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Environmental DNA and Traditional Approaches for Fish Diversity Assessment in Mesangat Ilir, East Kalimantan, Indonesia

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ABSTRACT

Traditional fish capture methods often provide incomplete data, particularly for species inhabiting hard-to-access environments. Environmental DNA (eDNA) metabarcoding offers a sensitive and non-invasive alternative for species detection. This study integrated traditional morphological surveys and eDNA analysis to obtain a more comprehensive assessment of fish diversity in the Mesangat Ilir River, East Kalimantan. eDNA metabarcoding detected six species, five of which overlapped with the 19 species captured by traditional methods, and one additional species (*Trichopsis vittata*) that was not captured. Based on the combined results of both methods, the family Cyprinidae was dominant (12 species; 60%), followed by Bagridae and Osphronemidae, each represented by two species (10%). Additionally, Cobitidae, Anabantidae, Helostomatidae, and Pristolepidae were each represented by a single species (5%). Therefore, integrating eDNA with traditional surveys is recommended to support long-term conservation strategies for endemic, threatened, and vulnerable freshwater species.

1. INTRODUCTION

Monitoring fish species diversity in riverine ecosystems presents considerable challenges, particularly with respect to the accuracy of species identification under dynamic environmental conditions. Conventional fish capture techniques, such as netting and trapping, often yield incomplete or biased datasets, especially for species that are rare, cryptic, or restricted to inaccessible habitats. In addition, these methods are labor-intensive, time-consuming, and may disrupt aquatic ecosystems [1-3]. To overcome these limitations, environmental DNA (eDNA) metabarcoding provides a more efficient and non-invasive alternative [4-8]. Through this method, the presence of fish species can be detected from collected and analyzed DNA fragments released by organisms into the environment, thereby rendering direct capture of individuals unnecessary [9]. eDNA technologies have proven effective in monitoring fish species in various ecosystems, such as rivers [10-16], lakes [17-19], and ponds [20, 21]. This method is particularly effective for detecting rare or cryptic species. Despite its popularity as an effective method for revealing biodiversity across various environments, several studies have highlighted the limitations of eDNA in tropical river systems. These limitations include the lack of comprehensive genetic barcode reference libraries and the taxonomic complexity, both of which can hinder accurate identification of local species, particularly in tropical regions that remain underrepresented in genetic databases [22]. In addition, environmental factors such as hydrological variability, rainfall, sediment load, and water chemistry can influence the distribution and degradation rates of eDNA, ultimately affecting the success of genetic species detection [23]. However, to obtain a more comprehensive description of fish biodiversity, the integration of traditional and molecular approaches can be a strategic step. This integrative taxonomic approach not only enhances the accuracy of species identification but also enriches understanding of aquatic ecosystem dynamics, including rivers [9, 24].

The Mesangat Ilir River is one of the areas that experiences significant fluctuations in water discharge, which in turn affects the overall environmental conditions. During the rainy season, high water discharge causes areas to become inundated, whereas in the dry season, the decline in water level leads to the drying of most of these areas. This hydrological instability exerts pressure on the fisheries resources relied upon by local fishermen and on their livelihoods, which depend on this ecosystem [25]. In addition, fluctuating water discharge also affects the native fish communities and other species inhabiting the river [26, 27]. Fish are key components of aquatic ecosystems, serving as indicators for evaluating the biological integrity of water and contributing to the balance of ecosystems [28]. However, freshwater ecosystems are now threatened by various anthropogenic pressures, including habitat loss [29], climate change [30], overfishing [31], and the introduction of invasive species [32]. The impacts of these activities have led to a global decline in freshwater fish populations [33]. Therefore, integrating eDNA metabarcoding with traditional methods is crucial for monitoring fish species diversity in the Mesangat Ilir River. This approach provides a more comprehensive understanding of fish diversity and supports conservation efforts aimed at the sustainable management of fisheries resources [34-36].

2. MATERIAL AND METHODS

2.1 Study area

Fish sampling was conducted along a ± 4.54 km segment of the Mesangat Ilir River, East Kalimantan, Indonesia (Figure 1). An active, continuous survey was performed along the entire study reach, following the river flow to capture fish communities across various habitat gradients [37]. The study was carried out in August 2024 during the dry season, when water levels were low and partial drying occurred in several river sections. The study area is characterized by flat and low-lying topography, with an elevation of approximately 20 m above sea level and relatively homogeneous habitat conditions throughout the surveyed segment.

