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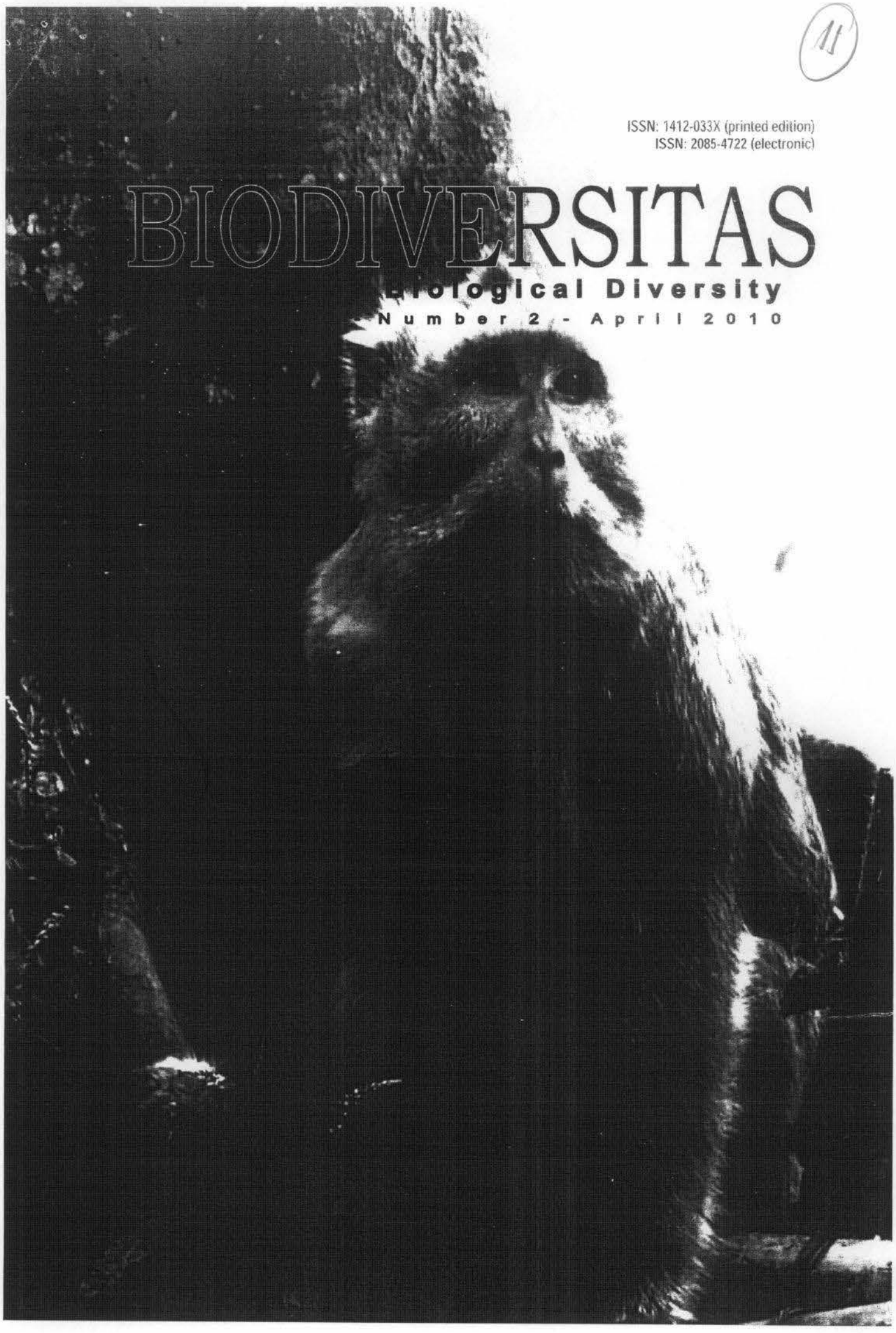

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Bacterial communities associated with white shrimp (*Litopenaeus vannamei*) larvae at early developmental stages

