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Choice of primer pairs and PCR polymerase affect the detection of fish eDNA

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Abstract

Efficient biomonitoring is essential for fish protection and management. Environmental DNA (eDNA) has become a promising tool for fish surveys, and its accuracy and robustness are closely related to the primer pairs and DNA polymerases, especially for different environmental samples. However, there is still a lack of sufficient efforts to assess the effects of both two factors on fish biomonitoring. Here, we selected ten primer pairs in the mitochondrial 12S rRNA gene region and three commercial DNA polymerases and analyzed their effects on fish eDNA monitoring in surface water and sediment samples of Dianchi Lake. We found that primer pairs and DNA polymerases significantly affected fish biomonitoring in surface water and sediments of Dianchi Lake. First, there were significant variations in annotated fish eDNA sequences in different groups of primer pairs and DNA polymerases, the percentage of fish sequences amplified by the groups related to primers Riaz-12S and 12S-V5 was more than 90% of the total sequences. Second, the composition of different classification levels of fish taxa varied considerably across groups of primer pairs and DNA polymerases, and the groups related to primers Riaz-12S (i.e., Taq Master–Riaz-12S, Rapid Taq–Riaz-12S) and 12S-V5 (i.e., Taq Master–12S-V5, Rapid Taq–12S-V5) identified more taxa than other groups. Third, primer pairs had greater impacts on the structure of fish communities than DNA polymerases, and the interactions between two factors had more significant effects than any single one. This study highlights that primer pairs and DNA polymerases play critical roles in fish biomonitoring, and this work aimed to provide methodological guidance for assisting the design of the fish eDNA survey scheme.

Keywords eDNA, Biomonitoring, Fish community, Dianchi Lake, Surface water, Sediment

Introduction

Human activities and climate change drive a sharp decline in freshwater fish biodiversity [3, 55]. Rapid and efficient biomonitoring is a prerequisite for decision-making in fish protection. eDNA-based species detection has proven to be an efficient, cost-effective, non-invasive monitoring method [12, 45, 59], which is widely used for target species monitoring such as invasive and endangered species [10, 27, 44], and community surveys such as fish, zooplankton and macroinvertebrates [36, 61, 65]. Currently, eDNA studies mainly focus on sampling optimization, the ecology of eDNA (e.g., the origin, state, transport) and bioinformatics tool development [24, 33, 47]. As a critical step of eDNA technology, the efficiency of PCR assays is affected by many factors such as the choice of primer pairs and DNA polymerases [39, 54]. However, the extent to which both factors affect fish biomonitoring remains unclear.

The choice of primer pairs determines the accuracy and validity of fish biomonitoring [40, 66]. An important aspect to consider when selecting primer pairs is their ability to form stable double-stranded pairs with specific

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sites on the target DNA, and no duplex formation with other primer pairs or no hybridization at any other target site, as this would severely reduce the primer efficiency [46, 54, 62]. Ideally, universal primer pairs should have high specificity, coverage and species identification capability to ensure the complete and accurate species monitoring of environmental samples [42, 52]. For targeted species monitoring, the efficiency of specific primer pairs directly affects the false negative/false positive detection rate. Indeed, the presence of eDNA in the environment, decay and components affect the specific binding between the primer pairs and DNA templates. For instance, a large part of eDNA in sediment is extracellular DNA, with short fragments and severe damage, and these eDNA fractions in sediments are complex due to suspension redeposition [9, 17, 22]. In comparison, eDNA in surface water is directly discharged by organisms, with relatively long DNA fragments that mainly exist in a free state and degrade rapidly [28, 34]. The inherent differences in eDNA itself in environmental samples can affect the applicability of primer pairs, such as primer pairs that amplify long DNA fragments are not suitable for highly degraded eDNA samples. However, how the choice of primer pairs affects fish biomonitoring in different environment samples, remains poorly explored.

DNA polymerase is another critical point affecting the efficiency of PCR assays [20], because the thermal stability, fidelity and specificity of DNA polymerase determine the accuracy and amplified fragment length of PCR assays [4, 8]. The high fidelity of DNA polymerase can ensure a high yield of target products and provide accuracy in sequence replication, and high thermal stability helps overcome some difficulties in PCR assays, such as secondary structure, GC-rich sequences and long DNA amplification [7, 15]. Inhibitors in the sample are important factors leading to reduced efficiency or failure of the PCR assays [26, 53]. For example, humic acid has been identified as a major PCR inhibitor in sediments [1, 56, 58], the main inhibitors in the water samples were sodium dodecyl sulphate (SDS), metal ions and Immunoglobulin G (IgG) [21, 50]. Although inhibitors affect PCR efficiency by acting directly on DNA polymerases, the impact of DNA polymerases on fish biomonitoring in different environments still lacks a clear picture.

Here, we selected ten primer pairs widely used by scholars in the mitochondrial 12s rRNA gene region (Additional file 1: Table S1) and three commercial DNA polymerases, a total of 30 groups of PCR assays were set up (Additional file 1: Table S2). Surface water and sediment eDNA samples were collected from Dianchi Lake in China (24° 29′–25° 28′ N, 102° 29′–103° 01′ E) and were analyzed with respect to the effects of primer pairs and DNA polymerases on fish biomonitoring. The purpose of this study is mainly achieved through the following three aspects: (1) the proportion of fish and non-fish eDNA sequences was calculated to assess the specificity with regard to fish in different groups of PCR assays; (2) the proportion of fish taxa at different classification levels was analyzed to compare the taxonomic specificity and richness; (3) the Jaccard dissimilarity matrix was calculated to reveal the structural differences of fish communities across different groups of PCR assays.

