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Metagenomic analysis reveals hidden links between gut microbes and habitat adaptation among cave and surface dwelling *Sinocyclocheilus* species

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ABSTRACT

Intestinal microbes are closely related to vital host functions such as digestion and nutrient absorption, which play important roles in enhancing host adaptability. As a natural “laboratory”, caves provide an outstanding model for understanding the significance of gut microbes and feeding habits in the habitat adaptability of hosts. However, research on the relationship between gut microbes, feeding habits, and the adaptability of troglobites remains insufficient. In this study, we compared the characteristics of the intestinal microbes of *Sinocyclocheilus* cavefish and surface fish and further established the relationship between intestinal and habitat microbes. Furthermore, we conducted environmental DNA (eDNA) (metabarcoding) analysis of environmental samples to clarify the composition of potential food resources in the habitats of the *Sinocyclocheilus* cavefish and surface fish. Results showed that the structure of the *Sinocyclocheilus* gut microbes was more related to ecological type (habitat type) than phylogenetic relationships. While horizontal transfer of habitat microbes was a source of gut microbes, hosts also showed strong selection for inherent microbes as dominant microorganisms. Differences in the composition and structure of gut microbes, especially dominant microbes, may enhance the adaptability of the two *Sinocyclocheilus* fish types from the perspectives of food intake, nutrient utilization, and harmful substance metabolism, suggesting that food resources, predation patterns, intestinal flora, digestive and absorptive capacity, and feeding habits and preferences are linked to habitat adaptability. These results should facilitate our understanding of the significance of fish gut microbes to habitat adaptation and provide a new perspective for studying the adaptive mechanisms of cavefish.

Keywords: Cavefish; Intestinal microbes; *Sinocyclocheilus*; Adaptive mechanism

INTRODUCTION

Considered the “second genome” of animals, intestinal microbes are closely related to host nutrient absorption, immunity, and development and have been studied in many fish species (Nikouli et al., 2021). The colonization of fish gut microbes is associated with multiple internal (e.g., feeding habits, intestinal structure, and phylogeny) and external factors (e.g., environmental microbes and water pH). Feeding habits, habitat microbes, and phylogeny are three major factors that determine the composition and structure of intestinal microbes in fish (Bakke et al., 2015; Rawls et al., 2004; Stephens et al., 2016; Sylvain et al., 2020). These intestinal microbes are mainly divided into “transient” and “adherent” types. In the juvenile stage, “transient” microbes may be indirectly obtained by fish (i.e., horizontally transferred) from habitat water when osmotic pressure is maintained (Reid et al., 2009). After ingestion, intestinal microbial structure and composition stabilize and widely converge according to feeding habit (specialized) (Ingerslev et al., 2014; Li et al., 2014). Moreover, gut microbes adjust with changes in host feeding preference and habitat factors and are considered an important parameter for fish feeding (energy intake) and habitat adaptation (Sullam et al., 2015).

The wild freshwater teleost genus *Sinocyclocheilus* (Cypriniformes: Cyprinidae) exhibits high species diversity and contains both cavefish and surface fish morphotypes (Yang et al., 2016). *Sinocyclocheilus* cavefish habitats are completely devoid of light, resulting in a lack of photosynthesis and limited food resources (Monro et al., 2018), whereas the surface habitats are mostly open water with strong sunlight, resulting in abundant food resources. Interestingly, we

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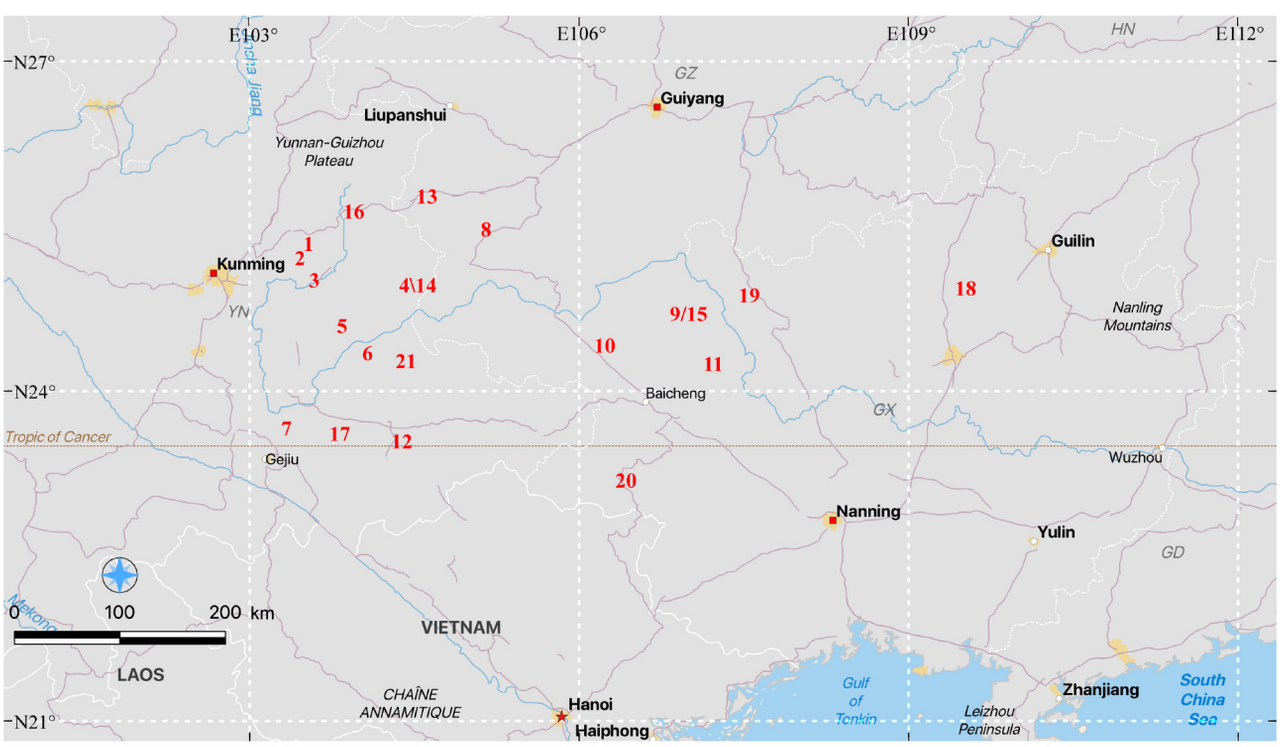
previously found that cavefish contain a higher gut microbial diversity than surface fish (Chen et al, 2020), although the reason why was unclear. More recent study has shown that the gut microbiome of *Sinocyclocheilus* fish can be affected by habitat water conditions, such as temperature, pH, and dissolved oxygen concentration (Zhou et al., 2022). Furthermore, half of the microorganisms found in the gut of cave-dwelling *Sinocyclocheilus* fish are associated with microorganisms in habitat water (Zhou et al., 2022). Thus, habitat appears to play a significant role in the structure and composition of the gut microbiome in *Sinocyclocheilus* fish. However, due to the limited number of species examined and inadequate exploration of food resources and habitat microbiome, the underlying mechanisms can only be inferred as being associated with fish feeding habits. Accordingly, this study sought to address the following questions: (1) Is the habitat microbiome the primary driver of differences in gut microbes between surface fish and cavefish? (2) Does the habitat microbiome or feeding habits account for the higher gut microbial diversity in cavefish? (3) What is the role of intestinal microflora in the habitat adaptation of the two *Sinocyclocheilus* fish types?

To explore the characteristics of intestinal flora and habitat microbes in different *Sinocyclocheilus* fish types, we first conducted high-throughput sequencing of the 16S ribosomal RNA (rRNA) gene (V4–V5) of intestinal microorganisms from 20 *Sinocyclocheilus* species and environmental microbes from the habitats of eight *Sinocyclocheilus* fish. We then examined environmental samples of one *Sinocyclocheilus* sympatric group to conduct environmental DNA (eDNA) identification using barcode primers *COI* and *mat*K (metabarcoding). We aimed to (i) obtain and compare the characteristics of the intestinal microbes of *Sinocyclocheilus* cavefish and surface fish, (ii) establish the relationship between intestinal and habitat microbes, (iii) investigate the composition of potential food resources in habitats to infer the feeding habits of the two fish types, and (iv) clarify the significance of gut microbes and feeding habits in the habitat adaptability of the two fish types.

MATERIALS AND METHODS

Intestinal content sample collection

In this study, 20 representative *Sinocyclocheilus* fish species distributed in three provinces (Yunnan, Guizhou, and Guangxi) were selected (Figure 1). Sampling sites were characterized by distance from cities. To ensure that the intestinal contents were not spoiled or excreted during long-distance transportation, the contents were immediately collected and processed after fish capture on-site using a portable table, liquid nitrogen, alcohol lamp, absolute ethanol, rechargeable table lamp, and pre-sterilized tools. To prevent contamination during gut content extraction, we followed strict sterility regulations: (1) Before the operation, the portable table was wiped with alcohol, and the surrounding environment was



1. *Sinocyclocheilus malacopterus (ZHAN YI)*
2. *Sinocyclocheilus qujingensis*
3. *Sinocyclocheilus lateristritus*
4. *Sinocyclocheilus rhinocerous*
5. *Sinocyclocheilus aluensis*
6. *Sinocyclocheilus qiubeiensis*
7. *Sinocyclocheilus purpureus*
8. *Sinocyclocheilus bicornutus*
9. *Sinocyclocheilus microphthalmus*
10. *Sinocyclocheilus altishoulderus*
11. *Sinocyclocheilus furcodorsalis*
12. *Sinocyclocheilus maculatus*
13. *Sinocyclocheilus angularis*
14. *Sinocyclocheilus malacopterus(XIAO MING VILAGE)*
15. *Sinocyclocheilus lingyunensis*
16. *Sinocyclocheilus wumengshanensis*
17. *Sinocyclocheilus wenshanensis*
18. *Sinocyclocheilus ronganensis*
19. *Sinocyclocheilus macrophthalmus*
20. *Sinocyclocheilus jinxiensis*
21. *Sinocyclocheilus aquihornes*

Figure 1 Map of sampling sites for 20 *Sinocyclocheilus* species (Map was generated using QGIS v3.10.)

disinfected using an alcohol sprayer; (2) Due to the small size of the fish, the entire intestinal segment was excised, and the contents were extracted into sampling tubes by squeezing the intestines, avoiding contact with the external environment; (3) After collection, the intestinal contents were snap frozen and immediately stored in liquid nitrogen.