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ABSTRACT

*Bacterial communities associated with white shrimp (*Litopenaeus vannamei*) larvae at early developmental stages. Biodiversitas 11 (2): 65-68.* Terminal Restriction Fragment Length Polymorphism (T-RFLP) was used to monitor the dynamics of the bacterial communities associated with early developmental stages of white shrimp (*Litopenaeus vannamei*) larvae. Samples for analysis were egg, hatching nauplii, 24 hours old nauplii, and 48 hours old nauplii which were collected from one cycle of production at commercial hatchery. T-RFLP results indicated that the bacterial community associated with early stages of shrimp development might be transferred vertically from broodstock via egg. There was no significant difference between bacterial communities investigated, except the bacterial community of 48 hours old nauplii. Diversity analyses showed that the bacterial community of egg had the highest diversity and evenness, meanwhile the bacterial community of 48 hours old nauplii had the lowest diversity. Nine phylotypes were found at all stages with high abundance. Those TRFs were identified as γ- proteobacteria, α-proteobacteria, and bacteroidetes group.

Key words: *Litopenaeus vannamei*, bacterial community, T-RFLP.

INTRODUCTION

White shrimp (*Litopenaeus vannamei*) is one of the major cultured shrimp species in the world. Since the year 2000, *L. vannamei* production is growing rapidly. In Indonesia, *L. vannamei* production increased five fold in five years between 2000 and 2005 and is expected to outpace the other species in the next few years. An increasing demand for white shrimp had forced intensive culture of this species, which brought many problems due to increasing disease outbreaks caused by microorganism that lead to mass mortality. A number of emerging reports indicated that microbial community plays a major role in aquaculture. Microbiota that lived in association with aquatic animal may enhance host growth and survival by producing some digestive enzymes (Sugita et al. 1995; Seeto et al. 1996; Izvekova 2006), out-competing pathogenic bacteria, and supplying essential compound important for host metabolism. Rapid growth of shrimp occurred in unfiltered pond water, which contained organic particle including bacteria (Moss and Pruder 1995).

Most of the works to study the microbial community of shrimp were done based on the culture method. This method has limitation, since less than 1% of bacteria that have been successfully cultured in artificial media until now (Amann et al. 1995; Rappe and Giovannoni 2003). Artificial medium and culture condition preferred the growth of particular group of bacteria. Molecular methods based on the amplification of 16S rRNA genes were used to overcome this problem. This gene is ubiquitous and highly conserved among procaryotic organisms; make it useful as molecular marker for microbial community analyses. One of the techniques that used this approach was terminal restriction fragment length polymorphism analysis (T-RFLP). T-RFLP provides several advantages over other techniques because it saves time and cost especially when there are many samples to be analyzed. T-RFLP has been suggested more sensitive and has a greater resolution than other fingerprinting techniques such as Denaturing Gradient Gel Electrophoresis (Marsh 1999). However, this technique has limitation in accurate identification of species in the community, since one terminal restriction fragment (TRF) can be generated from multiple taxa.

This research was aimed to characterize bacterial community associated with white shrimp larvae at early developmental stages. Characterization of typical microorganism profiles will provide a basis for future work to understand the host-microbe interaction and the efficacy of some practices in shrimp farming.

MATERIALS AND METHODS

Sample preparation

Samples of egg and nauplii were from a single cycle of production, obtained from the hatchery of PT. Central Pertiwi Bahari, Lampung, Indonesia. Nauplii were sampled 3 times, i.e. just after hatching, 24 hours after hatching, and 48 hours after hatching. Prior to DNA extraction, 0.1 g of each egg or larvae samples was washed 3 times in 0.85%

sterile NaCl on sterile filter paper to minimize non-associated microorganisms.

DNA isolation

Bacterial DNA was extracted from egg/larvae samples. DNA isolation was performed employing UltraClean Soil DNA Isolation kit (MoBio, California). Egg or larvae sample was homogenized in lyses buffer provided in the kit. Lysozyme with final concentration of 10 mg/ml was added to the homogenate and then incubated at 37°C for 1 hour. Further procedure followed the instruction suggested by manufacturer.

