PLOS WATER

RESEARCH ARTICLE

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

Prajwol Manandhar[1], Sulochana Manandhar[1], Adarsh M. Sherchan[1,2], Jyoti Joshi[1], Hemanta K. Chaudhary[1], Bimala Dhakal[1], Rajindra Napit[1], Bishwo Shrestha[1], Pragun G. Rajbhandari[1], Seily Shrestha[1], Saman M. Pradhan[1], Ashok Chaudhary[1], Dhiraj Puri[1], Smriti Khadka[1], Nikita Pradhan[1,3], Ajit Poudel[1], Jessie A. Moravek[1,4], Deepak Risal[5], Nilu Basnyat[5], Pablo Cardinale[6], David Philipp[7], Julie E. Claussen[7], David Gillette[8], Sushan M. Shakya[9], Genuine Prajapati[9], Laura Bertola[10], Dibesh Karmacharya[1,11]*

1 Center for Molecular Dynamics Nepal, Kathmandu, Nepal, **2** Biome and Ecosystem Solutions (Pvt.) Ltd., Lalitpur, Nepal, **3** The Australian National University, Canberra, Australia, **4** University of California—Berkeley, Berkeley, California, United States of America, **5** DAI Country Focal Point, Kathmandu, Nepal, **6** World Bank Group, Environmental and Social Advisor for South Asia, Washington, DC, United States of America, **7** Fisheries Conservation Foundation, Champaign, Illinois, United States of America, **8** University of North Carolina, Asheville, North Carolina, United States of America, **9** Tribhuvan University, Kathmandu, Nepal, **10** Department of Biology, University of Copenhagen, Kobenhavns, Denmark, **11** School of Biological Sciences, University of Queensland, Brisbane, Australia

* dibesh@cmdn.org

Citation: Manandhar P, Manandhar S, Sherchan AM, Joshi J, Chaudhary HK, Dhakal B, et al. (2023) Environmental DNA (eDNA) based fish biodiversity assessment of two Himalayan rivers of Nepal reveals diversity differences and highlights new species distribution records. PLOS Water 2(6): e0000099. https://doi.org/10.1371/journal.pwat.0000099

Editor: Bimlesh Kumar, Indian Institute of Technology Guwahati, INDIA

Received: January 31, 2023

Accepted: May 3, 2023

Published: June 7, 2023

Peer Review History: PLOS recognizes the benefits of transparency in the peer review process; therefore, we enable the publication of all of the content of peer review and author responses alongside final, published articles. The editorial history of this article is available here: https://doi.org/10.1371/journal.pwat.0000099

Data Availability Statement: Data associated to the study is submitted to NCBI Genbank database

Abstract

Although over 180 freshwater fish species have been reported from Nepal, little is known of their ecology and distribution. This information is needed because their diversity may be threatened by developments like hydropower constructions. We conducted Nepal's first environmental DNA (eDNA) based fish biodiversity assessment in two major river systems-Karnali River (KR), which is still pristine and Trishuli River (TR) with numerous hydropower plants. The eDNA was concentrated by filtering (0.45 μm pore size) two liters of water collected at different sampling points in each study site. A total of 224 eDNA samples (KR = 162 and TR = 62) were collected, from which fish species were identified by 12S rRNA metabarcording approach utilizing Illumina sequencing platform. Alpha and beta diversity of species between two river basins were compared. Also, in KR site, fish (N = 795) were caught, and identified using COI gene based DNA barcoding method- building Nepal's first fish DNA reference database. Field sampling identified 21 species through morphology and DNA barcoding, where *Barilius* spp. and *Schizothorax* spp. were the most abundant. From 244 eDNA samples, 24 Operational Taxonomic Units (OTUs) were identified in TR and 46 in KR with 19 being common to both sites, 27 being unique in KR, and five in TR only. Most fishes were of Cypriniformes and Siluriformes orders, with *Barilius* spp. and *Schizothorax* spp. being the most abundant. Long distance migratory fish (*Tor* spp, *Neolissochilus*

and information is provided in Table 1 in the manuscript.

Funding: This study was conducted with the financial support from the USFS (Nepal Fish Biodiversity Project, awarded to DBK), the USAID (PAANI project implemented by DAI, awarded to DBK) and the IFC (Trisuli eDNA project, awarded to DBK). Some members of the funding agencies were involved in research design and conceptualization, including manuscript preparation (NB and PC).

Competing interests: The authors have declared that no competing interests exist.

hexagonolepis) and non-native fishes were identified in eDNA samples as well. Alpha diversity in TR was significantly lower than in KR. High beta diversity between the two sites indicated low similarity in fish diversity between the TR and KR. This study demonstrated the utility of eDNA as a non-invasive technique for biodiversity assessment which is particularly useful in areas like Nepal with scarce data on fish species distribution.

Introduction

Nepal is rich in water resources with over 745,000 hectares of land being covered with water [1]. This has made Nepal a country with the highest per capita hydropower potentials in the world with estimated theoretical power potential of ~ 43,000 megawatts (MW), though operational output in 2015 was 516 MW only [2]. Additionally, rivers in Nepal serve as important income source for many low income communities living closer to river banks. Harvest fisheries are intricately woven into social, economic, and cultural fabrics of many Nepalese communities. Over 180 freshwater fish species have been reported in Nepal's major river systems [3, 4]. However, updated information on ecology, distribution and diversity of fish species found in Nepal is limited hindering their conservation efforts [5]. Recent research on fish populations in Nepal's Kaligandaki—Narayani River suggests that local diversity may already have been declining in some areas [6].

Various anthropogenic developments can have significant impact on aquatic biodiversity and the ecosystem. Depending on nature and scale of these developmental activities, the magnitude of impact may vary. Construction of hydropower dams, reservoirs, and other infrastructures particularly can have devastating impacts by directly affecting flow and quality of water, and thus altering, fragmenting or entirely destroying aquatic habitats [7]. An increasing demand for renewable energy has resulted in an accelerated growth in hydropower development across the world including Nepal, impacting the aquatic biodiversity of previously free-flowing rivers [8, 9]. Incorporation of various mitigation and management measures, such as carefully designed construction plan, a comprehensive environmental impact assessments (EIA), habitat restoration, along with stringently enforced conservation laws can prevent or mitigate potential harmful impact on aquatic ecosystems. For this, it is critical to assess status of river systems so as to generate robust baseline datasets that can be used for successful EIA to monitor potential impacts of human activities [10].

Environmental DNA (eDNA) analysis is a scientific technique that involves the use of genetic material collected from a given environment to identify and monitor presence and abundance of species in that ecosystem [11]. This analysis has been used as a rapid assessment tool not only to evaluate existing biodiversity but also to monitor the extent and magnitude of biodiversity loss. Fish species monitoring has traditionally been conducted through physical sampling followed by morphological species identification. This technique often involves sacrificing the specimens, is subject to misidentification (especially with little-known and cryptic species), and often requires taxonomic experts to work in remote field settings. Emerging genomics-based tools such as eDNA can bring ease, accuracy, and reliability to large aquatic biodiversity assessment studies. This technology is based on extracting DNA from environmental samples, such as from river water, and obtaining from those samples the DNA sequences of standardized gene biomarker(s) using next generation DNA sequencing technology. The species of fish present in the river upstream from those sampling sites are then identified by cross referencing those DNA sequences to those listed in some of the widely used public reference databases.

The objective of our study was to build Nepal's first fish species database using eDNA based meta-barcoding technique, thereby creating a baseline fish diversity profile of two important river systems of Nepal.