Materials and methods

Experimental design

To reveal the effects of primer pairs on fish eDNA biomonitoring, we retrieved 10 primer pairs located in the mitochondrial 12s rRNA gene region for fish eDNA biomonitoring retrieved from the literature (Additional file 1: Table S1). These primer pairs have been proven to have good amplification performance and have been widely used in fish surveys [25, 66]. Thirty groups generated by 10 primer pairs and 3 DNA polymerases (Additional file 1: Table S2), and 10 blanks (DEPC water as DNA template) were carried out PCR assays, the successful groups (i.e., the agarose gel electrophoresis have specific bands and correct amplification size) were performed to subsequent sequencing and data analysis. Each PCR assay was conducted in a 20 μl volume, including 1 μl forward primer, 1 μl reverse primer, 2 μl DNA template (collected from the Dianchi Lake), 10 μl 2 × DNA polymerases Master Mix (Vazyme Biotech) and 6 μl DEPC water. The target bands of PCR assays were checked by a 1.5–2% agarose gel electrophoresis. After dozens of attempts, the Mifish-U, AcMDB07, Elas02, Ac12S and Am12S failed to amplify specific bands in any reaction conditions (Additional file 1: Table S3). Finally, only five primer pairs (i.e., Mifish-E, Teleo, Tele02, Riaz-12S and 12S-V5, Table 1 were kept for subsequent high-throughput sequencing and statistical analysis. To analyze the effects of DNA polymerases on fish biomonitoring, we purchased three common commercial DNA polymerase mixes from the Vazyme Biotech Co., Ltd. (Nanjing, China, namely 2 × Taq Master Mix II (Dye Plus, 2 × Rapid Taq Master Mix and 2 × Phanta Flash Master Mix (Dye Plus. These three DNA polymerases are abbreviated as Taq Master, Rapid Taq and Phanta Flash, respectively. Among them, the Taq Master is easy to operate and has high stability; the Rapid Taq has a fast amplification speed (15 s/kb, simple operation and good stability; the Phanta Flash has a high amplification efficiency, fast amplification (4–5 s/kb, high fidelity (up to 81

Table 1 Summary of five primer pairs in the mitochondrial 12s rRNA gene region used for fish eDNA sequencing in the current study, including primer name and sequences, annealing temperature (Ta), amplicon size (bp) and original references

Primer pairs	Primer sequence (5′-3′)	Ta/℃	Size/bp	References
Mifish-E	Forward: GTTGGTAAATCTCGTGCCAGC Reverse: CATAGTGGGGTATCTAATCCTAGTTTG	53	171	[37]
Riaz-12S	Forward: ACTGGGATTAGATACCCC Reverse: TAGAACAGGCTCCTCTAG	55	106	[42]
Teleo	Forward: ACACCGCCCGTCACTCT Reverse: CTTCCGGTACACTTACCATG	52	100	[60]
Tele02	Forward: AAACTCGTGCCAGCCACC Reverse: GGGTATCTAATCCCAGTTTG	56.8	167	[57]
12S-V5	Forward: TAGAACAGGCTCCTCTAG Reverse: TTAGATACCCCACTATGC	55	106	[42]

Table 2 The amplification speed and advantages of three commercial DNA polymerases, and all DNA polymerases are premixes

Polymerase/mix	Amplification speed	Advantages
2×Taq plus Master Mix II (Dye Plus)	60 s/kb	Ready-to-use premixes, easy to handle
2×Rapid Taq Master Mix	15 s/kb	High amplification performance, high specificity, high stability and high yield
2×Phanta Flash Master Mix (Dye Plus)	4–5 s/kb	Broad template compatibility, excellent amplification speed and yield, stable crude amplification, perfect high GC suitability, ultra-high fidelity

times of the common Taq Polymerase and high specificity (Table 2).

Our experimental designs aim to answer the following three questions. First, to what extent do primer pairs affect fish biomonitoring, and are the results consistent across different samples (i.e., surface water and sediments)? To answer this question, we conducted PCR assays on surface water and sediment samples collected from Dianchi Lake in China, with three replicates and a blank control set for each primer pair. Second, how do DNA polymerases affect fish biomonitoring, and is the degree of influence different across different samples? To address this concern, three DNA polymerases were chosen to perform PCR assays of surface water and sediment samples, we set up three replicates and a blank control set for each DNA polymerase. Finally, based on the above experimental designs, we analyzed the dissimilarity of community structure and taxonomic richness across different groups of primer pairs and DNA polymerases, the interactions on fish biomonitoring were tested by the two-way ANOVA. All PCR assays with bright and specific bands were performed to high-throughput sequencing.

Sample collection and DNA extraction

Surface water and sediment eDNA samples were collected from 23 sites in Dianchi Lake in July 2022. At each site, three liters of surface water were sampled using sterile bottles (Thermo Fisher Scientific™, USA), and immediately transferred on cryogenic incubators with several ice packs (*ca.* 0 to 4 °C) until filtration treatment within 6 h. The sediment samples were collected by a gravity corer and were stored in sterile plastic bags at −80 °C until DNA extraction. Surface water was vacuum-filtered through a 0.45 μm hydrophilic nylon membrane (Merck Millipore, USA). In addition, *ca.* 300 ml of autoclaved tap water was performed as blank controls to monitor possible contaminants across different sites. All replicates of the eDNA samples and blank controls were individually stored in 5.0 ml centrifugal tubes and then frozen and stored at −20 °C until DNA extraction. eDNA from surface water and sediment samples (*ca.* 0.5 g dry weight) were extracted using a DNeasy Power Water Kit and DNeasy PowerSoil Kit (QIAGEN, Hilden, Germany) with the manufacturer's protocol, respectively. Extracted eDNA was quantified using a Qubit Flex Fluorometer (Thermo Fisher Scientific™, USA) and Equalbit dsDNA HS Assay Kit (Vazyme Biotech, China). Finally, the surface water and sediment eDNA samples from 23 sites were individually pooled into one water eDNA and one sediment eDNA sample for subsequent PCR assays.

PCR and sequencing

A unique 12-nt nucleotide fragment (also known as barcode) was added to the 5′ ends of the forward or reverse primers (Shanghai Generay Biotech Co., Ltd.) to allow the identification of different eDNA samples during the split processing of sequencing data. Three PCR replicates were

performed on pooled water and sediment eDNA samples to reduce potential PCR bias. PCR blank controls (i.e., nuclease-free water as DNA template) were used for all assays. The PCR was performed in a final volume of 20 μl, consisting of 1 μl of 10 μM forward and reverse primers, 2 μl of eDNA template (*ca.* 5–20 ng/μl), 10 μl of 2 × DNA polymerases Master Mix (Vazyme Biotech, China) and 6 μl of DEPC water. Details on the protocol of PCR amplification are shown in Additional file 1: Table S2. The target bands of PCR assays were checked by a 1.5–2% agarose gel electrophoresis. All PCR products were purified using the EasyPure PCR Purification Kit (TransGen Biotech, China), the purified products were quantified by a Qubit Flex Fluorometer (Thermo Fisher Scientific, USA) and pooled at equal DNA quantities of 200 ng/sample for library preparation. Two tagged PCR libraries were individually constructed using the VAHTS Universal DNA Library Prep Kit for Illumina (Vazyme Biotech, China). The library concentration was measured by a Qubit Flex Fluorometer. Before sequencing, each library was diluted to a final concentration of 100 pM. Finally, each library added with 10% of PhiX (control DNA) was sequenced on the Illumina MiSeq PE150 (Illumina, San Diego, California, USA).

