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Research Article

Association of Fungi in the Intestine of Black Carp and Grass Carp Compared with their Cultured Water

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The current study aimed to explore the intestinal fungal community characteristics of grass carp and black carp and their correlation with cultured water fungi. Grass carp, black carp, and their cultured water samples were collected from the same reservoir. Based on the Illumina HiSeq 2500 high-throughput sequencing platform, the fungal internal transcribed spacer (ITS) region sequences of each sample were determined and analyzed. The results showed that a total of 1,193,261 valid sequences with an average length of 235–251 bp were detected in the three groups of samples, which included 9 phyla, 27 classes, 65 orders, and 288 genera. Ascomycota, Basidiomycota, Mortierellomycota, and Chytridiomycota were the dominant phyla. *Mortierella*, *Thermoascus,* and *Thermomyces* were the main genera. Compared with cultured water samples, the abundance of major phyla and genera was significantly different from grass carp and black carp samples, but there was no significant difference between grass carp and black carp samples. Surprisingly, Ascomycota was enriched in CY and QY samples. In conclusion, the dominant fungi in grass carp, black carp, and cultured water samples were similar, but the relative abundance was significantly different compared with cultured water samples. The results will provide a basis for the tolerance of fish with different feeding habits to colonize water and provide a theoretical basis for the regulation and improvement of aquaculture water quality and the realization of healthy and green aquaculture of fish.

1. Introduction

Intestinal microbiota plays an important role in host health and nutrition and has attracted increasing attention recently [1]. As an important source of human protein, fish is undoubtedly the focus of research [2]. A large number of microorganisms are distributed in the intestinal tract of fish, which have formed a complex symbiotic relationship with the host in the long-term natural evolution process [3, 4]. Fish gut provides a reproductive environment for intestinal microorganisms, which in turn play an irreplaceable role in the development, nutrition, immune metabolism, and physiological health of the host [5, 6].

Sustainable development of aquaculture requires full consideration of the interaction between the environment and aquatic organisms [7–9]. As one of the most important aquaculture models, pond aquaculture plays an irreplaceable role in aquaculture. There are a large number of microorganisms such as bacteria and fungi in the pond ecosystem. As an important microorganism, fungi have a large number, diverse forms, and complex community structure [10, 11]. On the one hand, fungi participate in the decomposition of organic matter and provide nutrients for aquatic plants and fish [12]; on the other hand, many pathogenic fungi affect the growth and reproduction of plants, thus affecting the health of fish [13, 14]. Therefore, comparative studies of fungal microbial community characteristics in aquaculture waters

and intestinal tract of aquatic animals are beneficial to the regulation of aquaculture water quality.

Grass carp (*Ctenopharyngodon idella*) and black carp (*Mylopharyngodon piceus*), both of which belong to the "four major Chinese carps," are widely distributed in China and have been extended to more than 100 countries [15]. Grass carp is an herbivorous freshwater fish belonging to *Cyprinidae* (the carp family) and the genus *Ctenopharyngodon*. It often lives in the middle water body and mainly feeds on the stems and leaves of aquatic plants [16]. Black carp is a carnivorous freshwater fish belonging to *Cyprinidae* and the genus *Mylopharyngodon*. It often lives in the lower water body. Juvenile fish live on zooplankton, while adult fish feed on mollusks such as snails and clams [17]. With the excellent characteristics of large yield, delicious meat quality and high nutritional value, it is deeply loved by the majority of consumers [18, 19]. According to the statistics, the annual production of black carp was about 6.8×10^5 tons and the annual production of grass carp was about 5.53×10^6 tons [20].

At present, researchers have conducted a large number of studies on the structural characteristics of bacteria and archaea in the aquatic environment [21–24]. The studies on intestinal microorganisms of fish mainly focus on growth and metabolism, nutrient absorption, immune regulation, and the effect of bait on intestinal tract [25–27]. The correlation between intestinal fungi of two different predatory fishes and fungi in reproductive water environment has not been reported. In this study, we selected grass carp, black carp, and cultured water samples from the same aquatic culture environment. Based on Illumina Hiseq 2500 high-throughput sequencing platform, we analyzed the diversity and community structure of fungi in grass carp, black carp and their cultured water, and explored their correlation between fungi. These results will provide basic data for the tolerance of fish with different feeding habits to aquaculture water, so as to provide theoretical basis for the regulation and improvement of aquaculture water quality and the realization of healthy and green aquaculture.

