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1 # Environmental DNA (eDNA) based fish biodiversity assessment of
2 two Himalayan rivers of Nepal reveals diversity differences and
3 highlights new species distribution records

4

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23 **Short title**

24 eDNA based fish biodiversity assessment of Himalayan rivers of Nepal

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27 # Abstract

28 Although over 180 freshwater fish species have been reported from Nepal, little is known of their
29 ecology and distribution. This information is needed because their diversity may be threatened by
30 developments like hydropower constructions. We conducted Nepal's first environmental DNA
31 (eDNA) based fish biodiversity assessment in two major river systems- Karnali River (KR), which
32 is still pristine and Trishuli River (TR) with numerous hydropower plants. The eDNA was
33 concentrated by filtering (0.45 µm pore size) two liters of water collected at different sampling
34 points in each study site. A total of 224 eDNA samples (KR=162 and TR= 62) were collected,
35 from which fish species was identified by 12S rRNA metabarcording approach utilizing Illumina
36 sequencing platform. Alpha and beta diversity of species between two sites were compared. Also,
37 in KR site, fish (N=795) were caught, and identified using COI based DNA barcoding- building
38 Nepal's first fish DNA reference database. Field sampling identified 21 species through
39 morphology and DNA barcoding, where *Barilius* spp. and *Schizothorax* spp. were the most
40 abundant. From 244 eDNA samples, 24 Operational Taxonomic Units (OTUs) were identified in
41 TR and 46 in KR with 19 being common to both sites, 27 being unique in KR, and five in TR only.
42 Most fishes were of Cypriniformes and Siluriformes orders, with *Barilius* spp. and *Schizothorax*
43 spp. being the most abundant. Long distance migratory fish (*Tor* spp, *Neolissochilus*
44 *hexagonolepis*) and non-native fish (*Cyprinus carpio, Hypophthalmichthys molitrix,*
45 *Gymnocorymbuster netzi*, *Ctenopharyngo donidella*, *Clarias gariepinus)* were identified in eDNA
46 samples as well. Alpha diversity in TR was significantly lower than in KR. High beta diversity
47 between the two sites indicated low similarity in fish diversity between the TR and KR. This study
48 demonstrated the utility of eDNA as a non-invasive technique for biodiversity assessment which
49 is particularly useful in areas like Nepal with scarce data on fish species distribution.

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50 # Introduction

51 Nepal is rich in water resources with over 745,000 hectares of land being covered with water (1).
52 This has made Nepal a country with the highest per capita hydropower potentials in the world with
53 estimated theoretical power potential of ~ 43,000 megawatts (MW), though operational output in
54 2015 was 516 MW only (2). Additionally, rivers in Nepal serve as important income source for
55 many low income communities living closer to river banks. Harvest fisheries are intricately woven
56 into social, economic, and cultural fabrics of many Nepalese communities. Over 180 freshwater
57 fish species have been reported in Nepal's major river systems (3, 4). However, updated
58 information on ecology, distribution and diversity of fish species found in Nepal is limited
59 hindering their conservation efforts (5). Recent research on fish populations in Nepal's
60 Kaligandaki - Narayani River suggests that local diversity may already have been declining in
61 some areas (6).
62
63 Various anthropogenic developments can have significant impact on aquatic biodiversity and the
64 ecosystem. Depending on nature and scale of these developmental activities, the magnitude of
65 impact may vary. Construction of hydropower dams, reservoirs, and other infrastructures
66 particularly can have devastating impacts by directly affecting flow and quality of water, and thus
67 altering, fragmenting or entirely destroying aquatic habitats (7). An increasing demand for
68 renewable energy has resulted in an accelerated growth in hydropower development across the
69 world including Nepal, impacting the aquatic biodiversity of previously free-flowing rivers (8, 9).
70 Incorporation of various mitigation and management measures, such as carefully designed
71 construction plan, a comprehensive environmental impact assessments (EIA), habitat restoration,
72 along with stringently enforced conservation laws can prevent or mitigate potential harmful impact

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73 on aquatic ecosystems. For this, it is critical to assess status of river systems so as to generate
74 robust baseline datasets that can be used for successful EIA to monitor potential impacts of human
75 activities (10).
76
77 Environmental DNA (eDNA) analysis is a scientific technique that involves the use of genetic
78 material collected from a given environment to identify and monitor presence and abundance of
79 species in that ecosystem (11). This analysis has been used as a rapid assessment tool not only to
80 evaluate existing biodiversity but also to monitor the extent and magnitude of biodiversity loss.
81 Fish species monitoring has traditionally been conducted through physical sampling followed by
82 morphological species identification. This technique often involves sacrificing the specimens, is
83 subject to misidentification (especially with little-known and cryptic species), and often requires
84 taxonomic experts to work in remote field settings. Emerging genomics-based tools such as eDNA
85 can bring ease, accuracy, and reliability to large aquatic biodiversity assessment studies. This
86 technology is based on extracting DNA from environmental samples, such as from river water,
87 and obtaining from those samples the DNA sequences of standardized gene biomarker(s) using
88 next generation DNA sequencing technology. The species of fish present in the river upstream
89 from those sampling sites are then identified by cross referencing those DNA sequences to those
90 listed in some of the widely used public reference databases.
91
92 The objective of our study was to build Nepal's first fish species database using eDNA based meta-
93 barcoding technique, thereby creating a baseline fish diversity profile of two important river
94 systems of Nepal.
95

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96 # Materials and methods

97 ## Study areas and site selection

98 Our two study areas were contained within two of the major river basins of Nepal- i) the Gandaki
99 Basin, of which Trishuli River (TR) is one of the main tributaries, and ii) the Karnali basin, where
100 Karnali River (KR) is the main river stem. The Gandaki Basin lies in central Nepal with over seven
101 tributaries (e.g. Trishuli, Budhi Gandaki, Marsyangdi, and Kali Gandaki) which eventually drain
102 into the Narayani River on the south. Although this river basin spreads mostly across the Gandaki
103 province, its Trishuli catchment emanates from the western region of the Bagmati province before
104 joining the other tributaries. There are six operational hydropower projects along the Trishuli
105 River and its major tributaries that total 81 megawatts (MW).

106 The Karnali Basin in western Nepal is stretched across the Karnali and the Sudurpaschim
107 provinces. The west Seti, thuli-Bheri, Tila and Upper Karnali are its main tributaries, all of which
108 drain into the lower KR in the south. This river system is in a relatively pristine state and lies in a
109 rural and underdeveloped region of Nepal. There are only 42 MW of HPP in operation only in the
110 Sudurpaschim province. Most of these HPP are within the catchments of the Mahakali basin in the
111 far-western region, outside of the Karnali basin. Currently, only 3.75 MW is being produced at the
112 KR basin by the Dwarikhola hydroelectricity project.

113 Our eDNA sampling efforts were conducted through multiple projects, including Nepal Fish
114 Biodiversity Project (NFBP, 2016-18) and PAANI project (PAANI, 2018-19) in KR, and Upper
115 Trishuli eDNA assessment (2019-20, IFC funded) in TR. All of these projects assessed aquatic
116 (fish) biodiversity of river systems of Nepal and created Nepal's first baseline fish database. We

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117 collected samples from nine sites representing three seasons in three phases (two pre-monsoon and
118 one post-monsoon, 2016- 2017) from the lower KR region. We also collected samples from 15
119 sites in two phases from the upper Karnali- Seti and Bheri catchments (pre and post monsoon,
120 2018) (Fig 1). Our eDNA sampling in the Gandaki basin (TR) included seven sites in the Trishuli
121 catchment in two phases (pre and post monsoon, 2018) and from 12 sites in a single phase (pre-
122 monsoon, 2020) (Fig 2).

