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Optimization of DNA Extraction Methods for Reliable RAPD-PCR Analysis in *Harpadon nehereus* (Hamilton, 1822) from the Bay of Bengal: A Comparative Study for Fisheries Genetic Management

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Abstract: Effective genetic diversity assessment is pivotal for sustainable fisheries management, yet standardized DNA extraction methods for non-model marine species like *Harpadon nehereus* (Bombay duck) remain limited. This study optimizes DNA extraction protocols to enhance the reliability of Random Amplified Polymorphic DNA (RAPD)-PCR analysis for *H. nehereus* from the Bay of Bengal, a vital fishery resource. Four protocols—phenol-chloroform, liquid nitrogen-assisted lysis, and two modified methods—were evaluated for DNA yield, purity, and RAPD-PCR suitability using muscle, gill, fin, and ocular tissues. DNA quality was assessed via NanoDrop spectrophotometry (A260/A280: 1.43–1.95; A260/A230: 0.49–1.93) and agarose gel electrophoresis. Fourth optimized protocol (liquid nitrogen lysis with RNase and β-mercaptoethanol) yielded the highest DNA concentration (2546.68 ± 546.60 ng/µL) with optimal purity, producing consistent RAPD-PCR banding patterns using primer OPA-03 (5′-AGTCAGCCAC-3′). Modifications, including extended incubation, Proteinase K and RNase treatment, minimized contaminants, enabling clear detection of tissue-specific and population-level genetic variations. The optimized protocol reduced technical variability, offering a reproducible, cost-effective approach for genetic fingerprinting in resource-limited settings. This work establishes a robust framework for assessing genetic diversity, detecting population bottlenecks, and informing conservation strategies for *H. nehereus*, contributing to sustainable fisheries management in the Bay of Bengal. These findings advance molecular ecology by providing scalable solutions for genetic studies of commercially important marine species.

Keywords: DNA extraction optimization; RAPD-PCR; *Harpadon nehereus*; genetic diversity; fisheries management; molecular ecology; Bay of Bengal.

Research Paper

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1. INTRODUCTION

Harpadon nehereus (Hamilton, 1822), widely recognized as the Bombay duck, locally called 'Loitta fish' thrives in the estuaries and shallow nearshore waters of the Western Pacific and Indian oceans, with a prominent presence in the Bay of Bengal, which vast area is enriched with 475 fish species but only few of them are commercially harvested as target species (Barman *et al*., 2020; Yang *et al*., 2021; Hossain *et al*., 2022). Esteemed for its unique flavor and nutritional richness—up to 70% protein by dry weight, 19.5245% ω-3 PUFAs, and 1500–2500 mg/100 g calcium—this marine lizardfish supports vital commercial fisheries across Bangladesh, Pakistan, Southeast China, and India (Yang *et al*., 2021; Hossain *et al*., 2022; Ali *et al*., 2024). Its ecological resilience, driven by adaptability to salinity fluctuations and stable recruitment, supports populations in dynamic ecosystems like Tarakan waters and the Bay of Bengal (Sarker *et al*., 2017; Galib, 2011; Taqwa *et al*., 2022). However, genetic research on marine fish like *H. nehereus* lags behind terrestrial species, with molecular tools such as Random Amplified Polymorphic DNA (RAPD) underutilized compared to microorganisms, plants, and insects, underscoring a critical gap in

sustainable management (Satyaveer *et al*., 2023; Abied *et al*., 2014).

Understanding genetic diversity is essential for stock identification, selective breeding, and biodiversity conservation, bolstering resilience against pollution, diseases, and climate change in fisheries (Satyaveer *et al*., 2023; Chelomina *et al*., 2008; Ali *et al*., 2004; Barus *et al*., 2023). With fish representing over half of global vertebrate diversity (~34,800 of 68,582 species), molecular markers are indispensable, especially in regions like the Bay of Bengal (Satyaveer *et al*., 2023; Zhu *et al*., 2014). Genetic markers, particularly Random Amplified Polymorphic DNA (RAPD) markers, are essential for assessing genetic diversity and population structure in fish species, enabling stock identification and informing sustainable fisheries management strategies. Yet, there remains a significant knowledge gap regarding the genetic variation of *H. nehereus* in the coastal regions of Khulna and Chittagong, highlighting the need for targeted molecular studies (Ali *et al*., 2005; Megahed *et al*., 2010; Mahdi *et al*., 2018).

RAPD markers, requiring minimal DNA and no prior genomic knowledge, excel in detecting polymorphisms for phylogenetic studies, species identification, and population analysis across species like guppies, tilapia, carp, sturgeon, and catfish—where they have elucidated genetic variability, mapped pedigrees, and identified hybrids (Neekhra *et al*., 2014; Alege *et al*., 2021; Faddagh *et al*., 2012; Mojekwu & Megbowon, 2013; Foo *et al*., 1995; Ahmed *et al*., 2004; El-Zaeem *et al*., 2006; Comincini *et al*., 1998; Andrian *et al*., 2023). Beyond population genetics, RAPD has been employed to assess pathogen diversity, such as in *Plesiomonas shigelloides*, and to detect genotoxic effects of pollutants like arsenic and carbofuran in fish, highlighting its broader relevance to aquatic health (Gu *et al*., 2006; Jha *et al*., 2021; Tiwari *et al*., 2016).

