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ORIGINAL ARTICLE

Comparative study on intestinal bacterial communities of *Boleophthalmus pectinirostris* and *Periophthalmus magnuspinnatus* with different sexes and feeding strategies

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Abstract
Intertidal mudflats are unique, highly productive ecosystems. *Boleophthalmus pectinirostris* and *Periophthalmus magnuspinnatus* are common fish species that are distributed in the intertidal mudflats of the Yangtze Estuary in China. They perform important ecological functions and have different feeding strategies. Herein, we studied the intestinal microbial diversity and structure of wild *B. pectinirostris* and *P. magnuspinnatus* with different sexes and feeding strategies during their breeding season. Gut samples of *B. pectinirostris* and *P. magnuspinnatus* individuals (female:male ratio = 1:1) were collected and subjected to high-throughput DNA sequencing. The results showed Proteobacteria was the most dominant phylum in all the four sample groups: 73.5% in the males and 52.6% in the females of *B. pectinirostris* and 40.2% in the males and 40.9% in the females of *P. magnuspinnatus*. *Aeromonas*, *Shewanella*, *Halomonas*, and *Acinetobacter* of the phylum Proteobacteria were dominant genera in all the sample groups and accounted for 62.13% of the ten dominant genera. The diversity of the intestinal microflora in the omnivorous *P. magnuspinnatus* was significantly higher ($P < 0.05$) than that in the herbivorous *B. pectinirostris*. Beta diversity, including PCoA and UPGMA of unweighted UniFrac distances, showed that *B. pectinirostris* samples were clustered together, and *P. magnuspinnatus* samples were clustered together, implying the effect of the feeding habits on the microbial community structure is more considerable than that of sex.

Keywords Mudskippers · Intestinal microbiota · Sex · Feeding strategy

Introduction

Intertidal mudflats are a common feature of most estuaries and coastal plains all over the world. These coastal wetlands are highly productive ecosystems and provide a wealth of food resources for many fish, bird, and invertebrate species. However, as land-sea transitional zones, intertidal mudflats impose severe environmental challenges on their occupants by tidal oscillation and extreme shifts in habitat conditions. Mudskippers (Order Perciformes; Family Gobiidae) are amphibious fish that reside in mudflats (Ishimatsu et al. 2007; Ravi 2013). They are a highly specialized teleostean fish with a capacity for aerial respiration, which facilitates their adaptation to this harsh environment. During low tide, they actively feed on the mud surface, whereas during high tide, they stay in mudflat burrows and maintain their metabolism under severely hypoxic conditions (Lee et al. 2005). Mudskippers are common food resources for migratory waterfowl. As one of the few vertebrates that reside in mudflats, mudskippers are an important link between primary production or primary consumers and higher-level trophic consumers, and are key intermediates in the energy flow of estuarine systems. Therefore, the ecological roles of mudskippers are important to estuarine ecosystems.

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Boleophthalmus pectinirostris, belonging to the genus *Boleophthalmus*, and *Periophthalmus magnuspinnatus*, belonging to the genus *Periophthalmus*, are mudskipper species that are widely distributed in the estuary areas in China. They have a close phylogenetic relationship and have a considerable overlap in their spatial and temporal distribution. However, their diets are different, which contributes to their distinct ecological roles. *Boleophthalmus* are herbivorous and partially omnivorous fish (Yang et al. 2003), which usually obtain food by scraping the surface of intertidal mudflats using their teeth while wiggling their body. An earlier study on the food consumption patterns of *Boleophthalmus* found that more than a half of its diet consists of benthic diatoms throughout the year (Zhu and Zhang 1993; Yang et al. 2003; Ravi 2013). Gammaridae, polychaetes, algae, and fish eggs, even detritus and mud/sand particles, are also found to be consumed, but constitute a low percentage in the gut content of this fish (Ravi 2013). On the other hand, *Periophthalmus* are omnivorous and partially carnivorous fish that access food mainly by jumping in attack. They primarily consume gammaridae, crabs, other crustaceans, and benthic animals commonly distributed in mudflats and tidal channels (Baeck et al. 2008). *B. pectinirostris* and *P. magnuspinnatus* are dioecious creatures with an exceedingly long breeding season that can last from May to October in the estuary area of China. The external differences between male and female individuals are not obvious, but male individuals have a strong territorial awareness and egg-protective behavior (Brillet 1969). During the breeding season, female individuals lay their eggs in mudflat burrows. Then, male individuals reduce their food intake and protect the fertilized eggs in mudflat burrows.

In contrast to the abundance of investigations on food preference and sex difference, little is known about the intestinal flora structure of mudskippers which is closely related to their food consumption patterns and sexual characteristics. Intestinal microbes are an important part of living organisms. After a long period of co-evolution, intestinal microbes have formed mutually beneficial relationships with their host and thus exert exceedingly important functions in host physiological metabolism (Flint et al. 2008; Ley et al. 2008). Various factors influence the intestinal microbial composition (Sekirov et al. 2010; Sullam et al. 2012), including the environment (Jabari et al. 2016), physiological characteristics (Ley et al. 2008), diet (Brown et al. 2012), and host phylogeny (Sanders et al. 2015). Among them, diet is widely believed to be one of the most important factors that have an impact on the structure and abundance of intestinal bacterial communities. In contrast, the impact of different sexes on intestinal flora is not clear.

In this paper, we investigated the structural differences in the intestinal flora of wild *B. pectinirostris* and *P. magnuspinnatus* with different food consumption patterns and sexes, and examine the degree to which sex and food affect the diversity and abundance of intestinal microbial communities and also hope to provide substantial microecological information for estuarine ecosystems.

Materials and methods

Sample collection and ethical standards

The use of experimental animals in this study and the study protocol were reviewed and approved by the Ethics Committee of East China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences.

In July 2016, *B. pectinirostris* and *P. magnuspinnatus* individuals were collected by entrapment using bamboo shoots embedded in the intertidal mudflats of the Yangtze Estuary, China. All studied fish individual samples were collected at the same site to ensure consistent environmental conditions. Six *B. pectinirostris* and six *P. magnuspinnatus* (female:male ratio, 1:1) individuals were randomly selected from the collected fish. The stomachs of the collected mudskippers were filled with food, with contents similar to those established in previously reported studies (Baeck et al. 2008; Ravi 2013). The stomach content of *B. pectinirostris* consisted mainly of benthic diatoms, whereas the stomach content of *P. magnuspinnatus* was predominantly constituted of little crabs and shrimps. The intestinal tracts were collected and gently washed using sterile saline solution (6.8 g NaCl dissolved in 1000 mL of PBS buffer) after the intestinal content was removed. Then, the intestinal tracts of each of the samples were placed in sterile tubes and cryopreserved in liquid nitrogen until DNA extraction.

