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Phylogenetic analysis of bacterial communities in the shrimp and sea cucumber aquaculture environment in northern China by culturing and PCR–DGGE

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Abstract In this study, we investigated the bacterial communities in the shrimp and sea cucumber culture environment, including shrimp ponds (SP), sea cucumber ponds (SCP), mixed-culture ponds (MCP) and the effluent channel (EC) in Qingdao, China. Bacteria cultivation showed that the counts of heterotrophic, nitrate-reducing and sulfate-reducing bacteria in the sediment of SP were higher than that in the sediment of SCP and MCP, varying between 8.7×10^4 and 1.86×10^6, 2.1×10^4 and 1.1×10^5, and 9.3×10^1 and 1.1×10^4 CFU g^{-1}, respectively. In contrast, the counts of ammonium-oxidizing and nitrifying bacteria in the sediment of SP was lower than that in the sediment of SCP and MCP. Denaturing gradient gel electrophoresis (DGGE) of 16S rDNA gene and dendrogram analyses showed that bacterial diversity in the mixed-culture environment was higher than that in the monocultures. The similarity of bacterial community between EC and SCP or MCP was higher than that between EC and SP. These results indicated that sea cucumber culture played a significant role in influencing the environmental bacterial communities that were composed mainly of Flavobacteriaceae (64.3%), Bacteriodetes (21%) and delta proteobacteria (14.7%), including the genera of *Croceimarina*, *Lutibacter*, *Psychroserpens* and so on. The results explained the benefit of sea cucumber culture in shrimp ponds at the level of microbial ecology.

Keywords Bacteria · Biodiversity · Aquaculture · Shrimp · Sea cucumber · Environment · DGGE

Introduction

The composition of the bacterial community in an aquaculture environment has a strong influence on the internal bacterial flora of farmed animals, which is vital for their

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nutrition, immunity and disease resistance (Luo et al. 2006). At the same time, it also impacts, and is impacted by, the bacterial communities in the nearby marine environments that receive the aquaculture effluents (Guo and Xu 1994). Therefore, more attention has been paid recently to the study of bacterial communities associated with aquaculture environment.

Traditional culturing methods are time-consuming, costly and cannot reflect the actual situation due to the fact that an estimated 99.99% of microorganisms in the natural environment are currently non-culturable (Amann et al. 1995), which prevented the advancement of studies on the bacterial community in shrimp aquaculture environment. Thus, our understanding of the composition of bacterial communities in the complex aquaculture ecosystem is very limited (Guo and Xu 1994; Li et al. 2002a, b, c). More and more molecular techniques that were developed recently have been successfully applied in the microbial ecology studies. For example, denaturing gradient gel electrophoresis (DGGE) is used to study the diversity of microbes based on the sequence difference of PCR products of 16S rRNA gene amplified from different microbes. DGGE was initially designed to detect gene mutations in medical research. Since its first application in bacterial studies (Muyzer et al. 1993), DGGE has been widely used to examine the genetic diversity of uncharacterized microbial populations in a variety of eco-environments, e.g., explosive-polluted soil (Juck et al. 2003), estuaries (Thomas et al. 2006), deep-sea sediments, shrimp digestive organs (Luo et al. 2006) and macroalgae (Staufenberger et al. 2008). However, utilization of DGGE to monitor the bacterial communities in shrimp and sea cucumber culture environments has been less reported.

Since the occurrence of explosive epidemic disease in farmed shrimp in 1993, shrimp culture, as one of the main mariculture industries in China, has been challenged with the problems of disease and pollution (Li 2002; Wang 2003), which resulted in the economic losses in shrimp farming and environment pollution by the untreated effluent from the ponds. Scientists and farmers have been attempting to solve these problems by culturing sea cucumbers in the shrimp ponds (Jiang et al. 2006; Song et al. 2007). Sea cucumber (*Apostichopus japonicu*), as a benthic animal (Liao 1979), is traditionally recognized as a rare type of sea food and medicine. The commercial value of sea cucumber in China has been increasing because it possess sufficient bio-active substances, e.g., *Stichopus japonincus* acid mucopolysaccharide (SJAMP), saponin, phosphorous and proteinase, which are thought to play a role in resisting blood clotting and improving the immune system (Song et al. 2007). Studies have shown that culturing sea cucumbers (*Apostichopus japonicu*) in the shrimp ponds has positive effects on the growth of co-cultured shrimp, leading to a total economic profit increase from \$1.5 to \$56 per m^2 when compared with shrimp monoculture (Jiang et al. 2006). From the ecological point of view, it is generally believed that the benthic-feeding habit of the sea cucumbers contributes to the elevated growth rate of shrimp and economic yields (Liao 1979; Li et al. 1994; Chang et al. 2003; Liu et al. 2006). However, microbial ecology-based evidence for the role of sea cucumber on the growth of shrimp is currently unavailable. Therefore, we analyzed the composition of bacterial communities in the shrimp and sea cucumber culture environment as well as the nearby marine areas by utilization of both culturing and PCR–DGGE approaches. We expected to provide underlying mechanisms to explain the observed benefit of co-culturing sea cumber with shrimp.

