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Species-level monitoring of rare and invasive fishes using eDNA metabarcoding in the middle and upper Yarlung Zangbo River, Tibet

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ABSTRACT

Fish diversity of the Yarlung Zangbo River is very sensitive and vulnerable to biological invasion, anthropogenic activities and climate change, especially in the upper and middle reaches where several endemic fishes have become endangered and nearly ten invasive fishes have been established. Here, we used environmental DNA (eDNA) metabarcoding to monitor rare and invasive fishes, and to assess diversity in 25 sites from two wetlands (Lalu and Chabalang) and the main channel (YT), within the upper and middle reaches. To obtain a species-level resolution, we evaluated species discrimination potentials of three mitochondrial markers and found Cytb had the highest average genetic distance at each taxonomic level followed by COI and 12S. The 12S was unqualified for species assignment, as two species shared identical haplotypes. The newly designed Cytb primers used for metabarcoding showed an average mismatch of 0.28 and amplified well across species. In total, 8942 operational taxonomic units (OTUs) were obtained based on a 100% identity threshold, among which 98.1% were assigned to 24 fishes based on our custom-made database and the remaining were assigned to six fishes based on the NCBI nt database. Almost all captured fishes were detected by the eDNA method except for two species. However, 12 fishes detected by the eDNA method were not listed in catch data for several sites, including one endangered species (*Oxygymnocypris stewartii*), four previously recorded non-native species and two unrecorded non-native species (*Monopterus albus* and *Siniperca chuatsi*). The alpha diversities estimated by eDNA and capture-based methods were correlated for sites at Lalu. Both methods revealed significant differences in community composition between YT and the wetlands. Our results provide both basic information for conservation and management of rare and invasive fishes in the Yarlung Zangbo River and a framework of fish eDNA metabarcoding with a species-level resolution.

1. Introduction

The Yarlung Zangbo River (the upper Brahmaputa River) is the largest river on the Tibetan Plateau and one of the highest rivers in the world. It originates from the Chema Yongdrung glacier and flows through the southern Tibetan Plateau along the Himalayan ranges (He and Chen, 2009). Due to the fragility of this ecosystem and its unique fish fauna (which mainly comprises groups of the subfamily Schizothoracinae and the genus *Triplophysa*), the fish diversity of the Yarlung Zangbo River is very sensitive and vulnerable to biological invasion, anthropogenic activities and climate change (Favre et al., 2015; He et al., 2020; Jia et al., 2019; Tao et al., 2018). Within the upper and middle reaches, most of Schizothoracinae fishes are basin-specific species and now have been listed as Endangered (EN) or Vulnerable (VU) species by the red list of China's vertebrates, including *Oxygymnocypris stewartii*, *Schizothorax macropogon*, *Schizothorax waltoni* and *Ptychobarbus dipogon* (Jiang et al., 2016). The declined populations of these fishes are hard to be naturally recovered because of their inherent characteristics, e.g., slow growth rate, late maturity, low fecundity, and longevity (Chen et al., 2004; Chen and Cao, 2004; Feng et al., 2019). Hence the conservation of fish diversity in the upper and middle Yarlung Zangbo River has attracted considerable attention (Chen and Chen, 2010; Li et al., 2019; Yang et al., 2010).

With the rapid development of the economy and transport systems in Tibet, many non-native fishes have entered local waters owing to release activities and escape events, become successfully established, and are showing potential effects on the survival of native fishes (Chen and Chen, 2010; Jia et al., 2019). In recent years, several surveys of non-native

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fishes in the upper and middle Yarlung Zangbo River have been conducted using gill-netting and trapping and have revealed differences in the species list (Chen and Chen, 2010; Ding et al., 2014; Fan et al., 2011, 2016; Li et al., 2018; Yang et al., 2010). A total of 13 non-native fishes were recorded in these studies, including five commonly detected species (*Carassius auratus*, *Pseudorasbora parva*, *Micropercops swinhonis*, *Ctenopharyngodon idellus* and *Silurus asotus*), four detected in most studies (*Abbottina rivularis*, *Cyprinus carpio*, *Hypophthalmichthys nobilis* and *Misgurnus anguillicaudatus*) and four species that were detected in only one or two studies (*Paramisgurnus dabryanus*, *Channa argus*, *Oryzias latipes* and *Hypophthalmichthys molitrix*). These fishes show various body sizes and habitat types, thus huge efforts are needed to improve their capture efficiency. Furthermore, the conventional capture-based methods are laborious and destructive and are inefficient for the early detection of new non-native fishes present in low densities (Wang et al., 2022; Zou et al., 2020). Effective conservation of rare native fishes and management of non-native fishes require monitoring data of their distribution (Balasingham et al., 2018). To facilitate the long-term monitoring of rare and non-native fishes in the upper and middle Yarlung Zangbo River, there is an urgent need to develop convenient and accurate methods such as the analysis of environmental DNA (eDNA).

In aquatic environments, the eDNA of fishes can be derived from a variety of sources, e.g., shed skin, excreta, urine, faeces, eggs and sperm, thus allowing us to monitor the species by eDNA analysis. Recently, eDNA-based techniques have provided highly effective, economical, and non-invasive methods for biomonitoring, and have been used for fish monitoring in various aquatic ecosystems (Deiner et al., 2017; Goldberg et al., 2016; Thomsen et al., 2012; Xiong et al., 2022). eDNA-based approaches utilize the conventional, quantitative or digital droplet polymerase chain reaction (PCR, qPCR and ddPCR) for monitoring a single fish species and the metabarcoding method, which combines PCR and high-throughput next generation sequencing, for monitoring the whole fish community (Deiner et al., 2017; Wang et al., 2021; Xing et al., 2022). Many studies have successfully employed eDNA-based approaches for rare fish detection, such as European weather loach (Sigsgaard et al., 2015), Roanoke logperch (Strickland and Roberts, 2019), European eel (Cardás et al., 2020), Atlantic sturgeon (Plough et al., 2021), big-headed turtle (Lam et al., 2022) and three species at risk in the Sydenham and Grand River in Canada (Balasingham et al., 2018). eDNA methods have also been used for the detection of non-native fishes in many countries, such as Canada (Balasingham et al., 2018), USA (Pukk et al., 2021), Belarus (Jeunen et al., 2022) Turkey (Keskin et al., 2016), UK (Davison et al., 2017), and China (Lee et al., 2021).

