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Intestinal microbiota of mangrove red snapper (*Lutjanus argentimaculatus* Forsskål, 1775) reared in sea cages

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Abstract

Using polymerase chain reaction amplification of 16S rDNA coupled to denaturing gradient gel electrophoresis (DGGE) and sequencing of isolated amplicons, we investigated the microbiota of the intestinal digesta and mucosal surface in mangrove red snapper cultured in a cage aquaculture area in Daya Bay. A total of 14 sequences were characterized by phylogenetic analysis. Among the bacterial species determinated from sequences, the γ-*Proteobacteria* group (64.25%, nine species) dominated absolutely in fish intestines. Others belonged to *Spirochaetes* (14.3%, two species), *Cyanobacteria* (14.3%, two species) and *Firmicutes* (7.15%, one species). However, the bacteria were identified as uncultured accounting for 28.6% (four species). The apparent bacterial richness (calculated as the numbers of DGGE bands) was significantly higher in digesta than that in mucosal tissue samples ($P < 0.05$). There existed five dominant individual populations including one unknown species of *Firmicutes*, *Arthrospira* sp., *Vibrio metschnikovii*, *Vibrio harveyi* and *Vibrio* sp. in intestinal digesta, and in contrast, only three dominant individual populations including *Vibrio natriegens*, *V. harveyi* and *Vibrio* sp. in intestinal mucosal surface. The results indicated that the microbiota in intestinal digesta was significantly different from that in mucosal surface.

Keywords: mangrove red snapper, microbiota, DGGE, intestinal digesta, intestinal mucosal surface

Introduction

Mangrove red snapper *Lutjanus argentimaculatus*, belonging to the genus *Lutjanus* Bloch (Grimes, Manooch, Huntsman & Dixon 1977), is an important market species in southeast Asia (Estudillo, Duray, Marasigan & Emata 2000) and China (Zhang, Huang, Cai & Huang 2006). With firm, sweet-tasting and white flesh, mangrove red snapper commands a high market price and is in much demand in southeast Asia and China. In China, the artificial fry rearing of mangrove red snapper has succeeded (Xing, Yin, Chen & Zhang 2005). Mangrove red snapper is generally cultured in sea netcage. Pathogenic bacteria in the open production system are relatively difficult to control (Olafsen 2001) and thus potential threats against cultured animal's health at all times. The development of mangrove red snapper has mainly been hampered by bacterial disease problems, which have caused large economic loss. It is known that the gut is an important infection site in fish. The proliferation of pathogens can be suppressed by the commensal microbiota in fish (Olsson, Westerdahl, Conway &

Kjelleberg 1992; Ringø & Olsen 1999; Olafsen 2001). However, not much information regarding gut microbiota of mangrove red snapper is available.

The intestinal microbiota of fish can be classified as autochthonous or adherent, i.e. the bacteria are able to colonize the epithelial surface of the host or adhere to the microvilli, or as allochthonous or non-adherent, i.e. the bacteria in the gastrointestinal tract that are rejected after some time (Ringø & Birkbeck 1999), which play an important role in host health and nutrition (Liu, Zhou, Yao, Shi, He, Høvold & Ringø 2008). Earlier, most studies on fish intestines have concentrated on bacteria present in gut contents (Pond, Stone & Alderman 2006). However, intestinal mucosa is of important interest in examining the interaction of the microbiota and disease (Pond *et al.* 2006; Kim, Brunt & Austin 2007). More recently, many studies related to bacteria attached to the gut epithelium are consequently conducted on fish such as yellow grouper (*Epinephelus awoora*) (Zhou, Shi, He, Liu, Huang, Yao & Ringø 2009; Feng, Hu, Luo, Zhang & Chen 2010), emperor red snapper (*Lutijanus sebae* Cuvier) (Zhou, Shi *et al.* 2009), rainbow trout (*Oncorhynchus mykiss*) (Pond *et al.* 2006; Kim, Brunt *et al.* 2007), Atlantic cod (*Gadus morhua* L.) (Ringø, Sperstad, Myklebust, Refstie & Krogdahl 2006) and Atlantic salmon (*Salmo salar* L.) (Hovda, Lunestad, Fontanillas & Rosnes 2007; Liu *et al.* 2008). In order to obtain whole profile of gut microbiota in mangrove red snapper, both intestinal digesta and mucosal surface should be investigated.