Materials and methods

Description of the sites and sampling

The sampling sites were located at a shrimp culture facility in Qingdao, Shandong province in northern China. The facility, composed of shrimp monoculture aquaculture ponds, sea cucumber monoculture ponds and shrimp–sea cucumber mixed ponds, is a typical unit for shrimp farming in northern China for more than 20 years. The water in these ponds is exchanged with natural sea water based on the tide and the effluents from all these ponds flow into the Yellow Sea through a channel. The sediment and water samples were collected from a shrimp monoculture pond (Shrimp A and B), a sea cucumber monoculture pond (Sea cucumber), a shrimp–sea cucumber mixed farming pond (Shrimp + Sea cucumber) and the effluent channel (Shrimp Eff) in July 2006. Three samples were collected from each pond or effluent channel and were placed in a sterilized 50-ml Facol tube, respectively. Three water samples (500 ml) were collected from each kind of pond or channel. The samples were transferred to the laboratory on ice as soon as possible. Each sample was divided into several portions and inoculated to various media immediately. One portion of each sample was stored at −20°C for molecular biological analysis.

Detection of bacteria belonging to different physiological groups using culturing methods

A ten fold serial dilution was carried out for the sediment and water samples using the sterilized sea water, and aliquots (0.1 ml) of each dilution were spread onto Zobell's 2216E sea water medium for CFU counting of total heterotrophic bacteria. At the same time, aliquots (0.1 ml) of each dilution were spread onto selective media for ammonia-oxidizing bacteria and nitrifying bacteria counting (Li et al. 2002a). CFU was counted after incubation of the plate at 28°C for 2–3 days.

The sulfate-reducing and nitrate-reducing bacteria were detected by using "Most Possible Number" method as described previously (Chen et al. 1987; Li et al. 2002b). Briefly, 1-ml aliquots of a dilution series were added into 10 ml of the media with three tubes for each dilution. The results were recorded after incubating for 14 and 7 days at 28°C for sulfate- and nitrate-reducing bacteria, respectively. The numbers of bacteria were obtained by referring to a table based on the number of tubes with a positive outcome at each dilution. The three counting results for each pond or channel were averaged, and the standard deviations (STDEV) were shown.

Extraction of genomic DNA from various aquaculture environments

Genomic DNA was extracted using the chemical-enzymatic lyses protocol (Fortin et al. 2004) with some modifications. Briefly, 5 ml of sterilized distilled water was added to a 50-ml Falcon tube containing 10 g of each sample, and the tubes were vortexed at maximum speed for 5 min before addition of 1 ml of 100 mg/ml lysozyme dissolved in Tris–HCl (250 mM, pH 8.0) and 4-ml DNA extract buffer (100 mM Tris–HCl, 100 mM EDTA, 100 mM Na_3PO_4, 1.5 M NaCl, pH 8.0). After incubating in rotating inoculators for 30 min at 30 and 37°C, respectively, 20-μl proteinase k (100 mg/ml) was added. The samples were incubated for 1 h at 37°C, followed by addition of 100 μl of SDS (20%) and incubation in a water bath at 85°C for 30 min during which the samples were mixed gently by inversion

every 10 min. After centrifugation (4,100×*g*) for 15 min at room temperature, the supernatant was transferred to a new 50-ml Falcon tube with addition of half the volume of 7.5 M ammonium acetate followed by 15 min incubation on ice. After centrifugation (9,400×*g*) for 15 min at 4°C, the supernatant was transferred to a 50-ml Falcon tube, and the DNA was precipitated with addition of cold 2-propanol overnight at −20°C. The pellet was collected by centrifugation for 30 min at 4°C and washed once with 70 and 95% ethanol. The vacuum-speed-dried DNA was purified with 1 volume of phenol/chloroform/iso-propanol (25:24:1). After precipitating, washing and drying, the DNA was resuspended in sterilized distilled water. The crude extract of DNA was treated with polyvinylpolypyrrolidone (PVPP) and sephacryl S-400 spin columns as described by Berthelet et al. (1996) to remove the PCR inhibitors such as humic acids. Untreated and treated DNA (5 ml) were loaded onto a 0.7% agarose gel with SYBR safe™ for electrophoresis at 60 V for 2 h with HindIII-digested λ DNA as the molecular weight marker (Invitrogen, Carlsbad, CA). The gel was visualized with blue light on a MultiImage™ light Cabinet (Alpha Innotech Corporation, Fr).

