A Gastrointestinal Metagenomic Study On Mudskippers Supports Their Terrestrial Adaptation

Bo Dong (✉ dongbo@gdaas.cn)

GDAAS <https://orcid.org/0000-0002-6965-4585>

Jing Liu

Guangdong Academy of Agricultural Sciences

Bing Chen

Guangdong Academy of Agricultural Sciences

Yuqi Huang

Guangdong Academy of Agricultural Sciences

Peng Ai

Genedenove Company

Xinxin You

BGI

Qiong Shi

BGI

Wen Huang

Guangdong Academy of Agricultural Sciences

Research Article

Keywords: gastrointestinal microbiome, metagenomics, amphibious mudskipper, terrestrial adaptation

Posted Date: January 4th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1066801/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

-Purpose: The adaptability of blue-spotted mudskipper (*Boleophthalmus Boleophthalmus*; BP) and giant-fin mudskipper (*Periophthalmus magnuspinnatus*; PM), has been previously reported at the genome level to explain their amphibious life. However, the roles of GI microbiota in their adaptation to the terrestrial life are worth exploring.

-Methods: In this study, we mainly utilized metagenomic data from these two representative mudskippers and typical aquicolous fish species to obtain microbial composition, diversity, abundance and potential functions of GI microbiota for comparisons between amphibious and aquicolous fishes. Meanwhile, we summarized the GI microbiota results of representative seawater fishes, freshwater fishes, amphibians, and terrestrial animals by literature mining for comparing those of the mudskippers.

-Result: Interestingly the content for each dominant 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 life style of water-to-land and freshwater to seawater transition. More interestingly, certain bacteria strains like S24-7, previously thought to be specific in terrestrial animals, were also identified in both BP and PM.

-Conclusion: The various composite and diversity of mudskipper GI microflora are therefore considered to conduce to their terrestrial adaptation in these amphibious fishes.

1. Introduction

Amphibious mudskippers have evolved this trait independently of leaf-finned fish, serving as an extant model to probe into the symbiotic microbial associated mechanism of adapting to land life. (You et al., 2014). Terrestrial adaptations in mudskippers like aerial respiration, high ammonia tolerance, etc (You. et al., 2018) are believed to be caused by the genetic changes driven by the selection pressure (You et al., 2014). However, very little is known about the adaptations from a view of the gastrointestinal (GI) metagenome, the second set of genome in life beings (Dong et al., 2018).

Blue-spotted mudskipper (*Boleophthalmus pectinirostris*; BP) and giant-fin mudskipper (*Periophthalmus magnuspinnatus*; PM) encounter diverse environments and various pathogens during their transition from water to land. In addition to the genomes providing terrestrial adaptation for mudskippers, it is widely acknowledged that GI microbiota play important roles in their physiological traits like immunity (Flint et al., 2008; Dawood et al., 2016; Neu, 2014, Cerezuela et al., 2013). Therefore, the comparisons of GI microbiome between aquicolous fishes, land-dwelling animals, and amphibious fishes like mudskippers will help explain their particular traits for dwelling in water and on land. The GI microbiota (DeSantis et al., 2006; Fakruddin et al., 2013) co-evolve with their host and feed on common resources during ages of transition from water to land. Many functions of fishes were maintained or participated in by them, such

as the inhibition of pathogens, the interaction with enteric epithelium, and the education of immunity (John et al., 2004).

It has long been hypothesized that the host genetics and living niche of all fishes select for a “core microbiota” to keep some essential functions existing in all fish members (Turnbaugh et al., 2006). This proposal may be extrapolated to amphibians and terrestrial vertebrates. Hence the unique living niche of mudskippers will provide a good model to test this hypothesis and to examine how the core microbiota changed from water to land (Larsen et al., 2014; Ye et al., 2014). Meanwhile, beneficial autochthonous GI microbiota can be used to help investigate 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 with those in aquicolous fishes and terrestrial animals, which will help determine specific microbial strains for the terrestrial adaptation of amphibious fishes.

