Journal of Applied Biology & Biotechnology Vol. 11(5), pp. 105-111, Sep-Oct, 2023
Available online at http://www.jabonline.in
DOI: 10.7324/JABB.2023.11512-1

A metagenomic analysis of gut microbiome phylogeny among four economically important carp species from wild and aquaculture farms

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ARTICLE INFO

Article history:
Received on: January 13, 2023
Accepted on: May 16, 2023
Available online: August 10, 2023

Key words:
Aquaculture
Carp
Gut microbiome
Metagenomics
16S rRNA

ABSTRACT

The present study was carried out to compare the gut microbiome of Indian Major Carp and Common carp from wild and aquaculture setting through 16S rDNA sequencing. The library of 16S rDNA V3-V4 hypervariable regions of gut microbiota was amplified, followed by sequencing using Illumina MiSeq. The analysis of sequencing data carried out through the Quantitative Insights into Microbial Ecology pipeline suggests that *Proteobacteria*, *Firmicutes, Cyanobacteria* and *Actinobacteria* are the most dominant phyla. Some microbial phyla were found to be species-specific. Genera such as *Bacillus, Sphingomonas*, and *Clostridium* were highly abundant in cultured forms indicating their role in the survival of cultured forms under considerable ecological stress. The α-diversity and β- diversity indicators suggest that the gut microbiome of cultured forms shows more diversity and also resembles a great extent. Absence of *Bacillus spp*. in wild form, and low abundance of *Lactococcus spp.* indicates the need of finding alternatives for probiotics. The insights from the present study can be used for further exploring the role of the gut microbiome in aspects such as growth, immunity and other physiological functions of the fish.

1. INTRODUCTION

Aquatic foods are important because of their role in food security and nutrition [1]. As it is one of the major sources of food fish supply for the growing population, despite the wide range of aquatic organisms, the major carp were cultured extensively which include *Catla catla*, *Cirrhinus mrigala,* and *Labeo rohita*. The composite culture of common carp, grass carp and silver carp, which are exotic, is also practiced. India leads in the production of Indian major carp followed by Bangladesh [2-4]. During the past six to seven decades, *Cyprinus carpio* (Common carp) has also been introduced into the aquaculture systems of India. The common carp is now one of the major contributors to the fisheries in the state of Uttar Pradesh [5]. Yet, the Indian major carp represent most of the inland fish landing in India.

Due to the irrational exploitation of finite natural sources, to date, most of the world's demand for aquaculture was met with the aid of aquaculture farms. When cultured in a controlled environment, they will be subjected to an entirely different habitat type than that of natural. In such circumstances, what might be the impact of such habitat and diet changes on these Indian major carp needs to be studied. Fishes living in both wild and farmed environments exhibit not only morphological variances but also physiological changes and genetic variants, which may be a result of altered environmental conditions and dietary supplements. Despite a good number of reports on the above factors, there is a lack of literature stating the differential impact on the gut microbiome of the Indian carp.

The gut microbiome impacts the host fish with respect to the fish's size, metabolism, feeding habits, and immunity [6]. As the fish gut microbiome influences the digestive physiology of the host, the habitat and dietary influence on the microbiota of the host need to be explored. The habitat effect on the gut microbiome of aquatic organisms has been investigated [7]. The anticipated advantage of this host-microbiota interaction or association can change if antibiotics modify the gut microbiome. The application of antibiotics has caused the evolution of antibiotic-resistant gut microbes in the aquaculture industry that may disseminate to other farms and animals including the human population [8]. Before two decades, most of the microbial works were based on conventional methods. Due to current advancements, next generation sequencing (NGS) has been entitled to identify both culturable and unculturable organisms [9]. Metagenomic studies have mostly concentrated on microbiota composition and host-microbiota interaction. The gut microbiome composition keeps varying and their origin is seldom studied. Therefore, studies on the factors affecting the gut microbiome are important. Further, the diversity and functional

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prediction of the uncultured bacteria is difficult to ascertain. Such questions can be answered using metagenomics and functional genomics. Metagenomics can aid to the determination of gut microbiome diversity by studying the hypervariable region of the 16s rDNA of prokaryotes [10]. Through this study, the species-specific difference in gut microbiome composition concerning the wild and farm-based environment has been explored with the aid of NGS.

