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A Gastrointestinal Metagenomic Study on Amphibious Mudskippers Supports their Immunological Specificity

Bo Dong[1,2], Shi Liu[1], Zhilin Wang[1], Peng Ai[1,3], Yunhai Yi[2,4], Xinxin You[2,4], Qiong Shi[2,4] and Zhuang Chen[1]*

[1]*Agro-biological Gene Research Center, Shenzhen Shengya Environment Technology Ltd, Guangdong Academy of Agricultural Sciences, China*

[2]*Shenzhen Key Lab of Marine Genomics, BGI Academy of Marine Sciences, China*

[3]*Guangzhou Genedenovo Biotechnology Co. Ltd., University of Chinese Academy of Sciences, China*

[4]*BGI Education Center, University of Chinese Academy of Sciences, China*

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***Corresponding author:** Agro-biological Gene Research Center, Shenzhen Shengya Environment Technology Ltd, Guangdong Academy of Agricultural Sciences, China

Abstract

Terrestrial adaptation of blue-spotted mudskipper (Boleophthalmus Periophthalmodon; BP) and giant-fin mudskipper (Periophthalmus magnuspinnatus; PM), two representative amphibious fishes, has been previously reported by us at a genomic level. However, their metagenomics of gastrointestinal (GI) microbiome has not been studied yet, and the roles of GI microbiota in their adaptation to the terrestrial life and in their special immunity are worth exploring. In this study, we mainly utilized metagenomic data from these two representative mudskippers and three typical aquaholics fish species (including grass carp, silver carp, and bigheaded carp) to obtain microbial composition, diversity, abundance and potential functions of GI microbiota for comparisons between amphibious and aquicolous fishes. Meanwhile, we downloaded fish GI metagenomic data by literature mining for comparing the phylogenetic relationships with GI microbiota of mudskippers. Interestingly, for the first time, our results demonstrated the dominant presence of phyla Firmicutes, Proteobacteria, Bacteriodetes and Fusobacteria in the GI of mudskippers. However, the content for each main phylum was strikingly different among BP, PM and aquicolous fishes. We also observed that the profile of GI microbiota in mudskippers owned the typical bacterial families for the terrestrial animals, (freshwater and seawater) fishes, and amphibians at the same time, which is consistent with their water-to-land transition and residence at the interface of freshwater and seawater. More interestingly, certain bacteria strains like S24-7, previously thought to be specific in terrestrial animals, were also identified in both BP and PM. The various composite and diversity of mudskipper GI microflora are therefore considered to stimulate broader immunity in these amphibious fishes. Meanwhile, we identified antimicrobial peptide (AMP) genes in their GI metagenomes to help understand the special immunity in amphibious fishes through comprehensive comparisons.

Keywords: Gastrointestinal microbiota; Metagenomics; Amphibious mudskipper; Terrestrial adaptation; Immunological specificity

Introduction

Amphibious fishes such as mudskippers are an interesting group of vertebrates that can thrive in water as well as on land. They evolved independently and more recently than the lobe-finned fishes that made a successful transition from aquatic life to terrestrial living around 360 million years ago, resulting in the evolution of terrestrial tetrapods. Since the intermediary forms that existed during the transition from aquatic lobe-finned fishes to terrestrial tetrapods are represented currently only in fossils, amphibious fishes offer a critical model for understanding the genetic changes associated with the water-to-land transition of vertebrates [1]. Terrestrial adaptations in mudskippers include aerial respiration, high ammonia tolerance, modification of aerial vision, and terrestrial locomotion using modified pectoral fins [1,2]. These evolved phenotypes are believed to be the consequences of genetic changes driven by the selection pressure [1]. However, very little is known about the adaptations from a view of the gastrointestinal (GI) metagenome, the second set of genome in life beings. Immunity is a crucial system to help these amphibious fishes, such as blue-spotted mudskipper (*Boleophthalmus pectinirostris*; BP) and giant-fin mudskipper (*Periophthalmus magnuspinnatus*; PM) to cope with the more diverse environments and various pathogens during their transition from water to land. We reported an expansion of innate immune genes in the mudskippers after diverging from other teleost [1], which may provide extra defense against terrestrial pathogens. In fact, mudskipper genomes seem to possess the largest number (11 copies) of toll-like receptor 13 (*tlr13*) gene in sequenced vertebrates so far [1,2]. In addition to the genomes, it is well accepted that GI microbiota play important roles in the maturation of immunological system [3-6]. However, there are still no report on the comparisons of GI microbiome be-

tween aquicolous fishes and amphibious fishes like mudskippers to elaborate differences in GI microbiota to underly their particular immunity or traits for dwelling in water and on land. The resilient and thriving GI microbiota [7,8] co-evolve with their host and compete for common resources among them through millions of years during the transition from water to land. Many physiological functions in fishes were maintained or participated in by them, such as the antagonism of pathogens, the proliferation of enteric epithelium, and the maturation of immunity [9].

It has long been proposed that the host genetic background and living niche of all fishes select for a "core microbiota" to maintain some essential functions that are shared by all fish members [10]. This hypothesis may be extrapolated to amphibians and terrestrial vertebrates. Hence the unique living niche of water-to-land mudskippers will provide a good model to test this hypothesis and to examine how the core microbiota changed from water to land [11,12]. Meanwhile, beneficial autochthonous GI microbiota and/or their natural products (such as antimicrobial peptides, AMPs) deserve further investigation in amphibious fishes, due to the potential importance of GI microbiota in the fish adaptation to a terrestrial life. To explore these, we employed both 16S amplicon sequencing and metagenome sequencing techniques to compare the compositions of GI microbiota in mudskippers and the AMP genes in their metagenomes with those in aquicolous fishes, which will benefit for determination of microbial strains or AMP genes for the special immunity of amphibious fishes.

Materials and Methods

Sample collection

Pond-cultured grass carps (*Ctenopharyngodon idellus*; CI), silver carps (*Hypophthalmichthys molitrix*; HM) and bigheaded carps (*Aristichthys nobilis*; AN) were collected from a local hatchery in Guangzhou, Guangdong, China by trawl netting in the August of 2016. They were fed twice a day with a commercial feed from Shenzhen Alphafeed Co. Ltd., China. Among these cultivated fish, only those with body weight of 1-2 kg were selected.

Body surface of these fishes was rinsed with sterile distilled water and subsequently 70% ethanol to reduce contamination. Their GIs were dissected aseptically from their abdominal cavity, and the GI content and the epithelial GI mucosa were squeezed out for a separate harvest. The GI contents were obtained and stored at -80°C before use. Blue-spotted mudskippers (BP) and giant-fin mudskippers (PM) were captured wildly from Island Qi'ao, Zhuhai, Guangdong in the July of 2016 and 2017. Only big and healthy fishes were chosen for biopsy to obtain GI contents as described above. All experiments were performed in accordance with the guidelines of the Animal Ethics Committee and were approved by the Institutional Review Board on Bioethics and Biosafety of BGI (No. FT15103).