Materials and methods

Study areas and site selection

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

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

Our eDNA sampling efforts were conducted through multiple projects, including Nepal Fish Biodiversity Project (NFBP, 2016–18) and PAANI project (PAANI, 2018–19) in KR, and Upper Trishuli eDNA assessment (2019–20, IFC funded) in TR (S1 Table). All of these projects assessed aquatic (fish) biodiversity of river systems of Nepal and created Nepal's first baseline fish database. We collected samples from nine sites representing three seasons in three phases (two pre-monsoon and one post-monsoon, 2016–2017) from the lower KR region. We also collected samples from fifteen sites in two phases from the upper KR region—Seti and Bheri catchments (pre and post monsoon, 2018) (Fig 1). Our eDNA sampling in the Gandaki basin (TR) included seven sites in the Trishuli catchment in two phases (pre and post monsoon, 2018) and from twelve sites in a single phase (pre-monsoon, 2020) (Fig 2).

Water sample collection for eDNA analysis

Each river site was sampled by scooping two liters of water (within 1 feet deep from the surface) at four different points (upstream, downstream, pool, and riffle) located within a 100 meter stretch. The water sample was filtered in field through a cellulose membrane filter of 47mm diameter and 0.45 μm pore size (Whatman, UK) using battery operated electric filtration system. The filter membrane with residue was stored in 15 ml Longmire buffer solution. At each site, two liter of double distilled water filtered similarly was included as a negative control. The filtration assembly was thoroughly disinfected by immersing in a series of 10% sodium hypochlorite, 70% ethanol followed by sterile distilled water before, in between and after filtration to prevent any carryover contamination. A total of 224 eDNA samples (162 from KR during 2016–2018 over five different phases; and 62 from TR during 2018 and 2020 over three different phases) were processed.

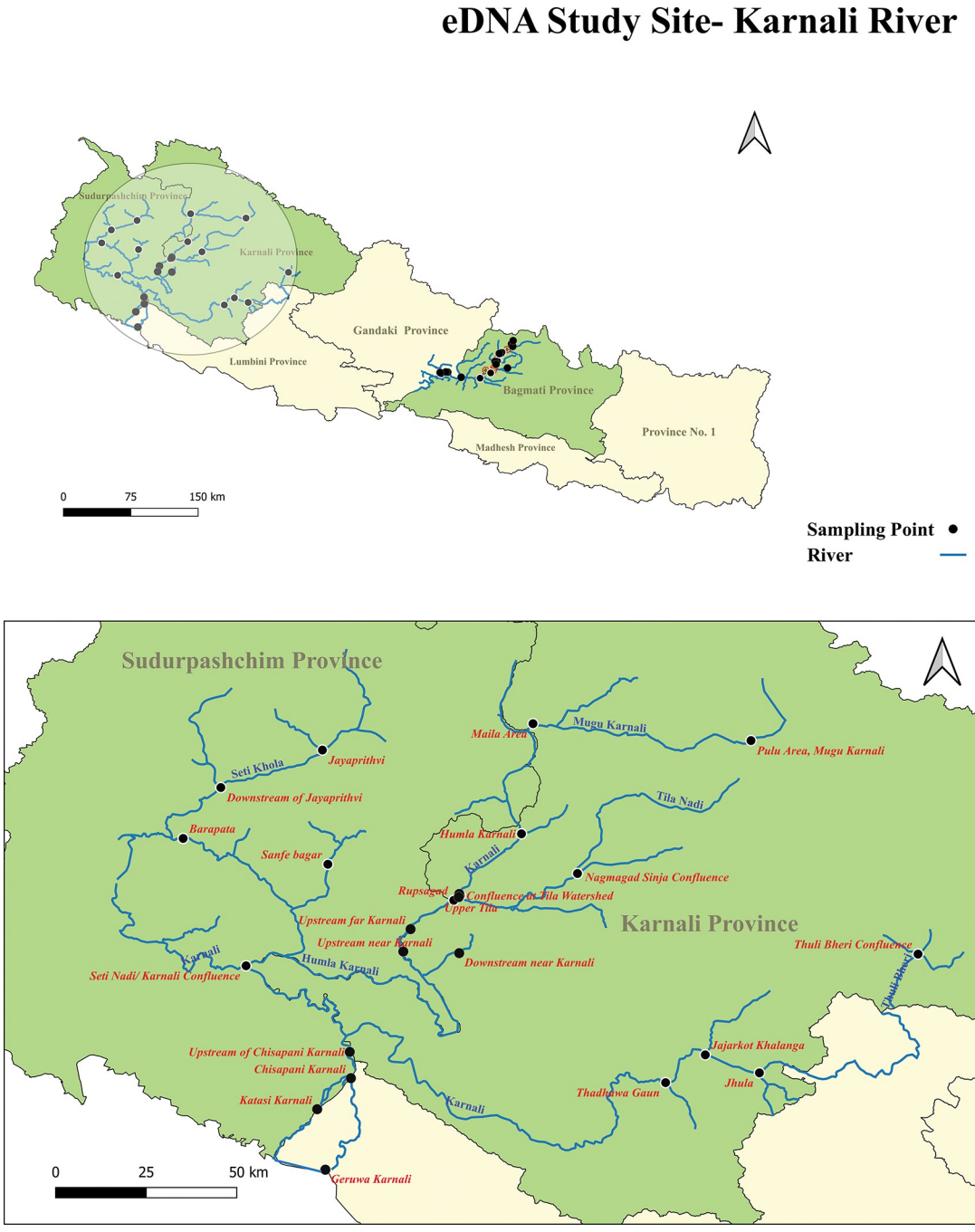

Fig 1. eDNA sample collection sites along major tributaries across Karnali River (KR). The sampling sites in KR cover catchments from Seti, Bheri, Upper Karnali and Lower Karnali tributaries. The map was created using QGIS v3.30.0 (https://www.qgis.org/en/site/), and the base map of Nepal administrative shape files obtained from Open Data Nepal (https://opendatanepal.com).

https://doi.org/10.1371/journal.pwat.0000099.g001

Building a freshwater fish reference database

At each water sample collection sites in KR, fish were also physically caught using a standard cast net (diameter = 4m, length = 2.2m) to build a local fish reference database. A total of 10 casts were performed at each sampling location, with the locations separated by a shoreline

