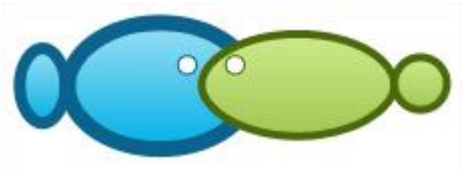

# Characterization of gut bacterial diversity of wild broodstock of *Penaeus monodon* and *Fenneropenaeus merguiensis* using PCR-DGGE

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**Abstract**. Black tiger shrimp (*Penaeus monodon*) and banana shrimp (*Fenneropenaeus merguiensis*) are commercially important aquatic species in South East Asia. However, there is a lack study on these shrimp gut bacterial community compared to Pacific white shrimp (*Litopenaeus vannamei*). Our study represents the first attempt to i) characterize the gut bacterial community of *P. monodon* and *F. merguiensis* broodstocks caught from the wild using PCR-DGGE (Denaturing Gradient Gel Electrophoresis) fingerprinting, and ii) compare the gut bacterial community of both species using cluster analysis. Gut bacterial community related with *P. monodon* was more diverse and dissimilar to those of *P. merguiensis* from the results of DGGE fingerprinting and cluster analysis. The sequences from DGGE bands showed that *Vibrio*, *Photobacterium*, and Fusobacteria were composed of core bacterial member in both shrimp gut, whereas Clostridia, Mollicutes and *Ferrimonas* spp. were only found in the gut of *P. monodon*. Our comparative analysis by means of cluster analysis revealed significant difference in the gut bacterial community between wild-caught *P. monodon* and *P. merguiensis* broodstocks, suggesting the possibility of host species-specific gut bacterial community. This study might be a stepping stone to develop host health management and improvement program, specifically for shrimp broodstock aquaculture.

**Key Words**: cluster analyse, gut bacterial community, *F. merguiensis*, *P. monodon*, black tiger shrimp, banana shrimp.

**Introduction**. The worldwide increasing consumption of shrimps has encouraged the expansion of shrimp farming in many tropical countries including Malaysia. In Malaysia, black tiger shrimp (*Penaeus monodon*) and banana shrimp (*Fenneropenaeus merguiensis*) are commercially important marine shrimp (FAO 2009). However, there is still lack of research on *F. merguiensis* and *P. monodon* in Malaysia especially on the aspect of microbiology including gut bacterial community. Most previous research on both species focused on the field of population biology and breeding (Loneragan et al 2005; Ikhwanuddin et al 2013; Nahavandi et al 2013).

Gut bacterial community constitutes a complex and dynamic ecosystem, and they play important roles in food digestion, nutrient absorption, and health of host aquatic organisms (Nayak 2010). Therefore, taxonomic studies and functional profiling of the gut bacterial community is essential to discover potential probiotics, pathogenic bacteria and other microorganisms that have profound influence on the overall host health and physiological functions (Wu et al 2010; Lamendella et al 2011). Moreover, environmental parameters such as geographical location, temperature and host physiological parameter also play major role in constructing the gut bacterial community (Wong & Rawls 2012; Pierce et al 2016).

Most microbiological research on shrimp focused on the effects of probiotics on growth and survival rate, digestive enzyme activities, disease resistance, and immune responses (Vaseeharan & Ramasamy 2003; Castex et al 2009; Zokaeifar et al 2012; Maeda et al 2014). Although there are increasing number of research on shrimp gut microbiota in recent years, knowledge on shrimp gut bacterial community is still scarce

when compared to fish (Huang et al 2014; Cheung et al 2015; Zhang et al 2016; Zheng et al 2016). Their results suggested that composition of gut bacterial community is profoundly influenced by life stages and health conditions of the host as well as environmental factors such as salinity.