PCR amplification

63f primer that 5' end labeled (5'-(6FAM) CAGGCCTAACACATGCAAGTC-3') and 1387r primer (5'-CCCGGGAACGTATTCACCGC-3') were used to amplify 16S rRNA gene (Marchesi et al. 1998). Reaction mixtures for PCR contained 100 ng DNA, 1x buffer (NEB, MA), 200 µM of each dNTP, 1 U Taq DNA Polymerase (NEB, MA), 5 pmol of each primer, in a final volume of 50 µL. DNA amplification was performed with specifications as follows: 3 minutes denature step at 94°C; 30 cycles of 1 minute at 94°C, 1 minute at 55°C, 1 minute at 72°C; final extension step at 72° C for 10 minutes. PCR product was treated with mung bean nuclease (NEB, MA) to eliminate pseudo TRF (Egert and Friedrich 2003). Then the PCR product was run on 0.8% agarose gel. The DNA band with approximately 1500 bp in size was excised prior to purification using Qiaquick Gel Extraction Kit (Qiagen, Germany).

Restriction enzyme digestion

Purified PCR product was single-digested with *Alu*I or *Rsa*I (NEB, MA) in separate tubes. Reaction mixtures contained 5U enzyme, 1x buffer, 100-200 ng DNA in total volume of 20 µl and incubated in 37°C overnight. Digested DNA was then purified with Qiaquick Nucleotide Removal Kit (Qiagen, Germany) and eluted with 30µl elution buffer.

T-RFLP analysis

1 µl of digested DNA was mixed with 0.5 µl of HD-400 [ROX] as internal standard and then denatured at 95°C for 5 minutes then placed on ice. The length of various TRF was analyzed using an ABIprism™ 3100 Automated DNA Sequencer and determined using GeneScan Programme (Perkin Elmer, Norwalk). The sizes of TRFs were compared with the database of Ribosomal Database Project to identify their closest relatives.

Diversity analyses

Bacterial phylotype richness (S) was expressed as total number of peaks within each sample. Shannon Wiener index (H') and the evenness (E) were calculated to describe the diversity of community and relative importance of each phylotype within the entire assemblage. H' was calculated as follows: $H' = -\Sigma (pi) (\ln pi)$ where pi is the relative abundance of fragment i. Evenness was measured based on equation: $E = H'/Hmax$ where $Hmax = \ln S$ (Margalef 1958).

RESULTS AND DISCUSSION

Results

T-RFLP was employed to monitor the changes in microbial community as the larvae undergo their nauplii developmental stages. Sample data consist of the size in base pair and peak area for each TRF peak in electrophoregram. One TRF is considered as a phylotype while each peak area shows the relative abundance of the TRF. Restriction enzyme *Alu*I yielded greater resolution (produced more TRFs) than *Rsa*I. Therefore, further analyses were conducted based on this enzyme TRFs.

The electrophoregram of T-RFLP result was shown in Figure 1. There were only minor changes in bacterial community composition after the hatching process until the larvae reach Nauplii 1 stage (24 hours after hatching). The