Illumina library preparation and sequencing

DNA samples were extracted from the obtained intestinal tract samples using the FastDNA™ Spin Kit for Soil (MP Biomedicals, USA), according to the manufacturer's instructions. The purity and quality of the extracted DNA were tested by 1% agarose gel electrophoresis. Then, the extracted DNA was diluted to a concentration of 1 ng/μL and used as a template for the amplification of the V4 regions of the 16S rRNA gene using the primer pair 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) with a specific barcode. Three PCR replicates of each sample were performed. The PCR products were mixed, and the quality of these mixed PCR products was examined via electrophoresis on a 2% agarose gel. PCR products were purified using the Qiaex II Gel Extraction Kit (Qiagen, Germany) and quantified by Qubit® version 2.0 (Invitrogen, USA). Subsequently, a

library was constructed using the TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA). High-throughput sequencing was carried out on an Illumina HiSeq PE 250 platform (Novogene Technology Co., Ltd., Beijing, China).

Sequencing data preprocessing and assembly

Paired-end sequence data from the Illumina HiSeq PE 250 platform were assembled based on the overlap relationship using Flash software, and the pairs of reads were merged into one sequence (Magoč and Salzberg 2011). The reads that could not be assembled were discarded. Then, the obtained sequence was processed for quality control using QIIME software (Caporaso et al. 2010). The process of quality control included cutting off the sequence from the first low-quality base site of the continuous low-quality base sites (default quality value ≤ 19, default length of the base ≥ 3) and removing sequences containing less than 75% of the original sequence. Subsequently, chimeras were removed (Edgar et al. 2011) to obtain effective data for subsequent analysis.

Statistical and bioinformatics analyses

The effective data of the samples were clustered into operational taxonomic units (OTUs) at a 97% identity threshold using Uparse software (Uparse v7.0.1001, http://drive5.com/uparse/) (Edgar 2013), and representative sequences (the highest-frequency sequence) of each OTU were picked. Further, the effective sample data were normalized based on the minimum effective data of each sample. These normalized data were mapped to OTU representative sequences to obtain the OTU cluster results of each sample. Meanwhile, rarefaction curves were generated based on normalized OTU numbers using Mothur software (Schloss et al. 2009). Subsequently, representative sequences were determined and classified taxonomically by species annotation based on RDP Native Bayesian Classifier using the Mothur software (Wang et al. 2007) and the SSU rRNA database of SILVA (http://www.arb-silva.de/; threshold: 0.8). The phylogenetic relationships of the representative sequences were analyzed using MUSCLE software (Version 3.8.31, http://www.drive5.com/muscle/) (Edgar 2004) to obtain the taxonomic information of intestinal bacteria at different classification levels. Further, a species classification tree and heatmap were produced using SVG and R software. In addition, diversity indices, including alpha diversity (the number of OTUs and Chao index), beta diversity (principal coordinate analysis (PCoA), and the unweighted pair-group method with arithmetic mean (UPGMA) based on unweighted UniFrac distances, were assessed using QIIME (Version 1.7.0) and R software. Analysis of molecular variance (AMOVA) was conducted to compare the differences in the microbial community structures of the experimental groups using Mothur software (Excoffier et al. 1992). Wilcoxon test was utilized to test the alpha diversity in the different sex groups using the agricolae package of R software. One-way ANOVA was carried out to test the alpha diversity of the different species using SPSS 19.0 software. LEfSe (LDA score > default values 4; removing non-bacterial sequences) was utilized to identify the species with significant differences between different sex of the same mudskipper species. Raw sequences were deposited at the NCBI Sequence Read Archive (Accession No. SRP122754).

Results

Sequencing depth and alpha diversity indices

After filtering out low-quality and short-length sequences and removing chimeras, we obtained 61,270–91,748 effective sequences from each sample (Table 1). The high-quality bases (quality value > 20) accounted for more than 99% of the effective sequences of all the samples. Meanwhile, the percentage ratio of the effective reads to that of the raw reads was more than 90% in most samples, except for samples BS1.1 and BS2.3. After normalization, 59,296 sequences remained for each sample and were clustered into a total of 5855 OTUs at a similarity level of 97%, showing the depth of sequencing was appropriate. In addition, the rarefaction curves (Fig. 1) tended to gradually reach a plateau with increasing the sequencing depth, indicating that the sequencing depth was sufficient for subsequent analysis.

Alpha diversity analysis (Fig. 2) is usually used to evaluate microbial community diversity. Here, we used the number of OTUs and the Chao index to assess the bacterial diversity. We found that the numbers of OTUs between male and female *B. pectinirostris* groups had no statistical difference ($P = 0.17 > 0.05$), and the average numbers were 1272 and 818, respectively. However, in *P. magnuspinnatus*, the average numbers of OTUs in male and female individuals were approximately 2397 and 1656, respectively, with a P value of 0.04 (< 0.05), indicating the intestinal microbial diversity of male individuals were higher than that of female individuals of *P. magnuspinnatus*. After combining the data of the same species including male and female samples, statistical analysis showed that the intestinal microbial diversity of *B. pectinirostris* was significantly lower than that of *P. magnuspinnatus* ($P < 0.05$), suggesting the intestinal microbial diversity of the herbivorous mudskipper was lower than that of the omnivorous mudskipper.

In addition, the Chao index was remarkably higher in *P. magnuspinnatus* than in *B. pectinirostris* ($P < 0.05$). It is

Table 1 Statistical results of data preprocessing

Sample name	Raw PE	Clean tags	Effective tags	Base (nt)	QV20 (%)	Effective (%)
BP1.1	70,158	63,582	61,270	15,532,299	99.18	87.33
BP1.2	97,342	92,940	89,935	22,746,705	99.21	92.39
BP1.3	99,565	92,777	90,847	22,883,498	99.21	91.24
BP2.1	96,870	89,098	87,282	22,070,125	99.23	90.10
BP2.2	97,181	92,817	91,748	23,156,291	99.18	94.41
BP2.3	84,645	62,495	60,946	15,426,463	99.00	72.00
PM1.1	94,535	88,251	86,565	21,866,417	99.19	91.57
PM1.2	86,163	82,289	80,311	20,360,892	99.15	93.21
PM1.3	80,279	78,998	75,111	19,009,286	99.39	93.56
PM2.1	84,877	80,313	78,893	19,772,141	99.21	92.95
PM2.2	90,750	86,136	84,915	21,344,846	99.17	93.57
PM2.3	87,003	83,650	82,452	20,652,184	99.21	94.77

Sample names BP1.1, BP1.2, and BP1.3 represent three parallel male samples of *B. pectinirostris*, respectively; BP2.1, BP2.2, and BP2.3 represent three parallel female samples of *B. pectinirostris*, respectively; PM1.1, PM1.2, and PM1.3 represent three parallel male samples of *P. magnuspinnatus*, respectively; PM2.1, PM2.2, and PM2.3 represent three parallel female samples of *P. magnuspinnatus*, respectively. Raw PE indicates the number of raw reads; clean tags indicate the sequence number after filtering out low-quality and short-length sequences; effective tags indicate the sequence number after filtering the chimera; effective tags were used for subsequent analysis. Base represents the base number of effective tags; QV20 (%) indicates the percentage of bases with a base quality > 20 in the effective tags; effective (%) indicates the percentage of the number of effective reads to the number of raw reads

noteworthy that no statistical difference was detected between the male and female individuals of the same species, implying that the between-species difference exerts a more pronounced impact on the diversity of the microbial community structure than the different sex within the same species.