2. MATERIALS AND METHODS

2.1. Sample Collection

For the present investigation, the fish were collected from Paithan, Aurangabad district, Maharashtra [Table 1]. *C. catla* (2 wild and 1 cultured), *C. carpio* (2 wild and 1 cultured), *C. mrigala* (1 wild and 1 cultured) and *L. rohita* (1 wild and 1 cultured) were investigated in the present study. Jayakwadi dam Nathsagar Jalashay (19°29'0.31"N, 75°22'24.63"E) was selected as the wild habitat. Cultured fish were procured from Fish seed Farm, Paithan (19°28'37.02"N, 75°22'6.07"E). Sterile Eppendorf tubes (1.5 mL) were used to collect the gut region of the carp. The tubes were transported in an ice-cold box. Before the DNA extraction, the samples were kept at −20°C.

2.2. DNA Extraction

DNA was extracted from the sample (50 mg each) with the aid of the NucleoSpin DNA stool kit (MACHEREY-NAGEL, Germany) as per the protocol provided with the kit. Elution buffer (50 µL) was used to dissolve the extracted DNA. Till further processing, the extracted DNA samples were kept at −80°C.

2.3. Amplicon Sequencing

The DNA extracts were amplified using PCR for 25 cycles with hypervariable V3–V4 regions of the 16S rDNA gene [11]. The gene-specific sequences were supplemented with the nucleotide sequences used in Illumina adapter overhangs. The following primer sequences were used to target the region:

F 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACG GGNGGCWGCAG**3'**

R 5 ' G T C T C G T G G G C T C G G A G A T G T G T A T A A G AGACAGGACTACHVGGGTATCTAAT**3'**

The overhang adapter sequences that were added are mentioned below.

Forward overhang: 5' TCGTCGGCAGCGTCAGATGTGTATAAG AGACAG-[locus specific target primer]

Reverse overhang: 5' GTCTCGTGGGCTCGGAGATGTGTATAAG AGACAG-[locus specific target primer]

A 1% agarose gel was used to visualize the amplicons. The library preparation was carried out using two-stage PCR. The successive cycles of PCR amplification were carried out as per the following details- denaturation (3 min at 95°C), 25 denaturation cycles (30 s at 95°C), annealing (30 s at 55°C), extension (30 s at 72°C) and a concluding extension (5 min at 72°C). After each PCR, PCR clean-up was done using AMPure XP Beads. Fluorometric quantification of the library was carried out through dsDNA binding dyes followed by the normalization. Before diluting the pooled library with a hybridization buffer, it was denatured using NaOH. After heat denaturation, the MiSeq v3 reagent kit was used to carry out Illumina MiSeq sequencing of the pooled library.

2.4. Sequence Analysis

To perform the sequence analysis, Quantitative Insights Into Microbial Ecology (QIIME2) software was employed [12]. Quality filtering of raw sequences was carried out. The sequence assignment to the samples was done based on barcodes. Primer sequences were removed. Chimeric sequences were detected. UCHIME [13] was used for the removal of these sequences. SILVA reference databases [14] were used for 16 s metagenomic analysis.

2.5. Statistical Analysis

Taxonomy-level reports in the output directory were created using QIIME 2. The taxonomic profiles at different levels were represented using MEGAN [15] and SILVA. The complexity of the species diversity was examined through Alpha diversity using Chao1, Faith's Phylogenetic diversity, Shannon and Pielou's evenness indices. The data was compiled into a spreadsheet. BioVinci data visualization package (Bioturing, San Diego, USA) was used to generate a violin plot. The beta diversity distance matrix was calculated from the OTU table and was presented in the form of an Unweighted Pair Group Method with Arithmetic mean (UPGMA) tree. Phylogenetic or count-based distance measurements were utilized to illustrate data similarities or differences using Principal Coordinates Analysis (PCoA). The analysis of the variations in each taxon's relative

Table 1: Sampling details for carps (culture ponds and wild).