The probability of detecting taxonomic groups in an eDNA metabarcoding study is strongly affected by the choice of marker region and primers (Alberdi et al., 2018; Bylemans et al., 2018; Zhang et al., 2020). Ideally, primers used for eDNA metabarcoding should: (1) be conservative thus amplifying the DNA from all target species, and (2) amplify a short DNA fragment (~200 bp) containing sufficient sequence variation to ensure accurate species assignments (Elbrecht and Leese, 2017; Coissac et al., 2012). Three commonly used molecular markers for fish eDNA metabarcoding are mitochondrial 12S, Cytb and COI genes, and some outstanding primers have been designed for these marker regions (Balasingham et al., 2018; Hänfling et al., 2016; Miya and Nishida, 2000; Taberlet et al., 2018; Zhang et al., 2020). For example, the universal primers MiFish were designed based on mitogenome sequences from 880 fish species and target a hypervariable region of the 12S which contains sufficient information to identify fishes to family-, genus- and species-level (Miya et al., 2015). However, the MiFish sequences still have inherent limitations to assign lower taxonomic ranks, such as genus and species. For fish eDNA metabarcoding at a species-level resolution, it is necessary to evaluate primer bias and the taxonomic resolution power of the amplified sequences.

In this study, by using an eDNA metabarcoding approach, we mainly investigated the spatial distribution of rare and non-native fishes at species-level resolution in the upper and middle Yarlung Zangbo River. To achieve this, we first collected sequences of three commonly used metabarcoding loci (12S, COI and Cytb) to evaluate the bias and taxonomic resolution of published universal primers and newly designed primers. We then performed eDNA metabarcoding with the most suitable primers to characterize the fish distribution and diversity, and compared the results with the data from conventional capture-based methods. Our results would provide both basic information for the conservation of rare native fishes and management of non-native fishes in the Yarlung Zangbo River and a framework of fish eDNA metabarcoding at a species-level resolution in a specific drainage basin.

2. Materials and methods

2.1. Fish and eDNA sampling

Sampling was conducted at 25 sites along the Yarlung Zangbo River in July 2020, including seven sites in the Lalu wetland (LL, an urban natural wetland), five sites in the Chabalang wetland (CBL, an artificial wetland), nine sites in the main river channel (YT), and other three sites along the Lhasa River, which is a main tributary (Fig. 1). The water depth at each site did not exceed 3 m. Fish samples were collected by using multi-mesh gillnets (mesh size from 1.5 to 7.5 cm) and fish trap nets (mesh size 1.5 mm). A small piece of fin tissue was sampled from each individual and stored in absolute ethyl alcohol.

At each sampling site, two or three replicate water samples were collected using sterile 500 mL plastic bottles and stored in sterile 2 L plastic bottles at 4 °C until filtration. Each water sample (1.5 L) was composed of equal volumes of water from the surface (about 20 cm below the top), middle and bottom (about 20 cm above the bottom) layers. After collecting each sample, the sampling equipment was sterilized by soaking in a commercial 10% bleach solution and rinsing with purified water. Within 6 h of collection, water samples were filtered through a mixed cellulose ester (MCE) membrane (47 mm diameter, 0.45 μm pore size) with a vacuum pump (Jinteng, Tianjin, China). After each filtration, the filtration equipment and forceps were soaked with 10% bleach solution and rinsed with purified water. Three filtration blanks were created by filtering 1.5 L of distilled and deionized water in the same way to check for contamination during field collection and water filtration. A total of 71 filter membranes were immediately placed into a sterile 2 ml microcentrifuge tube and stored in liquid nitrogen until transfer to an ultracold freezer (−80 °C) in the laboratory.

2.2. Collection of reference sequences and DNA barcoding analysis

A species list comprising 29 species (Supplementary Table S1) was built for the upper and middle Yarlung Zangbo River by combining published species records with our catch statistics. To construct reference sequence databases for better taxonomic assignment and primer assessment, the partial sequences of mitochondrial 12S and COI, and the complete sequences of Cytb, were obtained by Sanger sequencing for 180 captured fish samples belonging to 21 species. Total genomic DNA was extracted from fin tissues using a phenol-chloroform extraction method (Taggart et al., 1992). The PCR was performed in a total volume of 25 μL, including 30 ng genomic DNA, 0.4 μL of each gene-specific primer (Table 1) (10 μm), 12.5 μL 2 × FTaq PCR MasterMix (ZomanBio, Beijing, China), and the final volume was adjusted with sterile distilled water. The thermocycle profile consisted of an initial denaturation at 94 °C for 4 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 35 s and 72 °C for 40 s and a final extension at 72 °C for 8 min. In addition, the sequences of another eight species were mined from GenBank based on their mitogenomes (accession numbers can be found in Supplementary Table S2).

