CROSS SEASONAL INHERITANCE AND IMPACT OF AMBIENT WATER MICROBIOTA ON THE GUT MICROBIOTA OF *RHINOGOBIO CYLINDRICUS* GÜNTHER

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Abstract. The gut microbiota (GM) participates in various physiological processes in fish. Although there are many host- and environment-related factors affecting the composition of fish GM, there are relatively few reports on the inheritance of fish GM across seasons and the impact of environmental water microbiota (WM) on fish GM. Here, we aimed to identify the differences in the composition of *Rhinogobio cylindricus* GM (RGM) in autumn and summer, and to determine how the summer RGM affected the autumn RGM. Samples of R. cylindricus were collected in summer and autumn and the composition of GM was analyzed through high-throughput sequencing of 16S rDNA. Our results showed that the alpha diversity indices of RGM in autumn were significantly higher than those in summer. The RGM collected in summer and autumn showed significant differences. The relative abundances of the most dominant operational taxonomic units (OTUs) were significantly different between the summer and autumn RGM samples. The proportions of OTUs in autumn RGM from summer RGM were significantly lower than those in WM. Moreover, there was no sampling site difference in the proportion of RGM compared with that of WM. Our results provide important insight into understanding the maintenance mechanisms of RGM.
Keywords: *composition, dispersal limitation, habitat, inheritance, operational taxonomic unit, maintenance mechanism*

Introduction

The gut microbiota (GM) participates in various physiological processes, such as digestion (Ghanbari et al., 2015; Liu et al., 2016), growth (Li et al., 2019), metabolism (Butt and Volkoff, 2019), and immune response in fish (Galindo-Villegas et al., 2012; Stagaman et al., 2017). Although studies have established that the composition of GM is affected by many factors, such as habitat (Ni et al., 2012, 2014), feeding habits (Li et al., 2014), development (Li et al., 2017; Yukgehnaish et al., 2020), and host genetic variation (Li et al., 2014; Smith et al., 2015; Yukgehnaish et al., 2020), the underlying mechanisms are still unclear.

Rhinogobio cylindricus Günther is an endemic fish species in upper Yangtze River, widely distributed in the mainstream of the Yangtze River and its tributaries (Liu et al., 2012, 2019). *R. cylindricus* captured monthly from Yibin to Wanzhou river section of the upper Yangtze River from July 2010 to July 2012 showed that their main foods were algae, molluscs, and aquatic insects. In terms of quantity percentage, algae and molluscs

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are the majority (93.12%), whereas in terms of weight percentage, algae, molluscs, and aquatic insects account for the majority (78.38%) (Xiong et al., 2015). Historically, *R. cylindricus* is one of the most popular species in the Yangtze River region, and wild resources have decreased owing to overfishing, construction of hydraulic projects, and other anthropogenic influences (Liu et al., 2012). To protect wild *R. cylindricus*, its basic biology (Xiong, 2013), feeding habits (Xiong et al., 2013), population genetic diversity (Liu et al., 2012; Shao et al., 2013), population parameters and resources (Xiong et al., 2015), and morphological characteristics (Wang et al., 2012) have been studied. We analyzed the composition of *R. cylindricus* GM (RGM) collected from four different sampling sites in the upper Yangtze River and found that there were no significant differences in RGM among different sampling sites, although significant differences were noted in their habitat water microbiota (WM) (Chen et al., 2021).

Considering the important roles of GM in various fish physiological processes, maintaining the stability of the GM composition and clarifying the factors affecting its composition are of great significance for protecting wild *R. cylindricus*. Since there are seasonal differences in the food composition of *R. cylindricus* (Xiong et al., 2013), which are important factors affecting fish GM (Ni et al., 2014; Wang et al., 2018; Li et al., 2019), we speculated that there were significant differences in the composition of GM between autumn and summer. However, the extent to which the autumn RGM was inherited from the summer GM remains unclear. To identify any difference in the composition of RGM in autumn and summer, and to determine how the summer RGM affected the autumn RGM, we collected samples of *R. cylindricus* in summer and autumn and analyzed the composition of GM through high-throughput sequencing of 16S rDNA. Our results provide an important insight into the maintenance mechanisms of a wide range of RGM.

Materials and Methods

Sampling area and sample collection

R. cylindricus samples were collected from Mudong (29.577 °N, 106.843 °E), Jiangjin (29.348 °N, 106.429 °E), and Heijiang (28.805 °N, 105.843 °N) in June and July (summer) of 2019 and October and November (autumn) of 2020 (*Fig. 1*), as previously described (Chen et al., 2021). The total length and body length of each sample were measured using a vernier caliper, and the body weight was weighed using an electronic balance.