Extraction of Metagenomic DNAs

Each sample (250mg of intestinal content and mucosa) was thawed on ice, and then total bacterial DNA was extracted using a DNA extraction kit (CTAB method) as reported before [13]. DNA integrity and purity was monitored on 1% agarose gels. DNA concentration was measured by a Qubit Fluorometer (Thermo Fisher Scientific, USA). These extracted metagenomic DNAs were stored at -80 °C until use.

Library Construction and Validation

Total metagenomic DNA specimen was individually broken up into 350-bp fragments by the routine Covaris sheering. The fragmented DNAs were subsequently mixed with End Repair Mix (NEB, USA) before incubation at 20 °C for 30 min. The achieved end-repaired DNAs were purified with QIAquick PCR Purification Kit (Qiagen, USA), and then were added to A-Tailing Mix (NEB, USA). After ligation, the adapter-ligated DNAs were selected by running in a 2% agarose gel to recover those target fragments. After purification of the gels, we performed PCR amplification to enrich the adapter-ligated DNA fragments. PCR products were run in another 2% gel and purified using QIAquick PCR Purification Kit to recover the target fragments. The final libraries were constructed for quantification, including determination of the average molecule length using Agilent 2100 bioanalyzer with Agilent DNA 1000 Reagents (Agilent, USA), and measurement of each library by quantitative real-time PCR.

Library sequencing

The qualified libraries were firstly amplified with Hiseq 4000 PE Cluster Kit (Illumina, USA) for cluster generation. Subsequently, the clustered flow cells were loaded onto the Hiseq 4000 Sequencer for paired-end sequencing (Hiseq 4000 SBS Kit, Illumina) with sequencing reads of 100 or 150 bp in BGI-Wuhan, China.

Assembly and annotation of metagenomes

A total of 19 samples were sequenced by an Illumina Hiseq PE150 platform. Metagenomes were assembled by MEGAHIT [14]. Analyses were implemented as previously reported [13] by Guangdong Magigene Biotechnology Co. Ltd. In brief, annotation of deduced amino acid sequences was performed through BLASTP against the NCBI NR database by KAIJU with an E-value ≤ 1e-3 [15]. To determine the accurate phylogenetic composition of gut microbiota, all metagenomic reads were assigned to prokaryotic reference genomes that have been submitted to the Genome database of NCBI using BLASTN with default parameters. The aligned reads with sequence similarity ≥ 75% were filtered by BLAST against the genome of each corresponding host.

OTU statistics and Venn chart drawing

Tags were clustered to Operational Taxonomic Units (OUTs) by scripts of the software USEARCH (v7.0.1090) [16] as follows. (1) The tags were clustered with a 97% threshold by using UPARSE, and the OTU unique representative sequences were obtained. OTU number per sample primarily represents the degree of sample diversity. (2) Chimeras were filtered out by using UCHIME (v4.2.40); The 16S rDNA and sequences were screened for chimeras by mapping to gold database (v20110519) and UNITE (v20140703) separately; *de novo* chimera detection was also done for 18S rDNA

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sequences. (3) All tags were mapped to each OTU representative sequence using USEARCH GLOBAL, and then the tag number of each OTU in each sample were summarized as the OTU abundance table. OTU representative sequences were taxonomically classified using Ribosomal Database Project (RDP) Classifier v.2.2, which was trained on the Greengenes database with 0.8 confidence values as the cutoff. Based on the abundance, OTUs of each group were listed for comparison. A Venn diagram was drawn by R (v3.1.1), in which the common or specific OTU IDs were summarized. Different colors represent various samples or groups, and the interior of each circle symbolically represents the number of observed OTUs in the corresponding sample/group. The overlapping area or intersection represents the common set of OTUs presented in the counterparts. Likewise, the single-layer zone represents the number of OTUs uniquely identified in certain sample/group.

Prediction and identification of AMPs

We applied homology search with those reported AMPs from the established metagenome datasets of three Asian carps and two mudskippers. Previously validated AMPs were retrieved from the online Antimicrobial Peptides Database (APD3) [17] and were used as queries. Assembled metagenomes and raw reads of fish GI microbiota were used as a local index database. Subsequently, we applied TblastN with the threshold of E-value ≤ 10-5 to run the queries against the examined database. Those hits of nucleotides were then translated into peptide sequences and submitted to NCBI using the BLASTP tool for further verification. However, those AMPs with existence only in eukaryotes were removed.

Results

Summary of the achieved fish metagenome datasets

A total of 29Mb, 17Mb, 22Gb, 47Gb, and 12Gb of raw data were generated for each sample of 4 BP (BP1-BP4), 1 PM, 8 grass carps (C1-C9), 5 silver carps (L1-L5), and 1 bigheaded carp (Y1), respectively. All the sequence data were filtered to remove host contamination. Finally, from these clean metagenome data, we annotated a total of 4,966 species, 1,453 genera, 378 families, 178 orders, 76 classes and 54 phyla (Table 1). The GI microbiome of grass carp (Table 1) are dominated by members of phyla Proteobacteria (36.12%), Firmicutes (7.14%), Bacteroidetes (5.16%), Fusobacteria (3.82%) and Actinobacteria (1.31%). The highest-ranking phyla are consistent with previous reports [13,18], but the abundance varied perhaps due to different rearing conditions and fish ages. The well representative genera include Aeromonas (19.02%), Shewanella (3.79%), Cetobacterium (2.96%), Bacteroides (1.60%) and Clostridium (0.81%), respectively (Figure 1).

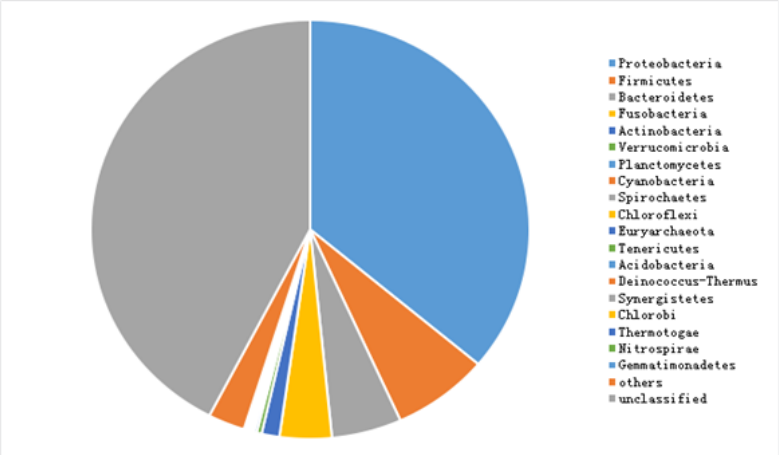

Figure 1: Relative Abundance of the Phyla annotated in the Metagenomes of Grass Carps.

Comparison of microbiome species and abundance between two mudskippers and three Asian carp species.

