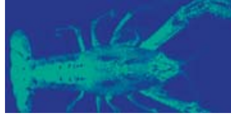
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Analysis of bacterial diversity in the intestine of grass carp (*Ctenopharyngodon idellus*) based on 16S rDNA gene sequences

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Abstract

In the current study, we assessed bacterial diversity in the gut content of pond-reared grass carp (*Ctenopharyngodon idellus*), in the associated habitat environments (pond water and sediment) and in the ingested food (commercial feed and the reed *Phragmites australis*) by analysing 16S rDNA sequences from clone libraries. The highest bacterial diversity was observed in the gut content and was determined by the total number of operational taxonomic units, Shannon diversity index (H), Shannon equitability index (E_H), Coverage (C_{good}) and rarefaction curves calculated from the 16S rDNA gene libraries. Our data indicated that allochthonous gut microbes of grass carp were distinctively different from the corresponding environmental microbes. The pairwise similarity coefficient (C_s) for microbe communities between gut content and ingested food was higher than for those between the gut content and habitats, indicating that the allochthonous microbiota identified in the intestines of grass carp were phylogenetically closer to those in the ingested food than to those in the habitat. Based on our study and previous research, we suggest that the digesta of grass carp harbours a microbiota phylogenetic core of Proteobacteria and Firmicutes and this observation deserves further investigations with respect to a potential pool of probiotics to grass carp.

Keywords: grass carp, *Ctenopharyngodon idellus*, intestinal bacterial diversity, 16S rDNA

Introduction

Gastrointestinal (GI) microbiota participate in several important physiological functions of the host, including digestion, development of the mucosal system, angiogenesis and protection against disease (Macfarlane & Macfarlane 1997; Hooper, Midtvedt & Gordon 2002). It is generally accepted that identification of the GI microbiota is undoubtedly important for understanding the functional mechanisms between the microbes and the host (Gómez & Balcázar 2008). Difficulties in analysing the complexity of bacterial community using classic methods of cultivation have necessitated the development of molecular methods. In order to overcome these problems, various methods such as denaturing gradient gel electrophoresis (DGGE) (Muyzer, Waal & Uitterlinden 1993), fluorescence *in situ* hybridization (Huber, Spanggaard, Appel, Rossen, Nielsen & Gram 2004), temporal temperature-gradient electrophoresis (Navarrete, Magne, Mardones, Riveros, Opazo, Suau, Pochart & Romero 2010) and clone libraries (Kim, Brunt & Austin 2007; Brons & Elsas 2008; Navarrete, Espejo & Romero 2009; Ward, Blaire, Penn, Methé & Detrich 2009) have been used in order to circumvent the need for microbial isolation.

The DGGE-based method is a useful tool for separating gene fragments but has strict length limitations (generally < 500 bp) (Myers, Fischer, Lerman & Maniatis 1985) and often fails to establish an exact identification of the fragments using the BLAST program (Altschul, Gish, Miller, Myers & Lipman 1990). In

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addition, it only detects the dominant bacterial species in the environments (Muyzer *et al.* 1993). The generation of 16S rDNA clone libraries that contain near-full-length 16S rDNA sequences would likely result in more precise sequence identification than sequences obtained from DGGE (Brons & Elsas 2008).

The grass carp (*Ctenopharyngodon idellus*) is a herbivorous freshwater fish of the Cyprinidae family and these fish are widely cultivated for food in China. The output was >4 million tonnes in 2008 and comprised $>20\%$ of the total freshwater-cultured fish annual output (Ministry of Agriculture, China 2009). Members of the Cyprinidae family have also been introduced to Europe and the United States for aquatic weed control (Chilton & Muoneke 1983).

During the last three decades, some papers have been published in which the gut microbiota of grass carp were identified using traditional methods such as freshwater agar and some selective culture media (Trust, Bull, Currie & Buckley 1979; Zhou, Chen, Zhang & Chen 1998; Luo, Chen & Cai 2001; He, Zhang, Xie, Hao, Wang & He 2008). Recently, Huang, Shi, Wang, Luo, Shao, Wang, Yang and Yao (2009) studied the intestinal bacterial community of grass carp by PCR amplification of the V3 region of 16S rDNA and by DGGE; to our knowledge, a 16S rDNA clone library has not been generated for the identification of grass carp gut microbiota, however.

The diversity of the GI microbiota of fish is influenced by environmental factors such as ingested food and habitat (Sugita, Oshima, Tamura & Deguchi 1983; Nieto, Toranzo & Barja 1984). However, the correlation between gut microbiota and its corresponding environmental microbiota is *per se* not fully understood, and whether the grass carp gut harbours a microbiota phylogenetic core (the common phyla within the gut contents of grass carp from different backgrounds) has not been addressed. In the present study, we identified the allochthonous intestinal microbiota of the grass carp by generating a 16S rDNA library comprised of sequences from samples of grass carp gut content, the associated habitat (pond water and sediment) and the ingested food (commercial feed and natural food).