DNA extraction and high-throughput sequencing

Gut microbial DNA was extracted using a PowerSoil DNA extraction kit (QIAGEN, Hilden, Germany). The V4-V5 hypervariable region of the prokaryotic 16S rDNA was amplified using primers 515F and 909R, as previously described (Xiang et al., 2018). Polymerase chain reaction (PCR) was performed and the amplicons were sequenced using a HiSeq system (Illumina, USA) at Guangdong Meilikang Bio-Science, Ltd. (Dongguan, China), as previously described (Ni et al., 2019; Chen et al., 2020).

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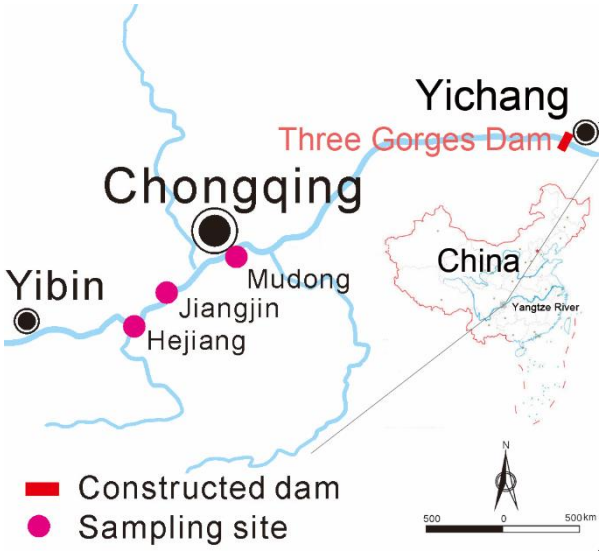

Figure 1. *Distribution of sampling sites*

The raw sequences were merged and quality-controlled and chimeric sequences were removed as previously described using FLASH version 1.2.8 (Magoč and Salzberg, 2011), QIIME 1.9.0 (Caporaso et al., 2010), and UCHIME 4.2.40 (Edgar et al., 2011), respectively. The remaining sequences were clustered into operational taxonomic units (OTUs) at 97% identity using UPARSE version 7.0.1090 (Edgar, 2013). Taxonomic assignment of each OTU was conducted using RDP classifier 2.2 (Wang et al., 2007) with the Greengene gg_13_8_otus dataset.

Merged sequences were deposited in the NCBI Sequence Read Archive database with accession number PRJNA824943.

Data analysis

Data are presented as the mean ± standard error for each group. The Wilcoxon rank-sum and Kruskal-Wallis tests were conducted using R version 4.0.3 (R Core Team, 2020). Permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2001) was used to test the significance of the differences between groups using the R vegan package (Dixon, 2003). Principal co-ordinates analysis (PCoA) based on weighted Unifrac distance was conducted using QIIME 1.9.0. Boxplots were constructed using the ggpubr R package (https://www.rdocumentation.org/packages/ggpubr/versions/0.4.0). Microbial source tracking analysis was conducted using SourceTracker (Knights et al., 2011). Statistical significance was set at $p < 0.05$.

Results

A total of 77 summer samples and 34 autumn samples were collected and measured total length, body length, and body weight. The total length (Wilcoxon test, $\chi 2 = 13.755$, $p < 0.001$; *Fig. 2A*), body length (Wilcoxon test, $\chi 2 = 14.784$, $p < 0.001$; *Fig. 2B*), and body weight (Wilcoxon test, $\chi 2 = 7.353$, $p = 0.007$; *Fig. 2C*) of autumn *R. cylindricus* samples were significantly larger than those of summer samples.

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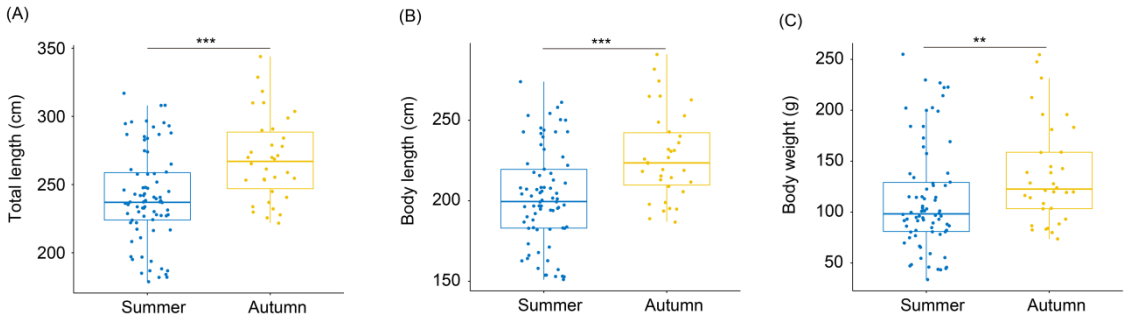