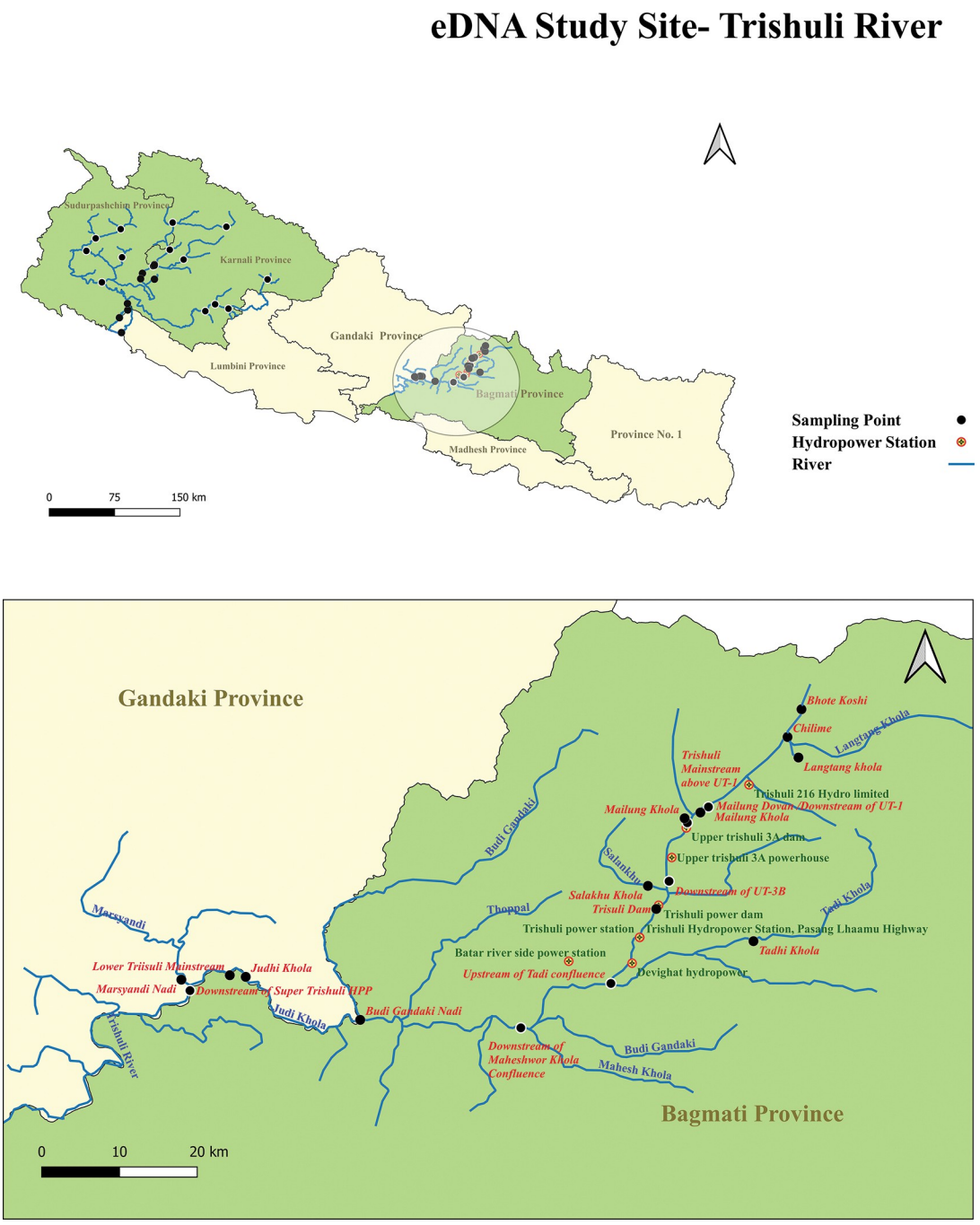

Fig 2. eDNA sample collection sites along major tributaries across Trishuli River (TR) basins in Nepal. TR is the eastern-most tributary of the Gandaki river basin. The map was created using QGIS v3.30.0 (https://www.qgis.org/en/site/), and the base map of Nepal administrative shape files obtained from Open Data Nepal (https://opendatanepal.com).

https://doi.org/10.1371/journal.pwat.0000099.g002

distance of about 100 m as per Trisuli Assessment Tool Field Manual [12]. The captured fishes from each cast net sample were collected, photographed, species identified using manuals from *Ichthyology of Nepal*, *2008* [13], measured by weight and length, and counted by species. Representative individuals of each species were transported to laboratory in 70% ethanol, where ~1 gram of tissue sample was excised from ventricle side for DNA barcoding, and then

whole fish was preserved as voucher specimens in 5% formalin. DNA was extracted from the excised fish tissue using DNeasy Blood and Tissue Kit (Qiagen, Germany) as per manufacturer's instruction. We selected the mitochondrial cytochrome *c* oxidase subunit 1 (COI) gene as a species identification gene biomarker. For genetic species identification, DNA barcoding was performed by amplifying COI region using M13 linked COI primer cocktail (VF2_t1, FishF2_t1, FishR2_t1 and FR1d_t1) at final concentration of 0.10 pMol/μL [14]. The 650 bp amplicon was subjected to Sanger sequencing. Finally, species identification was performed using BLAST tool on reference sequences in the NCBI GenBank database. DNA sequences of the identified fish species were deposited in NCBI database.

eDNA extraction, 12S PCR based metabarcoding and sequencing

In laboratory, the tube with filter membrane was vigorously vortexed to elute residue to the buffer solution. After removing the membrane, the buffer was then centrifuged at 8,000 rpm for 10 minutes to concentrate the residue. After decanting the supernatant, ~700 μL of pellet was used as a sample for eDNA extraction using GeneAll Exgene Tissue SV Mini Kit (GeneAll, South Korea) following manufacturer's instructions. For fish species identification, we amplified 170 bp region of fish mitochondrial 12S rRNA gene from the eDNA samples using MiFish primers [15] tagged with Illumina overhang adapters and sequenced in next generation sequencer (Illumina MiSeq). As per the Illumina protocol, the subsequent 8 cycle index PCR was performed using specific combinations of forward and reverse index primers (Nextera® XT Index Kit, Illumina, USA) with annealing at 55˚C for 30s to specifically allow multiplexing of samples in a single sequencing run. After AMPure XP magnetic beads based purification, the samples were pooled, library was quantified using Qubit dsDNA HS assay kit (Thermo Fisher Scientific, USA), normalized at 4nM, and finally the 10 pM library was subjected to pair-end sequencing on an Illumina MiSeq instrument using a MiSeq Reagent Kit v2 300 cycles (Illumina, USA).

Bioinformatics analysis

After initial quality assessment of raw MiSeq reads using FastQC v0.11.9 [16], and filtering using Trimmomatic v0.39 [17], the cleaned reads were processed using QIIME2 v2021.11.0 pipeline [18]. We performed de-noising of paired-end reads by trimming, merging and removing chimeric sequences using the DADA2 plugin [19]. We processed the denoised sequences for fish DNA filtering, which will only retain sequences belonging to fishes and filters out all other non-fish vertebrates, prokaryotes (bacteria, diatoms) etc. that could have been the products of non-target amplifications. For this, we utilized quality-control plugin in QIIME2 with percent identity 0.7 and percent query aligned 0.9 thresholds against a fish reference sequences. This method aligns our query sequences (denoised dataset) to the fish reference sequences and excludes any non-target sequences (eg. bacteria, diatoms, non-fish vertebrates etc.) from the input data. From the quality controlled sequences, we then generated sequence features (representative sequences) as amplicon sequence variants (ASVs), i.e., Operational Taxonomic Units (OTUs) at 100% sequence similarity, and produced a de-replicated feature table (with sequence counts) across the samples.

For taxonomy assignment, Mitohelper repository [20] was used as a reference which was curated for reference sequence analysis in the fish eDNA studies. This database consists of QIIME2-compatible datasets of fish 12S rRNA reference sequences and taxonomy classification information. The reference datasets of Mitohelper were compiled using complete and partial fish mito-genome sequences obtained from the MitoFish database, with further gene definition and taxonomic classification obtained from the NCBI nucleotide and taxonomy database.

The fish systematics data including order and family numbers were further retrieved and verified from the *Fishes of the World* for creating this curated fish reference database [21]. As of July 2022 release, 12S rRNA Mitohelper database consisted of 89 known taxonomic orders, 546 families, 3,444 genera and 12,335 species of fishes.

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

Fish diversity comparisons between two river systems

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

We analyzed the rarefied abundance data with a Kruskal-Wallis pairwise test to evaluate the alpha-significance of the diversity across river basins. For this purpose, we assessed the alpha diversity using metrics of Faith's phylogenetic diversity (PD) [22], the Shannon Diversity indices [23] and observed features or amplicon sequence variants. We visualized the alpha diversity boxplots using R package ggplot2 v3.34 [24].

For beta diversity, we calculated pairwise permutation analysis of variance (PERMANOVA) statistics by running 999 permutations based on the Bray-Curtis [25], Jaccard, Unweighted UniFrac and Weighted UniFrac dissimilarity metrices [26] in QIIME2. Bray-Curtis and Jaccard distance matrices assessed dissimilarities of samples based on relative abundance and presence/absence of ASVs respectively. Meanwhile Unweighted and Weighted UniFrac distance matrices calculated the dissimilarities of samples based on sequence distances and branch lengths weighted by relative abundances of ASVs. We, then, generated principal coordinate analysis (PCoA) plots of beta diversity distance matrices using Emperor Plugin in QIIME2 to visualize the similarities or dissimilarities among all of the samples across two river basins.