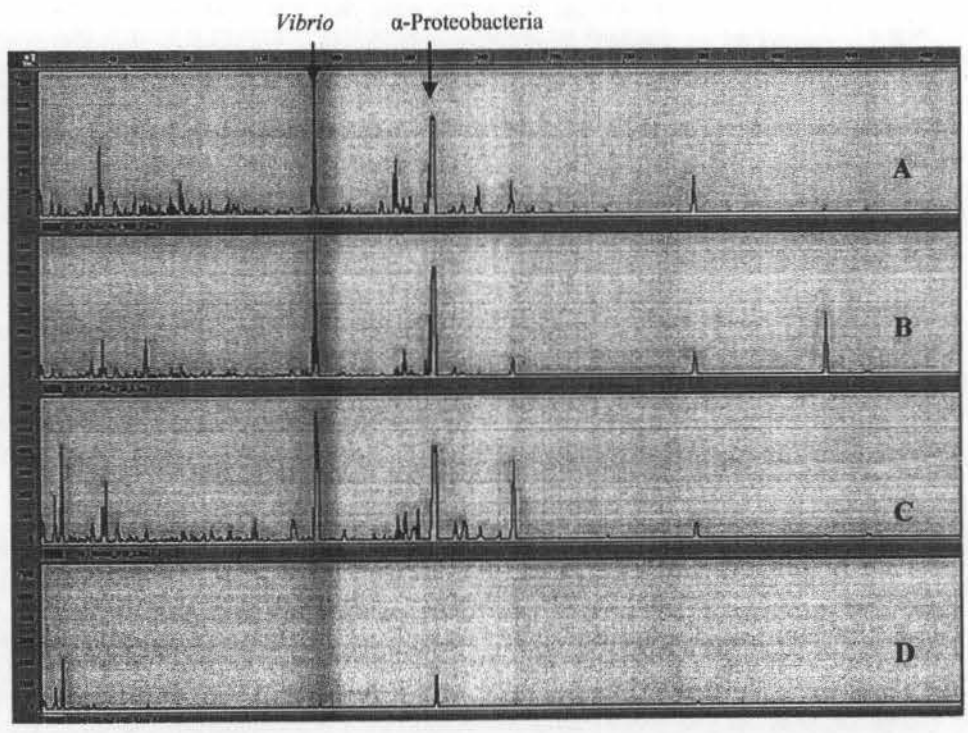

Figure 1. T-RFLP profiles of bacterial communities in early developmental stages of white shrimp larvae: (a) egg, (b) hatching nauplii, (c) 24 h old nauplii, and (d) 48 h old nauplii.

most abundant phylotypes in egg, hatching nauplii, and 24 hours old nauplii were the same, but in 48 hours old nauplii, this phylotype was not dominant.

Bacterial richness, Shannon-Wiener index, and evenness in every stage were shown in Table 1. Bacterial communities associated with early stage of white shrimp larvae development had high diversity and also had high evenness consistently. This result suggesting that the bacterial community in early larvae development was very diverse. High evenness value meant that all phylotype were distributed evenly. There was no phylotype that was really dominant comparing to the others here. However, the diversity of bacterial community which detected at 48 hours old nauplii was sharply decline. This might be related to the molting stage at which the sampling was conducted. The highest diversity and evenness was observed in egg while the lowest was in 48 hours old nauplii.

Table 1. Bacterial diversity in each stage of larvae development.

Stage	S	H'	E	Dominant *AluI* TRF size (bp)	Group
Egg	161	4.24	0.83	152	*Vibrio*
Hatching Nauplii	114	3.68	0.78	152	*Vibrio*
24 h old nauplii	139	3.97	0.81	152	*Vibrio*
48 h old nauplii	10	1.59	0.69	215	α-Proteo-bacteria

Note: S: bacterial richness; H': Shannon-Wiener index; E: evenness.

Some phylotypes could be found all stages of larval development that had been analyzed (Table 2). Nine *AluI* phylotypes were found consistently throughout the entire nauplii stages of larval development, i.e. 37 bp, 149 bp, 152 bp, 213 bp, and 215 bp, which were grouped into γ-proteobacteria class, while 36 bp belonged to bacteroidetes class. Three TRF, 58 bp, 259 bp, 357 bp did not match any species in database. Phylotype that was represented by 152 bp TRF was the most abundant phylotype in bacterial communities of egg and nauplii until 24 hour after hatching.

Table 2. Phylotypes found in all stages of larval development that were analyzed.

TRF size (bp)	Group
36	Bacteroidetes
37	*Pseudomonas*
58	No match in database
149	*Vibrio*
152	*Vibrio*
213	α-Proteobacteria
215	α-Proteobacteria
259	Bacteroidetes
357	No match in database

Discussion

Until now, studies about bacteria that lived associated with white shrimp were only conducted based on culture techniques. The bacteria that were commonly found in this organism were *Vibrio, Staphylococcus, Brevibacterium,* and *Micrococcus* (Goodwin 2005). Moss et al. (2000) found that *Vibrio, Aeromonas,* and *Pseudomonas* dominated the gut of juvenile *L. vannamei,* but according to Vandeberghe et al 1999 (Vandenberghe et al. 1999), *Vibrio* was not the dominant group in *L. vannamei.* To our knowledge, this is the first study to investigate the dynamics of microbial community associated with shrimp larvae employing molecular-based technique.

Each TRF could be identified by matching the size of TRF with database. Not all of TRFs could be unambiguously identified employing RDP database. The limitation of T-RFLP is its ability to identify phylotypes since only a small fragment of the 16S rRNA gene that is analyzed, i.e. the 5' terminal. Many genus of bacteria share the same TRF sizes, makes it difficult to obtain the real identity of TRF. The use of two or more restriction enzymes can reduce the possible identities of each TRF. In this study, the use of two restriction enzymes still gave many possibilities for phylotype identity, at the species level. Therefore, we identified the TRF at class level.