Given their scarcity, the collection of *Sinocyclocheilus* fish specimens was challenging. Furthermore, at least three biological replicates per species were required for a single sampling to avoid within-group differences (Table 1). Therefore, the sampling period lasted from 2016 to 2019 (last sampling year). The *Sinocyclocheilus* fish species used in this study were wild caught and no specific permissions were required. All animal experiments and procedures were conducted with the approval of the Ethics Committee of Yunnan University in accordance with local and international polices (Grant No: Ynucae 20190056) and with the support and approval of the local government.

Environmental sample collection

To mitigate the potential hazards involved in collecting environmental samples from *Sinocyclocheilus* fish habitats, the habitats of eight *Sinocyclocheilus* species were selected based on feasibility and representativeness to obtain environmental microorganisms (Table 2). Environmental material (water and soil) and fish samples were collected simultaneously to avoid spatiotemporal variation. In addition, the habitats of sympatric fish groups were targeted to collect water and soil at different depths in accordance with the ecological niches of the two fish types (Table 3).

DNA extraction, amplification, and sequencing

Genomic DNA of the gut microbial community of the *Sinocyclocheilus* fish was extracted from the intestinal content using a FastDNA® SPIN Kit (MP Biomedicals, USA) according to the manufacturer's instructions. After extraction, DNA was checked on 1% agarose gel and verified using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, USA) to ensure the required concentration and purity. The hypervariable V4–V5 region of the bacterial 16S rRNA gene was amplified using the primer pairs 515F (GTGCCAGC MGCCGCGG) and 907R (CCGTCAATTCMTTTRAGTTT) in an ABI GeneAmp® 9700 PCR Thermocycler (Applied Biosystems, USA). The PCR amplification of the 16S rRNA gene was performed as follows: initial denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s, extension at 72 °C for 45 s, and single extension at 72 °C for 10 min and 10 °C until termination. The PCR mixture contained 4 μL of 5×FastPfu buffer, 2 μL of 2.5 mmol/L dNTPs, 0.8 μL of each forward (5 μmol/L) and reverse primer (5 μmol/L), 0.4 μL of FastPfu DNA polymerase, 10 ng of template DNA, and up to 20 μL of ddH_2O. PCR was performed in triplicate. The PCR products were extracted from 2% agarose gel and purified using an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, USA) according to the manufacturer's instructions and quantified using a Quantus™ Fluorometer (Promega, USA). Thereafter, paired-end sequencing was conducted using the Illumina Miseq PE300 platform (Illumina, USA), and all data were deposited into the NCBI Sequence Read Archive (SRA) database (Accession Number: PRJNA896095; PRJNA542570 (this study) and Accession Number: SRP198202 (previous study)) (Chen et al., 2020).

A FastDNA SPIN Kit for Soil was used to extract eDNA from the habitat of the *S. rhinocerous* (XJ_sym)-*S. malacopterus* (RQ_sym) sympatric fish group. Two barcode genes (*CO1* and *mat*K) were amplified using primer pairs COIF (GGWACWGGWTGAACWGTWTAYCCYCC)-COIR (TANACYTCNGGRTGNCCRAARAAYCA) and *mat*K-XF (TAATTTACGATCAATTCATTC)-*matK*-MALPR (ACAAGAAAGTCGAAGTAT), respectively. PCR amplification conditions were set as follows: *COI*: initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 47 °C for 2 min, extension at 72 ℃ for 1 min, and single extension at 72 °C for 5 min and 4 °C until termination; *mat*K: initial denaturation at 94 °C for 3 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 48 °C for 40 s, extension at 72 °C for 1 min, and single extension at 72 °C for 10 min and 4 °C until termination. The PCR mixture contained 4 μL of 5×FastPfu buffer, 2 μL of 2.5 mmol/L dNTPs, 0.8 μL of each forward (5 μmol/L) and reverse primer (5 μmol/L), 0.4 μL of FastPfu DNA Polymerase, 10 ng of template DNA, and up to 20 μL of ddH_2O.

Bioinformatics analysis

We sequenced the different samples in batches and integrated the data for analysis. The raw 16S rRNA gene sequencing reads were demultiplexed, quality-filtered using fastp (v0.20.0), and merged using FLASH (v1.2.7). UPARSE (v7.1) was used to cluster operational taxonomic units (OTUs) under a 97% similarity cutoff and remove chimeric sequences. RDP Classifier (v2.2) was used for the taxonomic classification of OTUs against the Silva 138 database (BOLD database for barcode sequences (http://www.boldsystems.org/index.php/TaxBrowser_Home)) by rejecting sequences below 70% identity. The rarefaction curve and α-diversity index were plotted and calculated using Mothur (v1.40.5) and R software, respectively. The R statistics were tested for differences between groups. Kruskal-Wallis H and Wilcox tests were used for multi- and two-group tests, respectively. Venn diagrams of shared and unique OTUs or genera were calculated and constructed using R VennDiagram. Based on various distance algorithms (e.g., Jaccard and UniFrac), the R vegan and mixOmics packages were used to perform principal coordinate analysis (PCoA) and partial least squares discriminant analysis (PLS-DA), respectively, and the significance of the clusters was determined using permutational analysis of variance (PERMANOVA). Network analysis with NetworkX was used to calculate the correlation between the samples of each group. Phylogenetic investigation of communities by reconstruction of unobserved states2 (PICRUSt2) was used to predict the function and metabolic pathways of the OTUs. Multivariate association with linear models (MaAsLin2) was applied to explore the correlation between environmental food resources and sympatric fish group habitat (min_abundance: 0; min_prevalence: 0.1; max_significance: 0.25; normalization: TSS; transform: AST; analysis_method: default: correction: BH). Line, pie, and donut charts were constructed using GraphPad Prism (v8.0) (GraphPad Software, USA).

Phylogeny for representative *Sinocyclocheilus* species

To better understand the phylogenetic relationships among species within *Sinocyclocheilus* (including cavefish and surface fish) and representative species selected in this study, we analyzed the complete sequences of the mitochondrial cytochrome *b* (cyt *b*) genes of 46 recognized *Sinocyclocheilus* species and one outgroup species (*Barbodes laticeps*).

Table 1 Sampling details of each representative *Sinocyclocheilus* species

Species and abbreviation	Sampling time	Location	Type	Sample No.	Body length(cm)	Gender	Age
S.maculatus(MH)	August, 2016	N23°70′, E104°27′	Surface fish	MH01	12.00	Male	Adult
				MH02	11.50	Male	Adult
				MH03	11.00	Male	Adult
S.wenshanensis(WS)	January, 2019	N23°51′, E103°84′	Surface fish	WS01	11.80	Male	Adult
				WS02	12.30	Male	Adult
				WS03	10.10	Male	Adult
S.malacopterus(RQ_sym)	August, 2016	N24°77′, E104°28′	Surface fish	RQ_sym1	12.40	Male	Adult
				RQ_sym2	12.30	Male	Adult
				RQ_sym3	11.50	Male	Adult
S.malacopterus(RQ)	August, 2016	N25°40′, E103°56′	Surface fish	RQ01	12.20	Male	Adult
				RQ02	12.10	Male	Adult
				RQ03	12.40	Male	Adult
S.purpureus(ZS)	October, 2016	N23°45′, E103°35′	Surface fish	ZS01	7.50	Male	Adult
				ZS02	9.70	Male	Adult
				ZS03	12.00	Female	Adult
S.qiubeiensis(QB)	April, 2017	N24°05′, E104°13′	Surface fish	QB01	13.50	Male	Adult
				QB02	13.50	Male	Adult
				QB03	12.50	Male	Adult
S.wumengshanensis(WMS)	August, 2016	N26°00′, E104°35′	Surface fish	WMS01	12.60	Male	Adult
				WMS02	12.80	Male	Adult
				WMS03	11.20	Male	Adult
S.lateristritus(CT)	May, 2016	N25°00′, E103°59′	Surface fish	CT01	12.40	Male	Adult
				CT02	13.70	Male	Adult
				CT03	12.30	Male	Adult
S.aluensis(AL)	May, 2016	N24°53′, E103°76′	Surface fish	AL01	15.50	Male	Adult
				AL02	14.00	Male	Adult
				AL03	13.60	Male	Adult
S.qujingensis(QJ)	August, 2016	N25°27′, E103°48′	Surface fish	QJ01	15.00	Male	Adult
				QJ02	12.30	Male	Adult
				QJ03	13.00	Male	Adult
S.rhinocerous(XJ_sym)	August, 2016	N24°77′, E104°28′	Cavefish	XJ01	6.00	Male	Adult
				XJ02	5.10	Male	Adult
				XJ03	6.10	Male	Adult
S.aquihornes(YHJ)	April, 2017	N24°34′, E104°31′	Cavefish	YHJ01	8.20	Male	Adult
				YHJ02	8.10	Male	Adult
				YHJ03	8.60	Male	Adult
S.angularis(Jiao)	February, 2017	N25°24′, E104°44′	Cavefish	Jiao01	9.60	Male	Adult
				Jiao02	9.30	Male	Adult
				Jiao03	9.40	Male	Adult
S.bicornutus(SJ)	March, 2017	N25°29′, E105°14′	Cavefish	SJ01	13.60	Male	Adult
				SJ02	15.00	Male	Adult
				SJ03	14.00	Male	Adult
S.lingyunensis(LY_sym)	February, 2017	N24°25′, E106°36′	Cavefish	LY01	10.50	Male	Adult
				LY02	10.40	Male	Adult
				LY03	10.70	Male	Adult
S.macrophthalmus(DY)	January, 2016	N24°30′, E107°93′	Cavefish	DY01	11.00	Male	Adult
				DY02	11.40	Male	Adult
				DY03	11.20	Male	Adult
S.ronganensis(RA)	January, 2019	N24°99′, E109°46′	Cavefish	RA01	17.50	Male	Adult
				RA02	16.50	Male	Adult
				RA03	17.00	Male	Adult
S.microphthalmus(XY_sym)	February, 2017	N24°25′, E106°36′	Cavefish	XY01	10.50	Male	Adult
				XY02	10.20	Male	Adult
				XY03	13.00	Male	Adult
S.altishoulders(GJ)	February, 2017	N24°20′, E107°23′	Cavefish	GJ01	17.50	Male	Adult
				GJ02	17.00	Male	Adult
				GJ03	15.50	Male	Adult
S.furcodorsalis(CB)	February, 2017	N24°56′, E107°02′	Cavefish	CB01	14.00	Male	Adult
				CB02	13.90	Male	Adult
				CB03	14.50	Male	Adult
S.jinxiensis(JX)	October, 2016	N23°13′, E106°41′	Cavefish	JX01	16.70	Male	Adult
				JX02	16.30	Male	Adult
				JX03	16.90	Male	Adult