The limitations of culture-dependent methods have stimulated the application of culture-independent methods in investigations on the bacterial communities in many fish species such as haddock larvae (Griffiths, Melville, Cook & Vincent 2001), farmed and wild salmon (Holben, Williams, Saarinen, Särkilahti & Apajalahti 2002), abalone (*Haliotis discus hannai*) (Tanaka, Ootsubo, Sawabe, Ezur & Tajima 2004), zebrafish (*Danio rerio*) larvae (Rawls, Samuel & Gordon 2004), puffer fish (*Takifugu obscurus*) (Yang, Bao, Peatman, Li, Huang & Ren 2007), Atlantic cod eggs and larvae (McIntosh, Ji, Forward, Puvanendran, Boyce & Ritchie 2008) and yellow grouper (Zhou, Liu, Shi, He, Yao & Ringø 2009; Feng *et al.* 2010). Currently, one of the most widely used culture-independent methods in studying microbiota is polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), which is rapid and sensitive and can present more reliable information about the gut microbiota of fish investigated (Zhou, Liu *et al.* 2009).

The aim of this study is therefore to use PCR-DGGE and sequencing to investigate the microbiota in digesta and mucosal surface of the gut in mangrove red snapper cultured in cages and compare the microbial diversity in the two different sections, and the investigation will be valuable for application in areas such as selection of novel probiotic bacteria and detection of potential pathogens.

Materials and methods

Fish and rearing conditions

The experimental mangrove red snapper weighing about 900 g each reared in a cage aquaculture area in Daya Bay, near Key Lab of Field Marine Biology Research Station of CAS, were fed with natural diet (ice fresh fish and shrimp). Five fish were randomly collected by netting and then aseptically transferred to plastic bags. Water temperature was maintained at 30 ± 1 °C during the sampling period. The sampled fish were maintained on ice and immediately transported to the laboratory mentioned above with 0.5 h.

Sample preparation and collection of bacteria cell

The ventral belly surface of the fish was opened to expose the peritoneal cavity. The spleen, gall bladder, liver and fat deposits surrounding the gastrointestinal tract were removed. The whole digestive tracts were excised. The intestinal contents of fish were squeezed out in order to isolate non-adherent bacteria. Then, the gut sections were thoroughly rinsed three times using $\sim$ 3 mL of peptone water (PW) (1 g L^{-1} bacterial peptone and 8.5 g L^{-1} NaCl) to remove non-adherent and ensure capturing of the adherent bacteria, according to the recommendation of Ringø (1993). Thereafter, each sample associated with gut contents and mucosal surface was weighed into a porcelain mortar. In order to isolate bacterial cells, the sample was homogenized by hand for 3–5 min after addition of 6 mL of PW. The residue was discarded and the liquid mixture was transferred to a 10 mL graduated plastic-stoppered centrifuge tube. Bacterial cells were recovered from each sample by a differential centrifugation technique modified from Apajalahti, Särkilahti, Mäki, Heikkinen, Nurminen and Holben (1998) and described elsewhere (Feng *et al.* 2010).

DNA extraction and PCR amplification

Total bacterial genomic DNA was extracted using a method modified from Rivera, Lipp, Gil, Choopun, Huq and Colwell (2003) and described elsewhere (Feng *et al.* 2010). The PCR of 16S rDNA was conducted with a combination of primer 968f and 1401r corresponding to positions 968 to 1401 in *Escherichia coli* by the procedure of Evans, Rosado, Sebastian, Casella, Machado, Holmström, Kjelleberg, van Elsas and Seldin (2004) with modifications. The primer and GC clamp sequences are as follows: primer 968f, 5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGAACGCGAAGAACCTTAC-3′; primer 1401r, 5′-CGGTGTGTACAAGGCCC-3′. The primer 968f contains an additional 40-nucleotide GC-rich sequence (GC clamp) at its 5′ end. The GC clamp was designed here for DGGE analysis. Polymerase chain reaction amplification was carried out with the *TaKaRa Ex Taq*™ (TaKaRa Biotechnology, Dalian, China) according to the manufacturer's protocol by adding 1 μL of template DNA and 20 pmol of each primer in a 50 μL total reaction volume. The PCR amplification was performed using the PTC-100 Peltier Thermal Cycler (Bio-Rad, Hercules, CA, USA) with the following thermo-profiles: an initial denaturation for 4 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, annealing for 1 min at 55 °C and extension for 1 min at 72 °C, followed by a final extension step for 8 min at 72 °C. The PCR products were analysed by electrophoresis in 1.5% (w/v) agarose gel containing Gold View (0.5% v/v).