Taxonomic composition

The representative OTU sequences were classified and analyzed at different taxonomic levels. The results revealed some differences at the phylum level in the community composition between the different sample groups

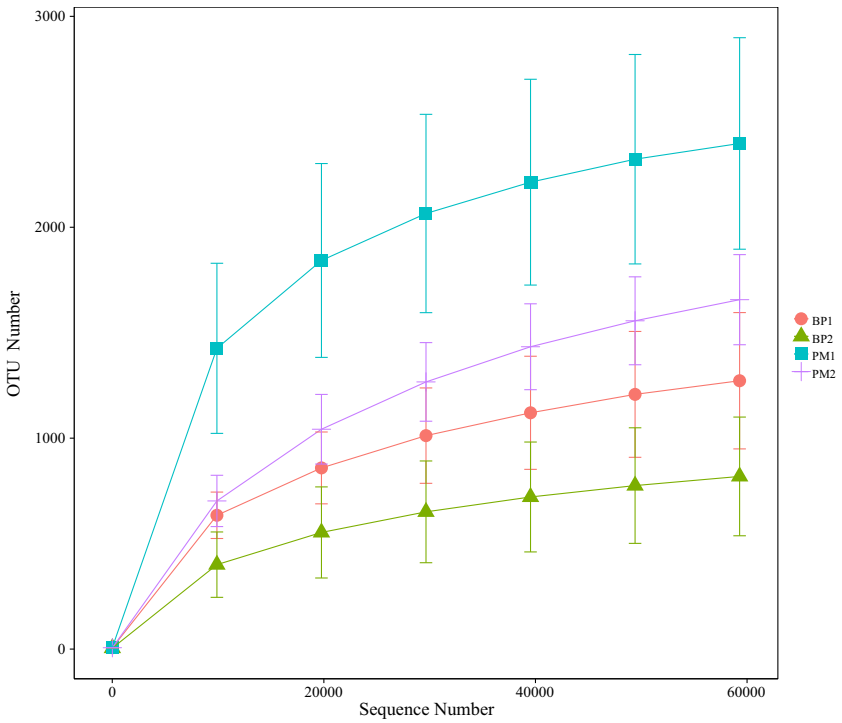
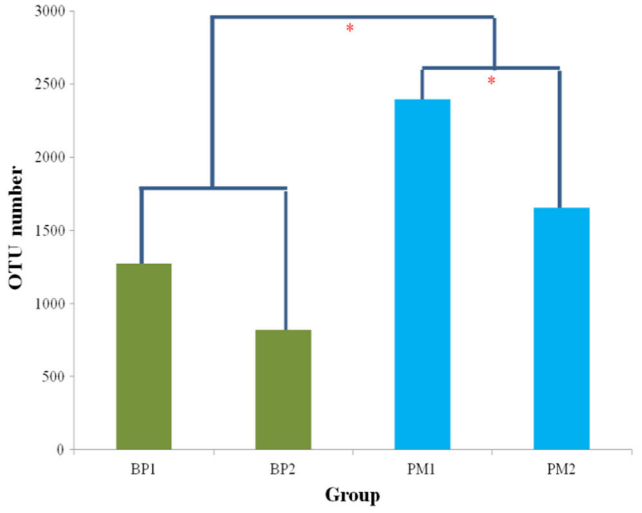


Fig. 1 Rarefaction curves of the different sample groups. Notes: BP1 and BP2 represent the male and female sample groups of *B. pectinirostris*, respectively; PM1 and PM2 represent the male and female sample groups of *P. magnuspinnatus*, respectively



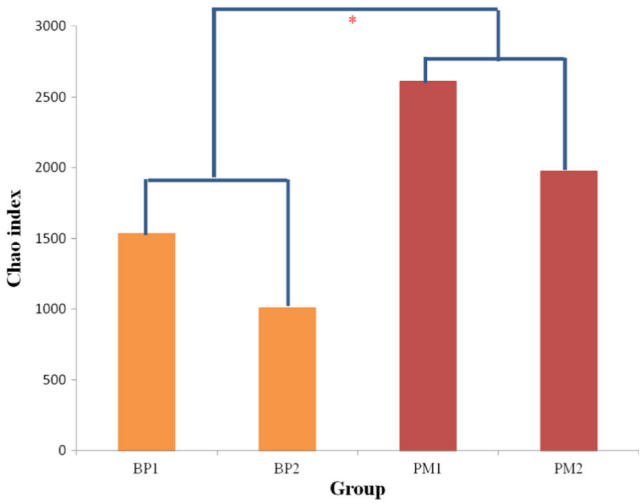


Fig. 2 Alpha diversity analysis of the different sample groups ($n = 3$). Note: BP1 and BP2 represent the male and female sample groups of *B. pectinirostris*, respectively; PM1 and PM2 represent the male and female sample groups of *P. magnuspinnatus*, respectively; * represents $P < 0.05$ and denotes a statistically difference between the two groups.

(Fig. 3). Proteobacteria were the dominant phylum in all the four sample groups (73.5 and 52.6% in *B. pectinirostris* males and females, respectively; 40.2 and 40.9% in *P. magnuspinnatus* males and females, respectively). However, Fusobacteria (18.2%) and Spirochaetes (10.5%) also were dominant bacteria (> 6.0%) in the *B. pectinirostris* female group, and less than 2% of Fusobacteria were present in the other groups. Besides, Firmicutes (20%), Actinobacteria (7.5%), Bacteroidetes (7.4%), and Acidobacteria (7.2%) were the dominant bacteria (> 6.0%) in the male gut samples of *P. magnuspinnatus*, whereas the proportion of Firmicutes was lower than 5% in the other groups. Meanwhile, Cyanobacteria (22.2%) followed by Spirochaetes (14.4%) and Tenericutes (9.8%) were the dominant phyla in the female *P. magnuspinnatus* gut. In the other groups, the proportions of

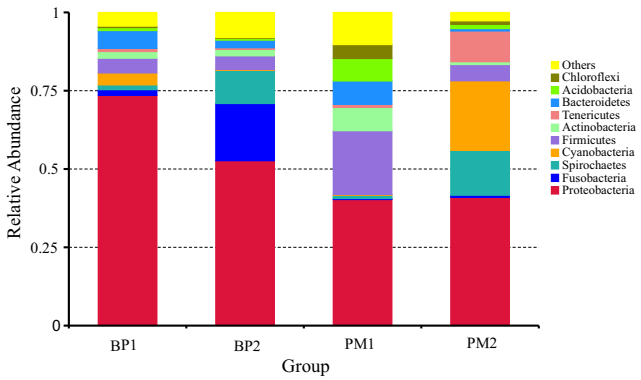


Fig. 3 Microbial community structure and relative abundance at the phylum level (top 10). Note: BP1 and BP2 represent the male and female sample groups of *B. pectinirostris*, respectively; PM1 and PM2 represent the male and female sample groups of *P. magnuspinnatus*, respectively

Cyanobacteria and Tenericutes were less than 4.0 and 1.0%, respectively.

