

Brief Report

# A Comparative Evaluation of eDNA Metabarcoding Primers in Fish Community Monitoring in the East Lake

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**Abstract:** East Lake in Wuhan, China, harbors a high number of freshwater fish species of great conservation value, concurrently serving as vital resources for local livelihoods. However, the ecosystem is threatened by an array of anthropogenic activities, thus requiring consistent monitoring of the local fish community to enable more efficacious conservation management. In place of conventional surveying methods, we undertook the first analysis of the fish distribution within East Lake via metabarcoding of environmental DNA (eDNA). The accuracy and efficacy of eDNA metabarcoding rely heavily upon selecting an appropriate primer set for PCR amplification. Given the varying environmental conditions and taxonomic diversity across distinct study systems, it remains a challenge to propose an optimal genetic marker for universal use. Thus, it becomes necessary to select PCR primers suitable for the composition of fish in the East Lake. Here, we evaluated the performance of two primer sets, Mifish-U and Metafish, designed to amplify 12S rRNA barcoding genes in fishes. Our results detected a total of 116 taxonomic units and 51 fish species, with beta diversity analysis indicating significant differences in community structure diversity between the six sampling locations encompassing East Lake. While it was difficult to accurately compare the species-level discriminatory power and amplification bias of the two primers, Mifish outperformed Metafish in terms of taxonomic specificity for fish taxa and reproducibility. These findings will assist with primer selection for eDNA-based fish monitoring and biodiversity conservation in the East Lake and other freshwater ecosystems.

**Keywords:** environmental DNA; metabarcoding; fish biodiversity; East Lake



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## 1. Introduction

East Lake, also known as Donghu, is a large, shallow freshwater lake located in Wuhan, China, that previously held the record as the largest urban lake in the country [1]. The lake has been intensively utilized for fish production, with silver carp (*Hypophthalmichthys molitrix*) and bighead carp (*H. nobilis*) introduced as the main species for fish stocking [2]. Yet, akin to other global freshwater and marine habitats, fish biodiversity and population in East Lake face a multitude of anthropogenic challenges, such as habitat disruption, overexploitation, climate change, pollution, infectious diseases, and foreign species invasion [3]. Located in the heart of the Yangtze River Delta, one of China's most prosperous regions, East Lake previously encountered severe environmental issues. This included lake eutrophication triggered by substantial domestic sewage, as well as oil waste from the catering industry and the discharge of industrial and agricultural pollutants over recent decades [4–6]. Nevertheless, since the early 2000s, the local government of Wuhan has vigorously promoted the sustainable development of the ecological environment. The establishment of the 3367-hectare East Lake National Wetland Park has curtailed any potential

lake-contaminating sewage intake and has concurrently planted contamination-absorbing aquatic vegetation, steadily restoring the previously ecologically compromised habitat. With the ongoing progress in East Lake's wetland conservation and ecological restoration, it is crucial to frequently and accurately evaluate the local fish community to facilitate effective fish conservation management [7,8].

Nonetheless, conducting regular monitoring of vast aquatic ecosystems, such as lakes, rivers, and reservoirs, poses significant difficulties. This is primarily due to the labor-intensive nature of fieldwork, which becomes increasingly complex when managing multiple sites and equipment. Additionally, the process can be intrusive to the biological communities being studied. Furthermore, a considerable lack of taxonomic expertise is presently available, which is necessary for accurate identification and assessment [9–11]. Furthermore, these approaches are hampered by systematic sampling bias, limitations in morphological identification, and an increased risk of false-negative results, leading to underestimations of species diversity [12–14]. In contrast, environmental DNA (eDNA)-based approaches offer non-invasive, efficient, and economical alternatives for characterizing marine and freshwater biodiversity [15].

Environmental DNA (eDNA)-based approaches are emerging as a tool for characterizing marine and freshwater biodiversity that can complement traditional surveys. eDNA denotes a composite of DNA molecules shed into the environment by organisms, primarily via their skin, saliva, and secretions, which are widely distributed in various environmental media, such as water, soil, sediment, and air [16]. The eDNA metabarcoding refers to a rapidly emerging tool for biomonitoring that involves direct extraction of total DNA from environmental samples, followed by Polymerase Chain Reaction (PCR) amplification using primers designed to amplify a barcoding gene (i.e., COI, 16S, 18S) across a specific taxonomic group, and subsequent identification of target species sequences through sequencing and bioinformatics analyses [13]. Several research endeavors have corroborated the efficacy of eDNA metabarcoding through High-Throughput Sequencing, demonstrating that it yields equivalent or superior species richness and uncovers biodiversity at a significantly lower cost compared to traditional surveys. Consequently, this technology promises immense potential as a complementary instrument for established monitoring methodologies in the realm of aquatic species ecology and conservation [17–19].

Given the relatively novel and rapidly evolving nature of the eDNA metabarcoding, numerous aspects of this technology remain to be validated and adapted for specific study systems, including the development of PCR primer pairs for DNA amplification, which is a crucial step in the process [20,21]. Universal primers target organisms that share close taxonomic relations and, therefore, possess conserved primer binding sequences, while amplified barcodes should encompass variable sites among distinct species for taxonomic classification [11]. Ideally, a well-designed universal PCR primer pair should fulfill the subsequent requirements: (1) exhibit high specificity and coverage for the target taxa (e.g., fishes); (2) ensure even amplification across species without PCR dropouts; and (3) demonstrate high discriminatory power for unambiguous taxonomic assignment [22–25]. Considering the trace amounts of highly degraded DNA from the study organisms in environmental samples, a small barcode size (usually <200 bp) is recommended for higher PCR success rates [11,26]. Furthermore, effective biodiversity analyses rely on comprehensive, accurate, and relevant reference databases to avoid limitations in taxonomic assignment for species without available information [27].

Generally, mitochondrial genes serve as standard markers for metabarcoding due to their taxonomic discriminatory power, abundant copies in cells shed by organisms, and slower degradation rates compared to nuclear genes [28]. Previous eDNA metabarcoding studies focusing on fish in both freshwater and marine environments have targeted mitochondrial cytochrome B (*cytb*), cytochrome oxidase subunit I (COI), 12S rRNA, and 16S rRNA genes [29]. Several of the most used primer pairs, including 12S-V5 (ca. 106 bp) [30,31], MiFish-U (ca. 170 bp) [23], and Teleo (ca. 65 bp) [19], target various regions of the 12S rRNA sequence. Nonetheless, although numerous research teams have designed