Despite its potential, RAPD-PCR efficacy hinges on high-quality DNA, challenged by inhibitors like lipids and polysaccharides, necessitating optimized extraction methods—saline, phenol-chloroform, or SureFood PREP (Cawthorn *et al*., 2011; Chowdhury *et al*., 2016; Muhammad *et al*., 2016; Pereira *et al*., 2016; Ramón-Laca *et al*., 2021; Li *et al*., 2012; Silva *et al*., 2023; Xiong *et al*., 2019). Rapid DNA extraction innovations, like Li *et al*.'s 25-minute fin method and high-yield Proteinase K protocols, enhances scalability for RAPD-PCR in fisheries, though optimization is critical to mitigate degradation and ensure reproducibility (Li *et al*., 2012; Silva *et al*., 2023; Xiong *et al*., 2019).

Previous studies underscore RAPD's utility in fisheries, with Azrita *et al*. (2014) and Sahabuddin *et al*. (2019) showing geographic structuring in *Osteochilus vittatus* and *Siganus canaliculatus*, respectively, while Zhu *et al*. (2014) reported 75.20% polymorphic loci and a Nei's gene diversity of 0.2478 for *H. nehereus* in the East and South China Seas, though Bay of Bengal populations remain underexplored (Azrita *et al*., 2014; Sahabuddin *et al*., 2019; Zhu *et al*., 2014; Nei, 1972).

Morphological identification, common in Bangladesh, falters with closely related species, necessitating molecular approaches for precision (Alege *et al*., 2021).

This study addresses this gap by targeting *H. nehereus* from Chittagong to: (1) optimize DNA extraction from multiple tissues, reducing inhibitors like humic substances (Mezzomo *et al*., 2021; Thomson-Laing *et al*., 2022; Valsecchi, 1998); (2) refine RAPD-PCR conditions, building on primer and $MgCl_2$ adjustments from guppy, sturgeon and suckermouth catfish studies (Foo *et al*., 1995; Comincini *et al*., 1998; Mezzomo *et al*., 2021); and (3) find species-specific RAPD marker to initiate a genetic database, aiding identification and conservation (Mulyasari *et al*., 2023; Velkova-Jordanoska *et al*., 2014).

Recent advancements in molecular techniques, including RAPD-PCR for genetic variation analysis and eDNA metabarcoding with optimized protocols to reduce stochastic template loss, have revolutionized fisheries management by improving pathogen detection and enabling sustainable resource utilization in dynamic marine ecosystems (Stoeckle *et al*., 2022; Behera *et al*., 2022; Han *et al*., 2017).

By integrating these efforts, this research not only advances genetic profiling of *H. nehereus* but also contributes to global Seafood production, aligning with molecular advancements in species like Atlantic salmon (*Salmo salar*) and Pacific white shrimp (*Litopenaeus vannamei*) (Anderson *et al*., 2017; Angthong *et al*., 2020). Reliable, scalable protocols will empower data-driven conservation, ensuring the Bombay duck's ecological and economic legacy endures.

2. MATERIALS AND METHODS

2.1 Sampling Site:

Fish samples used in this study were taken from Banskhali Upazilla of Chittagong District, which is a coastal area beside the Bay of Bengal (Figure 1). In total, 5 fish samples were collected from this area. Then, the samples are to be brought to the Genetic Engineering and Marine Biotechnology Laboratory, Bangladesh Maritime University, and stored in frozen conditions until the DNA extraction is carried out. ArcGIS Pro was used to create a sampling site map using data that was readily accessible.