2. Materials And Methods

2.1. Sample collection

Pond-cultured grass carps (*Ctenopharyngodon idellus*; CI) and silver carps (*Hypophthalmichthys molitrix*; HM) were selected as representatives for aquicolous fishes samples because of their vicinity to the sampled mudskippers. They were sampled from a local pond in Zhuhai, Guangdong, China in 2016. They fed on natural resources without human intervention. Besides these representative aquicolous fish, data of wild samples gained from other researches are also included in our study.

These fishes were washed with sterile water and 70% ethanol to reduce contamination. Their GIs were dissected aseptically, and the GI content and the epithelial GI mucosa were taken for a separate harvest. The GI contents were obtained and stored at -80°C before use.

Blue-spotted mudskippers (BP) (average length: 10 cm) and giant-fin mudskippers (PM) (average length: 3 cm) were also captured wildly from Island Qi'ao (E113.6°, N22.4°), Zhuhai, Guangdong in the July of 2016. Only big and healthy ones 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. FT15168).

2.2. Extraction of metagenomic DNAs

Each sample (250 mg of intestinal content and mucosa) was defrozen on ice, and then total bacterial DNA was extracted utilizing a conventional DNA extraction kit (CTAB method). DNA integrity and purity was assayed through 1% agarose gels. DNA concentration was quantified by a Qubit Fluorometer (Thermo Fisher Scientific, USA). The prepared metagenomic DNAs were stored at -80 °C prior to use.

2.3. Library construction and validation

Total metagenomic DNA preparation was evenly fragmented into 350bp using Covaris ruptor. The fragmented DNAs were then reacted with End Repair Mix (NEB, USA) prior to incubation at 20 °C for 30 min. The obtained end-repaired DNAs were purified with QIAquick PCR Purification Kit (Qiagen, USA), and were later applied to A-Tailing Mix (NEB, USA). The adapter-ligated DNAs were subsequently purified by running in a 2% agarose gel and PCR amplified to enrich the adapter-ligated DNA fragments. PCR products were applied to another 2% gel and selected using QIAquick PCR Purification Kit to generate the target fragments.

The final libraries were constructed for quantification, including assaying the average molecule length by Agilent 2100 bioanalyzer with Agilent DNA 1000 Reagents (Agilent, USA), and measurement of each library by quantitative real-time PCR.

2.4. 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.

2.5. Assembly and annotation of metagenomes

A total of 19 samples were sequenced by an Illumina Hiseq PE150 platform. Metagenomes were assembled by MEGAHIT (Li et al.,2016). Analyses were implemented as previously reported (Dong et al., 2017) by Shenzhen E-gene 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 $\leq 1e-3$ (Menzel et al.,2016). 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 $\geq 75\%$ were filtered by BLAST against the genome of each corresponding host.

2.6. OTU statistics and Venn chart drawing

Tags were clustered to Operational Taxonomic Units (OTUs) by scripts of the software USEARCH (v7.0.1090) (Edgar,2013) 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 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.

2.7 Prediction and identification of AMPs

We applied homology search with those reported AMPs from the established metagenome datasets of two Asian carps and two mudskippers. Previously validated AMPs were retrieved from the online Antimicrobial Peptides Database (APD3) (Wang et al., 2016) 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 $\leq 10^{-5}$ 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.

3. Results

3.1. Summary of the achieved fish metagenome datasets

A total of 29 Mb, 17 Mb, 22 Gb, and 47 Gb, and 12 Gb of raw data were generated for each sample of 6 BP (BP1-BP6), 5 PM (PM1-PM5), 8 grass carps (CI1-CI9), 5 silver carps (HM1-HM5), 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 S1).

The GI microbiome of grass carp (Table S1) 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 (Ni, 2014; Dong et al., 2017), 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).

3.2. Comparison of microbiome species and abundance between two mudskippers and two 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 to 5, the distribution histograms of taxonomic composition in each fish species was presented at the Phylum, Class, and Family 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 (Figs. 1-2). The species abundance less than 0.5% in all samples were classified into 'others'.

