega, USA), 1: SPD-2S4, 2: SPD-2P1, 3: SPD-2P2, 4: SPD-2P3, 5: SPD-2P4, 6: SPD-2P5, 7: SPD-2P6, 8: SPD-2P7, 9: SPD-2P8, 10: SPD-2P9, 11: SPD-2P10, 12: SPD-2W3, 13: SPD-S1, 14: SPD-S3, 15: SPD-S5, 16: SPD-S6, 17: SPD-S7, 18: SPD-S8, 19: SPD-S9, 20: SPD-S10, 21: SPD-P3, 22: SPD-P4, 23: SPD-P5, 24: SPD-P6, 25: SPD-P8, 26: SPD-25, 27: SPD-2S1, 28: SPD-2S3, 29: SPD-P1, 30: SPD-P7, 31: SPD-2W1, 32: SPD-2W4, 33: SPD-2W6, 34: SPD-2W8, 35: SPD-W1, 36: SPD-W2, 37: SPD-W3, 38: SPD-W4, 39: SPD-W6, 40: SPD-W7, 41: SPD- W9, 42: SPD-S2, 43: SPD-P10, 44: SPD-2S2, 45: SPD-2S9, 46: SPD-2S5, 47: SPD-2S6, 48: SPD-2S7, 49: SPD-2S8, NC: negative control.

high discrimination power when compared to other DNA fingerprinting or rep-PCR techniques. Above all, $(GTG)_5$ PCR primer was not designed based on specific sequence.

After the attempt of dendrographic analysis on the $(GTG)_5$ banding profiles, all the constructed dendrogram tree consists of two major clusters and many sub-clusters. As observed in the dendrograms (Figure 2a, 2b and 2c), some isolates from environment (water and sediment) samples were clustered

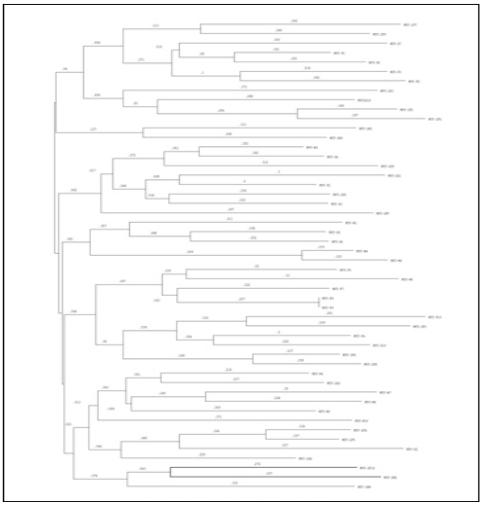

Figure 2a. Dendrogram of isolates from Miri.

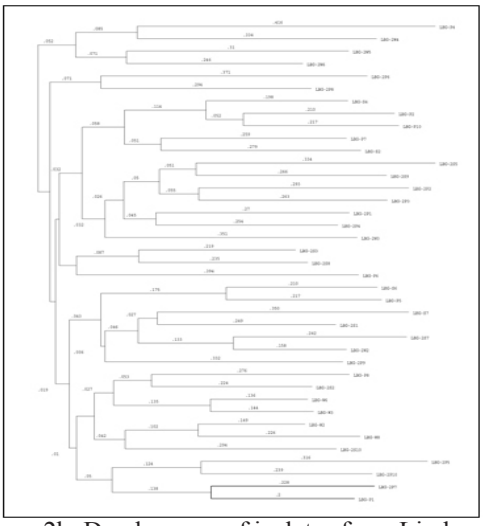

Figure 2b. Dendrogram of isolates from Limbang.