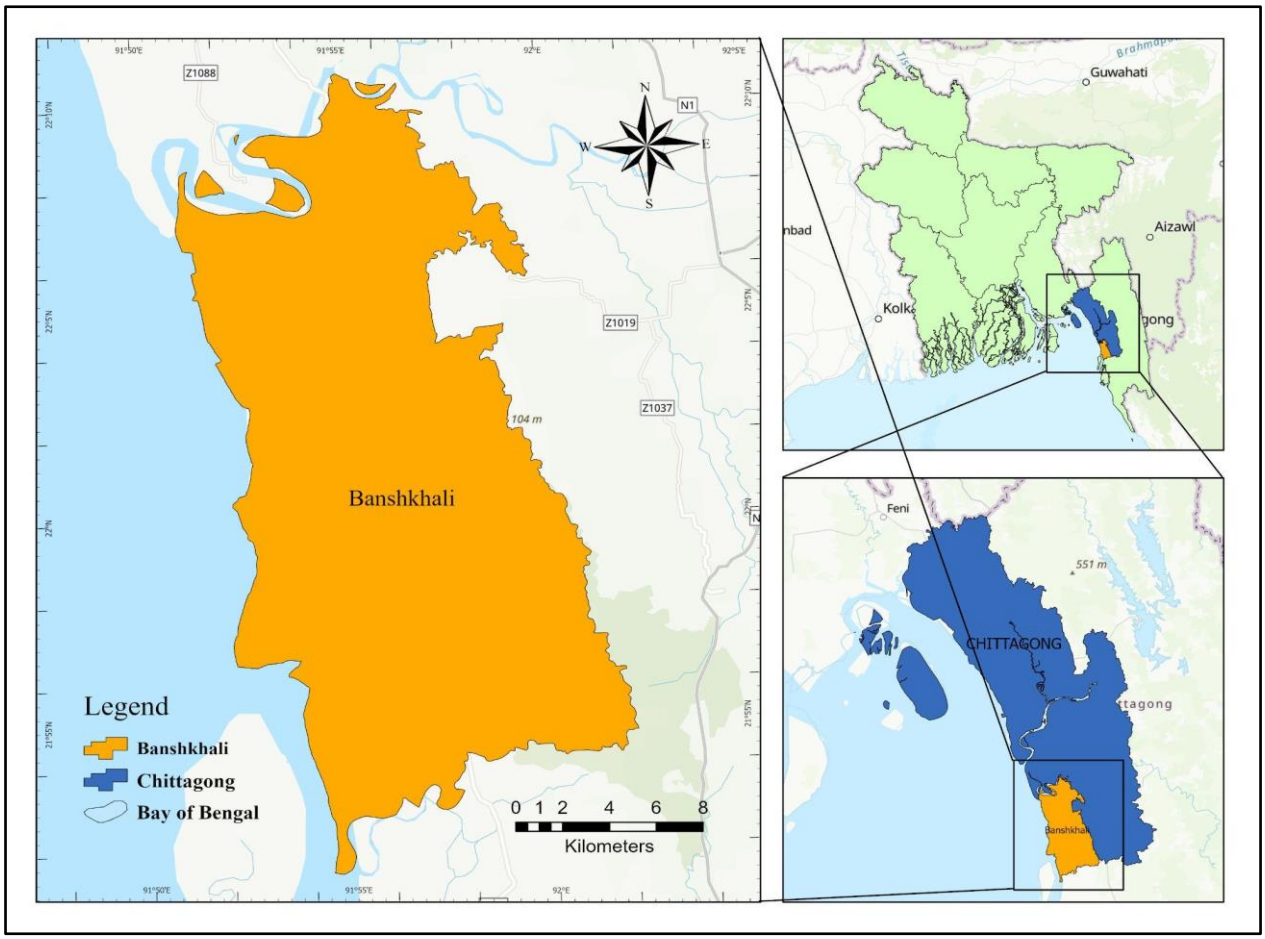


Figure 1: Geographical Location of the Sampling Area

2.2 DNA Extraction

All DNA extractions and quantifications were carried out at the Marine Biotechnology Laboratory, Department of Genetic Engineering and Marine Biotechnology, Bangladesh Maritime University (BMU). This study was primarily based on the methodologies described by Sahabuddin *et al*. (2019) and Mezzomo *et al*. (2021), which had been previously optimized for *H. nehereus* species. Building upon these, two additional protocols (Method 3 and Method 4) were proposed and evaluated by combining the most effective variables identified from the original methods for extracting high-quality DNA from *Harpadon nehereus*.

A total of six experimental variables were systematically tested to assess their influence on DNA concentration and purity:

1. **Temperature Conditions of Sample Preservation:** Tissues were stored at -4 °C, -10 °C, or -20 °C immediately after collection and transported in a frozen state to the laboratory.
2. **Forms of Sample Maceration:**
   Chemical lysis: Tissues were submerged in lysis buffer and incubated in a dry or water bath without mechanical agitation.
   Lysis with Liquid Nitrogen (LN_2): Tissues were triturated using liquid nitrogen before incubation in lysis buffer.
3. **Target Tissue Types:** Muscle, ocular tissue, gills, and fin samples were analyzed.
4. **Incubation Time in Extraction Buffer:** Two conditions were tested: 1 hour at 55 °C and 24 hours at 27 °C.
5. **RNase Treatment:** The presence or absence of RNase was evaluated for its effect on DNA purity.
6. **Storage Conditions After Extraction:** Extracted DNA was stored at either -40 °C or -20 °C.

Each variable was assessed for its effect on DNA yield and integrity. The outcomes were categorized as follows:

Optimal: Produced DNA with adequate concentration and purity.
Neutral: No significant effect observed.
Suboptimal: Resulted in low concentration, poor purity, or DNA degradation.

These variables were integrated into the optimization of Methods 3 and 4, which are fully described and compared in Table 3.