3.2.1 Amphibious mudskippers

In the metagenomes of mudskippers (BP and PM), we observed the dominated phyla of Firmicutes, Proteobacteria, Bacteroidetes, Fusobacteria in the GI microbiota (Fig. 1). 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 (Fig. 1A) (Kohl et al., 2014), which is consistent with the fact that BP is herbivorous while PM is omnivorous (You et al., 2018). Another distinction in the microbiome composition between the two mudskipper species is that *Cyanobacteria* takes a larger share in BP than in PM (Fig. 1).

3.2.2 Two Asian carp species

Metagenomic results (Fig. 2) of the two carp species, including silver carp (HM; Fig. 2A) and grass carp (CI; Fig. 2B), are consistent with certain previous reports (Wu et al., 2015, Ye et al., 2013, Ni et al., 2014, van Kessel et al., 2011, Li et al., 2014), in which the four phyla Firmicutes, Proteobacteria, Bacteroidetes 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 (Li et al., 2013).

3.2.3 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 4–27%) in carps, while no detection is available in mudskippers (Fig. 3).

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

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

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 kinds of 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), 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 (Fig. 3) are consistent with the results presented by pie charts (Fig. 4). From the bar data of the microbiota composition in fish samples at the level of class (Fig. 4B), 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. It's 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 (Fisher et al., 2009).

From Figure 4C, 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.

Likewise, Clostridiaceae, Moraxellaceae and 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. The same is true for the Comamonadaceae and 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%). The Comamonadaceae family will synthesize tetrodotoxin to protect a species of fish (Johnson, 2008).

3.3. Analysis of community patterns, OTU statistics and OTU Venn charts

The core microbiomes of different species could be determined once combined with the OTU representative species through the overlapping in Venn diagrams displaying the number of common/unique OTUs in multi-samples/groups. 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 Fig. 5, 134 OTUs were shared by the five animal groups, 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 (Fig. 5). From the stack comparison in Figure 6, we found that the OTUs in mudskippers and terrestrial animals differentiate from aquicolous fishes mostly in S24-7, Lachnospiraceae, Ruminococcaceae, and Rikenellaceae families. Detailed functions of these strains are worth further exploration.

3.4 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 (Fig. 7) with these relative abundance values.

From Fig. 7 and Fig. 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 (Fig. 7). That is to say, amphibians and amphibious fishes are the intermediate between aquatic 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 Bacteroides (Fig. 5).

3.5 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 Fig. 8, the function profiles of metagenomes in BP were substantially different from those in grass carps and bigheaded carps. The most striking difference was about 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 (You et al., 2014, 2018).

3.6. Different AMP genes identified from the GI metagenomes of amphibious mudskippers and aquatic 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 Fig. 9, we determined the overlapping of AMP genes between amphibious mudskippers and aquatic 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 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.

4. 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 along the established pedigree remains not addressed.

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

Mudskippers are characterized by their amphibious habits (You et al., 2014, You et al., 2018). We chose a more 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 profile 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.

4.2. 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 two representative freshwater fishes of the Cyprinidae family, and their annual outputs account for over 80% of the total cultured freshwater fish in China (Wu et al., 2012). In the present research, we sequenced the whole metagenomes of the GI microbiota of BP and two Asian carp species to compare the microbial composition, diversity, abundance and function predictions of GI microbiota among these fishes. Except these achieved metagenome and 16S data for mudskippers, we also collected 8 Gb 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 prevalent in carps but absent in mudskippers. It's reported that estrogen and other endocrine factors will affect CKC4 in GI microbiota significantly, suggesting that CKC4 may be involved in host lipid metabolism (Liu et al., 2016). 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 pectenica*, *V. penaeicida*, and *Photobacterium damsela* (Ghomrassiet al.,2016). 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.