Bioinformatic analysis

DNA sequencing data were pre-processed in the QIMME toolkit [6]. Raw sequence reads were filtered using a series of quality controls, first, the fastx-toolkit was used to assess the quality of sequence reads,second, the low-quality reads (e.g., series with average quality < 20, sequences contained ambiguous N, homopolymer and sequence length < 100 bp) were discarded using the *split-libraries.py* script with the parameter settings "*-s 25 -w 50 -l 100 -L 500 -H 6*" in the QIMME toolkit [6], and duplicate sequences were removed using the *–derep_fulllength* script in VSEARCH pipeline [43],then, the reads were sorted and distinguished by unique sample tag pairs, finally, sequence reads were clustered into operational taxonomic units (OTUs using UCLUST with 97% nucleotide similarity in UPARSE pipeline [16]. The OTUs were taxonomically assigned using BLAST against the mitochondrial genome database (i.e., Mitofish) [48] for fish detection. The relevant methods and criteria refer to previous studies, in brief, if the OTUs sequence matched one species with a max score and similarity > 97%, the species was assigned, and if the OTUs sequence matched one species with a max score but similarity < 97%, the genus was assigned [64, 66]. The assigned OTUs table was filtered referring to the following three criteria to exclude falsely positive and falsely negative OTU detections and establish a reliable dataset [31, 32]: (1) the OTUs with less than 50% detection frequency in all subsamples per site were discarded; (2) the OTUs with a relative abundance < 0.001% and a detection frequency < 10% were excluded; (3) the OTUs occurred in the blank controls were removed any taxa and. Finally, observed OTUs tables were obtained across the sampling sites (i.e., OTUs × sites) for diversity and composition analyses.

Effects of primer pairs and PCR polymerase

To assess the sequence specificity with regard to fish in different groups of PCR assays, the proportion of fish and non-fish eDNA sequences was calculated. The number of species at different taxonomic levels was calculated to identify the annotation resolutions for different groups. Non-metric multidimensional scaling (nMDS) analysis based on the Jaccard dissimilarity matrix was performed to reveal the structural differences of fish communities across different groups, the significance levels were tested by the Monte Carlo permutation tests with 999 permutations using the *vegdist* and *metaMDS* functions the R package *vegan* [14], and the figures were generated using the R package *ggplot2* [23]. The two-way ANOVA was analyzed using the SPSS 22 software to test the interaction effects of primer pairs and DNA polymerases on fish richness.

Results

Analysis of fish eDNA sequence specificity

A total of 1212 OTUs and 3,116,330 high-quality reads were obtained in 45 surface water eDNA samples (5 primer pairs × 3 DNA polymerases × 3 replicates), among which fish accounted for 2,428,746 reads (77.94%) that were assigned to 13 orders, 23 families, 53 genera and 51 species (Fig. 1), and the details of sequence reads are shown in Additional file 1: Table S4. The proportion of sequence reads assigned to fish had a considerable variation range (i.e., the maximum is 82 times the minimum) in 15 groups of primer pairs and DNA polymerases (Additional file 1: Figure S1a). Specifically, the group Pr14 (Rapid Taq–12S-V5) obtained 320,039 reads, of which 312,334 reads (97.59%) belong to fish, but only 1466 (1.19%) of the 123,402 reads of the group Pr12 (Phanta Flash–Teleo) was assigned to fish. Our data showed that the percentage of fish reads amplified by all groups related to primers Riaz-12S (i.e., Taq Master–Riaz-12S, Rapid Taq–Riaz-12S and Phanta Flash–Riaz-12S) and 12S-V5 (i.e., Taq Master–12S-V5, Rapid Taq–12S-V5 and Phanta Flash–12S-V5) was more than 90%, followed by the percentage of fish reads amplified by three groups of Tele02 (i.e., Taq Master–Tele02, Rapid Taq–Tele02 and Phanta Flash–Tele02) between 30.63% and 61.49%, and the percentage of fish reads successfully amplified by three groups of Mifish-E (i.e., Taq Master–Mifish-E, Rapid Taq–Mifish-E and Phanta Flash–Mifish-E) between 2.01% and 15.52%, the groups related to primer Teleo (i.e.,