The tag numbers of each taxonomic rank (Phylum, Class, Order, Family, Genus and Species) or OTU in different samples were summarized in a profiling table or histogram for convenient comparison. In Figures 1-5, the distribution histograms of taxonomic composition in each fish species was presented at the Phylum, Order, Class, Family, Genus and Species levels of GI microbiome respectively. The ratio of each category in certain sample is directly displayed. At Phylum, all species were used to draw the histograms (Figures 1-3). The species abundance less than 0.5% in all samples were classified into 'others'.

Amphibious mudskippers

In the metagenomes of mudskippers (BP and PM), we observed the dominated phyla of firmicutes, proteobacteria, bacteriodetes, fusobacteria in the GI microbiota (Figure 2). However, the abundance of each main phylum is strikingly different between BP and PM. For example, the phylum Firmicutes accounted for about 35% of the GI microbiota in BP (Figure 2(a)), whereas it was only 1% in PM (Figure 2(b)). On the contrary, the phylum *Fusobacteriain* in PM (43%) is much more than that in BP. Obviously, the diversity of intestinal bacteria increases from omnivory to herbivory [19,20], which is consistent with the fact that BP is herbivorous while PM is omnivorous [2]. Interestingly, in human GI microbiota, the ratio of firmicutes to *bacteriodetes* is an indicator of growth rate and

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adipose accumulation [21]; in one study on transgenic carp [22], the higher *firmicutes/bacteriodetes* ratio led to the higher growth rate. In our present work, we found that to some extent the ratio of firmicutes to *bacteriodetes* in BP is higher than PM, which may be related to the bigger size and more adipose accumulation in BP [2]. Another distinction in the microbiome composition between the two mudskipper species is that Cyanobacteria takes a larger share in BP than in PM (Figure 2), which may be related to the more aquicolous lifestyle and feeding preference for algae of BP [1,2]. In fact, Cyanobacteria are blue-green prokaryotic algae with prevalence in sea water [23].

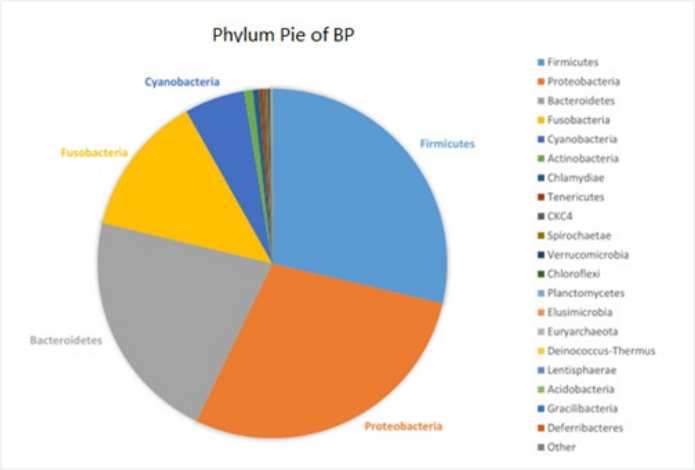

Figure 2 (a): BP.

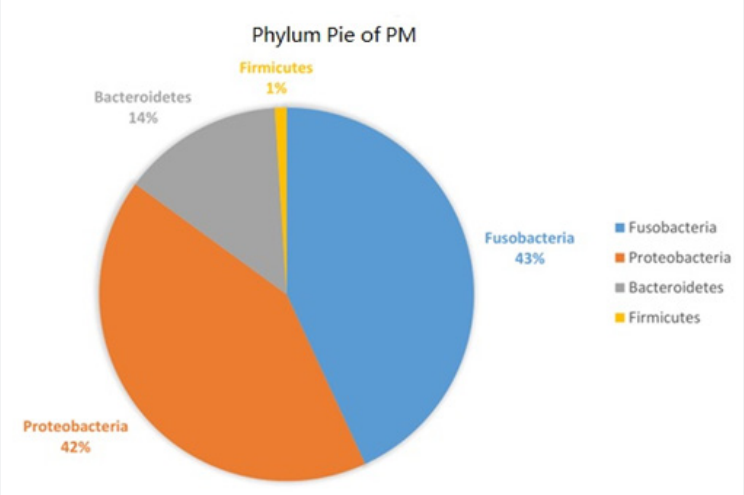

Figure 2 (b): PM.

Figure 2: Relative Abundance of the Phyla Annotated in the Mudskipper Metagenomes.

Three Asian carp species

Metagenomic results (Figure 3) of the three carp species, including bighead carp (AN; Figure 3(a)), silver carp (HM; Figure 3(b)) and grass carp (CI; Figure 3(c)), are consistent with certain previous reports [24-28], in which the four phyla Firmicutes, proteobacteria, bacteriodetes and fusobacteria dominated in the GI microbiota of these fish species. We found that the Bacteroidetes:-Firmicutes ratio increases from aquicolous carps , BP to PM, The relatively higher percentage of firmicutes in the aquicolous fishes is in accordance with the higher firmicutes ratio in the more aquicolous BP than the more terrestrial PM [21]

Differences between mudskippers and carps

The greatest difference of microbiota in carps from those in mudskippers is CKC4, which accounts for a bigger share (ranging from 4% to 27%) in carps, while no detection is available in mudskippers (Figure 4). Although the functional studies of CKC4, a phylum in SLIVA database, are very limited, previous results suggest that CKC4 may be sensitive to estrogen and its analogue. Endocrine exposure was reported to alter the abundance of CKC4 in the zebrafish intestinal microbiota significantly, suggesting that CKC4 may be related to changes in host lipid metabolism [29].

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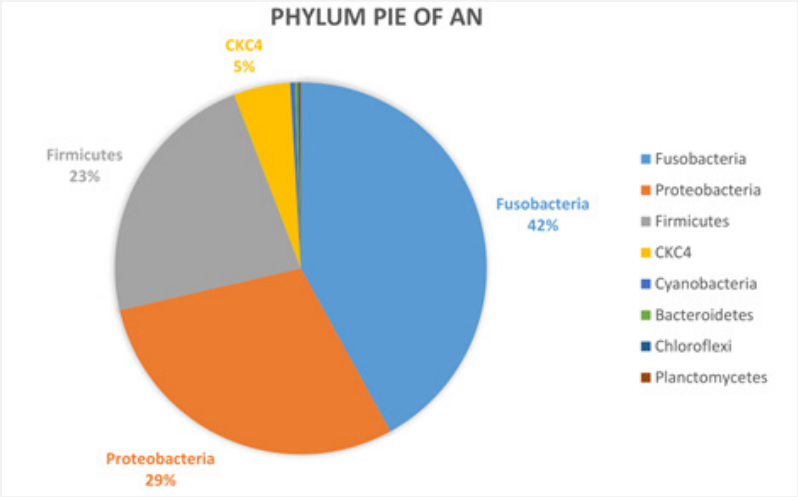

Figure 3(a): Bigheaded Carp.