DNA barcoding analysis was performed to determine the species discrimination potentials of the three markers. The collected sequences were aligned for each gene using the ClustalW algorithm (Thompson et al., 1994) implemented in MEGA 6 (Tamura et al., 2013). The Kimura 2-parameter (K2P) pairwise genetic distances were calculated using

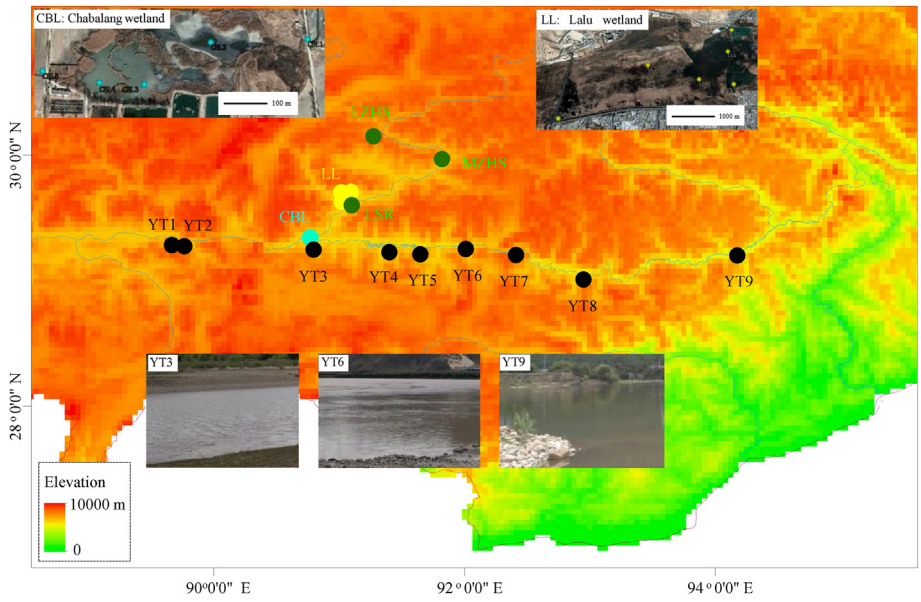

Fig. 1. Sampling sites in this study.

Table 1
PCR primer pairs used in this study for DNA barcoding and eDNA metabarcoding.

Primer name	Primer sequence (5'-3')	Usage
db_12S-F	TGTAAAACGACGGCCAGTTAYACATGCAAGTMTCCGC	DNA barcoding for 12S
db_12S-R	CAGGAAACAGCTATGACGCTACACCTCGACCTGACGT	
db_COI-F	TGTAAAACGACGGCCAGCAATCACRCGCTGATTYTTYT	DNA barcoding for COI
db_COI-R	CAGGAAACAGCTATGACCCYATRTANCCRAANGGYTCTT	
db_Cytb-F	TGTAAAACGACGGCCAGGRCTTGAARAACCACCGTTGT	DNA barcoding for Cytb
db_Cytb-R	CAGGAAACAGCTATGACCGGWTTACAAGACCGRYGCT	
edm_Cytb-F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTGRCTTGAARAACCACCGTTGT	eDNA metabarcoding
edm_Cytb-R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAARAAGAAWGADGCKCCRTTDGCRTG	
2nd-PCR-F	AATGATACGGCGACCACCGAGATCTACAC[index 2]TCGTCGGCAGCGTC	eDNA metabarcoding
2nd-PCR-R	CAAGCAGAAGACGGCATACGAGAT[index 1]GTCTCGTGGGCTCGG	

The shaded sequences in primer pairs for DNA barcoding and eDNA metabarcoding represent the M13 sequences and the Illumina overhang adapter sequences for NGS library preparation, respectively.

MEGA 6. The presence of a barcoding gap in morphospecies was assessed by species-level comparisons between the maximum intraspecific genetic distance and the minimum distance to the nearest neighbor (Chen et al., 2015). The operational taxonomic units (OTUs) were determined from the DNA barcodes using three sequence-based methods, i.e., Automatic Barcode Gap Discovery (ABGD) (Puillandre et al., 2012), General Mixed Yule-coalescent (GMYC) (Fujisawa and Barraclough, 2013), and Bayesian Poisson tree (bPTP) (Zhang et al., 2013).

2.3. Primer design and choice for eDNA metabarcoding

The primers used for eDNA metabarcoding were designed by searching a short hypervariable region (~200 bp) flanked by two conservative regions (~20 bp) based on aligned sequences. The information content (entropy H(x)) representing the sequence variability (Shenkin et al., 1991) was calculated at each nucleotide position of the sequence alignment and plotted for the visual inspection using the software BioEdit 7.0.9.0 (Hall, 1999). Some standard PCR primer design characteristics were also followed, such as (a) the GC proportion of a primer should be in the range of 40%–60%; (b) some G/C bases should be included at the 3' end of a primer, but a string of either Gs or Cs should be avoided; (c) primer dimers and hairpins should be avoided where possible. The primer-template match analysis for each species was performed to estimate the expected PCR amplification efficiencies of newly designed primers and published universal primers. The efficiency in species discrimination of amplicon sequences was re-assessed using the same methods as described above for the long fragment. To evaluate the amplification performance, newly designed primers were tested *in vitro* on 24 species which accounted for 82.8% of all species and 100% of all orders.

2.4. eDNA extraction, library preparation and sequencing

eDNA extraction and library preparation were conducted in dedicated laboratories where all workspaces were thoroughly decontaminated using UV irradiation and DNA-off reagents (Takara, Japan) before

experimental operation. The eDNA from MCE filters was extracted with the PowerWater DNA Isolation Kit (MO BIO Laboratories, Carlsbad, USA) following the manufacturer's protocol. Two-step PCR was employed to prepare paired-end libraries for the Illumina MiSeq platform following Miya et al. (2015). For the first-round PCR (1st PCR), the primer pair edm_Cytb-F/R (Table 1) was used for amplifying a 383 bp DNA fragment which contained a 246 bp region of the Cytb gene. The primers 2nd-PCR-F/R with the attached dual indices and sequencing adapters were employed for the second-round PCR (2nd PCR). The PCR was carried out in a total volume of 25 μL, comprising 12.5 μL Q5 Hot Start High-Fidelity 2 × Master Mix (New England Biolabs, USA), 0.5 μL of each primer (10 μm), 4.0 μL DNA template and 8.0 μL sterile distilled water. The thermocycle profile consisted of an initial denaturation at 95 °C for 4 min, followed by x cycles (25 cycles for 1st PCR and 12 cycles for 2nd PCR) at 95 °C for 30 s, y °C (54 °C for 1st PCR and 62 °C for 2nd PCR) for 35 s and 72 °C for 40 s and a final extension at 72 °C for 8 min. PCRs were replicated three times for each sample of eDNA, filtration blanks and negative controls. The 1st PCR products were purified using AMPure XP beads (Beckman Coulter, High Wycombe, UK) and used as templates for the 2nd PCR. The 2nd PCR products were purified using EZNA poly-Gel DNA Extraction kit (Omega Bio-Tek, Norcross, USA) and were then pooled in equimolar amounts to generate the final sequencing library which was sequenced on an Illumina MiSeq platform using the MiSeq Reagent Kit v2 (2 × 250 cycles) following the manufacturer's protocol.