Table 2 Sampling details of representative *Sinocyclocheilus* habitats

Location	Species	pH	Altitude (a.s.l., m)	Type	Abbreviations
N23°51′, E103°84′	*S.wenshanensis*	6.50	1447.20	Surface fish	WS_en
N23°70′, E104°27′	*S.maculatus*	7.00	1538.20	Surface fish	MH_en
N24°34′, E104°31′	*S.aquihornes*	6.50	1250.80	Cavefish	YHJ_en
N24°20′, E107°23′	*S.altishoulders*	6.50	303.00	Cavefish	GJ_en
N24°99′, E109°46′	*S.ronganensis*	6.50	297.00	Cavefish	RA_en
N24°30′, E107°93′	*S.macrophthalmus*	6.70	172.00	Cavefish	DY_en
N24°25′, E106°36′	*S.lingyunensis* *S.microphthalmus*	6.50	258.40	Cavefish Cavefish Sympatry	LY_en XY_en en_XY_LY_sym
N24°77′, E104°28′	*S.rhinocerous* *S.malacopterus*	7.70	1470.80	Cavefish Surfacefish Sympatry	XJ_en_sym RQ_en_sym Table 3

Table 3 Sampling details of *Sinocyclocheilus rhinocerous* and *S. malacopterus* (sympatry) habitats (niches)

Species	Location	Water temperature (°C, Surface)	pH	Altitude (a.s.l., m)
S.rhinocerous *S.malacopterus*	N24°77′, E104°28′	23.2	7.7	1470.8
No.	Sample types	Sampling location	Degree of depth (m)	Niche
DW 01-05	Water	Deep_water	>5.00	*S.rhinocerous*
MW 01-05	Water	Middle_water	3.00–5.00	*S.rhinocerous*
SW 01-05	Water	Surface_water	<1.00	*S.malacopterus*
DS 01-06	Soil	Deep_soil	>5.00	*S.rhinocerous*
SS 01-06	Soil	Surface_soil	<1.00	*S.malacopterus*

Phylogenetic trees were reconstructed using cyt *b* with the Bayesian inference (BI) approach. The optimal nucleotide substitution model was selected using likelihood ratio tests in jModelTest (v2.17) (Darriba et al., 2012). Likelihood settings from the best-fit model (TrN+I+G) were selected based on Bayesian information criterion (BIC) with jModelTest (v2.1.7). The BI analyses were performed with MrBayes (v3.2.7) (Ronquist et al., 2012). Analyses were run for 1×10^7 generations with four Monte Carlo Markov chains (MCMC) and sampling of trees every 100 generations with a burn-in of 25%. Samples prior to reaching stationarity (25 000 trees) were discarded as burn-in and the remaining trees were used to generate a majority-rule consensus tree. A clade was considered to be strongly supported if the posterior probability was equal to or greater than 95% (Ronquist et al., 2012).

RESULTS

Intestinal microbial characteristics and phylogenetic relationships of *Sinocyclocheilus* cavefish and surface fish

High-throughput sequencing of the 16S ribosomal RNA gene (V4–V5) in intestinal microorganisms from 20 *Sinocyclocheilus* fish species was used to explore the characteristics of the intestinal microbes of the two fish types. A total of 1 246 790 102 bases were sequenced, 3 261 731 effective quality-filtered sequences were acquired from 63 samples, and 8 715 OTUs were identified (52 bacterial phyla, 121 classes, 335 orders, 619 families, 1 461 genera, and 3 051 species). The rarefaction curve showed that the sequencing quality was appropriate and sufficient (Supplementary Figure S1). The abundance and diversity indices of the cavefish were significantly higher than those of the surface fish. Among species, *S. bicornutus* (SJ) showed the highest abundance, followed by *S. malacopterus* (RQ) and *S. microphthalmus* (XY_sym) (Table 4).

The Bayesian tree phylogeny revealed that the *Sinocyclocheilus* genus was a monophyletic group, with *S. jii* at the most basal position, with all species, except for *S. jii* and *S. ronganensis*, clustered into six major monophyletic clades (I, II, III, IV, V, and VI) with strong support (Supplementary Figure S2). The cavefish species were polyphyletic and occurred in all six clades. These findings suggest that adaptation to cave environments has occurred at least six times during the evolutionary history of *Sinocyclocheilus*. Accordingly, intestinal microbe similarity between the two fish types was determined based on PCoA. The scatter plot showed that the two *Sinocyclocheilus* fish types could be separated into two clusters according to ecotype (binary_jaccard and unweighted_unifrac) and PERMANOVA-supported grouping (R^2>0.25 and P<0.05, respectively) (Figure 2A, B) although some samples showed a mixed pattern. The PLS-DA results were consistent with the PCoA findings, but fish type clustering showed greater convergence (Figure 2C). Interestingly, different from the phylogenetic relationships, although belonging to different types, sympatric *S. rhinocerous* (XJ_sym; cavefish in clade III) and *S. malacopterus* (RQ_sym; surface fish in clade VI) showed higher similarity than that of the same species collected from different habitats (RQ_sym and RQ). In addition, sympatric cavefish species *S. microphthalmus* (XY_sym; cavefish in clade IV) and *S. lingyunensis* (LY_sym; cavefish in clade I) were close to each other (Supplementary Figure S3).

The composition and structure of intestinal microbes of *Sinocyclocheilus* species at each classification level are shown in Supplementary Figure S4. At the phylum level, Proteobacteria, Firmicutes, Fusobacteria, Actinobacteria, and Planctomycetes exhibited relatively high abundance (average) in the intestines of both surface and cavefish species. However, the abundance of the top 10 phyla did not differ significantly between the two types of *Sinocyclocheilus* fish (Supplementary Figure S4E, F).

At the genus level (Figure 3A; Supplementary Figure S5),

Table 4 α-diversity indices between two *Sinocyclocheilus* fish types

Sample	Sobs index means	Shannon index means	Simpson index mean	Ace index mean	Chao index mean
AL	385.67	2.89	0.21	439.82	432.30
CB	836.33	5.06	0.03	1053.76	1071.09
CT	246.00	1.68	0.50	300.23	288.68
DY	282.33	0.77	0.80	399.80	385.19
GJ	1559.33	4.31	0.18	2134.69	2091.86
jiao	185.67	1.63	0.40	403.91	293.78
JX	212.00	2.36	0.19	334.45	324.54
LY_sym	1049.33	4.03	0.06	1238.07	1212.15
MH	144.33	2.92	0.15	199.30	197.93
QB	93.67	1.25	0.50	183.81	140.84
QJ	49.33	1.12	0.54	111.97	86.00
RA	231.33	2.61	0.14	394.07	369.54
RQ	1724.00	5.52	0.02	2526.50	2453.45
RQ_sym	130.33	1.62	0.35	201.00	185.39
SJ	2064.67	5.68	0.03	3918.49	3176.27
WMS	809.00	3.60	0.10	1035.88	1009.04
WS	1076.67	3.78	0.13	1890.67	1563.53
XJ_sym	870.33	3.60	0.17	1340.07	1162.52
XY_sym	1312.33	4.60	0.06	2467.01	1996.99
YHJ	172.33	1.65	0.41	419.32	302.66
ZS	432.67	2.38	0.34	552.16	538.35
Estimators	Cave-Mean	Cave-*Sd*	Surface-Mean	Surface-*Sd*	*P*-value
Sobs index	797.54	580.64	509.16	513.69	>0.05
ACE index	1282.14	981.87	739.46	787.93	>0.05
Chao index	1126.05	844.57	684.88	734.04	>0.05
Shannon index	3.30	3.29	2.64	1.30	<0.05
Simpson index	0.22	0.22	0.28	0.18	<0.05

For abbreviations see Table 1. *SD*: Standard deviation.

the dominant genera (average abundance>5%) in the intestines of cavefish and surface fish were *Cetobacterium*, *Bacillus*, *Enterobacter*, and *Aeromonas*. Among the top 10 genera, *Bacillus* showed a significant difference between the two fish types (Figure 3B). Furthermore, linear discriminant analysis effect size (LEfSe) (LDA>2.5; P<0.05) showed that *Bacillus*, *Ochrobactrum*, and *Paenibacillus* had the highest contribution to cavefish clustering, whereas *Lactococcus*, *Lactobacillus*, and *Serratia* had the highest contribution to surface fish clustering (Figure 3C).