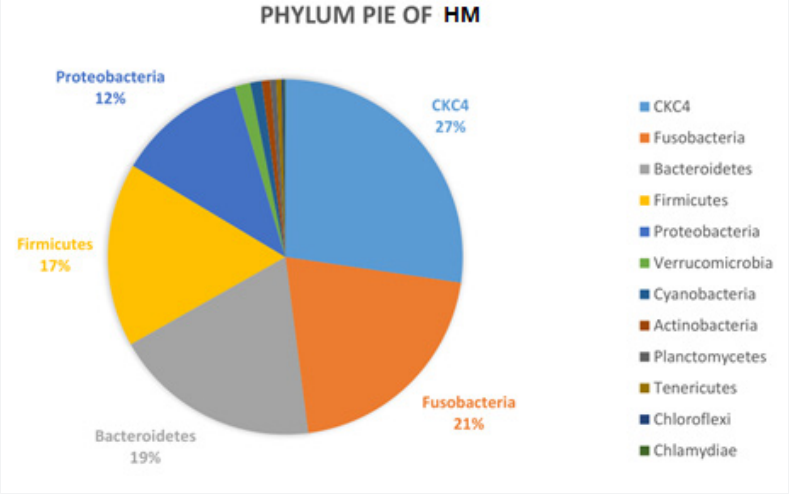

Figure 3(b): Silver Carp.

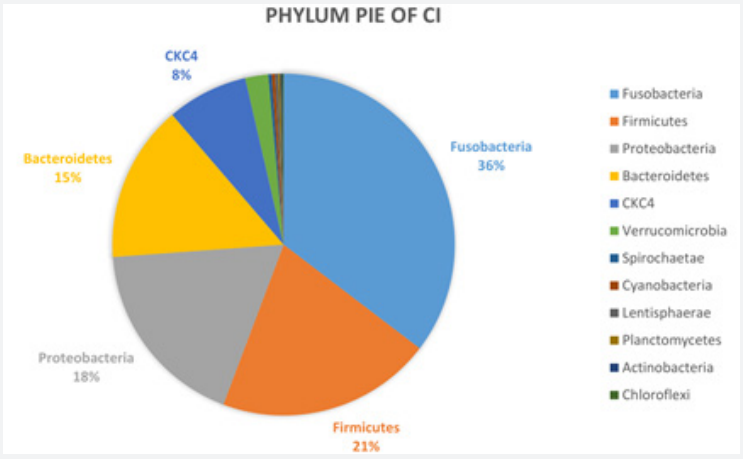

Figure 3(c): Grass Carp.

Figure 3: Relative Abundance of the Phyla Annotated in the Metagenomes of three Asian Carp Species. Cyanobacteria Are Capable of Synthesizing Vitamin B12, And They Have Symbioses With B12-Producing Bacteria [23].

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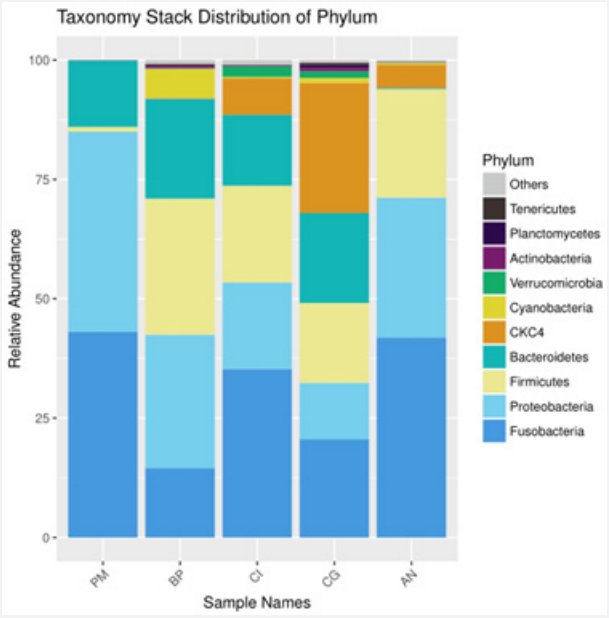

Figure 4: Comparison of Relative Abundance of the Phyla Annotated in the Metagenomes of Mudskippers (BP and PM) and Carps.

Comparison of microbiota composition and abundance among amphibious fishes, seawater fishes, freshwater fishes, amphibians and terrestrial animals.

Table 1: Summary of the 40 vertebrate species with available GI 16S rDNA data for comparisons.

Group	Vertebrate Species
freshwater fishes (11 species)	bigheaded carp *(Hypophthalmichthys nobilis)*, blue catfish *(Ictalurus furcatus)*, channel catfish *(Ictalurus punctatus)*, common carp *(Cyprinus carpio)*, common dace *(Leuciscus leuciscus)*, freshwater drum *(Aplodinotus grunniens)*, goldfish *(Carassius auratus)*, ide *(Leuciscus idus)*, roach *(Rutilus rutilu)*, silver carp *(Hypophthalmichthys molitrix)*, zebrafish *(Danio rerio)*
seawater fishes (6)	Atlantic cod *(Gadus morhua)*, pike perch *(Sander lucioperca)*, rabbit fish *(Siganus fuscescens)*, rainbow trout *(Oncorhynchus mykiss)*, sixbar grouper *(Epinephelus sexfasciatus)*, tiger grouper *(Epinepehlus fuscoguttatu)*
Amphibious fishes (2)	giant-fin mudskipper *(Periophthalmus magnuspinnatus; PM)*, blue-spotted mudskipper *(Boleophthalmus pectinirostris; BP)*
Amphibians (16)	Frogs *(Boophis narinsi, B. reticulatus; Dendropsophus minutus, D. sanborni; Hylodes asper, H. phyllodes; Hypsiboas albomarginatus, H. faber; Physalaemus cuvieri; Phasmahyla cruzi; Scinax fuscovarius, S. hayii, S. littoralis, S. trapicheroi; Thoropa taophora)*, fire salamander *(Salamandra salamandra)*
terrestrial animals (5)	Colobus monkey *(Colobus polykomos)*, human *(Homo sapiens)*, mouse *(Mus musculus)*, pig *(Susscrofa domestica)*, Rhesus monkey *(Macaca mulatta)*

We downloaded 16S rDNA sequencing data of 38 vertebrate species (Table 1) with available metagenomic results (reported in previous literatures or deposited in the NCBI databases) except for the two mudskippers from the present study. We aimed to recover as many as microbiota species to reflect the whole and fine landscape of GI microbiota in each evolutionary group. The 40 species are categorized into five groups (see more details in Table 1), including amphibious fishes (BP & PM), seawater fishes (such as Atlantic cod, rabbit fish and groupers), freshwater species (such as carps, goldfish and zebrafish), amphibians (such as frogs and salamanders), and terrestrial animals (such as human and mouse). Based on their various lifestyles, these animals are divided into aquicolous species (17 in total), amphibious species (18 in total, including 2 mudskippers and 16 amphibians), and land-dwelling species (5 mammals). The stack distributions in mudskippers and carps (Figure 4) are consistent with the results presented by pie charts (Figure 5). From the bar data of the microbiota composition in fish samples at the level of class (Figure 5(b)), we observed that Flavobacteria are much more in mudskippers than in other fishes. In fact, Flavobacteria are reported to be opportunistic pathogens with a wide distribution in both water and land, posing a serious threat to wild and cultured fish stocks [30]. Interestingly, we also found Enterococci strains (0.07%) in the GI microbiome of mudskipper BP by searching metagenomic annotation results (section 3.2.1). However, these Gram-positive bacteria are usually identified from terrestrial mammals [31]. From Figure 5(c), we observed that Ruminococcaceae and Lachnospiraceae families are relatively abundant in the mudskippers, amphibians and terrestrial mammals (0.16% and 0.61%, 7.0% and 4.4%, 10.3% and 11.9%, respectively). Especially the S24-7 family, uniquely abundant in terrestrial GI microbiota (14.6031%), can be identified as well in mudskippers (0.7842%); however, it was absent in the aquicolous fishes and amphibians.

