JOURNAL OF THE
WORLD AQUACULTURE SOCIETY

Vol. 46, No. 3
June, 2015
doi: 10.1111/jwas.12189

Comparison of the Intestinal Bacterial Flora in Healthy and Intestinal-diseased Seahorses *Hippocampus trimaculatus*, *Hippocampus erectus*, and *Hippocampus spinosissimus*

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Abstract

This investigation examined the intestinal microbial flora among healthy and intestinal-diseased seahorses *Hippocampus trimaculatus*, *Hippocampus erectus*, and *Hippocampus spinosissimus* by utilizing polymerase chain reaction-denaturing gradient gel electrophoresis (DGGE) and densitometric analysis. Results demonstrated that 16 disparate DGGE bands belong to six major bacterial groups, which were Vibrionace, Enterobacteriacea, Rhodobacteraceae, Sphingomonadaceae, Flavobacteriaceae, and Alcaligenaceae. It was found that Vibrionaceae was the dominant population among the healthy and intestinal-diseased seahorses. *Vibrio ponticus* strain XJ3 and *Vibrio neptunius* strain WT82, especially *V. ponticus* strain XJ3 of high abundance, were identified for the first time in seahorses and concluded to be intestinal disease pathogens because of their co-existence in three intestinal-diseased seahorse species and other reported fish or oyster. In comparison, uncultured bacterium clone Alcaligenaceae, *Vibrio sp.*, uncultured bacterium clone Rhodobacteraceae, *Serratia nematodiphila* strain, and *Serratia marcescens* strain comprised the basic intestinal bacterial flora of all healthy seahorses. This study is the first to report the presence of *S. nematodiphila* in seahorses.

Because of the global trade of seahorses to meet the strong demand from the Chinese traditional medicine, ornamental, and curio markets, the overexploitation of seahorses has exerted great pressure on wild stocks (Lourie et al. 1999; Vincent et al. 2011). As a result, seahorses have been listed in Appendix II of the Convention on the International Trade of Endangered Species of Wild Fauna and Flora (CITES) and the Red List of Threatened Species of the International Union for Conservation of Nature (IUCN 2006). In recent years, seahorse aquaculture has been deemed to be an important strategy to alleviate the pressure on the wild seahorse population (Woods 2000; Lin et al. 2009a, 2009b, 2009c, 2010; Celino et al. 2012; Qin et al. 2012, 2014). Fortunately, growing international interest on breeding and rearing seahorses has attracted increasing attention from farmers who traditionally fished seahorses in the wild (Koldewey and Martin-Smith 2010). Study of seahorse aquaculture has been performed throughout the world; however, focuses on the growth and survival

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of newborn seahorses and reproductive rates of parent seahorses are still challenging and have attracted substantial attention (Job et al. 2002; Woods 2003; Lin et al. 2008; Murugan et al. 2009). Numerous research works have determined that disease plays an essential role during seahorse farming and has caused a large number of deaths in some seahorse farms (Koldewey 2005; Lin et al. 2010).

Unbalanced intestinal bacterial flora often cause diseases and death of fish, as intestinal homeostasis can not only improve immunity against pathogens (Campos et al. 2006), but also provide abundant nutrient substances, such as vitamins, for the hosts (Sugita et al. 1991; Bairagi et al. 2002). Unfortunately, only a few researches have focused on isolating and identifying specific bacterium which cause seahorse diseases (Alcaide et al. 2001; Tendencia 2004; Balcázar et al. 2010a; Tanu et al. 2012), and there have been no reports concerning the intestinal bacterial flora of cultured seahorses. However, numerous diseases caused by intestinal bacteria have occurred in some fish (Koldewey 2005). The intestinal bacterial flora of cultured seahorses should be complex because of their mixed diet, that is, live and frozen food; this often increases the possibility of intestinal diseases in seahorses. Denaturing gradient gel electrophoresis (DGGE) has been shown to be a fast and effective method for investigating the diversity of the microbial community because of its independence of culture of microorganisms (Becker et al. 2008). Through DGGE, this study aimed to compare the difference of the composition and abundance of intestinal bacterial flora in both healthy and intestinal-diseased seahorses *Hippocampus trimaculatus, Hippocampus erectus*, and *Hippocampus spinosissimus*.