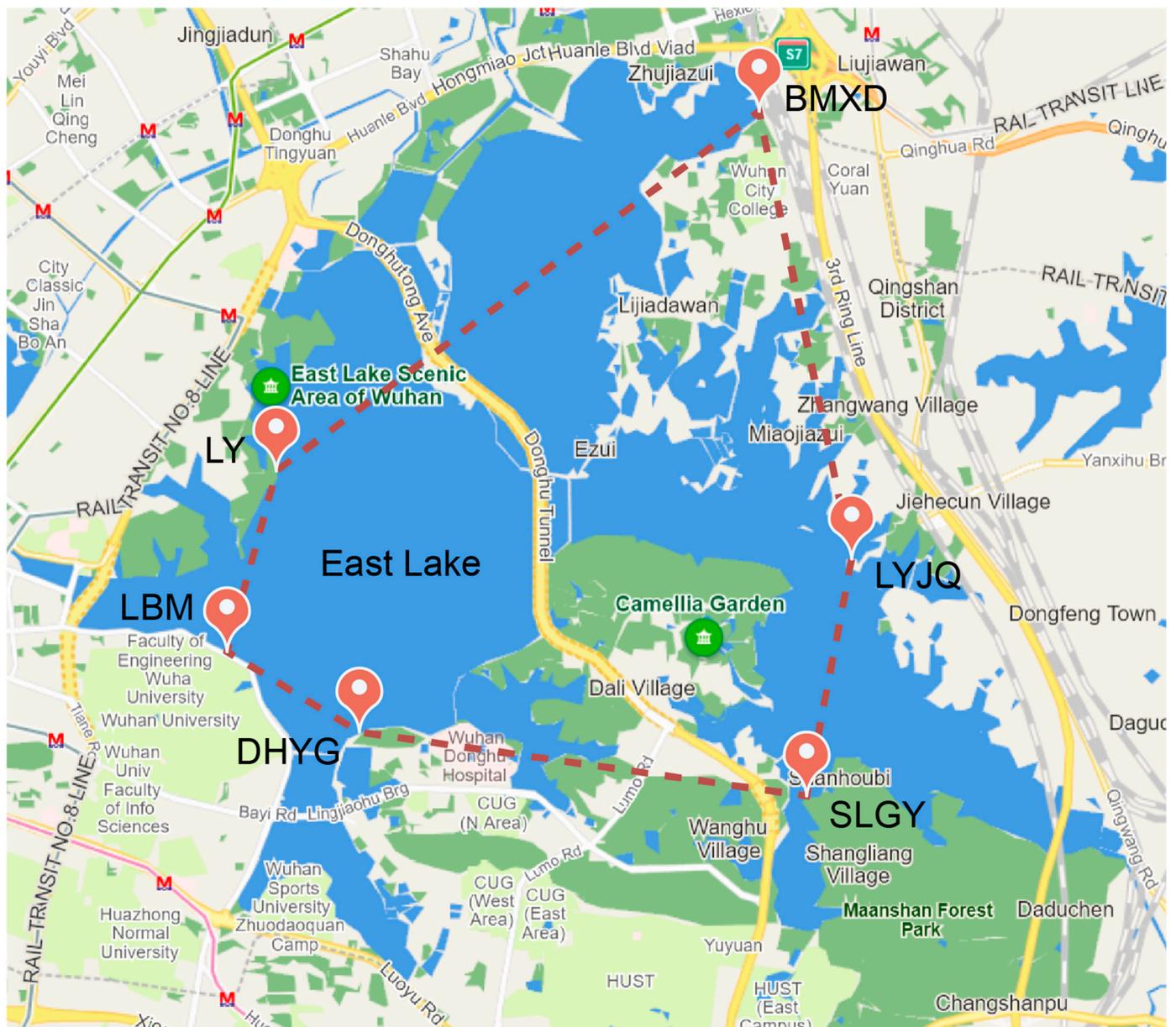
versatile primers for fish community assessments, all of which effectively illustrate regional fish diversity, comparing the efficiency of these metabarcoding primers across various studies and geographical areas remains challenging. Although some research has assessed the effectiveness of multiple universal primer sets, the majority of these assessments rely solely on *in silico* PCR without subsequent *in vitro* validation [19], which can lead to overly optimistic outcomes [21]. Furthermore, the scarce comparisons involving multiple primers often detect substantial discrepancies in the specificity of amplified taxa and the discrimination power of species, both *in silico* and *in situ* [11,23]. For instance, Zhang et al. [11] demonstrated that among 22 primer sets, the two longest pairs in the 12S region, Ac12S [32] and AcMDB07 [23], exhibited the best performance in terms of fish diversity amplified in China's freshwater river ecosystems. However, the efficiency of eDNA metabarcoding and primer pairs can significantly differ across diverse abiotic and biotic conditions of the studied ecosystems, as well as within species assemblages containing distinct lineage compositions or complexities [22,33]. Consequently, it is difficult to propose an optimal genetic marker or the most appropriate suite of primers for universal use, and for this reason, it remains necessary to select PCR primers for the composition of fish in the specific ecosystem to ensure an effective and accurate assessment of the community of interest [34].

As of now, the fish community of East Lake has yet to be analyzed via the technology of eDNA metabarcoding. It is, therefore, necessary to determine the suitable primers for the fish species in the local ecosystem. This study compares the performances of Mifish Universal Teleost Primers, the most frequently used primer pair so far [35], and Metafish, a new 12S metabarcoding primer set designed by Nanjing University based on the mitochondrial genome of common Chinese fish found in the middle and lower reaches of the Yangtze River [36]. The outcomes of this study will contribute to a better understanding of primer selection for future eDNA-based fish community surveys within the East Lake and other related freshwater systems.

## 2. Methods

### 2.1. Sample Collection

Water sampling was carried out in East Lake (30°32' N, 114°23' E) in June 2023. Sampling was conducted at 6 sites: Lingbo Gate (LBM), Liyuan (LY), Baima Road (BMXD), Luoyan Scenic Spot (LYJQ), Donghu Yangguang (DHYG), Ma'anshan Forest Park (SLGY). The location details of these samples are mapped out in Figure 1. At each site, 3 water samples, except for BMXD, were collected from the surface using a Tri-Mode eDNA Sampler, an equipment developed by the Institute of Hydrobiology that includes a filter head extended for filtration. This device automatically filters water samples until it reaches the maximum loading capacity of the filter membrane, at which point the machine stops filtering automatically. Because BMXD is located within the river bay and is relatively close to the highway interchange, its surrounding environment is complex. In order to minimize the sampling randomness-associated errors, we collected 7 samples at this location. The mean value was used at all sampling points in the subsequent data analysis. The filtered membrane samples were stored on ice and transported to the laboratory at the Institute of Hydrobiology for DNA extraction within 12 h.



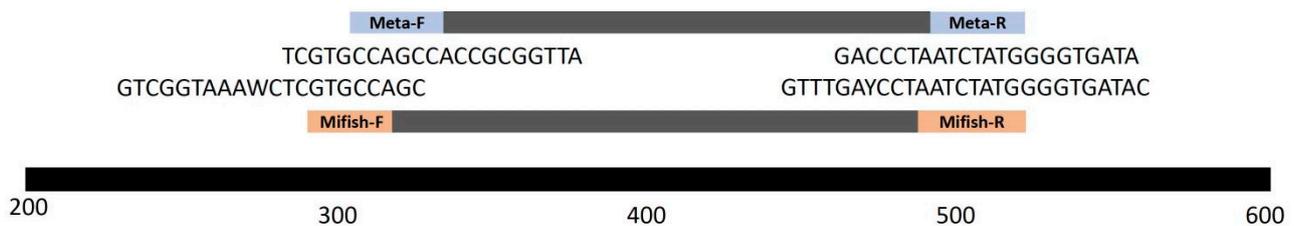
**Figure 1.** Map of East Lake showing the sampling sites (N = 6), including Lingbo Gate (LBM), Liyuan (LY), Baima Road (BMXD), Luoyan Scenic Spot (LYJQ), Donghu Yangguang (DHYG), Ma'anshan Forest Park (SLGY).

## 2.2. Metabarcoding of eDNA Samples

The analysis of our samples was conducted using two universal primer pairs, Mifish and Metafish, to amplify the V5 region of the mitochondrial 12S rRNA gene (Figure 2). eDNA metabarcoding employing the universal MiFish primer pairs has been demonstrated to generate short fragments of fish DNA from various taxa in environmental samples [24]. Metafish was created by Yang et al. [36], and it has been suggested to be included in the group standard of the China Society of Environmental Sciences. DNA extraction from sample filters was performed using the MGIEasy Stool Microbiome DNA Extraction Kit (MGI Tech, Wuhan, China) according to the corresponding Filtered Water Samples user manual. PCRs were performed on eDNA extracts and negative controls (including filtration, extraction, and no-template PCR blanks), each utilizing uniquely tagged barcode primer to facilitate the identification of individual PCR amplicons during the analysis of sequencing data [11]. In this study, the DNA input per sample was 50 ng, using the