Amplification of 16S rDNA

The bacterial universal primers, U341 and U758, were used to amplify a 418-bp fragment corresponding to position from 341 to 758 in the *Escherichia coli* 16S rDNA sequence (Muyzer et al. 1993). In order to stabilize the melting behavior of the amplified fragments in the DGGE, a GC-clamp (Sheffield et al. 1989) was included in the forward primer. The sequence for the primers was 5′341-357-GCGGGCGGGGCGGGGGGCACGGGGGGCG CCGGCGGGCGGGGC GGGGGCCTACGGGAGGCAGCAG-3′ and 5′758-740-CTAC-CAGGGTATCTAA TCC-3′, respectively. PCR protocol was optimized in order to achieve good DGGE results. The PCR was carried out in a 50-µl volume, including 5 µl of genomic DNA as template, 25 pmol of each primer, 200 µM of each dNTP, 1 mM $MgCl_2$, 2.5 U of Taq polymerase (Amersham Bioseiences, Piscataway, NJ) and 5 µl of 10× PCR buffer. Before addition of Taq DNA polymerase, the samples were predenatured for 5 min at 96°C and then a touchdown PCR (Don et al. 1991) was performed by using 65°C as initial annealing temperature and 1°C decrease for every cycle until it reached 55°C. The PCR parameters for each cycle were denaturation at 94°C for 1 min, annealing for 1 min and extension at 72°C for 3 min. Ten cycles were performed with annealing temperature decreasing from 65 to 55°C, and 20 cycles were performed with the annealing temperature at 55°C. Finally, 5 µl of PCR product and a 100-bp DNA ladder (MBI Fermentas, Amherst, NY) were loaded onto a 1.4% agarose gel. After electrophoresis, PCR products were stained with SYBR safe™ and visualized on a MultiImage™ light cabinet (Alpha Innotech Corporation, San Leandro, CA).

DGGE of amplified PCR fragment

The purified PCR product (approximately 600 µg) was loaded onto a lane of the denaturing gradient gel, and DGGE was performed on the Dcode Universal Mutation Detection System (Bio-Rad, Mississauga, Ont., Canada) according to the manufacturer's instruction. A 6% acrylamide-*N*,*N*-methylene/bisacrylamide (37.5:1) stack gel was added to avoid disturbing the gradient during comb insertion. Separation of PCR product was carried out on an 8% (W/V) acrylamide gel in 1× TAE (40 mM Tris acetate pH 8.0, 1 mM Na_2DETA) containing a linear gradient from 25 to 65% of denaturant [100% denaturant consisted of 7 M urea and 40% formamide as described by Muyzer et al. (1993)]. Electrophoresis was carried out for 16 h at 80 V, and the gel was stained for 30 min in the

staining solution containing 1:10000 dilution of Vistra Green (Amersham Pharmacia Biosciences Inc., Baie d'Urfe, QC, Canada) in 1× TAE. The gel was finally visualized on a FluorImager system model 595 (Amersham Pharmacia Biosciences Inc.) with a 488-nm excitation filter and a 530-nm emission filter.

Reamplification and sequencing of some DGGE bands

A total of 32 specific DGGE bands were carefully excised from the gel by using a sterile surgical scalpel. DNA was eluted by incubating the gel slices overnight at 37°C in sterilized deionized water (Rolleke et al. 1996) and purified by using a QIA quick PCR purification kit (Qiagen, Mississauga, Ont., Canada). The obtained DNA was used as a template for PCR reamplification. PCR was carried out in a 50-µl reaction volume containing 1 µl of DNA, 1.0 µl of U341 primer (25 pmol), 1.0 µl of U758 primer (25 pmol), 0.625 µl of BSA (10 mg/ml), 2.0 µl (25 mM), 8.0 µl of $MgCl_2$ (100 mg), 8.0 µl of dNTPs (1.25 mM), 32.4 µl of sterile deionized water, 0.5 µl of Taq polymerase and 5 µl of 10× PCR buffer. Taq polymerase was added separately when the temperature reach 80°C after initial denaturation for 5 min at 95°C. The standard PCR parameter for DGGE bands was 25 cycles of 1 min at 94°C, 1 min at 64°C and 1 min at 72°C. The quantity of template, annealing temperature and the number of cycles were optimized for each sample without satisfying result in order to obtain single band of PCR product for DNA sequencing. The single band observed in a 1.4% agarose gel after electrophoresis was purified with GFX Purification Kit (Amersham Biosciences) and quantified by loading 1 µl of the sample and serially diluted 100-bp DNA ladder onto a 1.4% agarose gel. The samples (20 µl, 2 ng/µl) were submitted to Laval University for sequencing.

Dendrogram analysis for the banding patterns of DGGE was performed using the Dendron 2.2 software package (Soll-tech Inc., Oakdale, LA). The unweighted pair group method, based on a similarity matrix calculated from the presence/absence of DGGE bands, was used to analyze the similarity between different samples.

Phylogenetic analysis of bacterial communities in different aquaculture environments

The obtained sequences were manually corrected by comparing the consensus of forward and reverse sequences with software Macvector 8.1. The length of the corrected sequences ranged from 352 to 387 bp. The sequences were initially aligned using the Clustal W function and then were compared with closely related sequences retrieved from http://www.ncbi.nlm.nih.gov//nucleic acid/BLAST. Identical sequences with the same migration on DGGE were treated as one operate unit. Further manual amendments to the alignment were performed using the multi-cluster function.

