The Journal of Microbiology (2012) Vol. 50, No. 1, pp. 29–37
DOI 10.1007/s12275-012-1340-1

Host Species as a Strong Determinant of the Intestinal Microbiota of Fish Larvae

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(Received July 18, 2011 / Accepted September 20, 2011)

We investigated the influence of host species on intestinal microbiota by comparing the gut bacterial community structure of four cohabitating freshwater fish larvae, silver carp, grass carp, bighead carp, and blunt snout bream, using denaturing gradient gel electrophoresis (DGGE) of the amplified 16S and 18S rRNA genes. Similarity clustering indicated that the intestinal microbiota derived from these four fish species could be divided into four groups based on 16S rRNA gene similarity, whereas the eukaryotic 18S rRNA genes showed no distinct groups. The water sample from the shared environment contained microbiota of an independent group as indicated by both 16S and 18S rRNA genes segments. The bacterial community structures were visualized using rank-abundance plots fitted with linear regression models. Results showed that the intestinal bacterial evenness was significantly different between species ($P<0.05$) and between species and the water sample ($P<0.01$). Thirty-five relatively dominant bands in DGGE patterns were sequenced and grouped into five major taxa: *Proteobacteria* (26), *Actinobacteria* (5), *Bacteroidetes* (1), *Firmicutes* (2), and *Cyanobacterial* (1). Six eukaryotes were detected by sequencing 18S rRNA genes segments. The present study suggests that the intestines of the four fish larvae, although reared in the same environment, contained distinct bacterial populations, while intestinal eukaryotic microorganisms were almost identical.

Keywords: intestinal microbiota, denaturing gradient gel electrophoresis, freshwater fish larvae, host species effects

Introduction

Prokaryotes are the most abundant form of life on the planet (Whitman *et al.*, 1998). Vast numbers of microbes are present in the animal gastrointestinal (GI) tract and are critical for the nutrition and health of the host (Bäckhed *et al.*, 2004; Gordon, 2005). The physiological role of microbiota in fish guts has been extensively studied (Rawls *et al.*, 2004, 2007; Bates *et al.*, 2006). The intestinal microflora of fish, primarily established at the yolk sac stage when ingestion of bacteria in ambient water starts (Hansen and Olafsen, 1999), are important for immune tolerance and for the successful rearing of several freshwater fish species in early life stages (Sugita *et al.*, 1994). In fish intestine, these microbiota are dominated by four bacterial phyla, *Proteobacteria*, *Fusobacterium*, *Actinobacteria*, and *Cyanobacteria*, but different hosts harbor distinct species and strains (Ley *et al.*, 2006). Species and other factors affecting intestinal community structure, such as diet, age, and environmental factors have been examined (Austin, 2006; Kim *et al.*, 2007; Navarrete *et al.*, 2009; Ward *et al.*, 2009).

Fish species is a major influence on intestinal microbiota (Camp *et al.*, 2009), but distinct environments and diets may obscure the true influence of fish species of intestinal microbiota. For example, different aquaculture farms and diets had a significant effect on the composition of the intestinal microflora in rainbow trout (*Oncorhynchus*) and abalone (*Haliotis discus hannai*) (Spanggaard *et al.*, 2000; Tanaka *et al.*, 2004). The influence of fish species on intestinal microbiota is revealed under similar environmental and dietary conditions.

In the present study, the intestinal microbial community structure of four freshwater fish species from the same hatchery were investigated: silver carp (*Hypophthalmichthys molitrix*), grass carp (*Ctenopharyngodon idella*), bighead carp (*Aristichthys nobilis*), and blunt snout bream (*Megalobrama amblycephala*). All larvae were sampled 4 d after hatching while in the pelagic phase. No artificial food was provided, and the fish thrived only on natural organisms in the hatchery water. Considering that all fish larvae shared the same water and were exposed to the same prey, it is reasonable to assume that all fish species had very similar diets.

A vast number of uncharacterized microorganisms restrict analysis of GI microbiota using traditional culture approaches. Alternative approaches, such as denaturing gradient gel electrophoresis (DGGE) based on 16S rRNA genes, have been widely used to study the microbial community composition in fish intestine (Tannock, 2001; Wielen *et al.*, 2002; De La Cochetière *et al.*, 2008; Zhou *et al.*, 2009). The genetic relationships of the four fish species were explored by sequencing the mitochondrial cytochrome c oxidase I (COI) gene, an efficient DNA "barcoding" technique for identifying freshwater fish species (Hubert *et al.*, 2008). The main aim of the study is to elucidate differences in the intestinal microbiota of the four fish species and to determine the impact of host species while controlling for dif-

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ferences in the environment and diet. Detailed phylogenetic information was obtained by cloning and sequencing bacterial 16S rRNA and 18S rRNA genes.

Materials and Methods

Sampling

Larvae of silver carp, grass carp, bighead carp, and blunt snout bream were collected from a commercial fish farm in Hubei Province, China. The first feeding larvae (8–15 mm long) were randomly collected from the same cyclical larval incubator and transported to the laboratory. A sample of freshwater (500 ml) was also taken directly from the aquifer access, the hatchery's water source.