Figure 2. *Wilcoxon tests of total length (A), body length (B), and body weight (C) of R. cylindricus samples collected in summer and autumn. **, $p < 0.01$; ***, $p < 0.001$*

A total of 112 samples (33, 23, and 22 samples collected from Hejiang, Jiangjin, and Mudong in summer, and 6, 13, and 15 samples collected from Hejiang, Jiangjin, and Mudong in autumn, respectively) were anlyzed their GM composition. After sequence quality control and chimera removal, 21,236 sequences were randomly re-sampled from each sample for subsequent analyses. A total of 34,949 OTUs were detected in the samples. The alpha-diversity indices of RGM in autumn were significantly higher than those in summer (Wilcoxon rank sum test, $p < 0.001$ for OTU number, Shannon, Simpson, and Chao1 indices; *Fig. 3A-3D*). Therefore, the coverage of sequencing of RGM in autumn was significantly lower than that in summer (Wilcoxon rank-sum test, $p < 0.001$; *Fig. 3E*). In summer, the OTU number and Chao1 index of RGM collected from Hejiang were significantly higher than those at other sampling sites (Kruskal-Wallis rank sum test, $p < 0.001$; *Fig. 3A and 3D*), whereas the OTU number and Chao1 index of RGM collected from Hejiang were significantly lower than those collected from Jiangjin and Mudong (Kruskal-Wallis rank sum test, $p < 0.05$; *Fig. 3A and 3D*). The alpha-diversity indices of autumn WM were between those of the RGM collected in summer and autumn (*Fig. 3A-3D*).

PCoA results based on weighted UniFrac distances also showed significant differences in RGM collected in summer and autumn (PERMANOVA, $F = 64.265$, $p = 0.005$; *Fig. 3F*), and they were significantly different from the WM composition (PERMANOVA, $F = 80.392$, $p = 0.005$; *Fig. 3F*). However, the composition of the RGM collected at different sampling sites was not significantly different in autumn (PERMANOVA, $F = 1.372$, $p = 0.090$), although the autumn WM composition among sampling sites was significantly different (PERMANOVA, $F = 2.214$, $p = 0.005$).

Except for a few OTUs whose phylum could not be determined, the other OTUs were divided into 78 prokaryotic phyla, among which AC1, Acidobacteria, Actinobacteria, Bacteroidetes, Chlorobi, Chloroflexi, Cyanobacteria, Elusimicrobia, Firmicutes, Fusobacteria, Gemmatimonadetes, KSB3, Nitrospirae, OP3, OP8, Planctomycetes, Proteobacteria, Spirochaetes, Tenericutes, WS3, and Thermi dominated the microbiota (*Fig. 3G*). The relative abundances of the dominant phyla in RGM were significantly different between the summer and autumn samples. The relative abundances of Firmicutes, Fusobacteria, Proteobacteria, and Tenericutes in the summer samples were significantly higher than those in the autumn samples, whereas those of AC1, Acidobacteria, Bacteroidetes, Chlorobi, Chloroflexi, Cyanobacteria, Elusimicrobia, Gemmatimonadetes, KSB3, Nitrospirae, OP3, OP8, Planctomycetes, Spirochaetes, and WS3 in the autumn samples were significantly higher than those in the summer samples

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(*Appendix1*). The relative abundances of the dominant phyla in the autumn WM were also significantly different from those in the autumn RGM (*Appendix 1*).