The classification tree up to the genus level (Fig. 4) illustrates the category and distribution of the top ten bacterial genera in each group. *Soonwooa* (1.35%), unidentified chloroplast (11.11%), Candidatus Arthromitus (3.99%), *Blautia*

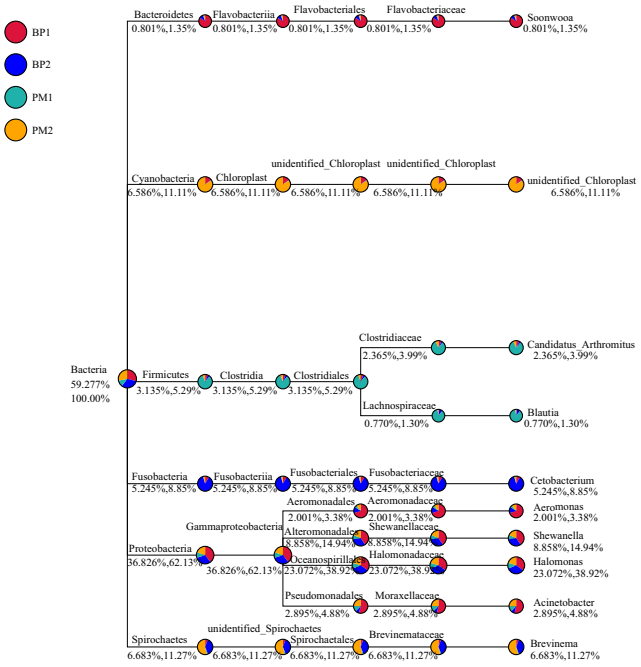


Fig. 4 Specific species classification tree in each group (top 10). Notes: BP1 and BP2 represent the male and female sample groups of *B. pectinirostris*, respectively; PM1 and PM2 represent the male and female sample groups of *P. magnuspinnatus*, respectively. The size of each sector represents the proportion of relative abundance in different groups; the numbers below the category name (the first number represents the percentage of all genera, and the second represents the percentage of the selected genus) indicate the average relative abundance of the classification

(1.30%), *Cetobacterium* (8.85%), *Aeromonas* (3.38%), *Shewanella* (14.94), *Halomonas* (18.92%), *Acinetobacter* (4.88%), and *Brevinema* (11.27%) were the dominant bacterial genera (top 10) in the gut samples analyzed in this research. The relative abundance of *Aeromonas*, *Shewanella*, *Halomonas*, and *Acinetobacter*, which belong to the phylum Proteobacteria, was high in all the sample groups and accounted for 62.13% of the ten dominant genera. However, the abundance of *Soonwooa*, which belongs to the phylum Bacteroidetes, was higher in the *B. pectinirostris* male gut samples than in those of the other groups. *Cetobacterium* of the phylum Fusobacteria was the most abundant genus in the gut of female *B. pectinirostris* individuals. *Candidatus* Arthromitus and *Blautia* of the phylum Firmicutes were predominant in the gut of males of *P. magnuspinnatus*, whereas unidentified chloroplast of the phylum Cyanobacteria was the most abundant in the gut of females of *P. magnuspinnatus*. These results suggest that the most dominant phylum and genus were similar in the different groups, but each species or gender group also had their unique bacterial structure at the phylum and genus levels. The heatmap of the abundance of top 35 species clustered also confirmed this conclusion (Fig. 5). Further analysis of the microbial community structure (Table 2) showed that statistically significant difference ($P < 0.05$) was present between the different species (*B. pectinirostris* and *P. magnuspinnatus*), whereas the samples of male and female individuals within the same species (*B. pectinirostris* or *P. magnuspinnatus*) exhibited no statistically significant difference ($P > 0.05$), implying that the influence of the species on the flora composition and relative abundance was more considerable than that of the sex. Sex exerted no significant impact on the overall bacterial community structure, but had a significant effect in certain specific microorganisms. LEfSe analysis (Fig. 6) showed that significant differences at different levels (phylum, class, order, family, or genus) were available between the male and female samples of the same species (*B. pectinirostris* or *P. magnuspinnatus*) in certain bacteria, suggesting that sex did have a certain influence on the bacterial community structure.

OTU-based beta diversity analysis

Smaller unweighted UniFrac distances (Fig. 7) were found in the analysis of the female to male distances (0.499 for *B. pectinirostris* and 0.504 for *P. magnuspinnatus*) compared to those between the two species (0.678 for *B. pectinirostris* and *P. magnuspinnatus* males, and 0.663 for *B. pectinirostris* and *P. magnuspinnatus* females), indicating the differences in the bacterial diversity are smaller within conspecific hosts (male and female) than non-conspecific hosts (between *B. pectinirostris* and *P. magnuspinnatus*). Furthermore, the PCoA plot and UPGMA phylogenic tree (Figs. 8 and 9) also confirmed that the male and female samples of *B. pectinirostris* cluster together, and the male and female samples of *P. magnuspinnatus* cluster together, suggesting that gut microbial assemblages of the same species (conspecific) hosts shared a greater similarity than those of different host species.

Discussion

The gut microbiota of fish, established during the initial feeding, is derived from the surrounding aquatic environment, soil, and food (Romero and Navarrete 2006; Ward et al. 2009; Li et al. 2014). During the growth and development of fish, intestinal flora usually undergoes dynamic changes (Stephens et al. 2016).The reproductive period is a critical developmental stage, during which fish of different sexes have significantly distinct physiological characteristics and behaviors (Liao et al. 2014). To the best of our knowledge, there are no published studies examining whether or not differences are present between the intestinal flora composition of male and female mudskipper individuals. In the present investigation, we explored the intestinal microbial characteristics of males and females of *B. pectinirostris* and *P. magnuspinnatus* during the breeding season. We found that in *P. magnuspinnatus*, the intestinal microbial diversity of male individuals was higher ($P < 0.05$) than that of female individuals. This finding is consistent with the findings of a previous report revealing that sex had a significant correlation with the intestinal microflora diversity in *Gasterosteus aculeatus* and *Perca fluviatilis* (Bolnick et al. 2014). However, in *B. pectinirostris*, the intestinal microbial diversity of male and female individuals displayed no significant differences, suggesting sex may exert different effects on the intestinal microbial diversity in various species. The specific reasons for this result are still unclear. The mechanisms of the influence of sex on the gut microbiota have not yet been completely elucidated (Freire et al. 2011), and apparently include hormone–microbe interactions and sex-specific immune responses (Bolnick et al. 2014). These potential mechanisms might be different in *P. magnuspinnatus* and *B. pectinirostris* and lead to the different effects of sex on microfloral structure.