123 **Fig 1. eDNA sample collection sites along major tributaries across Karnali River (KR).** The
124 sampling sites in KR cover catchments from Seti, Bheri, Upper Karnali and Lower Karnali
125 tributaries.

126 **Fig 2. eDNA sample collection sites along major tributaries across Trishuli River (TR) basins**
127 **in Nepal.** TR is the eastern-most tributary of the Gandaki river basin.

128

129 Water sample collection for eDNA analysis

130 Each river site was sampled by collecting two liters of water at four different points (upstream,
131 downstream, pool, and riffle) located within a 100 meter stretch. The water sample was filtered in
132 field through a membrane filter (0.45 µm pore size) using battery operated electric filtration
133 system. The filter membrane with residue was stored in 15 ml Longmire buffer solution. At each
134 site, two liter of double distilled water filtered similarly was included as a negative control. The
135 filtration assembly was thoroughly disinfected by immersing in a series of 10% sodium
136 hypochlorite, 70% ethanol followed by sterile distilled water before, in between and after filtration
137 to prevent any carryover contamination. A total of 224 eDNA samples (162 from KR during 2016-

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138 2018 over five sessions; and 62 from TR during 2018 and 2020 over three sessions) were
139 processed.

140

141 Building a freshwater fish reference database

142 At each water sample collection sites in KR, fish were also physically caught using a standard cast
143 net (diameter = 4m, length = 2.2m) to build a local fish reference database. A total of 10 casts were
144 performed at each sampling location, with the locations separated by a shoreline distance of about
145 100 m as per Trisuli Assessment Tool Field Manual (12). The captured fishes from each cast net
146 sample were collected, photographed, species identified, measured by weight and length, and
147 counted by species. Representative individuals of each species were transported to laboratory in
148 70% ethanol, where ~1 gram of tissue sample was excised from ventricle side for DNA barcoding,
149 and then whole fish was preserved as voucher specimens in 5% formalin. DNA was extracted from
150 the excised fish tissue using DNeasy Blood and Tissue Kit (Qiagen, Germany) as per
151 manufacturer's instruction. We selected COI as a species identification gene biomarker. For
152 genetic species identification, DNA barcoding was performed by amplifying COI region using
153 M13 linked COI primer cocktail (VF2_t1, FishF2_t1, FishR2_t1 and FR1d_t1) at final
154 concentration of 0.10 pMol/µL (13). The 650 bp amplicon was subjected to Sanger sequencing.
155 Finally, species identification was performed by use of BLAST tool on reference sequences in
156 NCBI GenBankdatabase. DNA sequences of the identified fish species were deposited in NCBI
157 database.

158

159 eDNA extraction, 12S PCR based metabarcoding and sequencing

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160 In laboratory, the tube with filter membrane was vigorously vortexed to elute residue to the buffer
161 solution. And removing the membrane, the buffer was then centrifuged at 8,000 rpm for 10 minutes
162 to concentrate the residue. After decanting the supernatant, ~700 µL of pellet was used as a sample
163 for eDNA extraction using GeneAll Tissue DNA extraction Kit (S. Korea) following
164 manufacturer's instructions. For metabarcoding based fish species identification, a ~170 bp
165 fragment of 12S gene was amplified on extracted each eDNA samples using specific MiFish
166 primers (14) with Illumina overhang adaptors. As per the Illumina protocol, the subsequent 8 cycle
167 index PCR was performed using specific combinations of forward and reverse index primers
168 (Nextera® XT Index Kit, Illumina, USA) with annealing at 55 °C for 30s. After AMPure XP
169 magnetic beads based purification, the samples were pooled, library was quantified using Qubit
170 dsDNA HS assay kit (Thermo Fisher Scientific, USA), normalized at 4nM, and finally the 10 pM
171 library was subjected to pair-end sequencing on an Illumina MiSeq instrument using a MiSeq
172 Reagent Kit v2 300 cycles (Illumina, USA).
173

174 Bioinformatics analysis

175 After initial quality assessment of raw MiSeq reads using FastQC v0.11.9 (15), and filtering using
176 Trimmomatic v0.39 (16), the cleaned reads were processed using QIIME2 v2021.11.0 pipeline
177 (17). We performed de-noising of paired-end reads by trimming, merging and removing chimeric
178 sequences using the DADA2 plugin (18). We processed the denoised sequences for fish DNA
179 filtering, which will only retain sequences belonging to fishes and filters out all other non-fish
180 vertebrates, prokaryotes (bacteria, diatoms) etc. that could have been the products of non-target
181 amplifications. For this, we utilized quality-control plugin in QIIME2 with percent identity 0.7
182 and percent query aligned 0.9 thresholds against a fish reference sequences. This method aligns

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183 our query sequences (denoised dataset) to the fish reference sequences and excludes any non-target
184 sequences (eg. bacteria, diatoms, non-fish vertebrates etc.) from the input data. From the quality
185 controlled sequences, we then generated sequence features (representative sequences) as amplicon
186 sequence variants (ASVs), i.e., Operational Taxonomic Units (OTUs) at 100% sequence
187 similarity, and produced a de-replicated feature table (with sequence counts) across the samples.

188 For taxonomy assignment, Mitohelper repository (19) was used as a reference which was curated
189 for reference sequence analysis in the fish eDNA studies. This database consists of QIIME2-
190 compatible datasets of fish 12S rRNA reference sequences and taxonomy classification
191 information. The reference datasets of Mitohelper were compiled using complete and partial fish
192 mito-genome sequences obtained from the MitoFish database, with further gene definition and
193 taxonomic classification obtained from the NCBI nucleotide and taxonomy database. The fish
194 systematics data including order and family numbers were further retrieved and verified from the
195 *Fishes of the World* for creating this curated fish reference database (20). As of July 2022 release,
196 12S rRNA Mitohelper database consisted of 89 known taxonomic orders, 546 families, 3,444
197 genera and 12,335 species of fishes.

198 We classified the de-replicated sequence features against the Mitohelper database for assigning
199 taxonomy using Blast+ search tool with parameters set for query coverage as 0.85, percent identity
200 as 0.97, maximum accepts as 10 and minimum consensus as 0.51 thresholds. The tool performs
201 local alignment between query and reference sequences in the database, then assigns consensus
202 taxonomy from among maximum accepts hits, minimum consensus of which share that taxonomic
203 assignment.

204

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205 **Fish diversity comparisons between two river systems**

206 We analyzed fish diversity within (alpha diversity) and between (beta diversity) the two study river
207 basins using QIIME2 based core-diversity plugins. For the diversity analysis, we categorized the
208 samples mainly based on KR and TR basins. The alpha diversity measures the fish species richness
209 in each of the river systems, whereas the beta diversity calculates the differences in diversity of
210 fish taxa between the two river systems. To make all of the data comparable, we normalized the
211 sampling/sequencing depth before performing these diversity analyses by applying rarefaction
212 with even sub-sampling of 8,287 sequences per sample based on rarefaction curve. All samples
213 having sequencing depth less than the diversity value were excluded from this diversity analyses.

214 We analyzed the rarefied abundance data with a Kruskal-Wallis pairwise test to evaluate the alpha-
215 significance of the diversity across river basins. For this purpose, we assessed the alpha diversity
216 using metrics of Faith's phylogenetic diversity (PD) (21), the Shannon Diversity indices (22) and
217 observed features or amplicon sequence variants. We visualized the alpha diversity boxplots using
218 R package ggplot2 v3.34 (23).