2.5. Bioinformatic and statistical analyses

The raw paired-end sequence reads were filtered using fastp 0.20.0 (−3 -W 4 -M 25 -q 25 -u 10 -c) (Chen et al., 2018) to eliminate reads of low quality and correct mismatched base pairs in overlapping regions of paired-end reads. The primer sequences and extra sequences (22 bp) not belonging to the Cytb gene were removed from the clean paired-end reads using cutadapt v3.0 (Martin, 2011). OTU clustering was subsequently conducted using VSEARCH 2.15.0 (Rognes et al., 2016) according to the following steps: (1) the fastq files from forward and reverse reads were merged into a single fasta file using the "–fastq_mergepairs" command; (2) the merged sequences of each sample were dereplicated using the "–derep_fulllength" command with –minuniquesize 3 to remove singletons and doubletons; (3) the dereplicated sequences of each sample were merged and re-dereplicated using the "–derep_fulllength" command; (4) potential chimeras and borderline sequences which have a small divergence from the closest parent but with a sufficiently high score for chimera detection were removed using the "–uchime_ref" command with the barcoding sequences as the reference; (5) OTUs were generated using the "–cluster_fast" command with –id 1, and the OTU table was obtained by using the "–usearch_global" command with –id 1. To obtain accurate species assignments, the non-chimera sequences were clustered into OTUs with a 100% identity threshold. The OTU sequences were mapped to a custom-made database using the Blastn tool (Camacho et al., 2009), and species-level taxonomic assignments were based on the best alignment with a minimum of 98% identity. Unassigned OTU sequences were then subjected to another Blastn search against the NCBI nucleotide (nt) database at a criterion of 98% identity.

2.6. Estimation of diversity indices using species abundance data from eDNA metabarcoding and capture-based methods

Alpha (species richness, Shannon-Wiener and Simpson) and beta (Bray-Curtis) diversity indices were calculated using the *vegan* R package (Oksanen et al., 2020) based on species abundance data from both eDNA metabarcoding and capture-based methods. The correlation analysis between values of each diversity index estimated from the two methods was performed by calculating the Spearman correlation coefficient. To determine the significance of community compositional differences among the three groups (CBL, LL and YT), a permutational multivariate analysis of variance (PERMANOVA) was performed with the *adonis2* function after checking for multivariate homogeneity of group dispersions with the *betadisper* function in the *vegan* R package. If *adonis2* analysis returned significant results, an additional pairwise adonis test was performed using the *pairwise.adonis* function in the pairwiseAdonis package (Martinez Arbizu, 2020). The beta diversity was visualized as a Bray-Curtis distance matrix in a NMDS plot.

3. Results

3.1. DNA barcoding sequences and species delimitation

In total, 219 sequences for each marker (12S, COI and Cytb) were collected from 29 species belonging to five orders, 10 families and 20 genera. The average K2P pairwise genetic distance within species, genera, families and orders were 0.063%, 0.821%, 5.598% and 9.910% for 12S sequences, 0.203%, 4.209%, 15.919% and 19.075% for COI gene, and 0.344%, 7.872%, 19.612% and 23.702% for Cytb gene, respectively. The number of haplotypes and percentage of informative sites were 97 and 49.61% for Cytb, 63 and 42.34% for COI, and 53 and 42.64% for 12S, respectively (Supplementary Table S3). For COI and Cytb, all species had a higher minimum interspecific distance than the maximum intraspecific distance, indicating the presence of DNA barcoding gaps (Fig. 2). However, for 12S, two species (*S. oconnori* and *S. waltoni*) shared identical haplotypes, indicating that 12S was unqualified for accurate species assignments. The species delimitation analyses by three methods (ABGD, GMYC and bPTP) with 12S sequences yielded 18–19 groups from all 29 morphospecies, showing that species within the genera *Schizothorax* and *Triplophysa,* and three Cyprinidae species (*H. molitrix*, *H. nobilis* and *C. idellus*), were separately clustered into one group (Supplementary Fig. S1). For COI and Cytb, each of the three methods generated 27 groups with each species constituting one group except for the three *Schizothorax* species which were clustered into one group. The phylogenetic trees based on COI and Cytb both showed that individuals from the genus *Schizothorax* formed three reliable clades corresponding to three species. Hence, COI and Cytb were potential loci for eDNA metabarcoding at a species-level resolution in this study.

3.2. Primers for eDNA metabarcoding

Because 12S was unqualified for species assignment at a species level, the new primer pairs for eDNA metabarcoding were only designed for COI and Cytb. The forward and reverse primers of edm_Cytb-F/R (Table 1) were located at upstream (−46 to −25) and coding region (247–272) of Cytb gene, respectively, amplifying a 246 bp region of the gene (Fig. 3a). The edm_COI–F and R were designed at the positions 195–215 and 508–533 of COI gene, respectively, amplifying a 292 bp region of the gene (Supplementary Fig. S2). The primer-template match analysis showed that the average number of mismatches between edm_Cytb-F/R and species sequences and was only 0.28 (Fig. 3b) which was much lower than that of other published universal primers (5.6 for L14912/H15149 in Miya and Nishida, 2000 and 3.1 for L14735/H15149 in Hänfling et al., 2016). Results of *in vitro* PCR showed that the primer pair edm_Cytb-F/R amplified consistently well across tested species (Fig. 3c), and the primers edm_COI–F/R showed biases in amplification efficiency among species (Supplementary Fig. S2). Based on amplicon sequences of edm_Cytb-F/R, all species still had DNA barcoding gaps (Fig. 2d), and the species delimitation analyses returned the same results as those based on long fragment sequences. Therefore, the newly designed primers edm_Cytb-F/R were used for fish eDNA metabarcoding in this study.