The larvae were processed immediately upon arrival in the laboratory. The GI samples of all fish were obtained by aseptic dissection and extraction of the entire GI tract under a stereomicroscope. The GI tract of first feeding larvae consisted mainly of a poorly developed tube from the mouth to the anus, with a slight enlargement in the foregut. After dissection, the entire GI tracts of 15 fish from the each species were pooled for analysis of bacterial communities, and three independent repetitions were performed for each species. Pooling of samples (15) is a common practice in studying the gut microbiota of fish (Thomas *et al.*, 1998; Hovda *et al.*, 2007). Furthermore, previous studies showed that individual microbiota are well represented by pooled samples (Romero and Navarrete, 2006).

DNA extraction

The DNA from each species was obtained from pooled tissue homogenates that were submerged overnight in 1 ml lysis buffer [30 mM EDTA, 10 mM Tris-HCl; pH 8.0, 50 µl 10% (w/v) SDS, 0.1 mg proteinase K, and 0.05 mg RNase A] at 55°C. Genomic DNA was subsequently extracted with phenol/chloroform and precipitated with ethanol as previously described (Li *et al.*, 2009). Pelletized DNA was resuspended in 50 µl ddH_2O and stored at -70°C until PCR amplification.

The DNA from the water sample was obtained by filtering 500 ml of water through glass fiber filters (Whatman type GF/C, 47 mm diameter) and membrane (0.22 µm, Millipore, USA). The filters were immediately cut into small pieces, soaked in 3 ml of the same lysis buffer, and extracted as described above.

PCR amplification

The variable V3 region of the bacterial 16S rRNA gene was amplified by PCR using bacterial domain-specific primers F357 (5′-CCTACGGGAGGCAGCAG-3′ with GC clamp in the 5′ end) and R518 (5′-ATTACCGCGGCTGCTGG-3′) as previously described (Muyzer *et al.*, 1993). PCR reactions were performed in 25 µl mixtures containing 80 µM dNTP, 2 mM $MgCl_2$, 0.25 µM of each primer, and 1.5 U of *Taq* DNA polymerase in 1× buffer. Touchdown PCR was performed on a S1000™ thermal cycler (Bio-Rad, USA) using an initial incubation of 5 min at 94°C, then 30 cycles of 30 sec at 94°C, 30 sec at annealing temperature (70–60°C for 10 cycles, then 59°C for 20 cycles), and 60 sec at 72°C, followed by a final extension step of 10 min at 72°C.

Amplification of the eukaryotic microorganisms in fish intestine was performed as described by Yu *et al.* (2008) using 18S rRNA gene primers F1427 (5′-TCTGTGATG CCCTTAGATGTTCTGGG-3′ with GC clamp in the 5′ end) and R1616 (5′-GCGGTGTGTACAAAGGGCAGGG-3′) (van Hannen *et al.*, 1998). A 652 bp COI gene was amplified by the following primers: FishF1 (5′-TCAACCAACCACAAA GACATTGGCAC-3′) and FishR1 (5′-TAGACTTCTGGG TGGCCAAAGAATCA-3′) (Ward *et al.*, 2005). All PCR amplifications of eukaryotic genes were performed in the same reaction mixture as described for prokaryotic gene amplification. The thermocycle protocol consisted of predenaturation at 94°C for 2 min, 35 cycles of 94°C for 30 sec, 52°C 40 sec, and 72°C for 1 min, and a final extension at 72°C for 10 min (Hubert *et al.*, 2008).

DGGE and statistical analysis

The PCR products obtained from F357 and R518 primers were separated on 9% polyacrylamide gels (the ratio of acrylamide to bisacrylamide was 37.5:1) in 1×TAE buffer and with a denaturing gradient from 35% to 65% of urea and formamide. The PCR products from F1427 and R1616 were separated on a denaturing gradient from 40% to 70%. Electrophoresis was run for 8 h at 130 V under a constant temperature of 60°C in a D-Code system (Bio-Rad, UK). After electrophoresis, the gels were stained with SYBR Gold (Molecular Probes) at room temperature for 30 min, washed with 1×TAE buffer, and visualized under UV light. The DGGE marker II (Wako, Japan) was used as a standard sample for between-gel analysis. Band and profile analysis and comparisons were performed with Quantity One (The Discovery Series™ software). The intensity and number of bands present in a sample were used to estimate the relative abundance and taxa richness as previously described (van der Gast *et al.*, 2006, 2008).

The unweighted pair-group method using arithmetic average (UPGMA) clustering was utilized to investigate the community similarity of bacterial and eukaryotic taxa among the different fish species and the water sample. The similarity coefficient among the treatments was calculated as the dice coefficient $S_D=(2n_{AB})/(n_A+n_B)$, where n_A is the number of bands in sample A, n_B is the number of bands in sample B, and n_{AB} is the number of bands common to both samples. Rank-abundance plots were used to determine bacterial community structures based on the relative abundance of each taxa (Lilley *et al.*, 1996). The relative abundance of each bacterial taxon (DGGE band) was standardized to percent values for each sample before construction of the rank-abundance plots. The taxa rank orders were plotted on the x-axis, while relative abundance ($\log_{10}$ transformed) was plotted on the y-axis. Each plot was fitted by the linear regression model $\log_{10}y=a+bx$, where "a" is the intercept and "b" is the slope of the plot. This slope ("b") was subsequently used as a descriptive statistic for changes in bacterial evenness in the intestines of the different species of fish (Ager *et al.*, 2010). Linear regressions, coefficients of determination (r), and one-way ANOVA test of evenness were calculated using SPSS 13.0 software.