Materials and Methods

Cultured Seahorses

F_4 generation seahorses *H. trimaculatus*, *H. erectus*, and *H. spinosissimus* were reared in the Zhanjiang Seahorse Center of the South China Sea Institute of Oceanology, Chinese Academy of Sciences (SCSIO-CAS) with animal ethics approval for experimentation granted by the Chinese Academy of Sciences. Seahorses were cultured in concrete tanks ($3.5 \times 6 \times 1.3$ m) each with 350–400 pairs, and seawater was pumped directly from the South China Sea and treated with double-sand filtration. Temperature, salinity, pH, light intensity, and dissolved oxygen were maintained at (mean ± SD) 25 ± 0.5 C, 32 ± 1.0‰, 7.9 ± 0.4, 2000 lx, and 6.5 ± 0.5 mg/L, respectively. Seahorses were fed three times a day (0900, 1200, and 1600 h) with live *Artemia* and frozen *Mysis spp.*, and feces and uneaten food were siphoned out daily.

All adult healthy and diseased seahorses in this experiment were directly collected from the concrete tanks from November 1 to December 30, 2013. The healthy seahorses were collected randomly from the culture tanks. During the culture, some seahorses, which suffered from the white protuberant anus (spreading outside 1–2.7 mm) and narrow abdomen and ceased to feed the diets for at least 3 d, were sampled and cultured separately. The abdomen of these seahorses was dissected after being scrubbed with 70% ethanol and anesthetized by MS-222 (Vivify Nature, Nanjing, China) to examine the intestinal diseases of seahorses. Finally, six individuals, about 10 g for each type with the same symptoms of intestinal diseases, were randomly collected for this experiment. The intestinal tract was sampled and homogenized utilizing a ceramic homogenizer (LeaMaster et al. 1997), and kept in eppendorf tubes at −20 C until analysis.

DNA Extraction and PCR Amplification

Two hundred milligrams of intestinal sample of each type was ground with liquid nitrogen, and the total genomic DNA was extracted by using Axyprep Bacterial Genomic DNA Miniprep Kit (China TaKaRa Biotechnology branch Co., Ltd., Dalian, China) according to the manufacturer's instructions. The total genomic DNA was analyzed by electrophoresis in 0.7% (w/v) agarose gel containing Gold View (0.5% v/v) (China TaKaRa Biotechnology).

The V3 region of 16S rDNA was amplified by polymerase chain reaction (PCR) using primers 341f (5′-CGCCCGCCGCGCGCGGCG

GGCGGGGCGGGGGCACGGGGGGCCTAC GGGAGGCAGCAG-3′) and 534r (5′-ATTACC GCGGCTGCTGG-3′) (Muyzer et al. 1993). PCR amplification was carried out using Takara Ex Taq (China TaKaRa Biotechnology) according to the manufacturer's protocol. The PCR volume is 50 μL in total, which consists of 5.0 μL of 10× PCR buffer (including Mg^{2+}), 0.4 μL of TaKaRa ExTaq, 3.75 μL of 10 mmol/L dNTP mixture , 2 μL of template DNA, 2.5 μL of upstream and downstream primers, and ddH_2O was added to make a final volume of to 50 μL. Response procedures adopted touch-down PCR amplification. Amplification consisted of 35 cycles, of which 10 cycles included denaturation for 1 min at 94 C, annealing for 45 sec at 65 C, touch-down at 55 C, and extension for 1 min at 72 C. Then, amplification was followed by annealing for 45 sec at 55 C, consisting of 25 cycles. The first cycle was preceded by an initial template denaturation step for 4 min at 94 C, and the last cycle was followed by a final extension step for 10 min at 72 C. All of the PCR products were analyzed using electrophoresis in a 1.5% (w/v) agarose gel containing Gold View (0.5% v/v; China TaKaRa Biotechnology) (Fig. S1).