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Likewise, *Clostridiaceae, Moraxellaceae & Fusobacteriaceae* families are most abundant in freshwater fishes (16.6%, 3.3% and 33.2%); they are the second in mudskippers too (3.6%, 4.0% and 14.2%), while they are correspondingly below 1.5%, 0.3% or 6.0% in the other three groups. Interestingly, it was reported that *Clostridium butyricum* could improve growth performance, increase body crude protein content, modulate intestine digestive capacity, and enhance intestine immune function of *Whiteleg shrimp (Litopenaeus vannamei)* against ammonia stress [32]; *C. perfringens* is an important foodborne pathogen in fish trade, as it has been implicated as the causative organism of two fish disease outbreaks [33]. The same is true for the *Comamonadaceae & Vibronaceae* families, which are most abundant in seawater fishes (25.6% and 39.6%), with their second abundance in mudskippers (3.0% and 2.3%). It was reported that rifampicin, a popular antibiotic, was selected from members of the family *Comamonadaceae* in the skin but not the gut microbiome of mosquito fish (*Gambusia affinis*) [34]. Members of this family also synthesize tetrodotoxin (TTX), an ancient marine alkaloid and powerful neurotoxin that serves to protect members of an order of fishes, the Tetraodontiformes (such as the puffer fish). Vibrionaceae bacteria are in symbiosis with many marine organisms. In the case of the puffer fish and other marine organisms harboring TTX-producing Vibrionaceae, the symbiosis is an ancient and powerful system, providing protection against predation for the marine organisms that harbor these bacteria, while supplying the bacteria a protected environment with plenty of nutrients for growth [35].

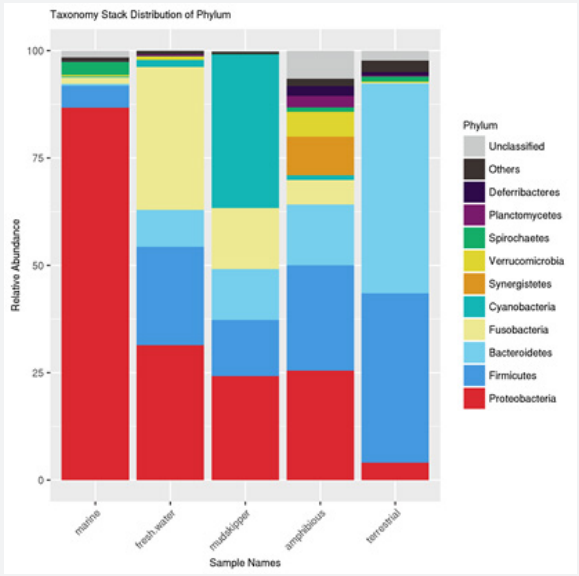

Figure 5(a): Class.

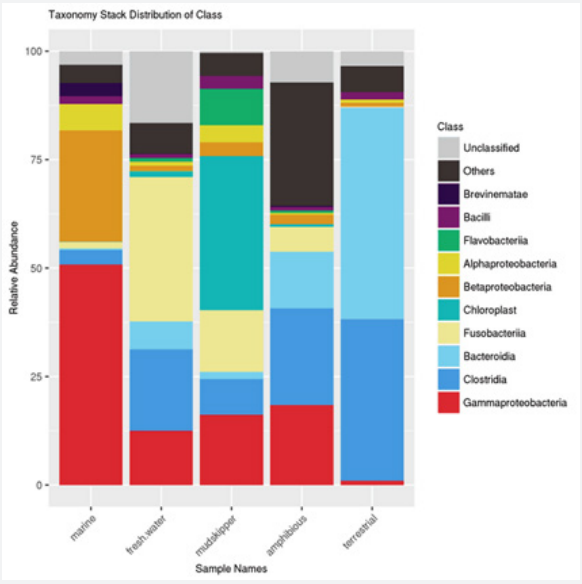

Figure 5(b): Family.

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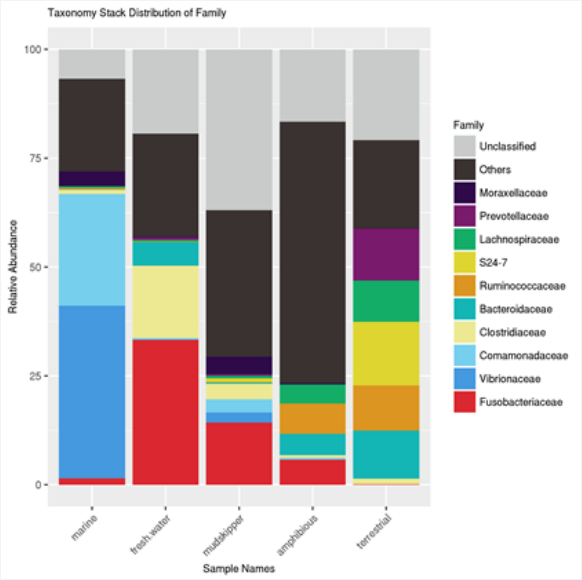

Figure 5(C): Level.

Figure 5: The Taxonomic Composition of GI Microbiota in the Five Groups of Examined 40 Species. They were Classified at a Phylum.

Analysis of community patterns, OTU statistics and OTU Venn charts

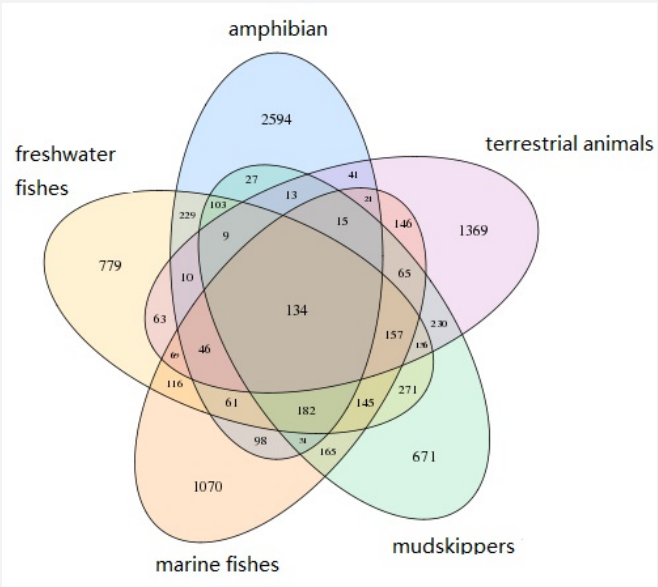

Figure 6(a): Overlaps between the Five Animal Groups.