Sequencing analysis

For cloning and sequencing, the dominant bacterial and eukaryotic bands derived from 16S rRNA and 18S rRNA gene DGGE patterns were excised and re-amplified. PCR was conducted as described above but with no-GC-clamp primers. Cloning was performed using the pGEM-T Easy vector system cloning kit and *Escherichia coli* DH-5α according to the manufacturer's instructions. In total, 41 clone libraries were constructed and three clones of each libraries were randomly selected and sequenced by Shanghai Sunny Biotechnology Co., Ltd. All partial 16S rRNA gene sequences were compared with those in the public Ribosomal Database Project II (Cole *et al*., 2005) in order to ascertain their closest relatives. Partial 18S rRNA gene sequences and COI sequences were compared with those in the NCBI database using BLASTN searches. Neighbor-joining phylogenetic trees were calculated for bacterial 16S rRNA gene sequences, 18S rRNA gene sequences, and COI sequences using CLUSTAL X (version 1.83) and MEGA (4.0) package (Tamura *et al*., 2007). Bootstrap analysis was performed with 1,000 repetitions, and values greater than 50% are shown in all phylogenetic trees.

The 41 sequences have been included in the GenBank nucleotide sequence database (GenBank accession nos. HM776316–HM776368).

Results

Comparisons of bacterial community structure among four fish species as determined by PCR-DGGE

The DGGE profiles of 16S rRNA and 18S rRNA gene composites of the four fish larvae and the water sample were compared to determine if there were any apparent differences in bacterial community structure (Figs. 1A and B). The number of 16S rRNA gene bands was much higher than 18S rRNA gene bands, indicating the presence of more bacterial taxa in the samples. In addition, there were definite

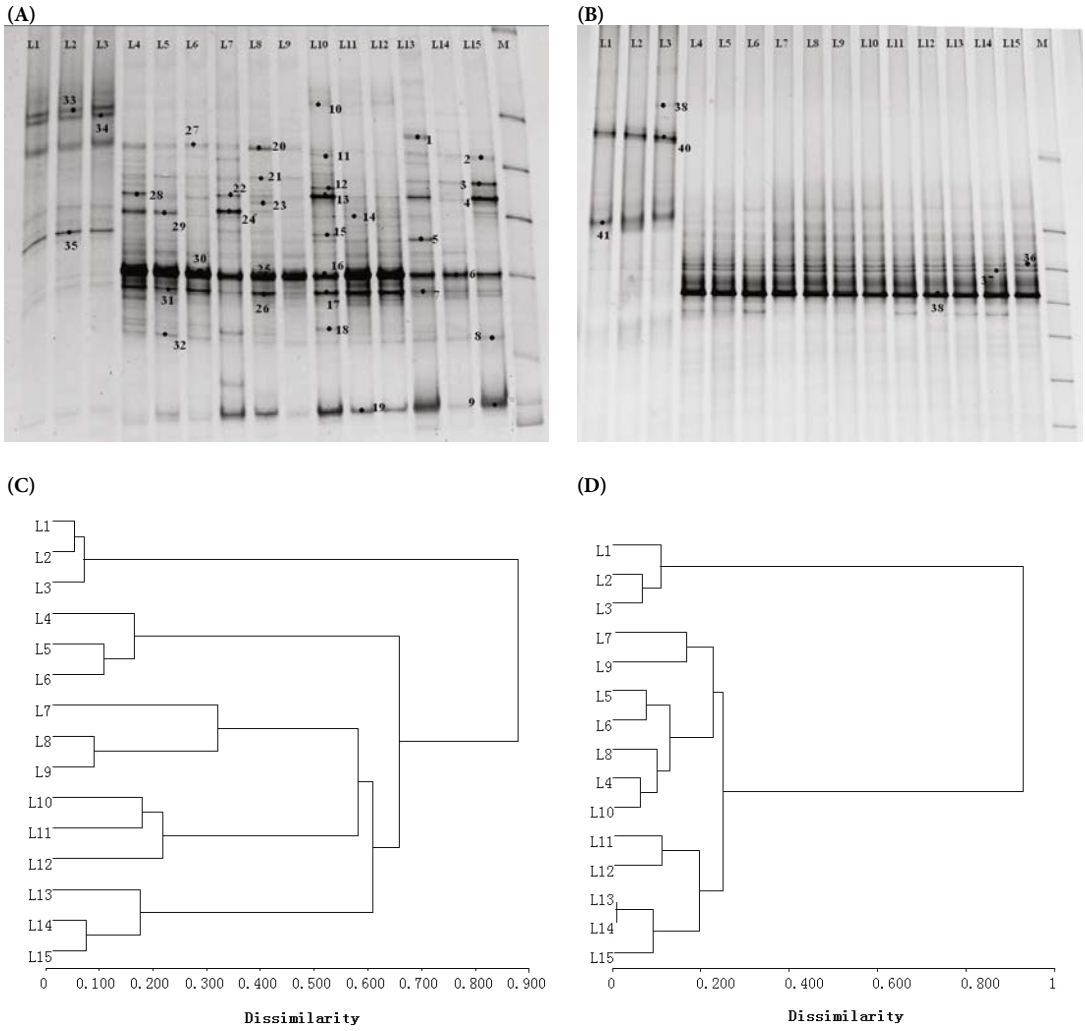

Fig. 1. DGGE profiles and UPGMA clustering of 16S rRNA genes (A, C) and 18S rRNA genes (B, D) based on the binary data set. Lanes: 1–3, (L1–L3) indicate samples collected from the water sample; L4–L6, from blunt snout bream; L7–L9, from bighead carp; L10–L12, from grass carp; and L13–L15, from silver carp. M is the DGGE marker.