2.2 Fish collection using traditional sampling and identification

Traditional fish sampling was conducted through a single intensive survey event to establish a baseline for comparison with eDNA results. To ensure comprehensive coverage of the study area, the survey was carried out across five distinct sampling points along the river. At each point, a stratified random sampling approach was employed to target various microhabitats. The sampling effort consisted of 15 cast-net throws (2.5-inch mesh size) within each habitat stratum at every sampling point. Additionally, scoop nets were utilized simultaneously to capture small-bodied or cryptic species along the riparian edges. The total active fishing effort was approximately 6 hours. Although the collection was conducted over only one day, this intensive effort across multiple strata and locations was designed to maximize species recovery at the site. Fish samples were sorted by species, and their abundances were recorded. Species identification was based on morphological characteristics, and morphometric measurements were taken using a digital scale (precision: 0.01 g) and a digital caliper (precision: 0.01 mm). After measurements, fish specimens were photographed in fresh condition with the head facing left. Then the fish samples were euthanized in MS-222 solution [38] and preserved in containers filled with 4% formalin, then labeled following standard procedures. Preserved specimens were then transported to the laboratory for further analysis. Reference guides for species identification included the official FishBase database (https://www.fishbase.se/). Local fish names were obtained through interviews with the local communities.

2.3 Water sampling and filtration

Before water sampling, all equipment, including sample bottles, was sterilized with a 10% bleach solution and rinsed three times with river water. Sampling was conducted while wearing gloves to prevent potential contamination. eDNA sampling was conducted at a single site in the Mesangat Ilir River (0°27'3.376" N, 116°41'7.433" E). A 1 L surface water sample [39, 40] was collected in a single event without biological replication. The sample was immediately stored in a cooler with ice packs prior to filtration [41]. Water samples were subsequently filtered using a Peristaltic Rocker 300 Vacuum Pump equipped with 0.45 µm Millipore filter papers [9]. Filter papers containing genetic material were cut into two parts using sterile tweezers, folded, and placed into 1.8 mL tubes containing DNA shield solution to preserve the samples and maintain DNA integrity. Water quality parameters were measured in situ during eDNA sampling. Water temperature was recorded using a digital thermometer (TP-101, China), pH was measured using a portable pH meter (China), and dissolved oxygen (DO) was measured using a DO meter (Lutron DO-5509, Taiwan).

2.4 Extraction, DNA amplification, and sequencing

eDNA was extracted using the Quick-DNA MagBead Plus Kit (Zymo Research, USA) following the manufacturer's instructions [42]. The extraction process involved adding reagents to the water samples, followed by the use of magnetic beads to separate the DNA from contaminants. The purity of the extracted eDNA was assessed using a NanoDrop spectrophotometer by measuring the A260/A280 ratio to ensure the absence of protein or other organic contaminants. DNA concentration was accurately quantified using the Qubit™ dsDNA HS Assay Kit (Invitrogen), which allows for reliable measurement of low-concentration DNA. To ensure data integrity, standard laboratory quality control was implemented, including the use of No Template Controls (NTC) during the PCR amplification phase to monitor for laboratory-acquired contamination. However, field blanks, filtration blanks, and extraction blanks were not implemented. To mitigate potential cross-contamination, we maintained strict physical separation between field sampling and laboratory processing, and all sampling equipment was pre-sterilized with 10% bleach followed by thorough rinsing with distilled water. Extracted DNA was stored at -20 °C until further analysis via metabarcoding. For eDNA amplification, the MiFish-U Adapt Forward (GTCGGTAAAACTCGTGCCAGC) and MiFish-U Adapt Reverse (CATAGTGGGGTATCTAATCCCAGTTTG) primers were used, targeting the mitochondrial 12S rRNA gene to amplify a 175 bp DNA fragment from the extracted eDNA samples [4].