Enrichment and comparison of the gut microbiome gene functions and metabolic pathways of the two fish types indicated higher enrichment in carbohydrate metabolism (including carbohydrate digestion and absorption, α-amylase, α-glucosidase, and cellulase) in the surface fish group than in the cavefish group, albeit not significantly. On the other hand, fat digestion and absorption were enriched in the cavefish intestinal microbes, with significantly higher "lipase" enrichment than found in the surface fish (Figure 4).

Characteristics of habitat microorganisms and their relationship with gut microbes

To characterize the environmental microbes in fish habitat and investigate the relationship between intestinal and habitat microbes, we performed high-throughput sequencing of the 16S ribosomal RNA gene (V4–V5) in microbes collected from the habitats of eight *Sinocyclocheilus* fish species. In total, 1 232 850 668 effective bases were sequenced, and 3 279 081 effective quality-filtered sequences were acquired from 48 samples. The rarefaction curve showed high sequencing quality (Supplementary Figure S1B). A total of 13 246 OTUs were identified, including 61 bacterial phyla, 151 classes, 436 orders, 772 families, 1 704 genera, and 4 163 species. The diversity indices (Sobs, Ace, and Chao indices) were significantly higher (P<0.05) in cavefish than in the surface fish (Table 5) and YHJ_en showed the highest abundance and diversity indices, followed by GJ_en and RA_en (Table 5). The habitat of the XJ_sym-RQ_sym sympatric fish group exhibited a higher α-diversity index in soil than in water, which increased with water depth (Table 6). Furthermore, a consistent trend in diversity and abundance indices was observed among environmental and intestinal microbes, except for YHJ and RA, indicating that fish living in high microbial α-diversity habitats (niches) also had higher gut microbe α-diversity. Based on PCoA and PLS-DA, the habitat and fish gut microbes could be divided into two clusters, without any mixing. The PERMANOVA results further supported the validity of the clustering (R^2>0.25; P<0.05) (Figure 5; Supplementary Figure S6). The composition and structure of the habitat microbes in *Sinocyclocheilus* species at each classification level are shown in Supplementary Figure S7. Proteobacteria was the dominant phylum, showing the highest proportion in all eight habitats (Supplementary Figure S7A, B). At the genus level, several genera with high abundance showed significant differences (P<0.05) between each habitat (Supplementary Figure S7C, D). For the habitat (niche) of the XJ_sym-RQ_sym sympatric fish group, each niche had a high proportion of Proteobacteria, Actinobacteria, and Bacteroidetes phyla (Supplementary Figure S7B). At the genus level, *Limnohabitans*, *unclassified_f_Burkholderiaceae*,

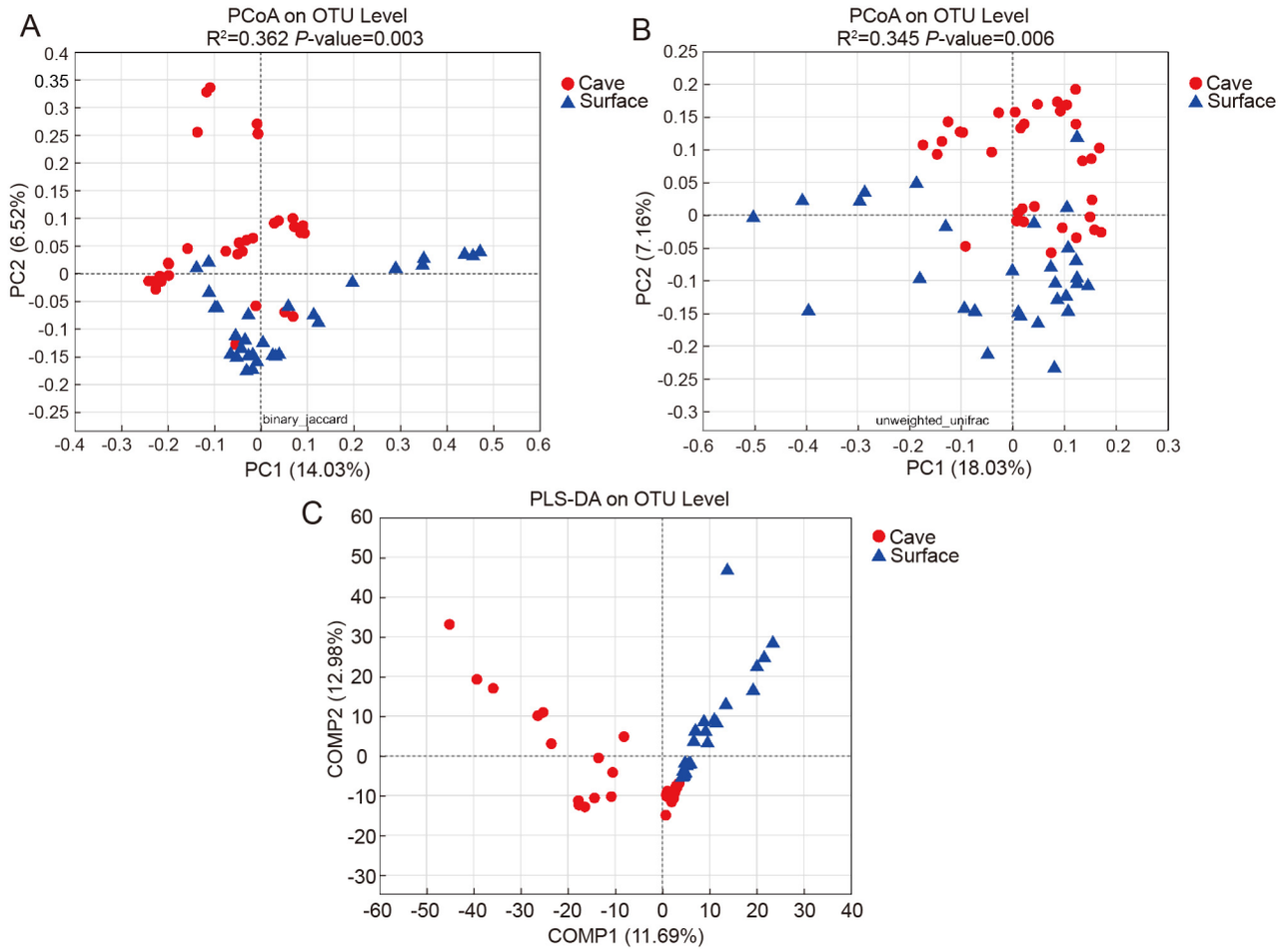


Figure 2 Inter-individual similarity in intestinal microbiota of *Sinocyclocheilus* cavefish and surface fish

A: PCoA of cavefish (cave) and surface fish groups (surface) by binary_jaccard distance. B: PCoA of cavefish (cave) and surface fish groups (surface) by unweighted_unifrac distance. C: PLS-DA of cavefish (cave) and surface fish groups (surface).

and *Bacillus* had higher average abundances, but showed significant differences between each niche sample (Supplementary Figure S7E, F). All fish gut samples shared microbes with their habitats, but these belonged to genera with low abundance (OTU>10). The number of OTUs shared between the gut and habitat decreased with increasing OTU threshold, with none shared at the threshold of the dominant genus level (OTU>200) (Supplementary Figures S8, S9).

Functional enrichment analysis of the fish habitat microbiome genes revealed that the most abundant level 1 function was “metabolism”, and the most abundant level 2 functions were “carbohydrate metabolism”, “global and overview maps”, and “amino acid metabolism”. A comparison of the microbiome gene functions between fish gut and habitat showed that the two groups exhibited significant differences in the abundance of several level 3 categories. In addition, 12 of the top 20 functions significantly differed between the fish gut (ALL_fish) and habitat (ALL_en) groups (P<0.05), with “biosynthesis of amino acids”, “carbon metabolism”, “oxidative phosphorylation”, and “carbon fixation pathways in prokaryotes” being significantly higher in the habitat group than in the intestinal group (Supplementary Figure S10).

Potential food resources in habitats (niches) of *Sinocyclocheilus* sympatric fish

To identify the composition of potential food resources in the cave and surface habitats (niches), we examined environmental samples (soil and water) from the habitats (niches) of the *Sinocyclocheilus* cavefish-surface fish sympatric group (*S. rhinocerous*-*S. malacopterus*) using high-throughput eDNA sequencing with barcode primers *COI* (animal) and *mat*K (plant). A total of 52 389 663 and 67 175 527 effective bases (168 147 and 195 174 sequences) were sequenced by *COI* and *mat*K, respectively. The rarefaction curve indicated the high quality of sequencing (Supplementary Figure S1C, D). The abundance and diversity indices of both resources were higher in soil than in water; deep water (D_W) and deep soil (D_S) showed the highest abundance and diversity, whereas surface water (S_W), surface soil (S_S), and middle water (M_W) showed relatively low abundance and diversity (Table 7).