In bacterial community of egg, hatching nauplii, and 24 hours old nauplii, the dominant phylotype belonged to while the dominant phylotype in bacterial community of 48 hours old nauplii belonged to. Overall, Proteobacteria group seemed to be dominant in bacterial community associated with white shrimp larvae. However, since all samples in this study were originated from single hatchery, the results obtained in this study might not necessarily reflect bacterial communities associated with white shrimp larvae derived from other hatcheries. Different environment and culture conditions could lead to different bacterial communities established in other places.

The composition of bacterial community was not significantly different between egg and nauplii stages of larvae. At early stages of development, the gut and immune system of shrimp larvae have not fully developed. The molting process of the host may also have a direct influence on the composition of bacterial community. Dempsey et al. (1989) found high individual variability in the types and the numbers of colony forming units that could be isolated from penaeid shrimp gut that might be attributed to the molting stage of the shrimp. During molting, the exoskeleton and also the chitinous hindgut lining is replaced. A study in millipede showed that the new hindgut lining was devoid of microbes (Crawford et al. 1983). After molting processes, new bacterial communities were established and the environment has great influence to determine the composition of new communities. This could explain why in 48 hours nauplii the diversity was very low. Possibly, the nauplii were sampled just after they undergo the molting process that only a small number of bacteria in the bacterial community newly established.

The establishment of bacterial community in *L. vannamei* larvae is still unclear. The composition of bacterial community at early developmental stages was very similar to the community at egg (sharing 83 phylotype which were the same), suggesting that there had been a vertical transmission from broodstock to larvae. The inner part of egg is sterile, but the surface might be colonized by bacteria that originated from broodstock during spawning process. In this case, the bacteria at the surface of egg are being transferred to nauplii when they hatch. Bacteria from

broodstock determine the composition of bacterial community of larvae, especially at early developmental stages where the additional feed has not introduced yet to the larviculture system. Even though we did not examine the status of bacterial community in broodstock, vertical transmission of bacteria might be one of the important processes for the establishment of bacterial community in *L. vannamei*.

It is also possible that the bacteria in the community associated with larvae at early developmental stage were originated from the water, since the shrimp larvae is a filter feeder. Large amount of water is always taken into the larval gut, makes it an important source of bacteria to occupy the gut. On the other hand, larval faeces are continuously released to the water, bringing bacteria from larval gut into rearing water.

TRFLP analysis showed that two phylotypes were very dominant in comparison to other phylotypes in communities, i.e. 152 bp, 213 bp, and 215 bp. Those phylotypes represented γ-Proteobacteria, α-Proteobacteria, and α-Proteobacteria group respectively. The dominant phylotypes in early developmental stages of larvae could play key roles in determining the survival of shrimp larvae. The 152 bp TRF identified as genus *Vibrio*, which is usually pathogenic to shrimp. However, in this study, the dominant phylotypes apparently did not harm their host as shown by high survival rate of the larvae (data not shown). This finding suggested that not all of *Vibrio* species are pathogenic to shrimp. The role of the diversity in shrimp bacterial community is to maintain the balance between harmful bacteria and the beneficial ones. High diversity could prevent the opportunistic bacteria to growth and cause the disease.

Despite of its limitation in precise identification, T-RFLP proved to be a useful tool for monitoring the population dynamics in complex bacterial community. This technique could be used to detect changes in bacterial community due to specific treatment, such as introduction of feed supplement or probiotics to improve the growth or survival of *L. vannamei* larvae. T-RFLP had been used to monitor the effect of *Lactobacillus acidophilus* NCFM supplementation in rats (Kaplan et al. 2001) or changes in human microbiota after antibiotic treatment and probiotic supplementation (Jernberg et al. 2005). We initiated to study on microbial community in the development of *L. vannamei* larvae employing T-RFLP technique. This study will provide critical data of bacterial community associated with *L. vannamei* larvae for future work in order to increase shrimp production and minimize problems associated with microorganism in aquaculture.

CONCLUSIONS

Terminal Restriction Fragment Length Polymorphism proved to be a useful tool to reveal the diversity in a complex bacterial community which might give information about the establishment of this bacterial community in early development of white shrimp larvae. Bacterial community associated with early developmental stage of white shrimp larvae was very diverse and contained phylotypes that were evenly distributed. Most bacteria in the bacterial community were acquired from vertical transmission via egg.

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