219 For beta diversity, we calculated pairwise permutation analysis of variance (PERMANOVA)
220 statistics by running 999 permutations based on the Bray-Curtis (24), Jaccard, Unweighted
221 UniFrac and Weighted UniFrac dissimilarity metrices (25) in QIIME2. We, then, generated
222 principal coordinate analysis (PCoA) plots of beta diversity distance matrices using Emperor
223 Plugin in QIIME2.

224

225

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226 # Results

227 ## General fish diversity in physically caught samples

228 Overall, 795 fish were caught in KR, of which 21 species were identified among the captured fish
229 through morphological characterization and COI DNA barcoding. Representatives of these 21
230 species are preserved as voucher specimens in our facility, and their DNA sequences were
231 deposited in the NCBI database (Table 1). Among the identified fish, the species that are currently
232 listed in the IUCN Red List included *Naziritor chelynoides*, *Schizothorax plagiostomus*,
233 *Neolissochilus hexagonolepis*, *Tor putitora,* and *Schizothorax nepalensis*. Of these fish species
234 identified, top five species based on their relative abundance were *Barilius* spp. (23.52%),
235 *Schizothorax* spp. (11.45%), *Schistura* spp. (8.3%), *Tor* spp. (8.0%), and *Acanthocobitis botia*
236 (5.54%). DNA sequence of all 21 identified species met 100% Query Coverage and 97%
237 Percentage Identity when compared with reference database.
238
239
240

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241 **Table 1: Fish species caught in KR and characterized with morphology and COI marker**
242 **based DNA barcoding technique.** Representatives of each were also preserved as voucher
243 reference specimens at the molecular laboratory of the Center for Molecular Dynamics Nepal
244 (Kathmandu, Nepal).

S.N.	Caught fish species	NCBI reference	IUCN Red List Status
1	*Acanthocobitis botia* *	MN178284	Least concern
2	*Barilius barna*	MN178260	Least concern
3	*Barilius bendelisis*	MN178258	Least concern
4	*Barilius vagra*	MN178261	Least concern
5	*Botia lohachata*	MN178273	Least concern
6	*Channa gachua*	MN178287	Least concern
7	*Crossocheilus*	MN178267	Least concern
8	*Glyptothorax gracilis*	MK993528	Data Deficient
9	*Glyptothorax trilineatus*	MN172316	Least concern
10	*Labeo bata*	MN178270	Least concern
11	*Labeo boggut*	MN172308	Least concern
12	*Mastacembelus armatus*	MN178296	Least concern
13	*Neolissochilus hexagonolepis* *	MN178268	Near threatened
14	*Opsarius shacra*	MN172306	Least concern
15	*Pseudecheneis sulcata*	MN178259	Least concern
16	*Puntius chelynoides*	MN172330	Vulnerable

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17	*Schizothorax plagiostomus*	MN178265	Vulnerable
18	*Tor putitora*	MN178263	Endangered
19	*Garraa nnandalei*	MK993526	Least concern
20	*Schizothorax nepalensis*	MN178262	Critically Endangered
21	*Garra* spp. *	MK962677	N/A

245 *represents caught species that were also found during eDNA analysis

246

247 ## General fish diversity in eDNA samples

248 A total of 179,224 de-replicated sequence features (Amplicon Sequence Variants-ASV) were
249 generated, out of which a total of 51 Operational Taxonomic Units (OTUs) were assigned a
250 taxonomy at either family, genus or species level (Fig 3, S1 table). About 16% of ASVs were
251 unassigned, because they did not meet consensus taxonomy assignment thresholds. We identified
252 24 OTUs in the TR and 46 OTUs in the KR. Among these, 19 OTUs were common in both river
253 basins, 27 were found only in KR and five were found only in TR (S1 Table and S1 Fig). The OTU
254 values were higher across the board in KR than TR.

255

256 **Fig 3**. Fish species and their relative frequency (%) identified in Karnali (KR) and Trishuli (TR)
257 sites by eDNA method.

258

259 Most of the freshwater fishes from both study systems belonged to the Cypriniformes and
260 Siluriformes orders. We identified the presence of some long distance migratory fishes such as
261 snow trout (*Schizothorax* spp.) and mahaseer (*Tor* spp.) in both river basins, while copper

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262 mahaseer (*Neolissochilus hexagonolepis*) was detected in KR only. Interestingly, we also found
263 Tibetan loach (*Triplophysa* spp), a new genus recently identified in the Upper Humla- a tributary
264 of KR basin. We also detected eight non-native commercial fish species. Common carp (*Cyprinus*
265 *carpio*) and silver carp (*Hypophthalmichthys molitrix*) were detected in both TR and KR, while
266 blue tilapia (*Oreochromis aureus*), and rainbow trout (*Onchorhynchus* spp.) were found in TR
267 only. Similarly, black tetra (*Gymnocorymbus ternetzi*), grass carp (*Ctenopharyngo donidella*), and
268 North African catfish (*Clarias gariepinus*) were detected only in KR.

269 *Barilius* spp. was the most abundant fish found in both KR and TR basins based on OTU values
270 (Fig 4). *Schizothorax* spp. was the second most abundant fish species. The relative frequency of
271 *Barilius* spp. in TR was 48% compared to 34% in KR.

272

273 **Fig 4.** Percentage relative OTU frequencies of common eDNA species found in KR and TR river
274 sites.

275

276 Alpha and Beta diversity of the two river systems

277 Our rarefaction analysis was based on 196 eDNA samples (Trishuli=44, Karnali=152), some
278 samples (n=28) failed quality control threshold and hence were excluded. When we inspected the
279 Faith's phylogenetic diversity (PD) across the river basins, we found that its average values were
280 0.93 in TR and 1.31 in KR. The PD differed significantly between the two river basins (Kruskal-
281 Wallis: H=30.702, p=3.009E-08). The average value of the Shannon diversity was 1.36 in TR and
282 2.29 in KR, values with significant differences (Kruskal-Wallis: H=31.509, p=1.984E-08). We
283 found the average value of observed features was 7.27 in TR and 14.82 in KR, and again the

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284 differences were highly significant (Kruskal-Wallis: H=42.226, p=8.129E-11). Overall, across all
285 tests, the alpha diversity in TR was significantly lower than KR (Fig 5).

286

287 **Fig 5**. Alpha diversity detected in the KR and TR river systems based on Faith's PD, Shannon
288 diversity, and observed features using Kruskal-Wallis analysis.

289 We found significant differences in pair-wise Beta diversity between two river basins based on all
290 the calculated matrices, Bray-Curtis (PERMANOVA; Pseudo-F=9.239; p=0.001), Jaccard
291 (PERMANOVA; Pseudo-F=5.491; p=0.001), Unweighted UniFrac (PERMANOVA; Pseudo-
292 F=11.219; p=0.001) and Weighted UniFrac (PERMANOVA; Pseudo-F=9.189; p=0.001)
293 distances. This large difference in beta diversity index between the two river systems indicates a
294 low level of similarity in fish diversity between TR and KR river systems (Fig 6).

295

296 **Fig 6.** Beta diversity as observed between KR and TR sites as determined in Bray-Curtis,
297 Jaccard, Unweighted UniFrac and Weighted UniFrac distances analysis.