Table 1: Reagents and Their Compositions/Concentrations for Method 1 (Sahabuddin *et al*., 2019)

Reagent/Buffer	**Composition/Concentration**
Buffer lysis	0.5 M NaCl, 0.001 M EDTA, 1% (w/v) SDS, 0.8% (v/v) Triton X-100, 0.1 M Tris-HCl (pH 9.0)
SDS	10% (w/v)
Proteinase K	20 mg/mL solution
RNase	20 mg/mL solution
Phenol:Chloroform:Isoamyl Alcohol (PCI)	25:24:1
Chloroform:Isoamyl Alcohol	24:1
Ethanol	70%
TE buffer	10 mM Tris, 1 mM EDTA (pH 8.0)

Table 2: Reagents and Their Compositions/Concentrations for Method 2 (Mezzomo *et al*., 2021) and optimized protocol 3 and 4

Reagent/Buffer	**Composition/Concentration**
STE Buffer	0.1 M NaCl, 10 mM Tris, 1 mM EDTA (pH 8.0)
Lysis Buffer	2.5 mM Tris-HCl (pH 8.0), 0.1 mM EDTA (pH 8.0), 0.1% SDS, 8.0 mM NaCl
Proteinase K	20 mg/mL
CIA (Chloroform:Isoamyl Alcohol)	1:24 (v/v)
TE Buffer	10 mM Tris (pH 8.0), 1 mM EDTA (pH 8.0)
1x TAE Buffer	Diluted from 50x TAE (see below for 50x composition)
50x TAE Buffer Components	
- EDTA disodium salt	50 mM (molecular weight: 372.24 g/mol)
- Tris	2 M (molecular weight: 121.14 g/mol)
- Glacial acetic acid	1 M (molecular weight: 60.052 g/mol)

Note: 50x TAE Buffer should be diluted to a working concentration of 1x TAE for use

Table 3: Description and Comparison of DNA Extraction Steps for *Harpadon nehereus*

General Step	**Method 1 (Sahabuddin *et al*., 2019)**	**Method 2 (Mezzomo *et al*., 2021)**	**Method 3 (Modified)**	**Method 4 (Modified)**
Tissue collection	Collect 20 mg of fish flesh and place it into a 1.5 mL Eppendorf tube.	Collect 20 mg of fresh tissue	Collect 30-50 mg of fresh tissue	Collect 50 mg of fresh tissue
Washing	-	Wash in 500 µl of cold STE buffer and discard	Wash tissues with STE buffer (cold) and discard	
Tissue disruption	Direct lysis		Pour liquid nitrogen and mesh it with a pestle in a mortar	
Lysis and incubation	Add 500 µL buffer lysis, 40 µL SDS, 20 µL Proteinase K, incubate at 55°C for 1 h (dry bath)	Add 500 µl of lysis buffer, 2 µL of β-Mercaptoethanol (inject into it & up down) and 13 µl of Proteinase K (into it & up down) in this order, and incubate at 55° C for 1 hour (water bath)	Add lysis buffer and mix with meshed tissues, take 500 µl, add 2 µL β-Mercaptoethanol, 13 µl Proteinase K, incubate at 27 ºC for 24 hours	Add lysis buffer and mix with meshed tissues, take 700 µL, add 2 µL β-Mercaptoethanol, 13 µl Proteinase K, incubate at 55 ºC for 1 hour
RNase treatment	Add 12.5 µL RNase (20 mg/mL), store at room temperature for 20 min	-		Add 5 µL RNase after pellet washing, before drying
Extraction	1. Add 500 µL PCI (25:24:1), vortex slowly until homogeneous, then leave at RT for 10 min,	Add 700 µL CIA (chloroform:isoamyl alcohol, 1:24), homogenized by inversion, centrifuge at	Similar to Method 2	Similar to Method 2

	centrifuge at 13,000 rpm for 4 min 2. Transfer 750 µL supernatant to a new 1.5 mL Eppendorf, then add 500 µL PCI (25:24:1), leave at RT for 10 min, centrifuge at 13,000 rpm for 4 min 3. Transfer 600 µL supernatant to a new 1.5 mL Eppendorf tube, add 600 µL chloroform:isoamyl alcohol (24:1), equal volume to the supernatant, and centrifuge at 13,000 rpm for 4 min	9,000 rpm for 10 min, transfer the supernatant to a new tube, repeat this step 3x		
Precipitation	Transfer 400 µL supernatant to a new tube, add 800 µL cold 70% ethanol (twice the sample volume), and centrifuge at 6,000 rpm for 30 min	Transfer aqueous phase to a new tube containing 900 µL absolute isopropanol (cold), homogenize by inversion, incubate at -20°C for 2 h, centrifuge at 9,000 rpm for 15 min and discard the supernatant		Transfer 500 µL aqueous phase to 900 µL absolute isopropanol (cold), homogenize, incubate at -20°C for 2 h, centrifuge at 9,000 rpm for 15 min
Pellet washing	Remove supernatant, add 800 µL 70% ethanol to the base layer (pellet), shake gently 3x, centrifuge at 6,000 rpm for 15 min, then centrifuge again at 6,000 rpm for 6 min. After that, remove the 70% ethanol, leaving the DNA sediment (pellet) at the bottom of the tube as a white substance.	Wash pellet with cold analytical-grade ethanol		
Additional treatment	Not included			Add 5 µL RNase, discard volume
Drying	Air-dry pellet at room temperature for ~24 h	Discard all the volume and incubate at 37 ºC for 15 minutes		Discard all the volume, incubate at 37°C for 15 min (after RNase treatment)
Resuspension	Resuspend dried pellet in 100 µL distilled water (pH 7.0) or TE buffer.	Add 100 µL of TE buffer and incubate for 1 hour at approximately 27 ºC		
Storage	Store the DNA at -20 °C until further use.			Store at -40°C

Schematic overview of the experimental program

These figures (2-5) present a schematic overview of the experimental design, detailing the key steps from tissue collection and emaciation to the evaluation of variables across multiple DNA extraction protocols. To standardize DNA extraction for the molecular certification of processed fish, we compared four extraction methods, assessing the effects of tissue type (four types), storage conditions (two forms), tissue amount, RNase addition, and incubation conditions on the quality and quantity of extracted DNA.