Materials and methods

Sample preparation

Grass carp was raised in a poly-culture pond of grass carp, gibel carp (*Carassius auratus gibelio*) and blunt-nose black bream (*Megalobrama amblycephala*) in the suburb of Nanjing City, Jiangsu Province, China. A description of the pond as well as the fish and pond water, sampling methods and sampling procedure is presented in Wang, Zhou, He, Liu, Cao, Shi, Yao and Ringø (2010). Twelve grass carp were randomly collected from the pond. Pond water and sediment samples were collected from the same pond locations ($n = 4$). Sediment samples were collected using a mud dredger (VG, Beijing Purity Instruments, Beijing, China) and were pooled before analysis. In addition, feed samples (~ 200 g) were obtained from the automatic feeder. Samples of the reed (*Phragmites australis*) available for ingestion by grass carp were collected from four randomly chosen sites in the pond. Samples were stored on ice for transport to the lab and then kept at -20 °C until analysis. After examining all fish (12), gut contents from six grass carp having identical gut fullness were used. The gut samples chosen were visually full of food ingested and the digesta were gently squeezed out under sterile conditions and pooled before analysis. Pooled samples were used to avoid erroneous conclusions due to individual variations in gut microbiota as described elsewhere (Spanggaard, Huber, Nielsen, Nielsen, Appel & Gram 2000; He, Zhou, Liu, Shi, Yao, Ringø & Yoon 2009).

DNA extraction

Total DNA was extracted from 5 g sediment or feed as described by Tsai and Olson (1991). Extracted DNA was purified using the Gel Cycle-Pure DNA kit (Takara, Tokyo, Japan) according to the manufacturer's instructions and used as template DNA for PCR amplification. DNA was extracted from pond water as described elsewhere (Gernert, Glöckner, Krohne & Hentschel 2005). DNA was extracted from fish gut content using the hexadecyltrimethylammonium bromide (CTAB) method, which involves a step of suspending the samples in CTAB extraction buffer (Thakuria, Schmidt, Mac Siúrtáin, Egan & Doohan 2008). To obtain reed DNA, 5 g reed sample was cut into small pieces, transferred to a sterile triangular flask containing 20 mL PBS buffer and 10 glass beads (0.5 cm diameter) and then agitated at 4 *g* for 30 min. The mixture was allowed to settle for 10 min, and the supernatant was transferred into a sterile tube and centrifuged at $14\,000 \times g$ for 15 min at 4 °C. Total DNA was extracted from the precipitate using the DNA extraction kit (Takara).

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PCR amplification and 16S rDNA library construction

Universal primers 27f and 1492r (Martin-Laurent, Philippot, Hallet, Chaussod, Germon, Soulas & Catroux 2001), which anneal at nucleotide positions 8–27 and 1492–1513 of the 16S rDNA gene (*Escherichia coli* numbering), respectively, were used for 16S rDNA library construction. PCR reaction conditions were as described by Martin-Laurent *et al.* (2001). PCR products (~ 1300 bp) were purified, cloned into the pGEM-T vector and transformed into *E. coli* XL1-blue (Promega, Southampton, UK) according to the manufacturer's instructions. Blue/white selection was used for clone screening. For each sample type, ~ 100 clones containing correct inserts (~ 1300 bp) were randomly selected, verified by PCR amplification using the 27f/1492r primer set and sequenced by Sunbiotech (Beijing, China).

Data analysis

The 16S rDNA clone library sequences were submitted to the CHECK.CHIMERA program of the Ribosomal Database Project to detect possible chimeric artefacts (Cole, Chai, Farris, Wang, Julam, McGarrel, Garrity & Tiedje 2005). All sequences were subjected to similarity searches using the BLAST program (Altschul *et al.* 1990) after removing unreliable sequences at the 3′ and 5′ ends.

Using the TSYS-PC program (version 2.1, Jandel Scientific, San Rafael, CA, USA), sequences identified in the current study were integrated into an annotated tree based on parsimony. The relative abundance (%) of an operational taxonomic unit (OTU; the clones with 100% sequence similarity), representing the ratio of the number of the clones of a specific OTU to the total number of clones, was considered to be significant when the value was more than 1.5-fold higher or less than 0.5-fold lower than the abundance of any other OTU. The Shannon diversity index was calculated using the equation $H = -\Sigma RA_i \ln(RA_i)$, and the Shannon equitability index was calculated using the equation $E_H = H/\ln(S)$ (where RA_i is the proportion of the ith OTU and S is the total number of OTUs) (Dethlefsen, Huse, Sogin & Relman 2008). The Coverage (C_{good}) was calculated according to Good (1953) using the equation $C_{good} = 1 - N_1$/the total number of OTUs (where N_1 is the number of OTUs with only one clone). Cluster analysis was based on the unweighted pair group method using the arithmetic mean algorithm (UPGMA). In this study, the bacterial communities with a pairwise similarity coefficient (C_s: the measure of the similarity of two samples by UPGMA) <0.60 were regarded as different, those with $0.60 \leq C_s < 0.80$ were considered to be marginally different and those with $C_s \geq 0.80$ were considered to be similar (Wang *et al.* 2010). Rarefaction curves were created using the species diversity function of the ECOSIM 700 statistical software (Gotelli & Entsminger 2002).