DGGE and Densitometric Analysis

DGGE was performed by utilizing a D-code System (Bio-Rad, Berkeley, CA, USA) to separate the PCR products. PCR samples were applied directly onto 8% (w/v) polyacrylamide gels in a 0.5× TAE buffer with a denaturing gradient ranging from 35 to 50% (100% corresponds to 7 M urea and 40% [v/v] formamide). Electrophoresis was performed with a constant voltage of 200 V at 60 C for 10 min and 80 V at 60 C for about 3.5 h. Gels were incubated for 30 min in 1× TAE buffer containing ethidium bromide (0.5 mg/L), and photographed with UV transillumination. Relative abundance of the predominant bands in the PCR–DGGE fingerprint was quantified by densitometric analysis (Wang et al. 2012).

Statistical Analysis

DGGE profiles were scanned by using DNAMAN software. The selected main DGGE bands were excised from the gels using a sterile knife (Fig. S2). Only one band representing the same bacterial 16S rDNA in different lanes was cut, excised from the DGGE gel, eluted by 30 μL of TE buffer at 4 C overnight, and then amplified again with the same primers 341f (without GC clamp) and 534r. The PCR product mixture was subsequently cloned into the pMD 18-T vector (China TaKaRa Biotechnology), and transferred into *Escherichia coli* DH5α and incubated at 37 C overnight. Five clones of each band were selected. Plasmid DNA was purified from the clones using the Qiagen Mini-Prep plasmid purification kit (Qiagen, Valencia, CA, USA) and sequenced Invitrogen (Shanghai Co., Ltd., Shanghai, China). All sequences were submitted for similarity searches using the BLAST program (Altschul et al. 1990), and checked for chimeric constructs by means of the Check Chimera program of the Ribosomal Database Project (Cole et al. 2005).

The closest relatives and phylogenetic affiliation of the obtained sequences were determined using the BLASTN program in GenBank at the NCBI website. The construction of the phylogenetic tree and bootstrap analysis of 1000 resamplings were performed using ClustalX (version 2.0) and MEGA (5.05) package (http://www.megasoftware.net/index.php; Larkin et al. 2007; Tamura et al. 2011).

Results

DGGE and Densitometric Analysis of V3 Regions

PCR–DGGE fingerprints of 16S rDNA-V3 were employed to compare the intestinal bacterial flora between healthy and diseased seahorses of three different species. Twenty-one bands were extracted from DGGE gel. Sixteen bands were successfully retrieved and numbered from 1 to 16, whereas the other five unsuccessfully retrieved bands were numbered from u1 to u5 because of their weak band density or difficulties in PCR as template after excision. Distribution patterns and relative abundance of all these bands are summarized in Table 1 and Figure 1, respectively. The order of number of bands of different samples, from high to low, is healthy