Schematic DNA Extraction Process (**Sahabuddin *et al*., 2019**).

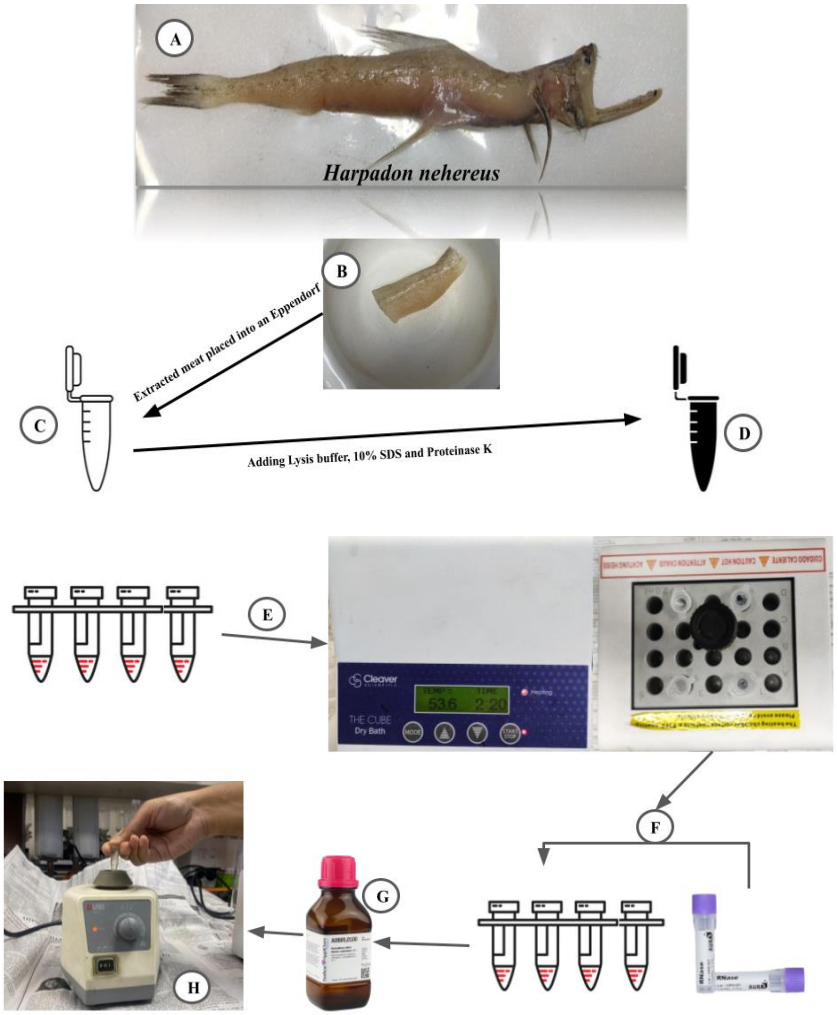
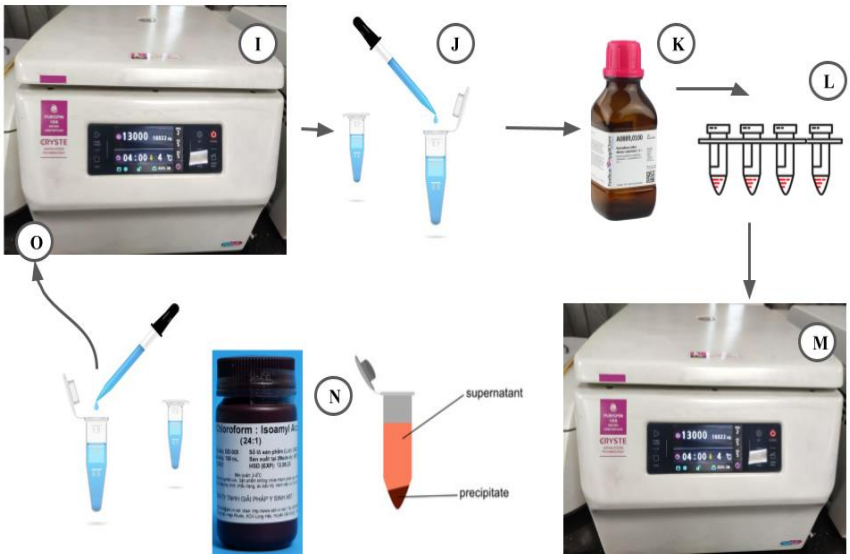