To date, gut microbiota research has expanded from the conventional culture dependent method to molecular method. Based on culture dependent method, Moss et al (2000) suggested that *Vibrio* spp. and *Aeromonas* spp. were predominant bacteria of Pacific white shrimp (*Litopenaeus vannamei*), whereas bacteria detection method using molecular technique by Zhang et al (2014) revealed that Proteobacteria and Tenericutes were the core gut bacteria. The outcome difference suggested that molecular method is far superior in detecting vast coverage of gut microbiota, and thus is a much robust and practical method. Other commercially important shrimp species, which have been studied so far, includes *Fenneropenaeus chinensis* (Liu et al 2011), *Penaeus notialis* (Dabadé et al 2016) and *Penaeus penicillatus* (Wang et al 2014). Notably, these studies come to the consensus that different host species composed of different gut bacterial community. Despite nominal research on gut bacterial community have been conducted on *P. monodon* (Chaiyapechara et al 2012; Rungrassamee et al 2014) and *F. merguiensis* (Oxley et al 2002) in other regions, no similar study has been carried out for wild-caught shrimp in Malaysia. In this study, we characterized and compared gut bacterial community of *P. monodon* and *F. merguiensis* wild-caught broodstocks using PCR-DGGE (Denaturing Gradient Gel Electrophoresis) fingerprinting.

The objective of this study is a particularly through characterization of the gut bacterial community to understand the relationship between host and gut bacterial community. This information is useful to provide insight to the host health and future experiment such as utilization of probiotics in shrimp aquaculture.

## Material and Method

***Sample collection***. Wild broodstock of *P. monodon* and *F. merguiensis* were collected from Setiu Wetland (Terengganu, Malaysia) and transported to the laboratory under continuously oxygenated condition. Average shrimp weight was 12.95±1.24 g for *P. monodon* and 36.79±1.06 g for *F. merguiensis*. Each shrimp sample (7 individuals/sample) was anaesthetized on ice for 5-10 min, and their gut were aseptically dissected and stored at –80$^{o}$C until DNA extraction. This study was also reviewed and approved by the Universiti Malaysia Terengganu ethics board.

***Extraction of bacterial genomic DNA and construction of denaturing gradient gel electrophoresis (DGGE) fingerprintings for PCR amplicons***. Bacterial genomic DNA from each section was extracted using Promega DNA purification system (Promega, Madison, WI, USA) according to the manufacturer's instruction. To obtain fingerprints of bacterial community from different samples, V3 region of the bacterial 16S rDNA was amplified using universal primer set 338F (5'- CCT ACG GGA GGC AGC AG -3') with 40 bp GC clamp and 519R (5'- CCG TCA ATT CCT TTG AGT TT -3') (Rungrassamee et al 2013) was used to amplify 16S rDNA from total community DNA (100 ng) extracted for each sample by PCR. The PCR reaction mixture (40 µL final volume) contained 1.25 U TaKaRa Ex Taq polymerase (Takara Bio, Shiga, Japan), 1 x PCR buffer, 200 µM for each dNTP, 10 pmol for each primer. The PCR cycling was performed using the following condition; 94°C for 5 min followed by 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 3 min, and terminated at 72°C 10 min. The reaction products were analyzed by electrophoresis in 2.0% (w v$^{-1}$) agarose gel containing SYBR® Safe (1 ng mL$^{-1}$) (Invitrogen, USA).
The PCR amplicons were analyzed by Denaturing Gradient Gel Electrophoresis (DGGE) using a Cipher Denaturing Gradient Gel Electrophoresis Systems Model DGGEK-2401 (C.B.S.Scientific, California, USA) according to the instruction of the manufacturer. The 16S rDNA amplicons (500 ng) were loaded onto 8% (v v$^{-1}$) polyacrylamide gel in 1 x TAE with 20 to 45% gradient urea-formamide (100% corresponded to 7 M urea and 30% (v v$^{-1}$) formamide). Electrophoresis was conducted with a constant voltage of 60V for 20 min and 200 V for 5.5 h in 1 x TAE buffer at 60ºC. The gel was stained in an SYBR safe

solution (2 µg $mL^{-1}$) in TAE buffer for 30 min and de-stained in distilled water for 20 min. The presence and absence of bands information was photographed and exported for further analysis using Bio-vision + 1000/26MX (Vilber Lourmat, France). Thereafter, DNA was obtained by elution of the excised bands into 20 µL of distilled water at 4ºC overnight. Approximately 2 µL elute from individual bands were re-amplified using 338F and 519R, and were run on 2.0% (w $v^{-1}$) agarose gel containing SYBR ® Safe (Invitrogen, USA).