3.3. eDNA metabarcoding data

No target-sized PCR bands or OTU sequences were detected for filtration blanks and negative controls, indicating no contamination

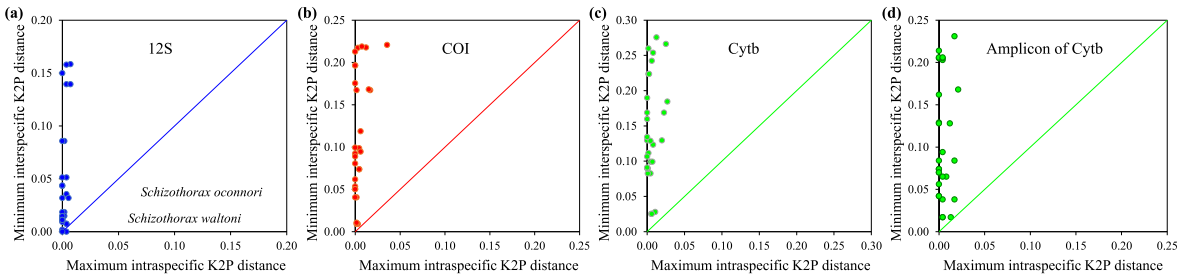

Fig. 2. Maximum intraspecific distance compared with minimum interspecific distance for each fish species based on sequences of mitochondrial 12S (**a**), COI (**b**), Cytb (**c**) and amplicons of Cytb (**d**).

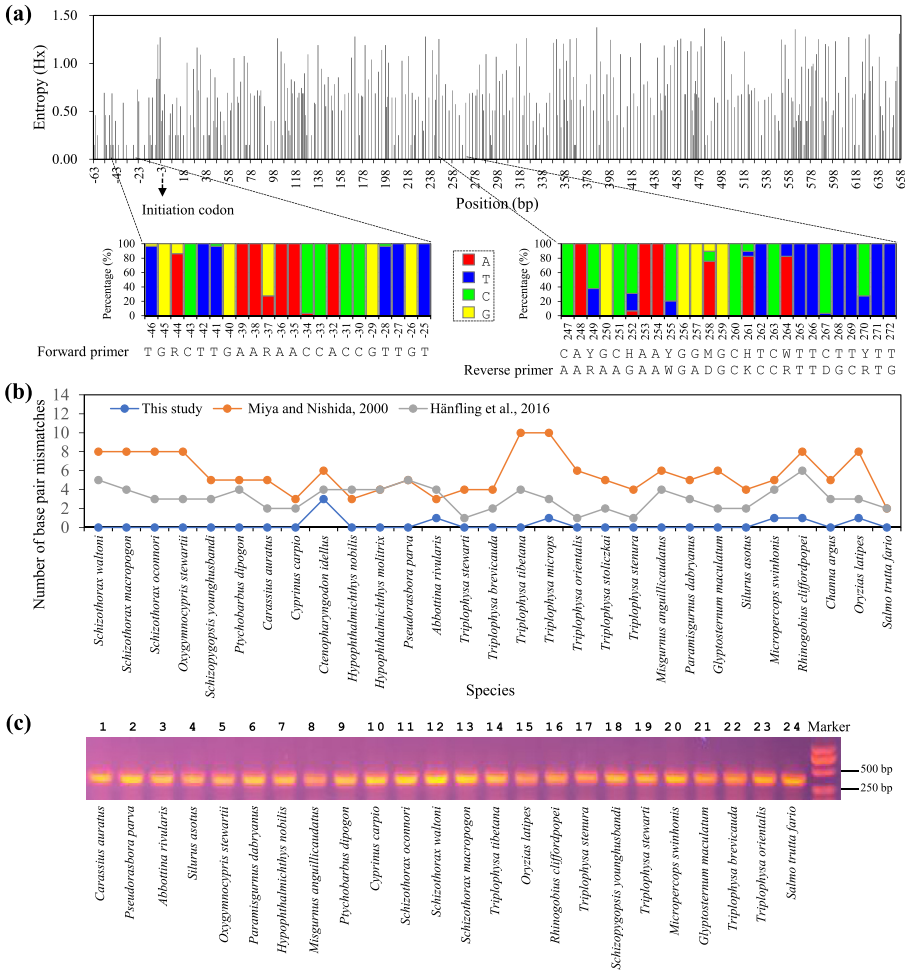

Fig. 3. Design of Cytb primers used for eDNA metabarcoding and evaluation of the amplification performance. (**a**) Primers were designed by searching a short hypervariable region (~200 bp) flanked by two conservative regions (~20 bp) based on 219 aligned sequences. The entropy H(x) represents the level of variability at each nucleotide position. (**b**) The number of base pair mismatches between sequences of primers and each species. For a degenerate site, no mismatch was counted when the base from species is the same as the corresponding base in the primer. (**c**) The amplification performance of the primer pair edm_Cytb-F/R in 24 fish species. PCR products were run on a 1.0% agarose gel and stained with ethidium bromide.

during sample collection and library construction. A total of 6.7 million clean reads were obtained after a strict quality control of the next generation sequencing data, with an average of 0.27 million reads per sampling site (Supplementary Table S4). After adapter trimming, read merging and length filtering, an average of 123,919 sequences were obtained per sampling site. The average number of non-redundant sequences per site was 1,257, with a total number of 10,928 across all sites. A total of 1600 (14.6%) chimeras and 44 (0.4%) borderline sequences were identified from all non-redundant sequences. After removal of chimeras and borderline sequences, 8942 OTUs were obtained based on a 100% identity threshold and assigned to all sites with the number of OTUs in each site ranging from 247 to 2253.