2. Materials and Methods

2.1. Sample Collection. Black carp (QY, Qīngyú), grass carp (CY, Cǎoyú), and cultured water samples (SY, Shuǐ yàng) were collected in the same pond of Loudi Fisheries Research Institute (27°43′47″N, 112°0′6″E), Hunan Province, China. The pond has a depth of 2.0 m and an area of 1.5 hm^2. The sampling time was 08:00 a.m. on January 19, 2021, with water temperature 17.8°C, pH 8.10–8.56 and dissolved oxygen > 4.35 mg/L. Five grass carps (1431.34 ± 33.25 g) and five black carps 2627.3 ± 42.69 g) of the same size without disease symptoms were randomly selected from the fish caught in the net and brought back to the laboratory together with water samples, and the others were put back into the pond. The fish used in the experiment came from natural ponds and were not fed any food. At the same time, 8 sampling points were randomly selected in the pond, and 10 mL equal volume water samples were collected at a depth of about 1.0 m under the water surface. After mixing, the samples were loaded into sterilization centrifuge tubes.

In order to minimize sample contamination, the fish surface was washed with sterile water and 70% ethanol successively before dissection. Grass carp intestinal contents samples (CY1-CY5), black carp intestinal contents samples (QY1–QY5), and water samples (SY1-SY5) were collected in a sterile operating tray with sterilized centrifuge tubes and refrigerated at −80°C for later use.

2.2. PCR Amplification and Illumina HiSeq Sequencing. The MN NucleoSpin 96 Soi kit was used to extract DNA from collected chyme samples. The PCR amplification was performed with ITS1_F and ITS2_R primers of fungal ITS, and high-throughput sequencing was performed using Illumina HiSeq 2500 platform. The primer, reaction system, and amplification conditions are as follows: Primer synthesis and sequencing were completed by Beijing Biomarker Technologies Co., Ltd (Beijing, China).

Amplification primers: ITS1_F (5′-CTTGGTCATTTA GAGGAAGTAA-3′) and ITS2_R (5′-GCTGCGTTCTTC ATCGATGC-3′). The amplification reaction was performed as follows: 5 μL KOD FX Neo Buffer, 0.3 μL (10 μM) of each forward primer and reverse primer, 2 μL (2 mM) of dNTPs, 0.2 μL KOD FX Neo, and 50 ng of DNA template, ddH_2O supplement to 20 μL. Reaction conditions: Initial denaturation at 95°C for 5 min, followed by 25 cycles consisting of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 40 s, with a final extension of 7 min at 72°C.

2.3. Microbial Composition Analysis. Quality filtering was performed on the original data (Trimmomatic, version 0.33) [28], primer sequences were identified and removed (Cutadapt, version 1.9.1) [29], and double-ended reads were spliced (FLASH, version 1.2.11) [30]. Finally, we removed chimeras using UCHIME version 8.1 to obtain high-quality sequences for subsequent analysis [31]. The sequences were clustered at the 97% similarity level (USEARCH, Version 10.0) [32], and operational taxonomic units (OTUs) were filtered with 0.005% of all sequenced sequences as the threshold [33]. QIIME2 software (https://qiime2.org/) was used to calculate alpha and beta diversity in the samples to comprehensively assess the overall diversity and reveal differences between samples. Alpha diversity includes Chao1 richness estimator and Ace richness estimator to measure the richness of the microbiota and Shannon–Wiener diversity index and Simpson diversity index to measure the diversity of the microbiota. Beta diversity analysis includes principal component analysis (PCA), principal coordinates analysis (PcoA), and nonmetric multidimensional scaling (NMDS), all belonging to ordination analysis, which is about rearrange these samples in a visualized low-dimensional space or plane, so that the distance between samples can reflect the relationship information between samples in the plane scatter plot to the maximum extent. Based on the four distance matrices obtained from beta diversity analysis, unweighted paired average (UPGMA) was used to perform hierarchical clustering of samples by R language tool to assess the similarity of species composition among samples.