***Sequencing and biodiversity analysis***. The PCR amplicons produced by re-amplification of the excised and eluted bands were purified and sequenced at an outsource company (First BASE Laboratories Sdn Bhd, Malaysia) with the same primers used to produce them. The resulting chromatograms of DNA sequences were examined using Chromas Lite (Technelysium Pty Ltd, Australia). The sequences were checked for chimeric constructs by using the Chimera Check program using Decipher (Wright et al 2012). Homology searches were performed using sequences of ~ 200bp for amplicons from DGGE bands and close relatives were determined in GenBank databases using BLAST available through the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/). Multiple alignments and calculation of distant matrixes were performed using MEGA version 6 (Tamura et al 2013). A phylogenetic tree was constructed using Neighbor-joining analysis function of MEGA version 6 with 1,000 replicates in the bootstrap analysis and distances were estimated with the Jukes-Cantor correction.

In order to investigate biodiversity analysis of DGGE profiles, Simpson's diversity index (1−λ), Chao1 index and Shannon–Wiener Index (*H'*) were calculated using PAST software (Hammer et al 2001). Cluster analysis was used to compare the bacterial communities based on Bray-Curtis dissimilarity index using PAST software. The sequences obtained in this study have been deposited in the DDBJ/EMBL/GenBank under the accession numbers LC114002- LC114016.

**Results and Discussion**. Recently, there is an increasing research trend on gut bacterial community of crustaceans (Meziti & Kormas 2013; Rungrassamee et al 2013). However, shrimp gut bacterial community in Malaysia has not been researched although shrimp aquaculture is one of the mainstream industries. PCR-DGGE fingerprinting technique is one of the culture independent method to overcome problems associated with cultivable methods and can be utilized with statistic and biodiversity analysis to compare bacterial community exposed to various factors such as different environmental condition and diet (Marzorati et al 2008). Recently, PCR-DGGE is widely applied to reveal bacterial community associated with aquatic invertebrates (Li et al 2012; Bekaert et al 2015). The DGGE profiles demonstrated the gut bacterial diversity of black tiger shrimp and banana shrimp in this study is given in Figure 1.

The result of Shannon–Wiener Index (*H'*) and Margalef species richness showed that gut bacterial community of *P. monodon* was significantly more diverse and rich than that of *F. merguiensis* (Figure 2). Similarly, DGGE banding patterns and cluster analysis indicated that the gut bacterial community composition is significantly different between *P. monodon* and *F. merguiensis* (Figure 1 and Figure 3). Interestingly, gut bacterial community composition was markedly similar when compared within the gut bacterial community of *P. monodon* broodstocks via cluster analysis (Figure 3). In contrast, the gut bacterial community of each *F. merguiensis* broodstock was significantly dissimilar. This suggests that the diversity of gut bacterial diversity in shrimp was largely independent of their host phylogeny. Our data is congruent with results from previous studies on other aquatic species including fish (Larsen et al 2014; Skrodenytė-Arbačiauskienė et al 2008) and shrimp (Rungrassamee et al 2016), which showed significant difference in bacterial community composition even though collected from individuals of similar populations and habitat. Indeed, host genetics have a greater influence on gut bacterial community composition than host habitats (Tzeng et al 2015). Thus, further investigation is needed to reveal the relationship between gut bacterial diversity and host phylogeny, habitats and ecological characteristics of both shrimp species.