ATOPlex MiFish Library Prep Set (MGI Tech, Shenzhen, China) for library preparation. This process involves two-step PCR and two-step purification, where the first-step PCR uses the MiFish PCR Primer Pool to amplify the target fragments, MiFish PCR Primer Pool including region-specific primer (Mifish-U-F: 5'-GTCGGTAAAWCTCGTGCCAGC-3'; Mifish-U-R: 5'-CATAGTGGGGTATCTAATCCYAGTTTG-3'; Metafish-F: 5'-TCGTGCCAGCCACCGCGGTTA-3'; and Metafish-R: 5'-ATAGTGGGGTATCTAATCCCAG-3') and adapter sequence two parts, the first-step PCR reaction volume was 25  $\mu$ L, including 12.5  $\mu$ L of PCR Enzyme Mix, 0.5  $\mu$ L of PCR Clean Enzyme, 2  $\mu$ L of MiFish PCR Primer Pool. The thermal cycling PCR process uses 95  $^{\circ}$ C for denaturation, 65  $^{\circ}$ C for annealing and 30 cycles for amplification. The second-step PCR was performed to add a sequencing adapter and barcode primer, and the operation procedure was performed according to the corresponding reagent kit instructions. Both step PCR products were purified using DNA cleaning beads. After purification, the Agilent 2100 Bioanalyzer (Agilent Technologies, Shanghai, China) was used to detect the fragment size. The results showed that the main peak size of the MiFish primer library product fragment was within the range of 350 bp  $\pm$  10 bp, while the main peak size of the Metafish primer library product fragment was within the range of 330 bp  $\pm$  10 bp. Library construction and sequencing were carried out by the Laboratory of MGI Tech in Wuhan, China, using 2  $\times$  150 bp paired-end sequencing on the DNBSEQ-G99 platform (MGI Tech, Wuhan, China).

#### 12S rRNA



**Figure 2.** Locations of the two fish metabarcoding primer pairs and amplicons on the 12S rRNA mitochondrial gene. Gene sequences of the grass carp (*Ctenopharyngodon idella*; GenBank Acc. No. MG827396.1) were used as templates. Note that amplicon sizes of the primer sets may vary depending on the fish species.

#### 2.3. Bioinformatics and Statistical Analyses

The quality assessment was carried out on paired-end reads in FASTQ format. To analyze the original double C-terminal sequencing data, a sliding window method with a window size of 10 bp was employed. The analysis revealed that the data started to shift at 1 bp from the 5' end of the first base position. A quality score of 30 (Q30) was required in the FASTQ data. The first value was below average quality due to a truncated sequence, which ended at 150 bp. No ambiguous bases (Ns) were allowed.

The sequence analysis was conducted using QIIME2 v2022.8 [37]. Qualified raw sequences were combined and sorted based on index and barcode information, removing barcode sequences in the process. Subsequently, sequences underwent quality control, denoising, combination, and chimera removal using DADA2 v3.16 [38]. Deduplicated sequences generated by DADA2 quality assurance were considered ASVs (amplicon sequence variants) [39]. ASVs are equivalent to OTUs with 100% similarity clustering [40]. ASVs with fewer than 20 reads were filtered out. Taxonomy was assigned using databases downloaded from NCBI (<https://www.ncbi.nlm.nih.gov/> (accessed on 28 February 2021)) and MitoFish (<http://mitofish.aori.u-tokyo.ac.jp> (accessed on 2 August 2022)). The classification of ASVs was carried out using QIIME 2's q2-feature-classifier plugin, setting the sequence similarity threshold at 99% [41]. The taxonomy dataset for each sample was utilized to calculate the observed species and Bray–Curtis indices, which were then employed to create non-metric multidimensional scaling (NMDS) plots using the vegan

2.3\_5 package in the statistical software R version 4.1.3 [42]. Data points were visualized in relation to the used primers and sampling locations. Functional regressions of the data points against each NMDS dimension were performed in MATLAB v2022a to assess the significance of the observed patterns [43]. The Bray–Curtis distance ranges from 0 to 1, with a value of 0 indicating identical community compositions and a value of 1 indicating that the communities have no shared taxa. The correlation between fish communities and sample properties (sampling locations and amplification primers) was calculated using the Mantel test [44]. The significance of the difference between the two sets of data was assessed using a T-test.