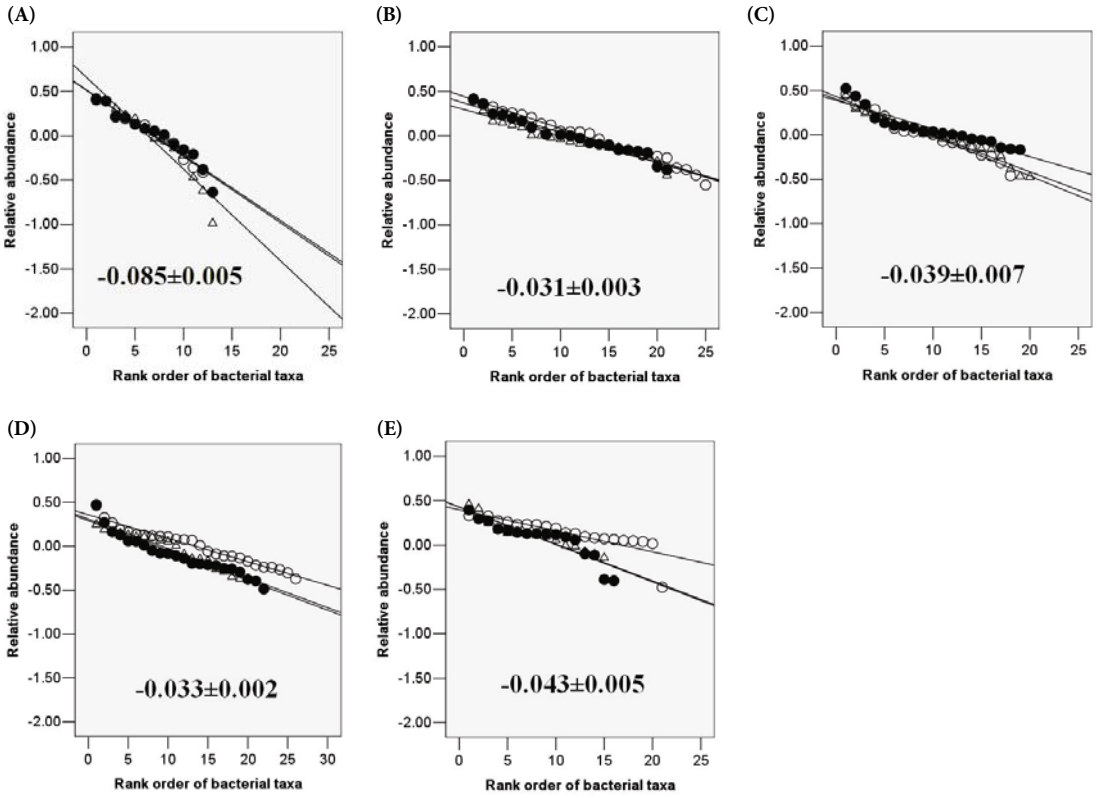

Fig. 2. Comparison of intestinal bacterial community structure between the four fish species and the water sample. The rank-abundance plots based on the DGGE bands of the water sample (A), blunt snout bream (B), bighead carp (C), grass carp (D), and silver carp (E) are presented. Plots from the three independent replicates per species (and water sample) are indicated by the open circles (○), closed circles (●), and open triangles (△). The value below the line is the mean slope and standard deviation (n=3).

differences in the bacterial communities between the four fish larvae species (Fig. 1A), whereas the eukaryotic taxa were similar in all. The water sample exhibited 16S rRNA and 18S rRNA gene bands distinct from the fish samples.

In the present study, UPGMA clustering of the 16S rRNA genes (Fig. 1C) revealed a clear separation among the fish species, indicating a distinct bacterial community structure in the intestine of each species. In contrast, the eukaryotic taxa (represented by 18S rRNA gene bands) were similar across the different species, while the water sample exhibited one distinct group (Fig. 1D).

The distribution of bacterial taxa abundance, based on the relative intensity of DGGE bands, was plotted as rank-abundance curves to examine differences in bacterial evenness (Fig. 2). To statistically assess differences in bacterial community structure across species, the slope values of the four species and the water sample were compared using one-way ANOVA (data not shown). Results showed that the bacterial evenness in the intestines of the four fish species were significantly different from one another ($P<0.05$). In addition, a marked change in the water sample ($P<0.01$) was observed.