3.4. Species assignments

Among all OTUs, 8771 (98.1%) were assigned to 24 fish species based on our custom-made database (Fig. 4). Among the remaining 171 (1.9%) OTUs mapped to the NCBI nt database, 78 were assigned to six fish species, including *T. dalaica* (60 OTUs in 21 sites), *Monopterus albus* (10 OTUs in 7 sites), *Siniperca chuatsi* (5 OTUs in 9 sites), *Microphysogobio tungtingensis* (1 OTU in 1 LL8), *Oreochromis niloticus* (1 OTU in CBL2 and YT8) and *Hemibarbus maculatus* (1 OTU in 1 CBL5) (Supplementary Table S5). For nonfish OTUs, one was assigned to *Homo sapiens* in three sampling sites (CBL1, LL8 and LZHS), other 92 OTUs showed no alignment or low alignment scores with bacteria. As all sites are connected within the river system, overall results of species detection by eDNA were compared with the catch data. Among the 30 species detected by eDNA

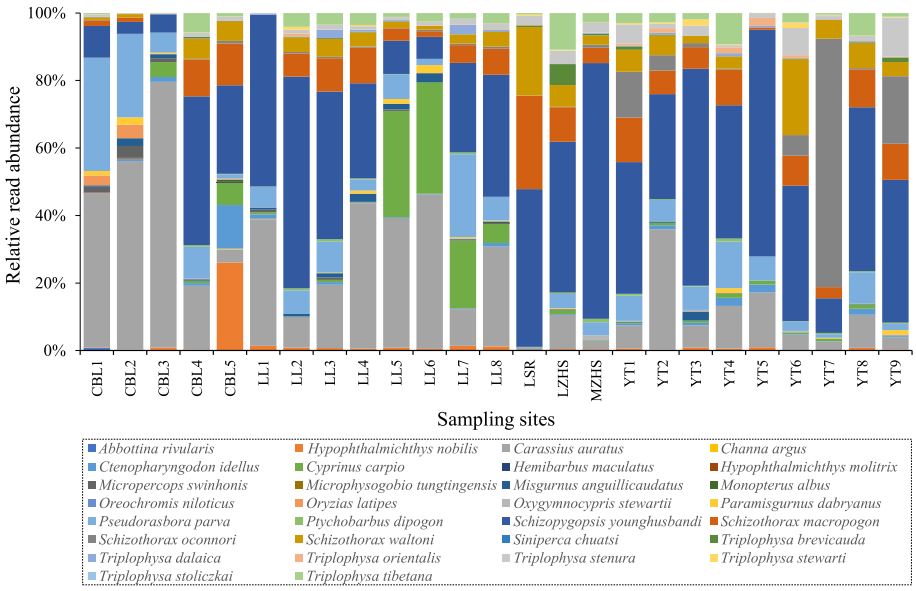

Fig. 4. Relative read abundance of fish species in each sampling site estimated by eDNA metabarcoding.

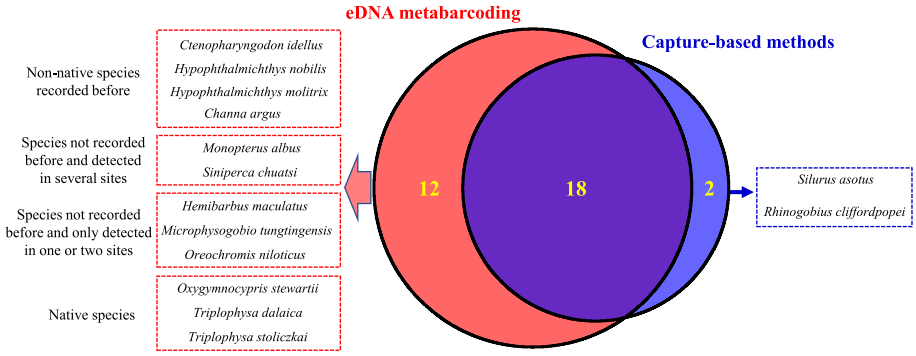

Fig. 5. Comparison between fishes detected by eDNA metabarcoding and capture-based methods.

metabarcoding, 12 species were not captured in this study, including four previously recorded non-native fishes, five non-native fishes not previously recorded and three native fishes (Fig. 5). The five newly recorded non-native fishes comprised two species (*M. albus* and *S. chuatsi*) detected in multiple sites and three species (*H. maculatus*, *M. tungtingensis* and *O. niloticus*) detected in only one or two sites. The number of sequences of these five fishes only accounted for 0.04% of the total. Only two captured fishes were not detected by eDNA metabarcoding, namely *R. cliffordpopei* and *S. asotus*. Among five native fishes listed on the red list of China's vertebrates, *Glyptosternum maculatum* was not detected by both eDNA and capture-based methods, *O. stewartii* was detected only by eDNA in 50% of sites, and other three Schizothoracinae fishes (*P. dipogon*, *S. macropogon* and *S. waltoni*) were detected by both eDNA and capture-based methods.

3.5. Species diversity

The alpha and beta diversity indices estimated based on species abundance data from eDNA metabarcoding were compared with those from capture-based methods. The number of fish species per site detected by eDNA metabarcoding ranged from 11 to 26 (mean = 19.8) which was significantly higher than that observed by capture-based methods (mean = 8.0) (Mann-Whitney-Wilcoxon Test, $P < 0.01$). Overall, no significant correlation was observed between the alpha diversity indices (Shannon-

