ASSESSING POPULATION AND DISTRIBUTION OF INDIAN MAJOR CARP FISH SPECIES IN KANGSABATI RIVER USING eDNA

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Abstract:

Successful management of native, endangered, and rare species requires the capability to detect and monitor their populations quickly and accurately, even at low densities. Identifying and protecting critical habitats is crucial to conserving these species and enhancing their survival and reproductive success. Immediate detection of invasive species is also essential for rapid response and potential eradication. eDNA is a method for detecting biological species by detecting DNA fragments shed by organisms. It's an effective tool for detecting rare and elusive species and has been tapped to monitor different aquatic organisms. In this study, we are pioneers in utilizing eDNA to assess the populations of three native major carp fish species in different layers of the aquatic body in the Kangsabati River in West Bengal, India. By analyzing water samples from ten sampling locations, the present study can detect the presence of *Labeo rohita*, *Catla catla*, and *Cirrhinus mrigala*, and determine their distribution patterns. PCR amplification success rates significantly differed among stations with different population densities (high, medium, and low), with higher success rates in higher-density stations ($p < 0.0002$). The success rates were 0.98 for high, 0.80 for medium, and 0.54 for low-density stations. Comparing traditional and eDNA surveys showed a 37.77% higher detection sensitivity of targeted species in eDNA results. This information is critical for conserving and managing targeted essential fish populations and protecting their habitats from potential threats. Overall, eDNA has excellent potential for enhancing the management and conservation of native and endangered species.

Keywords: native IMC species, environmental DNA (eDNA), species detection, freshwater environments, monitoring populations.

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Introduction:

One of the most crucial aspects of biodiversity studies is collecting accurate information on the species composition of a given area (Funk & Richardson, 2002). Numerous scientific studies indicate that several factors, including climate change, pollution, habitat destruction, overexploitation, and invasive alien species, contribute to the deterioration of biodiversity (Cardinale et al., 2012). These impacts are directly or indirectly influenced by anthropogenic activities (Meng et al., 2021). Therefore, it is essential to identify the species inhabiting a particular area to conserve biodiversity effectively (Dornelas et al., 2018). A proper assessment of the presence or absence of each species within a given time and effort scale is necessary.

Freshwater ecosystems account for less than 3% of the total water volume on Earth, and approximately 10,000 (40%) of the 25,000 identified fish species reside in freshwater for at least part of their lives (Moyle & Leidy, 1992). As a result, freshwater ecosystems provide high habitat and species diversity within small areas. However, these ecosystems are easily affected by anthropogenic actions, and the rate of species diversity loss is high-speed in freshwater ecosystems. Traditional species composition assessments, which require direct catches, are usually time-consuming and laborious (Kottelat & Whitten, 1996). Therefore, it is challenging to assess biodiversity losses accurately and routinely. Moreover, species detection in underwater environments is challenging, making it necessary to develop innovative, rapid methods for examining species composition (Deiner et al., 2016).

Recently, a non-invasive method for detecting aquatic species using the environmental DNA (eDNA) approach was used to identify targeted species such as Asian carp and bullfrog (Lin et al., 2019). This information motivated researchers to extend this approach to obtain a complete illustration of fish species composition. In this study, we used eDNA to detect native fish species found in a particular area by making molecular identifications at the species level from eDNA extracted from freshwater samples. The present study designed and amplified species-specific primers for mini barcode regions from the mitochondrial Cytochrome Oxidase I (COI) gene (Sharma & Kobayashi, 2014). Furthermore, this study is the first eDNA study targeting Kangsabati River West Bengal, India, and demonstrates the potential of eDNA as an effective tool for species detection and identification in freshwater ecosystems.

Methods:

Study area:

The Kangsabati River of West Bengal in India is selected as the study area because previously published monitoring studies in this region have reported the occurrence of native species, including *Labeo rohita* (Hamilton, 1822), *Catla catla* (Hamilton, 1822), and *Cirrhinus mrigala* (Hamilton, 1822) (Kar et al., 2017; Kisku et al., 2017), which were also target species in this study. However, to determine the distribution of the native carp species in the area surveys, samples have been supplied from the local fishermen engaged in sportive hunting and trade in the region besides catching fish using drag nets, gill nets, along with the hooks and lines and scoop nets.
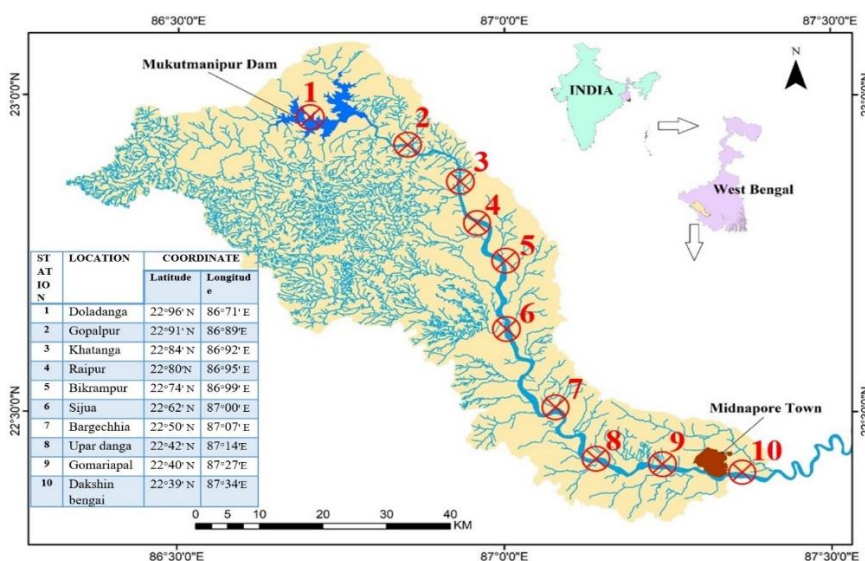

ST AT IO N	LOCATION	COORDINATE	
		Latitude	Longitude
1	Doladanga	22°96'N	86°71'E
2	Gopalpur	22°91'N	86°89'E
3	Khatanga	22°84'N	86°92'E
4	Raipur	22°80'N	86°95'E
5	Bikrampur	22°74'N	86°99'E
6	Sijua	22°62'N	87°00'E
7	Bargechhia	22°50'N	87°07'E
8	Upar danga	22°42'N	87°14'E
9	Gomariapal	22°40'N	87°27'E
10	Dakshin bengai	22°39'N	87°34'E

Figure 1: Locations of sampling stations on Kangsabati River.

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The availability of fish in different areas was assessed based on seasonal ratios and frequency of catches. If a species was the most caught during all two seasons, it was labeled as 'high.' If the species was not mostly caught in February, it was labeled as 'medium,' and if it was occasionally caught but not the same as the above two, it was labeled as 'low'. Species that were not caught in both seasons are referred to as 'unseen'. Ten sampling stations with distinct hydrological and ecological properties were selected (**Figure 1**). To determine the current status of the target population, all sampling stations were surveyed again (Emiroğlu, 2011). Population density information based on traditional monitoring methods is presented in **Table 1**.

STATION	LOCATION	COORDINATE		Density		
		Latitude	**Longitude**	*L. rohita*	*C. catla*	*C. mrigala*
1	Doladanga	22°96'14.38" N	86°71'95.95" E	Unseen	Unseen	Low
2	Gopalpur	22°91'45.08" N	86°89'46.38" E	Low	Unseen	Low
3	Khatanga	22°84'56.43" N	86°92'93.01" E	High	Unseen	unseen
4	Raipur	22°80'17.37" N	86°95'22.90" E	High	Unseen	High
5	Bikrampur	22°74'25.38" N	86°99'74.40" E	High	Unseen	High
6	Sijua	22°62'85.54" N	87°00'90.38" E	Unseen	Unseen	Medium
7	Bargechhia	22°50'41.62" N	87°07'51.53" E	Medium	Unseen	High
8	Upar danga	22°42'76.55" N	87°14'72.67" E	High	Medium	High
9	Gomariapal	22°40'66.67" N	87°27'79.06" E	High	Low	Low
10	Dakshin bengai	22°39'80.30" N	87°34'51.24" E	High	High	Medium

Table 1: Sampling stations and population density information of target species.

Sampling:

The present survey collected freshwater samples from 10 specific sampling stations in two different seasons, February and July 2019, with triplicate water samples taken at each location (Table-1). No positive or negative controls were used during the sampling process. Sterile containers were used to collect 2.0 liters of water during each sampling event, which were then transported to the laboratory on ice for further analysis. Three types of controls were implemented throughout the entire water sampling and transport process, including negative equipment control, negative field control, and negative transport controls, all of which utilized deionized water samples (Goldberg et al., 2013). In addition, a positive sample control was taken from a pond with an entire fisheries culture, which contained no target species. To understand the relationship between eDNA concentrations and seasonal changes in freshwater parameters, the current finding also measured dissolved oxygen, temperature, turbidity, alkalinity, salinity, and pH.