Analysis of the animal and plant composition in each sample demonstrated that animal genera *Polyarthra*, *Holopsis*, and *Adenomera* and plant genera *Melosira* and *Cryptomonas* were most abundant in the water habitat, while animal genera *Tanytarsus* and *Aspidiophorus* and plant genera *Navicula* and *Pinnularia* were more abundant in soil (Supplementary Figure S11). The abundance of *Tanytarsus* and *Synchaeta* in soil, especially bottom soil (D_S), was significantly higher than that in water. *Holopsis*, *Adenomera*, *Obelia*, *Cryptomonas*, *Synura*, and *Placoneis* were only identified in water samples. Furthermore, several genera showed distinct niche particularity. For example, the abundance of *Polyarthra* gradually increased with increasing water depth, whereas that of *Holopsis*, *Obelia*, and *Sceptonia* decreased (Supplementary

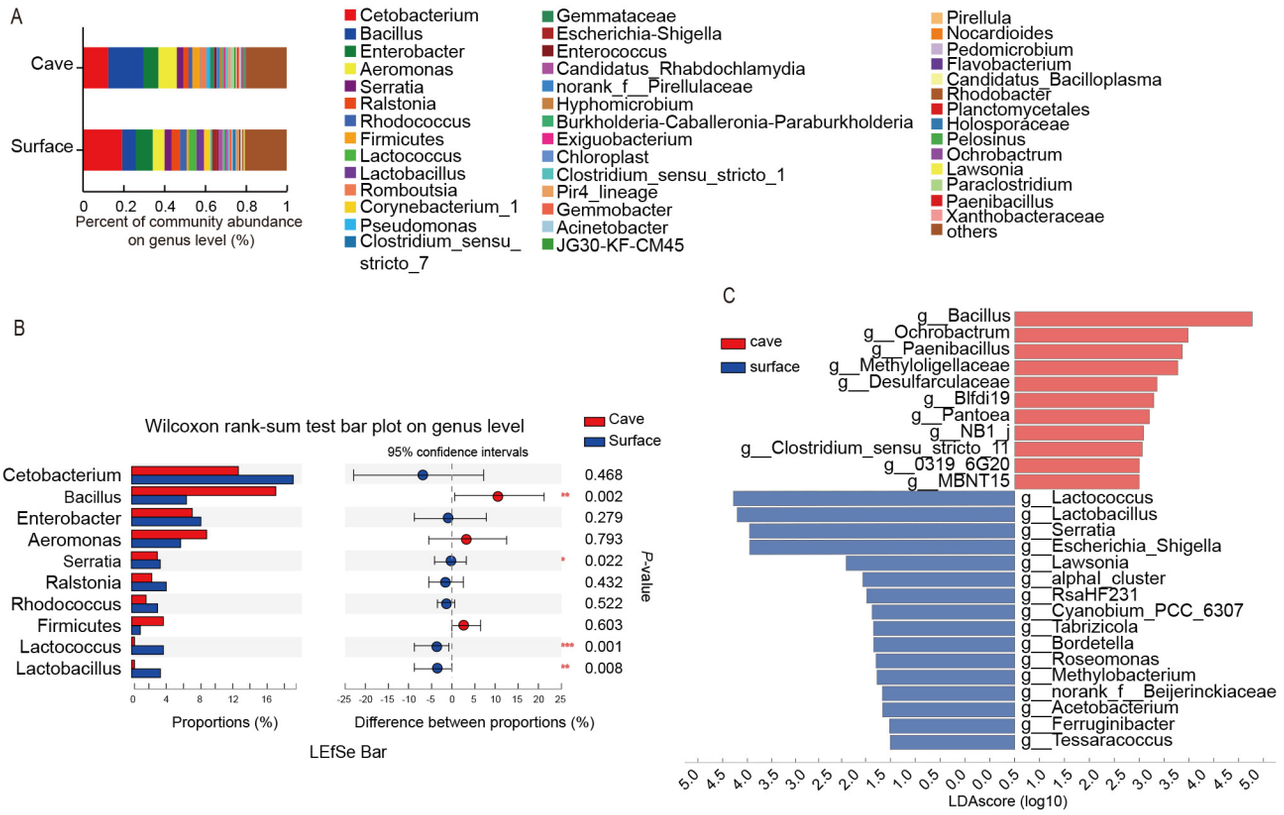


Figure 3 Composition of *Sinocyclocheilus* fish gut microbes at genus level

A: Compositions of gut microbiota communities of *Sinocyclocheilus* surface fish (surface) and cavefish groups (cave) at genus level. B: Differences in relative abundance of gut microbes (genus) between cave and surface fish species. *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$. C: Linear discriminant analysis effect size (LEfSe) of gut microbial composition in *Sinocyclocheilus* cave and surface fish groups (LDA>2.5, $P<0.05$).

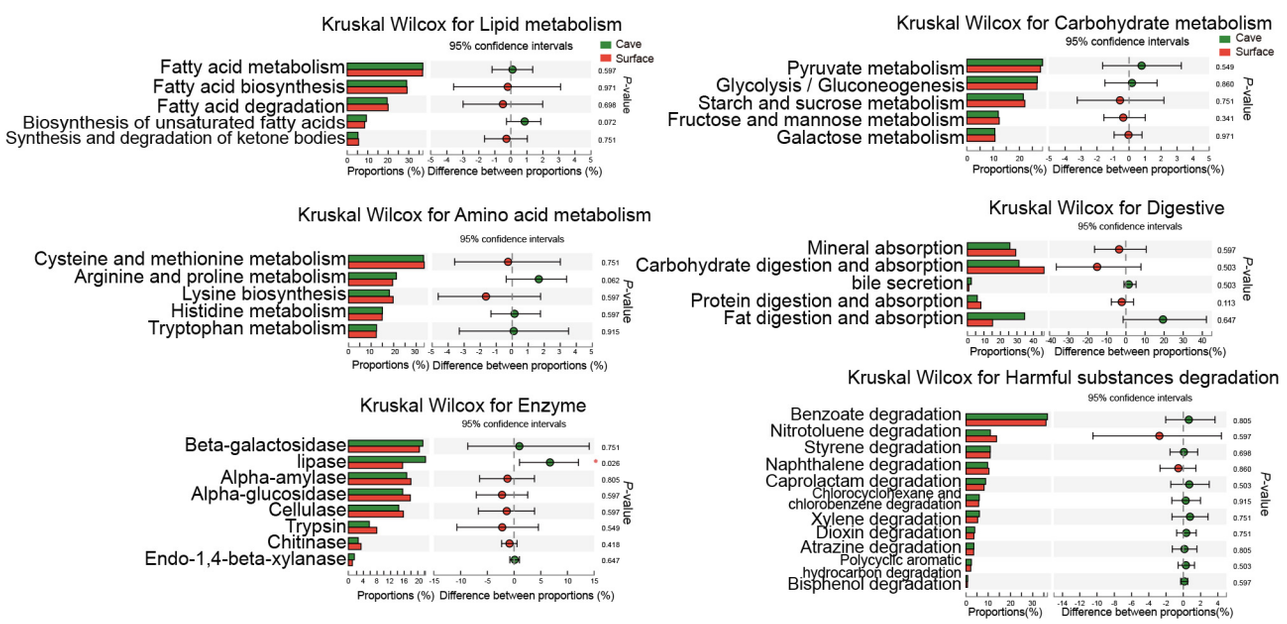


Figure 4 Comparison of relative abundance of PICRUSt2-generated functional profile of intestinal microbiota between *Sinocyclocheilus* cave and surface fish species. Functions are categorized at levels 2 and 3 (*: $P<0.05$)

Figure S12A, B). Niche-specific genera analysis using MaAsLin2 is shown in Supplementary Figure S13.

To fully understand the composition and structure of potential food resources in the niches of the two sympatric fish types, we classified them according to fish collection depth. Surface soil (S_S), middle water (M_W), and surface water (S_W) were classified as the surface fish niche group (Surface_fish_en), while bottom soil (D_S) and deep water (D_W) were classified as the cavefish niche group (Cavefish_en). Analysis showed that 10 of the 23 animal-based resources with relatively high abundance (>0.1%) exhibited significant differences between the two groups. Most (7/10) were significantly abundant in the cavefish niches. The composition of plant-based resources in the cavefish habitat

Table 5 Comparison of α-diversity indices between habitat samples from two *Sinocyclocheilus* fish types (excluding *S. rhinocerous* and *S. malacopterus* sympatric group)

Estimators	Encave-Mean	Encave-*Sd*	Ensur-Mean	Ensur-*Sd*	*P*-value	FDR
Sobs index	3263.30	680.17	2522.50	810.67	<0.05	>0.05
Shannon index	6.83	0.52	6.44	0.58	<0.05	>0.05
Simpson index	0.00	0.00	0.01	0.01	>0.05	>0.05
Ace index	5463.50	1449.00	3678.90	1352.10	<0.05	>0.05
Chao index	4858.60	1090.40	3653.90	1360.20	<0.05	<0.05
Coverage	0.94	0.02	0.96	0.02	>0.05	>0.05

en_cave: Cavefish habitats; en_sur: Surface-fish habitats; FDR: False discovery rates. *Sd*: Standard deviation.