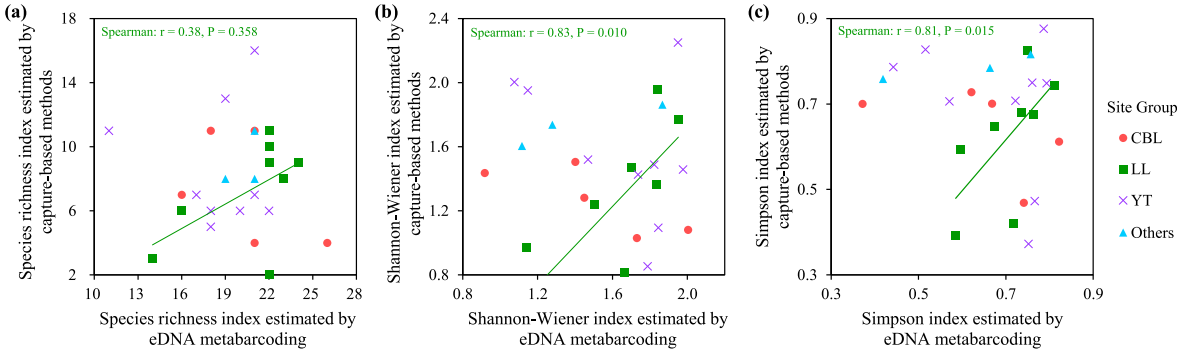

Fig. 6. Correlations between alpha diversity indices estimated by eDNA metabarcoding and capture-based methods, including species richness (**a**), Shannon-Wiener index (**b**) and Simpson index (**c**).

Wiener and Simpson index) estimated by the two approaches (Fig. 6). However, for sites from the Lalu wetland (group LL), diversity indices showed a significant correlation ($P < 0.05$) between estimates by the two approaches ($r = 0.83$ for Shannon-Wiener index and $r = 0.81$ for Simpson index). There was no significant difference in alpha diversity among the three groups (CBL, LL and YT). However, significant differences were detected in community composition among the three groups based on data from both eDNA (PERMANOVA, $R^2 = 0.31$, $P < 0.01$; betadisper, $P = 0.51$) and capture-based (PERMANOVA, $R^2 = 0.46$, $P < 0.01$; betadisper, $P = 0.65$) methods. The pairwise adonis test revealed that the YT group was significantly different ($P < 0.01$) in community composition from the LL and CBL groups (Supplementary Fig. S3).

4. Discussion

4.1. Markers and primers for eDNA metabarcoding

Mitochondrial DNA (mtDNA) are almost invariably chosen as genetic markers for eDNA metabarcoding due to the substantially greater copy number of mtDNA than nuclear DNA per cell (Jensen et al., 2021; Rees et al., 2014). An ideal marker is required to provide conservative regions for primer design, hypervariable regions and high-quality reference databases for taxonomic assignment (Miya et al., 2015; Taberlet et al., 2012). The most commonly used genetic markers are 12S, Cytb and COI. COI serves as the standard DNA barcode marker for animals and therefore has an extensive reference library (Hebert et al., 2003). However, it has been argued that COI does not contain suitable conserved regions to design primers for amplicon-based metabarcoding applications (Collins et al., 2019; Deagle et al., 2014). The primers within the short hypervariable regions of 12S have been recognized as the best for fish eDNA metabarcoding (Kumar et al., 2022; Zhang et al., 2020). However, recent studies have showed that 12S has inherent limitations for assignment to lower taxonomic ranks and still has a low species completeness in databases (Cantera et al., 2019; Hänfling et al., 2016; Miya et al., 2015). Cytb also has an extensive library as it serves as a main marker for phylogenetic study and exhibits a resolution that is comparable or higher than COI (Kartavtsev and Lee, 2006). In this study, we assessed the species discrimination potentials of the three markers and found Cytb had the highest average genetic distance at each taxonomic level followed by COI and 12S, which was consistent with the results of previous studies (Heras et al., 2009; Parhi et al., 2019; Webb and Moore, 2005). The 12S sequence was not qualified for the accurate species assignment, as *S. oconnori* and *S. waltoni* shared an identical haplotype. We also found it difficult to design suitable primers from COI sequences. After assessment, the optimal primer pair for eDNA detection in this study was the newly designed one for Cytb with the forward primer being located at the conserved regions from the upstream sequences, indicating a good alternative to design primers from the upstream and conserved regions of the metabarcoding gene (Hänfling et al., 2016). The universal primers of 12S are the most common choices for fish eDNA metabarcoding in biodiversity hotspots (Valdivia-Carrillo et al., 2021). However, for eDNA metabarcoding at a species-level resolution, it is necessary to evaluate the performance of the selected markers and primers.

4.2. Detection of rare native and non-native fishes

In this study, eDNA metabarcoding was more effective in detecting rare native and non-native fish species than capture-based methods. Half of native fishes in the upper and middle reaches of the Yarlung Zangbo River are basin-specific species including *G. maculatum* and species belonging to the subfamily Schizothoracinae, and most are at risk (Li et al., 2021; Jiang et al., 2016). *G. maculatum* is listed as a critically endangered species and was not detected by capture-based methods and eDNA metabarcoding. Among the six Schizothoracinae fishes, *O. stewartii* and *P. dipogon* are listed as endangered species, and the former is difficult to capture in the middle reach (Zhu et al., 2017). In this study, we obtained no *O. stewartii* and few *P. dipogon* specimens by capture-based methods. However, the eDNA of *O. stewartii* was detected in half of the sites and of *P. dipogon* in most sites. Because the six Schizothoracinae species have similar body sizes and close phylogenetic relationships, the numbers of eDNA sequences (3k in *O. stewartii*, 8k in *P. dipogon*, 161k in *S. waltoni*, 170k in *S. oconnori*, 222k in *S. macropogon* and 1 M in *S. younghusbandi*) were highly related to the numbers of captured individuals (0, 8, 142, 160, 163 and 289). Some OTUs widely detected in sampling sites were assigned to *T. dalaica* which is naturally distributed in the Yellow River and artesian waters in Inner Mongolia (Zhu, 1989). It may not be an invasive species because most non-native fishes are introduced to Tibet via aquaria and aquaculture trades, and *T. dalaica* is neither an aquarium fish nor a commercial fish. It is possible that its presence in these waters is a result of natural factors (e.g. bird carry) or it is a cryptic species that has not previously been discovered or described.