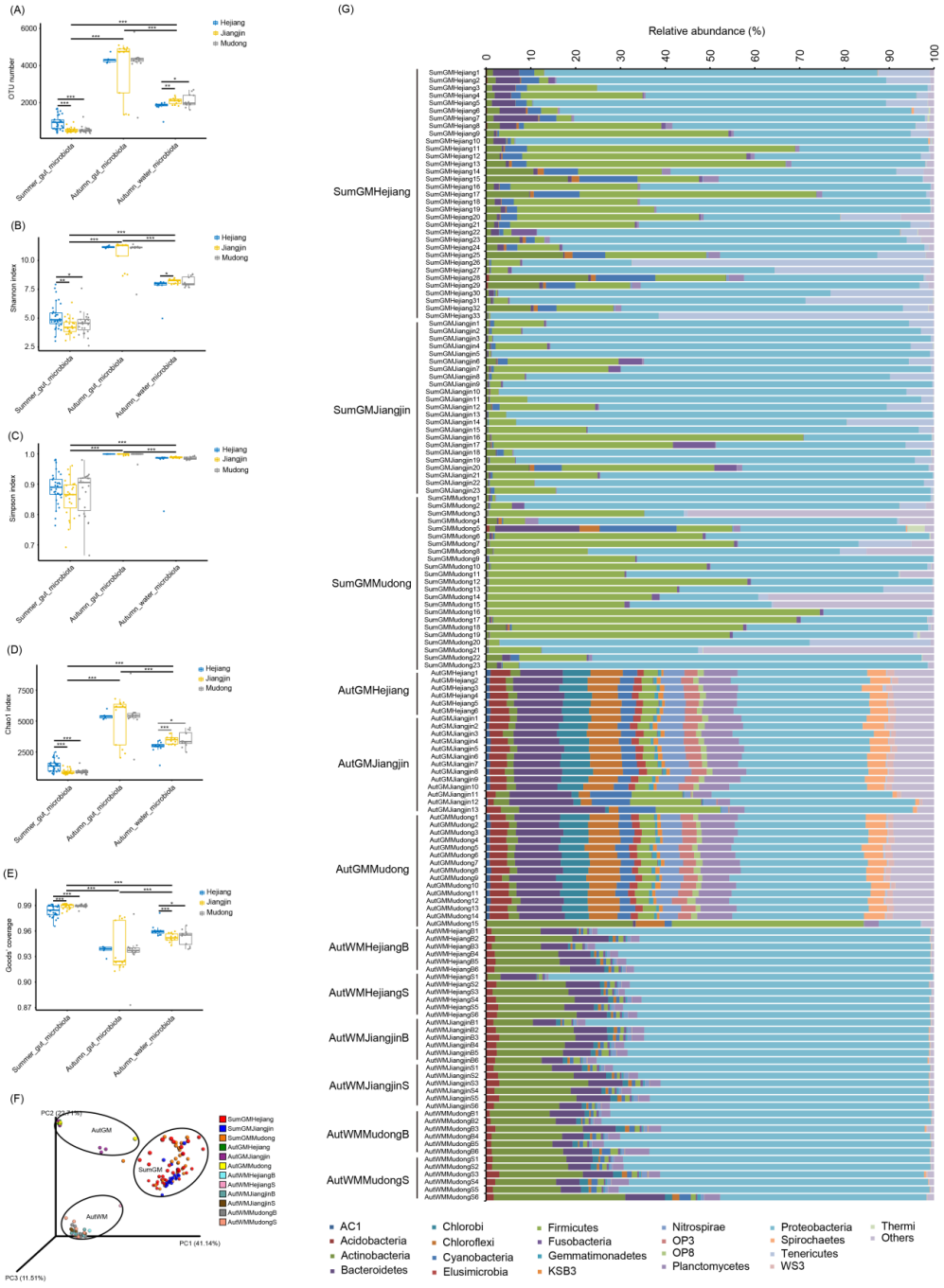

Figure 3. *Alpha-diversity indices and composition of Rhinogobio cylindricus gut microbiota and ambient water microbiota. (A), OTU number; (B), Shannon index; (C), Simpson index; (D), Chao1 index; (E), Good's coverage; (F), PCoA profile showed composition changes of R. cylindricus gut microbiota and ambient water microbiota; (G), dominant phylum composition of R. cylindricus gut microbiota and ambient water microbiota. SumGM, gut microbiota of Rhinogobio cylindricus collected in summer; WinGM, gut microbiota of Rhinogobio cylindricus collected in autumn; and WinWM, ambient water microbiota collected in autumn. *, p < 0.05; **, p < 0.01; ***, p < 0.001*

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LEfSe results showed that the relative abundances of the most dominant OTUs were significantly different between the summer and autumn *R. cylindricus* samples (*Fig. 4A*), whereas at the sampling time, the relative abundance of only a few dominant genera exhibited significant differences among sampling sites (*Fig. 4B*). Among the dominant genera that could be identified to the genus level, *Phormidium*, *Enterococcus*, *Lactococcus*, *Epulopiscium*, *Clostridium*, *Ochrobactrum*, *Sphingomonas*, *Ralstonia*, *Aeromonas*, *Escherichia*, *Plesiomonas*, *Vibrio*, *Stenotrophomonas*, *Mycoplasma*, *Deinococcus*, and *Mycobacterium* significantly enriched in the summer RGM; *Synechococcus*, *Ruminococcus*, *Oscillospira*, *Nitrospira*, *GOUTA19*, *LCP_6*, *Crenothrix*, *Halomonas*, *Treponema*, and *Bacteroides* significantly enriched in autumn RGM; whereas *Planctomyces*, *Rhodobacter*, *Aquabactenum*, *Comamonas*, *Hydrogenophaga*, *Limnohabitans*, *Rhodoferax*, *Polynucleobacter*, *Methylotenera*, *Acinetobacter*, *Perlucidibaca*, *Pseudoalteromonas*, and *Flavobacterium* significantly enriched in autumn WM (*Fig. 4A*). For autumn *R. cylindricus* GM, only LCP_6 and *Ralstonia* significantly enriched in Jiangjin, whereas *Bacillus* and *Hyphomicrobium* significantly enriched in Mudong (*Fig. 4B*).