PCR was performed in a total reaction volume of 25 µL, comprising 1 µL of each forward and reverse primer (10 µM), 1 µL of eDNA template, 19.9 µL of ultrapure water, 2.5 µL of 10× High-Fidelity PCR buffer, 0.5 µL of dNTP mix (10 mM), and 0.1 µL of Taq DNA polymerase (Invitrogen). The PCR program started with an initial denaturation at 95 °C for 10 minutes, followed by 35 cycles, each consisting of denaturation at 95 °C for 10 seconds, annealing at 56 °C for 20 seconds, and extension at 72 °C for 20 seconds. The program concluded with a final extension at 72 °C for 5 minutes to ensure complete DNA elongation. PCR products were then assessed by electrophoresis on a 1.5% agarose gel, stained with Fluoresafe [43], and DNA bands were visualized using a UV transilluminator. Library preparation was conducted following the protocol of the Native Barcoding Kit 24 v14 (SQK-NBD114.24, Oxford Nanopore Technologies, UK) [44].

The process began with the preparation of amplicons, which were quantified using a fluorometer to ensure accurate DNA input. Each sample was assigned a unique barcode, and ligation was performed to attach the barcode to the DNA. The purified DNA samples were then eluted and prepared for sequencing by adding Oxford Nanopore adapters, enabling recognition by the sequencer. Prior to sequencing, the flow cell was cleaned to ensure optimal performance. Sequencing data were subsequently analyzed to demultiplex individual samples based on the unique barcodes. Library preparation was performed using Oxford Nanopore Technologies kits. Nanopore sequencing was conducted with MinKNOW software version 24.02.16, and basecalling was performed using Dorado version 7.3.11 with the high-accuracy model [45]. FASTQ file quality was assessed and visualized using NanoPlot, and quality filtering was carried out with NanoFilt [46, 47]. The filtered reads were subsequently classified using the Centrifuge classifier [48]. For taxonomic assignment, a fish reference database was constructed from the MitoFish database (http://mitofish.aori.u-tokyo.ac.jp/) [49], and species identification was performed using a similarity threshold of ≥ 97% [50]. Further analyses and data visualization were conducted using Pavian (https://github.com/fbreitwieser/pavian) and RStudio version 4.3.3 (https://www.R-project.org/).