Serial number	Sample ID	Species	Habitat	Location from where the sample is collected
1	MF2	*L. rohita*	Wild	Godawari, Jayakwadi
2	CATLAOLD	*C. catla*	Wild	Godawari, Jayakwadi
3	MF7	*C. catla*	Wild	Godawari, Jayakwadi
4	MF1	*C. mrigala*	Wild	Godawari, Jayakwadi
5	CC4	*Cyprinus carpio*	Wild	Godawari, Jayakwadi
6	CYPRINUSOLD	*C. carpio*	Wild	Godawari, Jayakwadi
7	ROHU	*L. rohita*	Cultured	Paithan Fish Seed Farm
8	CATLA	*C. catla*	Cultured	Paithan Fish Seed Farm
9	MRIGAL	*C. mrigala*	Cultured	Paithan Fish Seed Farm
10	CYPRINUS	*C. carpio*	Cultured	Paithan Fish Seed Farm

L. rohita: Labeo rohita, C. catla: Catla catla, C. mrigala: Cirrhinus mrigala, C. carpio: Cyprinus carpio

abundance among the communities was done using Weighted UniFrac PCoA.

3. RESULTS AND DISCUSSION

3.1. Composition and Diversity in Gut Microbial Communities

In the present study, the total read count observed was 46,987, with the maximum and minimum reads of 8607 and 1383 respectively. In precise, 826 OTUs were assigned to various taxonomic levels. The taxonomic levels assigned included 18 phyla, 24 classes, 59 orders, 91 families, and 118 genera. *Firmicutes, Proteobacteria* and *Cyanobacteria* represented the three most dominant phyla in all carp species studied. Among the classified reads, Phylum *Firmicutes* represented in the range of 0–100%, *Proteobacteria* from 0% to 85%, and *Cyanobacteria* from 0% to 63%. Relative abundance in every sample at the phylum level is presented in Figure 1. Earlier reports have also highlighted a higher abundance of these phyla in the fish gut microbiome of *Labeo rohita, Hypophthalmichthys molitrix*, *Aristichthys nobilis*, *Ctenopharyngodon idellus*, *Oncorhynchus mykiss,* and *Carassius auratus.* Similar observations have been made in recent studies on grass carp, crucian carp, Rohu and Tilapia [10,16-21]. *Firmicutes* were predominant in the gut of cultured and wild Catla. The presence of *Proteobacteria* was variable in the species studied. *Cyanobacteria* were predominant in the gut of Rohu. Species-specific differences in gut microbiome were also noted. Some microbial phyla were species-specific which included *Acidobacteria*, *Fibrobacteria* and *Nitrospirae* in Catla; *Gemmatimonadetes* and *Nanoarchaeota* in the common carp.

The phylum *Proteobacteria* is involved in the metabolism of nutrients in fish and accounts for the major proportion of the gut of aquatic animals [22]. The bacteria from the phylum *Proteobacteria* have a key role in carbon, nitrogen and sulphur cycling from sludge and municipal wastes [20,23]. Some *Proteobacteria* contribute to digestive physiology through enzyme secretion while some play role in the biosynthesis of riboflavin and biotin [24]. *Firmicutes* which are important for the metabolism of carbohydrates in man [25], dominated the gut of wild as well cultured forms. The *Firmicutes* also affect lipid metabolism, produce digestive enzymes, promote host metabolism, and increase the bioavailability of fatty acids [26]. A higher relative abundance of *Firmicutes* was seen in the cultured forms and unlike *Bacteroidetes.* The faster growth rate of the cultured forms can be attributed to the presence and role of *Firmicutes* [27].