Results

The phylogenetic affiliations of the 16S rDNA genes isolated from the gut content of grass carp and from corresponding habitat and food samples are shown in Table 1. After removing unreliable sequence data, a total of 490 clones were identified, including 100 clones from gut samples, 102 clones from feed samples, 88 clones from reed samples, 100 clones from pond water samples and 100 clones from sediment samples. The dominant bacterial phylum identified in each sample type was Proteobacteria (Fig. 1). Specifically, the dominant class of bacteria in grass carp gut, feed, pond water and sediment samples was γ-Proteobacteria, which comprised 28.0%, 33.3%, 46.0% and 49.0%, respectively, of the total bacterial content. The dominant class in reed samples was Bacteroidetes, with a relative abundance of 18.2% (Table 1). However, unclassified bacteria comprised a large proportion of the bacteria in each sample type: 21.0%, 31.4%, 13.6%, 42.0% and 37.0% in gut content, feed, reed, pond water and sediment samples respectively. The OTUs with the greatest relative abundance in gut content, feed, reed, pond water and sediment were OTU36 (99% similarity to *Pseudomonas aeruginosa*; FM209186), OTU10 (99% similarity to *Bacillus* sp.; AY822760), OTU34 (98% similarity to uncultured β-Proteobacterium; EU753670), OTU64 (99% similarity to the uncultured bacterium, DQ394301) and OTU35 (100% similarity to *Enterobacter* sp.; EF175731) respectively (Table 1).

The diversity of the allochthonous intestinal bacterial community in the grass carp gut and in the corresponding ecosystem components is presented in Fig. 2. The total number of OTUs was the highest in the gut content (48), followed by pond water (29), reed (19), sediment (18) and feed (13). The Shannon diversity index (H) in the gut content was 3.465, higher than that in the associated habitat and food samples. Similar trends were observed in the Shannon equitability index (E_H) values and the Coverage

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Table 1 Phylogenetic affiliation of 16S rDNA gene phylotypes isolated from the gut content of grass carp and from associated food and habitat samples*