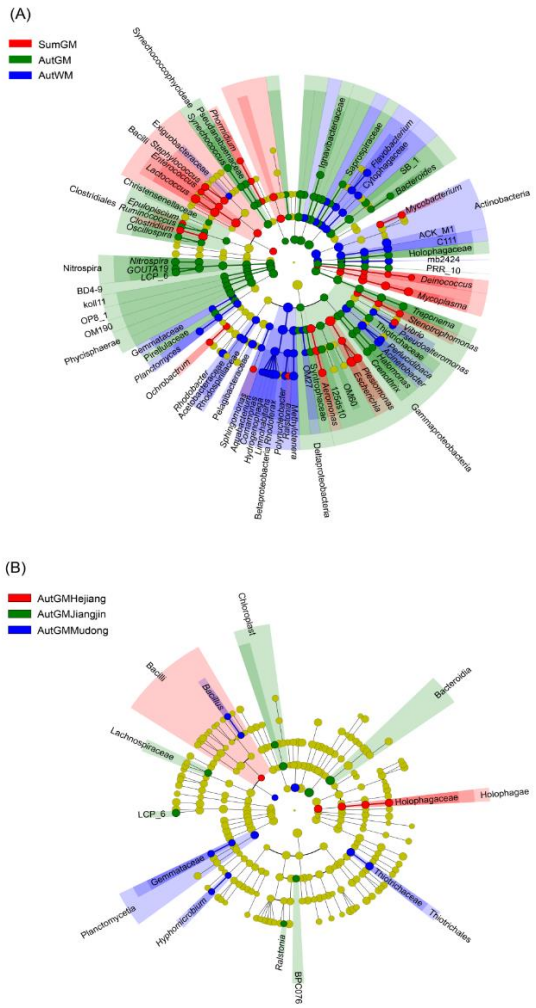

Figure 4. *Cladogram plots emerged LEfSe results. (A) different genera among SumGM, AutGM, and AutWM; (B) different genera of R. cylindricus gut microbiota at different sampling sites*

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Source tracking results showed that only a small number of OTUs in autumn RGM were from summer RGM and were significantly fewer than those from autumn WM (Wilcoxon test, $p < 0.001$; *Fig. 5* and *Appendix 2*). Only 0.45 ± 0.10%, 0.53 ± 0.06%, and 0.79 ± 0.06% OTUs in autumn RGM collected from Hejiang were from summer RGM collected from Hejiang, Jiangjin, and Mudong, respectively (*Fig. 5*). Only 4.71 ± 2.28%, 3.42 ± 1.66%, and 4.32 ± 2.02% OTUs in autumn RGM collected from Jiangjin were from summer RGM collected from Hejiang, Jiangjin, and Mudong, respectively (*Fig. 5*). Only 1.93 ± 1.22%, 1.27 ± 0.90%, and 0.96 ± 0.25% OTUs in autumn RGM collected from Mudong were from summer RGM collected from Hejiang, Jiangjin, and Mudong, respectively (*Fig. 5*). There were no significant differences in the proportions of OTUs from different sampling sites in the autumn RGM (Wilcoxon test, $p > 0.05$). The OTU proportion of RGM in the autumn from summer RGM collected from Jiangjin was significantly higher than that in the *R. cylindricus* samples collected from the other two sampling sites (Kruskal-Wallis test, $\chi 2 = 13.473$, $p = 0.001$; *Fig. 5* and *Appendix 2*). The OTU proportions of autumn RGM collected from Hejiang were 6.08 ± 0.33% and 5.65 ± 0.37% from surface and bottom WM, respectively (*Fig. 5*). The OTU proportions of autumn RGM collected from Jiangjin were 5.88 ± 0.58% and 6.36 ± 0.57% from surface and bottom WM, respectively (*Fig. 5*). The OTU proportions of autumn RGM collected from Mudong were 5.41 ± 0.39% and 5.66 ± 0.36% from surface and bottom WM, respectively (*Fig. 5*). The OTU proportions of autumn RGM from the surface and bottom WM were not significantly different (Wilcoxon test, $p > 0.05$; *Fig. 5* and *Appendix 2*). These results indicate that the proportions of OTUs in autumn RGM from summer RGM were significantly lower than those from habitat WM. Moreover, there was no sampling site difference in the proportion of RGM from WM.