298

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299 # Discussion

300 Fish biodiversity assessment is an important tool for understanding complexity and
301 interdependence of different species and their role in aquatic ecosystems. The biodiversity
302 assessment can help identify species and habitats at risk of extinction or degradation, and thus has
303 potential to inform conservation efforts to protect and restore such species and habitats. Such
304 assessment can be used to inform management of natural resources such as water ensuring that
305 these resources are used sustainably.

306

307 eDNA analysis is a relatively new biodiversity assessment tool that has been used in a variety of
308 fields, including ecology, conservation, and environmental management. eDNA analysis has
309 several advantages over traditional methods of species identification and monitoring, such as
310 visual observation by physical sampling methods. eDNA analysis being a non-invasive technique,
311 precludes possibility of direct negative impact on the study species or ecosystem in addition to
312 reducing resources and time. This technique is highly effective at delineating ranges of rare species
313 (26, 27) and documenting migration patterns of species that may only use habitats for short periods
314 of time (28-30). Such work can highly contribute EIA of the Himalayan rivers where movement
315 patterns of economically important migratory species such as mahseer and snowtrout are not yet
316 well understood. Thus, eDNA technique is a valuable tool with a potential to revolutionize an
317 understanding and management of an ecosystems and their species. It has a potential to timely
318 inform and alert resources managers of potential negative consequences impacted by various
319 factors such as dam construction.

320

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321 In this study, via eDNA analyses, we identified 24 OTUs in TR and 46 OTUs in KR sites.
322 Identification of these fish species through 12S DNA sequences depended on accuracy of these
323 DNA sequences and representative reference database such as the NCBI GenBank. Due to
324 substantial lack of references for Asian fishes on the NCBI database, species level resolution of
325 several fish such as *Schizothorax* and *Garra* could not be attained beyond genus level, reflecting
326 the need for further taxonomic clarity by assessing multiple gene segments.
327 In our study, for the most relatively abundant genera, *Schizothorax* and *Barilius*, the OTUs found
328 in the KR were almost 8 to 12 times higher than in the TR. Because a higher eDNA concentration
329 might be linked to greater fish biomass, it may infer to a greater abundance of fish in KR compared
330 to TR site. Several mesocosm studies have shown a positive correlation between amount of eDNA
331 and animal density (31-33), however, this relationship is not as strong in nature, and further
332 refinement is needed to correlate quantitative relative abundance values of eDNA and actual
333 species estimates in the ecosystem (34). Hydropower dam construction can have a negative impact
334 on fish biodiversity and population by changing various aspects of fish ecosystem like migration,
335 prey resources and breeding habitat due to changes in river morphology and quality (35, 36). In
336 this study, there were over 12 hydropower projects currently in operations or under construction
337 in the TR drainage, which may have likely impacted on the fish diversity and population. Our
338 results of significantly lower OTUs in hydropower rich TR site compared to relatively pristine KR
339 site may support the developmental differences. Although fish density and biomass is affected by
340 many factors, anthropogenic activities like dam construction can have negative impacts (35, 36),
341 and our study suggests this could play a role in driving differences between the two rivers. Further
342 assessment and experimentation is required to confirm those assertions that hydropower directly
343 impacts the abundance, migration, breeding habits, and reproductive success of aquatic organisms

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344 in the affected rivers. Further, our results showing *Barilius* spp. and *Schizothorax* spp. being
345 identified as the most abundant fish species by both conventional and eDNA methods also
346 highlights significance of eDNA method.
347
348 To our knowledge, our results on some fishes provide novel information on their occurrence in
349 Nepali rivers. Black tetra is a common aquarium species native to South America that has become
350 established in Asia, including India (37). It inhabits slow-flowing river sections, and therefore has
351 the potential to become established in downstream sections of the Himalayan rivers. The loach
352 genus *Triplophysa* occurs at high-altitudes throughout the Qinghai-Tibet Plateau and adjacent
353 areas as well as the upper and middle Yangtze River, Nujiang River, upper Mekong River, Red
354 River, Yellow River, and Pearl River drainages of China, upper Indus and Tigris River drainages
355 of West Asia, and in river drainages of Central Asia (38). Further investigation is needed to confirm
356 the distribution of this species in Nepal.
357
358 Our study has important implications for monitoring of aquatic non-native species in Nepal. Non-
359 native fishes such as common carp and rainbow trout are prevalent in the Himalayan rivers to the
360 west of Nepal (39), with documented negative effects on native fishes (39, 40) . A recent review
361 suggests that eDNA methods are now sufficiently mature for natural resources managers to use
362 them when controlling non-native species (41). Although long-term datasets on fish diversity in
363 Nepal are scarce, data collected across three decades from 40 sites in the Kaligandaki-Naryani
364 River in central Nepal suggest that non-native species have not yet become well-established (6).
365 Our detection of black tetra, grass carp and North African catfish in the KR, blue tilapia and
366 rainbow trout in the TR, and common carp and silver carp in both the TR and KR therefore are

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367 cause for concern. Because many of these species are food fishes, and eDNA can come from fish
368 carcasses and slime (42), we cannot completely rule out the possibility that our positive eDNA
369 results were influenced by disposal of fish waste from local fish markets. However, we believe
370 this is unlikely, given the larger number of OTUs we detected. Further research can help to clarify
371 this. It is important to continue monitoring of non-native aquatic species in Nepal, and eDNA
372 methods should be an important part of this effort.
373
374 In light of the factors discussed above, we strongly recommend expansion of eDNA surveys across
375 Nepal. The eDNA metabarcoding methods are often more effective at detecting rare species than
376 traditional survey methods, and are part of regular monitoring efforts in other parts of the world
377 (43, 44). As eDNA studies expand across Nepal, we anticipate that the DNA reference database
378 for Nepali fishes presented here will be extremely beneficial to facilitate species monitoring.
379

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394

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504

505

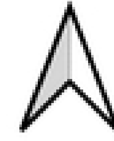
506 Supporting information

507 **S1 Table.** eDNA identified fish species in TR and KR along with their relative abundance

508 **S1 Fig. The variation of relative abundance of all the identified eDNA OTUs represented**
509 **on the basis of different phases of the eDNA projects in this study.** The pre-monsoon
510 sampling were conducted during April/May while the post-monsoon sampling were conducted
511 during September/October months.