OTU	Relative abundance (%) Gut content (100 clones)	Feed (102 clones)	Reed (88 clones)	Water (100 clones)	Sediment (100 clones)	Closest relative in GenBank (accession no.)	Similarity to the closest relative (%)	Phylogenetic group
OTU1	1.0^{b}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	*Actinomyces naeslundii* (AJ635359.1)	94	Actinobacteridae
OTU2	2.0^{b}	0.0^{a}	1.1^{b}	0.0^{a}	0.0^{a}	*Arthrobacter* sp. (AJ810894.1)	98	Actinobacteridae
OTU3	1.0^{b}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	*Curtobacterium* sp. (EF411134.1)	99	Actinobacteridae
OTU4	1.0^{b}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	*Curtobacterium flaccumfaciens* (AM410688.1)	99	Actinobacteridae
OTU5	2.0^{b}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	*Microbacterium phyllosphaerae* (EF143430.1)	98	Actinobacteridae
OTU6	2.0^{b}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	*Bacillus coagulans* (DQ297928.1)	99	Bacillales
OTU7	1.0^{b}	2.0^{b}	0.0^{a}	0.0^{a}	0.0^{a}	*Bacillus massiliensis* (DQ350816.1)	99	Bacillales
OTU8	4.0^{c}	1.0^{b}	0.0^{a}	0.0^{a}	0.0^{a}	*Bacillus megaterium* (DQ660362.1)	99	Bacillales
OTU9	1.0^{b}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	*Bacillus pumilus* (EU221329.1)	99	Bacillales
OTU10	2.0^{ab}	19.6^{c}	4.5^{b}	1.0^{a}	2.0^{ab}	*Bacillus* sp. (AY822760.1)	99	Bacillales
OTU11	1.0^{b}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	*Exiguobacterium* sp. (DQ019168.1)	99	Bacillales
OTU12	2.0^{b}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	*Geobacillus toebi* (AY608982.1)	99	Bacillales
OTU13	1.0^{b}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	*Staphylococcus kloosii* (DQ093351.1)	91	Bacillales
OTU14	1.0^{b}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	*Ureibacillus koreensis* (DQ348072.1)	99	Bacillales
OTU15	2.0^{b}	0.0^{a}	13.6^{c}	0.0^{a}	0.0^{a}	*Ureibacillus thermosphaericus* (AB101594.1)	99	Bacillales
OTU16	1.0^{b}	0.0^{a}	18.2d	3.0^{c}	0.0^{a}	Uncultured *Bacteroidetes* (EF612369.1)	94	Bacteroidetes
OTU17	1.0^{b}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	*Clostridium* sp. (AY188850.1)	99	Clostridia
OTU18	1.0^{b}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	Low G+C Gram-positive bacterium M54 (AB116132.1)	99	Firmicutes
OTU19	1.0^{b}	2.0^{b}	0.0^{a}	0.0^{a}	0.0^{a}	*Lactobacillus curvatus* (EU855223.1)	99	Lactobacillales
OTU20	1.0^{b}	1.0^{b}	2.3^{b}	0.0^{a}	0.0^{a}	*Lactobacillus fermentum* (AB362626.1)	99	Lactobacillales
OTU21	2.0^{b}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	*Lactococcus lactis* (AB008215.1)	99	Lactobacillales
OTU22	7.0^{c}	2.9^{bc}	1.1^{b}	0.0^{a}	0.0^{a}	*Leuconostoc citreum* (AB362721.1)	99	Lactobacillales
OTU23	1.0^{b}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	*Streptococcus* (AY232833.1)	94	Lactobacillales
OTU24	2.0^{b}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	*Streptococcus constellatus* (AF104676.1)	94	Lactobacillales
OTU25	2.0^{bc}	0.0^{a}	1.1^{b}	4.0^{c}	0.0^{a}	*Streptococcus iniae* (AF335572.1)	99	Lactobacillales
OTU26	1.0^{b}	0.0^{a}	3.4^{c}	0.0^{a}	0.0^{a}	*Streptococcus parauberis* (FJ009631.1)	99	Lactobacillales
OTU27	0.0^{a}	4.9^{b}	0.0^{a}	0.0^{a}	0.0^{a}	*Streptococcus salivarius* (AM157419.1)	93	Lactobacillales
OTU28	1.0^{b}	2.0^{b}	0.0^{a}	0.0^{a}	0.0^{a}	*Wiessella confuse* (DQ321751.1)	99	Lactobacillales
OTU29	1.0^{b}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	*Afipia geno* sp. (U87773.1)	99	α-Proteobacteria
OTU30	1.0^{b}	0.0^{a}	0.0^{a}	0.0^{a}	3.0^{c}	*Achromobacter xylosoxidans* (EU373389.1)	99	β-Proteobacteria
OTU31	1.0^{b}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	*Methylophilus leisingeri* (AB193725.1)	99	β-Proteobacteria
OTU32	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	6.0^{b}	Uncultured β-Proteobacterium (FM253602.1)	98	β-Proteobacteria
OTU33	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	3.0^{b}	Uncultured β-Proteobacterium (EF612408.1)	98	β-Proteobacteria
OTU34	3.0^{b}	0.0^{a}	25.0^{c}	4.0^{b}	0.0^{a}	Uncultured β-Proteobacterium (EU753670.1)	98	β-Proteobacteria
OTU35	3.0^{a}	15.7^{bc}	5.7^{ab}	2.0^{a}	22.0^{c}	*Enterobacter* sp. (EF175731.1)	100	γ-Proteobacteria
OTU36	17.0d	7.8^{c}d	2.3^{ab}	1.0^{a}	4.0^{bc}	*Pseudomonas aeruginosa* (FM209186.1)	99	γ-Proteobacteria
OTU37	2.0^{bc}	9.8d	0.0^{a}	1.0^{b}	3.0^{c}	*Pseudomonas putida* (CP000926.1)	99	γ-Proteobacteria
OTU38	1.0^{b}	0.0^{a}	0.0^{a}	0.0^{a}	2.0^{b}	*Serratia liquefaciens* (DQ123840.1)	99	γ-Proteobacteria
OTU39	3.0^{b}	0.0^{a}	2.3^{b}	0.0^{a}	13.0^{c}	*Shigella sonnei* (EU723822.1)	99	γ-Proteobacteria
OTU40	0.0^{a}	0.0^{a}	0.0^{a}	1.0^{b}	0.0^{a}	Uncultured γ-Proteobacterium (AF324537.1)	99	γ-Proteobacteria
OTU41	0.0^{a}	0.0^{a}	1.1^{b}	4.0^{c}	0.0^{a}	Uncultured γ-Proteobacterium (EU394575.1)	99	γ-Proteobacteria