DGGE and sequencing of 16S rDNA

The DGGE was performed with a D-code System (Bio-Rad) to separate the above PCR products. The DNA bands on the DGGE gel should represent the apparent bacterial diversity. PCR samples were applied directly onto 6% (w/v) polyacrylamide gels in a 0.5 × TAE buffer with a denaturing gradient ranging from 40% to 80% of denaturation [100% corresponds to 7 M urea and 40% (v/v) formamide]. Electrophoresis was performed with a constant voltage of 80 V at 60 °C for about 15 h. Gels were incubated for 20 min in 0.5 × TAE buffer containing ethidium bromide (0.5 mg L^{-1}), photographed with UV transillumination.

Selected main DGGE bands were excised from the gels for sequencing and identification. For the bands with same distance from the well in different lanes, only one band was cut representing the same bacterial 16S rDNA. The band was excised from the DGGE gel, eluted and amplified again with the same primers 968f (without GC clamp) and 1401r. The PCR product mixture was subsequently cloned into the pMD 18-T vector (TaKaRa Biotechnology). Plasmid DNA was purified from the resulting clones using the Qiagen Mini-Prep plasmid purification kit (Qiagen, Valencia, CA, USA). DNA sequence analysis was then performed on an ABI 3730 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Data analysis

The DGGE profiles were scanned with BIOCAPTMW software (Vilber Lourmat, Marne la Vallée, France). The banding patterns were carefully checked manually. Similarities between the PCR-DGGE banding patterns were analysed using the Nei & Li's coefficient and displayed graphically as a dendrogram. An unweighted pair-group clustering algorithm with arithmetic averages was used to calculate the dendrograms. Cluster analyses were performed using the MVSP package version 3.1. Statistical analysis on apparent bacterial diversity (calculated as numbers of DGGE bands) in two parts of the gut was subjected to one-way ANOVA. When signicant differences were detected ($P < 0.05$), Duncan's multiple range was used to compare mean values among digesta and mucosa. All statistical analyses were carried out using the statistic software SPSS version 15.0.

All sequences were submitted for similarity searches with the BLAST program (Altschul, Gish, Miller, Myers & Lipman 1990). The closest relatives and phylogenetic affiliation of the obtained sequences were determined by using the BLASTN program in GenBank at the NCBI web site. The construction of phylogenetic tree was performed using the neighbor-joining method (Saitou & Nei 1987). The topological stability of the neighbor-joining tree was evaluated by 1000 bootstrapping replications, and the bootstrapping values were indicated by numbers at the nodes (Felsenstein 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura, Nei & Kumar 2004) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Phylogenetic analyses were conducted in MEGA4.1 (Tamura, Dudley, Nei & Kumar 2007).

Results

DGGE pattern analysis

Nineteen bands (1–14 and u1–u5) were excised from the DGGE gel (Fig. 1). The DGGE band distribution patterns are summarized in Table 1. There were nine to 13 bands in all the digesta samples except B sample with five bands. There were only two or three bands in mucosal samples. The average band number of digesta samples was significantly higher than that of tissue samples ($P < 0.05$, Table 1). There were less common bands (five bands) including band 5, 9, 10, 11 and 12 between digesta and mucosal samples. In contrast, there existed more different bands between digesta and mucosal samples. Three bands (band 1, 7 and 13) were unique in mucosal samples, while 11 bands (band u1, u2, u3, u4, u5, 2, 3, 4, 6, 8 and 9) were only observed in digesta samples. These results indicated that there existed significant differences in microbiota between digesta and mucosal samples.