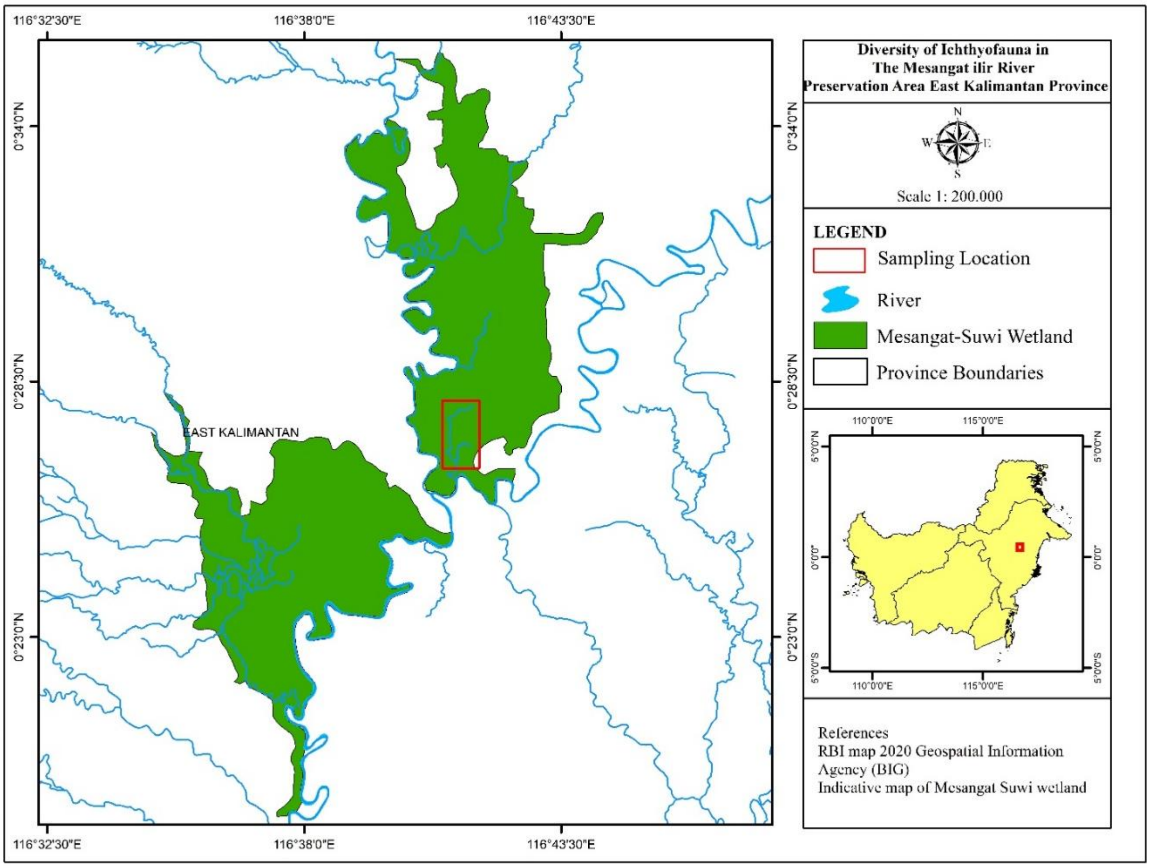

Figure 1. Map of research location (red rectangle) in the Mesangat Ilir River of East Kalimantan

3. RESULT

3.1 Fish species and composition

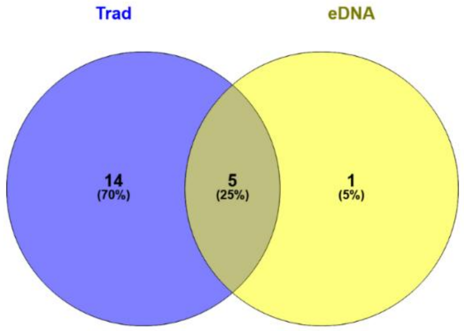

Figure 2. A Venn diagram showing the comparison of fish species detection using the traditional methods and eDNA in the Mesangat Ilir River

A total of 191,071 sequence fragments were obtained using the MiFish primer, of which 189,556 fragments were identified as originating from the class Actinopterygii, demonstrating the universal coverage of this primer for fish. Through eDNA analysis, six fish species were identified, belonging to two orders and two families, with a minimum percentage of identical matches of 97%. The family Cyprinidae accounted for four species: *Oxygaster anomalura, Cyclocheilichthys apogon, Barbonymus schwanenfeldii,* and *Osteochilus waandersii.* The remaining two species were *Trichopsis vittata* (Osphronemidae) and *Pristolepis fasciata* (Pristolepididae). In contrast, traditional methods identified 19 fish species, representing three orders and seven families (Table 1). Overall, the combination of eDNA and traditional methods detected a total of 20 species (Figure 2). Photographic documentation of the recorded fish species is presented in Figure 3. The family Cyprinidae was the most dominant (12 species; 60%), followed by Bagridae and Osphronemidae, each with 2 species (10%). Additionally, Cobitidae, Anabantidae, Helostomatidae, and Pristolepidae were each represented by a single species (5%) (Figure 4).