Nucleotide sequences from DNA bands in DGGE gels

A total of 41 bands from the four fish species and the water sample were excised (Figs. 1A and B), and the DNA fragments from these bands were sequenced. The bacterial and eukaryotic species identified from the sequenced bands are summarized in Table 1. The excised DGGE bands were compared with BLAST references based on the phylogenetic relationship of ~200 bp partial 16S rRNA gene sequence (Figs. 3 and 4). Of the 35 16S rRNA genes bands, 9 bands with DNA originating from silver carp larvae were associated with *Actinobacteria*, *Firmicutes*, α- and *γ-Proteobacteria* (>97% similarity). Grass carp and bighead carp intestine sequences were found to be affiliated with *α-*, *β-*, *γ-Proteobacteria*, and *Actinobacteria*. Sequences from blunt snout bream intestine were related to *β-* and *γ-Proteobacteria*. Three dominant sequences derived from the water sample were closely related to *β-Proteobacteria* (similarity=99%), *Cyanobacterial* (similarity=98%), and *Bacteroidetes* (similarity=97%). Six bands from the 18S rRNA gene sequences were affiliated with eukaryotic picoplankton (similarity=98%), *Gobiocypris rarus* (similarity=98%), *Cryptomonas marssonii* (similarity=100%), and *Acanthocyclops vernalis* (similarity=99%).

Discussion

Silver carp, grass carp, bighead carp, and blunt snout bream belong to the same family (Cyprinidae) and are currently the four most important aquaculture fish species in China. An understanding of the characteristics or features of the

Table 1. Nearest match identification of 16S rRNA and 18S rRNA gene sequences from fish larvae intestines and the water sample based on known sequences in the RDP II and NCBI databases

Number	Identity (%)	Accession no.	Affiliation phylum/class	Closest sequence
1	100	HM776316	*Actinobacteria/Actinobacteria*	*Kocuria* sp. ljh-7 (GU217694.1)
2	97	HM776317	*Proteobacteria/α-Proteobacteria*	Uncultured SAR11 cluster *α-Proteobacteria* (FN665731.1)
3	100	HM776318	*Proteobacteria/γ-Proteobacteria*	*Alteromonas* sp. a1s1 (FN811317.1)
4	99	HM776319	*Actinobacteria/Actinobacteria*	*Brachybacterium* sp. NH89-63 (FJ494788.1)
5	98	HM776320	*Actinobacteria/Actinobacteria*	Uncultured *actinobacterium* clone TG_FD0.2 _ AC09 (GU798073.1)
6	100	HM776321	*Firmicutes/Bacillus*	*Bacillus* sp. LMG 22260 (AY766326.1)
7	100	HM776322	*Actinobacteria/Actinobacteria*	*Kocuria* sp. ljh-7 (GU217694.1)
8	99	HM776323	*Proteobacteria/γ-Proteobacteria*	Uncultured *Xanthomonadaceae* bacterium clone LW18m-1-18 (EU642263.1)
9	100	HM776324	*Firmicutes/Bacillus*	*Bacillus* sp. LMG 22260 (AY766326.1)
10	97	HM776325	*Proteobacteria/β-Proteobacteria*	Uncultured *Comamonadaceae* bacterium clone Gap-4-51 (EU639803.1)
11	100	HM776326	*Proteobacteria/α-Proteobacteria*	*Erythrobacter* sp. JL1033 (DQ985037.1)
12	100	HM776327	*Proteobacteria/β-Proteobacteria*	Uncultured bacterium clone 11W_04e01 (FJ382055.1)
13	100	HM776328	*Proteobacteria/γ-Proteobacteria*	*Acinetobacter* sp. KRD8 (HM345977.1)
14	99	HM776329	*Proteobacteria/β-Proteobacteria*	*Hydrogenophaga* sp. C0015 (2010) (GU947879.1)
15	100	HM776330	*Proteobacteria/β-Proteobacteria*	Uncultured *Aquabacterium* sp. clone YJQ-2 (AY569280.1)
16	98	HM776331	*Proteobacteria/β-Proteobacteria*	Uncultured *Ramlibacter* sp. clone MAFB-C4-57 (AY435512.1)
17	99	HM776329	*Proteobacteria/β-Proteobacteria*	*Hydrogenophaga* sp. C0015 (2010) (GU947879.1)
18	98	HM776333	*Actinobacteria/Actinobacteria*	Uncultured *actinobacterium* (FN668302.2)
19	97	HM776334	*Proteobacteria/γ-Proteobacteria*	*Aeromonas veronii* strain CL0901 (HM240294.1)
20	100	HM776335	*Proteobacteria/γ-Proteobacteria*	*Acinetobacter* sp. BS6 (HM132103.1)
21	100	HM776336	*Proteobacteria/α-Proteobacteria*	*Erythrobacter* sp. RAS19 (FJ868599.1)
22	100	HM776337	*Proteobacteria/γ-Proteobacteria*	*Acinetobacter* sp. KRD8 (HM345977.1)
23	100	HM776338	*Proteobacteria/γ-Proteobacteria*	*Acinetobacter johnsonii* strain mol18 (HM031479.1)
24	98	HM776339	*Proteobacteria/β-Proteobacteria*	Uncultured *Ramlibacter* sp. clone MAFB-C4-57 (AY435512.1)
25	99	HM776340	*Proteobacteria/β-Proteobacteria*	*Hydrogenophaga* sp. C0015 (2010) (GU947879.1)
26	99	HM776341	*Proteobacteria/γ-Proteobacteria*	*Aeromonas hydrophila* (FN997627.1)
27	99	HM776342	*Proteobacteria/γ-Proteobacteria*	*Aeromonas media* strain 2CCH203 (GU174504.1)
28	98	HM776343	*Proteobacteria/β-Proteobacteria*	Uncultured bacterium clone NK2_612 (EU376208.1)
29	100	HM776344	*Proteobacteria/γ-Proteobacteria*	*Acinetobacter* sp. MB4 (2010) (GU566335.1)
30	97	HM776345	*Proteobacteria/β-Proteobacteria*	Uncultured *β-Proteobacteria* clone LF065 (EF417754.1)
31	98	HM776346	*Proteobacteria/β-Proteobacteria*	Uncultured bacterium clone NK2_612 (EU376208.1)
32	99	HM776347	*Proteobacteria/β-Proteobacteria*	*Hydrogenophaga* sp. C0015 (2010) (GU947879.1)
33	99	HM776348	*Proteobacteria/β-Proteobacteria*	Uncultured *β-Proteobacteria* clone IRD18G03 (AY947965.1)
34	98	HM776349	*Cyanobacterial/Cyanobacterial*	Uncultured *Cyanobacterium* clone SGSO748 (GQ347952.1)
35	97	HM776350	*Bacteroidetes/Bacteroidetes*	Uncultured *Balneola* sp. clone XZNMC81 (EU703220.1)
36	98	HM776351		Uncultured eukaryotic picoplankton clone BA300 (EF196700.1)
37	98	HM776352	*Chordata/ Cypriniformes*	*Gobiocypris rarus* (EF190321.1)
38	99	HM776353		Uncultured freshwater cryptophyte (FJ765405.1)
39	100	HM776354	*Cryptophyta/ Cryptophyceae*	*Cryptomonas marssonii* strain WCK01 (EU163586.1)
40	99	HM776355	*Arthropoda/Copepoda*	*Acanthocyclops vernalis* isolate AC10 (AY643532.1)
41	99	HM776356		Uncultured freshwater cryptophyte (FJ765405.1)