3.2. Sharing of Microbiota

Out of a total of 118 genera, *Clostridium* showed abundance in most gut samples. The other dominant genera were *Bacillus, Cyanobium, Aeromonas* and *Sphingomonas*. *Aeromonas* was significantly abundant in the gut of wild varieties of Catla (78.2%) and common carp (61.2%). *Sphingomonas* and *Bacillus* have represented the gut of only cultured fish. The abundance of *Cyanobium* was significantly more in wild Rohu (54.8%) and cultured Rohu (50.3%). The cultured group shared only a small number of bacterial genera with the wild forms.

Out of the 64 genera reported in cultured Catla, 60 were not reported in the wild forms [Figure 2a]. Four genera were shared with the wild forms. The shared genera included *Clostridium, Epulopiscium, Microcystis and Aeromonas.*

Out of the 38 genera reported from cultured Mrigal, only 1 (*Clostridium*) was shared with wild Mrigal [Figure 2b].

In Rohu, 7 unique genera belonged to the fish from aquaculture farms while 8 different genera represented the wild type. 3 genera (*Vibrio*, *Pirellula* and *Cyanobium*) were common in both varieties [Figure 2c].

The cultured Common carp shared 11 genera with the wild forms. The shared genera included *Aeromonas*, *Pasteurella*, *Pseudomonas*, *Corynebacterium*, *Mycobacterium*, *Brevibacterium*, *Kocuria*, *Rothia*, *Staphylococcus*, *Streptococcus,* and *Clostridium* [Figure 2d].

Clostridium represented both wild and cultured forms. *Clostridium* can have an important role in digesting plant food by fermenting cellulose [28]. The abundance of *Clostridium* in the GI tract of herbivores and omnivores is more than in carnivores. To ferment cellulose, *Clostridium* produces digestive enzymes [27]. *Clostridium butyricum* has been found to inhibit the multiplication of pathogens by altering the gut microbiome in common carp [29].

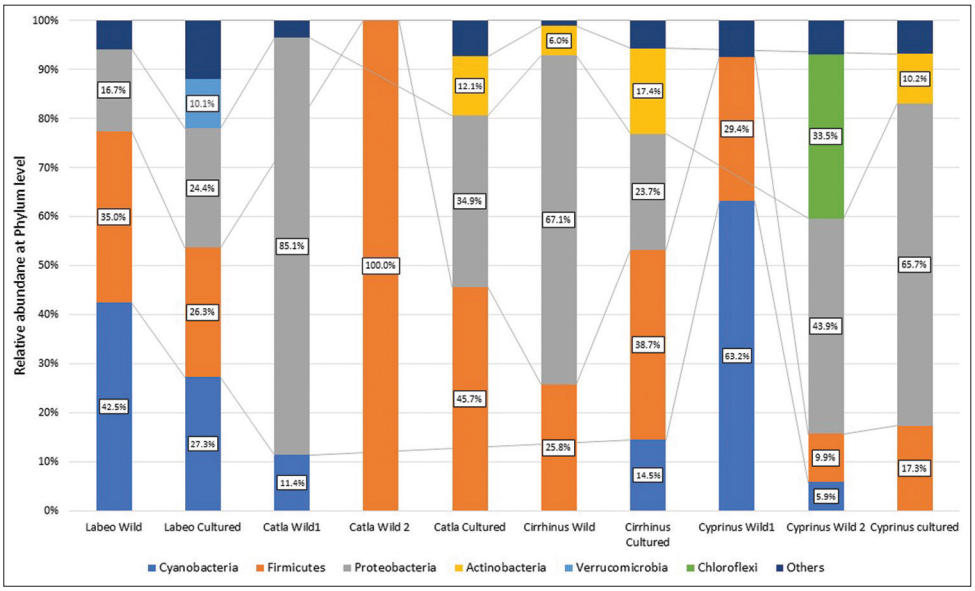


Figure 1: Relative abundance of phyla in wild and cultured carps of 4 groups.