Molecular and statistical analysis:

In this study, water samples remained filtered using a Sterivex-GP (Millipore, MA) unit with a 0.22 μm membrane pore size, and eDNA was extracted directly from the membrane pores using the PowerWater® Sterivex DNA Isolation Kits (MO BIO, CA). The extracted eDNA samples were kept at -20°C until the PCR phase. Species-specific primers were designed to amplify an approximately 150 bp fragment of the mitochondrial COI gene for each target species. The PCR reactions were performed using a multiple tubes approach, with each reaction being conducted in triplicate (Taberlet et al., 1996). The original sample was subjected to additional analyses if none of the three wells yielded amplification. The sample was classified as positive if any of the three wells showed positive amplification during this second phase. Amplified PCR products were checked using agarose gel electrophoresis, purified using Wizard SV Genomic DNA Purification Kit (Promega, CA) according to the manufacturer's protocol, and sequenced by using the ABI Prism 3130 Genetic Analyzer (Applied Biosystems, CA) platform. In silico, PCR tests were performed applying the ecoPCR (Dejean et al., 2011) and primer-BLAST (Jerde et al., 2011) to confirm the suitability of the primer pairs. The primary local alignment search tool (BLAST) analysis indicated that these primers did not show high scores of matchings with any other sequences stored in GenBank. Details of the primers, amplicon lengths, reaction volumes, and thermal cycler conditions were provided in **Table 2**.

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Sl No.	Species	Primers	Gen Bank database	Target amplicon length(bp)/ Expected amplicon size:	Tm	Thermal cycler condition	Reaction volumes
1	*Labeo rohita* (Hamilton, 1822)	Forward primer: 5'-ACTAAGCCAACCCGGATCAC-3' Reverse primer: 5'-TGGCACGAGTCAGTTTCCAA-3'	(Accession number JX983352)	133 bp	59.7°C	Initial denaturation: 95°C for 3 minutes Denaturation: 95°C for 30 seconds Annealing: 58°C for 30 seconds Extension: 72°C for 30 seconds Repeat steps 2-4 for 35 cycles Final extension: 72°C for 5 minutes Hold at 4°C	10 mM Tris-HCl (pH 8.3) 50 mM KCl 2.5 mM MgCl2 0.2 mM dNTP 0.8 µM each of forward and reverse primers 0.005 mg/µL BSA (bovine serum albumin) 0.5 U DNA polymerase 5 ng/µL DNA
2	*Catla catla* (Hamilton, 1822)	Forward primer: 5'-ACTAAGTCAACCCGGATCGC-3' Reverse primer: 5'-GGGGAATGCCATATCTGGGG-3'	(Accession number KX163998)	166 bp	59.7°C		
3	*Cirrhinus mrigala* (Hamilton, 1822)	Forward primer: 5'-CCCAGACATAGCATTCCCCC-3' Reverse primer: 5'-GCGTGGGCTAAATTTCCTGC-3'	(Accession number JX983258)	150 bp	60°C.		

Table 2: Primers, amplicon lengths, GenBank database Accession number, Tm, thermal cycler conditions and reaction volumes.

A Generalized Mixed Model (Ficetola et al., 2008) was employed to compare eDNA concentrations across different stations with varying population densities. Seasonal changes in water temperature's impact on eDNA concentration were assessed using a One-Way Analysis of Variance (ANOVA) with a significance level of 0.05. The ANOVA was performed with the use of SPSS version 19 software. The influence of other water parameters (conductivity, pH, and dissolved oxygen) on eDNA concentration was evaluated using a General Linear Model (GLM) with standardized and centered factors (Venables & Ripley, 2002). The collinearity of the factors was assessed using the Variance Inflation Factor (VIF). The GLM model that best fits the data set was selected based on the Akaike Information Criterion (Takahara et al., 2012).

Result:
The study successfully detected eDNA of the targeted three Indian major carp species *Labeo rohita* (Hamilton, 1822), *Catla catla* (Hamilton, 1822), and *Cirrhinus mrigala* (Hamilton, 1822) in all of the selected stations, and the distribution of these species based on eDNA detection rates is presented in **Figure 2**. This study aimed to investigate the presence and distribution of targeted freshwater fish species using environmental DNA (eDNA) detection. The results showed (**Table-3**) that the eDNA of all target species was successfully detected in all sampling stations, with positive results obtained from all three repetitions in 6/10 stations for *L. rohita*, 5/10 stations for *C. catla*, and 4/10 stations for *C. mrigala*. Comparing traditional and eDNA survey detection rates of target species revealed a 23.33% higher detection sensitivity of targeted species based on eDNA results (81/90 positive) compared to traditional survey results (60/90). Moreover, positive eDNA results were acquired from 2 and 5, out of 10 stations for *L. rohita and C. catla,* respectively, in which traditional surveys had not previously detected the species.