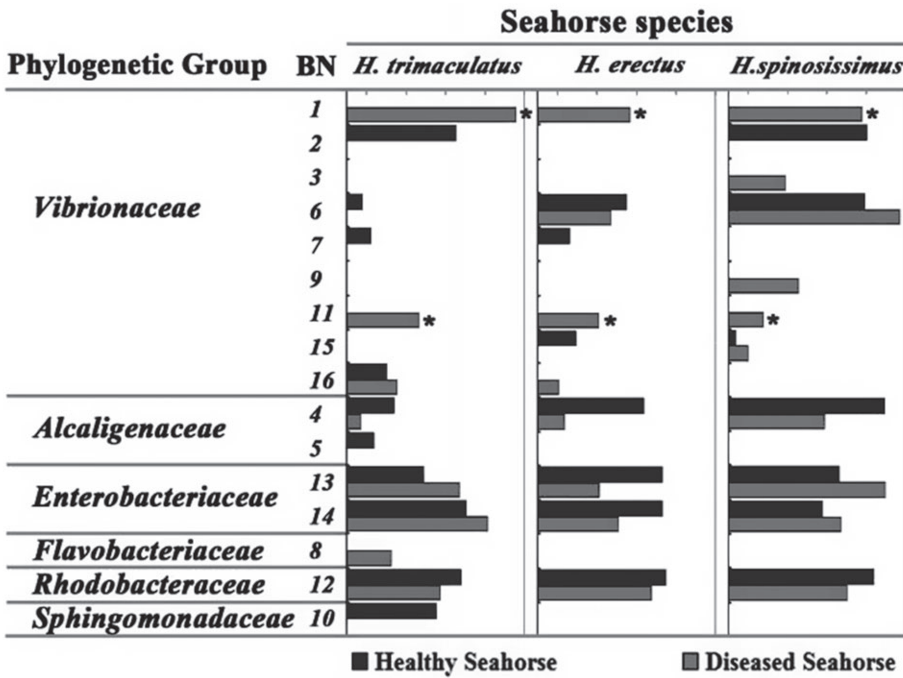

FIGURE 1. *Relative abundance of the intestinal bacterial flora in healthy and diseased seahorses of three species was determined by densitometric analysis, which was performed on the gray-level intensity of target bands derived from scanned images by using Gene Tools image analysis software (GeneTools, version 4.02; Syngene, Cambridge, UK) according to the manufacturer's instructions. * represents the bands specifically found in all diseased seahorse samples of three species.*

TABLE 1. *Band distribution of 16S rDNA fragments from the intestinal tract of three species of healthy and diseased seahorses.*

Sample[a]	Sample distribution	Total band
S1	2, 4, 5, 6, 7, 10, 12, 13, 14, 16, u1, u2, u3	13
S2	1, 4, 8, 11, 12, 13, 14, 16, u1, u2, u3	11
X1	4, 6, 7, 12, 13, 14, 15	7
X2	1, 4, 6, 11, 12, 13, 14, 16, u4	9
J1	2, 4, 6, 12, 13, 14, 15, u5	8
J2	1, 3, 4, 7, 9, 11, 12, 13, 14, 15	10

[a] S1 and S2: healthy and diseased *Hippocampus trimaculatus*; X1 and X2: healthy and diseased *Hippocampus erectus*; J1 and J2: healthy and diseased *Hippocampus spinosissimus.*

H. trimaculatus, diseased *H. trimaculatus*, diseased *H. spinosissimus*, diseased *H. erectus*, healthy *H. spinosissimus*, and healthy *H. erectus* (Table 1).

For healthy samples, five common bands were identified: 4, 6, 12, 13, and 14. For diseased samples, six were identified: 1, 4, 11, 12, 13, and 14. Notably, bands 4, 12, 13, and 14 were common in all healthy and diseased samples. Moreover, both bands 1 and 11 were specifically found in all diseased samples, especially for band 11 with high abundance (Fig. 1). In contrast to the above results, several bands were identified in one or two healthy species, such as band 2 in *H. trimaculatus* and *H. spinosissimus*, band 7 in *H. trimaculatus* and *H. erectus*, and band 10 in *H. trimaculatus* (Table 1 and Fig. 1).

Sequences of DNA Bands in DGGE Gel

Sixteen bands were successfully sequenced and identified by blasting (GenBank accession numbers KJ883014–KJ883029) (Table 2). Subsequently, a phylogenetic tree was constructed based on these sequences (Fig. 2). Six bacterial groups were identified as follows: Vibrionaceae (56.25% of the total; bands 1, 2, 3, 6, 7, 9, 11, 15, and 16), Alcaligenaceae (12.5% of the total; bands 4 and 5), Enterobacteriaceae (12.5% of the total; bands 13 and 14), Flavobacteriaceae (6.25% of the total; band 8), Rhodobacteraceae

(6.25% of the total; band 12), and Sphingomonadaceae (6.25% of the total; band 10) (Table 2). The ratios of different bacterial groups in healthy and diseased seahorses are summarized in Table 3. Notably, Vibrionaceae represented the dominant population of all healthy and diseased samples.