Figure 2: A) Specimen **B)** Extracted muscle **C)** Placed into a new Eppendorf **D)** Add buffer lysis, 10% (W/V) SDS and Proteinase K; **E)** Incubate at 55 °C for 1 hour; **F)** RNase was added and then stored at room temperature **G)** Added with 500 µL PCI: 25:24:1, **H)** Vortexed slowly until homogeneous, left at room temperature



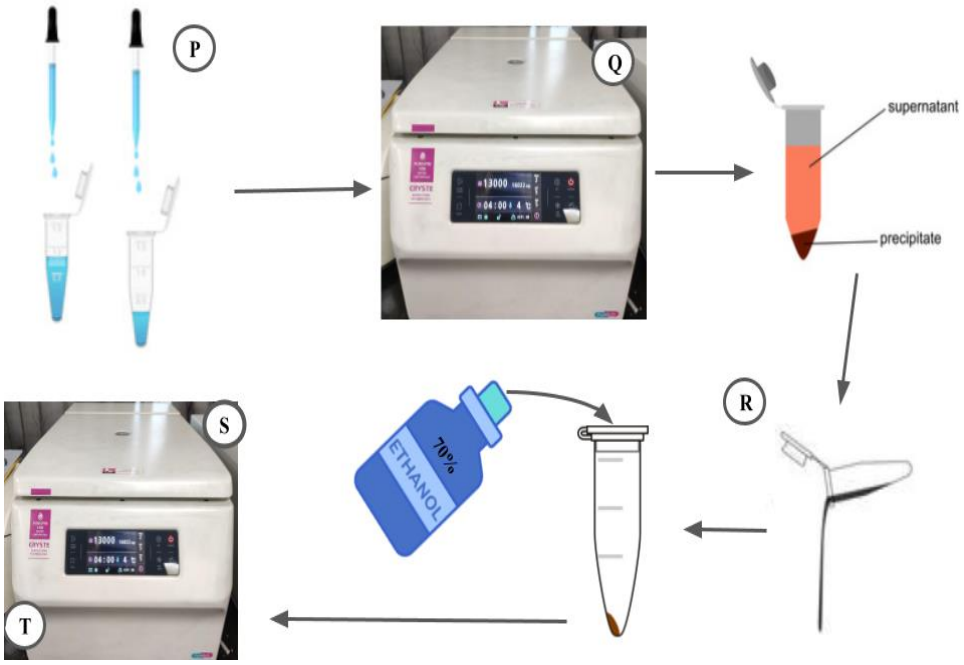


Figure 3: I) Centrifuged at a speed of 13,000 rpm for 4 minutes **J)** Liquid supernatant was transferred to a new Eppendorf tube **K)** PCI (25: 24: 1) was added **L)** The sample was left at room temperature **M)** Centrifuged at a speed of 13,000 rpm for 4 minutes; **N)** The supernatant was transferred to a new Eppendorf tube with a mixture of chloroform. Isoamyl Alcohol (24:1) was added to equal volume of supernatant **O)** Centrifuged at 13,000 rpm for 4 minutes **P)** Supernatant was added with a cold solution of 70% ethanol. **Q)** Centrifuged at a speed of 6,000 rpm for 30 minutes. **R)** The supernatant is removed, then the base layer (pellet) is added with 70% ethanol **S)** Gently shake the solution three times. Then, centrifuge it at 6,000 rpm for 15 minutes. **T)** Finally, centrifuge the resulting mixture again at 6,000 rpm for 6 minutes.