LefSe analysis [34] (https://huttenhower.sph-harvard.edu/lefse/), namely, the analysis of species with significant differences between groups, used linear discriminant analysis (LDA) to estimate the impact of each species abundance on the difference effect size and searched for species with significant differences between all groups.

2.4. Statistical Analysis. SPSS 25.0 statistical software (IBM Corp., Armonk, NY, USA) was used to analyze the data, and the measurement data was represented by means ± standard deviations, and independent sample T test was used for pair comparison. Differences between groups were considered statistically significant at $P < 0.05$ [35].

2.5. Data Storage. The original sequences obtained in this study have been submitted to the NCBI sequence read archive (accession number is PRJNA802701 https://www.ncbi.nlm.nih.gov/bioproject/PRJNA802701).

3. Results

3.1. Sequencing Characteristics and Microbial Diversity. After quality control, a total of 1193261 high quality sequences were obtained from 15 samples in CY, QY, and SY, and the proportion of effective sequences in each sample was between 0.9748 and 0.9911. The average sequence length was between 235–251 bp (Table 1). The average coverage index of each sample was 0.9986, between 0.9978 and 0.9997, which could reflect the real situation of species in the community (Table 2). As shown in the species Venn diagram, a total of 1099 OTUs were obtained in the three groups, and 651, 825, and 670 OTUs were found from the CY, QY, and SY groups. Among them, 282 were identical (Figure 1). It indicated that the richness and diversity of fungi in QY and SY were higher than that in CY, but there was no statistical difference.

Chao1 index, Ace index, Shannon index, and Simpson index were calculated to illustrate the diversity and richness of CY, QY, and SY. From the calculation results of richness index (Table 2), the Chao1 index in QY was the highest, the Ace index in SY was the highest, and the Chao1 index and Ace index in CY were the lowest. From the calculation results of diversity index, the Simpson index and Shannon index in QY were the highest, the Simpson index in SY was the lowest, and the Shannon index in CY was the lowest. Compared with SY, there was no significant difference in the other indexes except Shannon diversity index in CY ($P < 0.05$). These results indicated that the richness of fungi in cultured water was the highest, and the diversity of fungi in the intestinal tract of black carp was the highest, with no significant difference between them. These results indicated that the richness of fungi in SY was the highest, while the diversity of fungi in QY was the highest, with no significant difference between them.

3.2. Overall Microbial Community Structure. A total of 9 phyla and 288 genera were identified from 15 samples collected from CY, QY, and SY groups. Nine phyla and 210 genera were identified in CY, 9 phyla, and 253 genera in QY and 8 phyla and 188 genera in SY. Among the identified phyla, Ascomycota, Basidiomycota, Chytridiomycota, and Mortierellomycota had higher relative abundance. The total abundance of these four phyla accounted for 94.34%, 91.54%, and 66.77% in CY, QY, and SY, respectively (Figure 2).

3.3. Characteristics of Fungal Community Composition

3.3.1. Characteristic of Fungal Community Composition at Phylum Level. There were 9 phyla detected in 15 samples of the three groups, among which Calcarisporiellomycota was not detected in the SY samples. The dominant phyla in CY, QY, and SY samples were Ascomycota (68.29%, 68.67%, and 31.58%, respectively), Basidiomycota (13.40%, 12.37%, and 9.33%, respectively), Mortierellomycota (12.14%, 9.43%, and 4.14%, respectively), and Chytridiomycota (0.51%, 1.07%, and 21.72%, respectively). Among the 9 strains identified, Ascomycota, Chytridiomycota, Glomeromycota, and Mortierellomycota in CY and QY showed significant differences compared with SY ($P < 0.01$ or $P < 0.05$), and there was no significant difference in CY and QY between these bacteria (Figure 3, Table 3). In addition, Calcarisporiellomycota was not detected in the SY, and Chytridiomycota significantly increased in the SY, while all other phyla have different degrees of reduction.