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Table 1 Continued

OTU	Relative abundance (%) Gut content (100 clones)	Feed (102 clones)	Reed (88 clones)	Water (100 clones)	Sediment (100 clones)	Closest relative in GenBank (accession no.)	Similarity to the closest relative (%)	Phylogenetic group
OTU42	0.0^a	0.0^a	2.3^b	6.0^b	0.0^a	Uncultured γ-Proteobacterium (EU394575.1)	99	γ-Proteobacteria
OTU43	0.0^a	0.0^a	0.0^a	0.0^a	2.0^b	Uncultured Shigella (FJ193063.1)	100	γ-Proteobacteria
OTU44	1.0^b	0.0^a	0.0^a	10.0^c	0.0^a	Uncultured *Acinetobacter* (FJ192439.1)	99	γ-Proteobacteria
OTU45	0.0^a	0.0^a	0.0^a	1.0^b	0.0^a	Uncultured *Acinetobacter* (FJ192480.1)	99	γ-Proteobacteria
OTU46	1.0^b	0.0^a	0.0^a	12.0^b	0.0^a	Uncultured *Acinetobacter* (FJ192980.1)	100	γ-Proteobacteria
OTU47	0.0^a	0.0^a	0.0^a	1.0^b	0.0^a	Uncultured *Acinetobacter* (FJ192631.1)	99	γ-Proteobacteria
OTU48	0.0^a	0.0^a	0.0^a	2.0^b	0.0^a	Uncultured *Acinetobacter* (EU407207.1)	99	γ-Proteobacteria
OTU49	0.0^a	0.0^a	0.0^a	1.0^b	0.0^a	Uncultured *Acinetobacter* (AF467299.1)	99	γ-Proteobacteria
OTU50	0.0^a	0.0^a	0.0^a	1.0^b	0.0^a	*Acinetobacter calcoaceticus* (AM157426.1)	97	γ-Proteobacteria
OTU51	0.0^a	0.0^a	0.0^a	2.0^b	0.0^a	*Acinetobacter johnsonii* (DQ911549.1)	99	γ-Proteobacteria
OTU52	0.0^a	0.0^a	2.3^{bc}	1.0^b	3.0^c	*Acinetobacter* sp. (EU703817.1)	99	γ-Proteobacteria
OTU53	0.0^a	0.0^a	0.0^a	0.0^a	7.0^b	Uncultured bacterium (AJ487021.1)	99	Unclassified
OTU54	0.0^a	0.0^a	0.0^a	0.0^a	15.0^b	Uncultured bacterium (AM697120.1)	98	Unclassified
OTU55	0.0^a	0.0^a	0.0^a	0.0^a	5.0^b	Uncultured bacterium (AM745142.1)	89	Unclassified
OTU56	0.0^a	0.0^a	0.0^a	1.0^b	0.0^a	Uncultured bacterium (AY661997.1)	98	Unclassified
OTU57	1.0^b	0.0^a	0.0^a	0.0^a	0.0^a	Uncultured bacterium (DQ125520.1)	98	Unclassified
OTU58	1.0^b	0.0^a	4.5^c	11.0^c	1.0^b	Uncultured bacterium (DQ226081.1)	99	Unclassified
OTU59	0.0^a	0.0^a	4.5^b	0.0^a	0.0^a	Uncultured bacterium (DQ228365.1)	94	Unclassified
OTU60	1.0^b	0.0^a	0.0^a	0.0^a	0.0^a	Uncultured bacterium (DQ256349.1)	99	Unclassified
OTU61	0.0^a	0.0^a	0.0^a	2.0^b	0.0^a	Uncultured bacterium (DQ264533.1)	99	Unclassified
OTU62	0.0^a	0.0^a	0.0^a	1.0^b	0.0^a	Uncultured bacterium (DQ264605.1)	99	Unclassified
OTU63	0.0^a	0.0^a	0.0^a	4.0^b	0.0^a	Uncultured bacterium (DQ264645.1)	99	Unclassified
OTU64	0.0^a	0.0^a	2.3^b	13.0^c	0.0^a	Uncultured bacterium (DQ394301.1)	99	Unclassified
OTU65	0.0^a	0.0^a	0.0^a	0.0^a	2.0^b	Uncultured bacterium (DQ415787.1)	99	Unclassified
OTU66	2.0^b	0.0^a	0.0^a	0.0^a	0.0^a	Uncultured bacterium (DQ455576.1)	94	Unclassified
OTU67	1.0^b	0.0^a	2.3^{bc}	6.0^c	0.0^a	Uncultured bacterium (DQ532284.1)	98	Unclassified
OTU68	0.0^a	14.7^b	0.0^a	0.0^a	5.0^b	Uncultured bacterium (DQ675075.1)	99	Unclassified
OTU69	0.0^a	0.0^a	0.0^a	2.0^b	0.0^a	Uncultured bacterium (EF632913.1)	99	Unclassified
OTU70	0.0^a	16.7^c	0.0^a	1.0^b	2.0^b	Uncultured bacterium (EF655641.1)	99	Unclassified
OTU71	0.0^a	0.0^a	0.0^a	1.0^b	0.0^a	Uncultured bacterium (EF999404.1)	99	Unclassified
OTU72	1.0^b	0.0^a	0.0^a	0.0^a	0.0^a	Uncultured bacterium (EU024330.1)	99	Unclassified
OTU73	1.0^b	0.0^a	0.0^a	0.0^a	0.0^a	Uncultured bacterium (EU234087.1)	95	Unclassified
OTU74	6.0^b	0.0^a	0.0^a	0.0^a	0.0^a	Uncultured bacterium (EU358726.1)	99	Unclassified
OTU75	1.0^b	0.0^a	0.0^a	0.0^a	0.0^a	Uncultured bacterium (EU799211.1)	99	Unclassified
OTU76	6.0^b	0.0^a	0.0^a	0.0^a	0.0^a	Uncultured bacterium (FJ172868.1)	91	Unclassified

*Within each row, data marked with the same superscript reflect values within a 0.5–1.5-fold difference range.
OTU, operational taxonomic unit, the clones with 100% sequence similarity.