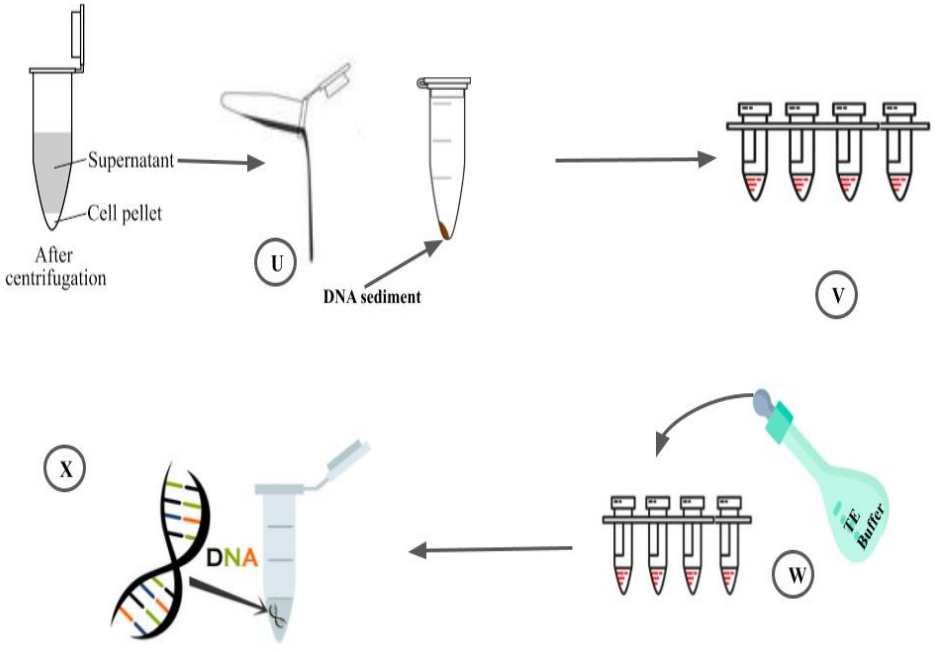
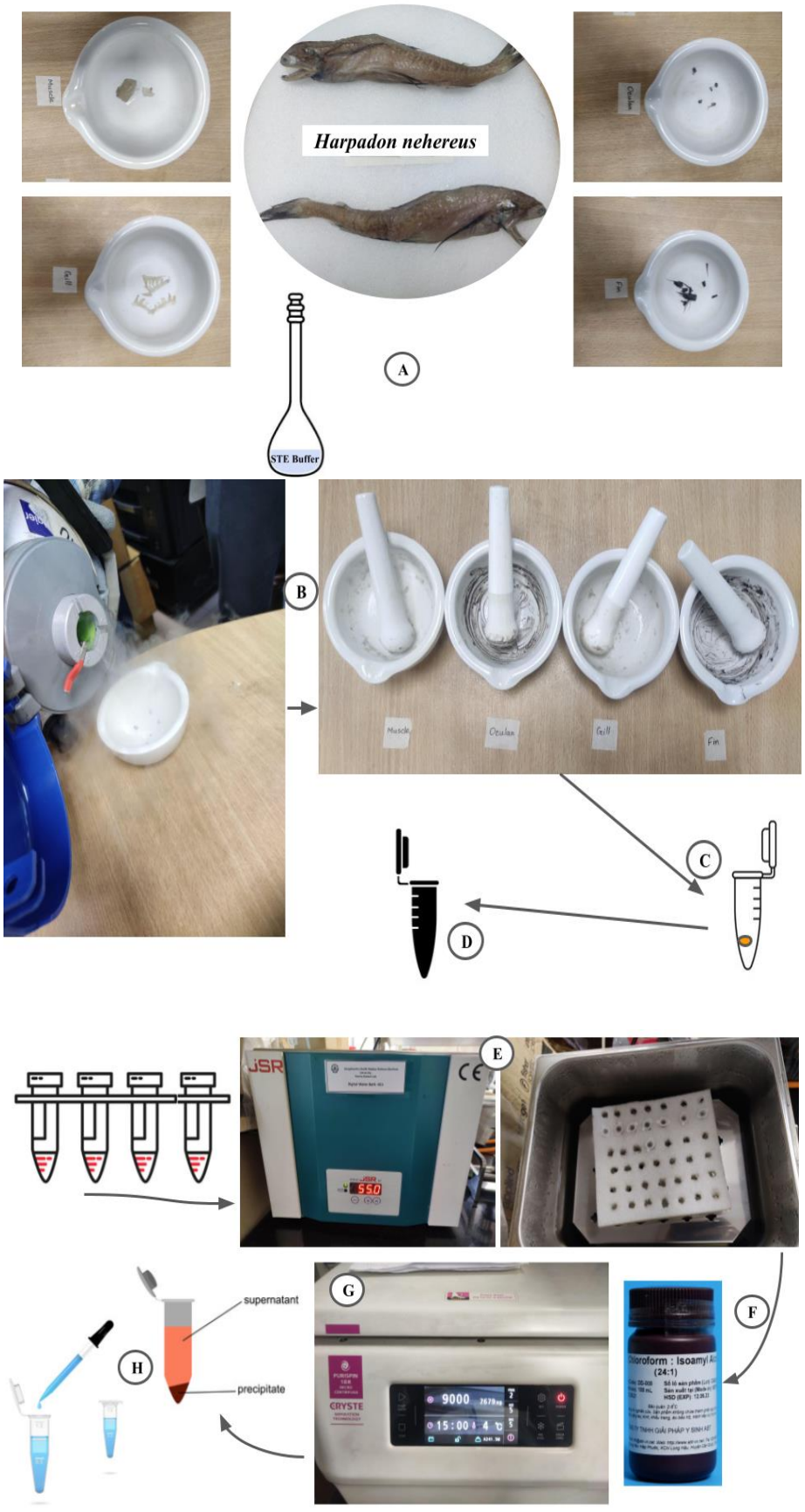


Figure 4: U) 70% ethanol solution is removed, leaving the DNA sediment at the bottom of the tube as a white pellet **V)** The DNA dried at room temperature for about 24 hours **W)** Adding TE buffer **X)** Store at -20 °C before being used for the next step

Schematic DNA Extraction Process (Mezzomo *et al*., 2021)