3.3.2. Characteristic of Fungal Community Composition at Genus Level. A total of 288 genera were detected in 15 samples of the three groups. 210, 253, and 188 genera were detected in CY, QY, and SY, respectively. The unclassified genera in the SY were the most, accounting for 66.87%. Of the 288 identified genera, the top 10 genera in relative abundance among these three groups of samples were *Mortierella*, *Thermoascus*, *Thermomyces*, *Aspergilleus*, *Penicillium*, *Fusarium*, *Saitozyma*, *Archaeorhizomyces*, *Alternaria*, and *Cladosporium* (Figure 4, Table 4). Compared with SY sample, there were 5 genera in the CY and QY samples that were significantly different from the SY sample ($P < 0.01$ or $P < 0.05$). In addition, *Aspergillus* and *Cladosporium* had significant differences in CY and QY samples ($P < 0.05$).

Further, as shown in Figure 5(a), UPGMA analysis showed that the microbial community similarity of each group was relatively high. According to LefSe analysis results, there were more fungal species with statistical differences in the CY but fewer in the QY, and only phylum Chytridiomycota and class Chytridiomycetes had statistical differences in the SY (Figure 5(b)).

4. Discussion

Pond aquaculture has become the most important and broadest breeding mode in China and the most important source of aquatic products [36]. The quality of aquatic products is closely related to the cultured environment, and the microbial composition in the cultured environment has become one of the most important factors. Therefore, the

Table 1: Characteristics of ITS sequences in each sample.

Sample ID	Raw reads	Clean reads	Effective reads	AvgLen (bp)	Effective (%)
CY1	80157	79447	79431	241	99.09
CY2	80094	79293	79023	244	98.66
CY3	79958	79163	78611	248	98.32
CY4	79886	79098	79088	242	99
CY5	79925	79153	77909	241	97.48
QY1	80158	79375	78295	242	97.68
QY2	80256	79354	79348	244	98.87
QY3	79967	79090	78033	242	97.58
QY4	73220	72620	71513	239	97.67
QY5	79987	79215	79040	235	98.82
SY1	79911	79179	79148	251	99.05
SY2	79799	79118	79090	248	99.11
SY3	79670	78967	78773	249	98.87
SY4	80190	79454	79075	249	98.61
SY5	80083	79374	78968	246	98.61

Note. CY stands for grass carp, QY stands for black carp; SY stands for water sample.

Table 2: Coverage and diversity indices of fungal species in each group.

Group	Chao1	Ace	Simpson	Shannon	Coverage
CY	467.7671 ± 159.2337	640.5842 ± 252.8119	0.9733 ± 0.0058	6.0649 ± 0.1492*	0.9985 ± 0.0007
QY	564.4470 ± 69.5352	870.1195 ± 204.2143	0.9768 ± 0.0050	6.4822 ± 0.3672	0.9983 ± 0.0003
SY	529.2817 ± 128.6672	1065.8730 ± 415.0554	0.9713 ± 0.0078	6.4572 ± 0.2899	0.9989 ± 0.0004

Note. CY stands for grass carp, QY stands for black carp; SY stands for water sample; compared with water sample. *stands for $P < 0.05$, **stands for $P < 0.01$.

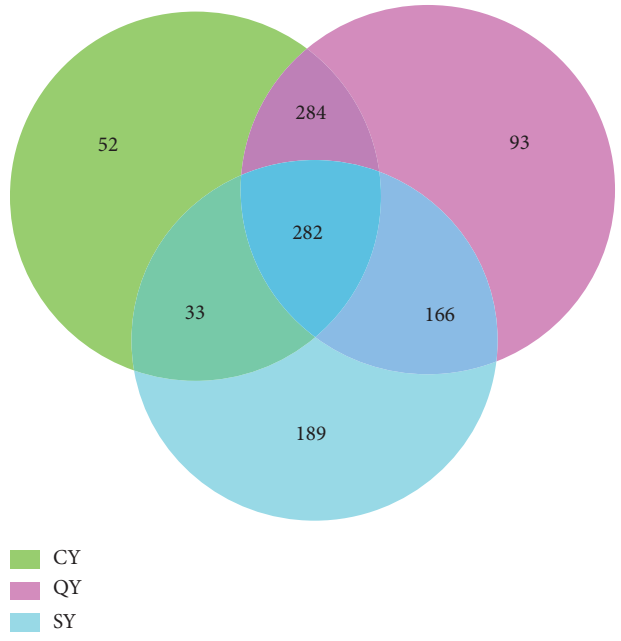

Figure 1: Fungi venn diagram in different test groups. Note: CY stands for grass carp, QY stands for black carp; SY stands for water sample.