Five dominant bands (bands 4, 6, 12, 13, and 14) of all healthy seahorses belonged to Enterobacteriacea (bands 13 and 14), Alcaligenaceae (band 4), Rhodobacteraceae (band 12), and Vibrionaceae (band 6), respectively. On the other hand, six dominant bands (bands 1, 4, 11, 12, 13, and 14) of all diseased seahorses belonged to Enterobacteriacea (bands 13 and 14), Vibrionaceae (bands 1 and 11), Alcaligenaceae (band 4), and Rhodobacteraceae (band 12). Bands 4, 12, 13, and 14 common in all samples showed 99, 100, 99, and 99% similarity to an uncultured Alcaligenaceae (KF791101.1), an uncultured Rhodobacteraceae (GQ413664.1), *Serratia nematodiphila* strain, and *Serratia marcescens* strain, respectively. Moreover, the specific bands 1 and 11 of all diseased seahorses belonged to the same genus *Vibrio spp.*, and showed 99 and 98% similarity to *Vibrio ponticus* strain XJ3 and *Vibrio neptunius* strain WT82, respectively. Moreover, bands 2, 7, and 10 showed 99, 98, and 100% similarity to an uncultured Vibrionaceae (KF760544.1), *Vibrio parahaemolyticus* UCM-V493, and an uncultured Sphingomonadaceae (KC712693.1), respectively.

Discussion

This study addressed the composition of the intestinal bacterial flora of cultured healthy and diseased seahorses, and then demonstrated the potential bacterial pathogens for intestinal disease of seahorses. PCR–DGGE and densitometric analysis were employed to determine the composition and relative abundance of the intestinal bacterial flora (Table 1 and Fig. 1). Genetic relationship was determined by constructing a phylogenetic tree after sequencing and blasting (Fig. 2). Our results showed that composition, relative abundance, and ratio of intestinal bacterial flora varied among both healthy and diseased seahorses of *H. trimaculatus*, *H. erectus*, and *H. spinosissimus* (Tables 1 and 3, Fig. 1). Of note, Vibrionaceae represents the dominant population in the intestinal tract of all healthy (40% in *H. trimaculatus*, 42.85% in *H. erectus*, and 42.85% in *H. spinosissimus*) and diseased (37.5% in *H. trimaculatus*, 50% in *H. erectus*, and 60% in *H. spinosissimus*) seahorses, which is consistent with that of healthy *Hippocampus guttulatus* (Balcázar et al. 2010b).

In this study, bands 1 and 11, which showed 99 and 98% similarities to *V. ponticus* strain XJ3 and *V. neptunius* strain WT82, respectively (Table 2), were specifically identified in all three species of diseased seahorses (Table 1 and Fig. 1). *V. ponticus* was first isolated from the marine aquaculture environment (Thompson et al. 2003), whereas *V. neptunius* was isolated from gilthead sea bream, mussels, and seawater (Macián et al. 2004). Subsequently, it has been declared as an oyster pathogen (Prado et al. 2005) and the pathogenic factor for cultured Japanese sea bass (Xie et al. 2007). Therefore, *V. ponticus* and *V. neptunius* identified from the diseased seahorses *H. trimaculatus*, *H. erectus*, and *H. spinosissimus* should be the pathogenic candidates for the intestinal disease of farmed seahorses, especially for *V. ponticus* because of its high abundance (Fig. 1).

Five bands (bands 4, 6, 12, 13, and 14) which showed 99, 98, 100, 99, and 99% similarities to uncultured bacterium clone Alcaligenaceae (KF791101.1), *Vibrio sp.*, uncultured bacterium clone Rhodobacteraceae (GQ413664.1), *S. nematodiphila* strain, and *S. marcescens* strain, respectively, were found to be dominant in all three species of healthy seahorses. Rhodobacteraceae has been identified in the intestinal tract and cutaneous mucus of healthy *H. guttulatus* (Balcázar et al. 2010b). Subsequently, Rhodobacteraceae was also identified in the intestinal tract of farmed healthy *Hippocampus kuda* (Tanu et al. 2012). Moreover, to the best of our knowledge, this study is the first to report the presence of *S. nematodiphila* in seahorses, and even in fish. *S. nematodiphila* has been shown to symbiotically associate with nematode (Zhang et al. 2009), and has bactericidal activity (Malarkodi et al. 2013) and insecticidal potency (Patil et al. 2012). *S. marcescens*, an

TABLE 2. *Representative bacteria or clones isolated from the intestinal tract of three species of healthy and diseased seahorses.*