512

eDNA Study Site- Karnali River



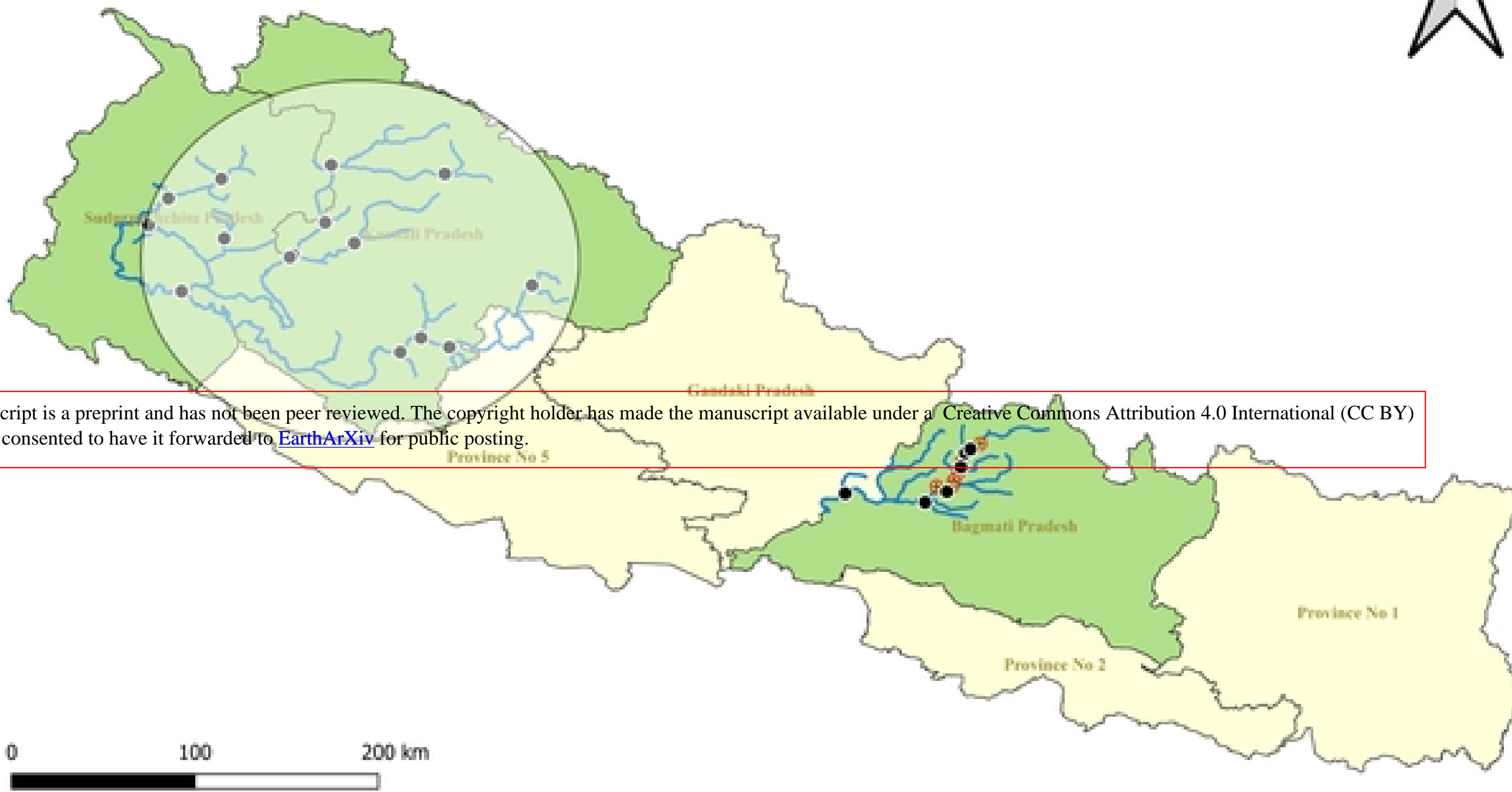


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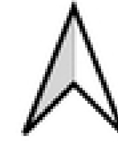
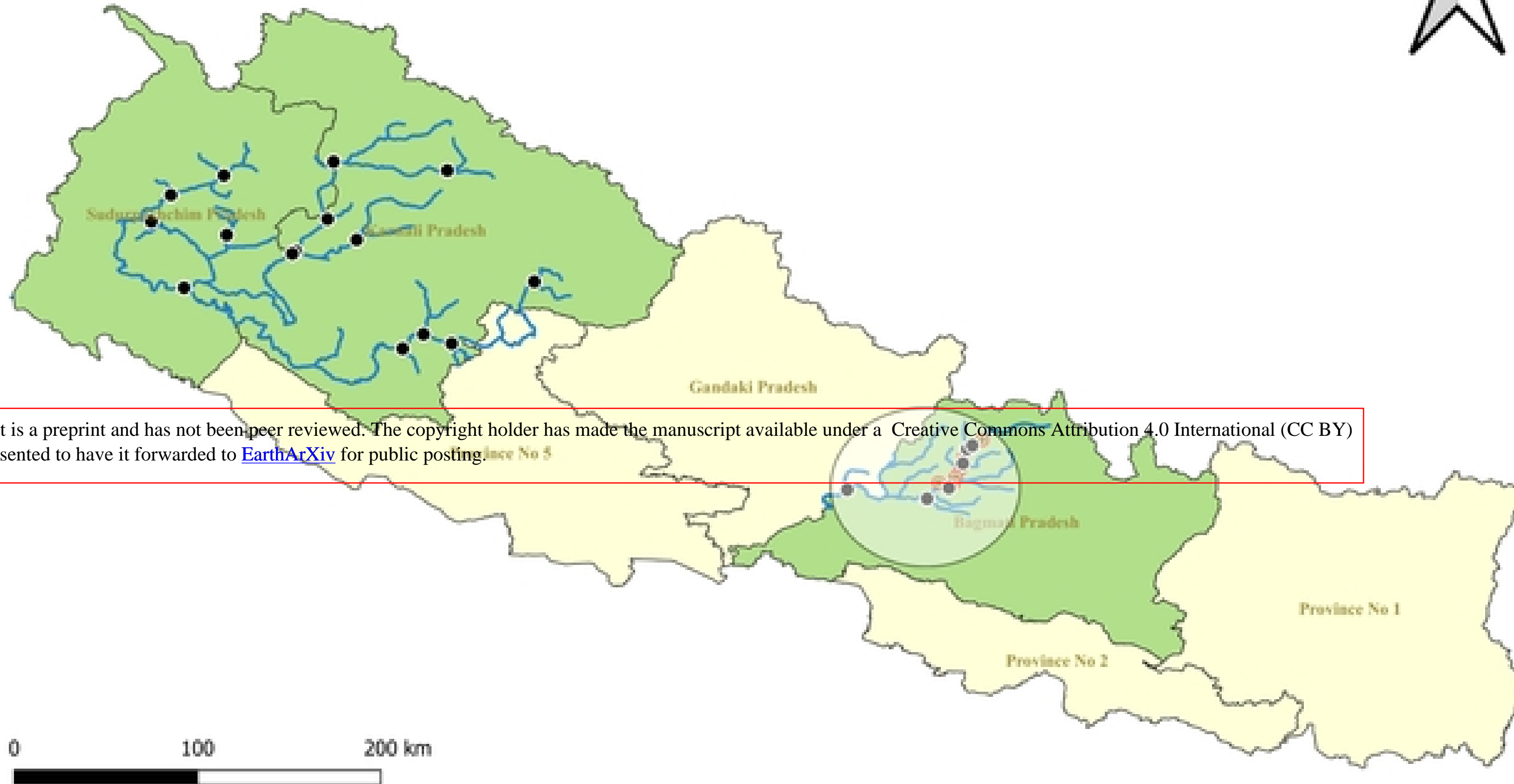
Fig1

eDNA Study Site- Trishuli River





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Sampling Point
Hydropower Station
River

Gandaki Pradesh
Bagmati Pradesh
Langtang Khola
Trishuli 216 Hydro limited
Mailun Khola Powerhouse
Mailung Khola
Upper trishuli 3A dam
Upper trishuli 3A powerhouse
Budi Gandaki
Salankhu
Downstream of UT-3B
Thoppal
Trishuli power dam
Trishuli power station
Tadi Khola
Trishuli Hydropower Station, Pasang Lhaamu Highway
Batar river side power station
Devighat hydropower
Upstream of Tadi confluence
Downstream of Super Trishuli HPP
Trishuli River
Kalphu Khola
Mahesh Khola
0
10
20 km