aquaculture environment of aquatic products should be highly valued. In the intestinal tract of freshwater fish, the dominant bacteria belong to *Pseudomonas*, *Bacteroides*, and *Aeromonas*, whose main functions can be summarized as improving the absorption and utilization of nutrients, promoting the maturation of the immune system, and protection against pathogenic microorganisms [37–40]. Its community structure varies with fish species, feeding habits, bait, and various environmental factors [41–43]. Under the same conditions, the composition of microbial community in reproductive water environment will directly affect the community structure of intestinal microorganisms of fish [43–46].

Fungi, as an important component of intestinal microorganisms, play an important role in maintaining the

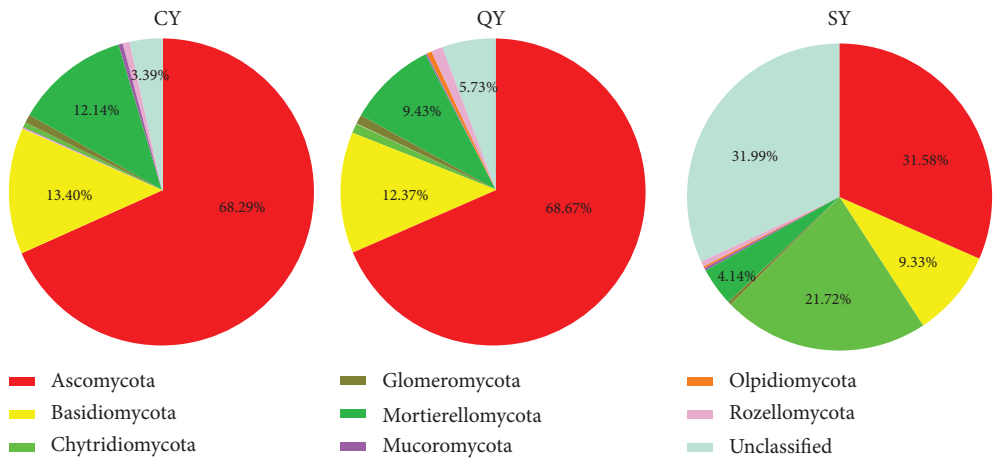

Figure 2: The fungal community in all samples at phylum level. Note: CY stands for grass carp, QY stands for black carp; SY stands for water sample.