16S rRNA gene DGGE bands from silver carp intestine (1-9), grass carp intestine (10-19), bighead carp intestine (20-26), blunt snout bream intestine (27-32), and influent (33-35); 18S rRNA gene DGGE bands from fish intestine (36-38) and influent (39-41).

intestinal microbiota of these fish larvae may help improve diets and incubation conditions for the intensive mass rearing of healthy fish (Olafsen, 2001). In the present study, the PCR-DGGE fingerprinting technique was used to analyze and compare the composition and structure of the intestinal bacteria and eukaryotic taxa of the four fish larvae. This approach allows for the detection of the relative dominant bacteria and eukaryotic taxa present in the samples by sequencing the excised DGGE gel bands.

For community structure analysis, the UPGMA dendrogram based on the 16S rRNA gene DGGE pattern clearly showed five clusters corresponding to the four species and the water sample (Fig. 1C). In contrast, the dendrogram of eukaryotic taxa showed no distinct clusters for the fish species, while the water had two distinct clusters (Fig. 1D). These results suggested the presence of unique bacterial populations in the larvae of different species, whereas the eukaryotic taxa in all fish intestines were similar. Given that these fish were reared under the same conditions (same environment and diet), these results indicate that species-specific selective factors like unique habitats within the intestine (dependent on host-specific immunity and physiology)