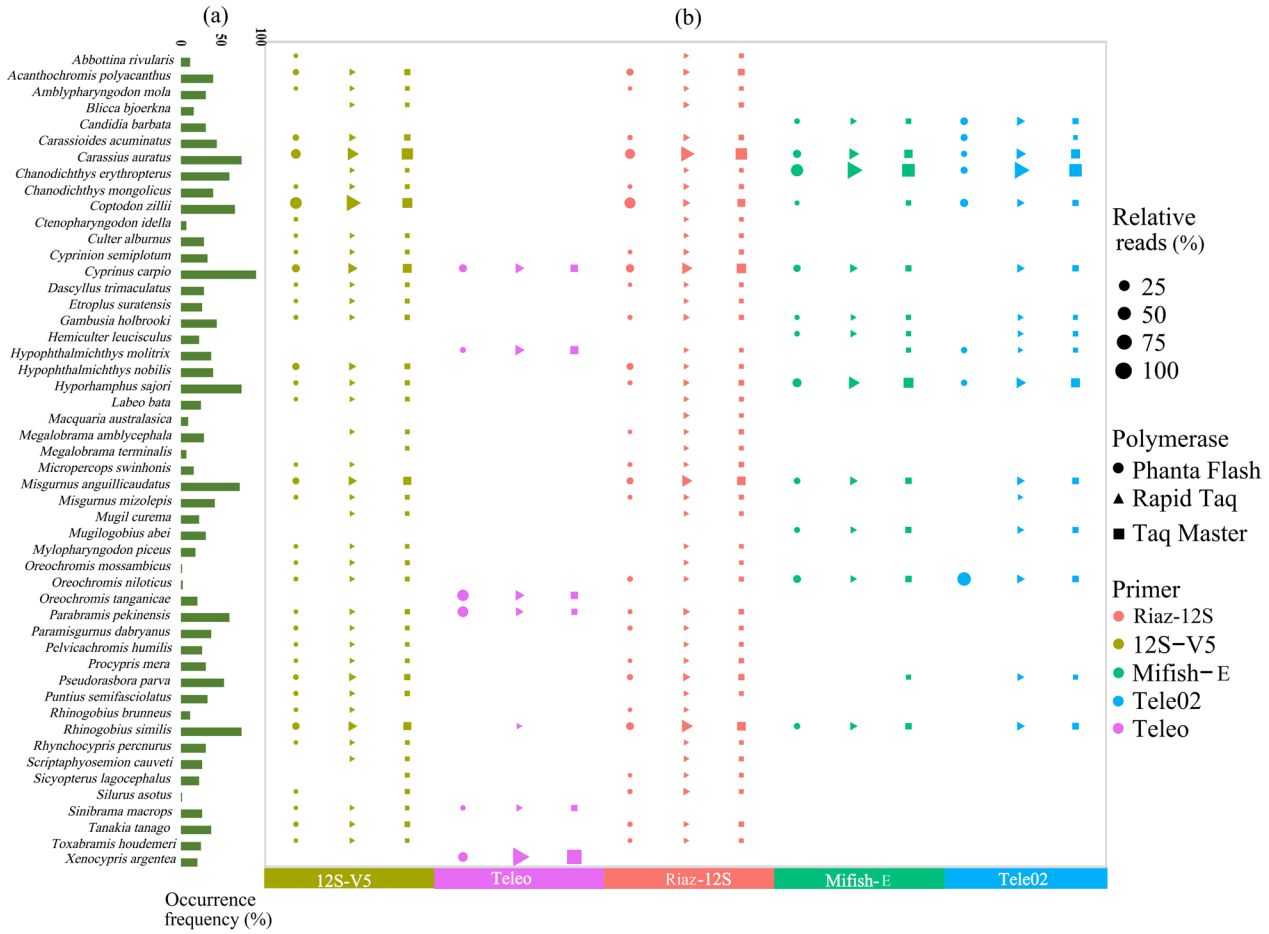

Fig. 1 Fish taxonomic diversity monitored by the eDNA technology in surface water samples. Occurrence frequency (**a**) and relative sequence reads (**b**) of each species among all 15 groups of primer pairs and DNA polymerases. The shapes represent different DNA polymerases, and the colors refer to different primer pairs. The bubble size reflects the relative sequence reads of each group, that is, the sum of sequence reads of three replicates in each group divided by the total sequence reads in all groups

Taq Master–Teleo, Rapid Taq–Teleo and Phanta Flash–Teleo) was the lowest, that is, the percentage of amplified fish reads was less than 4% (Additional file 1: Figure S1a). In addition, the proportion of sequence reads assigned to fish was considerably similar across three PCR replicates, such as the groups related to primers Riaz-12S and 12S-V5 (Additional file 1: Figure S2).

A total of 1826 OTUs and 3,707,983 high-quality reads were yielded in 45 sediment eDNA samples (5 primer pairs × 3 DNA polymerases × 3 replicates), among which fish accounted for 2,361,897 reads (63.7%) that were assigned to 12 orders, 24 families, 58 genera and 47 species (Fig. 2), and the details of sequence reads are shown in Additional file 1: Table S5. The highest percentage of fish sequences was the group Pr14 (Rapid Taq–12S-V5), accounting for 94.61% (382,264) of the total reads (404,034, Additional file 1: Figure S1b); followed by the group Pr13 (Taq Master–12S-V5, 92.51% of fish reads) and Pr15 (Phanta Flash–12S-V5, 90.71% of fish reads), the lowest was the group Pr11 (Rapid Taq–Teleo), which obtained 333,440 reads, while fish reads only accounted for 2.01%. In general, the data showed that the percentage of fish reads in all other groups was less than 50%, except for the groups related to primers Riaz-12S (i.e., Taq Master–Riaz-12S, Rapid Taq–Riaz-12S and Phanta Flash–Riaz-12S) and 12S-V5 (i.e., Taq Master–12S-V5, Rapid Taq–12S-V5 and Phanta Flash–12S-V5). In addition, the proportion of sequence reads assigned to fish in the groups related to primers Riaz-12S and 12S-V5 was considerably similar across three PCR replicates (Additional file 1: Figure S3).

Species composition and taxonomic richness

All groups of primer pairs and DNA polymerases in surface water samples showed significant differences in the composition of different classification levels of fish taxa