Table 1. List of species detected by eDNA metabarcoding and traditional method, status conservation, and potency in Mesangat Ilir River

Family	Species	Traditional (n)	Traditional (RA %)	eDNA (RRA %)	Total Read	Identity Matches (%)	IUCN	Potency
Cyprinidae	*Barbichthys laevis*	3	5.08	-	-	-	LC	C
	Barbonymus schwanenfeldii	3	5.08	14.05	9953	99.43	LC	C & O
	Crossocheilus nigriloba	1	1.69	-	-	-	LC	O
	Cyclocheilichthys apogon	5	8.47	18.14	12851	98.84	LC	C & O
	Labiobarbus fasciatus	3	5.08	-	-	-	LC	C
	Labiobarbus festivus	3	5.08	-	-	-	LC	C
	Osteochilus microcephalus	1	1.69	-	-	-	LC	C
	Osteochilus waandersii	1	1.69	8.17	5784	99.42	LC	C
	Oxygaster anomalura	3	5.08	26.74	18940	99.44	LC	C & O
	Puntioplites bulu	3	5.08	-	-	-	LC	C
	Rasbora argyrotaenia	3	5.08	-	-	-	LC	C & O
	Osteochilus vittatus	1	1.69	-	-	-	LC	C
Cobitidae	*Pangio alternans*	6	10.17	-	-	-	EN	O
Anabantidae	*Anabas testudineus*	3	5.08	-	-	-	LC	C
Pristolepididae	*Pristolepis fasciata*	3	5.08	15.21	10770	97.63	LC	C
Helostomatidae	*Helostoma temminckkii*	14	23.73	-	-	-	LC	C & O
Bagridae	*Hemibagrus nemurus*	1	1.69	-	-	-	DD	C
	Mystus nigriceps	1	1.69	-	-	-	LC	C
Osphronemidae	*Betta* sp.	1	1.69	-	-	-	-	O
	Trichopsis vittata	0	-	17.69	12531	97.6	LC	O