Table 6 Comparison of α-diversity indices between habitat samples of sympatric group *S. rhinocerous* and *S. malacopterus*

	Mean（average）					*Sd*				
Estimators	**D_S**	**S_S**	**D_W**	**M_W**	**S_W**	**D_S**	**S_S**	**D_W**	**M_W**	**S_W**
Sobs index	2848.70	2558.50	1586.00	821.80	680.00	883.08	692.42	76.14	107.94	39.72
Shannon index	6.37	5.86	5.01	4.50	4.35	0.75	0.67	0.14	0.19	0.09
Simpson index	0.01	0.02	0.02	0.03	0.03	0.01	0.01	0.01	0.01	0.01
Ace index	5316.80	4879.20	4424.90	2239.90	1502.40	1452.40	1363.30	489.67	351.32	193.28
Chao index	4385.00	3972.00	3016.20	1534.60	1088.80	1252.70	1063.00	194.82	190.24	115.79
Coverage	0.94	0.95	0.96	0.98	0.99	0.02	0.01	0.00	0.00	0.00

D_S: Deep soil; S_S: Surface soil; D_W: Deep water; M_W: Middle water; S_W: Surface water. *Sd*: Standard deviation.

was relatively simple, with just four resources comprising more than half of the total abundance. Plant resources, such as *Navicula*, *Gonium*, and *Chlorella*, were more abundant in the surface fish habitat. Furthermore, the composition of plant genera in the surface fish niche was more highly diverse, and the relative abundance (structure) was more balanced (Supplementary Figure S12).

DISCUSSION

Characteristics and relationships between intestinal and habitat microbes and phylogenetic relationships of *Sinocyclocheilus* cavefish and surface fish

Comparing the characteristics of intestinal microorganisms between the two types of *Sinocyclocheilus* fish, we found that the composition of dominant gut microbial genera (e.g., *Cetobacterium*, *Bacillus*, *Enterobacter*, and *Aeromonas*) was similar between the cavefish and surface fish. However, the relative abundance of intestinal *Lactobacillus* and *Lactococcus* was significantly higher in surface fish than in cavefish. *Lactobacillus* and *Lactococcus* are common lactic acid bacteria (LAB) found in the vertebrate gut (Sun et al., 2021). In the intestines of cultured clownfish (*Premnas biaculeatus*), *Lactobacillus* bacteria are involved in dietary energy harvesting and carbohydrate metabolism of the host, indicating that *Lactobacillus* diversity is related to dietary changes (Parris et al., 2019). Differences in the abundance (structure) of these microbes between the *Sinocyclocheilus* cavefish and surface fish reflect differences in energy utilization and regulation between the two fish types from the perspective of intestinal microbes (Zhao et al., 2020).

In contrast to previous studies in primates and reptiles (Amato et al., 2019; Mccauley et al., 2020), we found that the similarity in intestinal microbes among the *Sinocyclocheilus* fish species was inconsistent with their phylogenetic relationships, but rather related to habitat, in agreement with recent research (Zhou et al., 2022). The correlation between intestinal microbes and host phylogeny is still controversial due to a lack of generalizability among different species, such as cave-dwelling *Astyanax mexicanus* (Ornelas-García et al., 2018) and *Salmo salar* (Llewellyn et al., 2016). Species with closer phylogenetic relationships generally exhibit similar phenotypes, behaviors, and even habitats, which may result in similar gut microbial structure and composition. However, despite belonging to different clades in the phylogenetic tree (Supplementary Figure S2), sympatric *S. rhinocerous* (XJ_sym; cavefish) and *S. malacopterus* (RQ_sym; surface fish) showed greater similarity than that of the same species collected from different habitats (RQ_sym and RQ) (Supplementary Figure S3). This finding further confirmed that differences in gut microbes between *Sinocyclocheilus* cavefish and surface fish are primarily related to habitat (ecotype), which mitigates the effects of other pressures on intestinal microbes and does not conform to phylosymbiosis.

Research has demonstrated that environmental microorganisms can be horizontally transferred into fish intestines during ingestion or movement and can coexist with host microbes long-term. This may be important for the initial colonization of intestinal microbes in fish, particularly larvae (Mushegian et al., 2019; Spor et al., 2011; Voss et al., 2015). For example, in *Carassius auratus*, 60% of OTUs in intestinal microbes are shared with habitat microorganisms (Zhang et al., 2019). Interestingly, our results differed from previous research showing a stronger correlation between gut and habitat microbes in *Sinocyclocheilus* fish (Zhou et al., 2022). We found that the α-diversity trend of microbes at most sites was consistent with that of gut microbes in corresponding fish species. However, although some shared microbes were identified between the habitat and fish intestines, similarity was still low. For example, while Burkholderiaceae, Nitrosomonadaceae, and *Nitrospira* abundance was extremely low in the *Sinocyclocheilus* fish gut, they were highly abundant in the *Sinocyclocheilus* fish habitats. These microorganisms participate in the biogeochemical cycle and play important roles in the carbon, nitrogen, and phosphorus cycles (Jiao et al., 2017). *Nitrospira* is an autotrophic microorganism that catalyzes the second reaction of the nitrification process (Liu et al., 2020), and Burkholderiaceae can degrade environmentally harmful substances and purify water (Li et al., 2014).

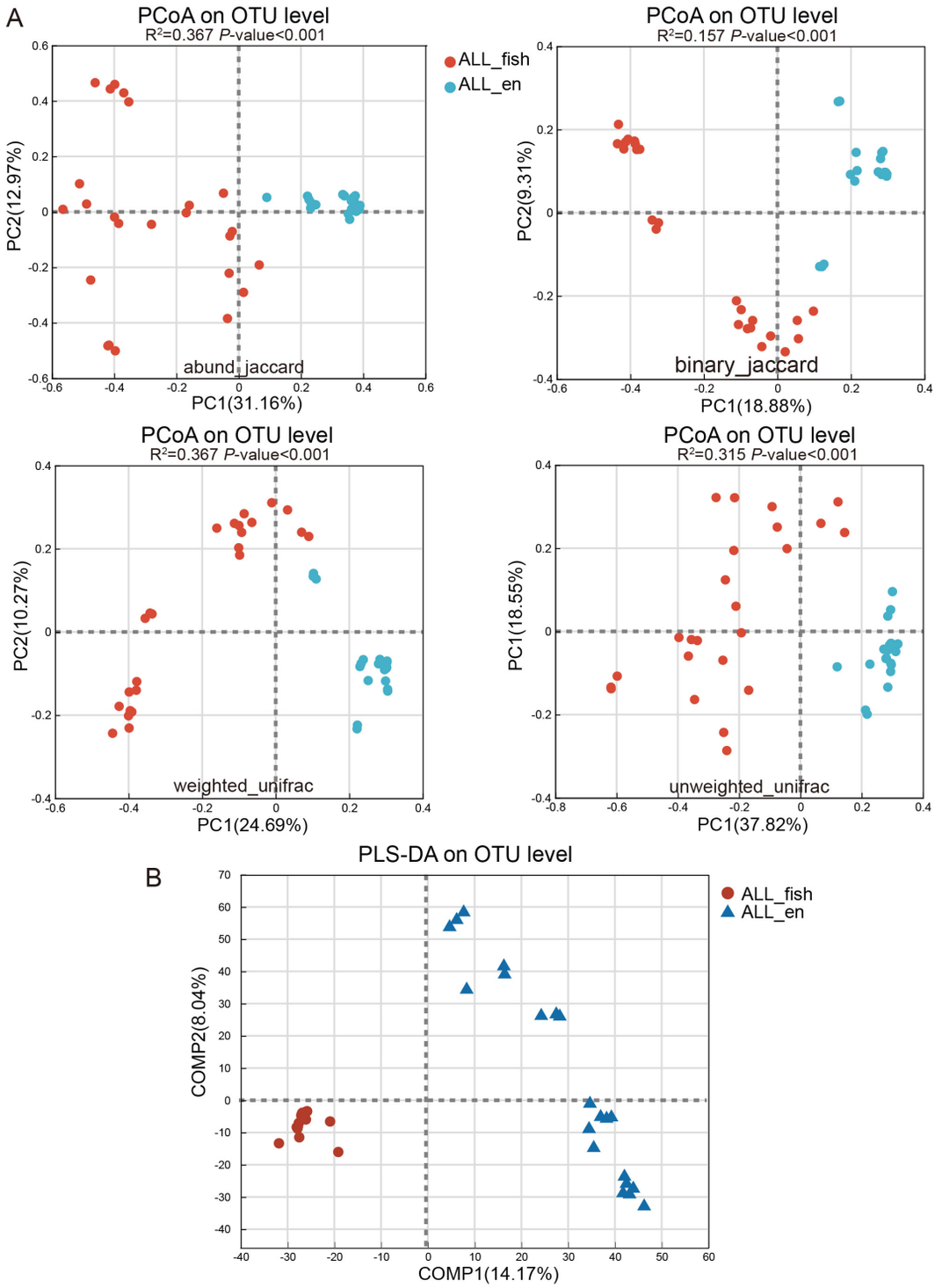


Figure 5 Inter-individual similarity between fish intestinal microbiota and habitat microbiota

Similarity analysis between fish intestinal microbiota (ALL_fish) and their habitat microbiome (ALL_en) (excluding *S. rhinocerous* and *S. malacopterus* sympatric group). R^2: Grouping interpretation value; *P*: Interpretative reliability value (P<0.05 reliable)

While horizontal transfer is considered a source of intestinal flora (Mazel et al., 2018), microbes that are beneficial for improving fitness are selectively recruited and eventually become stable and dominant microorganisms in the gut, instead of simply replicating habitat microorganisms (Suzuki & Ley, 2020). Weigel (2020) observed that the composition of microbes in the regenerated gut is highly similar to that pre-evisceration in sea cucumbers and is independent of habitat. Furthermore, Schmidt et al. (2015) found that intestinal microbial composition in *Poecilia sphenops* is affected by water salinity and is weakly correlated with habitat microorganisms. Thus, we speculate that, compared to habitats with low microbial diversity, more complex microorganisms can be transferred into the intestines of fish in habitats with high microbial diversity, which may partly explain the high microbial α-diversity observed in *Sinocyclocheilus* cavefish. However, although influenced by habitat, the microbes shared between the *Sinocyclocheilus* fish intestines and habitats were still low-abundance microorganisms. Moreover, the dominant intestinal bacterial genera were