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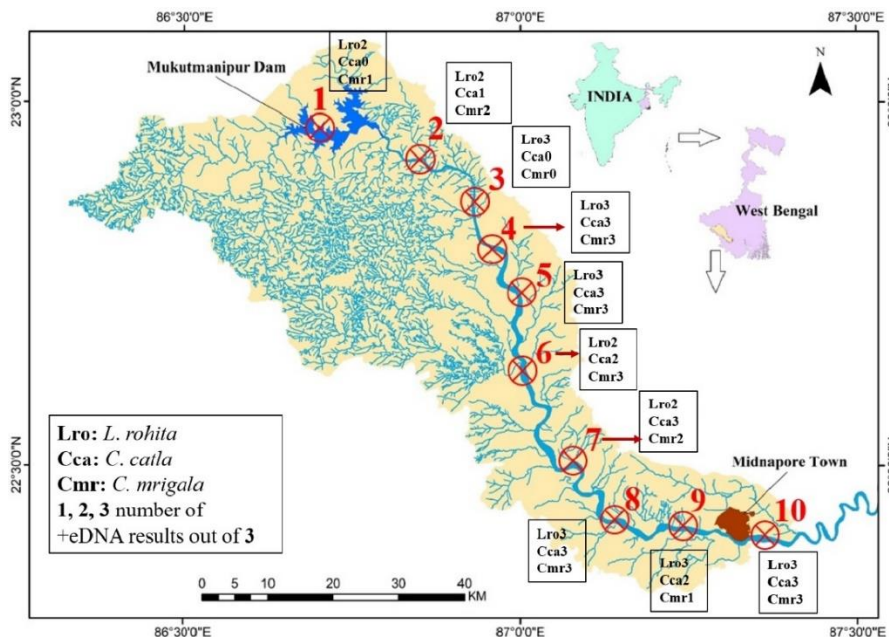
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Figure 2: Distribution of three target Indian major carp fish species based on eDNA detection.

Additionally, the effect of seasonal change on the detection of *L. rohita, C. catla,* and *C. mrigala* eDNA was significant, with positive results acquired only from samplings made in July from stations 8 to 10. To verify the specificity of our primers, we conducted three PCR replicates for each eDNA sample, and subsequently sequenced every positively amplified PCR product. Upon analysis, we confirmed that all sequences originated from the target species. The PCR amplification success rate (samples with at least one positive amplification) was 89.62%, 78.91%, and 79.28% for the three target species *L. rohita, C. catla,* and *C. mrigala*, respectively.

Sampling station	eDNA sampling						Traditional sampling					
	Detection			+ Water sample			Detection			Density		
	L. rohita	*C. catla*	*C. mrigala*	*L. rohita*	*C. catla*	*C. mrigala*	*L. rohita*	*C. catla*	*C. mrigala*	*L. rohita*	*C. catla*	*C. mrigala*
1	+	_	+	2/3	0/3	1/3	_	_	+	Unseen	Unseen	Low
2	+	+	+	2/3	1/3	2/3	+	_	+	Low	Unseen	Low
3	+	_	_	3/3	0/3	0/3	+	_	_	High	Unseen	Unseen
4	+	+	+	3/3	3/3	3/3	+	_	+	High	Unseen	High
5	+	+	+	3/3	3/3	3/3	+	_	+	High	Unseen	High
6	+	+	+	2/3	2/3	3/3	_	_	+	Unseen	Unseen	Medium
7	+	+	+	2/3	3/3	2/3	+	_	+	Medium	Unseen	High
8	+	+	+	3/3	3/3	3/3	+	+	+	High	Medium	High
9	+	+	+	3/3	2/3	1/3	+	+	+	High	Low	Low
10	+	+	+	3/3	3/3	2/3	+	+	+	High	High	Medium

Table 3: Occurrence of target species using traditional and eDNA surveys.

Furthermore, the amplification rate difference among stations with different population densities (low, medium, and high) was significant (p < 0.0002), with a higher amplification success rate found in stations with higher population densities) using a general linear model. The success of PCR amplification was determined to be 0. 98 for stations with high population densities, 0.80 for stations with medium population densities, and 0.54 for stations with low population densities. The overall occurrence of targeted species based on eDNA was calculated as 90%. The effect of water temperature on eDNA concentration was found to be significantly positive (p < 0.001), while the effects of other parameters, such as pH, dissolved oxygen, and conductivity, were found to be insignificant. These findings suggest that eDNA detection is a powerful tool for monitoring the distribution and abundance of target native fish species in freshwater systems, with higher sensitivity and specificity than traditional survey methods.