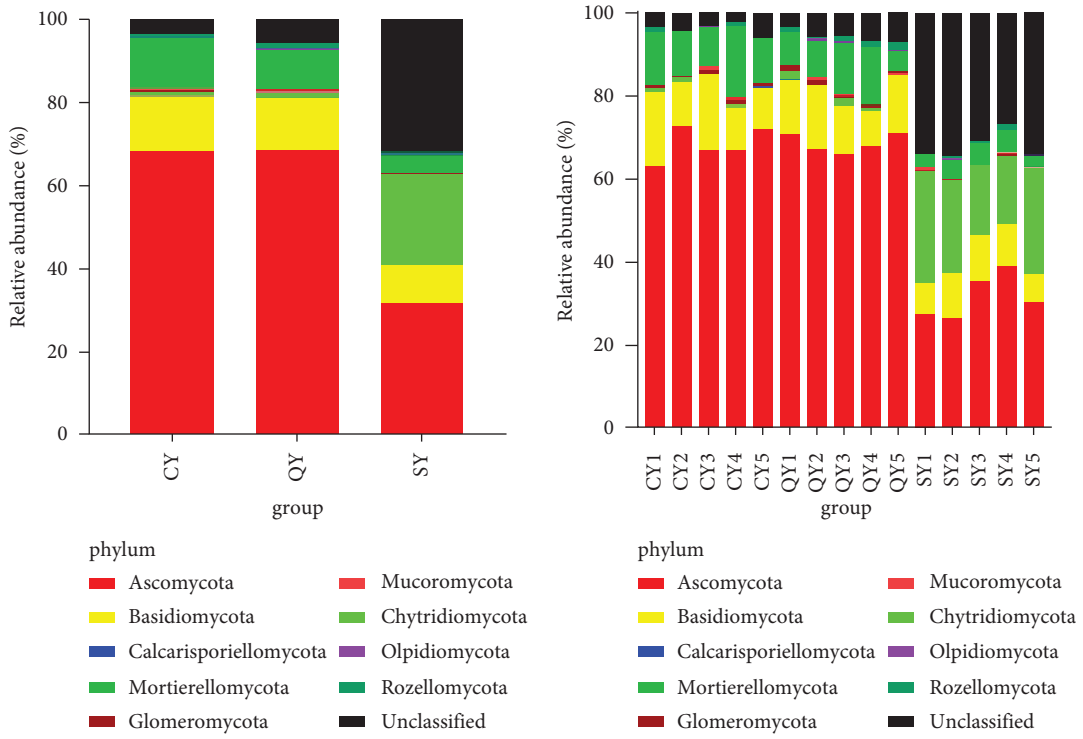

Figure 3: Relative abundance of fungi identified at phylum level in each group. Note: CY stands for grass carp, QY stands for black carp; SY stands for water sample.

Table 3: Relative abundance of fungi identified at phylum level in each group.

Phylum	CY	QY	SY
Ascomycota	0.6829 ± 0.0392**	0.6867 ± 0.0226**	0.3158 ± 0.0535
Basidiomycota	0.1340 ± 0.0415	0.1237 ± 0.0267	0.0933 ± 0.0213
Calcarisporiellomycota	0.0009 ± 0.0020	0.0003 ± 0.0006	0.0000 ± 0.0000
Chytridiomycota	0.0051 ± 0.0047**	0.0107 ± 0.0085**	0.2172 ± 0.0486
Glomeromycota	0.0096 ± 0.0030**	0.0092 ± 0.0053*	0.0023 ± 0.0016
Mortierellomycota	0.1214 ± 0.0319**	0.0943 ± 0.0366*	0.0414 ± 0.0109
Mucoromycota	0.0030 ± 0.0045	0.0021 ± 0.0028	0.0024 ± 0.0036
Olpidiomycota	0.0008 ± 0.0017	0.0037 ± 0.0032	0.0016 ± 0.0011
Rozellomycota	0.0085 ± 0.0099	0.0120 ± 0.0053	0.0061 ± 0.0066
Unclassified	0.0339 ± 0.0079**	0.0573 ± 0.0149**	0.3199 ± 0.0338

Note. CY stands for grass carp, QY stands for black carp; SY stands for water sample; compared with water sample. *stands for $P < 0.05$, **stands for $P < 0.01$.