Results

Numbers of bacteria in different samples detected with culturing methods

Bacteria belonging to five physiological groups were detected in the sediment and water samples. In the sediment samples, the numbers of heterotrophic bacteria, nitrate-reducing bacteria and sulfate-reducing bacteria ranged from 8.7×10^4 to 1.86×10^6, 2.1×10^4 to 1.1×10^5 and 9.3×10^1 to 1.1×10^4 cells/g, respectively (Table 1). The counts of these

Table 1 Numbers of various bacterial groups in sediment and water environments

Sampling sites	Total no. of heterotrophic bacteria (CFU/g)	No. of ammonium-oxidizing bacteria (CFU/g)	No. of nitrifying bacteria (CFU/g)	No. of sulfate-reducing bacteria (cells/g)	No. of nitrate-reducing bacteria (cells/g)
Sediment of shrimp pond	$1.64 \pm 0.04 \times 10^5$	$8.90 \pm 0.2 \times 10^3$	$4.30 \pm 0.05 \times 10^3$	$1.10 \pm 0.13 \times 10^4$	$1.10 \pm 0.01 \times 10^5$
Sediment of Sea cucumber pond	$8.70 \pm 0.12 \times 10^4$	$1.43 \pm 0.14 \times 10^4$	$9.80 \pm 0.03 \times 10^4$	$9.30 \pm 0.10 \times 10^1$	$2.90 \pm 0.06 \times 10^4$
Sediment of Shrimp + Sea cucumber pond	$9.30 \pm 0.01 \times 10^4$	$1.25 \pm 0.08 \times 10^4$	$8.70 \pm 0.12 \times 10^4$	$1.30 \pm 0.11 \times 10^2$	$5.60 \pm 0.14 \times 10^4$
Sediment of effluent channel	$1.86 \pm 0.30 \times 10^6$	$5.20 \pm 0.15 \times 10^4$	$4.20 \pm 0.18 \times 10^5$	$2.41 \pm 0.12 \times 10^3$	$2.10 \pm 0.30 \times 10^4$
Water of Sea cucumber pond	$1.20 \pm 0.04 \times 10^3$	$2.00 \pm 0.06 \times 10^2$	$4.40 \pm 0.16 \times 10^3$	$4.00 \pm 1.0 \times 10^0$	$2.40 \pm 0.05 \times 10^3$
Water of Shrimp pond	$6.30 \pm 0.13 \times 10^4$	$3.90 \pm 0.10 \times 10^2$	$2.10 \pm 0.03 \times 10^3$	$1.30 \pm 0.10 \times 10^1$	$2.40 \pm 0.01 \times 10^4$
Water of effluent channel	$1.50 \pm 0.22 \times 10^4$	$6.00 \pm 0.02 \times 10^2$	$4.00 \pm 0.07 \times 10^3$	$2.90 \pm 0.5 \times 10^0$	$2.30 \pm 0.15 \times 10^3$

three groups of bacteria in the shrimp ponds were higher than that in the sea cucumber + shrimp pond. In contrast, the counts of ammonium-oxidizing bacteria and nitrifying bacteria (8.9×10^3 and 4.3×10^3 CFU/g, respectively) in the shrimp ponds were lower than that in the sea cucumber pond and mix culture pond (1.25×10^4–1.43×10^4 CFU/g and 9.8×10^4–8.7×10^4 CFU/g, respectively) (Table 1). The population of bacteria in the water samples was significantly lower than that in the sediment samples. However, the bacterial distribution pattern in the water samples was similar to that in the sediment.

Extraction of bacterial genomic DNA from the sediment samples in various shrimp culture sites

The size of DNA isolated from the sediment samples in different ponds was approximately 23 kb (Fig. 1a). The brown color of the DNA crude extract disappeared after purification with PVPP and sephacryl columns. In addition, the DNA bands after separating in an agarose gel were much brighter and clearer than the crude extracts (compare Fig. 1a with b), suggesting that inhibitory factors in the crude extracts were efficiently removed after PVPP and sephacryl purification.

PCR amplification of 16S rDNA

A 417-bp fragment of 16S rRNA gene was amplified with universal bacterial primers GC U341 and U758 (Fig. 2). The touchdown PCR ensured that single specific band with expected size was amplified. The yield of PCR product was high as a bright band appeared after electrophoresis (Fig. 2).

Comparison of DGGE banding profiles between various environmental samples

DGGE analysis for the PCR products of 16S rDNA produced identical banding patterns. More than 20 bands for each sample suggested that a high diversity of bacteria was present in the mariculture environment. The highest number (28) of DGGE bands was obtained from the samples of shrimp + sea cucumber pond and the lowest (21) was obtained from the samples of shrimp pond B (Fig. 3). Furthermore, the migration pattern of DGGE bands varied significantly between the different sampling sites and with different dominant bands

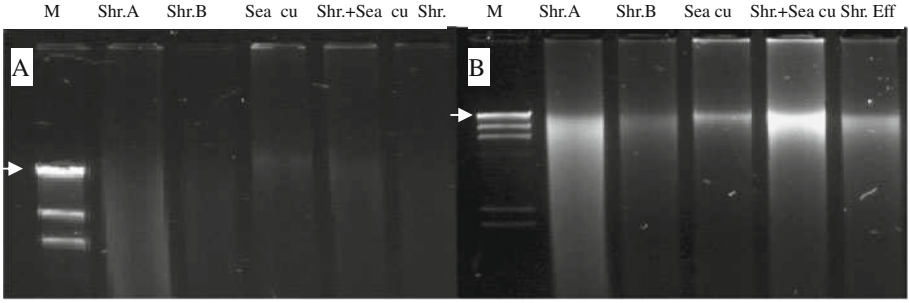

Fig. 1 Gel electrophoresis of genomic DNA extracted from sediment samples in shrimp and sea cucumber culture environments. (**a**) Crude DNA extract, (**b**) Purified DNA after PVPP and sephacryl treatment, *M* λDNA digested with *Hind* III (arrow indicates a 23.1-kb fragment), *Shr.* shrimp monoculture pond, *Sea cu* Sea cucumber monoculture pond, *Shri. Eff* sediment from the channel receiving the effluent from the ponds