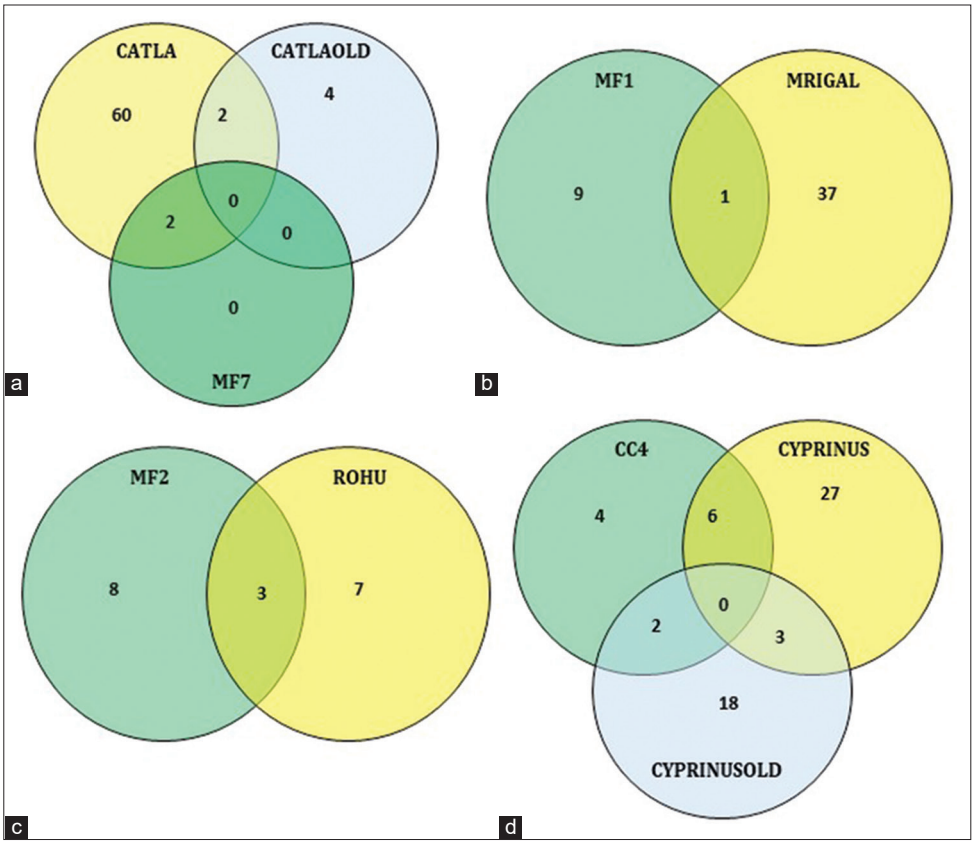


Figure 2: Venn diagram comparing the shared and unique gut microbiome at the genus level. (a) Wild and cultured Catla; (b) wild and cultured Mrigal; (c) wild and cultured Rohu; (d) wild and cultured common carp.

The most dominant phylum was *Cyanobacteria* in both cultured and wild Rohu. The abundance of *Cyanobacteria* can be related to its contribution as the major food source as reported in earlier studies too [9]. A notable presence of *Sphingomonas* in the cultured carp cultured groups was observed in our study. *Sphingomonas* have a role in the degradation of xenobiotics as per earlier reports [30,31]. *Pseudomonas,* a *Proteobacteria*, was another dominant genus in both cultured and wild carp. The role of *Pseudomonas* in microplastic degradation [32], and antimicrobial activity [33] has already been reported. *Bacillus* was also the dominant genus in the cultured carp while wild groups did not show the presence of *Bacillus.* The representation of *Bacillus* in the gut of cultured forms might be offering many health-related advantages due to their capacity to produce cellulase [34], their ability to provide probiotic benefits and their role as fish pathogen inhibitors [35]. These results indicate that the wild and aquaculture habitats lead to variation in the gut microbiome of fish. This variation may be associated with the fish species in addition to their habitat.