(C_{good}) values, indicating that the microbiota in the gut content of grass carp was more diverse than in samples from the associated microbial environments. Rarefaction curves generated for the 16S rDNA clone libraries confirmed that the bacterial diversity of the grass carp gut content was greater than in associated environmental samples (Fig. 3).

The bacterial communities in samples from the associated environment were significantly different ($C_s < 0.50$) from those in the gut content of grass carp (Table 2). Nonetheless, the C_s values between samples from ingested food (feed or reed) and gut content ($C_s = 0.46$ or 0.49 respectively) were higher than between samples from habitat (pond water or sediment) and gut content ($C_s = 0.28$ or 0.34 respectively), indicating that the allochthonous intestinal microbiota of the grass carp was relatively closer to ingested food than to the habitat (Table 2).

The relative abundance of 25 OTUs (1, 3–6, 9, 11–14, 17–18, 21, 23–24, 29, 31, 57, 60, 66 and 72–76) in

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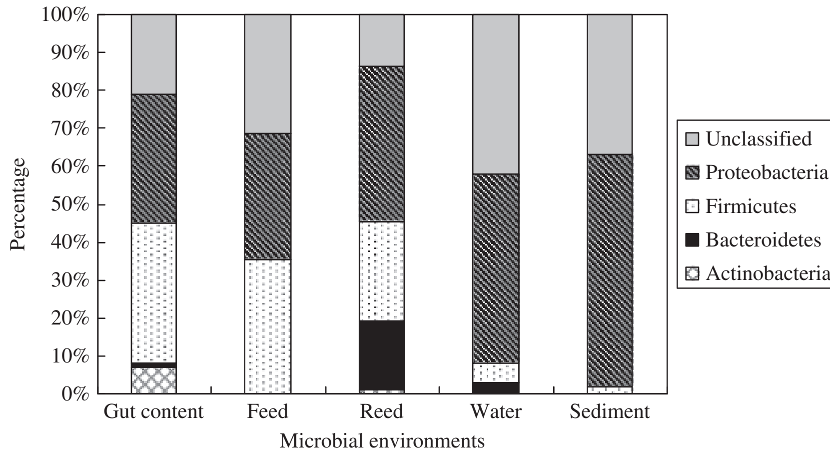

Figure 1 Bacterial phylum composition in the gut content of grass carp and in food and habitat samples. The bacterial phylum composition was calculated based on Table 1.

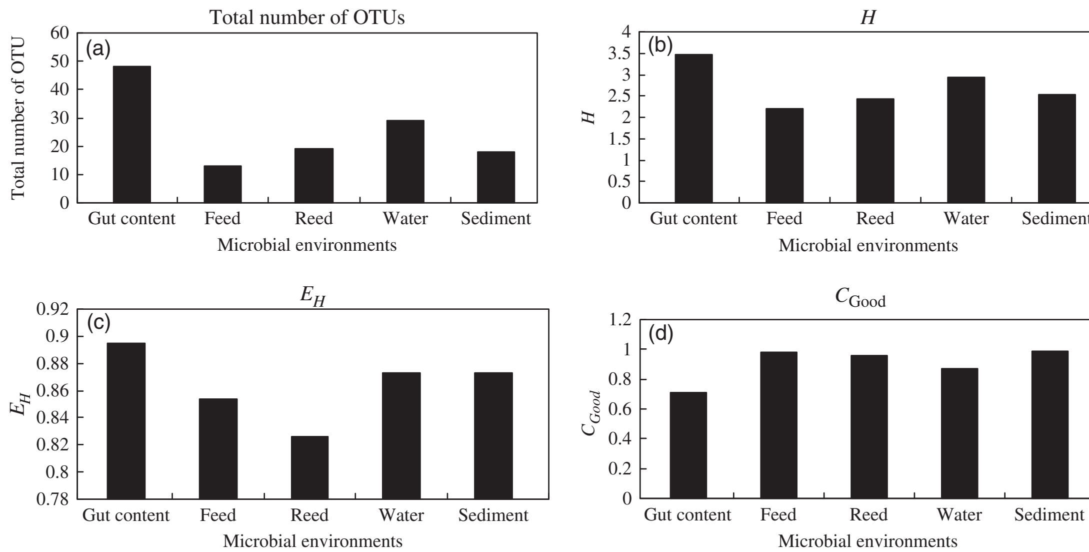

Figure 2 Bacterial diversity in the grass carp intestine and in the associated habitat and food samples (a) total number of operational taxonomic units (OTUs); (b) H; (c) E_H; (d) C_{good}. $H = -\Sigma RA_i \ln(RA_i)$; $E_H = H/\ln(S)$ (where RA_i is the proportion of the *i*th OTU and S is the total number of OTUs) (Dethlefsen *et al.* 2008); $C_{\mathrm{good}} = 1 - N_1$/total number of OTUs (where N_1 is the number of OTUs with only one clone) (Good 1953).