Note: Number of species (n); Relative abundance (RA); Relative reads abundance (RRA); Least concern (LC); Data deficient (DD); Endangered (EN); Consumption commodities (C); Ornamental commodities (O); Consumption & Ornamental commodities (C&O); Not detected (-). Percentage of Relative abundance (RA, based on individual counts) and Relative reads Abundance (RRA, based on DNA fragment proportions) represent distinct biological metrics.

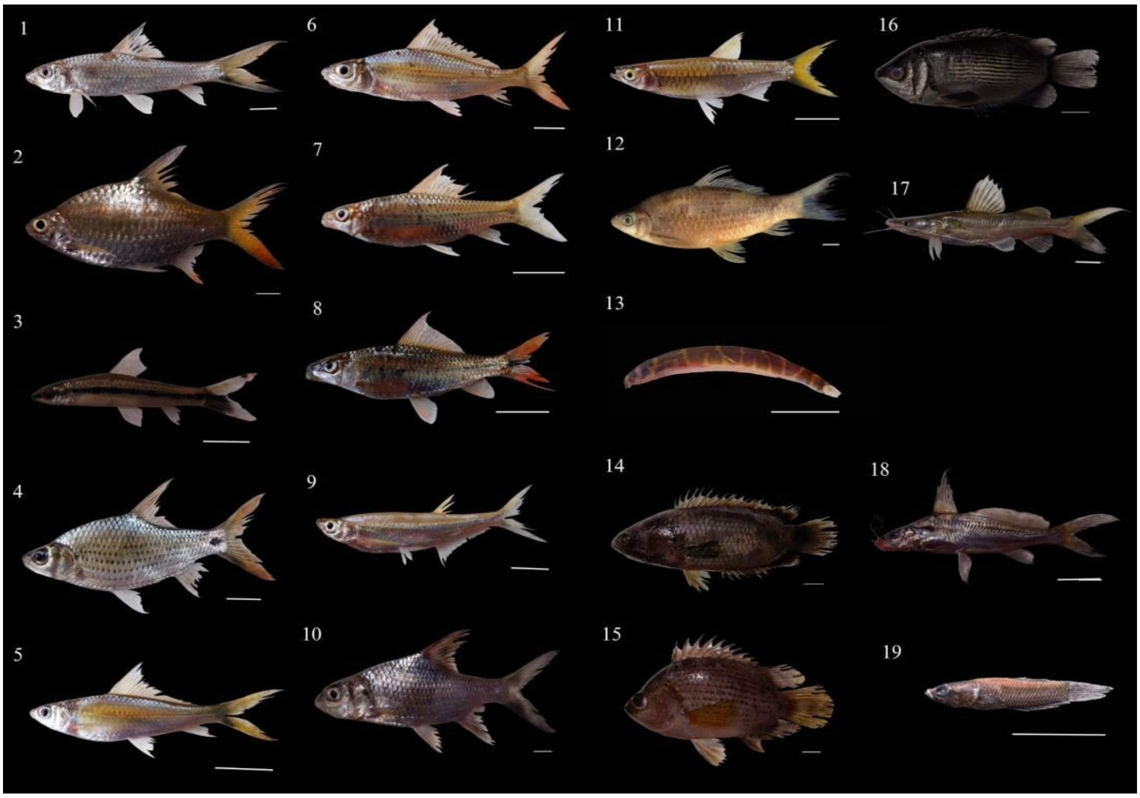

Figure 3. Fish species identified by traditional method in the Mesangat Ilir River of East Kalimantan, where (1) *Barbichthys laevis,* (2) *Barbonymus schwanenfeld*ii, (3) *Crossocheilus nigriloba*, (4) *Cyclocheilichthys apogon*, (5) *Labiobarbus fasciatus*, (6) *Labiobarbus festivus*, (7) *Osteochilus microcephalus*, (8) *Osteochilus waandersii*, (9) *Oxygaster anomalura*, (10) *Puntioplites bulu*, (11) *Rasbora argyrotaenia*, (12) *Osteochilus vittatus*, (13) *Pangio alternans*, (14) *Anabas testudineus*, (15) *Pristolepis fasciata*, (16) *Helostoma temminckkii*, (17) *Hemibagrus nemurus*, (18) *Mystus nigriceps*, (19) *Betta* sp., scale bar 10 mm

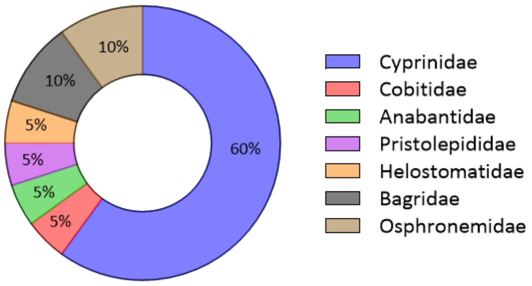

Figure 4. Percentage of the different families of fish in the Mesangat Ilir River of East Kalimantan

3.2 Relative abundance: eDNA vs. traditional catches

The comparison of relative abundance (RA, %) of fish species detected in the Mesangat Ilir River revealed variable detection patterns among species (Table 1 and Figure 5). Based on the RA analysis from eDNA read counts, the fish species with the highest detection proportions were *Oxygaster anomalura* (26.74%), *Cyclocheilichthys apogon* (18.14%), *Trichopsis vittata* (17.69%), *Pristolepis fasciata* (15.21%), *Barbonymus schwanenfeldii* (14.05%), and *Osteochilus waandersii* (8.17%). Meanwhile, a total of 59 individuals representing 19 fish species were captured using traditional methods (nets and scoop nets). The RA of these catches showed a dominance of *Helostoma temminckii* (n = 14), followed by *Pangio alternans* (n = 6) and *Cyclocheilichthys Apogon* (n = 5). Most of the remaining species exhibited lower RA, with the majority below 5 individuals, such as *Anabas testudineus* (n = 3) and *Barbonymus schwanenfeldii* (n = 3). Some species, including *Osteochilus vittatus*, *Osteochilus microcephalus*, *Osteochilus waandersii*, *Hemibagrus nemurus*, *Mystus nigriceps*, *Crossocheilus nigriloba*, and *Betta* sp., were recorded with very low RA (n = 1).

3.3 Water quality

Water quality measurements in the Mesangat Ilir River during the study period showed that water temperature ranged from 23.8 to 24.7 °C, pH values ranged from 5.75 to 6.65, and DO concentrations ranged from 5.9 to 6.4 mg/L.