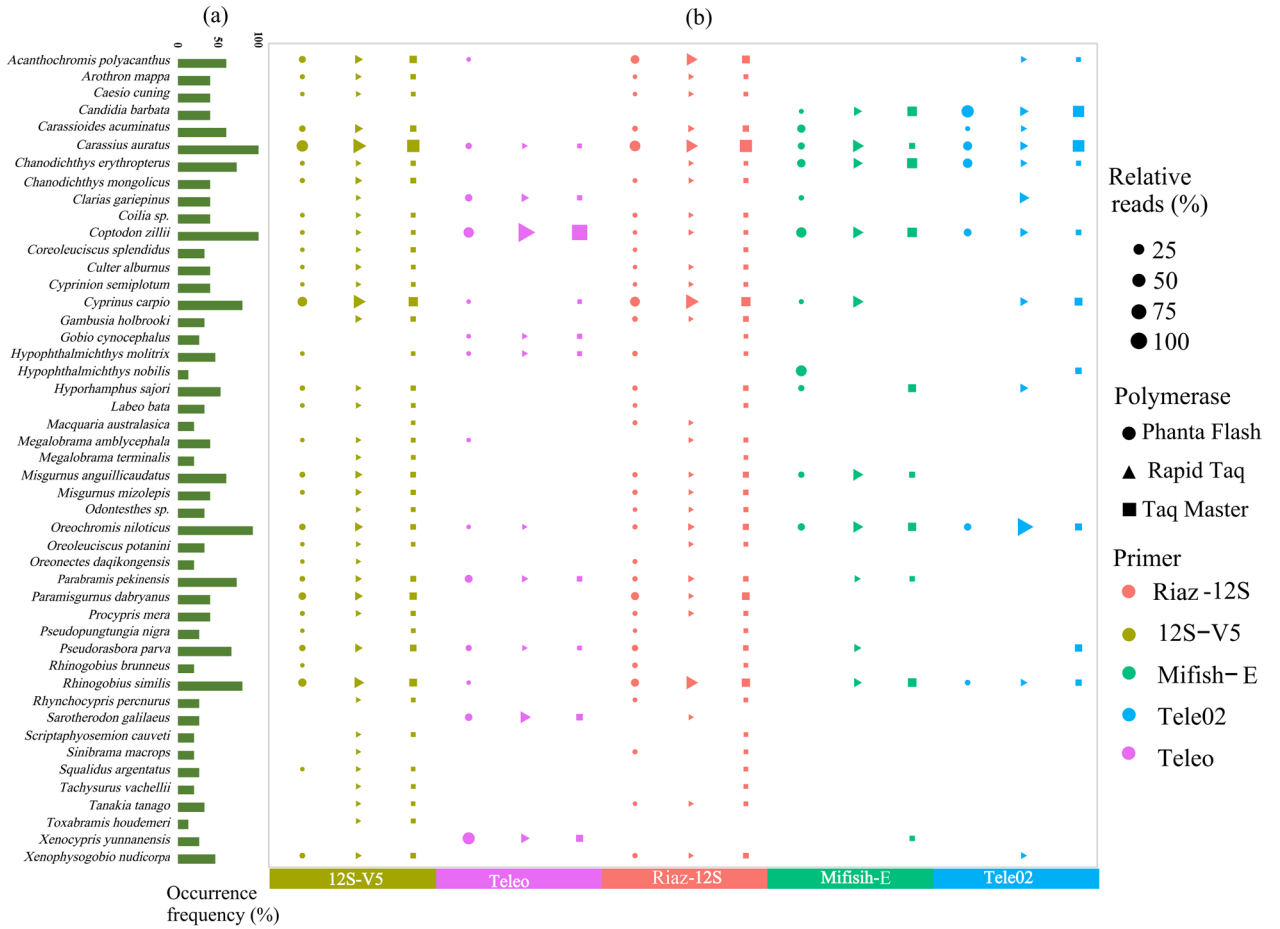

Fig. 2 Fish taxonomic diversity monitored by the eDNA technology in sediment samples. Occurrence frequency (**a**) and relative sequence reads (**b**) of each species among all 15 groups of primer pairs and DNA polymerases. The shapes represent different DNA polymerases, and the colors refer to different primer pairs. The bubble size reflects the relative sequence reads of each group, that is, the sum of sequence reads of three replicates in each group divided by the total sequence reads in all groups

(Fig. 3a), among which the group Pr08 (Rapid Taq–Riaz-12S) detected the most fish species (44 species), followed by the group Pr07 (Taq Master–Riaz-12S) and Pr13 (Taq Master–12S-V5) monitoring 43 and 40 fish species, respectively, with the group Pr12 being the least (Phanta Flash–Teleo, only 6 species). In addition, the groups related to primers Tele02 (i.e., Taq Master–Tele02, Rapid Taq–Tele02 and Phanta Flash–Tele02), Teleo (i.e., Taq Master–Teleo, Rapid Taq–Teleo and Phanta Flash–Teleo) and Mifish-E (i.e., Taq Master–Mifish-E, Rapid Taq–Mifish-E and Phanta Flash–Mifish-E) detected fewer fish species than the other groups (only 6–15 species). Cypriniformes accounted for the largest proportion of fish taxa detected in all groups (50%–88.89%) of fish taxa, followed by Cichliformes, Gobiiformes and Beloniformes (Fig. 3b). In terms of taxonomic richness, the groups related to primers Riaz-12S and 12S-V5 were significantly outperformed to other groups (Fig. 3c), for example, the group Pr07 (Taq Master–Riaz-12S), Pr08 (Rapid Taq–Riaz-12S), Pr13 (Taq Master–12S-V5) and Pr14 (Rapid Taq–12S-V5) showed significantly higher richness than other groups.

The results of sediment samples showed that the monitored fish in all groups of primer pairs and DNA polymerases were between 7 and 41 species (Fig. 4a). Specifically, the group Pr07 (Taq Master–Riaz-12S), Pr13 (Taq Master–12S-V5) and Pr14 (Rapid Taq–12S-V5) had better monitoring performance, with 41, 40 and 40 fish species detection, respectively. The monitoring rate of the groups related to primers Mifish-E (i.e., Taq Master–Mifish-E, Rapid Taq–Mifish-E and Phanta Flash–Mifish-E), Teleo (i.e., Taq Master–Teleo, Rapid Taq–Teleo and Phanta Flash–Teleo) and Tele02 (i.e., Taq Master–Tele02, Rapid Taq–Tele02 and Phanta Flash–Tele02) was low, with a maximum of 14 fish species amplified. Among the monitored fish taxa in all groups of primer pairs and DNA polymerases, Cypriniformes accounted for the largest proportion