3.3. Analysis of Alpha and Beta Diversities

The complexity of the species diversity was analyzed through Alpha diversity by means of Chao1, Shannon, Pielou's evenness index and Faith's Phylogenetic diversity. Alpha diversity data indicate significant differences (*P*-values: 0.019016474, 0.010515246, 0.010515246, and 0.055008834), respectively, among the examined four carp samples [Table 2]. The gastrointestinal microbial communities in carp from various habitats vary in diversity and richness, according to our findings.

Table 2: Analysis of α-diversity.

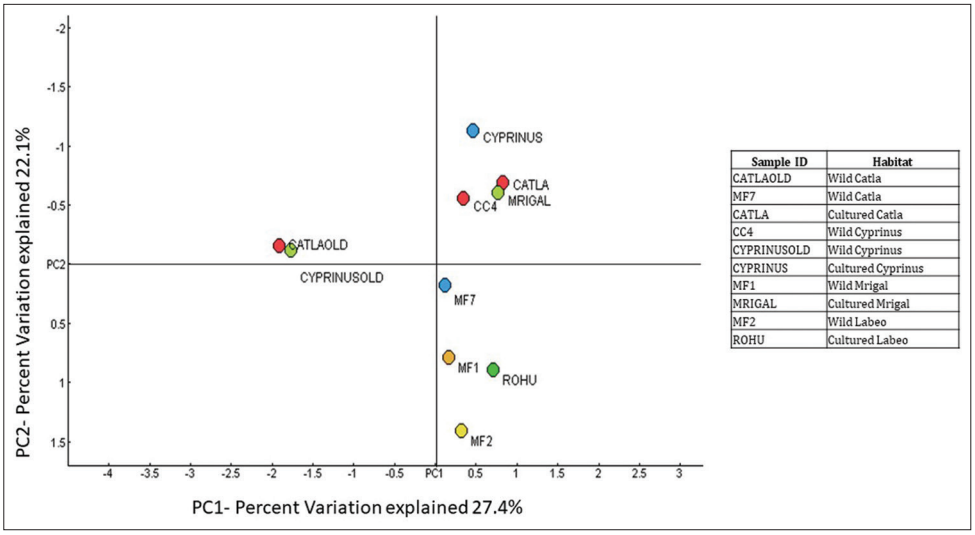
ID	Habitat	Chaos 1	Shannon entropy	Faith PD	Pielou's evenness
CC4	wild	276	2.29	4.60	0.45
MF1	wild	25	4.19	3.19	0.84
MF2	wild	35	4.36	3.88	0.83
MF7	wild	153	2.62	1.416	0.65
CATLAOLD	wild	99	2.24	2.47	0.50
CYPRINUSOLD	wild	31	4.42	8.867	0.69
CATLA	culture	38	6.74	17.26	0.86
ROHU	culture	16	5.64	16.12	0.88
MRIGAL	culture	238	6.34	35.16	0.84
CYPRINUS	culture	83	5.52	23.34	0.77

The Chao1 index reflects that the captive carp gut microbiome had slightly higher species richness than the wild carp studied. The Shannon index for cultured carps was higher than wild carps indicating that the species diversity is higher in fish that live in the captive environment. We observed the highest diversity in cultured Catla (Shannon index of 6.742358309) and Mrigal (Shannon index of 6.344573932) and the lowest in wild Catla (Shannon index of 2.244818315). Maximum phylogenetic diversity was observed in the case of cultured Mrigal (Faith's Phylogenetic diversity of 35.16157526) and minimum in wild Catla (Faith's Phylogenetic diversity of 41604475). This reflects that gut microbiome diversity in fish from aquaculture farms is higher than that in wild fish. This can be a result of the various feeds and habitat

types. A similar observation has been made by Bereded *et al.* [10]. Ringo *et al.* [36] reviewed the impact of various dietary nutrient compositions and forms on the gut microbiome of fish. Some workers have observed higher microbial diversity in wild forms compared to cultured forms of fish such as Atlantic Salmon [37] and Malaysian Mahseer [38].