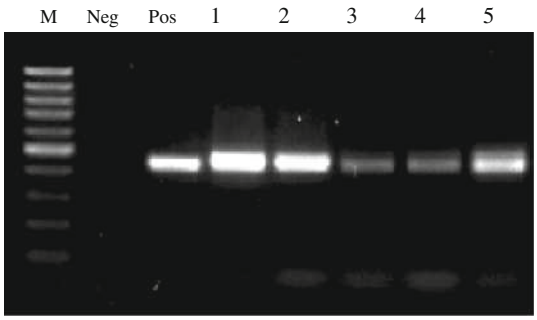

Fig. 2 Gel electrophoresis of PCR product of 16S rDNA amplified from DNA extracted from the sediment samples in shrimp and sea cucumber culture environments. *M* 100-bp DNA ladder, *Neg* negative control, *Pos* positive control, *1* shrimp monoculture pond A, *2* shrimp monoculture pond B, *3* Sea cucumber monoculture pond, *4* shrimp and sea cucumber mixed-culture pond, *5* the channel receiving the effluents from the ponds

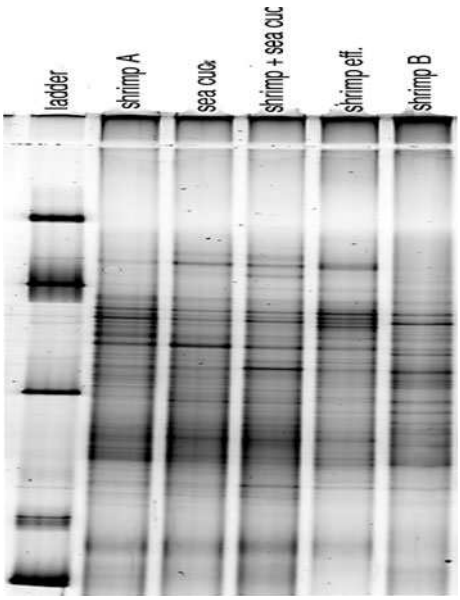

Fig. 3 DGGE analysis of 16S rDNA fragments generated by PCR amplification of total bacterial DNA from sediment environments

(Fig. 3). These results suggested that the composition of bacterial communities varied in different sampling sites.

The dendrogram analysis showed that DGGE pattern in the samples from the same environment shared higher similarity than that in different environments, suggesting that the same environment had similar bacterial community compositions. For example, the samples from Shrimp A and Shrimp B were clustered into one group (S_{AB}, 0.70), whereas shrimp + sea cucumber and sea cucumber were clustered into a different one (S_{AB}, 0.78). The composition of bacterial community in different environments varied significantly. For example, the S_{AB} of Shrimp A and B to other samples was only 0.38. The S_{AB} between

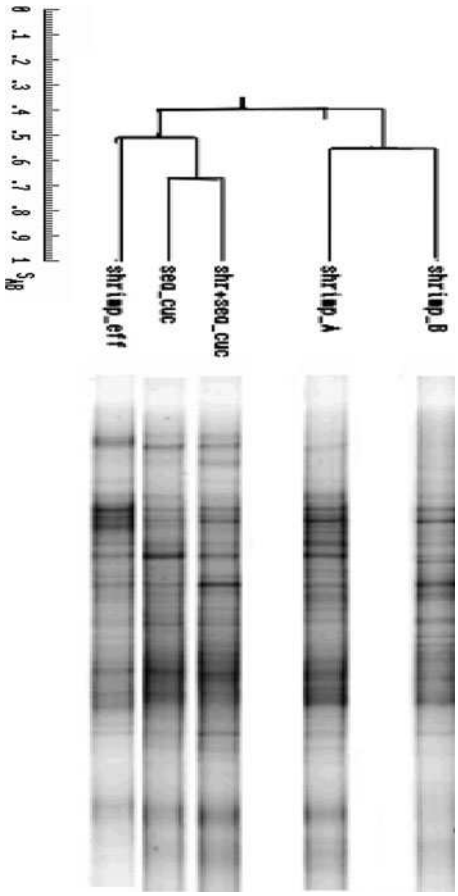

Fig. 4 UPGMA dendrogram analysis for the assessment of similarity between DGGE profiles illustrated in Fig. 3. Samples from Shrimp A and Shrimp B were clustered into one group with an S_{AB} value of 0.70. Samples from shrimp + sea cucumber and sea cucumber were clustered into a different group

Shrimp eff. and Shrimp A and B was 0.38, which was lower than that between Shrimp eff and the mixed-culture or sea cucumber monoculture environments (S_{AB}, 0.58) (Fig. 4).

Reamplification and sequencing of some DGGE bands

Some of the unique or common bands in different samples were excised from the DGGE gel. A total of 32 bands were selected, and 28 bands were reamplified with primers U341 and U758. Of these 28 bands, 14 samples yielded good sequencing results. BLAST analysis was performed for these 14 DNA sequences as shown in Table 2. The similarity between the obtained sequences and the reference sequences retrieved from the databases ranged from 92 to 99%.