Diet and phylogeny are other important factors that shape the structure of the intestinal microbial community (Muegge et al. 2011a; Yun et al. 2014). Some mammals with diets atypical of their clade, such as the herbivorous panda, possess taxonomically more similar microbial gut communities to those of their close relatives than to other mammals with similar diets (Ley et al. 2008). In our study, *B. pectinirostris* and *P. magnuspinnatus* individuals were collected from their native habitats, where both species shared the same aquatic environment. In addition, *B. pectinirostris* and *P. magnuspinnatus* both

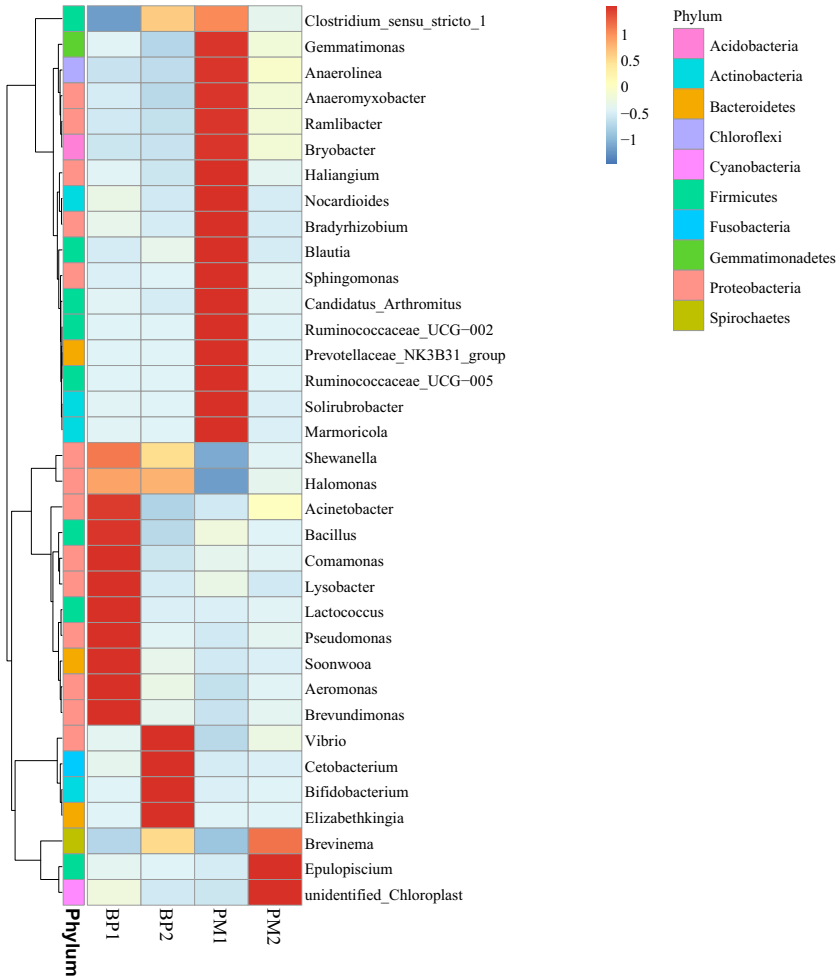


Fig. 5 Heatmap showing species abundance clustering of each group (top 35). Notes: BP1 and BP2 represent the male and female sample groups of *B. pectinirostris*, respectively; PM1 and PM2 represent the male and female sample groups of *P. magnuspinnatus*, respectively

belong to the family Gobiidae and the order Perciformes with a close host phylogenetic relationship (Murdy 1989; Zeehan et al. 2016). These facts partially explain the similarity in the microbial gut communities in *B. pectinirostris* and *P. magnuspinnatus*. Meanwhile, *B. pectinirostris* and *P. magnuspinnatus* samples collected from their same natural habitat partly represent how different feeding habits affect the microbial community structure of the intestine. Feeding habits are an essential factor that has an impact on the gut microflora (Filippo et al. 2010; Muegge et al. 2011b). Ley et al. (2008) reported that bacterial diversity in terrestrial mammals increased from carnivory to omnivory to herbivory. In insects, the bacterial diversity of cockroaches was higher than that of wasps and honeybees, implying that the bacterial diversity increases from omnivorous to herbivorous species (Mrázek et al. 2008). In our experiment, the number of OTUs and the Chao index of *B. pectinirostris* samples were lower than those of *P. magnuspinnatus* samples, suggesting that the herbivorous *B. pectinirostris* had a lower gut microbial diversity than the omnivorous *P. magnuspinnatus*. These findings are consistent with results obtained for insects but are inconsistent with those for terrestrial mammals (Ley et al. 2008; Mrázek et al. 2008). One possible explanation is that the influence of the feeding habits on the intestinal bacterial diversity is species-specific. *B. pectinirostris* and *P. magnuspinnatus* are aquatic creatures

Table 2 Statistical analysis of the microbial community structure of the different groups

Groups	*P* value	Significance
BP1-BP2	0.515	-
PM1-PM2	0.101	-
BP-PM	< 0.001	*

BP1 and BP2 represent the male and female sample groups of *B. pectinirostris*, respectively; PM1 and PM2 represent the male and female sample groups of *P. magnuspinnatus*, respectively; BP represents all the samples (male and female) of *B. pectinirostris* samples; PM represents all the samples (male and female) of *P. magnuspinnatus*; BP1-BP2 denotes the male and female sample of the groups of *B. pectinirostris*; PM1-PM2 indicates the male and female sample groups of *P. magnuspinnatus*; BP-PM indicates *B. pectinirostris* and *P. magnuspinnatus* groups; *P* value represent the value of *P*; "-" indicates the lack of statistical difference between the two groups; "*" indicates the presence of a statistical difference between the two groups