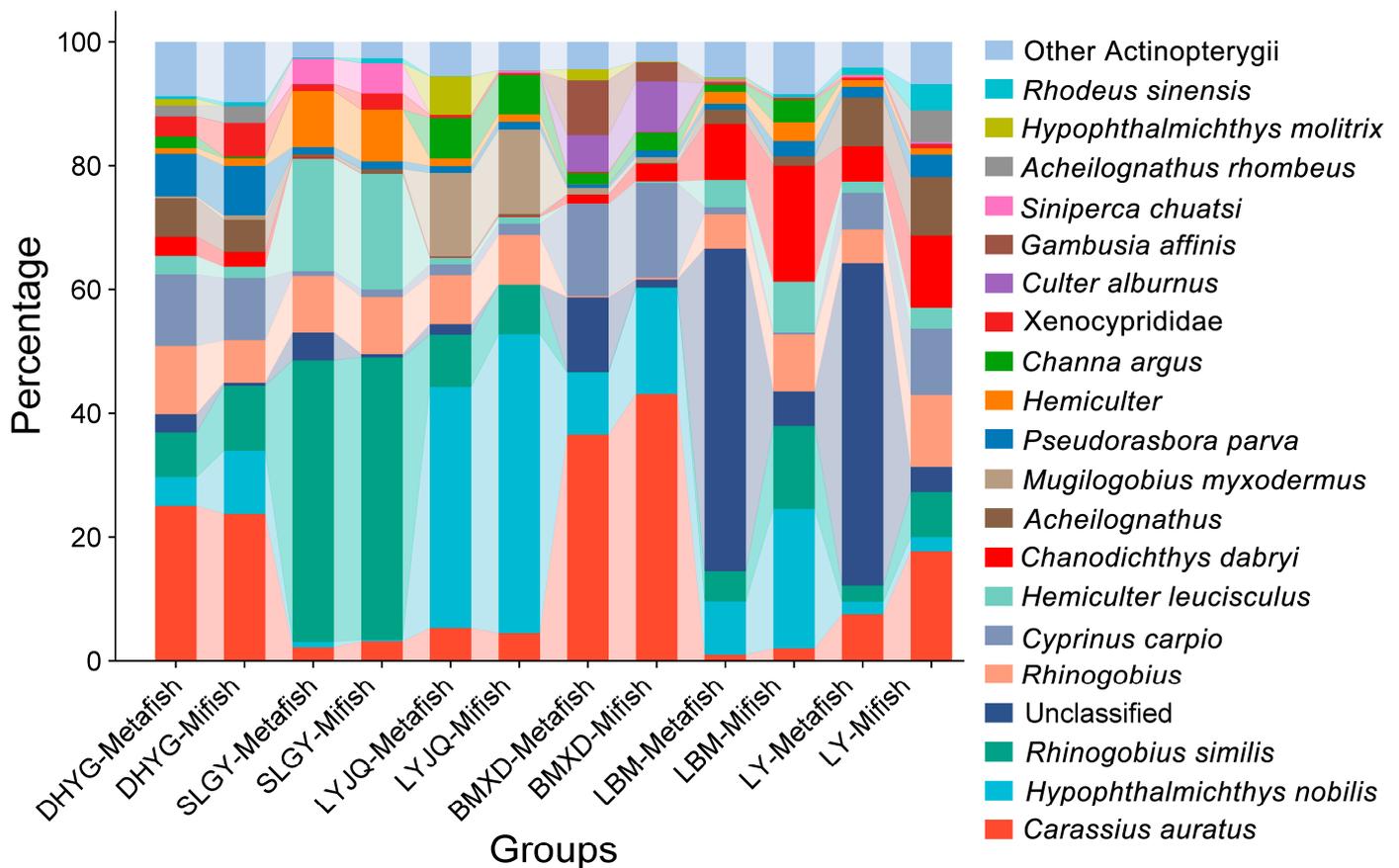
### 3. Results

#### 3.1. Species Composition and Diversity

Following sequence pairing, clustering, and quality filtering steps, 489,089–1,374,792 total sequence reads were retained for each library. A total of 72 taxonomic units were identified from six sampling sites (Tables 1 and S1), with no difference in the total number of detected species between the two sets of primers for the same location (Figure S1). Detected taxa encompassed 51 fish species, 36 genera, and 16 families, with high percentages of Xenocyprididae, Cyprinidae, Oxudercidae, Gobiionidae, Channidae, and Poeciliidae. An analysis by taxonomic order revealed that Cypriniformes consistently accounted for the largest portions of fish taxa for both primers (30.4–91.1%), and Gobiiformes was overall the second most abundant order. Other frequently detected orders included Anabantiformes, Cyprinodontiformes, and Perciformes. The 10 most common species detected in East Lake were *Carassius auratus*, *H. nobilis*, *Rhinogobius similis*, *Cyprinus carpio*, *Hemiculter leucisculus*, *Chanodichthys dabryi*, *Pseudorasbora parva*, *Mugilogobius myxodermus*, *Channa argus*, *Culter alburnus*. Certain invasive species, like *Gambusia affinis*, were also detected. The dominant species in the basin, alongside smaller-sized fish like *Rhinogobius cliffordpopei*, also comprise economically valuable fish, such as *C. carpio*, *H. nobilis*, and *C. auratus* (Figure 3).

**Table 1.** Table of the number of species, genera, and families detected by Metafish and Mifish at each sampling site and in total.

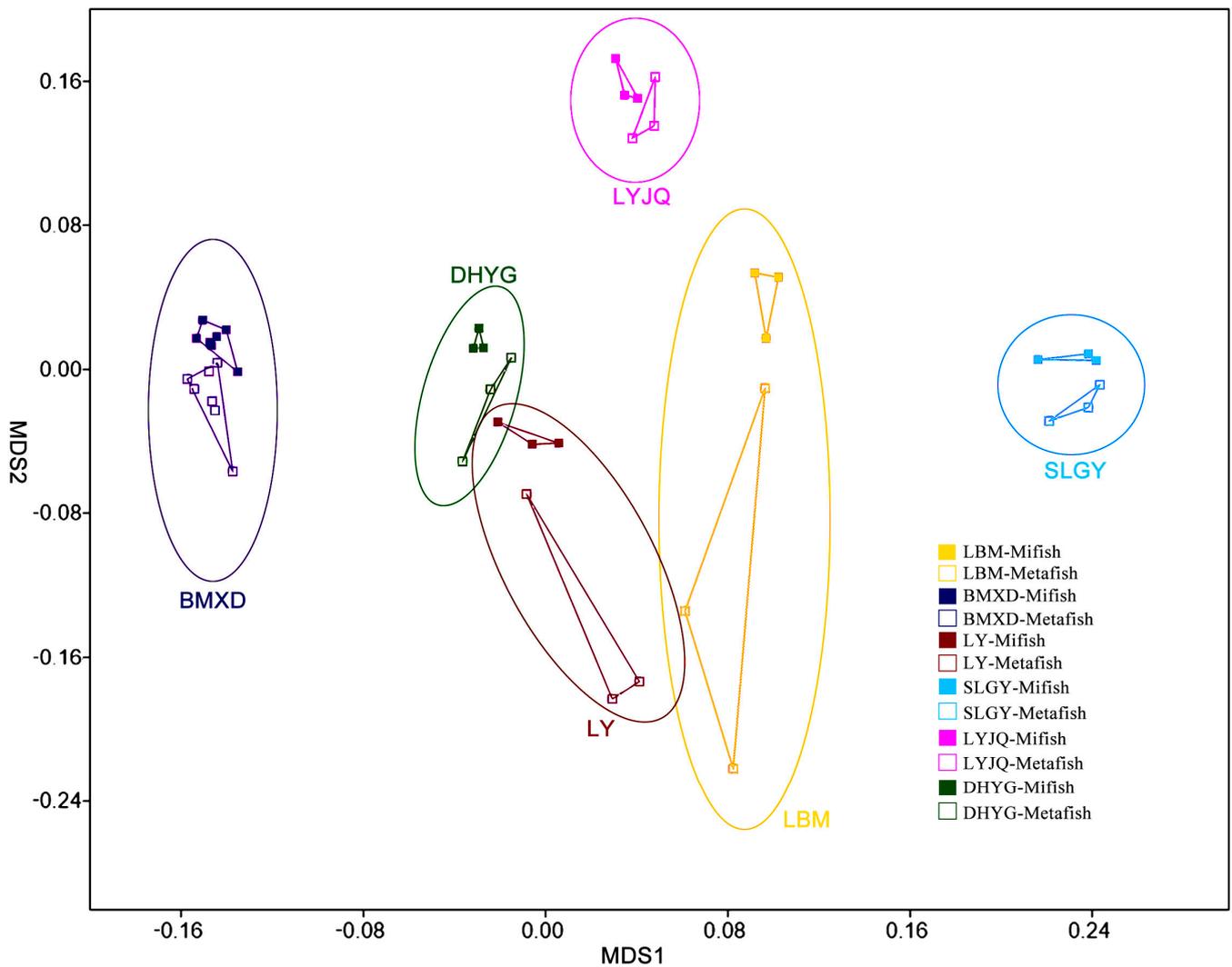
Location-Primer	Species	Genus	Family
DHYG-Metafish	29	8	4
SLGY-Metafish	22	6	2
LYJQ-Metafish	30	7	2
BMXD-Metafish	20	5	1
LBM-Metafish	23	9	3
LY-Metafish	25	10	1
DHYG-Mifish	30	11	2
SLGY-Mifish	18	7	2
LYJQ-Mifish	28	7	2
BMXD-Mifish	22	7	1
LBM-Mifish	21	10	2
LY-Mifish	24	10	2
Total-Metafish	44	32	14
Total-Mifish	45	34	15
Total-Metafish and Mifish	51	36	16



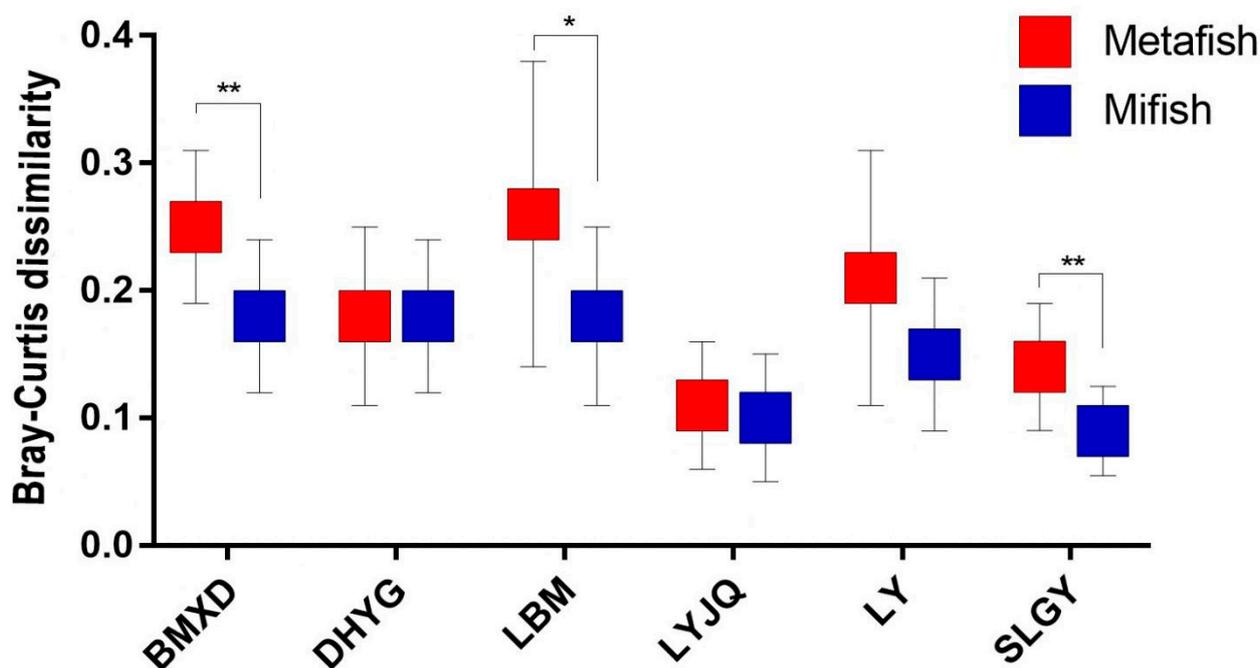
**Figure 3.** Bar plots showing average taxonomic distributions of amplified sequences for the six different locations and two metabarcoding primer sets.