Phylogenetic analysis of the sequences showed that the bacteria in various sediment samples were mainly composed of *Flavobacteriaceae, Bacteriodetes* and *delta Proteobacterium. Flavobacteriaceae* was observed in 64.3% of the samples, and *Bacteroidetes* was observed in 21% of the samples (Fig. 5). Only delta subgroup of *Proteobacterium* was detected from the samples collected in the current study (14.7%).

Table 2 BLAST analysis-retrieved closest relative to 16S rRNA genes of bacteria in the culture environments

Sampling sites	DGGE bands	Closest relative	BLAST closest match accession number	% identity	Reference
Shrimp pond A	A1	*Croceimarina litoralis strain* IMCC1993	EF108214.1	95	Flavobacteriaceae
	A2	*Lutibacter* sp.	AY177723	99	Flavobacteriaceae
	A3	Uncultured Bacteroidetes bacterium	DQ351797	99	Flavobacteriaceae
Shrimp pond B	E1	Uncultured *Sphingobacteria bacteri*	AY711530	92	Bacteroidetes
	E2	*Maribacter polysiphoniae* 1481	AM497875	98	Bacteroidetes
	E3	Uncultured *Desulfuromonas* sp.	AY177801	97	Delta proteobacterium
Sea cucumber pond	B1	*Psychroserpens mesophilus* strain K	DQ001321	98	Flavobacteriaceae
	B2	Uncultured Bacteroidetes bacterium	DQ200581	99	Flavobacteriaceae
	B3	Uncultured Bacteroidetes bacterium	DQ351797	99	Flavobacteriaceae
Shrimp + Sea cucumber Pond	C1	*Formosa algae* KMM 3553	AY228461	97	Flavobacteriaceae
	C3	Bacteroidetes bacterium ANT9105	AY167316	98	Bacteroidetes
	C4	Uncultured delta proteobacterium	DQ351798	98	Delta proteobacterium
Shrimp effluent	D2	*Lacinutrix copepodicola*	AB261015	96	Flavobacteriaceae
	D3	*Lacinutrix copepodicola*	AB261015	97	Flavobacteriaceae

Discussion

In this study, we determined the number and composition of bacteria in the sediment environments associated with shrimp and sea cucumber culture in northern China. China is one of the main countries for shrimp culturing in the world. During the past several years, many studies have been conducted in order to understand the distribution of heterotrophic bacteria, *Vibrios* and other bacteria belonging to some physiological groups in the shrimp culture environment. Specifically, investigators were interested in deciphering how bacterial communities vary with environmental factors and how they influence the health of farmed shrimp (Guo and Xu 1994; Yu et al. 1995; Liu et al. 2000; Li et al. 2002a, b, c). However, the compositions of the bacterial communities in the shrimp culture environment have not been studied thoroughly due to the limitations of traditional methods. Only

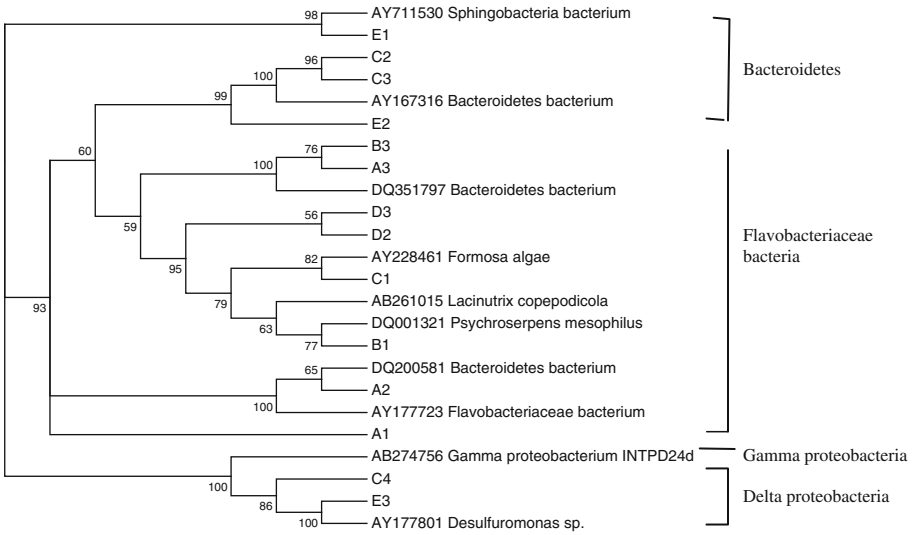

Fig. 5 Phylogenetic analysis for sequences of 16S rDNA fragments separated by DGGE. Reference sequences are shown with their respective Genbank accession numbers. The tree was constructed by MEGA bootstrap 1,000 using neighbor-joining method. Bacteria in the various sediment samples were mainly composed of *Flavobacteriaceae*, *Bacteriodetes* and *delta Proteobacterium*