4.3. Core microbiota and critical factors shaping teleost GI microbiota

After comparison of the examined vertebrate groups (seawater and freshwater fishes, amphibious mudskippers, amphibians, and land-dwelling animals), we identified 134 common OTUs among these five groups (Fig. 7). 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 (Fig. 7), 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 (Wilson et al., 2008). Discovering core microbiome, that is to say, 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 (Turnbaugh et al. 2007, Roeselers et al., 2011; Hennersdorf et al., 2016). While some researchers have proposed that host phylogeny was the determinant factor in shaping those microbial communities, It was noted that fish-associated microbiomes were more similar between freshwater fishes, regardless of phylogeny (Roeselers et al.2011; Sullam et al. 2012), 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 (Hennersdorf et al., 2016) and freshwater species (Larsen et al., 2014a; Eichmiller et al., 2016; Liu et al., 2016), seem to be dominated by the phylum Proteobacteria, followed by Fusobacteria and Firmicutes, and in a lesser percentage of Bacteroidetes,

Actinobacteria and Verrucomicrobia (Llewellyn et al., 2014). These phyla, representing up to 90% of the total communities, were identified in both transient and 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 (You et al., 2018). 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 groups, and the amphibious hosts at the transitional stage would harbor both sets to cope with the aquatic and terrestrial environments.

4.4. Microbial strains related to terrestrial adaptation for mudskippers

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 some microbial strains in their terrestrial adaptation.

Mudskippers are amphibious fishes, however few systematic studies on their components of innate system were 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, which may release DNAs or other substances into the mucus layer to influence host innate immune cells through specific receptors, such as TLR9 (Liu et al., 2017). It was substantiated that S24-7 can modulate immune functions by interaction with the gut mucus layer (Qi et al., 2017).

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 (11 copies) in teleost fishes (You et al., 2014). Their large TLR repertoire will recognize more pathogen categories through pattern recognition receptors (PRRs) and facilitate the downstream activation of immune factors and AMP expression (Yi et al., 2017), which will greatly improve the education of immune system and health of the enteric epithelia (Foureau et al., 2010, Stockinger et al., 2014). 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 (Fig. 9). The AMP data combined with our

previous finding about TLR expansion (You et al., 2014) 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 adaptations from water to land. Our present metagenomics study on amphibious mudskippers will lend some hint to the evolution of GI microbiota from water to land.

Declarations

o Ethics approval and consent to participate:

Not applicable

o Consent for publication:

Not applicable

o Availability of data and materials:

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

o Competing interests:

The authors declare no competing interest.

o Funding:

This work was supported by Science and Technology Program of Guangdong Province (2014B020201002), Special Fund for Rural Revitalization Strategy (Enhancement of Agricultural Science and Technology Capability) (TS-1-6-17), Guangzhou Rural Science and Technology Special Commissioner Project (GZKTP202034) the Key Laboratory of Modern Biological Seed Industry in South China, Ministry of Agriculture and Rural Affairs(2105-000000-20-03-457451), and projects subsidized by special funds for science technology innovation and industrial development of Shenzhen Dapeng New District Grand No. KJYF202101-13) .

o Authors' information:

Affiliations

Guangdong Provincial Key Laboratory for Crop Germplasm Resources Preservation and Utilization, Agro-biological Gene Research Center, Guangdong Academy of Agricultural Sciences, Guangzhou, China

Laboratory of Aquatic Sciences, Key Laboratory of Animal Nutrition and Feed Science in South China, Ministry of Agriculture in Rural Affairs, Guangdong Key Laboratory of Animal Breeding and Nutrition,

Institute of Animal Science, Guangdong Academy of Agricultural Sciences, Guangzhou, China.

Shenzhen Key Lab of Marine Genomics, Guangdong Provincial Key Lab of Molecular Breeding in Marine Economic Animals, BGI Academy of Marine Sciences, BGI Marine, BGI, Shenzhen, China

Guangzhou Genedenovo Biotechnology Co. Ltd., Guangzhou, China

BGI Education Center, University of Chinese Academy of Sciences, Shenzhen, China

Contributions

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

Corresponding author

Wen Huang

o Acknowledgements:

We appreciate all efforts made by all authors in GDAAS and BGI research.