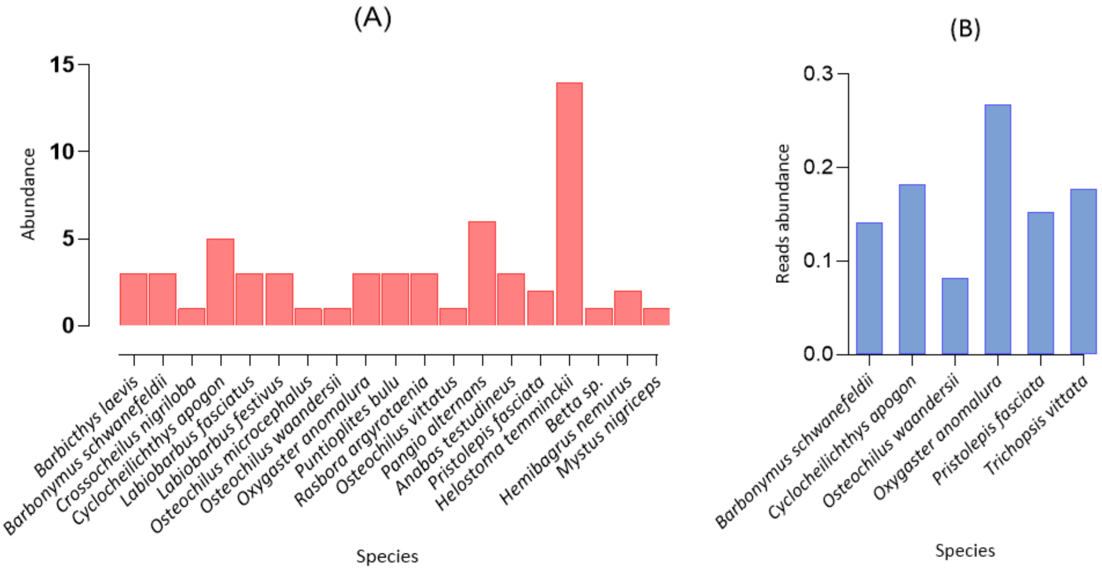

Figure 5. Comparison of fish abundance detected by traditional methods (number of individuals) and eDNA metabarcoding (number of sequences) in the Mesangat Ilir River of East Kalimantan

4. DISCUSSION

This study highlights the application of an integrative taxonomic approach to reveal fish species diversity in the Mesangat Ilir River, East Kalimantan, through a combination of eDNA metabarcoding and traditional methods (nets and scoop nets). Integrating eDNA metabarcoding with traditional sampling provided complementary insights into fish diversity. While traditional methods captured a greater number of species, eDNA was able to reveal cryptic taxa not recorded by nets, such as *Trichopsis vittata*. This highlights the importance of combining both approaches, particularly in dynamic tropical river systems. These results indicate that traditional methods still play a crucial role in species detection, particularly due to their ability to sample across different water layers and microhabitats around the benthic substrate. Compared to other eDNA metabarcoding studies in freshwater systems in Indonesia, this study shows variation in the number of species detected. The study reported 18 fish species in Lake Singkarak, West Sumatra [19], while research recorded 11 species in the Opak River, Yogyakarta [41]. Floodplain waters of Bangka Island [51] and the Batanghari River [52] reported up to 25 species using eDNA alone.

Nonetheless, eDNA metabarcoding has significant advantages, particularly in detecting species that are difficult to capture using traditional methods. These findings contrast with the study by Wibowo et al. [16], which reported that eDNA and traditional methods in the Kumbe River, Papua, were able to detect 23 species, including 18 species that had never been recorded at the site, whereas traditional methods alone detected only 5 species. The family Cyprinidae was the most dominant, both in species richness and DNA fragment abundance. These findings are consistent with studies conducted in the Kumbe River [16], Lake Singkarak [19], Merbau River [53], Lake Siawan [54], and peatland waters [55]. This suggests that species within Cyprinidae are not only visually abundant or frequently caught but also release substantial amounts of DNA fragments into the aquatic environment. In contrast, other families, such as Bagridae (*Hemibagrus nemurus, Mystus nigriceps*), Cobitidae (*Pangio alternans*), and Osphronemidae (*Betta* sp.), were represented by only one or two species and were not detected through eDNA. As seen in Table 1, some species with high RA, such as Helostoma temminckii, were not detected in RRA, illustrating the non-linear relationship between physical capture and DNA shedding/detection.

Figure 6. Environmental condition of the Mesangat Ilir River during the dry season

The relatively low detection of certain species using eDNA in this study may be attributed to a combination of technical and environmental factors. Methodological limitations, specifically the use of a 1 L water volume at a single site without replicates, present inherent constraints on detection probability. Previous studies have demonstrated that eDNA is often patchily distributed in aquatic environments, and limited sampling volume combined with the absence of replication can reduce the likelihood of detecting low-abundance or rare species, potentially leading to false-negative results [56, 57]. Consequently, the biodiversity richness reported here should be interpreted as a conservative estimate.

Environmental conditions during the dry season, characterized by low water levels and partial drying of the river channel (Figure 6), likely influenced eDNA concentration and distribution. Reduced water volume may increase DNA concentration locally but can also enhance spatial heterogeneity. In addition, factors such as low pH, reduced DO levels, and high organic matter content (leaves, roots, and sediments) are known to inhibit DNA extraction and PCR amplification [58]. Additionally, other technical constraints, including suboptimal primer selection, incomplete reference databases, and primer bias, may have further reduced detection efficiency [59, 60].