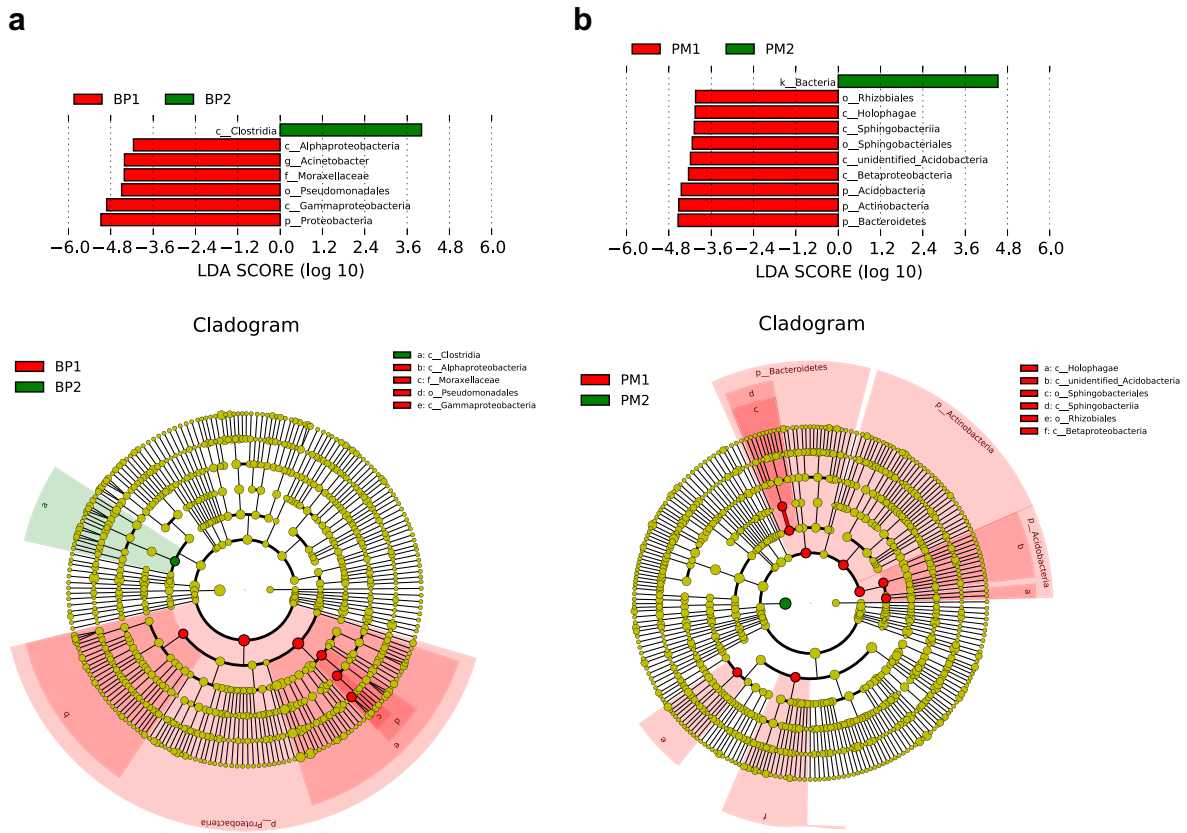


Fig. 6 LEfSe identified the species with statistical difference between the male and female sample groups in the same mudskipper species. Notes: the histogram shows the linear discriminant analysis (LDA) scores computed for the most discriminating species, based on their relative abundance in the gut microbiota of different male and female mudskippers. The cladogram shows the species phylogenic relationship of the most discriminating species. BP1 and BP2 represent the male and female sample groups of *B. pectinirostris*, respectively. PM1 and PM2 represent the male and female sample groups of *P. magnuspinnatus*, respectively. "A" denotes LEfSe analysis of the male and female sample groups of *B. pectinirostris*. "B" denotes the LEfSe analysis of the male and female sample groups of *P. magnuspinnatus*. "p" represents the analysis at the phylum level; "c" represents the analysis at the class level; "o" represents the analysis at the order level; "f" represents the analysis at the family level; "g" represents the analysis at the genus level

residing in tidal beach wetlands, in which, the aquatic ecosystem is more complex and changeable than terrestrial habitats. The microbial community structure of aquatic creatures is highly dependent on the aquatic environment. *B. pectinirostris* and *P. magnuspinnatus* have specific mechanisms to adapt to the conditions of this particular environment. Although these mechanisms are not yet clear, they may affect the influence of food on the intestinal flora diversity. On the other hand, mammalian fecal samples were collected from the posterior intestine contents (feces), whereas the samples of *B. pectinirostris* and *P. magnuspinnatus* in our investigation were collected from the intestinal wall after removal of the entire intestinal contents. The microbial communities present in the intestinal contents could reflect the structure of the intestinal microbial community. However, the microbial flora of the intestinal content varies due to occasional feeding. Our intestinal samples were collected after the intestinal contents were cleared, and were thus more likely to reflect the structure of the intestinal microbiota. In addition, herbivorous mammals have powerful stomachs enabling food digestion and are classified into two major groups: foregut fermenters (such

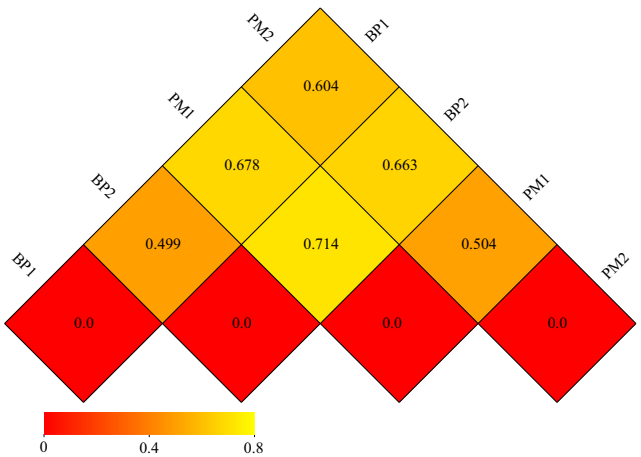


Fig. 7 Heatmap of unweighted UniFrac distances. Note: BP1 and BP2 represent the male and female sample groups of *B. pectinirostris*, respectively; PM1 and PM2 represent the male and female sample groups of *P. magnuspinnatus*, respectively

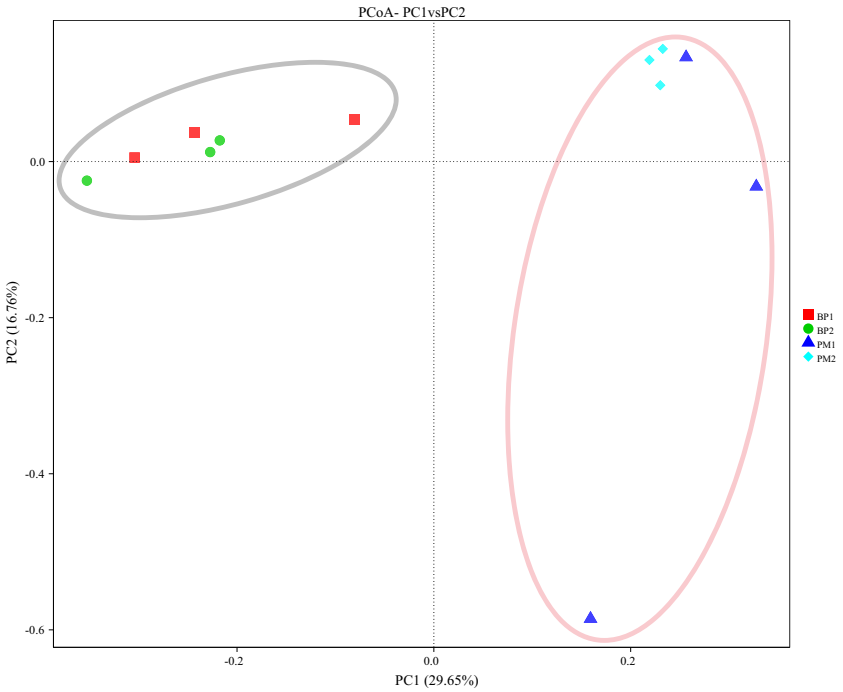


Fig. 8 Principal coordinate analysis (PCoA) based on unweighted UniFrac distances. Note: BP1 and BP2 represent the male and female sample groups of *B. pectinirostris*, respectively; PM1 and PM2 represent the male and female sample groups of *P. magnuspinnatus*, respectively