Fig2

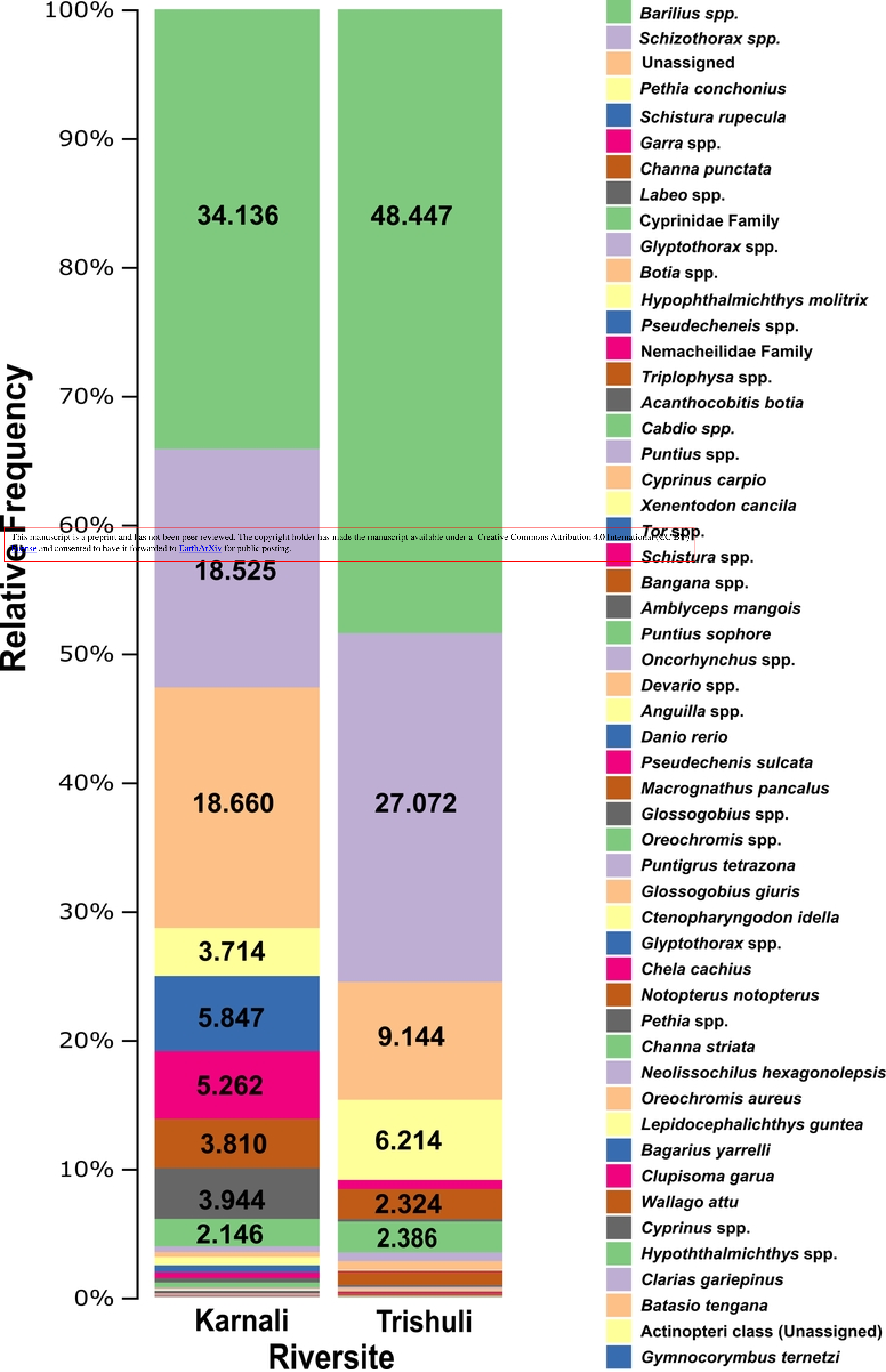

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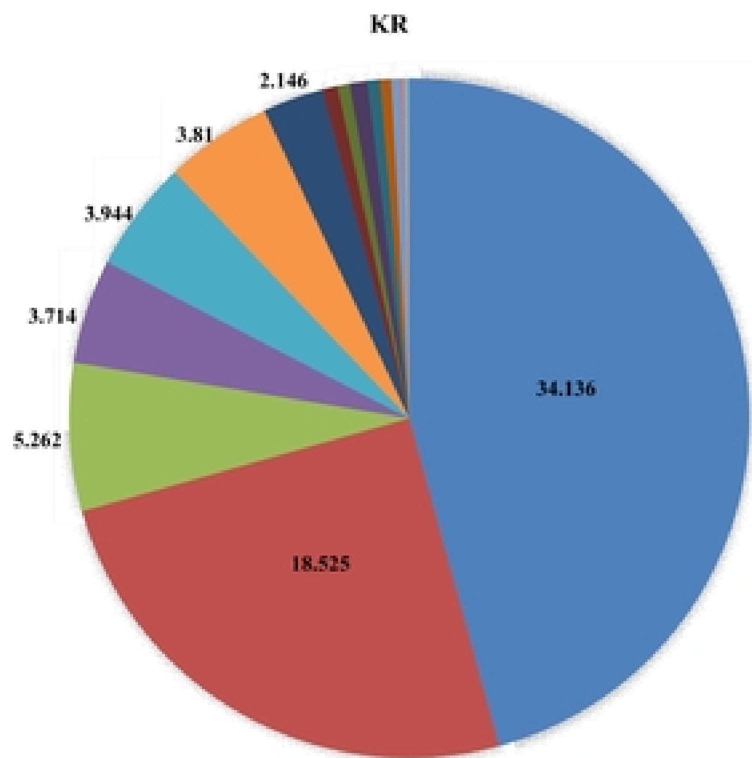
Fig3