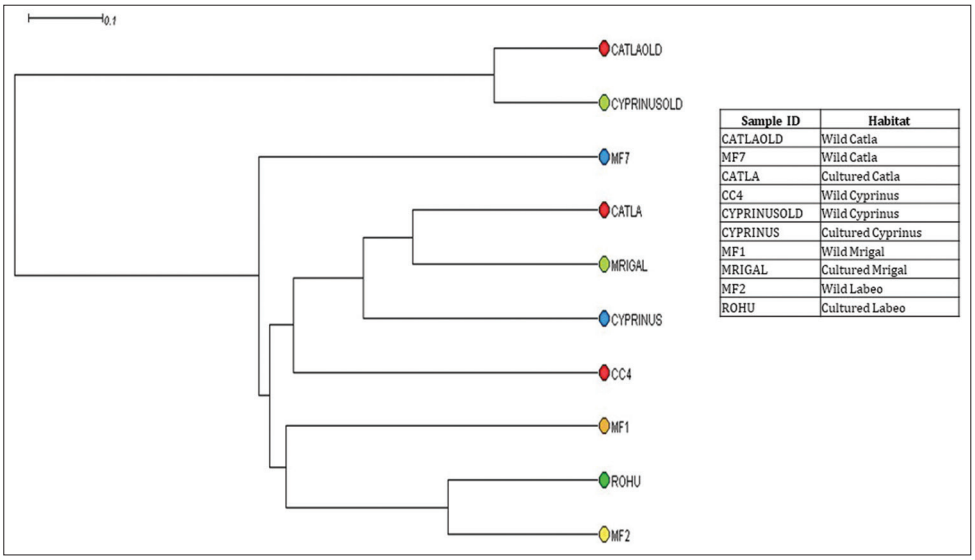
PCoA analysis showed significant differences in fish gut samples from wild and aquaculture settings. The PCoA plot [Figure 3] shows that the gut microbiome from the wild and the cultured samples are separated along PC1 representing 27.4% of the overall variation, except for one sample each of wild Catla (CATLAOLD) and Wild Cyprinus (CYPRINUSOLD). This can be an outcome of differences at the individual level. The gut microbiome of all the cultured and wild Rohu (ROHU and MF2, respectively) along with wild Catla (MF7) and Mrigal (MF1) were above PC2. The cultured fish gut samples of Catla (CATLA), Mrigal (MRIGAL), Cyprinus (CYPRINUS) and wild Catla (CATLAOLD), wild Cyprinus (CYPRINUSOLD) were below PC2, representing 22.1% of the overall variation. In conclusion, the two PCoA axes were able to account for more than 49% of the difference between the various communities. Clusters found in the UPGMA tree [Figure 4] of unweighted UniFrac distances were similar to the PCoA analysis. The cultured varieties of Catla, Mrigal and Common carp (CATLA, MRIGAL and CYPRINUS) clustered together with a wild variety of Common carp (CC4). The wild varieties of Catla, Mrigal and Rohu (MF7, MF1, and MF2, respectively) clustered together with the cultured variety of Rohu (ROHU). Unweighted unifrac PCoA [Figure 5] based on the number of features among the wild and cultured fishes shows that the gut microbiome of cultured forms clusters together while that of the wild forms cluster separately.

From the analysis of the α-diversity indicators, it is clear that the gut microbiome of cultured fish showed more diversity than that of the group of wild carp studied. The β- diversity indicators (PCoA analysis) suggest that the gut microbiome of cultured fish resembles to a great extent. However, the gut microbiome of Rohu has striking dissimilarity with the wild and cultured forms of the other three groups.



Sample ID	Habitat
CATLAOLD	Wild Catla
MF7	Wild Catla
CATLA	Cultured Catla
CC4	Wild Cyprinus
CYPRINUSOLD	Wild Cyprinus
CYPRINUS	Cultured Cyprinus
MF1	Wild Mrigal
MRIGAL	Cultured Mrigal
MF2	Wild Labeo
ROHU	Cultured Labeo

Figure 3: PCoA of Taxonomy using Bray-Curtis PC1 (27.4%) versus PC2 (22.1%) at the genus level.