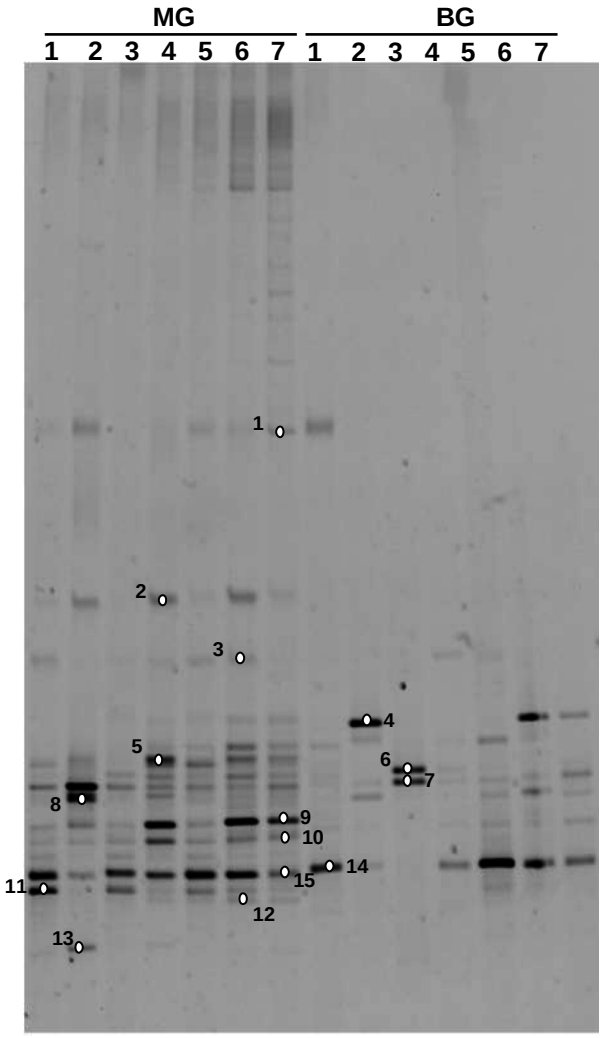


Figure 1. Denaturing gradient gel electrophoresis band profile of gut bacterial community associated with *Penaeus monodon* (MG) and *Fenneropenaeus merguiensis* (BG). Each lane shows the bacterial composition of one individual. The circles with number show the sequenced bands.

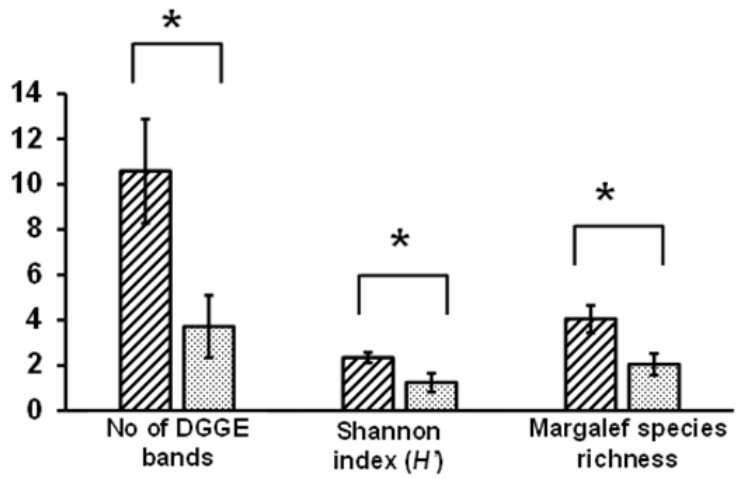


Figure 2. Number of DGGE bands as OTUs, Shannon index H', and Margalef species richness of the gut bacterial community of *P. monodon* (MG: ▨) and *P. merguiensis* (BG: ▩). Error bars represent standard error (n = 7) and * indicates significant difference ($P<0.05$).