Discussion:
eDNA has become a widely applied survey tool in molecular ecology (Dejean et al., 2012), allowing for the precise detection of aquatic species in rivers (Antognazza et al., 2021), streams (Curtis et al., 2021) and wetlands (Goldberg et al., 2018; Saenz-Agudelo et al., 2022) with success rates surpassing

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visual surveys. However, the success of eDNA detection is mainly dependent on sampling and analysis procedures rather than environmental factors (Dejean et al., 2011) such as pH, dissolved oxygen, and temperature. Precautions are necessary when using eDNA due to its easy degradation (Taberlet et al., 1996), and detecting eDNA from no longer present species is unlikely (Matsui et al., 2001). When transporting samples to the laboratory, it is essential to perform DNA isolations with blank controls to identify potential contaminant DNA, and testing primer specificity is crucial. To ensure primer specificity, it is necessary to test them first in silico. Next, high-quality DNA extracted from tissue samples of the target species should be used to test the reliability of the primers and optimize PCR conditions. Finally, eDNA samples should be tested to confirm primer reliability (Dejean et al., 2011; Ficetola et al., 2010). Furthermore, the study proved that traditional surveys cost 250% more in expenditure and time than eDNA surveys.

Triplicate samples were collected from each station. The average amplification success rates were determined to be 53.93% at stations with low population densities, 80% at stations with medium population densities, and 97.96% at stations with high population densities. At stations where target species were not detected through traditional surveys, the mean amplification success rate was 66.57%. Moreover, Ficetola et al. show an amplification success rate of 37% for the ponds where the target species were found at low densities and 79% for the ponds where the target species were found at high densities (Ficetola et al., 2008). Dejean et al. reported an amplification rate of 53% for the target species (Dejean et al., 2012). According to Takahara et al. (2013), eDNA was successfully amplified from all ponds where the target species were visually observed, and from 17.74% of ponds where the target species were not visually observed (Takahara et al., 2013).

To enhance the reliability of the PCR process, it's recommended to analyze more water samples and use a multi-tube approach (Taberlet et al., 1996). False positives can arise from various factors, but the specificity and reliability of primers are confirmed by the absence of amplification in false positives (Ficetola et al., 2015). Sampling and molecular technique optimization should also be maintained according to the ecosystem and target species. This study achieved eDNA detection using only 2.0 L of water sample (Seymour et al., 2018).

Estimates of the occurrence of the target native species were discovered to be higher than what was previously reported in a study that relied on traditional survey methods. This difference in estimates could be attributed to two possibilities: either the species densities have increased during the two-year period, or population densities were underestimated in the previous surveys that relied on traditional field methods (Thomsen et al., 2012). However, it is more likely that the underestimation occurred due to traditional surveys, as the results from this study were consistent with those from the previous one. This conclusion is further supported by comparing eDNA survey results with electrofishing, in which low densities of Indian major carp were only detected using the eDNA approach. After detecting Indian major carp's eDNA, the species presence was also confirmed using traditional surveys, only after 84 person-days of electrofishing effort. This finding underscores the effectiveness and accuracy of the eDNA method (Belle et al., 2019).

This study suggests that eDNA sampling is more accessible in summer and should be modified based on the target species' behavior and optimal water temperature. eDNA approach is a fast and cost-effective way to detect invasive species and should be considered a biological species monitoring tool (Harper et al., 2019). Future studies on detecting different species from various ecosystems will expand our understanding of the eDNA survey's applicability (Takahara et al., 2013). Precise and efficient monitoring tools are needed to detect invasive species and take appropriate action before populations become established.

Conclusion:
The study found that using eDNA to detect native Indian carp species is more effective and accurate than traditional survey methods like electrofishing. Estimates of invasive species occurrence were higher with eDNA sampling, and the method proved useful in detecting low densities of native Indian major carp fish. However, eDNA analysis could be more exceptional in its limitation, and it is also sensitive to environmental factors and requires careful consideration of sampling protocols. Overall, eDNA analysis is a promising tool for monitoring fish populations in freshwater ecosystems and can potentially revolutionize fish population management.

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Acknowledgements:
Authors are highly thankful to Kousik Das, Research Scholar Dept. of Geography and environment management, Deep sankar Chini, Pratik Ghosh and Prasanta Patra, Research Scholar, Dept. of Zoology, Vidyasagar University for adding their valuable information and help in writing this article.

Conflict of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

Availability of data and material
All data generated or analyzed during this study are included in this article.

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*Eur. Chem. Bull. **2023**, 12(Special Issue 10), 3776 - 3783*

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