Invasive fishes can pose a threat to native biodiversity via competition, predation and disease transmission (Chen and Chen, 2010; Jia et al., 2019). Among the ten captured non-native fishes, eight were detected by eDNA metabarcoding, the exceptions being *R. cliffordpopei* and *S. asotus* of the failure to detect *R. cliffordpopei* may be due to its small size and low abundance with only two individuals captured in one site. For *S. asotus* which was captured in ten sites, the metabarcoding primers showed no mismatch and amplified well in the species. The reason for the failure to detect these species by eDNA should be further investigated. The eDNA method detected four non-native fishes (*H. nobilis*, *C. idellus*, *H. molitrix* and *C. argus*) which were not captured in this study but have been previously recorded (Fan et al., 2011, 2016; Yang et al., 2010). The failure to catch the three Cyprinidae fishes could be because the gillnets with small mesh sizes were unsuitable for capturing large fishes (Hoover et al., 2017). The eDNA method also detected five non-native fishes that were neither captured in this study nor previously recorded. Two (*M. albus* and *S. chuatsi*) are worthy of attention as they were detected in multiple sites. These two species are not easy to capture because they live in special habitats and are nocturnal (Li et al., 2013; Liu et al., 2018). Three other species (*H. maculatus*, *M. tungtingensis* and *O. niloticus*) were only detected at one or two sites with negligible read counts. The detected eDNA of these fishes may come from wastewater from nearby restaurants and aquatic product markets.

4.3. Estimation of diversity

eDNA metabarcoding provides information on sequence reads count of each fish species, thus allowing the quantification of relative species abundances which is a crucial component of biodiversity monitoring (Di Muri et al., 2020). However, the relationship between abundance estimates in natural waters from eDNA metabarcoding data and conventional capture methods is still controversial (Bakker et al., 2017; Balasingham et al., 2018; Czeglédi et al., 2021). In this study, the read counts of fishes were strongly related to the numbers of captured individuals ($r = 0.91$, $P < 0.01$). However, the alpha diversity indices estimated by the two methods were only significantly correlated for sites at the Lalu wetland, but not correlated for sites overall. It is noteworthy that the factors influencing the diversity estimates differ for the two methods. The diversity estimation by conventional capture-based methods is easily influenced by the fishing tools employed (Xu et al., 2016) and fishing effort (Arreguín-Sánchez, 1996). By contrast, the main factors affecting the diversity estimation by eDNA metabarcoding include: (i) eDNA decay, which is influenced by the physical, chemical and biological properties of water, such as temperature, pH, salinity and flow rate (Eichmiller et al., 2016; Jo et al., 2019; Strickler et al., 2015); (ii) the read count of one species can be affected by the amplification efficiency of primers (Kelly et al., 2019); (iii) eDNA shedding rates differ among fish species due to their distinct metabolic rates and activities (Thalinger et al., 2021). The water in the main river channel (YT) has a high flow rate and possesses different physical and chemical properties from upstream to downstream (Zhu et al., 2017), resulting in strong

influences on the eDNA abundance and distribution (Bylemans et al., 2019; Deiner et al., 2016). The Chabalang wetland (CBL) is very shallow (<1 m) and small (20 hm^2) (Ding et al., 2014), easily causing a bias of the fish catch among the sampling sites. Therefore, the alpha diversity estimates were not correlated between the two methods in YT and CBL. In spite of this, the beta diversity estimates were correlated between the two methods, and both revealed significant differences in community composition between the YT and LL and CBL groups.

In summary, eDNA metabarcoding in this study has some advantages in fish diversity monitoring: (i) the custom-made database of Cytb sequences provides a good reference for accurate and rapid species identification; (ii) the newly designed Cytb primers for eDNA metabarcoding are highly effective in PCR amplification across fishes in the studied area and the amplicon sequence provides informative sites for taxonomic assignment at species-level resolution; (iii) eDNA metabarcoding is more effective than capture-based methods for detecting rare native and non-native fish species; (iv) eDNA metabarcoding provides information of relative read abundance of each species which can be used for diversity estimation. The eDNA method in this study may faces two challenges: (i) *R. cliffordpopei* and *S. asotus* may not be detected by eDNA metabarcoding with the newly designed Cytb primers, which should be further investigated; (ii) water properties (e.g., temperature, salinity and flow rate) affect the shedding, decay and flow of eDNA from fish, which may cause biases in diversity estimation when comparing different water habitats.

5. Conclusions

eDNA metabarcoding with a species-level resolution needs prior evaluation both of the taxonomic resolution power of markers and of primer bias. Here we used eDNA metabarcoding to monitor the distribution and diversity of fishes in the upper and middle reaches of Yarlung Zangbo River where several endangered and invasive fishes are in urgent need of conservation and management. Our results demonstrated that the commonly used marker 12S was not qualified for the species assignment for the fish fauna. Using newly designed Cytb primers, the eDNA metabarcoding detected 30 fishes among which 12 were not captured by gillnets including one endangered species and several non-native species only some of which had been previously recorded. The alpha diversities estimated by the eDNA and capture-based methods were significantly correlated and both methods revealed significant differences in community composition between the main channel and the two wetlands. These findings suggest that eDNA metabarcoding is more effective in monitoring fish species than conventional capture-based methods and can be used as an alternative or supplementary approach for biodiversity assessment.

Ethical statement

All experimental animal programs involved in this study were approved by the Animal Care and Use Committee at the Institute of Hydrobiology, Chinese Academy of Sciences.

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Authors' contributions

X.F., Y.C. and X.S. conceived and designed the study; X.F., X.S., R.Z. and Y.J. collected fish and water samples and conducted the experiments; X.F. and B.L. performed the data analyses; X.F. wrote the manuscript and Y.C. and X.S. revised the manuscript. All authors read and approved the final manuscript.

Data accessibility

Raw Illumina sequencing data used for metabarcoding analysis were deposited in the NCBI Sequence Read Archive (SRA) under accession number PRJNA796829. Sequences of three barcoding genes used for DNA barcoding analysis were deposited in figshare (https://doi.org/10.6084/m9.figshare.18320012).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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