as sheep) and hindgut fermenters (such as horses) (Ley et al. 2008). Due to host digestive physiology, a part of the microbiota is digested by foregut fermenters, whereas fermentative microbes are more likely to be excreted in the feces by hindgut fermenters. The digestion of the food in the herbivorous *B. pectinirostris* is facilitated by increasing the retention time of the stomach contents in their long intestine. In mudskippers, the intestinal length of *B. pectinirostris* is two to three times the length of its body length. However, in *P. magnuspinnatus*, the length of the intestinal tract is only 0.5–0.9 times the length of its body length. We speculate that the stomachs and intestines of *B. pectinirostris* probably digested a portion of microbiota during their long digestion passage, which resulted in a low bacterial diversity in the samples. Moreover, the difference in the bacterial diversity might have been caused by the more likely exposure of the omnivorous *P. magnuspinnatus* to bacteria from other organisms than that of the herbivorous *B. pectinirostris.*

In the taxonomic classification, Proteobacteria was found to be the dominant phylum in all the sample groups (73.5 and 52.6% in *B. pectinirostris* male and female samples, respectively; 40.2 and 40.9% in *P. magnuspinnatus* male and female samples, respectively) in the present study. This finding is similar to the reports on *Aristichthys nobilis* (Li et al. 2014) and *Coreius guichenoti* (Li et al. 2016) and may be related to the widespread presence of Proteobacteria in the surrounding environment (González and Moran 1997; Cottrell et al. 2000). Apart from Proteobacteria, Fusobacteria (18.2%) was dominant in the males and Bacteroidetes (5.7%) in the females of *B. pectinirostris*. In *P. magnuspinnatus*, Proteobacteria was followed by Cyanobacteria (22.2%) in females and Firmicutes (20.4%) in males, indicating that distinct bacterial phyla are present in different hosts. Further, the statistical analysis (AMOVA) showed that there were statistically significant differences ($P < 0.05$) between the microbial community structures of *B. pectinirostris* and *P. magnuspinnatus*, but no

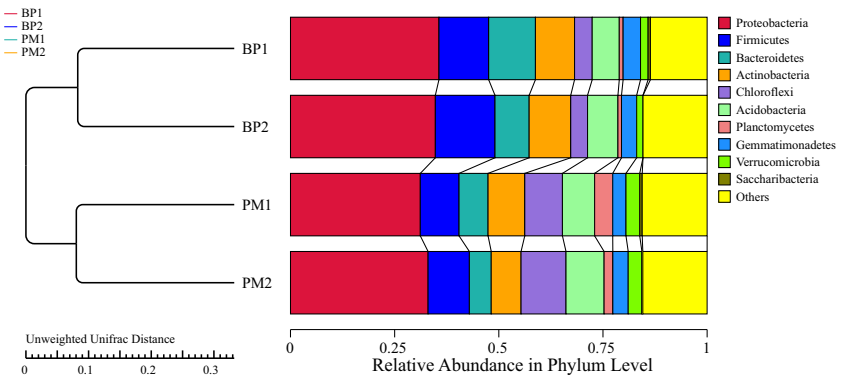


Fig. 9 UPGMA phylogenic tree based on unweighted UniFrac distances. Note: BP1 and BP2 represent the male and female sample groups of *B. pectinirostris*, respectively; PM1 and PM2 represent the male and female sample groups of *P. magnuspinnatus*, respectively

significant difference was observed between the male and female individuals of *B. pectinirostris* or *P. magnuspinnatus*, implying that the feeding habits significantly influence the microbiota composition and are a stronger determinant than sex. Beta diversity (PCoA and UPGMA of unweighted UniFrac distances) showed that the gut microbial assemblages of the same species (conspecific) hosts were more similar to each other than to those of different host species, which also supports the conclusion that the influence of feeding habits is more pronounced than that of sex. LEfSe analysis revealed the presence of considerable differences at the phylum level between the data obtained from male and female sample of *B. pectinirostris* or *P. magnuspinnatus*, suggesting that sex does influence fish gut microbiota composition and supports the view that diet has sex-dependent effects on vertebrate gut microbiota (Bolnick et al. 2014). At the genus level, the abundances of *Soonwooa* in *B. pectinirostris* males, *Cetobacterium* in *B. pectinirostris* females, Candidatus Arthromitus and *Blautia* in *P. magnuspinnatus* males, and unidentified chloroplast in *P. magnuspinnatus* females were higher than those in the other samples. LEfSe analysis results showed considerable differences in the abundances of *Acinetobacter* between the samples obtained from males and females of *B. pectinirostris*. The functions of these unique bacteria are currently unknown and worthy of further study. Meanwhile, *Aeromonas*, which not only harbors cellulase genes (Jiang et al. 2011), but is also a well-known fish pathogen (Beaz-Hidalgo and Figueras 2013), was detected at high levels in *B. pectinirostris* and *P. magnuspinnatus* gut samples. Both fish species have been considered to possess strong disease resistance. Whether *Aeromonas* is pathogenic to mudskippers or affects positively the digestion of food similarly to the favorable effect of *Aeromonas* in the processes of food digestion in the herbivorous *Ctenopharyngodon idella* (Jiang et al. 2011) is still unclear, and further investigation is warranted.

In summary, our findings indicate that sex-dependent differences are present in the gut microbial diversity of *P. magnuspinnatus*, whereas no sex-related differences were detected in *B. pectinirostris*. Meanwhile, the diversity of the intestinal microflora in the omnivorous *P. magnuspinnatus* was higher than that in the herbivorous *B. pectinirostris*. The effect of food on the microbial community structure is more considerable than that of sex.

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Compliance with ethical standards The study was reviewed and approved by the Ethics Committee of East China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences.

Conflict of interest The authors declare that they have no conflict of interest.