Table 7 α-diversity indices between *COI* and *mat*K barcodes

COI (Animal-based bardode)						
Sample	Sobs index	Shannon index	Simpson index	Ace index	Chao index	Coverage
S_S01	650.00	3.14	0.18	1194.03	993.13	0.98
S_S02	868.00	4.70	0.03	1085.70	1098.85	0.98
S_S03	909.00	4.38	0.05	1324.80	1286.36	0.98
D_S01	878.00	3.97	0.10	1238.76	1184.33	0.98
D_S02	967.00	4.45	0.04	1304.47	1221.74	0.98
D_S03	762.00	3.12	0.23	1400.62	1149.60	0.98
S_W	142.00	2.73	0.12	177.81	172.27	1.00
M_W	124.00	1.08	0.19	126.19	117.43	0.98
D_W	282.00	2.82	0.13	387.59	371.30	0.99
*Mat*K (Plant-based bardode)						
Sample	Sobs index	Shannon index	Simpson index	Ace index	Chao index	Coverage
S_S01	220.00	0.68	0.75	225.34	220.38	0.99
S_S02	250.00	0.84	0.67	254.17	250.82	0.99
S_S03	240.00	0.46	0.85	383.71	260.53	0.99
D_S01	440.00	1.59	0.44	442.80	442.39	0.99
D_S02	240.00	1.70	0.31	324.01	250.83	0.99
D_S03	300.00	0.84	0.68	310.36	320.33	0.99
S_W	150.00	0.64	0.72	154.66	158.93	0.99
M_W	120.00	0.35	0.94	122.71	132.48	0.99
D_W	200.00	0.85	0.60	210.47	203.13	0.99

specialized, suggesting strong selective effects of fish intestines on certain microorganisms. Although habitat microorganisms can be horizontally transferred into the *Sinocyclocheilus* fish intestine, the fish gut is highly selective and favors microbes closely related to host life activities, resulting in the high abundance of genera such as *Cetobacterium*, *Bacillus*, *Enterobacter*, and *Aeromonas*. Thus, horizontal transfer of habitat microorganisms is not the main reason for the difference in gut microbes between the surface and cavefish. We speculate that sampling method may contribute to the discrepancies found between our study (gut contents) and that of Zhou et al. (2022) (fecal collection). Environmental exposure of feces to water can result in contamination with habitat microorganisms, especially in fish habitats. Additionally, as the developmental stage of the collected subjects was not recorded and juvenile fish are more heavily colonized by environmental microorganisms than adult fish, other potential factors need to be considered.

Feeding habits and potential food resources of *Sinocyclocheilus* cavefish and surface fish

Feeding habits are shaped by long-term interactions between animals and their habitats (niches), which include feeding preference, food resources, and predation behavior (Han et al., 2019). However, the feeding process of *Sinocyclocheilus* fish, especially cavefish, is difficult to observe directly. Furthermore, we found that the food residue in the intestinal contents of *Sinocyclocheilus* fish was mostly liquefied and insufficient to support morphological identification. Diet is closely associated with the intestinal microbes that assist the host in nutrient digestion and absorption, which relate to feeding habits (David et al., 2014; Hughes et al., 2019). This can provide new insights and feasibility in studying *Sinocyclocheilus* fish feeding habits.

In this study, we found that dominant gut microbes in the two *Sinocyclocheilus* fish types were mostly related to nutrient utilization. *Bacillus* and *Cetobacterium* play a role in carbohydrate fermentation, plant polysaccharide decomposition, and cellulose degradation (Navarrete et al., 2009; Rawls et al., 2006; Ray et al., 2010) and are highly abundant in the intestines of many herbivorous and omnivorous fish, including *Ctenopharyngodon idellus*, *Cyprinus carpio* (Van Kessel et al., 2011), *Megalobrama amblycephala* (Li et al., 2014), and *Poecilia reticulata* (Sullam et al., 2015). High *Aeromonas* and *Enterobacter* abundance has been observed in the guts of carnivorous fish, including *Salvelinus alpinus* (Ringø et al., 2006), *Salmo salar* (Ringø et al., 2008), *Gadus morhua*, and *Culter alburnus* (Fjellheim et al., 2007), and omnivorous fish, including *Carassius auratus* and *Hypophthalmichthys molitrix* (Li et al., 2014). However, functional enrichment of gut microbes in both types of *Sinocyclocheilus* fish was primarily related to metabolism, including lipid, carbohydrate, and amino acid metabolism, which is associated with high abundance of cellulase, amylase, glucosidase, lipase, and trypsin. Consumed food, which serves as a substrate for gut microbes, corresponds to the specialized enzymes found in the host gut (e.g., carbohydrate, cellulase, phosphatase, lipase, and trypsin) (O' Grady & Shanahan, 2021; Perry et al., 2020). In summary, referring to the gut microbial characteristics of fish species with known feeding habits as an indicator for identifying the feeding habits for *Sinocyclocheilus* fishes. Consistent with previous studies, we believe that neither of the two *Sinocyclocheilus* fish types has obvious monophagous characteristics (Chen et al., 2018; Yang, 1994), but are omnivorous and could have different feeding preferences (Brown et al., 2012).

While multiple factors influence fish ingestion, such as dietary niches (Rantin & Bichuette, 2015) and predation patterns (Waraniak et al., 2019), the relative abundance of edible resources (encounter frequency) is a primary driver (Kelling et al., 2016). For instance, the preferred feeding resources of *Neosalanx taihuensis*, *Hypophthalmichthys molitrix*, and *Aristichthys nobilis* are consistent with the high-abundance species in the niches of each species (Hu, 2014).

Similarly, blackside darter (*Percina maculata*), logperch (*Percina caprodes*), and rock bass (*Amblopites rupestris*) exhibit feeding preferences that are closely related to high-abundance resources in their habitat (Waraniak et al., 2019). For example, blackside darters show a preference for mayfly larvae when the abundance of mayflies in their niche is high and adjust their feeding accordingly after changes in resource structure (Waraniak et al., 2019). In the sympatric *S. rhinocerous*-*S. malacopterus*, most animal-based resources identified in the habitats, including zooplankton species such as *Polyarthra*, *Aspidiophorus*, and *Tanytarsus*, were more abundant in the cavefish niche than in the surface fish niche. Furthermore, many resources were only found in deep-water niches (*Exechia*, *Ormyrus*, *Tubifex*, and *Limnodrilus*). Conversely, the composition of plant-based resources showed lower diversity in the cavefish niche than in surface fish niche, with only four high-abundance genera identified (i.e., *Melosira*, *Navicula*, *Pinnularia*, and *Cryptomonas*). Zooplankton are usually benthic and hide in the bottom sediment of water or on the benthophyte surfaces, and their diversity and abundance are directly proportional to water depth (Yin et al., 2020). Rotifers are rich in protein, vitamins, carbohydrates, and minerals and are a fundamental component of fish diets (Samat et al., 2020). Over 400 rotifer species have been identified in China, with the majority distributed in freshwater habitats, such as lakes and reservoirs. *Polyarthra* species are the most abundant and are characterized by slow movement and high nutrition, making them a prime target for many fish (Fernando et al., 1990; Snell & Carrillo, 1984; Start & Gilbert, 2017). *Tanytarsus* larvae are also entirely benthic and hide in sedimentary mud (Wang et al., 2020). Phytoplankton (*Melosira*, *Navicula*, and *Pinnularia*) that dominate freshwater ecosystems are often ingested by fish (Bae et al., 2021; Davey, 1987) and are mostly distributed on the water surface to obtain adequate light for photosynthesis (Bright & Walsby, 2000; Porat et al., 2001). The habitat of *S. malacopterus*, a surface fish species, is located in open lakes with high illumination efficiency and no perturbance, providing a suitable environment for the growth and aggregation of phytoplankton (Ge et al., 2021). Thus, we believe that differences in the composition and abundance of potential food resources lead to differences in food availability (encounter frequency) for the two fish species. Cavefish are considered to be more efficient at predation than surface fish due to enlargement of their olfactory organ and developed lateral line system (Espinasa et al., 2014; Hinaux et al., 2016; Hüppop, 1987; Yamamoto et al., 2009). Small zooplankton, which move at a frequency of 30–40 Hz in the water, meet the optimal detection range (35 Hz) of the vibration attraction behavior (VAB) of cavefish, making it easier for them to prey (Rantin & Bichuette, 2015; Yoshizawa et al., 2010). Based on the gut microbes, potential food resources, and predation abilities of the *Sinocyclocheilus* cave-surface sympatric fish, we speculate that *S. rhinocerous* cavefish broaden their diets and become generalists to ensure energy intake under substantial survival stress and stronger predation ability. Thus, a more diverse community of gut microorganisms is necessary for sufficient digestion. Furthermore, the intestinal flora of *S. rhinocerous* showed enrichment in functions related to protein, mineral digestion and absorption, lipase, and chitinase, reflecting their high utilization of abundant animal-based foods. Compared with cavefish species, the surface species *S. malacopterus* displays normal eye function, different feeding patterns, and lower feeding pressure, which may lead to stronger feeding preference (Neiße et al., 2020) and simplification of the composition and structure of intestinal flora. Moreover, the functional enrichment of carbohydrate digestion and absorption in the intestinal microorganisms of *S. malacopterus* indicated a preference for plant-based food resources.