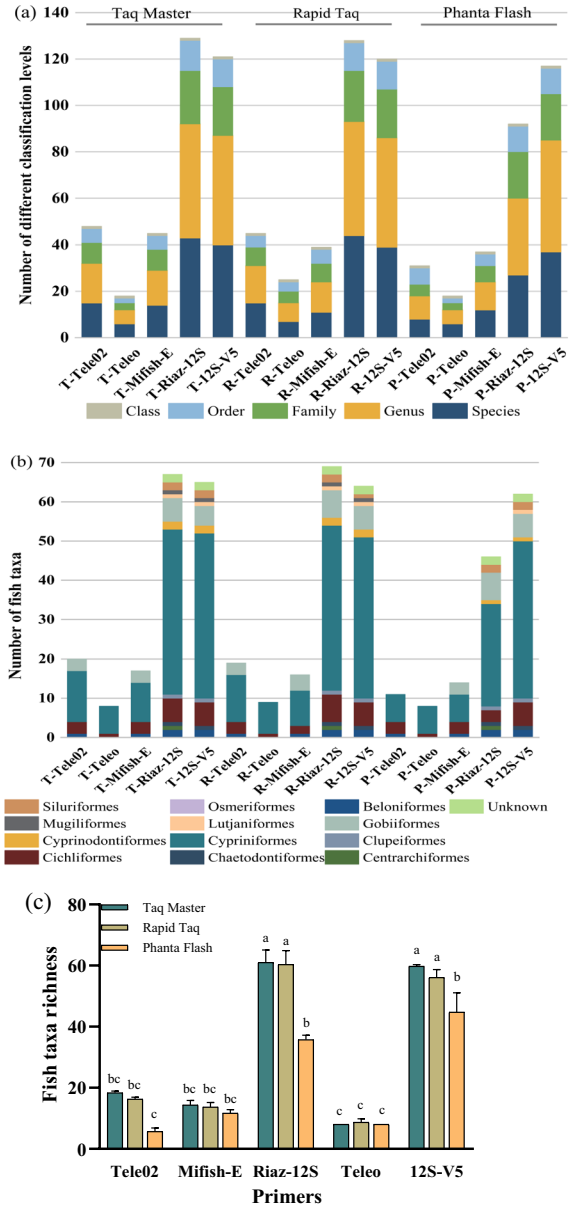

Fig. 3 Distribution of taxonomic classification levels of fish (**a**), the composition of taxa at the order level (**b**), and fish taxonomic richness of all 15 groups of primer pairs and DNA polymerases in surface water samples (**c**). 'Unknown' indicates reads not assigned to known taxonomic taxa, and the "**a**", "**b**" and "**c**" in panel C are the significance test by the one-way ANOVA, respectively

(53.3%–77.7%) of fish taxa for all groups, followed by Gobiiformes and Cichliformes (Fig. 4b). Similar to surface water, the groups related to primers Riaz-12S and 12S-V5 were significantly outperformed to other groups in sediment samples (Fig. 4c), for example, the group Pr07 (Taq Master–Riaz-12S), Pr13 (Taq

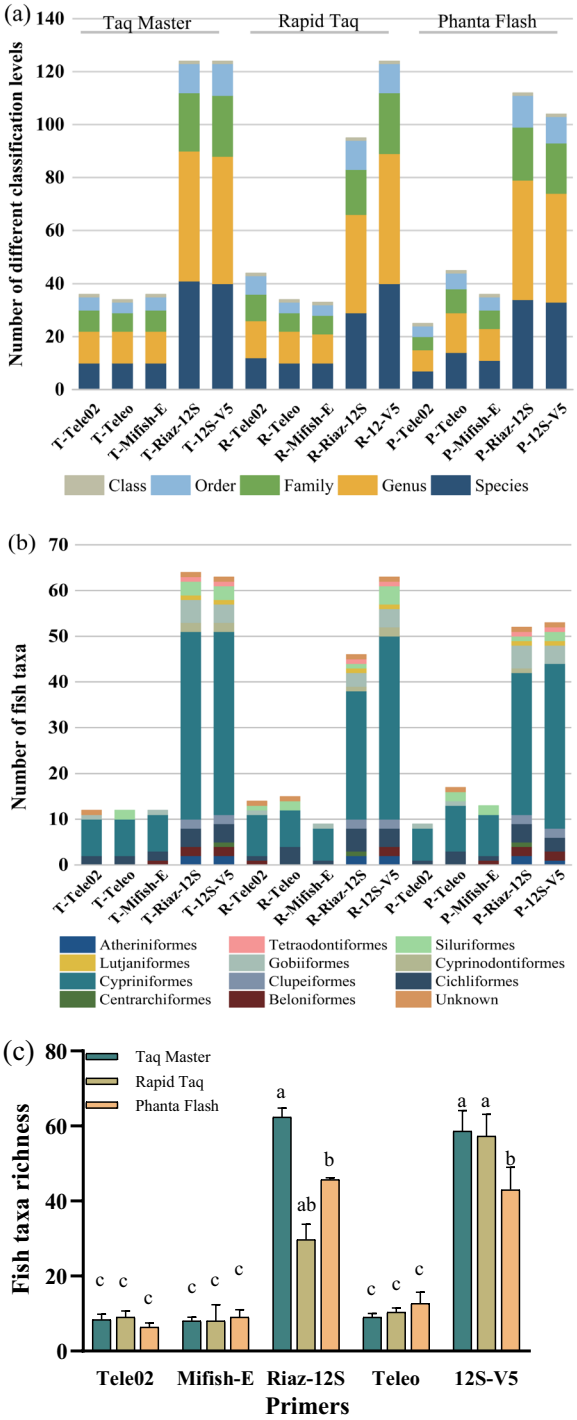

Fig. 4 Distribution of taxonomic classification levels of fish (**a**), the composition of taxa at the order level (**b**), and fish taxonomic richness of all 15 groups of primer pairs and DNA polymerases in sediment samples (**c**). 'Unknown' indicates reads not assigned to known taxonomic taxa, and the "a", "b" and "c" in panel C are the significance test by the one-way ANOVA, respectively

Master–12S-V5) and Pr14 (Rapid Taq–12S-V5) showed significantly higher richness than other groups.

Community structure and interaction judgment

The nMDS ordination plots showed that the primer pairs generated different structures of fish communities in surface water, as shown by the presence/absence-based Jaccard matrix (Fig. 5a), and had higher effects than DNA polymerases. In particular, the structural dissimilarity of fish communities in the primers Teleo was significantly different from other primer sets, the sample dots of primers Riaz-12S and 12S-V5 were closer in the ordination plots, indicating that the monitored structure of fish communities was more similar between each other. In contrast, while the primer pairs still had greater effects on fish communities in sediment samples, the effect intensity of DNA polymerases increased (Fig. 5b). For example, the sample dots of primer Teleo were discrete from other primer pairs in the ordination plots, but the sample dots of different DNA polymerases also had obvious spatial dispersion when zooming in space, which was obviously different from that in surface water. For the primers Mifish-E and Tele02, it was not easy to summarize the structural dissimilarity of fish communities among samples through primer pairs, but samples can be more clearly distinguished in the nMDS ordination plots based on DNA polymerases.

Our data showed that the primer pairs and polymerase had jointly significant effects on monitoring fish richness in both surface water and sediment (Table 3). Specifically, the effects of primer pairs on surface water ($P<0.0001$) and sediment ($P=0.0015$) were higher than those of DNA polymerases ($P=0.0079$ and $P=0.0016$). For the interactions, the effects of primer pairs and DNA polymerases on fish richness in sediment ($P=0.0062$) were higher than that of surface water ($P=0.0272$).