Phylogenetic group	Ratio (%)	Band number	Closest sequence (obtained from BLAST search)	Identity (%)	Accession number
Vibrionaceae	56.25	1	*Vibrio ponticus* strain XJ3 (KC884659)	99	KJ883014
		2	Uncultured *Vibrio sp.* clone CD22 (KF760544.1)	99	KJ883020
		3	*Vibrio tubiashii* strain GR09 (KF784850.1)	99	KJ883027
		6	*Vibrio sp.* VibC-Oc-085 (KF577017.1)	98	KJ883028
		7	*Vibrio parahaemolyticus* UCM-V493 (CP007005.1)	98	KJ883023
		9	*Vibrio alginolyticus* strain POLY-S11 (KF155265.1)	99	KJ883024
		11	*Vibrio neptunius* strain WT82 (KC884657.1)	98	KJ883019
		15	*Enterovibrio sp.* EF4A-B638 (KC545318.1)	99	KJ883017
		16	*Vibrio sp.* R7B1 (KC439250.1)	98	KJ883029
Alcaligenaceae	12.5	4	Uncultured bacterium clone 08-BS78 (KF791101.1)	99	KJ883021
		5	*Achromobacter sp.* I12B-00880 (KC589242.1)	99	KJ883022
Enterobacteriaceae	12.5	13	*Serratia nematodiphila* strain faro7_41A (KF792276.1)	99	KJ883015
		14	*Serratia marcescens* strain S-5 (KF831003.1)	99	KJ883016
Flavobacteriaceae	6.25	8	Uncultured bacterium clone K4H (JF789310.1)	98	KJ883018
Rhodobacteraceae	6.25	12	Uncultured bacterium clone RefT1c10_F06 (GQ413664.1)	100	KJ883026
Sphingomonadaceae	6.25	10	Uncultured bacterium clone ORV1F_8F_a04 (KC712693.1)	100	KJ883025

opportunistic bacterial pathogen with a broad range of hosts, was found to be a pathogen of diseased tilapia fish (Chan et al. 2013). Based on this evidence and the variation of the abundance in healthy and diseased seahorses (Fig. 1), we suggest that these dominant bacteria basically make up the normal intestinal bacterial flora of three species of healthy seahorses.

V. parahaemolyticus is known as an important cause of foodborne disease, linked to the consumption of contaminated seafood (Bisha et al. 2012). It was identified from the intestinal tract of farmed *H. kuda* (Tanu et al. 2012). In this study, band 7, which showed 98% similarity to *V. parahaemolyticus* UCM-V493, was only found in the intestinal tract of healthy *H. trimaculatus* and *H. erectus*. The completion of whole-genome sequencing of *V. parahaemolyticus* UCM-V493 will be helpful for revealing its role in seahorse intestinal tract.

Vibrio alginolyticus and *Vibrio splendidus* were first isolated and found to be dominant in lesions of captive-bred seahorses (Balcázar et al. 2010a). Band 9, which was only found in diseased *H. spinosissimus*, showed 99% similarity to *V. alginolyticus* strain POLY-S11. However, the virulence-associated genes of *V. alginolyticus* from the mariculture systems have been verified in *Epinephelus coioides* (Ren et al. 2013). Whether or not it is one of the intestinal pathogens of *H. spinosissimus* needs to be confirmed.

Vibrio tubiashii sp. nov. was identified to be a pathogen of bivalve mollusks (Hada et al. 1984), and its severe episodes caused great threat for shellfish aquaculture along the west coast of North America (Elston et al. 2008). Its extracellular metalloprotease was verified to be the virulence factor for pacific oyster (Hasegawa et al. 2008). Band 3, the other band especially found in diseased *H. spinosissimus*, showed 99% similarity to *V. tubiashii* strain GR09. To the best of our knowledge, this is the first time that *V. tubiashii* has been identified in diseased seahorses.