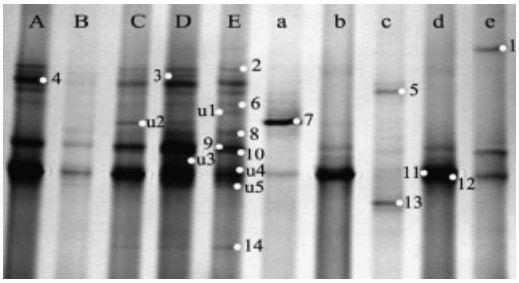

Figure 1 Denaturing gradient gel electrophoresis profiles of 16S rDNA fragments amplified from culture-independent bacteria in intestinal digesta and mucosa in mangrove red snapper ($n = 5$). Digesta: A, B, C, D, E; mucosa: a, b, c, d, e (corresponding to A, B, C, D, E).

Sequences and phylogenetic analysis

A total of 14 bands (band 1–14) were successfully sequenced from the DGGE gel (Fig. 1 and Table 2), these sequences were obtained (named MSR1 to MSR14, corresponding to band 1–14) and their respective closest sequences obtained from BLASTN searches in the GenBank database were used to construct phylogenetic trees (Figs 2–4). These sequences of approximately 430bp (GenBank accession numbers GU385014–GU385027) were compared using the online classifier tool in RDP and also are shown in Table 2. The bacteria identified from mangrove red snapper were closely related to one of the following groups: *γ-Proteobacteria* (64.25%, nine sequences), *Spirochaetes* (14.3%, two sequences) *Cyanobacteria* (14.3%, two sequences) and *Firmicutes* (7.15%, one sequence). However, the bacteria were identified as uncultured accounting for 28.6% (four sequences). MRS1 and MRS2 showed the similarities to the closest relatives obtained with 95% and 93% only, respectively, indicating that they might represent two new species. The bacteria identified from digesta included all the above four groups, whereas only three groups (*γ-Proteobacteria*, *Spirochaetes* and *Cyanobacteria* except *Firmicutes*) were observed in intestinal mucosa, indicating differences of microbiota between two parts of the gut.

Bacterial diversity in the intestines in mangrove red snapper

Common bacteria between intestinal digesta and mucosal surface (Fig. 2) included four species of *γ-Proteobacteria* (80%) and *Arthrospira* sp. (MRS9) of *Cyanobacteria* (20%). *γ-Proteobacteria* included four *Vibrio* spp., i.e. *Vibrio proteolyticus* (MRS5), *Vibrio*

Table 1 Band distribution of 16S rDNA fragments from culture-independent bacteria in intestinal digesta and mucosa

Fish	Sample	Band number	Band number	Average band number*
1	A	u4,2,3,4,5,6,9,11,12	9	11.2^{m}
2	B	u5,4,8,9,11	5	
3	C	u2,u4,2,3,4,5,6, 8,9,10, 11,12,14	13	
4	D	u2,u3,u4,u5,2,3,4,5,6,8,9,10,11,12, 14	15	
5	E	2,3,4,5,6,u1,8,9,10,u4,11,12,u5,14	14	
1	a	7,11	2	2.4^{n}
2	b	9,11	2	
3	c	5,13	2	
4	d	9,11,12	3	
5	e	1,10,11	3	

*Values (means) in the same column not sharing a common superscript are significantly different (Duncan's multiple range test, $P < 0.05$).

Table 2 Representative of bacteria or clones isolated from intestinal digesta and mucosa in mangrove red snapper

Phylogenetic group	Band number	Closest sequence (obtained from BLAST search)	Identity (%)	Accession number
Spirochaetes	1	Uncultured spirochaete clone TP-1 (DQ340184.1)	95	GU385014
	2	*Streptococcus henryi* strain 126 (EF364097.1)	93	GU385015
Firmicutes	4	Uncultured marine bacterium clone A6-1-13 (FJ826221.1)	99	GU385017
γ-Proteobacteria	5	*Vibrio proteolyticus* A2G-5B (DQ995521.1)	98	GU385018
	6	Uncultured bacterium clone aab65b04 (DQ814188.1)	98	GU385019
	7	*Vibrio natriegens* strain T4-05 (GU143898.1)	100	GU385020
	8	*Vibrio vulnificus* clone 05_D09 (EF546285.1)	98	GU385021
	10	*Vibrio metschnikovii* strain Fowl (NR_029258.1)	100	GU385023
	11	*Vibrio harveyi* (AY928014.1)	99	GU385024
	12	*Vibrio* sp. VS-59 (FJ497680.1)	100	GU385025
	13	*Vibrio parahaemolyticus* strain J-C2-34 (EU652251.1)	99	GU385026
	14	*Vibrio vulnificus* clone 09_B08 (EF546295.1)	98	GU385027
Cyanobacteria	3	*Brevibacillus agri* strain Z1-12 (GQ927168.1)	99	GU385016
	9	Uncultured *Arthrospira* sp. clone LAII-24 (EU872303.1)	100	GU385022