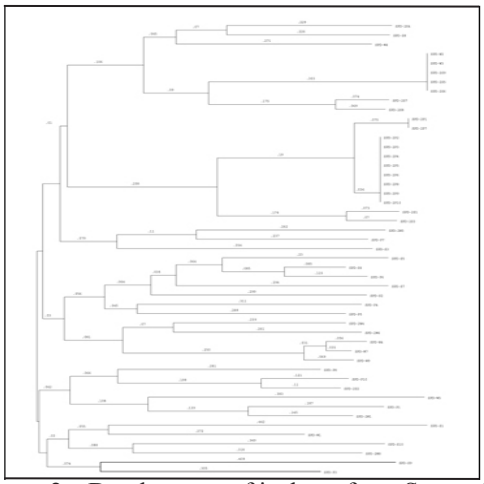

Figure 2c. Dendrogram of isolates from Sampadi.

together with the isolates from the cultured organism indicating that these isolates were closely related with one another. This is expected as these bacteria belong to the same niche. The cultured organism may be contaminated with the bacteria from the sediment and the water in the aquaculture farm.

The bacteria distribution in aquaculture pond and its environment is highly diverse. There are many factors contributing to the high bacterial diversity in the aquaculture pond and its environment such as human activity and animal activity. The bacterial isolated from Sampadi aquaculture farm is more homogeneous than other locations because the aquaculture system in Sampadi was less exposed to human activities. The Sampadi aquaculture farm is located in a remote area. The management does not allow individuals to enter the farms without permission and without wearing the provided boots and transportation. This is to reduce contamination from being introduced into the aquaculture system. In the aquaculture pond itself, nettings were installed on top of every pond to prevent birds from feeding on the cultured shrimps. Birds may also be the carrier of bacterial contamination. The aquaculture system in Limbang and Miri were more exposed to human and animal activities.

Table 1. Bacteria identification using 16S rRNA sequences

Isolates	Bacteria Identity	% similarity	Isolates	Bacteria Identity	% similarity
MYY-2S4	*Bacillus cereus*	99%	**LBG-P6**	*Bacillus pumilus*	99%
MYY-W6	*Staphylococcus sciuri*	99%	**LBG-2W5**	*Serratia marcesencs*	100%
MYY-W9	*Enterobacter hormaechei*	99%	**LBG-W8**	*Bacillus vietnamensis*	99%
MYY-2W4	*Acinetobacter* sp.	99%	**LBG-W6**	*Stenotrophomonas maltophilia*	99%
MYY-2W7	*Bacillus* sp.	99%	**LBG-S4**	*Bacillus subtilis*	99%
MYY-F9	*Escherichia coli*	99%	**LBG-2P6**	*Serratia liquefaciens*	97%
MYY-2F4	*Aeromonas punctata*	99%	**LBG-2P9**	*Microbacterium* sp.	99%
MYY-2F7	*Bacillus megaterium*	99%	**SPD W7**	*Staphylococcus* sp.	99%
MYY-S10	*Bacillus megaterium*	99%	**SPD W5**	*Vibrio* sp.	99%
MYY-2S5	*Bacillus* sp.	100%	**SPD W3**	*Staphylococcus* sp.	99%
MYY-S9	*Bacillus cereus*	99%	**SPD 2W4**	*Bacillus cereus*	99%
MYY-S3	*Bacillus pumilus*	99%	**SPD-W1**	*Staphylococcus saprophyticus*	99%
MYY-2W3	*Aeromonas jandaei*	99%	**SPD-S8**	*Bacillus* sp.	99%
MYY-S5	*Pseudomonas* sp.	99%	**SPD-2S4**	*Bacillus cereus*	99%
MYY-2F8	*Aeromonas jandaei*	99%	**SPD-S7**	*Exiguobacterium profundum*	99%
MYY-2F2	*Staphylococcus xylosus*	99%	**SPD-2S3**	*Bacillus cereus*	99%
LBG-2S8	*Bacillus jeotgali*	99%	**SPD-S6**	*Vibrio fischeri*	99%
LBG-2W2	*Chryseobacterium* sp.	99%	**SPD-2P1**	*Acinetobacter* sp.	99%
LBG-2S5	*Bacillus infantis*	99%	**SPD-2P8**	*Acinetobacter calcoaceticus*	99%
LBG-2W3	*Acinetobacter* sp.	99%	**SPD-P4**	*Bacillus cereus*	99%
LBG-S7	*Bacillus pumilus*	99%	**SPD-P7**	*Vibrio rotiferianus*	99%
LBG-2S10	*Bacillus* sp.	94%	**SPD-P10**	*Bacillus cereus*	99%
LBG-2P7	*Staphylococcus xylosus*	99%	**SPD-P1**	*Staphylococcus* sp.	99%
LBG-P2	*Staphylococcus* sp.	99%	**SPD-S9**	*Staphylococcus saprophyticus*	99%
LBG-2P3	*Staphylococcus saprophyticus*	99%			