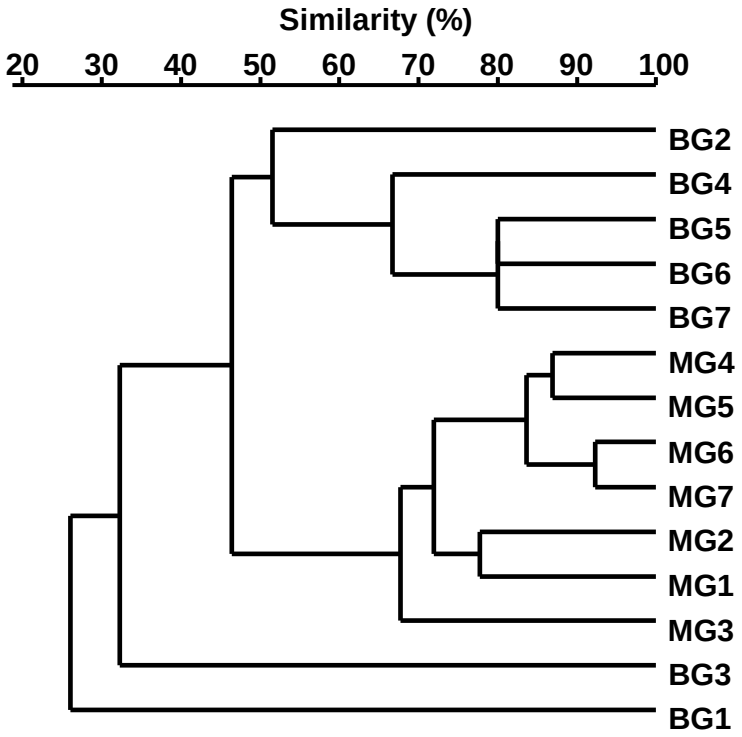


Figure 3. Cluster analysis of 16S rDNA PCR-DGGE profiles of individual shrimp gut sample. Cluster analysis was performed using UPGMA method based on Bray-Curtis dissimilarity index.

The 16S rRNA gene sequence data showed that 8 out of 15 bands (S2, S5, S6, S10, S11, S12, S14, and S15) were affiliated to γ-Proteobacteria; bands S7 and S8 were affiliated to Fusobacteriia; bands S9 and S3 were affiliated to Clostridia and Mollicutes, respectively (Table 1 and Figure 3). Some DGGE bands such as S1, S4-S8, S10, and S15 were composed of gut microbiota of both *P. monodon* and *F. merguiensis*. These bands were similar by 95-100% to *Vibrio neptunius*, *Photobacterium damselae*, uncultured *Propionigenium* sp. (class Fusobacteriia), *Vibrio* sp., uncultured Fusobacteria, uncultured Firmicutes, and uncultured bacterium clones. *Vibrio* spp. and *P. damselae* have been associated with shrimp gut bacterial community (Rungrassamee et al 2014; Oxley et al 2002), in which *P. damselae* is known as opportunistic pathogen for marine animals (Rivas et al 2013). To date, *Propigenium* spp. have been found in the gut of abalone (*Haliotis discus hannai*) (Tanaka et al 2004), sea cucumber (*Holothuria scabra*) (Plotieau et al 2013) and sea squirt (Dishaw et al 2014). *Propioigenium* spp. are detected in anoxic marine sediment and thrived in that oxygen deprived condition through decarboxylation of succinate to propionate (Schink 2006).

Our DGGE profiles demonstrated unique banding patterns for *P. monodon*, which could be used to differentiate them from other shrimp species. The bands of S2, S3, S9, and S11-S14 were only found in *P. monodon* gut, and displayed high similarity of between 93 and 100% with *Vibrio brasiliensis*, uncultured Mycoplasmataceae (class Mollicutes), Clostridia bacterium, *Ferrimonas* spp. We observed consistency of our data with previous findings, in which Mollicutes, Clostridia, Fusobacteriia, and *Ferrimonas* spp. composed the majority of the gut bacterial community associated with black tiger shrimp, suggesting that these bacteria are exclusively specific to black tiger shrimp (Chaiyapechara et al 2012).