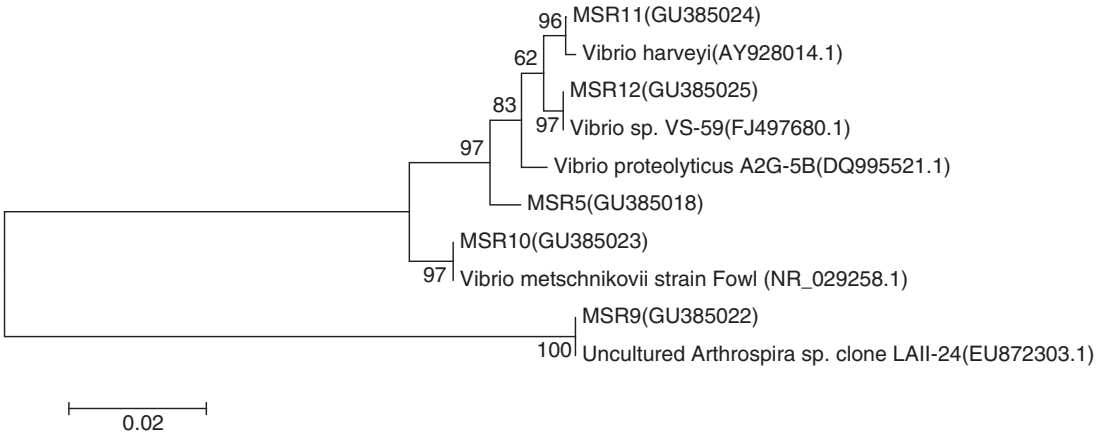

Figure 2 Neighbour-joining phylogenetic tree showing the relationship of common bacterial 16S rDNA gene sequences retrieved from denaturing gradient gel electrophoresis profiles between intestinal digesta and mucosal surface. The scale bar represents 2% sequence variation.

metschnikovii (MRS10), *Vibrio harveyi* (MRS11) and *Vibrio* sp. (MRS12).

The digesta-specific bacteria (Fig. 3) included six species belonging to four phyla, of which *Vibrio vulnificus* (MRS8) and one species of unidentified bacterium (MSR6) belonged to *γ-Proteobacteria*, *Brevibacillus agri* (MRS3) and *Arthrospira* sp. (MRS9) to *Cyanobacteria*, *Streptococcus henryi* (MRS2) to *Spirochaetes* and one unidentified bacterium species (MRS4) to *Firmicutes*.

A total of 11 bacteria species consisting of four phyla were identified in intestinal digesta of fish (Figs 2 and 3), of which seven species belonged to the *γ-Proteobacteria* (63.6%), two species to *Cyanobacteria* (18.2%), one species (MRS2, *S. henryi*) to *Spirochaetes* (9.1%) and one unidentified bacterium species (MRS4) to *Firmicutes* (9.1%).

The mucosa-specific bacteria (Fig. 4) included three species belonging to two phyla, of which *Vibrio natriegens* (MRS7), *Vibrio parahaemolyticus* (MRS13) belonged to *γ-Proteobacteria* and one uncultured species (MRS1) to *Spirochaetes*.

Intestinal adherent bacteria of all fish (Figs 2 and 4) included six species of *γ-Proteobacteria* (75%) and one species (MRS1) of *Spirochaetes* (12.5%), and one species (MRS9, *Arthrospira* sp.) of *Cyanobacteria* (12.5%), which belonged to three phyla.