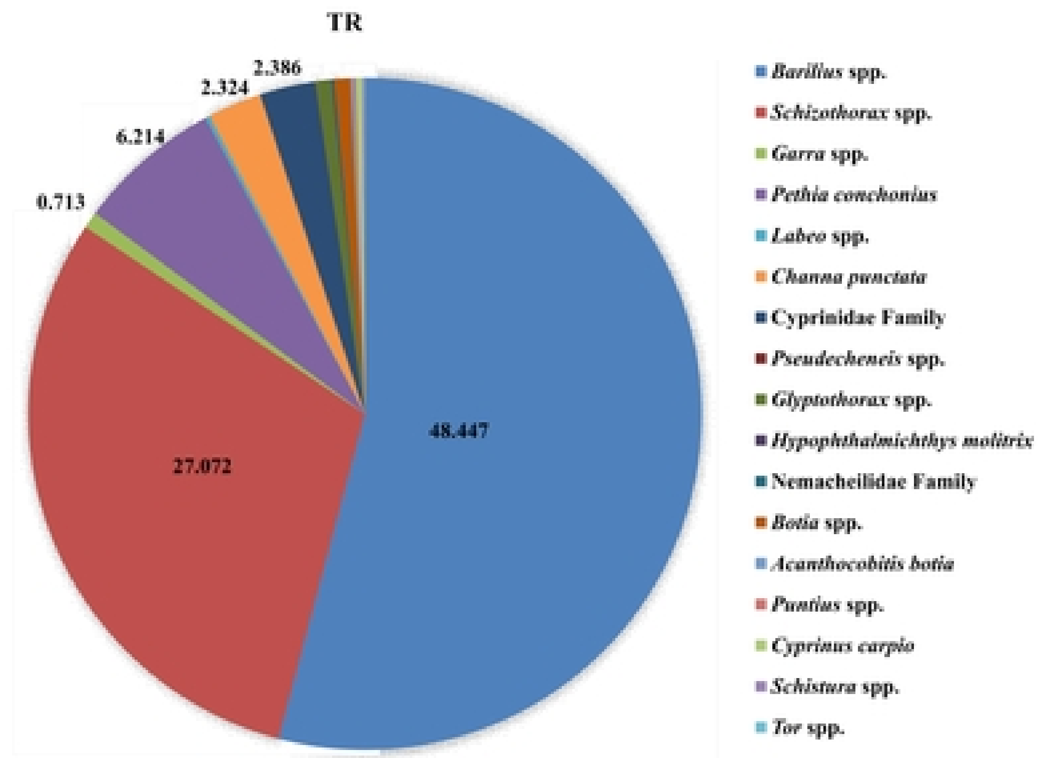
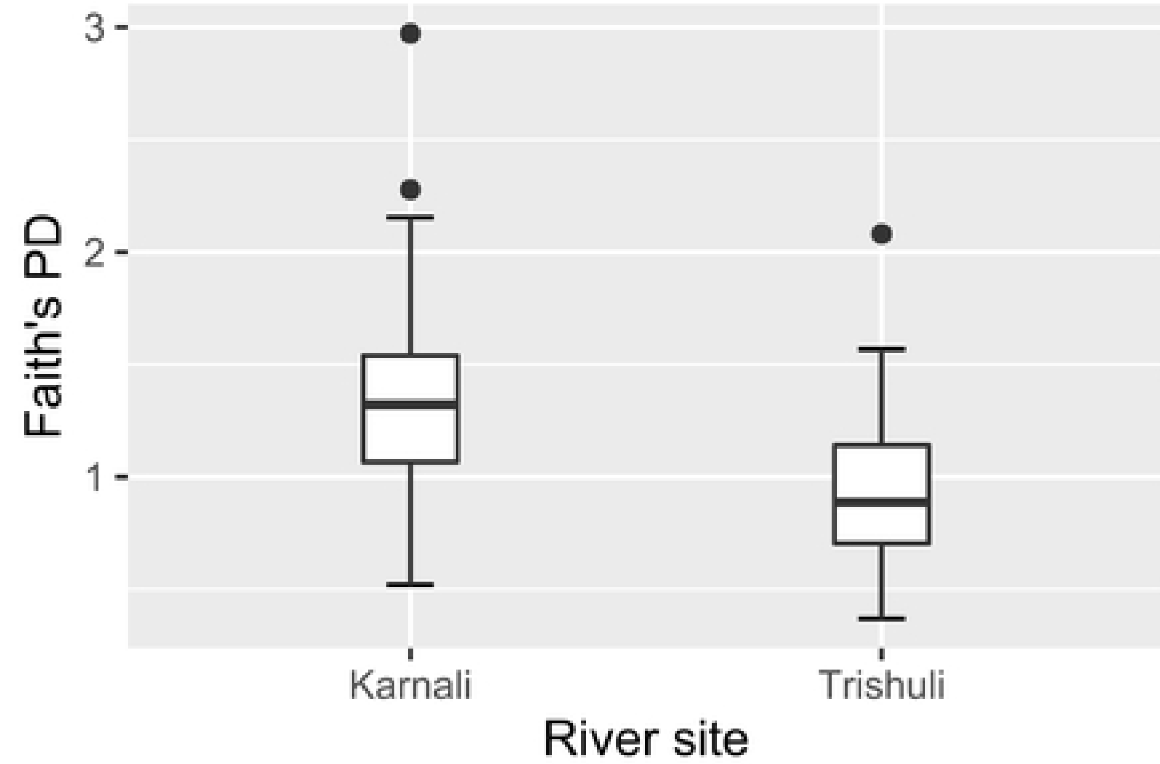
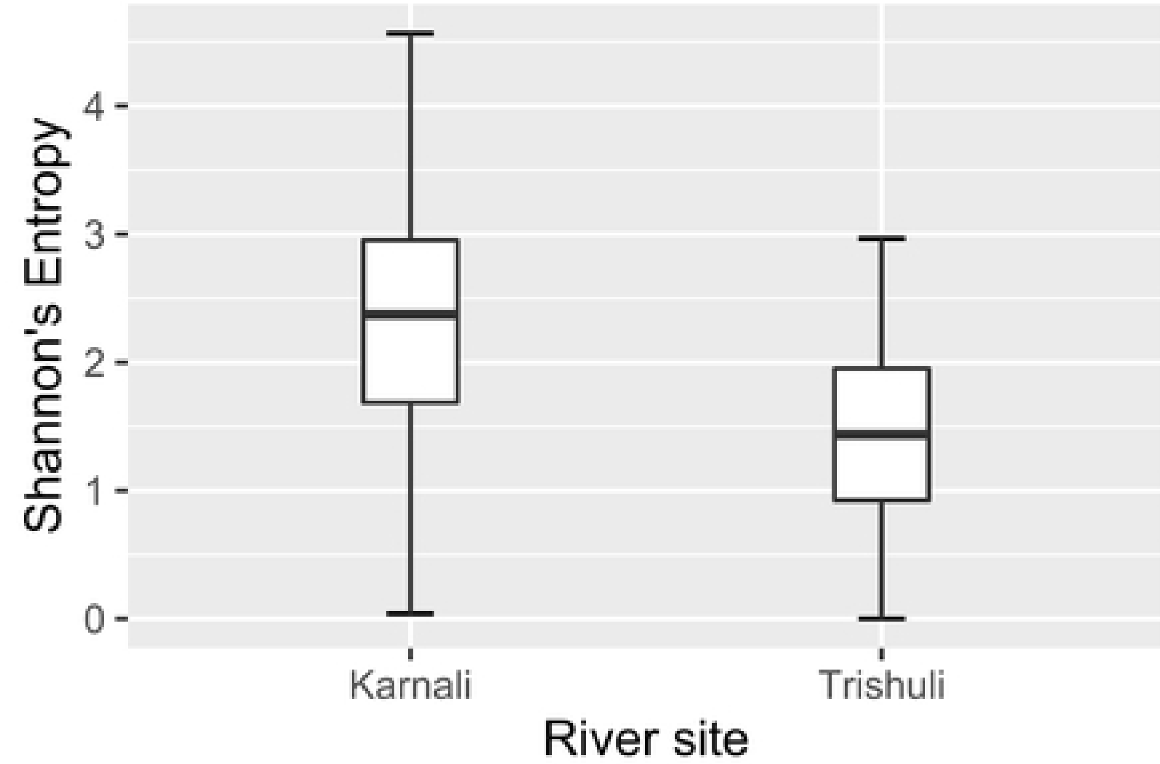