Venn diagrams could visually display the number of common/unique OTUs in multi-samples/groups. The core microbiomes of different species could be determined once combined with the OTU representative species. We downloaded the available GI metagenome data of 40 vertebrate species through literature mining and categorized these species into five groups as above-mentioned. The microbiota annotation results in each animal of these five groups were summed up to make a non-redundant OTU set. Related Venn displays of the five OTU sets facilitate our understanding of the relationships between each two of the five OTU sets. They also reflect GI microbiota changes during the evolutionary transition from water, through amphibian and/or amphibious fishes (like mudskippers) to land-dwelling animals. In these Venn displays, 134 OTUs were shared by the five animal groups (Figure 6(a)), mainly belonging to the families of *Bradyrhizobiaceae, Moraxellaceae, Fusobacteriaceae, Comamonadaceae, Aeromonadaceae, Peptostreptococcaceae, Enterobacteriaceae, Turicibacteraceae, Streptococcaceae*, respectively with a descending order in abundance. They may form the core microbiota for these animals. However, when we compared amphibious mudskippers and terrestrial animals (amphibians and land-dwelling mammals), 759 OTUs were revealed to be overlapped (Figure 6(c)). From the

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stack comparison in Figure 7, we found that the OTUs in mudskippers and terrestrial animals differentiate from aquicolous fishes mostly in S24-7, *Lachnospiraceae, Ruminococcaceae, & Rikenellaceae* families. Detailed functions of these strains are worth further exploration.

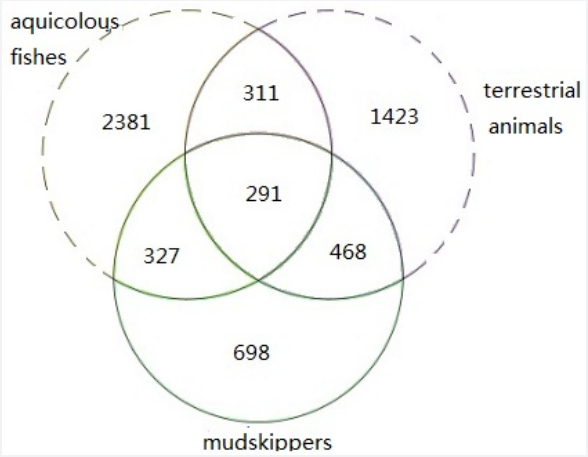

Figure 6(b): Overlaps between Mudskippers, Aquicolous Fishes, and Terrestrial Animals.

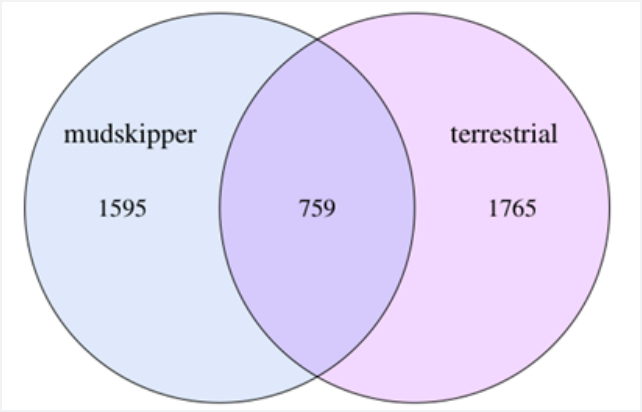

Figure 6(c): Overlaps between Mudskippers and Terrestrial Animals.

Figure 6: A Venn Display of Various OTUs in the Metagenomes of Five Animal Groups. Please Note the Overlapping Between Amphibious Mudskippers and Terrestrial Animals.

PCA analysis of OTUs

In order to examine the differences of OTU composition in various animal groups, we employed the principal component analysis (PCA) to construct a 2-D graph for summary of mainly responsible factors. Based on the OTU abundance, we calculated the relative amount of each OTU in every sample and drew the PCA chart of OTUs (Figure 8) with these relative abundance values. From the PCA chart (Figure 8) and stack bar (Figure 5) based on the metagenomic data of the examined animal species, we observed that the composition profile of BP and PM microbiota is at the transitional stage between the aquicolous fishes and the land-dwelling animals, with a closer location to that of amphibians (Figure 8). This is consistent with the phylogenetic relations proposed from our genome data [1]. Amphibians and amphibious fishes are the intermediate between auqicolous fishes and terrestrial animals. Interestingly, fishes generally possess higher amount of Fusobacteria and Proteobacteria compared to land-dwelling animals (including amphibians and mammals), while land-dwelling animals have more Bacteriodes (Figure 5).

Functional annotation of metagenomes: comparisons between BP and carps

Based on our functional annotations and abundance information of the metagenomic data for BP and mudskipper samples, we selected the top 35 function categories and their abundance in each sample to draw heat maps and clustered for functional differences. As shown in the function cluster map of Figure 9, the function profiles of metagnomes in BP were substantially different from those in grass carps and bigheaded carps. Interestingly, the abundance of enzyme and metabolic activity in BP was much higher than that in carps, suggesting the water to land transitional lifestyle of mudskippers may demand more energy (usually generated from glycolysis) [1]. The most striking issue happened to

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an ammonia channel protein, AmtB, which was significantly more abundant in BP microbiota than in carps, and its high ammonia transport capacity is associated with the ammonia resistance feature of mudskippers during an intertidal life history [1,2].

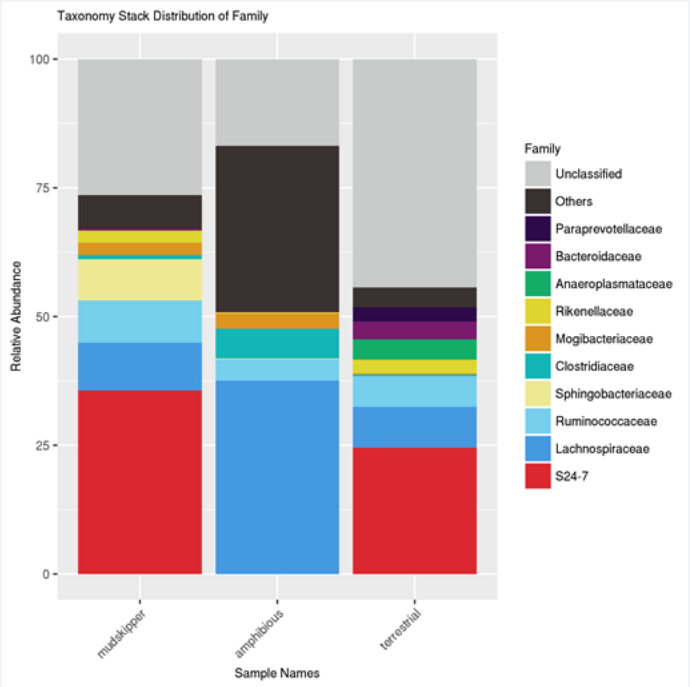

Figure 7: Taxonomic Distributions of Various Typical OTUs in Mudskippers, Amphibians and Terrestrial Animals at Family Level.