DGGE bands with higher intensity represented the dominant individual populations. Based on the intensity of DGGE bands (Fig. 1) and the phylogenetic

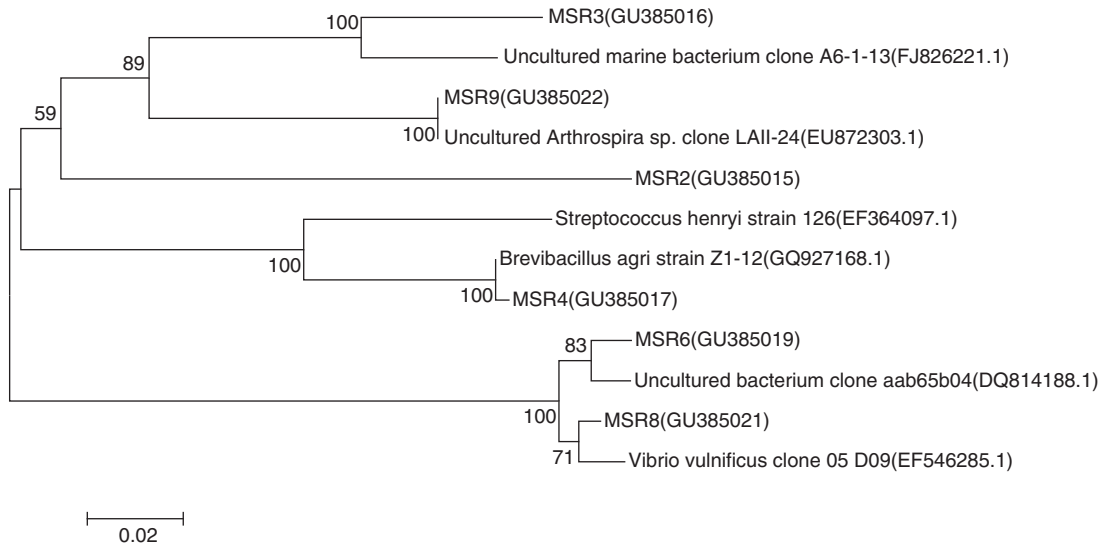

Figure 3 Neighbour-joining phylogenetic tree showing the relationship of bacterial 16S rDNA gene sequences uniquely retrieved from denaturing gradient gel electrophoresis profiles in intestinal digesta. The scale bar represents 2% sequence variation.

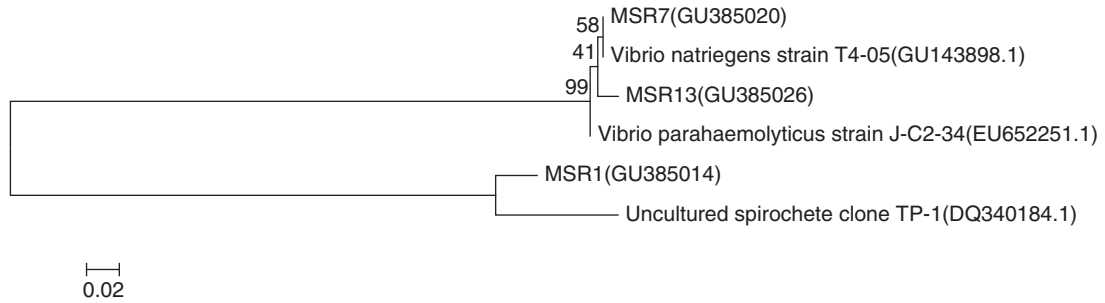

Figure 4 Neighbour-joining phylogenetic tree showing the relationship of bacterial 16S rDNA gene sequences specifically retrieved from denaturing gradient gel electrophoresis profiles in intestinal mucosa. The scale bar represents 2% sequence variation.

results (Table 2), we concluded that the dominant individual populations determined were one unknown species of *Firmicutes*, *Arthrospira* sp., *V. metschnikovii*, *V. harveyi* and *Vibrio* sp. in intestinal digesta and *V. natriegens*, *V. harveyi* and *Vibrio* sp. in intestinal mucosal surface. From the viewpoint of the population, members of the gamma subclass of *Proteobacteria* dominated absolutely the microbial community in the intestines of mangrove red snapper.

Discussion

Gut bacteria have antagonistic activity against pathogenic bacteria and might have a protective potential against colonization of pathogens (Ringø, Schillinger & Holzapfel 2005; Ringø, Sperstad, Myklebust, Myklebust, Refstie & Krogdahl 2006). The microbiota in intestinal mucosal surface facilitated beneficial functions including induction of the host innate immune response and nutrition exchange (Sonnenburg, Angenent & Gordon 2004). However, the information available regarding the mucosal microbiota in fish was scarce. Therefore, this study divided fish intestines into digesta and mucosal tissue samples in order to investigate the composition of microbiota in two parts of the gut.