gut content samples was substantially higher than that in samples from the associated environments (Table 1). Twenty-eight OTUs (27, 32, 33, 40–43, 45, 47–56, 59, 61–65 and 68–71) were identified in the associated environment samples. These OTUs were not detected in the digesta samples. Of the eight OTUs with a relative abundance of $\geq 3\%$ in the gut content samples, six were identified in both gut content and its corresponding environment samples (OTUs 8, 22, 34–36 and 39) and two were identified only in the gut content samples (OTUs 74 and 76).

Discussion

To our knowledge, 16S rDNA clone library has been used in four studies to evaluate the intestinal microbial diversity in fish (Kim *et al.* 2007; Navarrete *et al.* 2009; Ward *et al.* 2009; the current study). In the present study, we used a universal primer set to construct 16S rDNA gene libraries for identification of the allochthonous gut microbiota of grass carp and of the habitat and food samples. Many researchers have suggested that methods based on 16S rDNA

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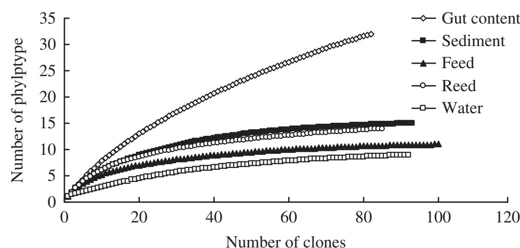

Figure 3 Rarefaction curves from 16S rDNA clone libraries from the gut content of grass carp and from associated habitat and food samples. Rarefaction curves were created using the species diversity function of the ECOSIM 700 statistical software (Gotelli & Entsminger 2002).

Table 2 Pairwise similarity coefficients (C_s) matrix for bacterial communities identified in the gut content of grass carp and in food and habitat samples*

	Gut content	Feed	Reed	Water	Sediment
Gut content	1.00				
Feed	0.46**	1.00			
Reed	0.49**	0.71†	1.00		
Water	0.28**	0.58**	0.68†	1.00	
Sediment	0.34**	0.75†	0.67†	0.57**	1.00

*In this study, $C_s < 0.60$ is regarded as a significant difference; that of $0.60 \leq C_s < 0.80$ is a marginal difference; and that ≥ 0.80 is very similar.
**Significantly different.
†Marginally different.

gene sequences using universal primers may not accurately reflect the true underlying diversity of a given environment (Marchesi, Sato, Weightman, Martin, Fry, Hiom & Wade 1998; Suzuki & Giovannoni 1996). In addition, technical challenges such as PCR bias, varying ribosomal DNA copy numbers and the efficiency of DNA extraction procedures all have the potential to significantly skew abundance estimates; therefore, assumption of a direct relationship between the number of sequences of a particular type in a clone library and the number of organisms in the environment may be inaccurate (Marchesi *et al.* 1998; Suzuki & Giovannoni 1996). However, generation of a 16S rDNA clone library using sequences that are almost full length improves the accuracy of species identification (Brons & Elsas 2008). The current study used different methods to extract DNA from different sample types, which allowed for the better recovery of DNA (He, Zhou, Yao & Bai 2009) and, subsequently, for identical PCR amplification reaction conditions. Thus, bias towards any individual sample would be similar across all sample types and would consequently be minimal when comparing the relative abundance of a specific OTU (Zhou, Liu, He, Shi, Gao, Yao & Ringø 2009). Furthermore, relative abundance differences were considered to be significant only when the relative OTU abundance in any one sample type was ≥ 1.5-fold higher or ≤ 0.5-fold lower than that in any other sample type.

In the present study, Proteobacteria, Firmicutes and Actinobacteria were the dominant allochthonous microbiota in the gut content of grass carp cultured in pond, while Huang *et al.* (2009) reported three bacterial phyla, Proteobacteria, Firmicutes and Cyanobacteria, in the gut digesta of grass carp when the 16S rDNA V3 DGGE method was used. In previous studies using classic cultivation, Luo *et al.* (2001) identified Proteobacteria, Firmicutes, Bacteroides and Actinobacteria as the dominant allochthonous bacteria in the intestine of grass carp fed a commercial feed containing diverse components and nutrients, while Zhou *et al.* (1998) reported Proteobacteria, Bacteroides and Firmicutes in the gut content of grass carp fed either a commercial feed or *Spirodela polyrhiza*. Although different food types obviously change the bacterial composition of the gut (Zhou *et al.* 1998), the gut studies of grass carp indicate that Proteobacteria and Firmicutes comprise the microbiota phylogenetic core (the common phyla).