References

1. Beek AA, Fransen F, Meijer B, de Vos P, Knol EF, Savelkoul HFJ (2018) Aged mice display altered numbers and phenotype of basophils, and bone marrow-derived basophil activation, with a limited role for aging-associated microbiota. *Immun Ageing* 15:32. doi: 10.1186/s12979-018-0135-6
2. Bledsoe JW, Waldbieser GC, Swanson KS, Peterson BC (2018) Small. Comparison of Channel Catfish and Blue Catfish Gut Microbiota Assemblages Shows Minimal Effects of Host Genetics on Microbial Structure and Inferred Function. *Front Microbiol* 9:1073. doi: 10.3389/fmicb.2018.01073
3. Cerezuela R, Meseguer J, Esteban MA (2013) Effects of dietary inulin, *Bacillus subtilis* and microalgae on intestinal gene expression in gilthead seabream (*Sparus aurata* L.). *Fish Shellfish Immunol* 34(3):843–848. doi: 10.1016/j.fsi
4. Chen W, Liu F, Ling Z, Tong X, Xiang C (2012) Human intestinal lumen and mucosa-associated microbiota in patients with colorectal cancer. *PLoS ONE* 7(6):e39743. doi: 10.1371/journal.pone.0039743
5. Dawood MA, Koshio S, Ishikawa M, Yokoyama S, El Basuini MF, Hossain MS (2016) Effects of dietary supplementation of *Lactobacillus rhamnosus* or/and *Lactococcus lactis* on the growth, gut microbiota and immune responses of red sea bream, *Pagrus major*. *Fish Shellfish Immunol* 49:275–285. doi: 10.1016/j.fsi

6. DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K et al (2006) Green genes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl Environ Microbiol* 72(7):5069–5072. doi: 10.1128/aem.03006-05
7. Dong B, Yi Y, Liang L, Shi Q (2017) High throughput identification of antimicrobial peptides from fish gastrointestinal microbiota. *Toxins* 9(9):266. doi: 10.3390/toxins9090266
8. Edgar RC (2013) UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat Methods* 10(10):996–998. doi: 10.1038/nmeth.2604
9. Eichmiller JJ, Hamilton MJ, Staley C, Sadowsky MJ, Sorensen PW, Sorensen (2016) Environment shapes the fecal microbiome of invasive carp species. *Microbiome* 4(1):44. doi: 10.1186/s40168-016-0190-1
10. Fakruddin M, Mannan KS, Andrews S (2013) Viable but nonculturable bacteria: food safety and public health perspective. *ISRN Microbiol* 2013:703–813. doi: 10.1155/2013/703813
11. Fisher K, Phillips C (2019) The ecology, epidemiology and virulence of *Enterococcus*. *Microbiology* 155:749–757. doi: 10.1099/mic.0.026385-0
12. 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(2):121–131. doi: 10.1038/nrmicro1817
13. Foureau DM, Mielcarz DW, Menard LC, Schulthess J, Werts C, Vasseur V et al (2010) TLR9-dependent induction of intestinal alpha-defensins by *Toxoplasma gondii*. *J Immunol* 184(12):7022–7029. doi: 10.4049/jimmunol.0901642
14. Ghomrassi H, Ben BO, Choiset Y, Haertlé T, Hani K, Chobert JM, Ghrairi T (2016) Evaluation of marine AMPogenic enterococci strains with inhibitory activity against fish-pathogenic Gram-negative bacteria. *Dis Aquat Organ* 118(1):31–43. doi: 10.3354/dao02953
15. Hennersdorf P, Kleinertz S, Theisen S, Abdul-Aziz MA, Mrotzek G, Palm HW, Saluz HP (2016) Microbial diversity and parasitic load in tropical fish of different environmental conditions. *PLoS One* 11(3):e0151594. doi: 10.1371/journal.pone.0151594
16. Kessel MA, Dutilh BE, Neveling K, Kwint MP, Veltman JA, Flik G et al (2011) Pyrosequencing of 16S rRNA gene amplicons to study the microbiota in the gastrointestinal tract of carp (*Cyprinus carpio* L.). *AMB Express* 1:41. doi: 10.1186/2191-0855-1-41
17. Kohl KD, Amaya J, Passemment CA, Dearing MD, McCue MD (2014) Unique and shared responses of the gut microbiota to prolonged fasting: a comparative study across five classes of vertebrate hosts. *FEMS Microbiol Ecol* 90(3):883–894. doi: 10.1111/1574-6941.12442
18. Larsen AM, Mohammed HH, Arias CR (2014) Characterization of the gut microbiome of three commercially valuable warmwater fish species. *J Appl Microbiol* 116:1396–1404. doi: 10.1111/jam.12475
19. Ley RE, Turnbaugh PJ, Klein S, Gordon JI (2006) Microbial ecology: human gut microbes associated with obesity. *Nature* 444(7122):1022–1023. doi: 10.1038/4441022a