Members of the gamma subclass of *Proteobacteria* dominated absolutely the microbial communities investigated in this study, which was in agreement with

previous reports (Huber, Spanggaard, Appel, Rossen, Nielsen & Gram 2004; Kim, Brunt *et al.* 2007; Zhou, Liu *et al.* 2009). γ-*Proteobacteria* accounted for 63.6% and 75% of the total bacterial richness in intestinal digesta and mucosal surface respectively. Vibrios (belonging to γ-*Proteobacteria*) were the predominant family in intestines in mangrove red snapper and accounted for 57.1% of the total bacterial species determinated.

V. harveyi (MRS11) and a *Vibrio* sp. (MRS12) dominated in both intestinal digesta and mucosal surfacesa in this study. Numerous strains of *V. harveyi* had been recognized as the most significant pathogens in cultured marine fish (Alvarez, Austin, Alvarez & Reyes 1998; Soffientino, Gwaltney, Nelson, Specker, Mauel & Gomez-Chiarri 1999; Zhang & Austin 2000; Pujalte, Sitja-Bobadilla, Maclan, Belloch, Alvarez-Pellitero, Perez-Sanchez, Uruburu & Garay 2003; Zorrilla, Arijo, Chabrillon, Diaz, Martinez-Manzanares, Balebona & Morinigo 2003). However, it was generally considered that this species was an opportunistic pathogen of marine fish and some infections related were only reported in stressed or experimental situations (Owens & Busico-Salcedo 2005). Phylogenetic analysis indicated that *Vibrio* sp. was the closest relative to *V. harveyi* (Fig. 2).

Other two vibirios that dominated in digesta and mucosa were *V. metschnikovii* and *V. natriegens* respectively. *V. metschnikovii* has been isolated previously from river water, clams, oysters, fish and birds that have died of a choleralike illness, and it may cause disease in both aquatic animals and humans (Buck 1991; Wallet 2005; Austin 2010). *V. natriegens*, with the capability of rapid reproduction and N_2-fixation, is a facultative anaerobic and ubiquitous in marine and estuarine environments (Coyer, Cabello-Pasini, Swifi & Alberte 1996). In China, this species has been reported able to infect cultured marine invertebrates including *Penaeus chinensis* and bivalves (Wang, Li & He 1993; Zhang, Liao, Li, Ji & Xu 1998; Deng, Chen, Shu, Zhang & Ma 2004; Li, Yan, Sun, Lin, Ma & Chang 2009).

Other two dominant species in the intestinal digesta were one unknown species of *Firmicutes* and *Arthrospira* sp. respectively. The bacteria of *Firmicutes* group including some potential probiotics and pathogens were frequently observed in intestines of marine fish (Olafsen, Mikkelsen, Giæver & Hansen 1993; Gildberg, Mikkeisen, Sandaker & Ringø 1997; Zhou, Liu *et al.* 2009; Feng *et al.* 2010), indicating their good adaptation to fish gut. *Arthrospira* is a microscopic and filamentous cyanobacterium, which had a long history of use as food and animal diets (Vonshak 1997) and the ability to modulate immune functions and could exhibit anti-inflammatory properties (Karkos, Leong, Karkos & Sivaji 2011). Seppola, Olsen, Sandaker, Kanapathippillai, Holzapfel and Ringø (2006) found members of the taxonomic group, with antagonistic activity to two pathogens *Aeronmonas salmonicida* and *Vibrio anguillarum* (Ringø *et al.* 2006), to be dominant in intestines of Atlantic cod. In addition, vibrios *V. proteolyticus*, *V. vulnificus* and *V. parahaemolyticus* among the γ-*Proteobacteria* have commonly been found as pathogenic bacteria for fish (Verschuere, Heang, Criel, Sorgeloos & Verstraete 2000; Marques, Thanh, Verstraete, Dhont, Sorgeloos & Bossier 2006; Austin 2010).