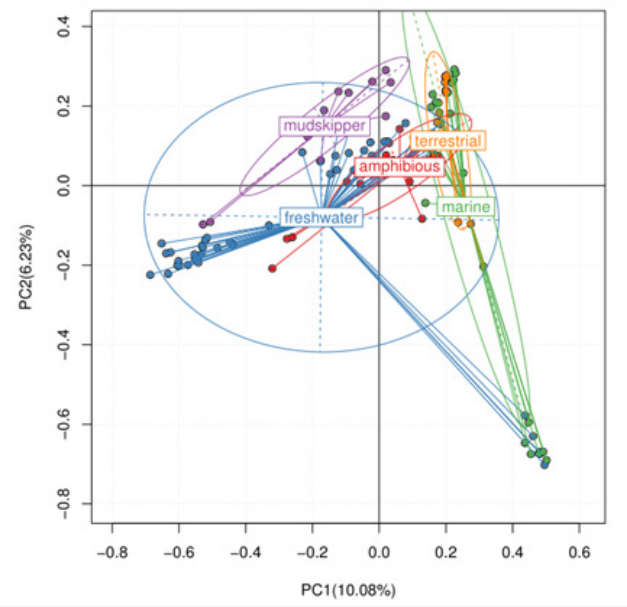

Figure 8: PCA Of OTU Abundance in the Five Animal Groups. X-Axis Represents The 1st Principal Component; Y-Axis Represents The 2nd Principal Component. Numbers in the Brackets Stands for Contributions of The Principal Components to Differences among Various Samples. Each Dot Represents A Sample, And Different Colors Stand for Different Groups.

Different AMP genes identified from the GI metagenomes of amphibious mudskippers and aquicolous fishes

We assigned the assembled metagenomes to the online Antimicrobial Peptides Database to screen AMP genes for comparisons of categories and numbers between mudskippers, seawater fishes and freshwater fishes. From the diagram in Figure 10, we determined the overlapping of AMP genes between amphibious mudskippers and aquicolous fishes (freshwater and seawater fishes). However, there are no overlaps between the freshwater fishes and the seawater fishes, and the categories and numbers of AMP genes

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in mudskippers is more than in other single fish species. It is more like that the GI microbiota of mudskippers may produce the intermediate AMP types (55), between those in freshwater fishes (141) and seawater fishes (44), to cope with pathogens both in seawater and in freshwater. These data indicate that the GI metagenomes of these amphibious fishes harbor relatively more diverse AMP genes than other fish species that lives in relatively stable and unitary residential environments. It was reported that the AMPs synthesized by GI microbiota help the host to defend exogenous pathogens and to benefit the immunological maturation in enteric epithelial cells through interaction with enteric epithelial receptors and TLR signaling pathways. The mudskippers' enteric epithelial cells exposed to relatively diverse microbial strains (even some strains specific to terrestrial animals like S24-7) and AMPs will enhance and diversify mudskippers' immunological responses to the changeable intertidal environments.

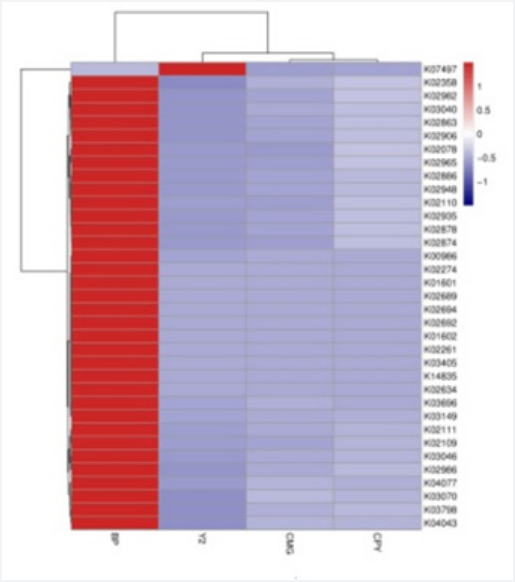

Figure 9: A heatmap of function abundance clusters from the metagenomes of BP and carps. CMG and CPY denote two samples of grass carps, and Y2 denotes a sample of bigheaded carp. X-axis: sample name; Y-axis: function annotation items. The function cluster tree is located to the right of the heatmap. Corresponding value of the middle heat map is the Z value obtained by normalizing the relative abundance in each row.

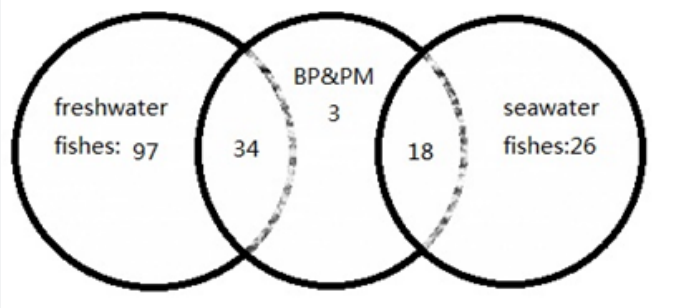

Figure 10: A Venn Display of AMP Gene Numbers Identified from Various Fishes. Please Note that there Are No Overlaps between Freshwater Fishes and Seawater Fishes.

Discussion

The fish taxa are currently underrepresented among gastrointestinal microbiome studies, especially for amphibious mudskippers that have distinctive physiological and biochemical differences compared to other animal hosts. The present work therefore provides the first investigation into the GI microbiota differences or overlaps between fishes including mudskippers, amphibians and land-dwelling vertebrates. The evolution pedigree of the five examined animal groups is clear based on available genome sequences; however, the evolution of the GI microbiota and corresponding metagenomes remains unresolved.

Differences in GI microbiota composition between BP and PM for the various habitat preference and feeding habits.

Mudskippers are characterized by their amphibious habits [1,2]. We chose a mor land-dwelling species PM and the relatively more aquicolous mudskipper BP to collect GI contents and subjected them to the subsequent 16S rDNA amplicon sequencing. Despite their close genetic relationship between the two mudskippers, there are striking distinctions between them in GI microbial categories, diversity and abundance, possibly due to different habitats and feeding preferences. In fact, we observed that the pro-

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file of GI microbiota in diversity and Cyanobacteria content in BP coincides with the aquicolous preference and herbivorous feeding habits of BP, suggesting the roles of diverse bacteria required for metabolizing biological materials in feed. Meanwhile, there are more firmicutes in BP than PM, which is in part consistent with the larger body size of BP than PM.