### 3.2. Community Diversity

The NMDS plots revealed that fish communities at the different sampling sites have different fish compositions and that communities revealed by both markers in most locations are more similar between them than between sampling sites (Figure 4). A regression of the Bray–Curtis data points against the NMDS axes revealed a significant relationship with dimension 1 ( $R^2 = 0.563$ ,  $p < 0.001$ ) but less so with dimension 2 ( $R^2 = 0.210$ ,  $p = 0.028$ ). To evaluate the impact of sampling location and amplification primers on the fish community, the Mantel test (i.e., Bray–Curtis distance) was performed. The results showed that the dissimilarity of fish communities was strongly correlated with sampling locations ( $r = 0.653$ ,  $p < 0.001$ ) and amplification primers ( $r = 0.167$ ,  $p < 0.05$ ). This result indicated that both sampling locations and amplification primers had a significant influence on fish communities, with the former exhibiting a stronger correlation with community structure. The Bray–Curtis dissimilarity among samples from the same location (all six locations) was lower when amplified with Mifish primers than that amplified with Metafish primers, with three locations (BMXD, LBM, and SLGY) showing significant difference with  $p$ -value  $< 0.05$  (Figure 5).



**Figure 4.** NMDS plots of eDNA data based on Bray–Curtis indices (stress = 0.181). Bray–Curtis indices are based on log-transformed data. The filled squares indicate samples amplified with “Mifish” primer, and the hollow squares indicate samples amplified with “Metafish” primer. The colors indicate sampling locations.



**Figure 5.** The Bray–Curtis dissimilarity among sub-samples of a sampling location. The symbol “\*\*\*” indicates a significant difference as  $p < 0.01$ , “\*\*” indicates  $p < 0.05$ .

### 3.3. Species Distribution by Primers

There were distinctions in taxonomic identity and relative abundance of taxa between primer pairs. Notably, for samples collected at LBM and LY, Metafish detected a considerably higher percentage of unclassified non-fish taxa (52.7% and 53.5%, respectively) compared to Mifish primers (5.44% and 4.38%, respectively). Several dominant fish species were detected with differing relative read proportions between the two primer sets, including *C. auratus* (17.5% for Mifish and 7.28% for Metafish in LY samples); *H. nobilis* (22.1% for Mifish and 8.13% for Metafish in LBM samples); *R. similis* (13.1% for Mifish and 4.68% for Metafish in LBM samples); *C. dabryi* (19.2% for Mifish and 8.69% for Metafish in LBM samples); and *H. molitrix* (0.04% for Mifish and 6.26% for Metafish in LYJQ samples). Some fish species were also detected by only one primer set at a low relative frequency (<1%) but were absent entirely from the other primer set in certain sampling sites, such as *C. dabryi* (detected only by Metafish for SLGY); *G. affinis* (detected only by Metafish for LBM); *C. alburnus* (detected only by Metafish for SLGY and by Mifish for LY and LYJQ); and *Acheilognathus rhombeus* (detected only by Mifish for LBM and BMXD and by Metafish for SLGY). In addition, certain fish species were exclusively detected by only one of the primer sets across all sampling sites. Fish species observed only by Mifish include *Saurogobio xiangjiangensis* (native), *Saurogobio lissilabris* (native), *Paramisgurnus dabryanus* (native), *Misgurnus bipartitus* (native), *Pagellus bellottii* (invasive), *Coptodo zillii* (invasive), and *Gambusia holbrooki* (invasive). On the other hand, fish species that were detected only by Metafish include *Mystacoleucus marginatus* (native), *Misgurnus anguillicaudatus* (native), *Hyporhamphus intermedius* (native), *Hemibarbus barbus* (native), *Carassius carassius* (native), and *Acheilognathus tonkinensis* (native) (Table 2). In total, Mifish and Metafish detected similar numbers of total fish species (45 vs. 44) (Table 1).

**Table 2.** Table of the relative frequency of fish species that vary across sampling sites or by primer choice.

Species	DHYG-Metafish	SLGY-Metafish	LYJQ-Metafish	BMXD-Metafish	LBM-Metafish	LY-Metafish	DHYG-Mifish	SLGY-Mifish	LYJQ-Mifish	BMXD-Mifish	LBM-Mifish	LY-Mifish
Unclassified	3.0	4.5	1.7	12.1	52.1	52.1	0.5	0.5	<0.1	1.3	5.6	4.1
<i>Acheilognathus rhombeus</i>	1.7	<0.1	<0.1	0	0	<0.1	2.7	0	<0.1	<0.1	<0.1	5.1
<i>Acheilognathus tonkinensis</i>	<0.1	0	0	0	0	<0.1	0	0	0	0	0	0
<i>Culter alburnus</i>	0	<0.1	0	5.9	0	0	0	0	<0.1	8.2	0	<0.1
<i>Carassius auratus</i>	25.1	2.2	5.3	36.6	1.0	7.6	23.8	3.2	4.5	43.1	2.0	17.7
<i>Carassius carassius</i>	0	0	0	0	<0.1	0	0	0	0	0	0	0
<i>Channa argus</i>	1.6	0	6.6	1.8	1.2	0	0.2	0	6.4	1.8	3.6	0
<i>Chanodichthys dabryi</i>	3.1	0.2	<0.1	1.5	9.1	5.7	2.4	0	0.13	2.8	18.8	11.7
<i>Coptodon zillii</i>	0	0	0	0	0	0	<0.1	<0.1	0	<0.1	<0.1	<0.1
<i>Gambusia affinis</i>	0	0	0	9.0	0.3	0	0	0	0	3.0	0	0
<i>Gambusia holbrooki</i>	0	0	0	0	0	0	0	0	0	<0.1	0	0
<i>Hemibarbus barbus</i>	0	0	<0.1	0	0	0	0	0	0	0	0	0
<i>Hypophthalmichthys molitrix</i>	1.1	0	6.3	1.9	0.4	<0.1	<0.1	0	<0.1	0.2	<0.1	<0.1
<i>Hypophthalmichthys nobilis</i>	5.1	0.9	38.7	9.6	8.1	1.9	10.2	0.24	48.2	16.9	22.1	2.4
<i>Hyporhamphus intermedius</i>	0	0	<0.1	0	0	0	0	0	0	0	0	0
<i>Misgurnus anguillicaudatus</i>	0	0	<0.1	0	0	0	0	0	0	0	0	0
<i>Misgurnus bipartitus</i>	0	0	0	0	0	0	0	0	<0.1	0	0	0
<i>Mystacoleucus marginatus</i>	<0.1	0	0	0	0	0	0	0	0	0	0	0
<i>Pagellus bellottii</i>	0	0	0	0	0	0	<0.1	0	0	0	0	0
<i>Paramisgurnus dabryanus</i>	0	0	0	0	0	0	<0.1	0	<0.1	0	0	0
<i>Rhinogobius similis</i>	7.6	45.6	8.2	<0.1	4.7	2.5	10.5	45.5	7.9	<0.1	13.1	7.2
<i>Sarcocheilichthys sinensis</i>	0	0	<0.1	0	0	0	0.3	0	0	0	0	0
<i>Saurogobio lissilabris</i>	0	0	0	0	0	0	<0.1	0	0	0	0	0
<i>Saurogobio xiangiangensis</i>	0	0	0	0	0	0	<0.1	0	0	0	0	0

### 3.4. Species Distribution by Location

Several dominant fish species displayed substantial variation in relative read abundances across different sampling sites or were prominently present in one specific location. For instance, *C. auratus* accounted for 36.6–43.5% of relative read frequency for BMXD but less than 8% for LBM, SLGY, and LYJQ; *R. similis* represented 45% of detected fish for SLGY but less than 0.2% for BMXD; *H. nobilis* contributed to 38.7–48.2% for relative frequency for LYJQ but less than 5% for SLGY and LY. Moreover, fish species of the Oxudercidae family accounted for significant percentages for SLGY (54.9%) but less than 1.5% in BMXD. Conversely, fish species of the Cyprinidae family were found to have higher relative frequencies (52.3–60.1%) for BMXD but less than 7% for SLGY (Table 2). Overall, the highest number of total fish taxa detected by Metafish and Mifish was from samples collected at DHYG (43 and 46, respectively), while the fewest taxa units were found at SLGY and BMXD (32 and 28; 28 and 32, respectively) (Figure S1).