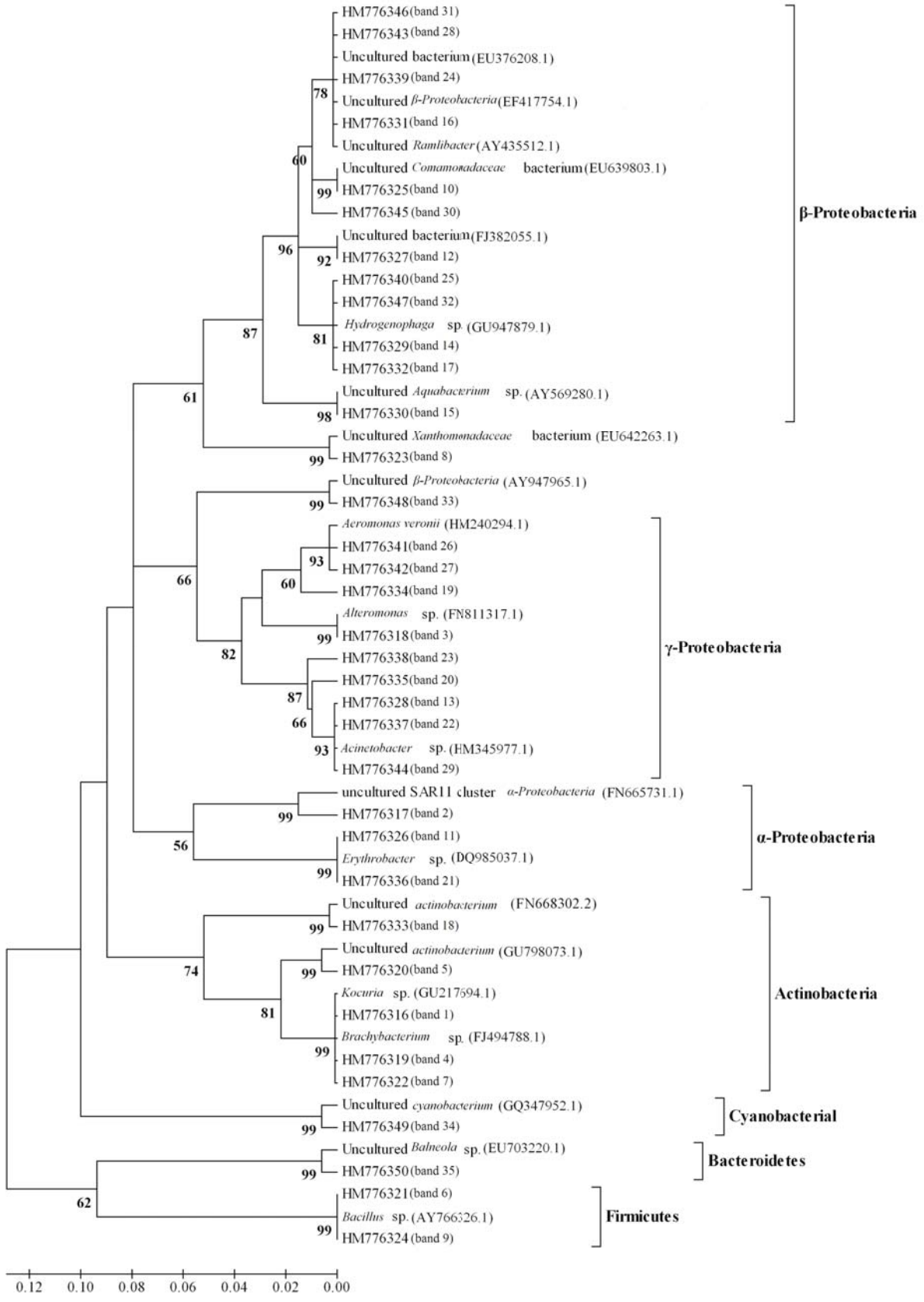

Fig. 3. **Neighbor-joining phylogenetic tree showing the relationship between 16S rDNA sequences retrieved from the DGGE profiles and their closest relative sequences deposited in the NCBI database.**

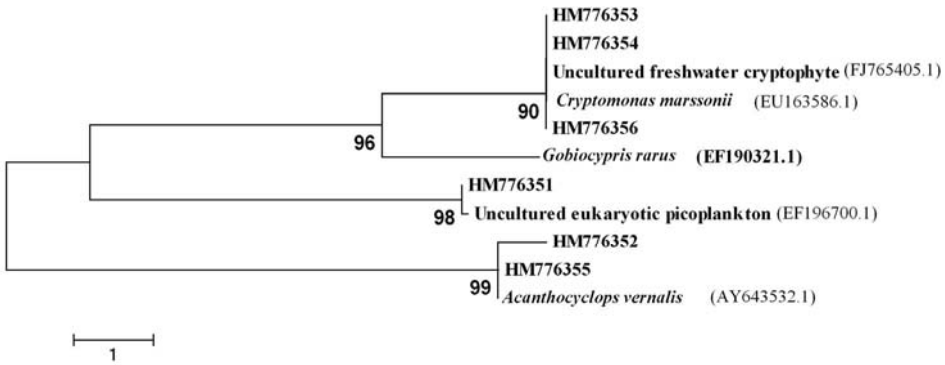

Fig. 4. **Neighbor-joining phylogenetic tree showing the relationship between 18S rRNA gene sequences retrieved from the DGGE profiles and their closest relative sequences deposited in the NCBI database.**

shape the community structure of the intestinal microbiota. Camp *et al.* (2009) reported that the GI habitat within different hosts helps shape microbial community composition in distinct ways. As predicted, bacterial rank-abundance plots based on comparative taxa abundances (Fig. 2) also revealed significant differences ($P<0.05$) among the intestinal bacterial structures of the four species, suggesting that each had different relative abundances of dominant bacteria. Although the DGGE technique showed biases in the detection of bacterial taxa (Woodcock *et al.*, 2007), band abundances as reflected by bacterial rank-abundance plots can indicate ecological shifts in the characteristics of bacterial communities (Loisel *et al.*, 2006; van der Gast *et al.*, 2006; Woodcock *et al.*, 2007). Diet and the environment affect the intestinal microflora of fish and mammals (Holben *et al.*, 2002; Savas *et al.*, 2005; Ley *et al.*, 2008), but the same environment and same natural food in the present study did not result in similar intestinal bacteria. Conversely, the same environment and natural food may account for the similar eukaryotic taxa in different fish intestines (Dabrowski, 1984).

In previous studies, the bacterial population in healthy fish was shown to reflect the aqueous environment (Nieto *et al.*, 1984; Cahill, 1990; Austin, 2006). The current results revealed that both bacterial and eukaryotic taxa were distinct between the fish intestines and the water sample, consistent with a previous investigation that found no significant similarity among the bacterial groups isolated from water, intestine, and fish diet (Sakata *et al.*, 1980). The rank-abundance plots based on band relative abundance supported this lack of association. The bacterial community structure of the water sample was significantly different ($P<0.01$) from that of the fish intestines (Fig. 2), indicating that only a small number of bacterial species within the water sample proliferated significantly in the intestinal tract.