Discussion

The results showed that primer pairs and DNA polymerases significantly affected fish biomonitoring in both surface water and sediment samples of Dianchi Lake. We found that eDNA data and historical fish records had almost 70% overlap (at the genus level, Additional file 1: Table S6). The groups related to Riaz-12S and 12S-V5 had consistently higher taxonomic specificity, fish coverage and species resolution than others, and the effects of primer pairs on communities' structure were higher than DNA polymerases. The critical role of primer pairs in fish biomonitoring has been highlighted in previous studies [63, 64], and the primer pairs Riaz-12S and 12S-V5 show relatively high fish sequence proportion and fish diversity [66]. Here we further explored another important factor in the PCR assays, and found that the role of DNA polymerases in sediment samples for fish biomonitoring would be slightly higher than that of surface water. We provide performance assessments of primer pairs and DNA polymerases across different environmental samples, and these findings could provide methodological guidance for assisting the design of the fish eDNA survey scheme in aquatic systems such as rivers and lakes.

Our data showed that Riaz-12S and 12S-V5 have a higher fish sequence percentage and a more specific classification resolution than other primer pairs in both water and sediment samples. Two reasons can explain this result, on the one hand, the degradation of DNA released by organisms into environmental media, primer pairs targeting relatively short fragments have a higher amplification success rate [2, 13]. A previous study has also shown that primer pairs that amplify longer DNA fragments do not necessarily produce more target sequence reads than shorter ones with the same GC content [38]. On the other hand, the primer pairs Riaz-12S and 12S-V5 have a higher annealing temperature (Ta), which makes them more stable and better combined with DNA templates [46]. Satterfield et al. suggested that a lower Ta value may cause non-amplification [49]. Given that eDNA is often stored in environmental media as shorter DNA fragments [5], we suggest that priority should be given to selecting primer sets with shorter target DNA fragments for fish biomonitoring, especially for highly degraded samples such as soils, sediments and samples from tropical regions [11, 18]. In addition, the Riaz-12S and 12S-V5 had similar results may also be due to their same reverse primer [42]. Although the primer pairs Riaz-12S and 12S-V5 showed high performance, primer pairs showed different classification ranges. For a comprehensive "health checkup" of the fish composition in ecosystems, we recommend multiple primer pairs to increase the probability and reliability of species detection [19, 51]. As we know, the biodiversity complexity of the study system and completeness of the reference databases can also complicate the effect of barcode size on taxonomic assignments [29, 66], the completeness and quality of reference databases are known to be geographically and taxonomically biased, so the construction of high-quality reference databases of local biological communities should be a priority in eDNA biodiversity biomonitoring.

We found that the DNA polymerase Taq Master and Rapid Taq showed high amplification performance. This is mainly because, as the optimized products of Taq DNA polymerase, these two have a strong 5'–3' DNA synthesis ability, high amplification performance and low mismatch rate, and they also add 3′–5′ exonuclease activity, hence their fidelity is six times higher than

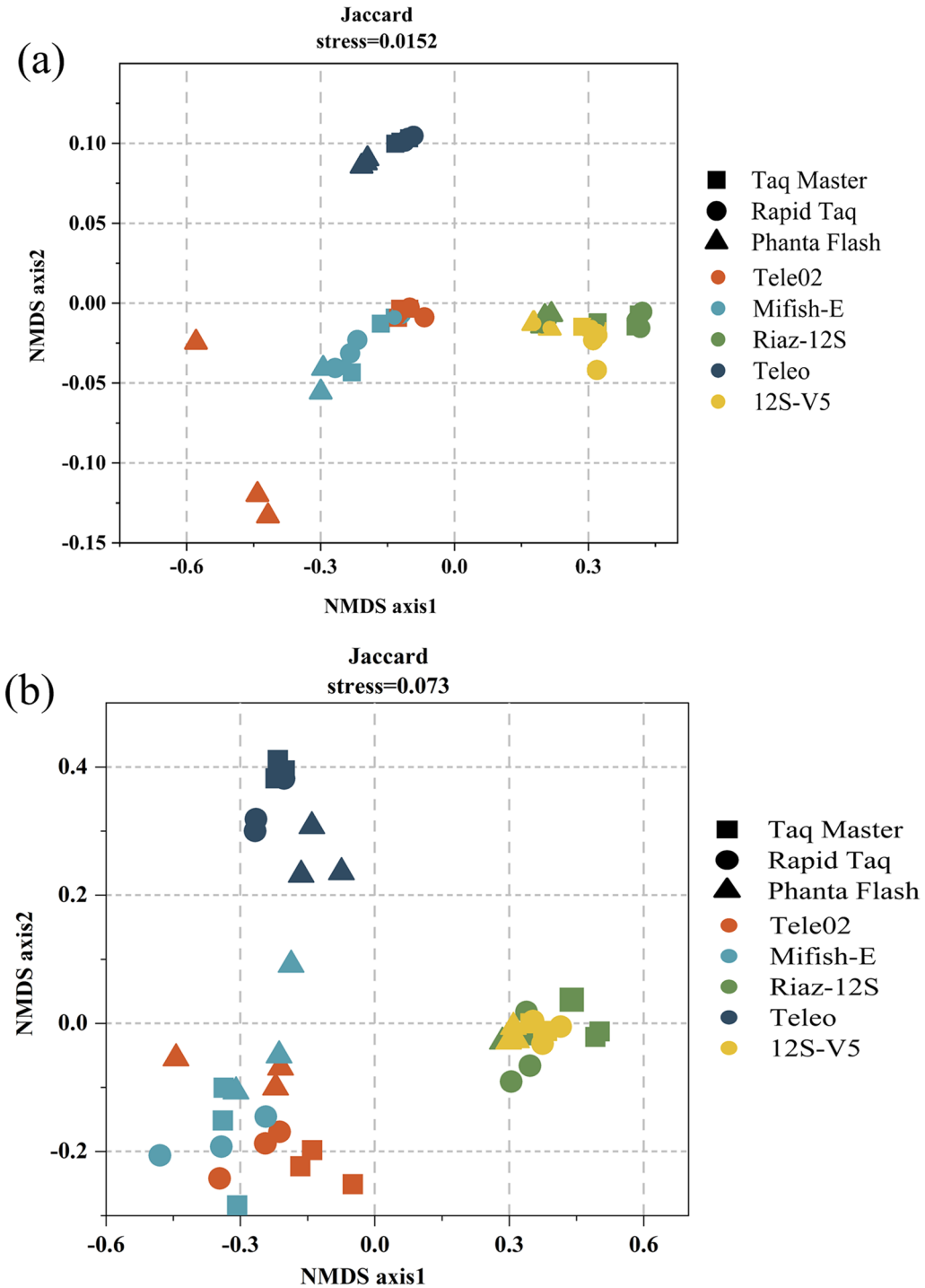

Fig. 5 Non-metric multidimensional scaling (NMDS) plots showing structural dissimilarities of fish communities across the groups of primer pairs and DNA polymerases in surface water (**a**) and sediment samples (**b**). The Jaccard dissimilarity matrix was performed to reveal the structural differences of fish communities across different groups, the significance levels were tested by the Monte Carlo permutation tests with 999 permutations