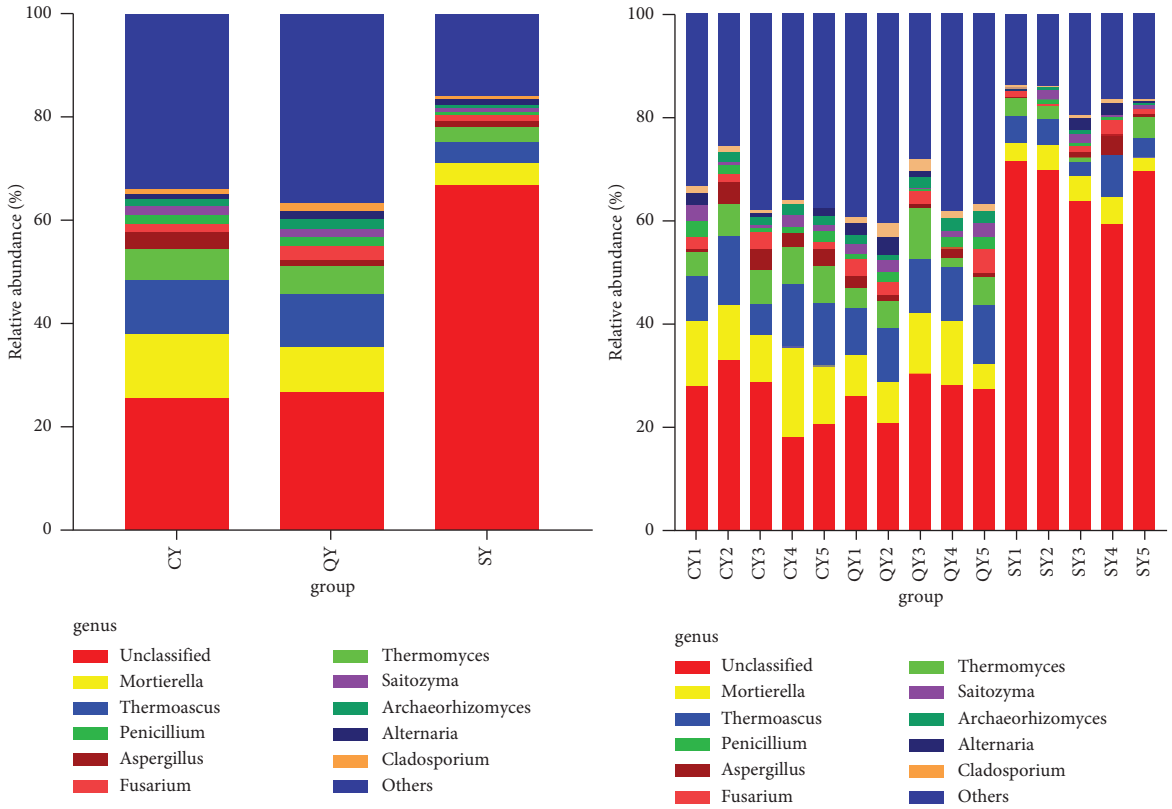

Figure 4: Relative abundance of fungi identified in each group for the most abundant 10 genera. Note: CY stands for grass carp, QY stands for black carp; SY stands for water sample.

Table 4: Relative abundance of fungi in each group for the 10 most abundant genera.

Genus	CY	QY	SY
Unclassified	0.2568 ± 0.0617**	0.2665 ± 0.0358**	0.6687 ± 0.0519
Mortierella	0.1214 ± 0.0319**	0.0879 ± 0.0309*	0.0414 ± 0.0109
Thermoascus	0.1055 ± 0.0299**	0.1045 ± 0.0085**	0.0425 ± 0.0100
Thermomyces	0.0628 ± 0.0119**	0.0525 ± 0.0305	0.0287 ± 0.0126
Aspergillus	0.0305 ± 0.0140	0.0138 ± 0.0071	0.0113 ± 0.0157
Penicillium	0.0187 ± 0.0085**	0.0157 ± 0.0086*	0.0040 ± 0.0041
Fusarium	0.0167 ± 0.0112	0.0259 ± 0.0169	0.0136 ± 0.0098
Saitozyma	0.0154 ± 0.0099	0.0167 ± 0.0102	0.0096 ± 0.0069
Archaeorhizomyces	0.0142 ± 0.0084*	0.0189 ± 0.0063**	0.0043 ± 0.0037
Alternaria	0.0095 ± 0.0103	0.0139 ± 0.0156	0.0111 ± 0.0109
Cladosporium	0.0074 ± 0.0055	0.0183 ± 0.0066**	0.0048 ± 0.0037
Others	0.3411 ± 0.0510**	0.3655 ± 0.0494**	0.1598 ± 0.0251

Note. CY stands for grass carp, QY stands for black carp; SY stands for water sample; compared with water sample. *stands for $P < 0.05$, **stands for $P < 0.01$.

balance of intestinal microecology [47]. Under normal circumstances, there is a stable synergistic, antagonistic, or symbiotic relationship between intestinal fungi, intestinal bacteria, and other intestinal microorganisms to jointly stabilize the intestinal microbiota environment and maintain the intestinal mucosal barrier function [16]. However, when intestinal microbiota is disturbed, fungi will play a negative role and become the source of pathogenic bacteria for fungal infection [48]. Studies have confirmed that pathogenic fungi *Fusarium*, *Aphanomyces*, and *Lagenidium* were identified in *Oreochromis niloticus* aquaculture pond [49].