The UPGMA and rank-abundance plots indicate DGGE band richness and abundance, but do not provide information about specific species comprising the microbiota community. To this end, we sequenced the major DGGE bands, consisting of 35 bacterial taxa and 6 eukaryotic taxa (Table 1). Indeed, the results showed different dominant intestinal bacteria in the four fish species. Proteobacteria, a common bacteria in freshwater fish intestine (Austin, 2006), were most prevalent. Eukaryotic taxa were affiliated with picoplankton, *Gobiocypris rarus*, *Cryptomonas marssonii*, and *Acanthocyclops vernalis*, which may have come from the environment.

The species-specific community structure was not the result of environment and diet, so we examined the relationship between intestinal microbiota and fish genotype by sequenc-

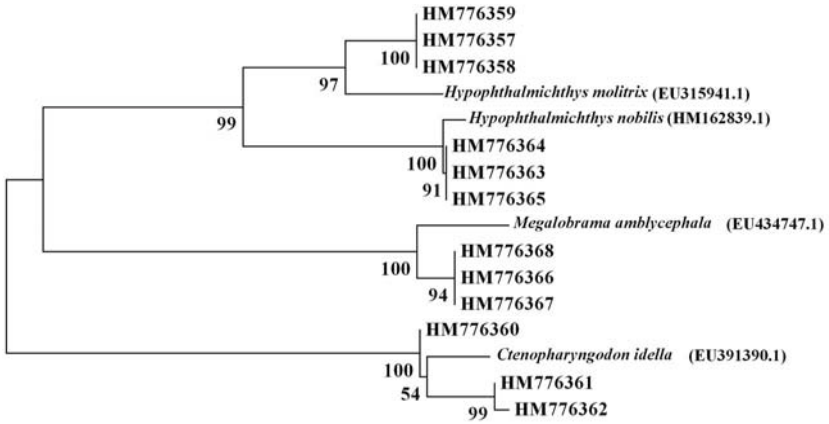

Fig. 5. **Neighbor-joining tree of the COI sequences in the four fish species (three replicates for each fish species) and their closest relative sequences deposited in the NCBI database.**

ing the fish COI gene and creating a phylogenetic tree (Fig. 5). The results showed that silver carp and bighead carp were the most closely related species, followed by blunt snout bream and grass carp. This phylogenetic relationship was not reflected by a similar dendrogram and phylogenetic tree of the intestinal microbiota (Figs. 1C and 3), suggesting that fish genotype and intestinal bacteria show no co-evolutionary relationship, at least in the larval stage. This was also contrary to results showing that genotype had a significant effect on gut microbiota composition in humans and mice (Zoetendal *et al.*, 2001; Kovacs *et al.*, 2011). This finding may be attributed to the incomplete larvae development of the four fish species, which influences the composition of the intestinal microbiota.

Denaturing gradient gel electrophoresis of the 16S rRNA gene has been successfully used to characterize bacterial populations associated with fish larvae (Jensen *et al.*, 2004; Brunvold *et al.*, 2007; Navarrete *et al.*, 2009). Indeed, the banding patterns can reflect the composition of the dominant bacterial community. However, the heterogeneity between 16S rRNA gene copies and the possibility of heteroduplex formation (Moreno *et al.*, 2002; Navarrete *et al.*, 2010) led us to observe multiple bands for a single strain in the present study, such as those observed for *Kocuria* sp. (bands 1 and 7, similarity=100%), *Bacillus* sp. (bands 6 and 9, similarity=100%), *Erythrobacter* sp. (bands 11 and 21, similarity=100%), and *Hydrogenophaga* sp. (bands 14, 17, 25, and 32, similarity=99%). The same phenomenon was also found in eukaryotic taxa (bands 38 and 41, similarity=99%) (Table 1). Additionally, the co-migration of some bands representing different bacteria was also observed, for example bands 17, 26, and 31 (Fig. 1A). Magne *et al.* (2006) reported that amplicons with the same melting behavior but different sequences can co-migrate to the same position in a gel. Therefore, the diversity index deduced from DGGE banding patterns must be interpreted as an indication but not an absolute measure of the degree of diversity in a microbial community (Eichner *et al.*, 1999). The development of more accurate methodologies is necessary to present a more accurate picture of species-specific community structure.

In summary, the present study demonstrated distinct dominant intestinal microbiota in four fish larvae reared in the same environment. Therefore, species is a strong determinant of the intestinal microbiota. The eukaryotic taxa were almost the same in the different fish intestines due to the shared natural food source (zooplankton). Further studies are needed to determine the physiological role of intestinal microbiota and to explore whether fish intestinal bacteria vary during development in fish reared in the same environment.

Acknowledgements

This work was supported by the Major State Basic Research Development Program of China (2009CB118705) and the National Natural Science Foundation of China (31172084). The authors thank Dr. Jorge Cuéllar-Anjel for his review of the manuscript and suggestion to improve the English.

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