Table 3 Two-way ANOVA of the effects of primer pairs and DNA polymerases on fish richness detection in surface water and sediment samples

Factors	SS	df	MS	F (DFn, DFd)	P value
Water samples					
Primer pairs	18,548	4	4637	F (1.502,3.004) = 1233	< 0.0001
DNA polymerases	1115	2	557.3	F (1.006,2.012) = 122.0	0.0079
Primer * polymerases	793.7	8	99.21	F (1.734,3469) = 12.38	0.0272
Sediment samples					
Primer pairs	17,966	4	4429	F (1.271,2.542) = 220.4	0.0015
DNA polymerases	3819	2	191.0	F (1.022,2.004) = 60.51	0.0160
Primer * polymerases	1706	8	213.2	F (1800,3600) = 29.18	0.0062

$P < 0.05$ were considered significant. All data were tested for normal distribution before testing the two-way ANOVA. SS is the sum of squares, *df* is the degree of freedom, MS is the mean square and *F*(DFn, DFd) is *F*-statistics

that of common Taq DNA polymerase. Previous studies have shown that Platinum HiFi (similar to Phanta Flash with High-fidelity polymerase) had a high bias in taxonomic coverage and mismatch rate [39]. In addition, we found that the effect of DNA polymerases on community structure was slightly higher in sediment samples than in surface water, which may be due to the high sensitivity of DNA polymerases to sediment characteristics such as high humus and humic acid. Previous studies have shown that sediment samples appear to contain more or higher concentrations of inhibitors, and DNA polymerases have poor resistance to the main inhibitor (e.g., humic acid) in sediment [1, 35], resulting in greater effects in sediment samples than in surface water. For the inhibitors in environmental samples, we suggest (1) extensive sample processing and purification; (2) reducing the amount of sample matrix, thereby removing or diluting matrix-derived inhibitors; (3) adding bovine serum albumin (BSA), T4 gene32 protein (GP32) Master Mix or using other DNA polymerases [1, 30], (4) model calibration in data analysis (e.g., PMMoV assay as a model system to study the effect of inhibitors of PCR in environment matrices) [41]. The applicability of DNA polymerases depends on different inhibitors and sample characteristics, and selecting DNA polymerases for different samples can effectively reduce PCR inhibition.

In summary, we compared the effects of different primer pairs and DNA polymerases on fish eDNA biomonitoring, and suggested that both two factors are essential to generate reliable and comprehensive fish eDNA datasets. We insist on emphasizing to managers and stakeholders that candidate primer pairs must be screened before conducting eDNA surveys, especially in unknown biodiversity regions (e.g., biodiversity hotspots or developing countries). If rudeness or copying others' methods can cause significant errors in fish biomonitoring datasets, this is also the design intention and core purpose of our current study, rather than determining which one or two primer pairs are more suitable for fish eDNA biomonitoring, as no single or few primer pairs can be applied to all ecosystems. In addition, all primer pairs analyzed in this study have their own advantages and disadvantages, but they may also complement each other. Multiple primer pairs should be considered to increase species detection probability in an unknown or unexplored ecosystem. With the decrease in sequencing costs, optimized multi-primer methods should gradually become the standard for future eDNA research.

Supplementary Information

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Additional file 1: Figure S1. The proportion of eDNA sequences annotated (Success, blue box) and unassigned (Failure, grey box) into fish communities in 15 groups of three DNA polymerases and five primer pairs in surface water (**a**) and sediment samples (**b**). Prefixes T-, R- and P- in the abscissa are the Taq master polymerase, the Rapid Taq polymerase and the Phanta Flash polymerase, respectively. **Figure S2.** The proportional of fish eDNA sequences at the order level among three repeated samples in surface water samples. Prefixes T-, R- and P- in the abscissa are the Taq master polymerase, the Rapid Taq polymerase and the Phanta Flash polymerase, respectively. **Figure S3.** The proportional of fish eDNA sequences at the order level among three repeated samples in sediment samples. Prefixes T-, R- and P- in the abscissa are the Taq master polymerase, the Rapid Taq polymerase and the Phanta Flash polymerase, respectively**. Table S1.** Summary of 10 primer pairs in the mitochondrial 12 s rRNA gene region for fish eDNA biomonitoring retrieved from the literature and analyzed in this study, including primer name, target group, amplicon size, original references and primer sequences. **Table S2.** Summary of the reaction conditions for PCR assays on random groups of retrieved 10 primer pairs and 3 DNA polymerases, including the set time and temperature of the denaturation, annealing, and extension processes. All PCR assays run 35 cycles. **Table S3.** Results of successful PCR assays between primer pairs and DNA polymerase, namely, the agarose gel electrophoresis have specific bands and correct amplification size. The symbols "√" and "×" represent successful and failed PCR assays, respectively. **Table S4.** The number and proportion of eDNA sequences successfully annotated different taxonomic classifications of fish communities in surface water samples. Three replicates in each group are analyzed together, and percentages in parentheses are the After quality filtering/All sequences, Class seq/After quality filtering, Order-seq/After quality filtering and so on. **Table S5.** The number and proportion of eDNA sequences successfully annotated different taxonomic classifications of fish communities in sediment samples. Three replicates in each group are analyzed together, and percentages in parentheses are the After quality filtering/All sequences, Class seq/After quality filtering, Order-seq/After quality filtering and so on. **Table S6.** Comparison between historical records of common fish species in Dianchi Lake over the past decade and eDNA data in this current study. Blue shadows represent consistency between each other.

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Author contributions
XM and FL wrote the main manuscript text. FG, XZ and FZ reviewed the manuscript. FL and YZ supported the project.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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