Comparisons of GI microbiota profiles among mudskippers (representative BP), aquicolous fishes (Asian carps) and terrestrial animals

The grass carp, silver carp and bigheaded carp are three representative freshwater fishes of the Cyprinidae family, and their annual outputs account for over 80% of the total cultured freshwater fish in China [36]. In the present research, we sequenced the whole metagenomes of the GI microbiota of BP and three Asian carp species to compare the microbial composition, diversity, abundance and function predictions of GI microbiota among these fishes. Except these achieved metagenomes and 16S data for mudskippers, we also collected 8-Gb 16S dataset from previous publications and the NCBI database. It seems that microbial categories in the GI content of fishes (mudskippers and carps) are relatively conserved at the phylum level. The most abundant phyla are firmicutes, bacteroidetes, fusobacteria, and proteobacteria, although the abundance of each phylum varied greatly in different fishes. However, the remarkable exception to this is the CKC4 phylum, which was only identified prevalently in carps but absent in mudskippers. To confirm the differences at the family level, we examined many microbial strains and found that some were only presented in amphibious mudskippers and terrestrial animals (not in the aquicolous carps), such as ruminococcaceae and lachnospiraceae, especially S24-7, indicating that the amphibious mudskippers select bacteria for their terrestrial adaptation. We also found that flavobacteria was much more in BP than in other fishes. For example, we detected enterococci strains with an abundance rate of 0.07 in the GI microbiota of BP. In fact, enterococci strains were reported to produce a AMP-like substance and exhibited a broad spectrum of inhibition against pathogenic bacteria (isolated from diseased fish), in particular against the Gram-negative bacteria *Flavobacterium frigidarium, Vibrio pectenicida*, *V. penaeicida*, and *Photobacterium damselae* [37]. There may be an equilibrium between the enterococci strains and the pathogenic flavobacterium in the GI of mudskippers, hence mudskippers can endure relatively high abundant of flavobacterium in the adverse environments. The relative high abundance of this pathogen in healthy mudskipper individuals suggests that amphibious fishes may have developed special resistance strategies to deadly pathogens in diverse residential habitats. This strong immune trait of mudskippers is under further investigation.

Core microbiota and critical factors shaping teleost GI microbiota

After comparison of the examined vertebrate groups (seawater & freshwater fishes, amphibious mudskippers, amphibians, and land-dwelling animals), we identified 134 common OTUs among these five groups (Figure 8). Corresponding bacteria may be involved in many essential functions, such as vitamin synthesis, immunity maturation, or development of GI tract epithelial cells The 243 OTUs that were identified in terrestrial animals and amphibious mudskippers but were absent in aquicolous fishes may be related to some special adaptive traits, such as terrestrial metabolism, ammonia resistance and air exposure. Among them, 13 OTUs appeared in amphibians, while the rest 230 were only identified in the terrestrial animals. Meanwhile, 27 OTUs were present in amphibians and amphibious mudskippers, but absent in the aquicolous fishes (Figure 8), suggesting their involvement in physiological activities of amphibious lifestyle. Many factors contribute to the composition diversity of the GI microbiome in teleost, including host genetics, surrounding environments, GI physiology, bacterial symbionts, and feed nutrition [38]. Discovering core microbiome members of the microbial community present in all individuals of fish species across various environments, has been a primary goal for many researchers with interests in understanding of teleost GI microbial communities [39-41]. While some researchers have proposed that host phylogeny was the determinant factor in shaping those microbial communities, Roeselers *et al.* [40] & Sullam *et al.* [42] noted that fish-associated microbiomes were more similar between freshwater fishes, regardless of phylogeny, than to those of fishes inhabiting in marine environments. This is also true in our present work.

Overall, the GI microbiome of fishes, including seawater [41] and freshwater species, [29,43,44] seem to be dominated by the phylum Proteobacteria, followed by Fusobacteria and Firmicutes, and in a lesser percentage of Bacteroidetes, Actinobacteria and Verrucomicrobia [45]. These phyla, representing up to 90% of the total communities, were identified in both allochthonous (transient) and autochthonous (adherent) microbial communities, although the content for each main phylum was strikingly different in various species. Interestingly, we found that the profile of GI microbiota in mudskippers owned the typical families of the terrestrial, freshwater, marine and amphibious groups at the same time, which coincides well with mudskippers' features of water-to-land transition and residence at the interface of freshwater and marine water [2]. To a certain extent, this finding provides solid evidence to support the popular hypothesis of core GI microbiota in that different ecological environments and living habits select for some common microbiota group(s), and the amphibious hosts at the transitional stage would harbor both sets to cope with the aquatic and terrestrial environments.

Microbial strains related to terrestrial adaptation for mudskippers' special immunity

Our present research is the first report to integrate the metagenomic data from sequencing and published data across different representative vertebrate taxa to summarize the changes of GI microflora during the evolution from water to terrestrial environments. Especially, we focused on the outstanding amphibious mudskippers, with a major aim at elucidation of their special immunity. Mudskippers are amphibious fishes, however few systematic studies on their components of innate system were

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reported, especially when the immunity power was conferred by the important barrier against pathogens from GI microbiota. Here, for the first time, we analyzed the GI microbiota in two representative mudskippers (BP and PM), and annotated their microbes therein to explore whether special bacterial stains or antimicrobial peptides in the GI tract for the adverse (such as high ammonia) and fickle intertidal environments. The microbial strains uniquely presented in mudskippers and terrestrial animals, like the above-mentioned Bacteroidales-S24-7, can alter the local immune system in the host gut. Bacterial cells do not contact with enterocytes in normal physiological status; however, they may release DNAs into the mucus layer to influence host innate immune cells through specific receptors, such as TLR9 [46]. It was reported that S24-7 can modulate immune functions by interaction with the gut mucus layer [47]. The higher diversity of GI microbiota in mudskippers, compared with the aquicolous fishes, is consistent with our previous report that the mudskippers own the largest set of TLR expansion (11copies) in teleost fishes [1].

Their large TLR repertoire will recognize more pathogen categories though pattern recognition receptors (PRRs) and facilitate the downstream activation of immune factors and AMP expression [48], which will greatly improve the immune power and health of the enteric epithelia [49,50]. Accordingly, we mined AMPs and AMP-producing bacteria in the GI metagenomes based on big data like metagenomics sequencing, online AMP database and literature mining, and further compared the bacterocins genes identified in examined animal species. We observed that the potential AMPs in GI microbiota of mudskippers are very diverse and comprise with presence in both freshwater and seawater fishes, although there are no overlaps between the freshwater fishes and the seawater fishes (Figure 10). The AMP data combined with our previous finding about TLR expansion [1] support the high resistance of these amphibious species to the harsh and diverse intertidal living conditions. Altogether, these interesting data help us understand the fish GI microbiota during the evolutionary adaptions from water to land. Our present metagenomics study on amphibious mudskippers supports their immunological specificity. More investigations on the bacterial communities functioning in the immunity and pathogen antagonism of mudskippers will promote host health and well-being in fish themselves.

Author Contributions

Q.S. and B.D. conceived and designed the project; B.D., Z.W. and Y.Y. analyzed the data; P.A. and X.Y. participated in the data analysis and figure preparation; B.D. and Y.Y. wrote the manuscript; Q.S. and Z.W. revised the manuscript.

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