References

Baeck GW, Takita T, Yang HY (2008) Lifestyle of Korean mudskipper Periophthalmus magnuspinnatus with reference to a congeneric species Periophthalmus modestus. Ichthyol Res 55:43–52

Beaz-Hidalgo R, Figueras MJ (2013) Aeromonas spp. whole genomes and virulence factors implicated in fish disease. J Fish Dis 36:371–388

Bolnick DI, Snowberg LK, Hirsch PE, Lauber CL, Org E, Parks B, Lusis AJ, Knight R, Caporaso JG, Svanbäck R (2014) Individual diet has sex-dependent effects on vertebrate gut microbiota. Nat Commun 5:4500

Brillet C (1969) Etude du comportement constructeur des poissons amphibies Periophtalmidæ. FRA 4:496

Brown K, Decoffe D, Molcan E, Gibson DL (2012) Diet-induced dysbiosis of the intestinal microbiota and the effects on immunity and disease. Nutrients 4:1552–1553

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R (2010) QIIME allows analysis of high-throughput community sequencing data. Nat Methods 7:335–336

Cottrell MT, Wood DN, Yu L, Kirchman DL (2000) Selected chitinase genes in cultured and uncultured marine bacteria in the α- and γ-subclasses of the proteobacteria. Applied & Environmental Microbiology 66:1195

Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32:1792–1797

Edgar RC (2013) UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat Methods 10:996

Edgar RC, Haas BJ, Clemente JC, Christopher Q, Rob K (2011) UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27:2194–2200

Excoffier L, Smouse PE, Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131:479

Filippo CD, Cavalieri D, Paola MD, Ramazzotti M, Poullet JB, Massart S, Collini S, Pieraccini G, Lionetti P (2010) Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. Proc Natl Acad Sci U S A 107:14691

Flint HJ, Bayer EA, Rincon MT, Lamed R, White BA (2008) Polysaccharide utilization by gut bacteria: potential for new insights from genomic analysis. Nat Rev Microbiol 6:121–131

Freire AC, Basit AW, Choudhary R, Piong CW, Merchant HA (2011) Does sex matter? The influence of gender on gastrointestinal physiology and drug delivery. Int J Pharm 415:15

González JM, Moran MA (1997) Numerical dominance of a group of marine bacteria in the alpha-subclass of the class Proteobacteria in coastal seawater. Applied & Environmental Microbiology 63:4237–4242

Ishimatsu A, Yoshida Y, Itoki N, Takeda T, Lee HJ, Graham JB (2007) Mudskippers brood their eggs in air but submerge them for hatching. J Exp Biol 210:3946–3954

Zeehan J, Gianluca P, Yuliadi Z (2016) Description of a new species of Periophthalmus (Teleostei: Gobiidae) from the Lesser Sunda Islands. Raffles B Zool 64:278–283

Jabari L, Khelifi E, Gannoun H, Fardeau ML, Godon JJ, Hamdi M (2016) Effect of salinity and temperature on the bacterial diversity shift of anaerobic batch cultures treating abattoir wastewater. Desalination & Water Treatment 57:13909–13915

Jiang Y, Xie C, Yang G, Gong X, Chen X, Xu L, Bao B (2011) Cellulase-producing bacteria of Aeromonas are dominant and indigenous in

the gut of Ctenopharyngodon idellus (Valenciennes). Aquac Res 42: 499–505

Lee HJ, Martinez CA, Hertzberg KJ, Hamilton AL, Graham JB (2005) Burrow air phase maintenance and respiration by the mudskipper Scartelaos histophorus (Gobiidae: Oxudercinae). J Exp Biol 208: 169–177

Ley RE, Hamady M, Lozupone C, Turnbaugh PJ, Ramey RR, Bircher JS, Schlegel ML, Tucker TA, Schrenzel MD, Knight R (2008) Evolution of mammals and their gut microbes. Science 320:1647–1651

Li XM, Zhu YJ, Yan QY, Ringø E, Yang DG (2014) Do the intestinal microbiotas differ between paddlefish (Polyodon spathala) and bighead carp (Aristichthys nobilis) reared in the same pond? J Appl Microbiol 117:1245–1252

Li T, Long M, Ji C, Shen Z, Gatesoupe FJ, Zhang X, Zhang Q, Zhang L, Zhao Y, Liu X (2016) Alterations of the gut microbiome of largemouth bronze gudgeon (Coreius guichenoti) suffering from furunculosis. Sci Rep 6:30606

Liao C, Yu D, Chen Y, Reichard M, Liu H (2014) Reproductive behaviour of female rosy bitterling Rhodeus ocellatus in response to a female-biased operational sex ratio. Behaviour 151:755–768

Magoč T, Salzberg SL (2011) FLASH: fast length adjustment of short reads to improve genome assemblies. Bioinformatics 27:2957–2963

Mrázek J, Strosová L, Fliegerová K, Kott T, Kopecný J (2008) Diversity of insect intestinal microflora. Folia Microbiol 53:229–233

Muegge BD, Kuczynski J, Knights D, Clemente JC, González A, Fontana L, Henrissat B, Knight R, Gordon JI (2011a) Diet drives convergence in gut microbiome functions across mammalian phylogeny and within humans. Science (New York, NY) 332:970–974

Muegge BD, Kuczynski J, Knights D, Clemente JC, González A, Fontana L, Henrissat B, Knight R, Gordon JI (2011b) Diet drives convergence in gut microbiome functions across mammalian phylogeny and within humans. Science 332:970

Murdy EO (1989) A taxonomic revision and cladistic analysis of the oxudercine gobies (Gobiidae: Oxudercinae). Records of the Australian Museum Supplement 11:1–93

Ravi V (2013) Food and feeding habits of the mudskipper, Boleophthalmus boddarti (Pallas, 1770) from Pichavaram Mangroves, Southeast Coast of India. International Journal of Marine Science 3:98–104

Romero J, Navarrete P (2006) 16S rDNA-based analysis of dominant bacterial populations associated with early life stages of coho salmon (Oncorhynchus kisutch). Microb Ecol 51:422–430

Sanders JG, Beichman AC, Roman J, Scott JJ, Emerson D, Mccarthy JJ, Girguis PR (2015) Baleen whales host a unique gut microbiome with similarities to both carnivores and herbivores[J]. Nat Commun 6:8285

Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ (2009) Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Applied & Environmental Microbiology 75: 7537

Sekirov I, Russell SL, Antunes LC, Finlay BB (2010) Gut microbiota in health and disease. Physiol Rev 90:859

Stephens WZ, Burns AR, Stagaman K, Wong S, Rawls JF, Guillemin K, Bohannan BJM (2016) The composition of the zebrafish intestinal microbial community varies across development. ISME J 10:644

Sullam KE, Essinger SD, Lozupone CA, O'Connor MP, Rosen GL, Knight R, Kilham SS, Russell JA (2012) Environmental and ecological factors that shape the gut bacterial communities of fish: a meta-analysis. Mol Ecol 21:3363

Wang Q, Garrity GM, Tiedje JM, Cole JR (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Applied & Environmental Microbiology 73: 5261

Ward NL, Steven B, Penn K, Methé BA, Iii WHD (2009) Characterization of the intestinal microbiota of two Antarctic notothenioid fish species. Extremophiles 13:679–685

Yang KY, Lee SY, Williams GA (2003) Selective feeding by the mudskipper (Boleophthalmus pectinirostris) on the microalgal assemblage of a tropical mudflat. Mar Biol 143:245–256

Yun JH, Roh SW, Whon TW, Jung MJ, Kim MS, Park DS, Yoon C, Nam YD, Kim YJ, Choi JH (2014) Insect gut bacterial diversity determined by environmental habitat, diet, developmental stage, and phylogeny of host. Applied & Environmental Microbiology 80:5254–5264

Zhu Y, Zhang Q (1993) On feeding habits and histological structure of digestive tract of the mudskipper, *Boleophthalmus pectinirostris*, in intertidal zone of Jiulong River Estuary. J Oceanography Taiwan Strait 12:225–232