After the dendrograms were generated based on the $(GTG)_5$ PCR analysis, the representative isolates were selected for bacteria identification by 16S rRNA PCR analysis. In fact, the 16S rRNA is particularly notable, even though other genes, for instances 23S rRNA, 16S-23S intergenic spacer region and the *gyrB* gene, have been used (Dorsch *et al*., 1992; Venkateswaran *et al*., 1998; Chun *et al.*, 1999; Gomez-Gil *et al*., 2004). There were several reasons that make the 16S rRNA chosen as the gene to sequence. The 16S rRNA gene sequence is 1542 bp in length and consists of both conserved and variable regions (Clarridge, 2004). The gene is adequate with interspecific polymorphisms of 16S rRNA, which is essential in contributing a discriminatively and statistically authentic measurement (Clarridge, 2004). Usually, universal primers are selected as complementary to the conserved regions and the sequence of the variable region is used for the comparative taxonomy (Chen *et al*., 1989; Relman,

1999; Clarridge, 2004). In addition, the 16S rRNA gene sequence has been widely used to determine a large amount of bacterial strains and there are many deposited sequences for comparing the sequence of an unknown strain (Clarridge, 2004). Besides, the 16S rRNA gene allows the relationships among all bacteria to be measured, since it is a universal gene in all bacteria (Woese, 1987; Woese *et al*., 1985). Generally, the 16S rRNA gene sequences comparison allows the discriminating of bacteria at the genus level and the classifying strains at multiple levels (Clarridge, 2004).

Among the 138 screened bacteria isolates, only 49 isolates were selected for bacteria identification using the 16S rRNA PCR analysis and DNA sequencing. The list of isolates along with its identity is tabulated in Table 1. The identification of the bacteria partial 16S rRNA gene revealed thirteen bacterial genera. *Bacillus* sp., mainly isolated from sediment samples, dominated the aquaculture environment with 41%, followed by *Staphylococcus* sp. (21%), *Acinetobacter* sp. (8%), *Vibrio* sp. and *Aeromonas* sp. (6%) and *Serratia* sp. (4%). There was 14% of the isolated bacteria were categorized as other genera, which includes *Escherichia* sp., *Chryseobacterium* sp., *Microbacterium* sp., *Stenotrophomonas* sp., *Pseudomonas* sp., *Enterobacter* sp. and *Exiguobacterium* sp.. In fact, *Bacillus* sp. was a common soil bacterium and can be found abundantly in the soil. This explains the domination of this bacterial species in the aquaculture environment.

Conclusion

Phylogenetic analysis of the $(GTG)_5$ PCR was conducted to screen for clonal isolates for every location. This is to prevent sequencing of the same clonal isolates, thus, reducing the cost for DNA sequencing. Through $(GTG)_5$ PCR, it was revealed that the Limbang and Miri bacteria isolates were highly diverse. Sampadi bacteria isolates were less diverse when compared to Limbang and Miri. This may be due to the different type of activities and practices carried out at the aquaculture farm. Selected bacteria isolates from the $(GTG)_5$ PCR analysis were successfully identified using 16S rRNA PCR and DNA sequencing. Molecular identification has revealed thirteen bacteria genera isolated from the aquaculture farms and their environment.

Acknowledgement

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