B. agri have been isolated from oil field (Li, She, Zhang, Zheng, Li, Xia & Wang 2006) and bioreactors containing natural gas (Bothe, Møller Jensen, Mergel, Larsen, Jørgensen, Bothe & Jørgensen 2002), fish meal wastewater (Kim, Kim, Cho & Hong 2007) and industrial effluents (Lara-Mayorga, Durán-Hinojosa, Arana-Cuenca, Monroy-Hermosillo & Ramirez-Vives 2010), indicating that this species was well adapted to different environments. In the present study, a sequence (MRS3) showed 99% identity to *B. agri*, yet phylogenetic analysis indicated that it belonged to *Cyanobacteria* and it was the dominant species in intestinal digesta.

In this study, two sequences (MRS1, MRS2) showed 95% and 93% similarities to an uncultured spirochaete (DQ340184.1) and *S. henryi* (EF364097.1) and belonged to *Spirochaetes*, which were observed previously in intestines of yellow grouper (Zhou, Liu *et al.* 2009) and puffer fish including *Takifugu niphobles* (Shiina, Itoi, Washio & Sugita 2006) and *T. obscurus* (Yang *et al.* 2007). *Spirochaetes* were famous because many members of the phylum caused a variety of poultry, human and mammalian diseases (Swayne, Eaton, Stoutenburg, Trott, Hampson & Jensen 1995; Lux, Moter & Shi 2000).

Previously, a study based on the culture-dependent method showed that the general population level of bacteria associated with the digesta was higher than that of bacteria adherent to the gut wall in Atlantic cod (*G. morhua* L.) fed soybean meal (Ringø *et al.* 2006). Our study indicated that there were significant differences of the microbiota between intestinal digesta and mucosal surface in mangrove red snapper. At first, the apparent bacterial richness (calculated as the mean number of DGGE bands) was significantly higher in digesta than in mucosal surface ($P < 0.05$). This was in support of the report of Kim,

Brunt *et al.* (2007), indicating that the intestinal mucus harboured a comparatively lower bacterial diversity than digesta. It was speculated that this may be attributed to the differences of environmental conditions between intestinal digesta and mucosal surface in fish. The gut contents may be favourable environments for most bacterial species. Whereas the intestinal mucosal surface with bile salt and low pH (Sera & Ishida 1972) may be harsh environments for most bacteria, so only a few bacterial species that adapted the environments could survive and settle. Further study should be conducted to confirm this speculation. Furthermore, there were unique bacterial species in both parts of the gut. By comparison, an uncultured spirochaetes (MRS1), *V. natriegens* (MRS7) and *V. parahaemolyticus* (MRS13) were only observed in intestinal mucosal surface, whereas *S. henryi* (MRS2), *B. agri* (MRS3), one unidentified bacterium species (MRS4, belonging to Firmucutes), an unidentified bacterium (MSR6, belonging to γ-*Proteobacteria*), *V. vulnificus* (MRS8), *Arthrospira* sp. (MRS9) and *Vibrio* sp. (MRS12) were uniquely present in digesta. The third difference of the microbial community structures between intestinal digesta and mucosal surface was that there were different dominant individual populations in both parts of the gut. There existed five dominant individual populations including one unknown species of *Firmicutes*, *Arthrospira* sp., *V. metschnikovii*, *V. harveyi* and *Vibrio* sp. in intestinal digesta and in contrast, only three dominant individual populations including *V. natriegens*, *V. harveyi* and *Vibrio* sp. in intestinal mucosal surface. It should be noted, however, that the individual dominant populations were correlative with the intestinal digesta or mucosal surface and might differ when seen from the whole intestine. For example, Spanggaard, Huber, Nielson, Nielson, Appel and Gram (2000) found that the abundance of bacteria in intestinal contents was two to three orders of magnitude higher than in the gut wall in freshwater rainbow trout by using the direct count method and concluded that the dominant microbial populations for the whole intestine existed in the digesta.

In conclusion, the results of the present study based on the culture-independent technique PCR-DGGE indicated that there existed diverse bacterial species in the intestine of mangrove red snapper. There were both potential pathogenic and beneficial bacteria in fish gut. The autochthonous microbiota in intestinal mucosal surface was significantly different from the allochthonous microbiota in digesta. In order to obtain more information such as physiological and biochemical characteristics as well as pathogenicity on bacterial species related, traditional culture methods and infection experiments should be included in future studies.

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