In previous investigations, it has been proposed that water and food are the sources of some of the bacteria present in the GI tract of fish (Verschuere, Rombaut, Sorgeloos & Verstraete 2000; Olafsen 2001; Romero & Navarrete 2006). Similar findings were observed in the present study; 75% of the OTUs with a relative abundance $\geq 3\%$ in the gut content were identified in feed and habitat samples. However, we observed that the similarity coefficients between gut microbiota and microbiota from the associated environment were quite low ($C_s < 0.50$), indicating that a substantial number of grass carp gut microbiota are distinct from the corresponding environmental microbiota. Furthermore, the gut content of grass carp raised in the pond showed the highest bacterial diversity compared with its surrounding environments, supporting by the total number of OTUs, Shannon diversity index (H), Shannon equitability index (E_H), Coverage (C_{good}) and rarefaction curves, which might reflect the uniqueness of the host gut environment. In addition, the C_{good} values observed in all the samples were larger than 0.70, which indicates that the clone number analysed in each sample

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in the present study is accepted as valid in microbial diversity analysis (Pace 1997).

β-Proteobacteria have been reported to predominate in freshwater and freshwater sediment (Bissett, Bowman & Burke 2006). However, in the present study, γ-Proteobacteria were the most abundant bacteria in all samples, except for reed samples (Bacteroidetes, still not β-Proteobacteria). As γ-Proteobacteria are usually found in oligotrophic environments such as marine sediments and seawaters (Grey & Herwig 1996; Urakawa, Kita-Tsukamoto & Ohwada 1999; Bowman & McCuaig 2003; Kawahara, Nishi, Hisano, Fukui, Yamaguchi & Mochizuki 2009), we suggest that the sampling pond was nutrient deficient. Actually, this pond was recently converted from a natural reed pond to an artificial feed-based rearing pond for poly-cultured fish species including grass carp (Wang *et al*. 2010).

Westerdahl, Olsson, Kjelleberg and Conway (1991) suggested that all fish had indigenous bacteria with inhibitory effects in protecting the host against pathogens. Probiotics are thought to be beneficial for the host by improving the intestinal microbial balance via inhibition of pathogens and toxin-producing bacteria (Lilly & Stillwell 1965; Fuller 1989; Irianto & Austin 2002). Therefore, the fish intestinal microbiota might be a key pool of potential probiotics for cultured fish species. *Bacillus* spp. (*B. megaterium*, *B. polymyxa*, *B. subtilis*, *B. lichenifomis*), lactic acid bacteria (*Lactobacillus* spp., *Carnobacterium* spp., *Streptococcus* spp.), *Pseudomonas* sp. (*P. fluorescens*) and *Vibrio* sp. (*V. alginolyticus*, *V. salmonicida* like) have been examined as probiotics for aquaculture (Gatesoupe 1999; Verschuere *et al*. 2000). In the present study, several potential probiotic strains of *Bacillus* spp. were detected: *B. coagulans* (OTU6, identity 99%), which has been reported to have the ability to ferment biomass-derived sugars to lactic acid (Patel, Ou, Harbrucker, Aldrich, Buszko, Ingram & Shanmugam 2006); *B. massiliensis* (OTU7, identity 99%), having thermostable hydantoinase and carbamoylase activity (Mei, He, Liu & Ouyang 2009); and *B. megaterium* (OTU8, identity 99%), which has been recommended as a probiotic in aquaculture by Gatesoupe (1999). Furthermore, *B. pumilus* (OTU9, identity 99%), isolated firstly from penaeid shrimp (*Penaeus monodon*) and found to be inhibitory against marine pathogens such as *V. alginolyticus*, *V. mimicus* and *V. harveyi* (Hill, Baiano & Barnes 2009), was also identified in the gut digesta of grass carp.

Lactobacillus curvatus-like, *Lactobacillus fermentum*-like, *Streptococcus* sp.-like (Gatesoupe 1999; Verschuere *et al*. 2000) and *Lactococcus lactis*-like (Itoi, Yuasa, Washio, Abe, Ikuno & Sugita 2009) bacteria, which have been suggested previously to be potential probiotic candidates in aquaculture, were also identified from the gut content of grass carp. Although one or several probiotic characterizations of these bacteria were suggested in the concerned studies, further studies are required to clarify whether these bacteria are suitable as probiotics to cultured grass carp. *Streptococcus iniae*-like bacterium, previously isolated from diseased fish and identified as fish pathogen (Bachrach, Zlotkin, Hurvitz, Evans & Eldar 2001), was identified in the gut digesta of grass carp. Based on our results, this bacterium might originate from the pond water. To clarify this hypothesis, additional studies are necessary.

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