## 4. Discussion

In this research, we explored the possibility of utilizing eDNA-based monitoring methods to assess freshwater fish communities in East Lake. Repeated monitoring is essential for safeguarding these populations and addressing the anthropogenic pressures these habitats are subjected to. We employed two 12S primer sets, which have been proven to outperform other gene region-targeting assays in evaluating fish communities [11,23,45]. Although the two primer sets produced similar results in some aspects (such as taxonomic coverage and community characterization), the Mifish primer demonstrated superior taxonomic specificity and reproducibility compared to the Metafish primer.

High taxonomic specificity for the target taxonomic group is a crucial consideration when choosing metabarcoding primers. Insufficient specificity for the target group can

lead to the excessive amplification of non-target sequences, causing the desired taxa to be overwhelmed and resulting in inefficient utilization of sequencing resources. Our research demonstrates that both primers amplified sequences of non-fish organisms at all sampling sites. However, for LBM and LY sampling sites, Metafish primers detected significantly higher relative proportions of unclassified taxa (52.7% and 53.5%, respectively) compared to Mifish primers (5.4% and 4.4%). This discrepancy suggests that the Mifish primer offers a higher degree of specificity for fish taxa, making it a more suitable choice for fish community assessments.

The high read abundances of unclassified taxa from water samples collected at LBM and LY could potentially be attributed to the increased level of human activity present at these sites: LBM is situated at Wuhan University, while LY encompasses the Liyuan Hospital. As a result, the extracted eDNA cannot avoid being contaminated with various human DNA-containing waste products. The amplification of human sequences using metabarcoding primers from environmental samples has been previously reported [23,45]. Efforts have been made to reduce human DNA amplification by employing general fish primers in combination with a blocking oligonucleotide. However, Zhang et al. [11] demonstrated that blocking oligos might also prevent primers from binding to or amplifying certain desired fish sequences. Therefore, they suggested increasing sequencing depth as a preferred alternative to compensate for non-target amplification.

In addition to having a high taxonomic specificity for the target species, it is essential to have broad taxonomic coverage within the target group and a high ability to assign species levels for generating comprehensive and accurate biodiversity data using metabarcoding primers alone detected four native and three invasive fish species, while the Metafish primers uniquely detected six native fish species. Thus, both primers showed similar performance in our study system regarding taxonomic coverage and species-level assignment (45 for Mifish and 44 for Metafish). This consistency is probably due to the comparable barcode size and significant overlap in the barcode sequence of the two primers. As shown in Figure 2 in our result, Metafish is essentially a modified version of Mifish, with the reverse barcode sequence of the Metafish primer entirely encompassed by that of the Mifish primer set.

Regarding the amplification bias of the two primers, several dominant fish species displayed substantial variation in the relative frequency of taxa between assays amplified by these primers. Prior studies have suggested a positive correlation between taxa relative abundance or biomass and taxa sequence counts [46–48], which supports the potential of using metabarcoding sequencing data for multispecies quantitative estimations. Sequence read counts cannot be relied upon as an accurate measure of fish abundance or biomass, as they are prone to biases originating from multiple factors such as sampling methods, laboratory procedures, and analytical stages (including amplification, sequencing, and bioinformatics), apart from the source and fate of species eDNA in various environments [48,49]. Moreover, the presence of unclassified taxa, particularly at high percentages in samples collected at LBM and LY, complicates the comparison of Mifish and Metafish primer performance in terms of even amplification across species. Given that most of the differences in the relative abundance of dominant fish taxa were identified among the LBM and LY samples, it is even more difficult to ascertain which designed universal PCR primer has unbiasedly amplified 12S gene fragments across the target taxa without PCR dropouts. Consequently, it becomes challenging to accurately compare the species distribution across sampling locations, particularly for fish species detected with very low relative frequency by both primers. Ushio et al. [48] suggested incorporating an internal standard DNA (i.e., a known copy number of short DNA fragments from non-target species) into eDNA samples to detect potential biases within metabarcoding data for quantitative fish eDNA analysis. This method was effectively employed by Stoeckle et al. [50], who discovered that Riaz 12S gene metabarcoding with an internal DNA standard can quantify marine bony fish eDNA across a range of approximately 10–5000 copies per reaction, indicating no significant PCR bias among teleost species.

Nevertheless, analysis by proportional reads-based Bray–Curtis indices indicated that eDNA amplified by both primer sets generated different profiles of fish diversity for samples collected from different locations. Although both primer choices for amplification and sampling location appeared to contribute to the significant relationship observed between the regression of Bray–Curtis data points and the NMDS axes, the Mantel test revealed that the latter had a stronger correlation with the dissimilarity of fish communities. More importantly, while it was difficult to determine which diversity profile detected by the two primers represented that of East Lake most accurately, the Bray–Curtis dissimilarity among samples from the same location (all six sites) was lower when amplified with Mifish primers compared to Metafish primers. This suggests that Mifish primers possess greater reproducibility than Metafish. However, it is worth noting that unlike observed species, which are based on the presence/absence of taxa, Bray–Curtis indices take into account read abundances, a factor influenced by PCR biases [45] and eDNA dynamics [51].

In the future, to select a suitable primer set for eDNA-based monitoring in East Lake, it is necessary to gain a more comprehensive and comparative evaluation of a wider selection of metabarcoding primers. Since different primers may have varying taxonomic ranges in amplification, employing multiple primer sets in combination can enhance taxonomic coverage and species discriminatory power [32]. For instance, as proposed by Zhang et al. [11], besides primers aimed at Actinopterygii species, those effective in amplifying Chondrichthyes species from environmental samples should also be included. Moreover, the success of metabarcoding applications in recovering biodiversity and assigning accurate taxonomy depends on the comprehensiveness and sequence quality of corresponding reference databases. Therefore, constructing high-quality reference databases for local biological communities should be a priority in DNA-based biodiversity monitoring. The combined use of both local and global databases can increase the detection probability of native, invasive, and rare species.

## 5. Conclusions

In this study, we examined the primer performance of Mifish-U and Metafish in assessing fish eDNA composition of samples collected from 6 different locations across East Lake. While it was difficult to compare the species-level discriminatory power and even amplification of the two primers, our results revealed that Mifish outperformed Metafish in terms of taxonomic specificity and reproducibility. These findings will contribute to the usage of eDNA technology in future fish biodiversity assessments, particularly for other lakes in the Yangtze basin. However, it is crucial to remember that the community composition and complexity can vary significantly between geographical locations and different ecosystems, which may imply that the primer performance in this study might not be entirely applicable to other situations.

**Supplementary Materials:** The following are available online at <https://www.mdpi.com/article/10.3390/w16050631/s1>. Figure S1: Barplots showing the number of total taxonomic units detected by both primers at the 6 sampling sites. Darker blue bars represent Metafish, and lighter blue bars represent Mifish. Table S1: The raw counts of each taxonomic distribution in each sample. Table S2: The raw data of average taxonomic distributions of amplified sequences for the six different locations and two metabarcoding primer sets.