Fig4





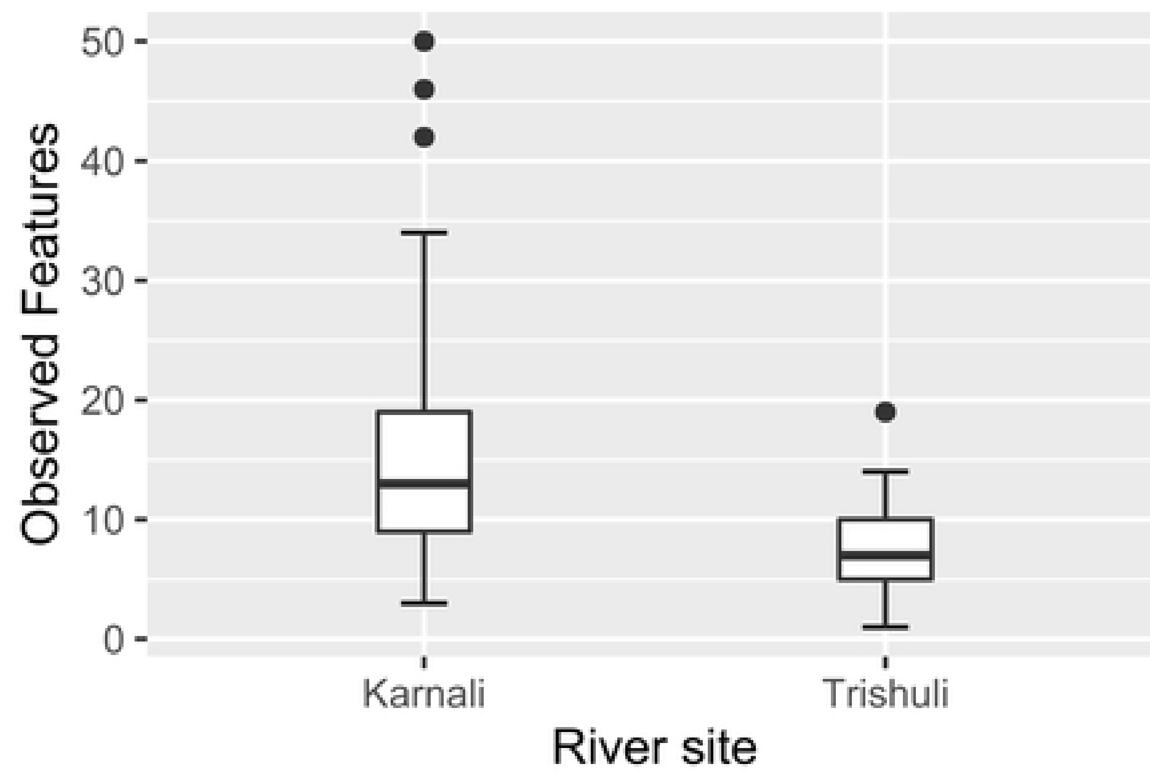
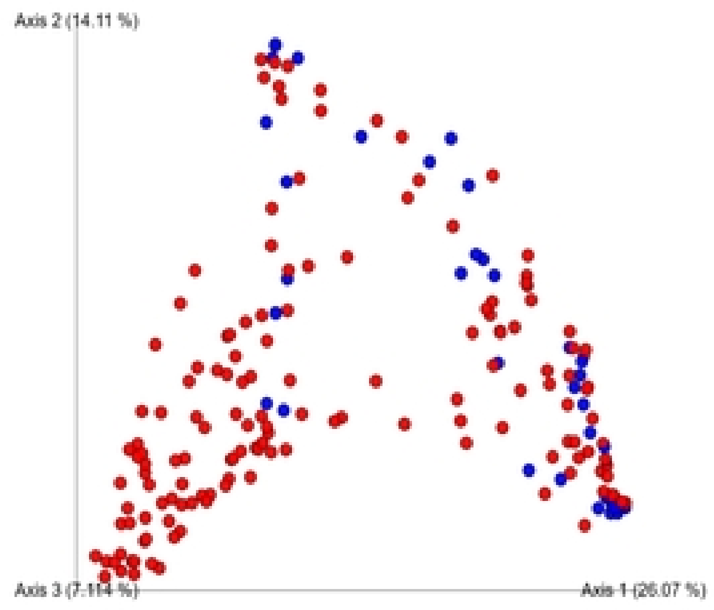
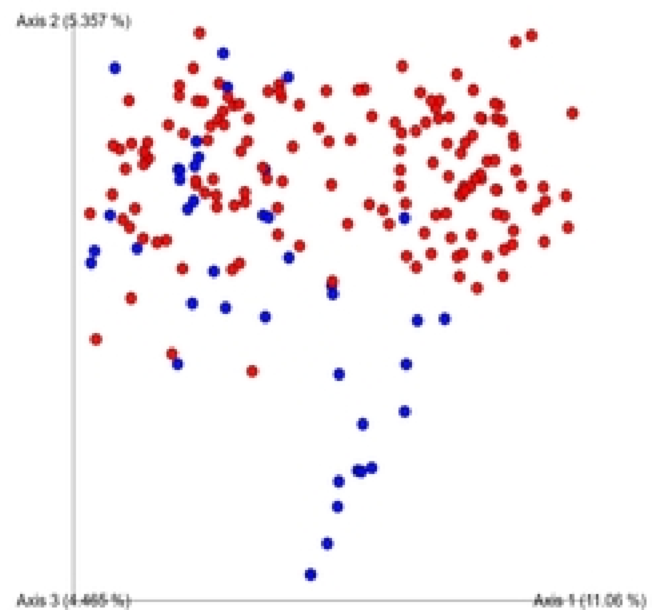


Fig5

Bray curtis



Jaccard

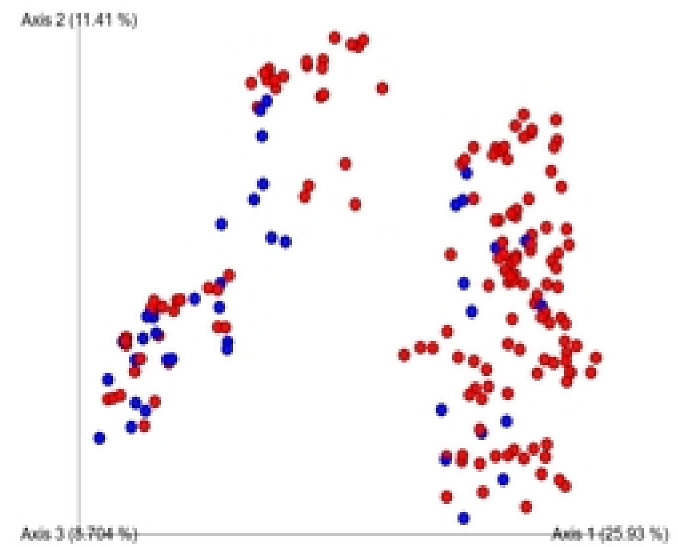


Legend

Karnali

Trishuli

Unweighted UniFrac



Weighted UniFrac

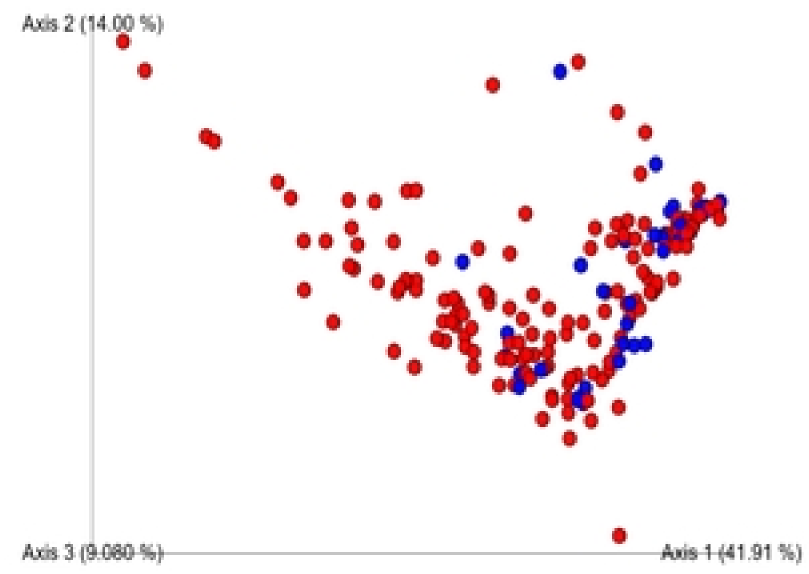


Fig6