Table 1

Representative DGGE band sequences from the shrimp gut samples

| *DGGE band* | *Base pair* | *Affiliation phylum/class* | *Closest relative* | *Similarity (%)* |
|---|---|---|---|---|
| S1 | 153 | Firmicutes | Uncultured *Firmicutes* bacterium clone 21G-6 (KC918335) | 95 |
| S2 | 199 | Proteobacteria/γ-proteobacteria | *Vibrio brasiliensis* strain FD W9 (JN871709) | 98 |
| S3 | 198 | Tenericutes/Mollicutes | Uncultured Mycoplasmataceae bacterium clone 17G-27 (KC918211) | 96 |
| S4 | 197 | Unidentified | Uncultured bacterium clone G7DUZBG01BKJZN (KF327838) | 98 |
| S5 | 198 | Proteobacteria/γ-proteobacteria | Uncultured *Vibrio* sp. clone HA_39 (KF859576) | 99 |
| S6 | 199 | Proteobacteria/γ-proteobacteria | *Photobacterium damselae* strain P12 (KR075017) | 99 |
| S7 | 177 | Fusobacteria | Uncultured Fusobacteria bacterium clone 2-F6 (HE611101) | 99 |
| S8 | 178 | Fusobacteria/Fusobacteriia | Uncultured *Propionigenium* sp. clone 16G-6 (KC918186) | 100 |
| S9 | 178 | Firmicutes/Clostridia | Clostridia bacterium TSAR14 (KC854383) | 93 |
| S10 | 196 | Proteobacteria/γ-proteobacteria | *Vibrio* sp. DZ141002 (KU245721) | 99 |
| S11 | 199 | Proteobacteria/γ-proteobacteria | *Ferrimonas* sp. NBRC 104251 (AB682171) | 99 |
| S12 | 194 | Proteobacteria/γ-proteobacteria | *Ferrimonas gelatinilytica* strain CJ24 (JQ806740) | 95 |
| S13 | 198 | Unidentified | Uncultured bacterium clone GXTJ5A301AE5HJ (KF334436) | 99 |
| S14 | 200 | Proteobacteria/γ-proteobacteria | *Vibrio neptunius* strain ZS15 (HQ538769) | 97 |
| S15 | 198 | Proteobacteria/γ-proteobacteria | *Vibrio brasiliensis* strain G9C_35m_05 (KM041167) | 100 |

Mycoplasma were known as dominant bacteria in oyster (*Crassostrea virginica*) gut (King et al 2012) and octopus (*Octopus mimus*) gut (Iehata et al 2015), with possibility in utilizing cytoplasmic secretion to produce lactic and acetic acids in host gut (Holben et al 2002). Clostridia were recovered from *Centroscyllium fabricii* gut (Bindiya et al 2015) and temperate marine herbivorous fishes (Clements et al 2007), with the capability of breaking down proteins as well as producing amylase and cellulase (Ray et al 2012; Mengoni et al 2013; Tzeng et al 2015). Given the lack of information on the functional roles of these gut bacteria in shrimp, further study in this aspect is necessary.

To our knowledge, this study provides the first report on gut bacterial community of two important shrimp species (*P. monodon* and *F. merguiensis*) using PCR-DGGE fingerprinting technique. Our result suggests that some bacteria such as Mollicutes, Clostridia, and *Ferrimonas* spp. are the core bacteria of *P. monodon* gut, although their functional roles in the host gut is poorly understood. Interestingly, besides showing overlapping DGGE banding patterns with *P. monodon*, *F. merguiensis* displayed marked discrepancy in the gut bacterial community despite similar host taxa and habitats. This divergence showed inconsistent relationship between *F. merguiensis* and gut microbiota, suggesting the deviation from the host-specific gut bacteria community model. Our study also prompts the need to proceed with deep investigation on host-gut bacteria relationship and the main roles played by the gut bacterial community in maintaining the health of shrimp broodstocks.

**Conclusions**. In conclusion, by means of PCR-DGGE fingerprinting method, this study reveal that the different gut bacterial community composition found from the different shrimp species and host phylogeny might not largely involve in gut bacterial community composition. These finding suggest that different shrimp has unique gut bacterial community and provides the possibility of host species specific gut microbiota. In addition, integration of PCR-DGGE fingerprinting and biodiversity analysis is suitable for comparative analysis of gut microbiota.

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