Results

General fish diversity in physically caught samples

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

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

*represents caught species that were also found during eDNA analysis

https://doi.org/10.1371/journal.pwat.0000099.t001

General fish diversity in eDNA samples

A total of 179,224 de-replicated sequence features (Amplicon Sequence Variants-ASV) were generated, out of which a total of 51 Operational Taxonomic Units (OTUs) were assigned a taxonomy at either family, genus or species level (Fig 3 and S2 Table). About 16% of ASVs were unassigned, because they did not meet consensus taxonomy assignment thresholds. We identified 24 OTUs in the TR and 46 OTUs in the KR. Among these, 19 OTUs were common in both river basins, 27 were found only in KR and five were found only in TR (S2 Table and S1 Fig). The OTU values were higher across the board in KR than TR.

Most of the freshwater fishes from both study systems belonged to the Cypriniformes and Siluriformes orders. We identified the presence of some long distance migratory fishes such as snow trout (*Schizothorax* spp.) and mahaseer (*Tor* spp.) in both river basins, while copper mahaseer (*Neolissochilus hexagonolepis*) was detected in KR only. Interestingly, we also found Tibetan loach (*Triplophysa* spp), a new genus recently identified in the Upper Humla- a tributary of KR basin. We also detected eight non-native commercial fish species. Common carp (*Cyprinus carpio*) and silver carp (*Hypophthalmichthys molitrix*) were detected in both TR and KR, while blue tilapia (*Oreochromis aureus*), and rainbow trout (*Onchorhynchus* spp.) were found in TR only. Similarly, black tetra (*Gymnocorymbus ternetzi*), grass carp (*Ctenopharyngo donidella*), and North African catfish (*Clarias gariepinus*) were detected only in KR.

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

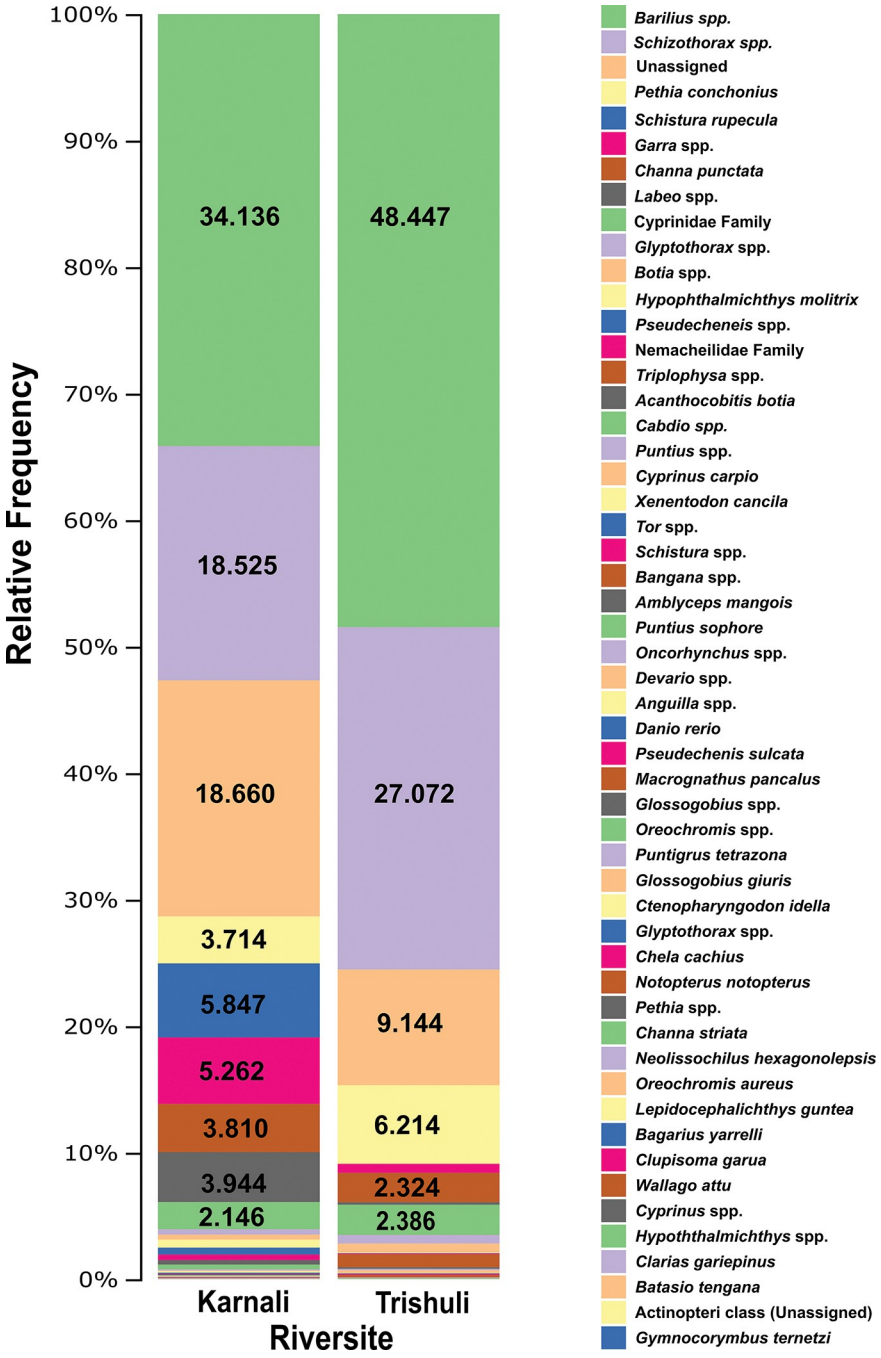

Fig 3. Fish species and their relative frequency (%) identified in Karnali (KR) and Trishuli (TR) sites by eDNA method.

https://doi.org/10.1371/journal.pwat.0000099.g003

Alpha and beta diversity of the two river systems

Our rarefaction analysis was based on 196 eDNA samples (Trishuli = 44, Karnali = 152), some samples (n = 28) failed quality control threshold and hence were excluded. When we inspected the Faith's phylogenetic diversity (PD) across the river basins, we found that its average values were 0.93 in TR and 1.31 in KR. The PD differed significantly between the two river basins (Kruskal-Wallis: H = 30.702, p = 3.009E-08). The average value of the Shannon diversity was

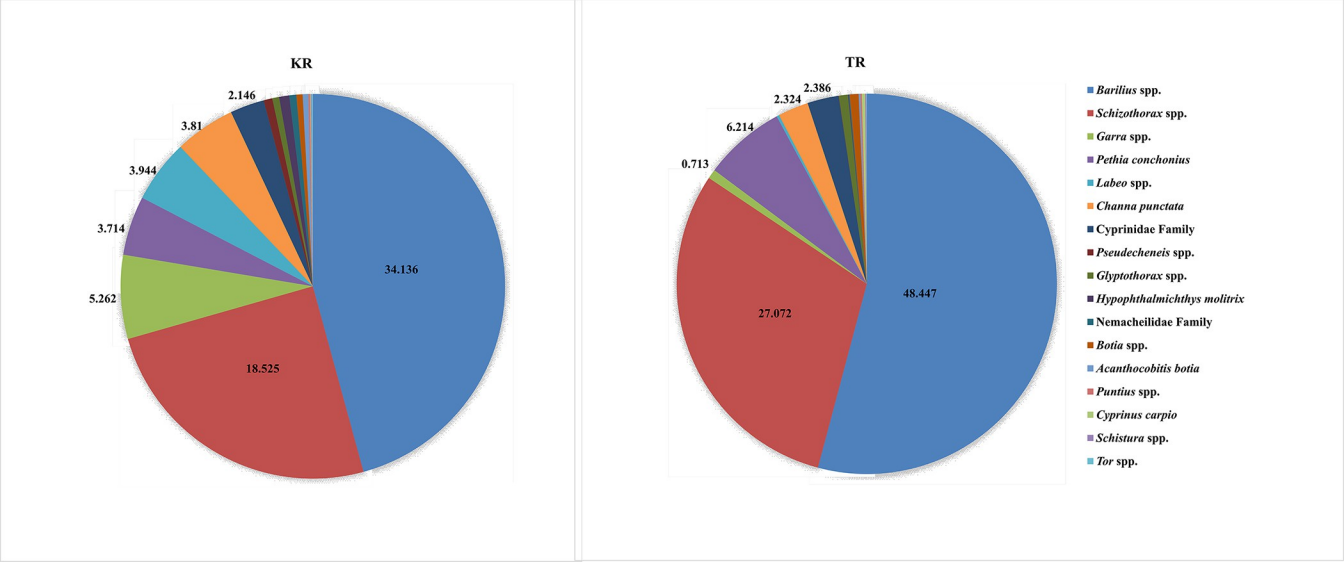

Fig 4. Percentage relative OTU frequencies of common eDNA species found in KR and TR river sites.

https://doi.org/10.1371/journal.pwat.0000099.g004

1.36 in TR and 2.29 in KR, values with significant differences (Kruskal-Wallis: H = 31.509, p = 1.984E-08). We found the average value of observed features was 7.27 in TR and 14.82 in KR, and again the differences were highly significant (Kruskal-Wallis: H = 42.226, p = 8.129E-11). Overall, across all tests, the alpha diversity in TR was significantly lower than KR (Fig 5).