Achromobacter sp. I12B-00880 (band 5, 99% similarity) was only identified in healthy *H. trimaculatus* with relatively low abundance (Fig. 1). It was only reported in a study by

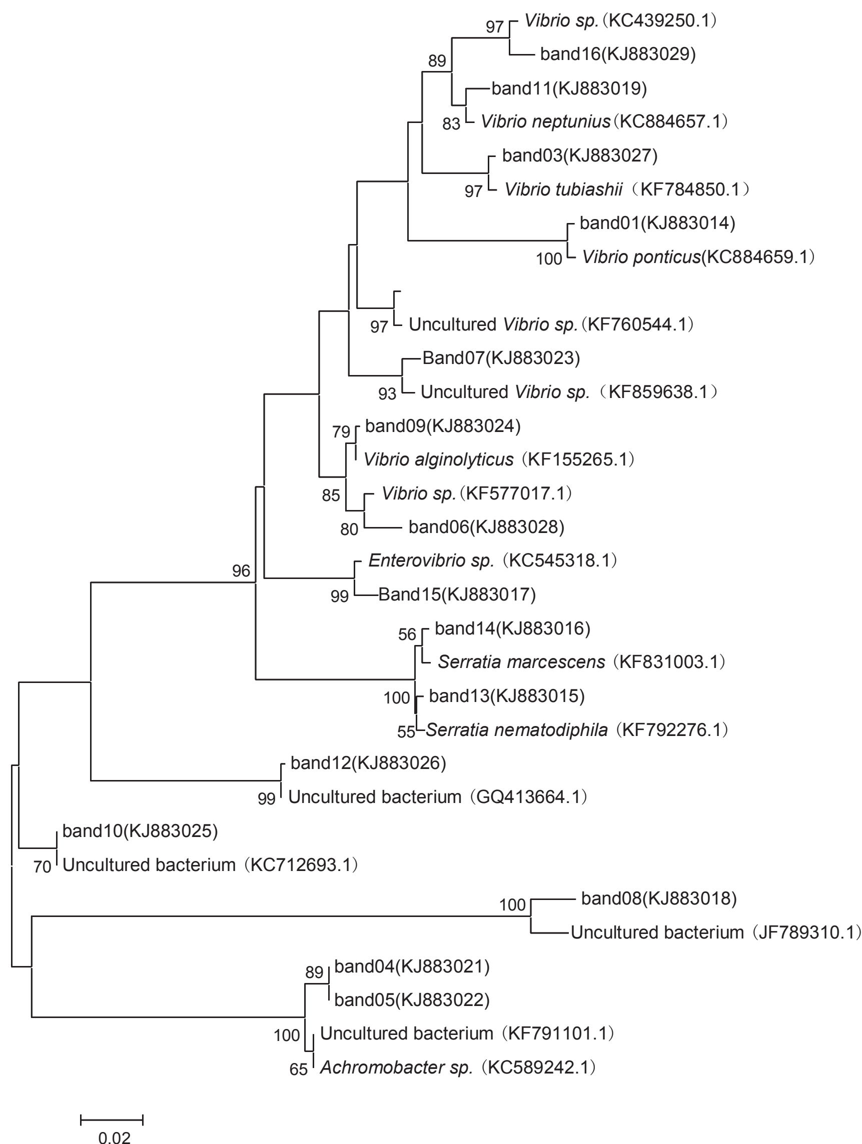

FIGURE 2. *Neighbor-joining phylogenetic tree revealing the relationship of 16 16S rDNA gene sequences retrieved from denaturing gradient gel electrophoresis profiles. It was constructed by using ClustalX (version 2.0) and the neighbor-joining method within the MEGA (5.0) package. Bootstrap values based on 1000 resamplings display the significance of the interior nodes, and are shown at branch points. Only values that display >50% are given. The scale bar represents 2% sequence variation. Sequences of known species are shown in italics.*

Fang et al. (National Center for Biotechnology Information [NCBI], http://www.ncbi.nlm.nih.gov/). It may represent one of the normal intestinal bacteria of *H. trimaculatus*.

Band 15 was only identified in diseased *H. erectus* with low abundance (Fig. 1) and showed 99% similarity to *Enterovibrio sp.* EF4A-B638 (KC545318.1), which was first isolated from *Eunicea fusca* (Pike et al. unpublished data, NCBI). In addition, band 16 showed 98% similarity to *Vibrio sp.* R7B1 (KC439250.1), which was first isolated from *Narcine bancroftii* from the Gulf of Mexico (Tao and Arias, unpublished data, NCBI).