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**Data Availability Statement:** All the data required to assess the findings of the paper are provided in the main text and/or the Supplementary Materials. The raw sequencing data associated with this study can be accessed on the NCBI's SRA database under BioProject ID: PRJNA957488 ([www.ncbi.nlm.nih.gov/bioproject/957488](http://www.ncbi.nlm.nih.gov/bioproject/957488) (accessed on 1 November 2023)).

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## References

- Ge, J.; Liu, M.; Yun, X.; Yang, Y.; Zhang, M.; Li, Q.; Wang, J. Occurrence, distribution and seasonal variations of polychlorinated biphenyls and polybrominated diphenyl ethers in surface waters of the East Lake, China. *Chemosphere* **2014**, *103*, 256–262. [[CrossRef](#)]
- Liu, J.; Chen, S. An assessment of the impact of fish stocking on lake eutrophication in China. *SIL Commun.* **1994**, *24*, 231–235. [[CrossRef](#)]
- Yao, M.; Zhang, S.; Lu, Q.; Chen, X.; Zhang, S.Y.; Kong, Y.; Zhao, J. Fishing for Fish Environmental DNA: Ecological Applications, Methodological Considerations, Surveying Designs, and Ways forward. *Mol. Ecol.* **2022**, *31*, 5132–5164. [[CrossRef](#)]
- Gan, Y.; Guo, Y. Evaluation analysis and remedy strategy for eutrophication in Wuhan lake Donghu. *Resour. Environ. Yangtze* **2004**, *13*, 277–281.
- Du, Y.; Xue, H.P.; Wu, S.J.; Ling, F.; Xiao, F.; Wei, X. Lake area changes in the middle Yangtze region of China over the 20th century. *J. Environ. Manag.* **2011**, *92*, 1248–1255. [[CrossRef](#)] [[PubMed](#)]
- Yang, Y.; Yun, X.; Liu, M.; Jiang, Y.; Li, Q.X.; Wang, J. Concentrations, distributions, sources, and risk assessment of organochlorine pesticides in surface water of the East Lake, China. *Environ. Sci. Pollut. Res.* **2014**, *21*, 3041–3050. [[CrossRef](#)]
- Rees, H.C.; Maddison, B.C.; Middleditch, D.J.; Patmore, J.R.M.; Gough, K.C. The detection of aquatic animal species using environmental DNA—A review of eDNA as a survey tool in ecology. *J. Appl. Ecol.* **2014**, *51*, 1450–1459. [[CrossRef](#)]
- Clements, C.F.; Blanchard, J.L.; Nash, K.L.; Hindell, M.A.; Ozgul, A. Body size shifts and early warning signals precede the historic collapse of whale stocks. *Nat. Ecol. Evol.* **2017**, *1*, 188. [[CrossRef](#)]
- Hopkins, G.; Freckleton, R. Declines in the numbers of amateur and professional taxonomists: Implications for conservation. *Anim. Conserv.* **2002**, *5*, 245–249. [[CrossRef](#)]
- Hering, D.; Borja, A.; Jones, J.I.; Pont, D.; Boets, P.; Bouchez, A.; Bruce, K.; Drakare, S.; Hänfling, B.; Kahlert, M.; et al. Implementation options for DNA-based identification into ecological status assessment under the European Water Framework Directive. *Water Resour.* **2018**, *138*, 192–205. [[CrossRef](#)] [[PubMed](#)]
- Zhang, S.; Zhao, J.; Yao, M. A comprehensive and comparative evaluation of primers for metabarcoding eDNA from fish. *Methods Ecol. Evol.* **2020**, *11*, 1609–1625. [[CrossRef](#)]
- Yamamoto, S.; Minami, K.; Fukaya, K.; Takahashi, K.; Sawada, H.; Murakami, H.; Tsuji, S.; Hashizume, H.; Kubonaga, S.; Horiuchi, T.; et al. Environmental DNA as a ‘snapshot’ of fish distribution: A case study of Japanese Jack Mackerel in Maizuru Bay, sea of Japan. *PLoS ONE* **2016**, *11*, e0149786.
- Wang, S.; Yan, Z.; Hänfling, B.; Zheng, X.; Wang, P.; Fan, J.; Li, J. Methodology of fish eDNA and its applications in ecology and environment. *Sci. Total Environ.* **2021**, *755*, 142622. [[CrossRef](#)]
- Burian, A.; Bruce, K.; Tovela, E.; Bakker, J.; Balcells, L.; Bennett, R.; Chordekar, S.; Costa, H.M.; Crampton-Platt, A.; Boer, H.; et al. Merging two eDNA metabarcoding approaches and citizen-science-based sampling to facilitate fish community monitoring along vast Sub-Saharan coastlines. *Mol. Ecol. Resour.* **2023**, *23*, 1641–1655. [[CrossRef](#)] [[PubMed](#)]
- Osathanunkul, M.; Minamoto, T. A molecular survey based on eDNA to assess the presence of a clown featherback (*Chitala ornata*) in a confined environment. *PeerJ* **2020**, *8*, e10338. [[CrossRef](#)] [[PubMed](#)]
- Bohmann, K.; Evans, A.; Gilbert, M.T.P.; Carvalho, G.R.; Creer, S.; Knapp, M.; Yu, D.W.; Bruyn, M. Environmental DNA for wildlife biology and biodiversity monitoring. *Trends Ecol. Evol.* **2014**, *29*, 358–367. [[CrossRef](#)] [[PubMed](#)]
- Port, J.A.; O'Donnell, J.L.; Romero-Maraccini, O.C.; Leary, P.R.; Litvin, S.Y.; Nickols, K.J.; Yamahara, K.M.; Kelly, R.P. Assessing vertebrate biodiversity in a kelp forest ecosystem using environmental DNA. *Mol. Ecol.* **2016**, *25*, 527–541. [[CrossRef](#)]
- Shaw, J.L.A.; Clarke, L.J.; Wedderburn, S.D.; Barnes, T.C.; Weyrich, L.S.; Cooper, A. Comparison of environmental DNA metabarcoding and conventional fish survey methods in a river system. *Biol. Conserv.* **2016**, *197*, 131–138. [[CrossRef](#)]
- Valentini, A.; Taberlet, P.; Miaud, C.; Civade, R.; Herder, J.; Thomsen, P.F.; Bellemain, E.; Besnard, A.; Coissac, E.; Boyer, F.; et al. Next-generation monitoring of aquatic biodiversity using environmental DNA metabarcoding. *Mol. Ecol.* **2016**, *25*, 929–942. [[CrossRef](#)]
- Collins, R.A.; Bakker, J.; Wangensteen, O.S.; Soto, A.Z.; Corrigan, L.; Sims, D.W.; Genner, M.J.; Mariani, S. Non-specific amplification compromises environmental DNA metabarcoding with COI. *Methods Ecol. Evol.* **2019**, *10*, 1985–2001. [[CrossRef](#)]