The PCoA of beta-diversity analyses showed that KR and TR samples were different in overall diversity. KR samples, collected from larger KR landscape compared to landscape in TR basin, had higher and broader beta-diversity than that found in TR samples. We found significant differences in pair-wise Beta diversity between two river basins based on all the calculated matrices, Bray-Curtis (PERMANOVA; Pseudo-F = 9.239; p = 0.001), Jaccard (PERMANOVA; Pseudo-F = 5.491; p = 0.001), Unweighted UniFrac (PERMANOVA; Pseudo-F = 11.219; p = 0.001) and Weighted UniFrac (PERMANOVA; Pseudo-F = 9.189; p = 0.001) distances. This large difference in beta diversity index between the two river systems indicates a low level of similarity in fish diversity between TR and KR river systems (Fig 6).

Discussion

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

eDNA analysis is a relatively new biodiversity assessment tool that has been used in a variety of fields, including ecology, conservation, and environmental management. eDNA analysis has several advantages over traditional methods of species identification and monitoring, such as visual observation by physical sampling methods. eDNA analysis being a non-invasive technique, precludes possibility of direct negative impact on the study species or ecosystem in addition to reducing resources and time. This technique is highly effective at delineating ranges of rare species [27, 28] and documenting migration patterns of species that may only use habitats for short periods of time [29–31]. Such work can highly contribute to EIA of the

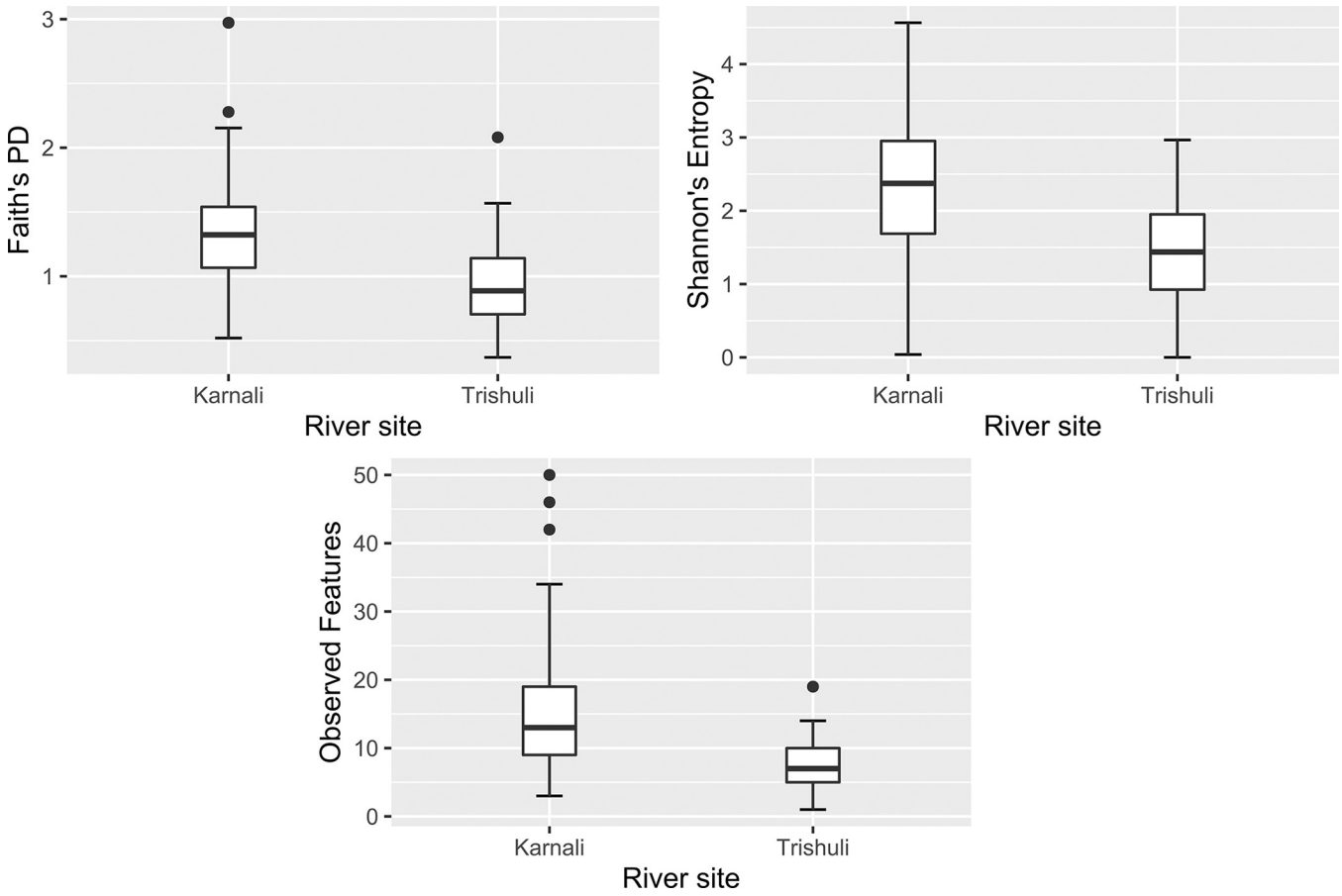

Fig 5. Alpha diversity detected in the KR and TR river systems based on Faith's PD, Shannon diversity, and observed features using Kruskal-Wallis analysis.

https://doi.org/10.1371/journal.pwat.0000099.g005

Himalayan rivers where movement patterns of economically important migratory species such as mahseer and snowtrout are not yet well understood. Thus, eDNA technique is a valuable tool with a potential to revolutionize an understanding and management of ecosystems and their species. It has a potential to timely inform and alert resource managers of potential negative consequences impacted by various factors such as dam construction.

In this study, via eDNA analyses, we identified 24 OTUs in TR and 46 OTUs in KR sites. Identification of these fish species through 12S DNA sequences depended on accuracy of these DNA sequences and representative reference database such as the NCBI GenBank. Due to substantial lack of references for Asian fishes on the NCBI database, species level resolution of several fish such as *Schizothorax* and *Garra* could not be attained beyond genus level, reflecting the need for further taxonomic clarity by assessing multiple gene segments.