Our results confirmed the significant role of gut microbiota in facilitating food digestion and nutrient absorption in both types of *Sinocyclocheilus* fish. The difference in intestinal microbiota composition and function between the cavefish and surface fish was related to feeding preferences driven by the composition of highly abundant food resources in the habitat (niche), as well as the predation pattern of the host. However, as *Sinocyclocheilus* fish habitats are scattered and diverse, habitat conditions may vary, even for the same type of fish (Lunghi & Zhao, 2020). Moreover, the composition of food resources in the habitat is susceptible to seasonal variations (Du et al., 2018). Therefore, the feeding habits of *Sinocyclocheilus* fish still require further research.

Significance of intestinal microbes and feeding habits in habitat adaptability of *Sinocyclocheilus* fish

To understand the mechanisms of adaptive evolution, it has been suggested that the host and gut microbiota should be regarded as a whole symbiotic unit shaped by natural selection (hologenome). Although controversial, this theory reflects the importance of gut microbes in studying the adaptive evolution of species (Alberdi et al., 2016). By changing microbial structure and composition (metagenomic plasticity), intestinal flora can regulate gene expression products in response to host adaptation to physiological and external environmental changes (Nicholson et al., 2012). For example, diet-treated mice can regulate the intestinal proportion of *Bacteroides*, bacteria with high polysaccharide-degrading activity, to obtain additional energy from food and provide a buffer for the host during periods of food scarcity (Murphy et al., 2010; Xu et al., 2003).

Intestinal microbes can indirectly benefit host fitness (Foster et al., 2017), while hosts can also recruit beneficial microorganisms to enhance their adaptations to diet, pathogens, and climate change, with further selection also acting on microbes to drive host traits (Suzuki & Ley, 2020). Gut microbes produce metabolites for host utilization, while hosts provide stable habitat and substrates for gut microbes, leading to potential niche specialization (Sommer & Bäckhed, 2013). Thus, diet plays a critical role in shaping gut microbe composition, function, and structure (Brown et al., 2012). Intestinal microbes have greater metabolic potential than their host, which assists the host in forming a wider feeding niche and enhanced adaptability (Moeller & Sanders, 2020). To increase their food acquisition, many species rely on the capacity of intestinal microbes to degrade dietary toxins. For example, the woodrat (*Neotoma lepida*) can consume the highly toxic creosote bush (*Larrea tridentata*) (Kohl & Dearing, 2016). The transfer of gut microbes from koalas with distinct feeding habits to newborn koalas can alter their ability to consume new eucalyptus species, leading to changes in feeding behavior and potentially fitness (Blyton et al., 2019). In the current study, we found that both *Sinocyclocheilus* fish types experienced horizontal transfer of microbes from their habitat to gut, but also showed obvious selection in the colonization of dominant gut microbes. The distribution of potential food resources in sympatric fish niches revealed varying food availability. Thus, differences in the diversity and

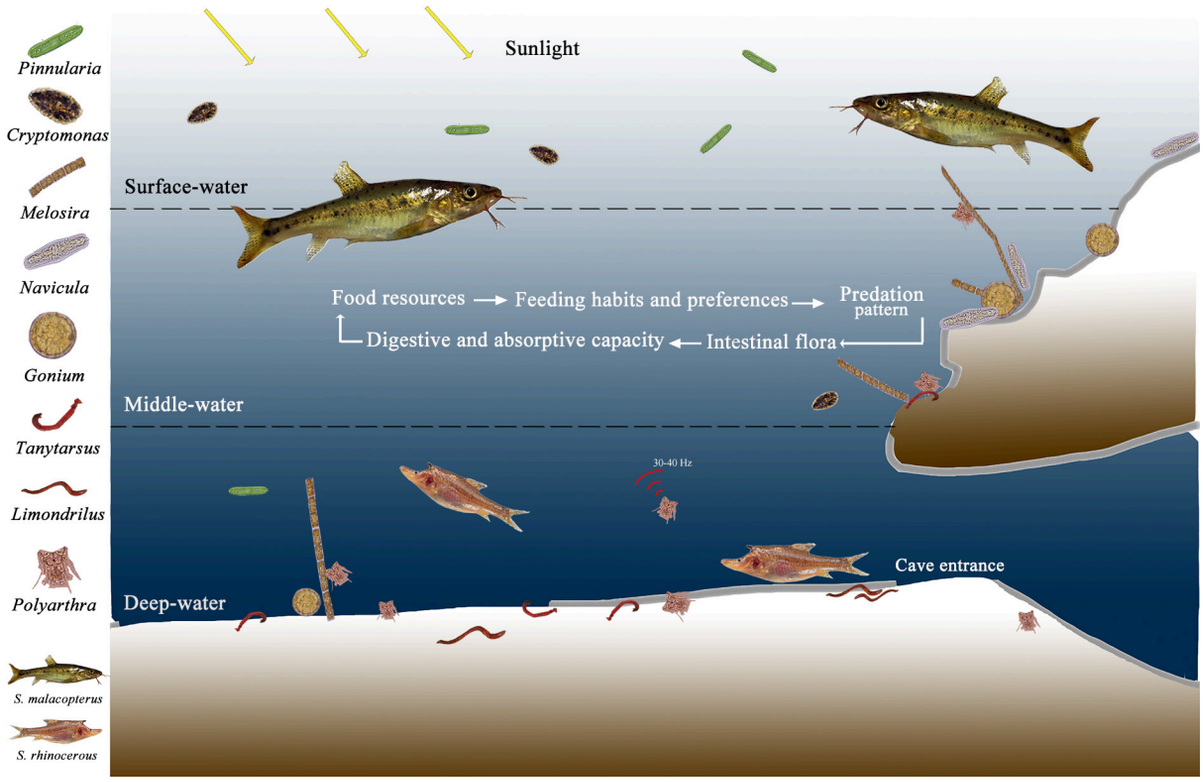


Figure 6 Diagram of sympatric fish group habitats

abundance of gut microbes, especially dominant species, may reflect their role in enhancing host digestion and nutrient utilization adaptability under different food substrates. This suggests that food resources, predation patterns, intestinal flora, digestive and absorptive capacity, and feeding habits and preferences are linked to habitat adaptability (Figure 6). For example, in *Sinocyclocheilus* cavefish, opportunistic feeding is necessary for greater microbial diversity, while highly diverse gut microbes (varied digestive enzymes) form the basis for broadening the dietary niche and adaptability of cavefish (Parris et al., 2019).

Although fertilizers and insecticides are necessary for modern agriculture, only a limited amount of pesticide residue can be degraded by soil microbes and sunlight (Zhang et al., 2019), with long-term accumulation causing serious environmental damage (Fevery et al., 2016). Industrial sewage and pollutants also contaminate wild fish habitats, impairing fish organs and affecting growth and development. However, intestinal flora can play a crucial role in degrading environmental chemicals and toxic pollutants, reducing their potential harm and enhancing host adaptability to the environment (Claus et al., 2016; Feng et al., 2020). Our study showed that the intestinal microbiota of the *Sinocyclocheilus* fish, especially cavefish, was functionally enriched in the degradation of harmful substances, such as benzoate, nitrotoluene, styrene, and naphthalene. Polycyclic aromatic hydrocarbons (styrene, naphthalene, and nitrotoluene) and halogenated compounds degrade slowly and remain persistent in nature, leading to water pollution (Godheja et al., 2016; Wang et al., 2012). Benzoate is a chemical additive used in the production of detergents, soaps, shampoos, and pesticides, posing a significant risk to aquatic organisms (Tan et al., 2021). Styrene and styrene polymers are released into the environment during the process of synthetic rubber and plastic production and incineration (Kwon & Moon, 2019; Mooney et al., 2006). During our field investigation, we found that *Sinocyclocheilus* fish habitats are seriously affected by sewage discharge, tourism development, and incineration of harmful substances (tobacco, straw, and plastics), which has resulted in a gradual decline in species number. Functional enrichment of harmful substance degradation in the intestinal microbes of both *Sinocyclocheilus* fish types can protect against the ingestion of such substances and enhance adaptability to worsening habitat pollution. We speculate that lower disturbance and circulation of cave water may result in greater deposition of harmful substances, which may partially explain the higher functional enrichment of harmful substance degradation in the intestinal flora of cavefish. However, further physicochemical analyses of the habitats of *Sinocyclocheilus* fish species are needed to obtain more environmental hydrological data.

To the best of our knowledge, this study is the first to investigate the mechanisms underlying *Sinocyclocheilus* fish species adaptation by combining 16S rRNA high-throughput sequencing of intestinal and environmental microbes and DNA metabarcoding. This study should improve our understanding of the significance of fish gut microbes in habitat adaptation and provide new perspectives for studying the adaptive mechanisms of cavefish.

DATA AVAILABILITY

The raw sequence data reported in this paper were deposited in the Genome Sequence Archive of the National Genomics Data Center, China National Center for Bioinformation/Beijing Institute of Genomics, Chinese Academy of Sciences (GSA: PRJCA014133) and are publicly accessible at https://ngdc.cncb.ac.cn/gsa. The raw sequence data were also submitted to the Science Data Bank database (DOI: 10.57760/sciencedb.07026) and NCBI Sequence Read Archive (SRA) database (Accession No.: PRJNA896095, PRJNA542570, SRP19820).

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

S.Y.C. and H.X. contributed to conceptualization, project administration, and funding acquisition. H.Y.C. and C.Q.L. carried out the investigations and performed formal analyses. C.Q.L. contributed to methodology and resources. H.Y.C., C.Q.L., S.Y.C., and H.X. wrote the manuscript. H.Y.C. and S.Y.C. reviewed and edited the manuscript. All authors read and approved the final version of the manuscript.

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