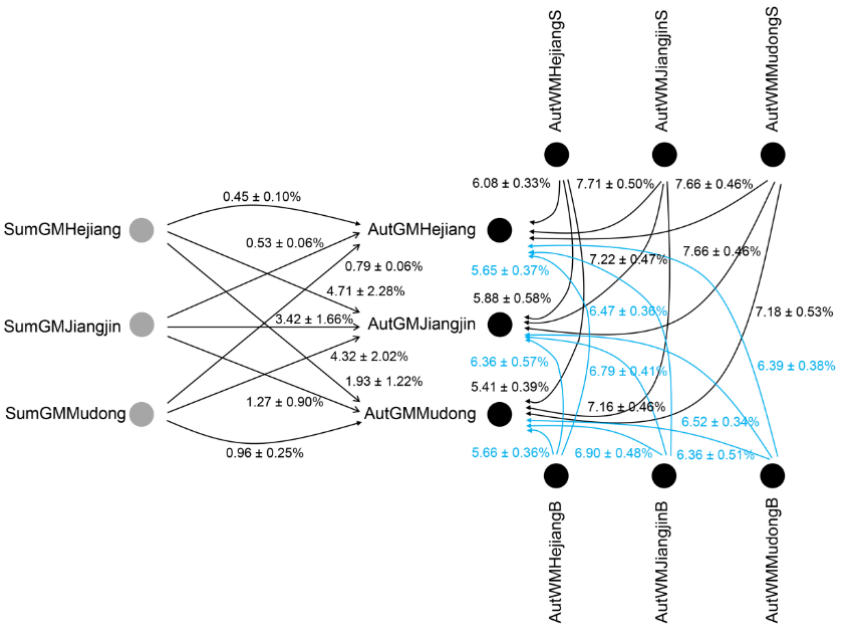

Figure 5. *Proportions of autumn R. cylindricus gut microbiota from summer R. cylindricus gut and autumn water microbiota. The proportions were calculated using SourceTracker script. Light blue indicates the proportions of autumn R. cylindricus gut microbiota from bottom water microbiota*

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Discussion

GM plays important roles in fish growth, development, immunity, and health (Pérez et al., 2010; Galindo-Villegas et al., 2012; Stagaman et al., 2017; Xiong et al., 2019). Dysbiosis of the GM often leads to diseases in fish (Nie et al., 2017; He et al., 2017). Therefore, understanding the maintenance mechanism and influencing factors of fish GM not only has important ecological and theoretical value but is also significant for its production (Liu et al., 2021). At present, it has been confirmed that habitat (Ni et al., 2012, 2014; Kuang et al., 2020; Kim et al., 2021), feeding habit (Li et al., 2014), development (Yan et al., 2016; Li et al., 2017), season (Tarnecki et al., 2017; Egerton et al., 2018), and species (Li et al., 2014; Huang et al., 2020) affect the composition of fish GM. Our results showed that RGM composition collected in summer and autumn exhibited significant differences, indicating that there were significant seasonal differences in the composition of RGM (*Fig. 3F*). This may be due to differences in the dietary niche breadth and consumed detritus of R. cylindricus between summer and autumn (Liu et al., 2019).

Although there are many host- and environment-related factors affecting the composition of fish GM, there are relatively few reports on the its inheritance across seasons, and the impact of environmental WM on fish GM. Liu et al. (2021) reported that 12.69 ± 3.63% OTUs of largemouth bass (*Micropterus salmoides*) GM came from ambient WM in ponds, and the proportion increased with an increase in culture time. They also found that the proportion of ambient sediment microbiota was 7.03 ± 3.47%. Our results showed that 5.41% to 7.71% of the OTUs of autumn RGM came from ambient WM, and less than 5% of the OTUs of autumn RGM came from summer RGM (*Fig. 5*). These results showed that RGM underwent reconstruction greatly from summer to autumn, which might be caused by the significant decrease in water temperature upstream of the Yangtze River and changes in the food composition of *R. cylindricus* after autumn.

Fish GM contains a variety of opportunistic pathogens such as *Aeromonas*, *Flavobacterium*, and *Vibrio* (Ni et al., 2012; Derome et al., 2016; Xiong et al., 2019; Emam et al., 2019); these pathogens were the dominant genera identified in this study. Moreover, *Aeromonas* and *Vibrio* were significantly enriched in summer RGM, whereas *Flavobacterium* was significantly enriched in autumn WM. *Clostridium* species have been reported to attenuate inflammation and allergic diseases effectively owing to their distinctive biological activities (Guo et al., 2020), and *Clostridium* was significantly enriched in summer RGM. These results imply that *R. cylindricus* has a higher risk of bacterial diseases caused by opportunistic pathogens from GM in summer than in autumn, which is consistent with the fact that fish are more prone to bacterial diseases in summer (Toranzo et al., 2005; Gauger et al., 2006; Marcos-López et al., 2010; Loch and Faisal, 2015).