In our study, for the most relatively abundant genera, *Schizothorax* and *Barilius*, the OTUs found in the KR were almost 8 to 12 times higher than in the TR. Because a higher eDNA concentration might be linked to greater fish biomass, it may infer to a greater abundance of fish in KR compared to TR site. Several mesocosm studies have shown a positive correlation between amount of eDNA and animal density [32–34], however, this relationship is not as strong in nature, and further refinement is needed to correlate quantitative relative abundance values of eDNA and actual species estimates in the ecosystem [35]. Hydropower dam construction can have a negative impact on fish biodiversity and population by changing various

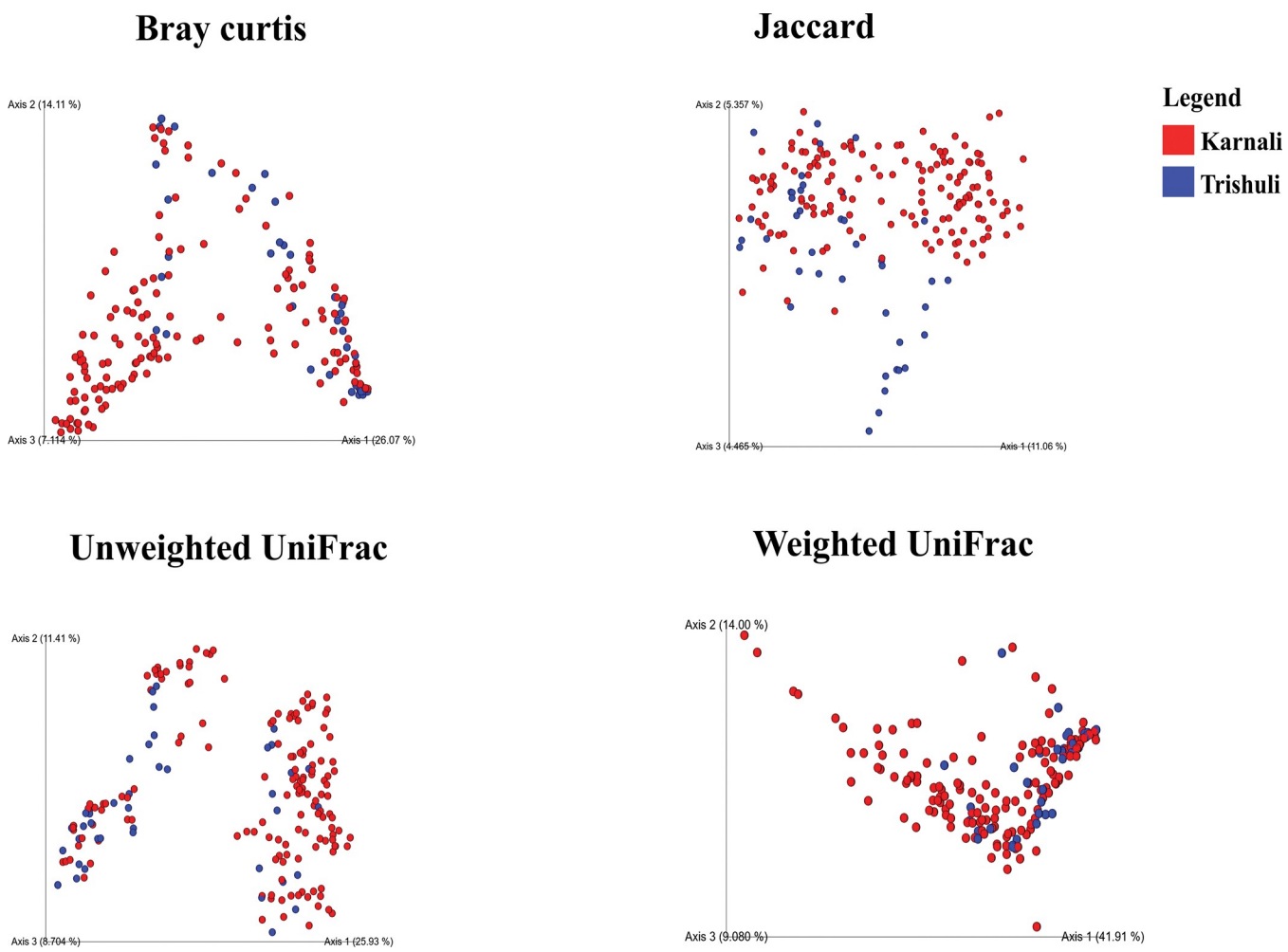

Fig 6. Beta diversity as observed between KR and TR sites as determined in Bray-Curtis, Jaccard, Unweighted UniFrac and Weighted UniFrac distances analysis.

https://doi.org/10.1371/journal.pwat.0000099.g006

aspects of fish ecosystem like migration, prey resources and breeding habitat due to changes in river morphology and quality [36, 37]. In this study, there were over 12 hydropower projects currently in operations or under construction in the TR drainage, which may have likely impacted on the fish diversity and population. Our results of significantly lower OTUs in hydropower rich TR site compared to relatively pristine KR site may support the developmental differences. Although fish density and biomass is affected by many factors, anthropogenic activities like dam construction can have negative impacts [36, 37], and our study suggests this could play a role in driving differences between the two rivers. Further assessment and experimentation is required to confirm those assertions that hydropower directly impacts the abundance, migration, breeding habits, and reproductive success of aquatic organisms in the affected rivers. Further, our results showing *Barilius* spp. and *Schizothorax* spp. being identified as the most abundant fish species by both conventional and eDNA methods also highlights significance of eDNA method.

To our knowledge, our results on some fishes provide novel information on their occurrence in Nepali rivers. Black tetra is a common aquarium species native to South America that has become established in Asia, including India [38]. It inhabits slow-flowing river sections, and therefore has the potential to become established in downstream sections of the

Himalayan rivers. The loach genus *Triplophysa* occurs at high-altitudes throughout the Qinghai-Tibet Plateau and adjacent areas as well as the upper and middle Yangtze River, Nujiang River, upper Mekong River, Red River, Yellow River, and Pearl River drainages of China, upper Indus and Tigris River drainages of West Asia, and in river drainages of Central Asia [39]. Further investigation is needed to confirm the distribution of this species in Nepal.

Our study has important implications for monitoring of aquatic non-native fish species in Nepal. Non-native fishes such as common carp and rainbow trout have infiltrated rivers in Indian Himalayan region to the west of Nepal [40, 41]. Most of these non-native fishes were also introduced in Nepali rivers through accidental or intentional release from sources like fish farms, religious and cultural practices, and ornamental aquarium trade [42–44]. Information obtained on non-native fish species from eDNA analysis can help control the problem [45]. Although long-term datasets on fish diversity in Nepal are limited, data from 40 sites collected over three decades in the Kaligandaki-Narayani River in central Nepal shows non-native fish species have yet to establish themselves in critical numbers in the infested rivers [6]. Our detection of black tetra, grass carp and North African catfish in the KR, blue tilapia and rainbow trout in the TR, and common carp and silver carp in both the rivers, therefore are cause for concern. For example, black tetra, native to tropical regions in South America, are known to tolerate cold water [46, 47], therefore, could survive and thrive in cold Himalayan winters and becoming a threat to native fish species and ecosystem.

Leftover from fish consumption (food) can be detected in eDNA analysis [48], our sampling sites close to fish markets or households consuming fish can have false positive results, detecting imported, farmed or natural fish species [49]. This limitation can be resolved by mapping fish markets and households living close to river banks and routinely monitoring the fish biodiversity using eDNA.

The eDNA metabarcoding technique is often more effective at detecting rare species of fishes compared to traditional survey methods, is getting popular as a regular monitoring tool [50, 51]. Our eDNA studies have been building reference database of Nepali fish species and helping in long-term monitoring of fish and aquatic biodiversity of Nepal.

Supporting information

S1 Table. Geographic information about the eDNA sampling locations across Karnali and Trishuli river basins.
(DOCX)

S2 Table. eDNA identified fish species in TR and KR along with their relative abundance.
(DOCX)

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

Acknowledgments

We would like to thank the Department of Forest (Nepal) and the Department of National Park and Wildlife Conservation (Nepal) for their cooperation and guidance on permitting the research study. The Nepal Fish Biodiversity Project was implemented by the US Forest Services; we would like to thank Mr. Nicolai Stoehr and Mr. Justin Green of the US Forest Services and Ms. Karolyn Upham and Ms. Bronwyn Llewellyn of USAID Nepal for assisting us.