Furthermore, the interpretation of these results must be approached with caution regarding quality control. As emphasized by research of Schenekar et al. [61], rigorous quality assurance, including field, filtration, and extraction blanks, is critical for mitigating contamination risks and precluding the definitive exclusion of environmental or laboratory cross-contamination. In this study, such controls were not implemented due to extreme logistical constraints during the expedition to the remote areas of the Mesangat Ilir River. Although we maintained strict physical separation between field sampling and laboratory processing and followed rigorous sterilization protocols, the absence of these blanks remains a recognized limitation of this preliminary assessment. Future monitoring in these remote wetland systems must prioritize the inclusion of a full suite of negative controls to further validate the reliability of rare species detections.

A notable finding in this study is the detection of Kalimantan's endemic species, *Pangio alternans* (Cobitidae) and *Crossocheilus nigriloba* (Cyprinidae), which were only captured using traditional methods. *P. alternans* was found along the riverbanks, closely associated with the benthic substrate**.** Beyond potential gaps in reference databases, these behavioral and habitat preferences likely influenced eDNA detectability. *P. alternans* exhibits fossorial behavior, burrowing into substrates such as mud, sand, or leaf litter [62]. Its occurrence within the substrate at the river's edge likely restricts the release of DNA into the body of water. Both species have a highly restricted geographic distribution, and low population density or limited spatial occurrence may further reduce eDNA detectability. This species is known to experience population declines in its natural habitat due to its restricted distribution range and environmental pressures. At the study site, we observed the presence of artificial dams and the use of electrofishing, which are anthropogenic activities that can alter hydrology, including increased sedimentation, changes in water flow patterns, impacts on the genetic composition of populations, and reduction of available habitat for endemic species. Similar threats to endemic species have been reported by the study of Divya et al. [63], where two endemic species from the Western Ghats in India, *Hemibagrus punctatus* and *Horabagrus brachysoma*, face significant threats due to habitat degradation, overfishing, and dam construction, altering their natural environments. The findings concerning *Pangio alternans* and *Crossocheilus nigriloba* in this study underscore the importance of site-specific habitat management in East Kalimantan. Given the species' endemic status and vulnerability to environmental changes, in-situ conservation strategies and continuous population monitoring are necessary. Collaboration among local government authorities, communities, and researchers should be strengthened to mitigate the anthropogenic pressures that threaten this species.

In terms of conservation status and potential, the fish species in the Mesangat Ilir River are categorized into three groups according to the IUCN Red List: Least Concern (LC, 89.5%), Data Deficient (DD, 5.3%), and Endangered (EN, 5.3%). Regarding economic and ornamental potential, 25% of species have the potential to serve both as ornamental and food fish, 20% as ornamental fish, and 55% as food fish. Economically valuable food fish include *Oxygaster anomalura*, *Hemibagrus nemurus*, *Helostoma temminckii*, *Cyclocheilichthys apogon*, *Rasbora argyrotaenia*, and *Anabas testudineus*. Species such as *Trichopsis vittata*, *Crossocheilus nigriloba*, and *Betta* sp. are commonly regarded as ornamental species. However, they are currently not utilized as ornamental fish by local communities in this area. This indicates the presence of underutilized local biological resources that warrant further assessment, taking sustainability and conservation considerations into account. It also highlights the conservation value of the study area and the potential role of local fish species in supporting sustainable livelihoods and biodiversity preservation in the Mesangat Ilir River.

5. CONCLUSIONS

In conclusion, this study provides several key insights into the effectiveness of fish diversity monitoring in the Mesangat Ilir River: (1) under the specific dry season conditions of this study, the traditional method proved superior to the limited eDNA sampling scheme in the detection of total species richness, likely due to the concentrated fish populations in receding waters; (2) eDNA serves as a vital supplement for detecting individual cryptic species like *Trichopsis vittata*; (3) the instances of missed eDNA detections in this study may be related to insufficient sampling volume, environmental conditions and specific species characteristics; (4) in order to achieve comprehensive monitoring, it is necessary to optimize the eDNA sampling strategy (increase water volume, multi-point replication) and combine it with the traditional method.

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