recently, molecular approaches, such as PCR, DGGE and RFLP, have been applied, for example, for the analysis of microbial communities in the gastrointestinal tract of shrimp and their culture environment in China (Luo et al. 2006; Li et al. 2005). The number of bands separated by DGGE in this study ranged from 20 to 28, which is consistent with the typical DGGE band number (10–40) for the sample collected from aquatic environments (Moesendeder et al. 1999; Murray et al. 1996; Scharer et al. 2000). Similar band numbers (19–21) were obtained when the samples collected from the environment of great scallop at early stage were analyzed by DGGE (Sanda et al. 2003). The presence of abundant DGGE bands in the samples of current study suggested that the mariculture environments had a highly diversified bacterial community including *Bacteriodetes*, *Flavobacteriaceae* and *Delta Proteobacteria*. In this study, gamma-subclass of the *Proteobacteria* was not detected, and *Aeromonadaceae*, *Pseudomonadaceae* and *Vibrionaceae*, usually considered as the predominant bacteria population in mariculture environments by traditional culturing studies, were either not detected. This is possibly due to the utilization of highly selective medium that allows different bacterial species to grow in different studies. Although the number of culturable bacterial species can be increased by adding different electron acceptors and decreasing the nutrient etc., utilization of PCR and DGGE could further increase the bacterial diversity in the enriched cultures (Beate et al. 2005).

It was found that the bacterial composition in the two shrimp ponds was similar. In contrast, the bacterial composition of the mixed-culture pond and the effluent channel was similar to that of the sea cucumber pond. These results suggested that sea cucumber culture played a more significant role on the composition of bacterial population than the shrimp did. We also found that the bacterial diversity in the shrimp + sea cucumber pond was higher than that in the shrimp monoculture ponds (28 DGGE bands versus 20 DGGE bands). In addition, these two types of ponds had different dominant bacterial species. These differences might be attributed to the feeding characteristics of the sea cucumbers.

For example, sea cucumber can eat organic residues, benthic algae and protozoans in the sediment of the mariculture area (Liao 1979; Li et al. 1994; Chang et al. 2003; Liu et al. 2006) and tend to select sediments with higher nutrients (Uthicke and Karez 1999). This feeding characteristic prevents accumulation of organic materials, leading to the occurrence of an oxygen-deficient and oligotrophic sediment environment that is more conducive to the development of microbial diversity (Beate et al. 2005). The high diversity of bacteria in the mixed-culture environment is instrumental to maintain the balance of ecosystem, thereby supplying various nutrients and sufficient oxygen for the farmed animals and inhibiting the explosive growth of some potentially pathogenic bacteria. This beneficial effect of sea cucumbers is also reflected by the distribution of the five physiological groups of bacteria in the sediment. High level of nitrifying and ammonium-oxidizing bacteria in the sea cucumber culture environment is indicative of oxic condition, whereas high level of nitrate-reducing and sulfate-reducing bacteria in shrimp monoculture environments represents an increased redoxic condition.

We failed to obtain the sequence of several reamplified DGGE bands due to high level of background in the sequencing reaction. It is possible that these bands were actually composed of two or more distinct sequences with similar migration ability in the DGGE gel (Beate et al. 2005). Thus, the bacterial diversity in the samples would be underestimated if the total number of discernible bands were used to estimate the diversity (Juck et al. 2000). To obtain complete list of sequences, cloning of these bands into a vector and resequencing of individual clone are needed. During the database searches, we found that several sequences, such as A3, B2, B3 and E1, had highest similarity to sequences obtained from uncultured bacteria of the marine environments in Japan, Korea or Yellow Sea areas in China. These results suggest that these bacterial species may be ubiquitous and predominant species in the Yellow Sea but have not yet been introduced into culture collection, and we do not know their characters. Since the Gene Bank currently lacks sufficient data on marine bacteria, it will be useful to further investigate these potentially novel marine bacteria to enrich the database and further understand the marine bacterial world.

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References

Amann RI, Ludwig W, Schleifer KH (1995) Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. Microbiol Rev 59:143–169

Beate K, Reinhard W, Bert E, Heribert C, Henrik S (2005) Microbial diversity in coastal subsurface sediments: a cultivation approach using various electron acceptors and substrate gradients. Appl Environ Microbiol 71(12):7819–7830

Berthelet M, Whyer L, Greer C (1996) Rapid, direct extraction of DNA from soils for PCR analysis using polyvinylpolypyrrolidon spin columns. FEMS Microbiol Lett 138:17–22

Chang Z, Yi J, Mu K (2003) Factors influencing growth and survival of *Apostichopus japonicus*. Hebei Fish 2:32–36

Chen S, Bao W, Li B (1987) Pollution situation of Rushan Bay and analysis of heterotrophic microorganisms. J Ocean Coll Shandong 17(4):86–94

Don R, Cox P, Wainwright B, Baker K, Mattick J (1991) Touchdown PCR to circumvent spurious priming during gene amplification. Nucleic Acid Res 19:4008

Fortin N, Beaumier D, Lee K, Greer C (2004) Soil washing improves the recovery of total community DNA from polluted and high organic content sediments. J Microbiol Methods 56:181–191

Guo P, Xu M (1994) The bacterial variation in the water environment of cultured prawn pond. Oceano et limno Sinica 25:625–629

Jiang W, Zhang S, Liu Y (2006) Try of culturing sea cucumber, *Apostichopus japonicus* in shrimp ponds. Shandong Fish 23(9):23–24