The PAANI project covered larger catchments in Karnali basin and was implemented by DAI. The Trisuli eDNA study was implemented by the International Finance Corporation (IFC); we would like to show our gratitude to Dr. Leeanne Alonso for her technical guidance. We would like to thank all our funding agencies and implementing partners. Finally, we would like to thank all research associates, field personnel and intern students of Intrepid Nepal Pvt, Ltd., Center for Molecular Dynamics Nepal, Kathmandu University, Tribhuvan University and all other project partners for their help both in the field as well as in the lab.

Author Contributions

Conceptualization: Sulochana Manandhar, David Gillette, Dibesh Karmacharya.

Data curation: Prajwol Manandhar, Sulochana Manandhar, Adarsh M. Sherchan, Jyoti Joshi, Hemanta K. Chaudhary, Bimala Dhakal, Pragun G. Rajbhandari, Seily Shrestha, Nikita Pradhan, Sushan M. Shakya, Genuine Prajapati.

Formal analysis: Prajwol Manandhar, Jyoti Joshi, Hemanta K. Chaudhary, Pragun G. Rajbhandari, Dibesh Karmacharya.

Funding acquisition: Deepak Risal, Nilu Basnyat, Pablo Cardinale.

Investigation: Sulochana Manandhar, Adarsh M. Sherchan, Jyoti Joshi, Saman M. Pradhan, Ashok Chaudhary, Nikita Pradhan, Ajit Poudel, Jessie A. Moravek, David Philipp, Julie E. Claussen, David Gillette, Laura Bertola, Dibesh Karmacharya.

Methodology: Prajwol Manandhar, Sulochana Manandhar, Adarsh M. Sherchan, Jyoti Joshi, Hemanta K. Chaudhary, Bimala Dhakal, Rajindra Napit, Bishwo Shrestha, Pragun G. Rajbhandari, Seily Shrestha, Ashok Chaudhary, Dhiraj Puri, Nikita Pradhan, Jessie A. Moravek, Laura Bertola, Dibesh Karmacharya.

Project administration: Adarsh M. Sherchan, Jyoti Joshi, Hemanta K. Chaudhary, Saman M. Pradhan, Deepak Risal, Nilu Basnyat, Pablo Cardinale, David Philipp, Dibesh Karmacharya.

Resources: Bishwo Shrestha, Ashok Chaudhary, Dhiraj Puri, Dibesh Karmacharya.

Software: Prajwol Manandhar, Pragun G. Rajbhandari, Seily Shrestha, Smriti Khadka.

Supervision: Sulochana Manandhar, Adarsh M. Sherchan, Rajindra Napit, Jessie A. Moravek, David Philipp, Julie E. Claussen, David Gillette, Laura Bertola, Dibesh Karmacharya.

Validation: Adarsh M. Sherchan, Rajindra Napit, Bishwo Shrestha, Nikita Pradhan, David Philipp, Julie E. Claussen, David Gillette, Sushan M. Shakya, Laura Bertola, Dibesh Karmacharya.

Visualization: Hemanta K. Chaudhary, Bimala Dhakal, Rajindra Napit, Pragun G. Rajbhandari, Seily Shrestha, Smriti Khadka.

Writing – original draft: Prajwol Manandhar, Laura Bertola, Dibesh Karmacharya.

Writing – review & editing: Prajwol Manandhar, Sulochana Manandhar, Jessie A. Moravek, Pablo Cardinale, David Philipp, Julie E. Claussen, David Gillette, Laura Bertola, Dibesh Karmacharya.

References

1. Sharma CM. Freshwater fishes, fisheries, and habitat prospects of Nepal. Aquatic Ecosystem Health and Management. 2008; 11(3):289–97. https://doi.org/10.1080/14634980802317329

2. Chinnasamy P, Bharati L, Bhattarai U, Khadka A, Dahal V, Wahid S. Impact of planned water resource development on current and future water demand in the Koshi River basin, Nepal. Water International. 2015; 40(7):1004–20. https://doi.org/10.1080/02508060.2015.1099192
3. Gautam N. Challenges of Freshwater Fisheries in Nepal: A Short Overview. International Journal of Applied Sciences and Biotechnology. 2015; 3(4):579–83. https://doi.org/10.3126/ijasbt.v3i4.13524
4. Froese R, Pauly D. FishBase: World Wide Web electronic publication; 2022. Available from: https://www.fishbase.se/Country/CountryCheckList.php?showAll=yes&c_code=524&ext_pic=y.
5. Edds DR. Fish Assemblage Structure and Environmental Correlates in Nepal's Gandaki River. Copeia. 1993;1993(1):48-. https://doi.org/10.2307/1446294
6. Gillette DP, Edds DR, Jha BR, Mishra B. Thirty years of environmental change reduces local, but not regional, diversity of riverine fish assemblages in a Himalayan biodiversity hotspot. Biological Conservation. 2022; 265(0006–3207). https://doi.org/10.1016/j.biocon.2021.109427.
7. Wu H, Chen J, Xu J, Zeng G, Sang L, Liu Q, et al. Effects of dam construction on biodiversity: A review. Journal of Cleaner Production. 2019; 221:480–9. https://doi.org/10.1016/j.jclepro.2019.03.001.
8. Winemiller KO, Nam S, Baird IG, Darwall W, Lujan NK, Harrison I, et al. Balancing hydropower and biodiversity in the Amazon, Congo, and Mekong. Science. 2016; 351(6269):128–9.
9. Grill G, Lehner B, Thieme M, Geenen B, Tickner D, Antonelli F, et al. Mapping the world's free-flowing rivers. Nature. 2019; 569(7755):215–21. https://doi.org/10.1038/s41586-019-1111-9 PMID: 31068722
10. Couto TBA, Olden JD. Global proliferation of small hydropower plants–science and policy. Frontiers in Ecology and the Environment. 2018; 16(2):91–100. https://doi.org/10.1002/fee.1746
11. Kelly RP, Port JA, Yamahara KM, Martone RG, Lowell N, Thomsen PF, et al. Harnessing DNA to improve environmental management. Science. 2014; 344(6191):1455–6.
12. IFC. Trishuli Assessment Tool Field Manual. Washington, D.C., USA: 2021.
13. Shrestha TK. Ichthyology of Nepal: A study of fishes of the Himalayan waters. 2008.
14. Ivanova NV, Zemlak TS, Hanner RH, Hebert PDN. Universal primer cocktails for fish DNA barcoding. Molecular Ecology Notes. 2007; 7(4):544–8. https://doi.org/10.1111/j.1471-8286.2007.01748.x
15. Miya M, Sato Y, Fukunaga T, Sado T, Poulsen J, Sato K, et al. MiFish, a set of universal PCR primers for metabarcoding environmental DNA from fishes: detection of more than 230 subtropical marine species. Royal Society open science. 2015; 2(7):150088. https://doi.org/10.1098/rsos.150088 PMID: 26587265
16. Andrews S. FastQC: a quality control tool for high throughput sequence data. Babraham Bioinformatics, Babraham Institute, Cambridge, United Kingdom; 2010.
17. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics. 2014; 30(15):2114–20. https://doi.org/10.1093/bioinformatics/btu170 PMID: 24695404
18. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nature biotechnology. 2019; 37 (8):852–7. https://doi.org/10.1038/s41587-019-0209-9 PMID: 31341288
19. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. Nature methods. 2016; 13(7):581–3. https://doi.org/10.1038/nmeth.3869 PMID: 27214047
20. Lim SJ, Thompson LR. Mitohelper: A mitochondrial reference sequence analysis tool for fish eDNA studies. Environmental DNA. 2021; 3(4):706–15. https://doi.org/10.1002/EDN3.187
21. Nelson JS, Grande TC, Wilson MVH. Fishes of the World. 5 ed. Hoboken, USA: John Wiley and Sons; 2016.
22. Faith DP. Systematics and Conservation: on Predicting the Feature Diversity of Subsets of Taxa. Cladistics. 1992; 8(4):361–73. https://doi.org/10.1111/j.1096-0031.1992.tb00078.x PMID: 34929967
23. Shannon CE. A Mathematical Theory of Communication. The Bell System Technical Journal. 1948; 27 (3):212–4.
24. H W. ggplot2: Elegant Graphics for Data Analysis. New York: Springer-Verlag 2016.
25. Beals EW. Bray-curtis ordination: An effective strategy for analysis of multivariate ecological data. Advances in Ecological Research. 1984; 14(C):1–55. https://doi.org/10.1016/S0065-2504(08)60168-3
26. Lozupone C, Knight R. UniFrac: A new phylogenetic method for comparing microbial communities. Applied and Environmental Microbiology. 2005; 71(12):8228–35. https://doi.org/10.1128/AEM.71.12.8228-8235.2005 PMID: 16332807
27. Sigsgaard EE, Carl H, Møller PR, Thomsen PF. Monitoring the near-extinct European weather loach in Denmark based on environmental DNA from water samples. Biological Conservation. 2015; 183:46–52. https://doi.org/10.1016/j.biocon.2014.11.023