Sample ID	Habitat
CATLAOLD	Wild Catla
MF7	Wild Catla
CATLA	Cultured Catla
CC4	Wild Cyprinus
CYPRINUSOLD	Wild Cyprinus
CYPRINUS	Cultured Cyprinus
MF1	Wild Mrigal
MRIGAL	Cultured Mrigal
MF2	Wild Labeo
ROHU	Cultured Labeo

Figure 4: Unweighted pair group method with arithmetic mean tree at genus level obtained after OTU table rarefied.

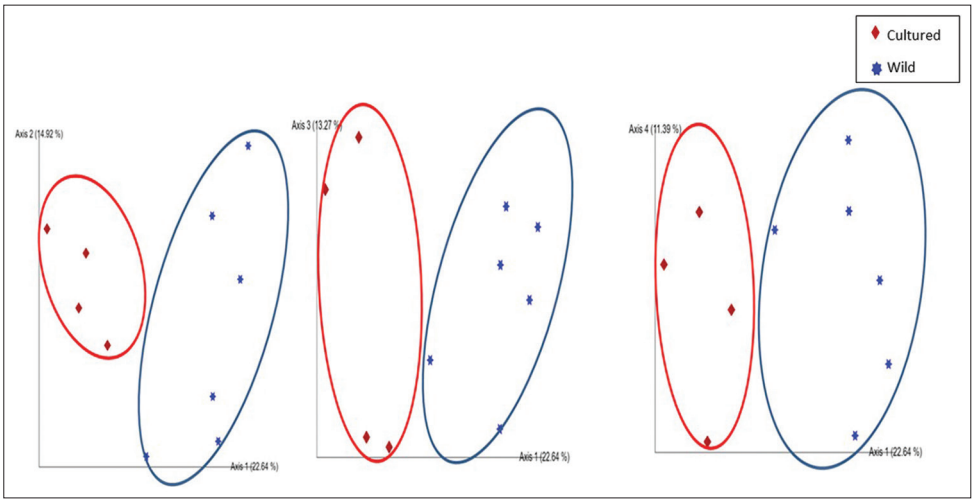


Figure 5: Unweighted unifrac PCoA based on the number of features among the wild and cultured fishes.

4. CONCLUSION

This is the first report on the comparative account of the gut microbiome of Catla, Mrigal, Rohu and common carp together from wild and aquaculture settings. The gut microbiome analysis in the 4 fish groups from the aquaculture farms and wild was carried out using NGS. The wild and cultured groups exhibited a prominent dissimilarity in the composition and diversity of the gut microorganism. Our investigation reveals that the gut microbiome make-up varies in wild and cultured carp. The diversity of gut microbiome also varies considerably in the wild and cultured fish groups. The cultured carps exhibit more diversity of microbial communities. Furthermore, the occurrence of some genera such as *Sphingomonas* and *Bacillus* was comparatively high in cultured fish groups suggesting that they may offer survival benefits to cultured fish and may have colonized from the habitat. Since this is a pilot study, further investigations are needed on a larger sample size to derive concrete conclusions.

5. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

6. FUNDING

There is no funding for the report.

7. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

8. ETHICAL APPROVALS

The ethical approval is not required for the experiments on fishes.

9. DATA AVAILABILITY

The authors confirm that the data supporting the findings of this study are available within the article and no supplementary data is required.

10. PUBLISHER'S NOTE

This journal remains neutral with regard to jurisdictional claims in published institutional affiliation.

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How to cite this article:
Pingle SA, Khandagle AJ. A metagenomic analysis of gut microbiome phylogeny among four economically important carp species from wild and aquaculture farms. J App Biol Biotech. 2023;11(5):105-111.
DOI: 10.7324/JABB.2023.11512