In this study, we applied high-throughput sequencing technology for the first time to fungal microbial communities in cultured water bodies and the intestinal tracts of aquatic animals. The results showed that 9 phyla were identified from the DNA metabarcodes, among which Ascomycota, Basidiomycota, and Mortierellomycota were the dominant phyla in CY and QY samples. Ascomycota, Basidiomycota, Mortierellomycota, and Chytridiomycota were the dominant phyla in SY samples. At the phylum level, the fungal microbial composition in CY and QY samples was similar, and there was no significant difference in abundance. However, Ascomycota, Chytridiomycota, Glomeromycota, and

Figure 5: Similarity and difference analysis of fungi in each group. (a) UPGMA analysis, (b) LefSe analysis. Note: CY stands for grass carp, QY stands for black carp; SY stands for water sample.

Mortierellomycota in CY and QY were significantly different from those in SY, and Calcarisporiellomycota was not detected in SY. Moreover, the abundance of Chytridiomycota was relatively high in SY. This result indicated that Ascomycota, Basidiomycota, and Chytridiomycota were the main phyla in the freshwater fungal community, with Ascomycota and Chytridiomycota as the dominant phyla; these are consistent with the research results of domestic

and foreign scholars [50–52]. Surprisingly, Ascomycota was enriched in CY and QY samples. Ascomycota is the largest category in the fungal community in the study, mainly by the *Dothideomycetes*, *Sordariomycetes*, and *Eurotiomycetes*. These three fungi were widely distributed in all habitat types, among which *Dothideomycetes* had diverse ecological functions and environmental adaptability [50]. *Basidiomycota* is the main component of the terrestrial fungal community, and the abundance of Basidiomycota in sediments is closely related to the input of exogenous organic matter [51, 53]. Chytridiomycota feeds mainly on aquatic plant residues [54], which may be the reason for its relatively high abundance in SY. Furthermore, Ascomycota and Basidiomycota are not only the main decomposers of organic matter in the soil fungal community but also participate in the nitrogen cycle and play an extremely important role in the material cycle and energy flow of the biosphere [55, 56].

At the genus level, a total of 288 genera were detected, of which 210 were detected in CY samples, 253 in QY samples, and 188 in SY samples. *Mortierella*, *Thermoascus*, and *Thermomyces* were the three genera with the highest relative abundance in each group. Among the unclassified fungi detected, CY and QY samples accounted for 25.68% and 26.65%, respectively. There was little difference between the two groups of samples, but they accounted for 66.87% in SY samples. These results indicated that the fungal species of QY sample were richer than CY sample, and the fungal diversity of SY sample was richer than CY and QY samples, mainly belonging to unclassified fungi. Compared with SY samples, 5 of the 10 dominant fungal genera in CY and QY samples showed significant differences, but these genera did not show significant differences in CY and QY. These results indicated that the species and abundance of intestinal fungi in QY and CY samples were basically the same, which were related to cultured water. *Mortierella* is an important component of soil microbial community. Some species form symbiotic relationships with plants [57, 58], while some are hosts themselves and benefit from the symbiotic bacteria in the process of growth and reproduction [59–61]. Both *Thermoascus* and *Thermomyces* are thermophilic fungi with unique survival ability and can secrete a variety of enzymes such as amylase and cellulase, which can provide nutrients for the growth of microorganisms [62, 63].

In conclusion, based on the community characteristics of fungi in CY, QY, and SY samples, our work identified the similarity of fungal microbes in CY and QY, and the correlation between fungal microbes in CY, QY, and SY. The present study showed that species composition of abundance of intestinal fungi in two cyprinid species were remarkably similar despite their contrasting feeding habits (herbivorous vs. carnivorous). This suggests that intestinal fungi have similar roles to the digestive ability of the two carp species. These results will provide a theoretical basis for the regulation and improvement of aquaculture water quality, the realization of healthy and green fish aquaculture, and provide reference for the research and development of probiotic products.

Data Availability

The data used to support the findings of this study have been uploaded to the NCBI sequence read archive (accession number is PRJNA802701).

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Zhou-Jin Tan designed the study; Jie-Qi Wu collected the data; Jia-Lin Liu analyzed the data; Cheng-Xing Long wrote the manuscript. All authors reviewed the manuscript.

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