20. Li D, Luo R, Liu CM, Leung CM, Ting HF, Sadakane K et al (2016) MEGAHIT v1.0: A fast and scalable metagenome assembler driven by advanced methodologies and community practices. *Methods* 102:3–11. doi: 10.1016/j.ymeth
21. Li T, Long M, Gatesoupe FJ, Zhang Q, Li A, Gong X (2015) Comparative analysis of the intestinal bacterial communities in different species of carp by pyrosequencing. *Microb Ecol* 69(1):25–36. doi: 10.1007/s00248-014-0480-8
22. Li X, Yan Q, Xie S, Hu W, Yu Y, Hu Z (2013) Gut microbiota contributes to the growth of fast-growing transgenic common carp (*Cyprinus carpio* L.). *PLoS ONE* 8(5):e6457. doi: 10.1371/journal.pone.0064577
23. Liu H, Guo X, Gooneratne R, Lai R, Zeng C, Zhan F, Wang W (2016) The gut microbiome and degradation enzyme activity of wild freshwater fishes influenced by their trophic levels. *Sci Rep* 6:24340. doi: 10.1038/srep24340
24. Liu J, Bian G, Sun D, Zhu W, Mao S (2017) Starter feeding supplementation alters colonic mucosal bacterial communities and modulates mucosal immune homeostasis in newborn lambs. *Front Microbiol* 8:429. doi: 10.3389/fmicb
25. Llewellyn MS, Boutin S, Hoseinifar SH, Derome N (2014) Derome. Teleost microbiomes: the state of the art in their characterisation, manipulation and importance in aquaculture and fisheries. *Front Microbiol* 5:207. doi: 10.3389/fmicb.2014.00207
26. Menzel P, Ng KL, Krogh A (2016) Fast and sensitive taxonomic classification for metagenomics with Kaiju. *Nat Commun* 7:11257. doi: 10.1038/ncomms11257
27. Neu J (2014) The developing intestinal microbiome: probiotics and prebiotics. *World Rev Nutr Diet* 110 110:167–176. doi: 10.1159/000358465
28. Ni J, Yan Q, Yu Y, Zhang T (2014) Factors influencing the grass carp gut microbiome and its effect on metabolism. *FEMS Microbiol Ecol* 87(3):704–714. doi: 10.1111/1574-6941.12256
29. Ni J, Yan Q, Yu Y, Zhang T Factors influencing the grass carp gut microbiome and its effect on metabolism. *FEMS Microbiol Ecol*. 2014(3):704–714. doi: 10.1111/1574-6941
30. Rawls JF, Samuel BS, Gordon JI (2004) Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. *Proc Natl Acad Sci USA* 101(13):4596–4601. doi: 10.1073/pnas.0400706101
31. Roeselers G, Mittge EK, Stephens WZ, Parichy DM, Cavanaugh CM, Guillemin K, Rawls JF (2011) Evidence for a core gut microbiota in the zebrafish. *ISME J* 5(10):1595–1608. doi: 10.1038/ismej
32. Stockinger S, Albers T, Duerr CU, Ménard S, Pütsep K, Andersson M, Hornef MW, Hornef (2014) Interleukin-13-mediated paneth cell degranulation and antimicrobial peptide release. *J Innate Immun* 6(4):530–541. doi: 10.1159/000357644
33. 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(13):3363–3378. doi: 10.1111/j.1365-294X.2012.05552.x

34. Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI (2006) An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* 444(7122):1027–1031. doi: 10.1038/nature05414
35. Turnbaugh PJ, Ley RE, Fraser-Liggett CM, Knight R, Gordon JI (2007) The human microbiome project. *Nature* ;449:804–810. doi: 10.1038/nature06244
36. Wang G, Li X, Wang Z (2016) APD3: the antimicrobial peptide database as a tool for research and education. *Nucleic Acids Res* 44(D1):1087–1093. doi: 10.1093/nar/gkv1278
37. Wilson B, Danilowicz BS, Meijer WG (2008) The diversity of bacterial communities associated with Atlantic cod *Gadus morhua*. *Microb Ecol* 55(3):425–434. doi: 10.1007/s00248-007-9288-0
38. Wu S, Ren Y, Peng C, Hao Y, Xiong F, Wang G, Li W, Zou H, Angert ER (2015) Metatranscriptomic discovery of plant biomass-degrading capacity from grass carp intestinal microbiomes. *FEMS Microbiol Ecol* 91(10):107. doi: 10.1093/femsec/fiv107
39. Wu SG, Wang GT, Angert ER, Wang WW, Li WX, Zou H (2012) Composition, diversity, and origin of the bacterial community in grass carp intestine. *PLoS ONE* 7(2):e30440. doi: 10.1371/journal.pone.0030440
40. Ye L, Amberg J, Chapman D, Gaikowski M, Liu WT (2014) Fish gut microbiota analysis differentiates physiology and behavior of invasive Asian carp and indigenous American fish. *ISME J* 10(8):541–551. doi: 10.1038/ismej
41. Yi Y, Liang L, Wang Z, Ai P, You X, Bian C, Shi Q, Dong B (2019) A Comparative Metagenomics Study on Gastrointestinal Microbiota in Amphibious Mudskippers and Other Vertebrate Animals. *Animals (Basel)* 9(9). doi: 10.3390/ani9090660. pii: E660
42. Yi Y, You X, Bian C, Chen S, Lv Z, Qiu L, Shi Q (2017) High-throughput identification of antimicrobial peptides from amphibious mudskippers. *Mar Drugs* 15(11):364. doi: 10.3390/md15110364
43. You X, Bian C, Zan Q, Xu X, Liu X, Chen J et al (2014) Mudskipper genomes provide insights into the terrestrial adaptation of amphibious fishes. *Nat Commun* 5:5594. doi: 10.1038/ncomms6594
44. You X, Sun M, Li J, Bian C, Chen J, Yi Y et al (2018) Mudskippers and their genetic adaptations to an amphibious lifestyle. *Animals* 8(2):24. doi: 10.3390/ani8020024
45. Yu CF, Yu PH, Chan PL, Yan Q, Wong PK (2004) Two novel species of tetrodotoxin-producing bacteria isolated from toxic marine puffer fishes. *Toxicon* 44(6):641–647. doi: 10.1016/j.toxicon.2004.07.021

Figures

Figure 1

Relative abundance of the phyla annotated in the mudskipper metagenomes. **(A)** BP, **(B)** PM.

Figure 2

Relative abundance of the phyla annotated in the metagenomes of two Asian carp species. **(A)** silver carp, and **(B)** grass carp. Cyanobacteria are capable of synthesizing vitamin B12, and they have symbioses with B12-producing bacteria (Kamennaya et al., 2018).

Figure 3

Comparison of relative abundance of the phyla annotated in the metagenomes of mudskippers (BP and PM) and carps.

Figure 4

The taxonomic composition of GI microbiota in the five groups of examined 40 species. They were classified at a Phylum (A), Class (B) or Family (C) level.

Figure 5

A Venn display of various OTUs in the metagenomes of five animal groups. Please note the overlapping between amphibious mudskippers and terrestrial animals.

Figure 6

Taxonomic distributions of various typical OTUs in mudskippers, marine, fresh water, amphibians and terrestrial animals at a family level.

Figure 7

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.

Figure 8

A heatmap of function abundance clusters from the metagenomes of BP and carps. CMG and CPY denote two samples of grass carps. 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 9

A Venn display of AMP gene numbers identified from various fishes. Please note that there are no overlaps between freshwater fishes and seawater fishes.