Although we found that RGM was significantly different in summer and autumn, and the proportion of bacteria in autumn RGM from autumn WM was significantly higher than that from summer RGM in this study. We did not clarify the entire changing process of RGM from summer to autumn because of the large sampling time interval. Additionally, owing to the limitations tied to sampling, we did not study the annual change pattern of the RGM. These issues require further investigation.

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Conclusions

The alpha-diversity indices of the RGM in autumn were significantly higher than those in summer. The RGM collected in summer and autumn showed significant differences. The relative abundances of the most dominant OTUs were significantly different between summer and autumn RGM. The proportions of OTUs in autumn RGM from summer RGM were significantly lower than those in habitat WM. Moreover, there was no sampling site difference in the proportion of RGM compared with that of WM. These results implied that RGM had weak cross seasonal inheritance, and was more affected by ambient water microbiota than cross seasonal inheritance.

Ethical approval. All experimental protocols were approved by the Ethics Committee of the Institute of Hydroecology, Ministry of Water Resources, and the Chinese Academy of Sciences (approval number IHE[2019]030001). Fishing for studies was approved by the local fishery administrations of the Department of Agriculture Affairs of Sichuan province and Chongqing city.

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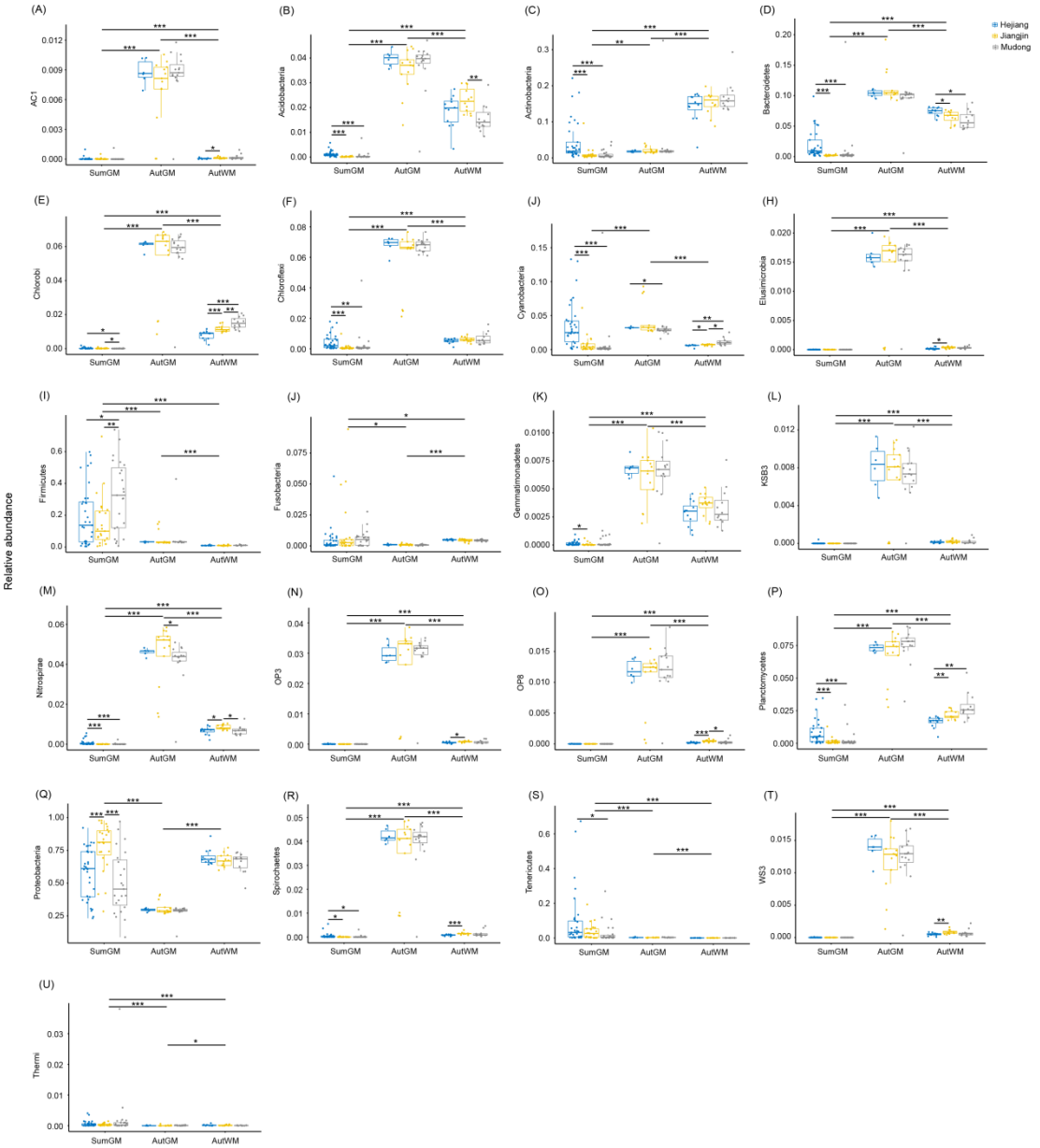
APPENDIX