Juck D, Charles T, Whyte L, Greer C (2000) Polyphasic microbial community analysis of petroleum hydrocarbon-contaminated soils from two northern Canadian communities. FEMS Microbiol Ecol 33:241–249

Juck D, Driscoll BT, Charles T, Greer CW (2003) Effect of experimental contamination with the explosive hexahydro-1,3,5-trinitro-1,3,5-trazine on soil bacterial communities. FEMS Microbiol Ecol 43:255–262

Li X (2002) The impact of effluent from shrimp ponds to near-shore marine culture and strategies to prevent it. J Chin Fish Econ (6):40–42

Li Y, Wang Y, Wang L (1994) The choose of environment and multiplication sea region *Apostichopus japonicus* of sea cucumber. Bull Oceanol Limnol 4:42–47

Li Q, Chen B, Qu K, Yuan Y, Li J, Sun X, Zhao F (2002a) Variation of bacteria numbers in fish-shrimp mix-culturing ecosystem. Chin J Appl Ecol 13:731–734

Li Q, Qu K, Chen B, Sun X, Li J, Zhao F (2002b) Seasonal variation of some main bacteria groups in old shrimp pond ecosystem. Mar Fish Res 23(2):45–49

Li Q, Chen B, Qu K, Xin F, Li J, Zhao F (2002c) Vertical distribution of three main bacteria groups in sediment of shrimp ponds. J Fish Sci China 9:367–370

Li Z, He L, Wu J, Chen J (2005) Study on predominant bacteria community in prawn based on 16S rDNA PCR–DGGE fingerprint. J Fish China 32(3):82–86

Liao Y (1979) Sea cucumber in China. Mar Sci 3:54–58

Liu G, Li D, Dong S, Wang K (2000) Numerical dynamics of sediment bacteria in shrimp polycultural ecosystems. Chin J Appl Ecol 11(1):138–140

Liu S, Yang H, Zhou Y, Zhang T, Yuan X (2006) Simulative studies on utilization efficiency of *Apostichopus japonicus* on the biodeposit in the raft culture system in shallow sea. Mar Sci 30(12):21–24

Luo P, Hu C, Xie Z, Zhang L, Ren C, Xu Y (2006) PCR–DGGE analysis of bacterial community composition in brackish water *Litopenaeus vannamei* culture system. J Trop Oceanogr 25(2):49–53

Moesendeder MM, Arrieta JM, Muzer G, Winter C, Hernd GJ (1999) Optimization of terminal-restriction fragment length polymorphism analysis for complex marine bacterioplankton communities and comparison with denaturing gradient gel electrophoresis. Appl Environ Microbiol 65:3518–3525

Murray AE, Hollibaugh J, Orrego TC (1996) Phylogenetic compositions of bacterioplancton from two California estuaries compared by denaturing gradient gel electrophoresis of 16S rDNA fragments. Appl Environ Microbiol 62:2676–2680

Muyzer G, de Waal EC, Uitterlinden AG (1993) Profiling of complex micirobial populations by denaturing gradient gel electrophoresis analysis of ploymerase chain reaction-amplified geens coding for 16S rRNA. Appl Environ Microbiol 59:695–700

Rolleke S, Muyzer G, Wanner G, Lubitz W (1996) Identification of bacteria in a biodegraded wall painting by denaturing gradient gel electrophoreses of PCR-amplified gene fragments coding for 16S rRNA. App Environ Microbiol 62:2059–2065

Sanda R, Magnesen T, Torildsen L, Bergh O (2003) Characterisation of the bacterial community associated with early stages of great scallpo (*Pecten maximus*), using denaturing gradient gel electrophoresis (DGGE). Syst Appl Microbiol 26(2):302–309

Scharer M, Massana R, Pedros-Alio C (2000) Spatial differences in bacterioplankton composition along the Catalan coast NW Mediterranean assessed by molecular finger-printing. FEMS Microbiol Ecol 33:51–59

Sheffield V, Cox D, Lerman L, Myers R (1989) Attachment of a 40-base-pair G+C-rich sequence (GC-clamp) to genomic DNA fragments by the polymerase chain reaction result in improved detection of single-base changes. Proc Natl Acad Sci 86:139–151

Song Z, Wang S, Xu J, Liu Z, Wang H (2007) Study on reform of the sea cucumber farming pattern in shrimp pond. Hebei Fishies 4:16–18

Staufenberger T, Thiel V, Wiese J, Imhoff J (2008) Phylogenetic analysis of bacteria associated with *Laminariasaccharina*. FEMS Microbiol Ecol 64:65–77

Thomas EF, Chang L, Presser JI (2006) Changes in the community structure and activity of beta proteobacterial ammonia-oxidizing sediment bacteria along a freshwater-marine gradient. Environ Microbiol 8(4):684–696

Uthicke S, Karez R (1999) Sediment patch selectivity in tropical sea cucumber (*Holothurioidea Aspidochirotida*) analysed with multiple choice experiments. J Exp Mar Biol Ecol 236:69–87

Wang R (2003) The environmental pollution by marine culture and its appropriate prevention and cure. J Zhejiang Ocean Univ (Nat Sci) 22(3):60–62

Yu Z, Lin F, He J (1995) Relationship between heterotrophic bacteria and shrimp disease. Acta Oceanologica Sinica 17(3):85–90