28. Boothroyd M, Mandrak NE, Fox M, Wilson CC. Environmental DNA (eDNA) detection and habitat occupancy of threatened spotted gar (Lepisosteus oculatus). Aquatic Conservation: Marine and Freshwater Ecosystems. 2016; 26(6):1107–19. https://doi.org/10.1002/aqc.2617

29. Pfleger MO, Rider SJ, Johnston CE, Janosik AM. Saving the doomed: Using eDNA to aid in detection of rare sturgeon for conservation (Acipenseridae). Global Ecology and Conservation. 2016; 8:99–107. https://doi.org/10.1016/j.gecco.2016.08.008

30. Yatsuyanagi T, Ishida R, Sakata MK, Kanbe T, Mizumoto H, Kobayashi Y, et al. Environmental DNA monitoring for short-term reproductive migration of endemic anadromous species, Shishamo smelt (Spirinchus lanceolatus). Environmental DNA. 2020; 2(2):130–9. https://doi.org/10.1002/edn3.50

31. Holm P, Nicholls J, Hayes PW, Ivinson J, Allaire B. Accelerated extractions of North Atlantic cod and herring, 1520–1790. Fish and Fisheries. 2022; 23(1):54–72. https://doi.org/10.1111/faf.12598

32. Takahara T, Minamoto T, Yamanaka H, Doi H, Kawabata Zi. Estimation of fish biomass using environmental DNA. PLoS ONE. 2012; 7(4):3–10. https://doi.org/10.1371/journal.pone.0035868 PMID: 22563411

33. Evans NT, Olds BP, Renshaw MA, Turner CR, Li Y, Jerde CL, et al. Quantification of mesocosm fish and amphibian species diversity via environmental DNA metabarcoding. (1755–0998 (Electronic)).

34. Robson HLA, Noble TH, Saunders RJ, Robson SKA, Burrows DW, Jerry DR. Fine-tuning for the tropics: application of eDNA technology for invasive fish detection in tropical freshwater ecosystems. Molecular ecology resources. 2016; 16(4):922–32. https://doi.org/10.1111/1755-0998.12505 PMID: 26849294

35. Yates MC, Fraser DJ, Derry AM. Meta-analysis supports further refinement of eDNA for monitoring aquatic species-specific abundance in nature. Environmental DNA. 2019; 1(1):5–13. https://doi.org/10.1002/edn3.7

36. Ziv G, Baran E, Nam S, Rodríguez-Iturbe I, Levin SA. Trading-off fish biodiversity, food security, and hydropower in the Mekong River Basin. Proceedings of the National Academy of Sciences of the United States of America. 2012; 109(15):5609–14. https://doi.org/10.1073/pnas.1201423109 PMID: 22393001

37. Agostinho AA, Gomes LC, Santos NCL, Ortega JCG, Pelicice FM. Fish assemblages in Neotropical reservoirs: Colonization patterns, impacts and management. Fisheries Research. 2016; 173:26–36. https://doi.org/10.1016/j.fishres.2015.04.006

38. Chaudhary A, Verma C, Singh HS. First report on the molecular characterization of Diaphorocleidus armillatus Jogunoori et al. 2004 (Monogenea: Dactylogyridae) infecting the gills of introduced fish, Gymnocorymbus ternetzi in India. Acta Parasitologica. 2016; 61:639–44. https://doi.org/10.1515/ap-2016-0086 PMID: 27447232

39. Ren Q, Yang JX, Chen XY. A new species of the genus Triplophysa (Cypriniformes: Nemacheilidae), Triplophysa longliensis sp. Nov, from Guizhou, China. Zootaxa. 2012; 194(3586):187–94. https://doi.org/10.11646/zootaxa.3586.1.17

40. Gupta N, Everard M. Non-native fishes in the Indian Himalaya: an emerging concern for freshwater scientists. International Journal of River Basin Management. 2019; 17(2):271–5. https://doi.org/10.1080/15715124.2017.1411929

41. Sharma A, Dubey VK, Johnson JA, Rawal YK, Sivakumar K. Introduced, invaded and forgotten: allopatric and sympatric native snow trout life-histories indicate brown trout invasion effects in the Himalayan hinterlands. Biological Invasions. 2021; 23(5):1497–515. https://doi.org/10.1007/s10530-020-02454-8

42. Gurung TB, Basnet SR. Introduction of rainbow trout Onchorynchus mykiss in Nepal: constraints and prospects. Aquaculture Asia. 2003; 8(4):16–8.

43. Husen MA. Impact of invasive alien fish, Nile Tilapia (Oreochromis niloticus) on native fish catches of sub-tropical lakes (Phewa, Begnas and Rupa) of Pokhara Valley, Nepal. Invasive Alien Species Management. 2014.

44. Shrestah T. Cold water fisheries development in Nepal. FAO Fisheries Technical Paper. 2002:47–58.

45. Sepulveda AJ, Nelson NM, Jerde CL, Luikart G. Are Environmental DNA Methods Ready for Aquatic Invasive Species Management? Trends in Ecology & Evolution. 2020; 35(8):668–78. https://doi.org/10.1016/j.tree.2020.03.011 PMID: 32371127

46. Leggatt R, Dhillon R, Mimeault C, Johnson N, Richards JG, Devlin RH. Low-temperature tolerances of tropical fish with potential transgenic applications in relation to winter water temperatures in Canada. Canadian Journal of Zoology. 2018; 96(3):253–60.

47. Tuckett QM, Ressel KN, Ritch JL, Lawson KM, Hill JE. Domestication and feralization influence the distribution and phenotypes of escaped ornamental fish. Biological Invasions. 2021; 23:1033–47.

48. Merkes CM, McCalla SG, Jensen NR, Gaikowski MP, Amberg JJ. Persistence of DNA in carcasses, slime and avian feces may affect interpretation of environmental DNA data. PLoS ONE. 2014;9(11). https://doi.org/10.1371/journal.pone.0113346 PMID: 25402206

49. Husen MA. Fish Marketing System in Nepal: Present Status and Future Prospects. International Journal of Applied Sciences and Biotechnology. 2019; 7(1):1–5. https://doi.org/10.3126/ijasbt.v7i1.22938

50. Amberg JJ, Grace McCalla S, Monroe E, Lance R, Baerwaldt K, Gaikowski MP. Improving efficiency and reliability of environmental DNA analysis for silver carp. Journal of Great Lakes Research. 2015; 41 (2):367–73. https://doi.org/10.1016/j.jglr.2015.02.009

51. Zaiko A, Pochon X, Garcia-Vazquez E, Olenin S, Wood SA. Advantages and limitations of environmental DNA/RNA tools for marine biosecurity: Management and surveillance of non-indigenous species. Frontiers in Marine Science. 2018; 5(SEP). https://doi.org/10.3389/fmars.2018.00322