Appendix 1. *Changes of dominant phyla of Rhinogobio cylindricus gut microbiota and ambient water microbiota. SumGM, gut microbiota of R. cylindricus collected in summer; WinGM, gut microbiota of R. cylindricus collected in autumn; and WinWM, ambient water microbiota collected in autumn. *, p < 0.05; **, p < 0.01; ***, p < 0.001*

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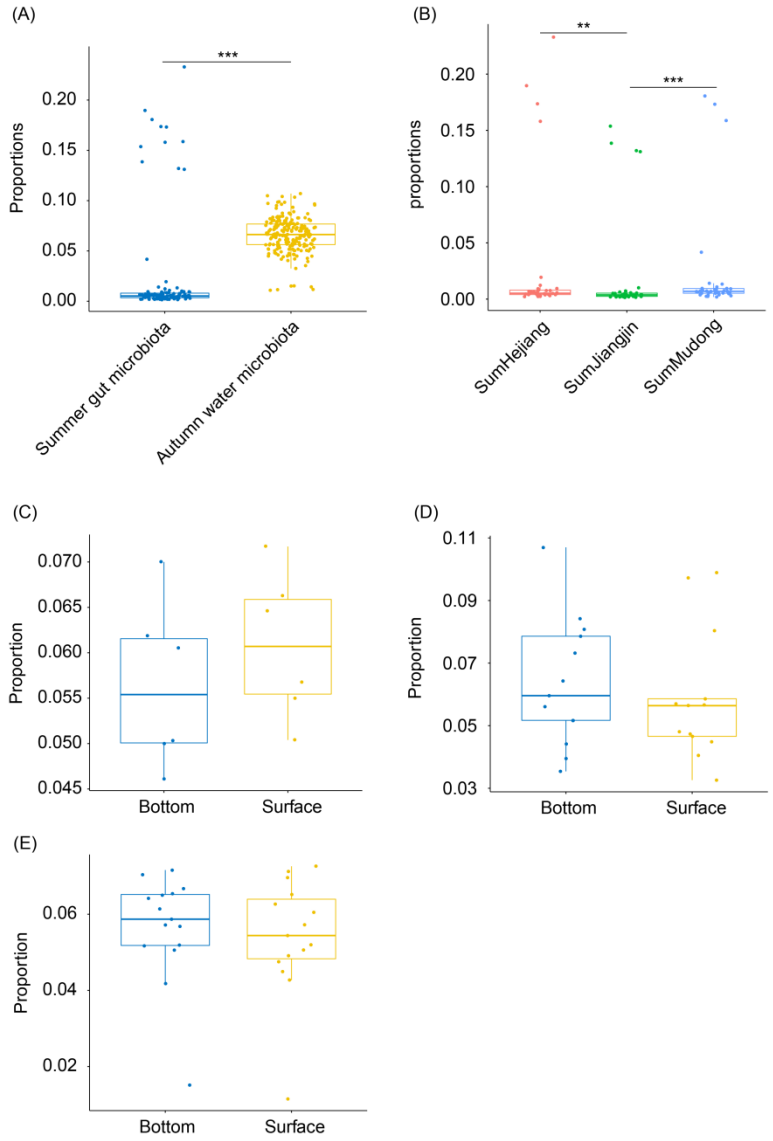

Appendix 2. *Proportion of autumn R. cylindricus gut microbiota (RGM) from summer RGM and autumn water microbiota. (A) Proportion of autumn RGM from summer RGM and autumn water microbiota, (B) Proportion of autumn RGM from summer RGM collected from different sampling sites, (C) Proportion of autumn RGM from autumn water microbiota collected from Hejiang, (D) Proportion of autumn RGM from autumn water microbiota collected from Jiangjin, and (E) Proportion of autumn RGM from autumn water microbiota collected from Mudong. **, $p < 0.01$; ***, $p < 0.001$*

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