21. Polanco, A.; Richards, E.; Flück, B.; Valentini, A.; Altermatt, F.; Brosse, S.; Walser, J.C.; Eme, D.; Marques, V.; Manel, S.; et al. Comparing the performance of 12S mitochondrial primers for fish environmental DNA across ecosystems. *Environ. DNA* **2006**, *3*, 1113–1127. [[CrossRef](#)]
22. Clarke, L.J.; Soubrier, J.; Weyrich, L.S.; Cooper, A. Environmental metabarcodes for insects: In silico PCR reveals potential for taxonomic bias. *Mol. Ecol. Resour.* **2014**, *14*, 1160–1170. [[CrossRef](#)] [[PubMed](#)]
23. Miya, M.; Gotoh, R.O.; Sado, T. Mifish metabarcoding: A high-throughput approach for simultaneous detection of multiple fish species from environmental DNA and other samples. *Fish. Sci.* **2020**, *86*, 939–970. [[CrossRef](#)]
24. Bylemans, J.; Gleeson, D.M.; Hardy, C.M.; Furlan, E. Toward an ecoregion scale evaluation of eDNA metabarcoding primers: A case study for the freshwater fish biodiversity of the Murray-Darling Basin (Australia). *Ecol. Evol.* **2018**, *8*, 8697–8712. [[CrossRef](#)] [[PubMed](#)]
25. Wilcox, T.M.; McKelvey, K.S.; Young, M.K.; Jane, S.F.; Lowe, W.H.; Whiteley, A.R.; Schwartz, M.K. Robust detection of rare species using environmental DNA: The importance of primer specificity. *PLoS ONE* **2013**, *8*, e59520. [[CrossRef](#)] [[PubMed](#)]
26. Freeland, J.R. The importance of molecular markers and primer design when characterizing biodiversity from environmental DNA. *Genome* **2017**, *60*, 358–374. [[CrossRef](#)] [[PubMed](#)]
27. Marques, V.; Guérin, P.-É.; Rocle, M.; Valentini, A.; Manel, S.; Mouillot, D.; Dejean, T. Blind assessment of vertebrate taxonomic diversity across spatial scales by clustering environmental DNA metabarcoding sequences. *Ecography* **2020**, *43*, 1779–1790. [[CrossRef](#)]
28. Jo, T.S.; Tsuru, K.; Yamanaka, H. Can nuclear aquatic environmental DNA be a genetic marker for the accurate estimation of a species abundance? *Sci. Nat.* **2022**, *109*, 38. [[CrossRef](#)]
29. Shu, L.; Ludwig, A.; Peng, Z. Environmental DNA metabarcoding primers for freshwater fish detection and quantification: In silico and in tanks. *Ecol. Evol.* **2021**, *11*, 8281–8294. [[CrossRef](#)]
30. Riaz, T.; Shehzad, W.; Viari, A.; Pompanon, F.; Taberlet, P.; Coissac, E. ecoPrimers: Inference of new DNA barcode markers from whole genome sequence analysis. *Nucleic Acids Res.* **2011**, *39*, e145. [[CrossRef](#)]
31. Kelly, R.P.; Port, J.A.; Yamahara, K.M.; Crowder, L.B. Using environmental DNA to census marine fishes in a large mesocosm. *PLoS ONE* **2014**, *9*, e86175. [[CrossRef](#)]
32. Evans, N.T.; Olds, B.P.; Renshaw, M.A.; Turner, C.R.; Li, Y.; Jerde, C.L.; Mahon, A.R.; Pfrender, M.E.; Lamberti, G.A.; Lodge, D.M. Quantification of mesocosm fish and amphibian species diversity via environmental DNA metabarcoding. *Mol. Ecol. Resour.* **2016**, *16*, 29–41. [[CrossRef](#)]
33. Bellemain, E.; Carlsen, T.; Brochmann, C.; Coissac, E.; Taberlet, P.; Kausserud, H. ITS as an environmental DNA barcode for fungi: An in silico approach reveals potential PCR biases. *BMC Microbiol.* **2010**, *10*, 189. [[CrossRef](#)]
34. García-Machado, E.; Normandeau, E.; Côté, G.; Bernatchez, L. How eDNA data filtration, sequence coverage, and primer selection influence assessment of fish communities in northern temperate lakes. *Environ. DNA* **2023**, *5*, 1216–1233. [[CrossRef](#)]
35. Xiong, F.; Shu, L.; Zeng, H.; Gan, X.; He, S.; Peng, Z. Methodology for fish biodiversity monitoring with environmental DNA metabarcoding: The primers, databases and bioinformatic pipelines. *Water Biol. Secur.* **2022**, *1*, 100007. [[CrossRef](#)]
36. Yang, J.; Zhang, X.W. Universal Metabarcoding Amplification Primers for Freshwater Fish Mitochondria 12S and Application Method Thereof. China Patent CN109943645B, 14 April 2023.
37. Bolyen, E.; Rideout, J.R.; Dillon, M.R.; Bokulich, N.A.; Abnet, C.C.; Al-Ghalith, G.A.; Alexander, H.; Alm, E.J.; Arumugam, M.; Asnicar, F.; et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* **2019**, *37*, 852–857. [[CrossRef](#)]
38. Callahan, B.J.; McMurdie, P.J.; Holmes, S.P. Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. *ISME J.* **2017**, *11*, 2639–2643. [[CrossRef](#)]
39. Katoh, K.; Misawa, K.; Kuma, K.; Miyata, T. MAFFT: A novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Res.* **2002**, *30*, 3059–3066. [[CrossRef](#)]
40. McDonald, D.; Price, M.N.; Goodrich, J.; Nawrocki, E.P.; DeSantis, T.Z.; Probst, A.; Andersen, G.L.; Knight, R.; Hugenholtz, P. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *ISME J.* **2012**, *6*, 610–618. [[CrossRef](#)]
41. Bokulich, N.A.; Kaehler, B.D.; Rideout, J.R.; Dillon, M.; Bolyen, E.; Knight, R.; Huttley, G.A.; Caporaso, J.G. Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. *Microbiome* **2018**, *6*, 90. [[CrossRef](#)]
42. Oksanen, J.; Simpson, G.L.; Blanchet, F.G.; Kindt, R.; Legendre, P.; Minchin, P.R.; O'Hara, R.B.; Solymos, P.; Steven, M.H.H.; Szoecs, E.; et al. *Vegan: Community Ecology Package*, R package version 2.4-3; R Foundation for Statistical Computing: Vienna, Austria, 2016.
43. Ricker, W.E. Linear Regressions in Fishery Research. *J. Fish. Res. Board Can.* **1973**, *30*, 409–434. [[CrossRef](#)]
44. Goslee, S.C.; Urban, D.L. The ecodist package for dissimilarity-based analysis of ecological data. *J. Stat. Softw.* **2007**, *22*, 7. [[CrossRef](#)]
45. Kelly, R.P.; Gallego, R.; Jacobs-Palmer, E. The effect of tides on nearshore environmental DNA. *PeerJ* **2018**, *6*, e4521. [[CrossRef](#)] [[PubMed](#)]
46. Evans, N.T.; Lamberti, G.A. Freshwater fisheries assessment using environmental DNA: A primer on the method, its potential, and shortcomings as a conservation tool. *Fish. Res.* **2018**, *197*, 60–66. [[CrossRef](#)]

47. Klobucar, S.L.; Rodgers, T.W.; Budy, P. At the forefront: Evidence of the applicability of using environmental DNA to quantify the abundance of fish populations in natural lentic waters with additional sampling considerations. *Can. J. Fish. Aquat. Sci.* **2017**, *74*, 2030–2034. [[CrossRef](#)]
48. Ushio, M.; Murakami, H.; Masuda, R.; Sado, T.; Miya, M.; Sakurai, S.; Yamanaka, H.; Minamoto, T.; Kondoh, M. Quantitative monitoring of multispecies fish environmental DNA using high-throughput sequencing. *Metabarcoding Metagenom.* **2018**, *2*, e23297.
49. Lacoursiere-Roussel, A.; Deiner, K. Environmental DNA is not the tool by itself. *J. Fish Biol.* **2019**, *98*, 383–386. [[CrossRef](#)]
50. Stoeckle, M.Y.; Ausubel, J.H.; Coogan, M. 12S gene metabarcoding with DNA standard quantifies marine bony fish environmental DNA, identifies threshold for reproducible detection, and overcomes distortion due to amplification of non-fish DNA. *Environ. DNA* **2022**, *6*, e376. [[CrossRef](#)]
51. Allan, E.A.; Zhang, W.G.; Lavery, A.C.; Govindarajan, A.F. Environmental DNA shedding and decay rates from diverse animal forms and thermal regimes. *Environ. DNA* **2020**, *3*, 492–514. [[CrossRef](#)]

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