TABLE 3. *Ratio of different bacterial groups of healthy and diseased seahorses.*[a]

G. S.	S1 (%)	S2 (%)	X1 (%)	X2 (%)	J1 (%)	J2 (%)
Vibrionaceae	40	37.5	42.85	50	42.85	60
Alcaligenaceae	20	12.5	14.29	10	14.29	10
Enterobacteriaceae	20	25	28.57	20	28.57	20
Flavobacteriaceae		12.5				
Rhodobacteraceae	10	12.5	14.29	10	14.29	10
Sphingomonadaceae	10					

[a] S. refers to sample; G. refers to group; S1 and S2 refer to healthy and diseased *Hippocampus trimaculatus,* respectively; X1 and X2 refer to healthy and diseased *Hippocampus erectus*, respectively; J1 and J2 refer to healthy and diseased *Hippocampus spinosissimus*, respectively.

In this study, some bands showed high similarity to uncultured bacteria. Band 2 showed 99% similarity to uncultured *Vibrio sp.* clone CD22 (KF760544.1) and was found in healthy *H. trimaculatus* and *H. spinosissimus*. It may be one component of the normal intestinal bacterial flora of *H. trimaculatus* and *H. spinosissimus*. Band 8, which was identified only in diseased *H. trimaculatus*, showed 98% similarity to uncultured bacterium clone K4H (JF789310.1) reported in a study by Godoy and Wittwer (unpublished data, NCBI). Band 10 showed 100% similarity to uncultured bacterium clone ORV1F_8F_a04 (KC712693.1), which was documented in a study of Colman et al. (unpublished data, NCBI) and was identified only in healthy *H. trimaculatus*. Thus, we suggest that it may be one of the normal intestinal bacteria of *H. trimaculatus*.

So far, the intestinal diseases have already been an important threat for the seahorse aquaculture, and *H. kuda* suffers high intestinal disease symptoms while being fed by the frozen food or non-sterile live food, and maintenance of culture water and diet quality is the main strategy for preventing the occurrence of the intestinal diseases in seahorses (Lin et al. 2010; Tanu et al. 2012). In comparison with the cultured seahorses, the wild seahorses *H. guttulatus* also carry some disease bacteria *V. alginolyticus* and *V. splendidus*, so the environmental condition is also important for the health of seahorse population (Balcázar et al. 2010a). Among the three different seahorse species in this study, the genus *Vibrio spp*. represented the dominant group of intestinal bacterial flora, which was consistent with that in other seahorse species (Balcázar et al. 2010b; Tanu et al. 2012). However, the particular composition of the intestinal bacterial flora between healthy and intestinal-diseased seahorses, and among different seahorse species varied significantly to different extents. Two potential pathogens, *V. ponticus* strain XJ3 and *V. neptunius* strain WT82, should be responsible for the intestinal disease of three seahorse species, although reinfection experiments need to be carried out to confirm their virulence. In comparison with other species of *Vibrio spp.*, *Vibrio hippocampi spp.* nov. in *H. guttulatus* (Balcázar et al. 2010c) and *V. alginolyticus* and *V. splendidus* in *H. guttulatus* and *H. hippocampus* have been isolated and shown to cause lesions in parts of diseased seahorses other than the intestinal tract (Balcázar et al. 2010a). Moreover, in addition to the diseases in the intestinal tract of seahorses, the study of diseases and pathogens in seahorses would be essential along with increasing interest in seahorse culture.

Acknowledgments

We thank Geng Qin for collecting the seahorse samples. This study was funded by the Outstanding Youth Foundation in Guangdong Province (S2013050014802), the Development of Special Biology Industry in Shenzhen City (HY20130205001), the Guangdong Oceanic and Fisheries Science and Technology Foundation (A201201I03 and A201208G02), and the Science Foundation of Ludong University (LY2014042).

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Supporting Information

Additional Supporting information may be found in the online version of this article.