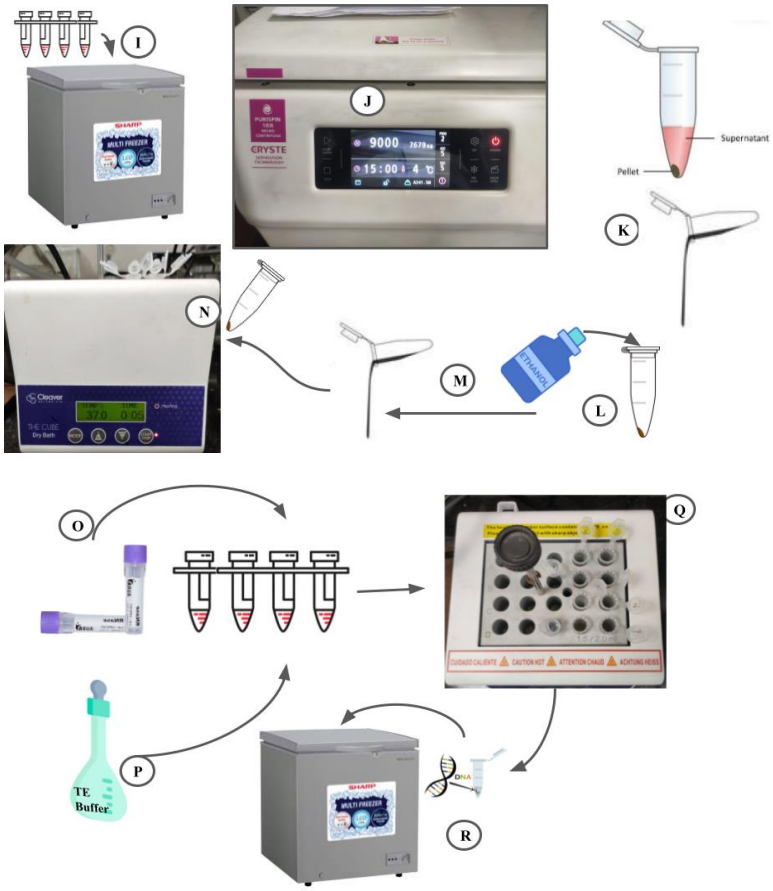


Figure 5: A) Collect sample tissues and Wash in 500 µl of STE (cold) and discard **B)** Lysis with liquid nitrogen (LN2): Samples triturated **C)** Fragments placed in tubes with lysis buffer **D)** Add 500 µl of lysis buffer, 2 µL of β-Mercaptoethanol (inject into it & up down) and 13 µl of Proteinase K (inject into it & up down) in this order, and **E)** Incubate at 55° C for 1 hour (water bath) **F)** Add 700 µl of CIA and homogenized by inversion. **G)** Centrifuge for 10 minutes at 9,000 rpm **H)** Transfer the aqueous phase (supernatant) to a new tube containing 900 µl of absolute isopropanol (cold); homogenized by inversion **I)** Incubate for 2 hours at -20°C **J)** Centrifuge for 15 minutes at 9,000 rpm K) and discard the supernatant **L)** Wash the resulting pellet with ethanol analytical grade (cold) **M)** Discard all the volume **N)** Incubate at 37°C for 15 minutes (dry bath) **O)** Add 100 µL of TE buffer **P)** Add 100 µL of TE buffer **Q)** Incubate for 1 hour at approximately 27°C (dry bath) **R)** Store at -20° C

DNA Concentration, Purity and Quantity Level Evaluation

The quantification (ng/µL) and the degree of DNA purity were evaluated using a NanoDrop (OPTIZEN™ NanoQ, Microvolume Spectrophotometer). Usually, values between 1.7 to 2.1 for the A260/A280 ratio and from 1.8 to 2.2 for the A260/A230 ratio are from pure DNA samples (Cawthorn *et al*., 2011; Lutz *et al*., 2023).

The concentration and purity of DNA were also determined for cross-checking by measuring the absorbance of diluted DNA solution at 260 nm and 280 nm by a spectrophotometer (1900i Shimadzu spectrophotometer, double beam, 190-1100 nm) according to the literatures' instructions (Tiwari *et al*., 2016; Cawthorn *et al*., 2011).

RAPD-PCR Technique and Gel Electrophoresis Method

The final quality assessment of the samples was verified through their amplification with RAPD marker. Samples that reached the above parameters of DNA concentration and yield were submitted to amplification with a primer of PCR-RAPD. Since the RAPD technique is sensitive to changes in reaction parameters (e.g., master mix, RAPD primer, NF water), the same reaction conditions were used for all samples. 1 µL of genomic DNA was amplified in 10.5 µL of NF water, 1 µL of RAPD primer (S51 or OPA-03), and 12.5 µL of master mix. The sequence of the OPA-03 primer is 5′-AGTCAGCCAC-3′.

With a final volume of 25 µL, the reaction was carried out under the following conditions: 40 cycles of denaturation at 95 ºC for 30 seconds, annealing at 50 ºC

for 1 minute, and extension at 72 ºC for 5 minutes (Mezzomo *et al*., 2021).

After applying the amplification results to a 2.5% agarose gel, they were stained with 1 mg/ml of ethidium bromide. At 150 V, the response lasts for 50 minutes. Band patterns were visualized and captioned in a picture under ultraviolet (UV) light.

The differential genomic patterns of the amplified DNA generated by using random primers were evaluated for the fish as well as marine fish. Primers were used to amplify the random sequences, and clearly detectable bands were recorded on agarose gel (Tiwari *et al*., 2016).

A flow diagram summarizing the experimental design of RAPD-PCR protocol and Gel Electrophoresis in Figure 6.

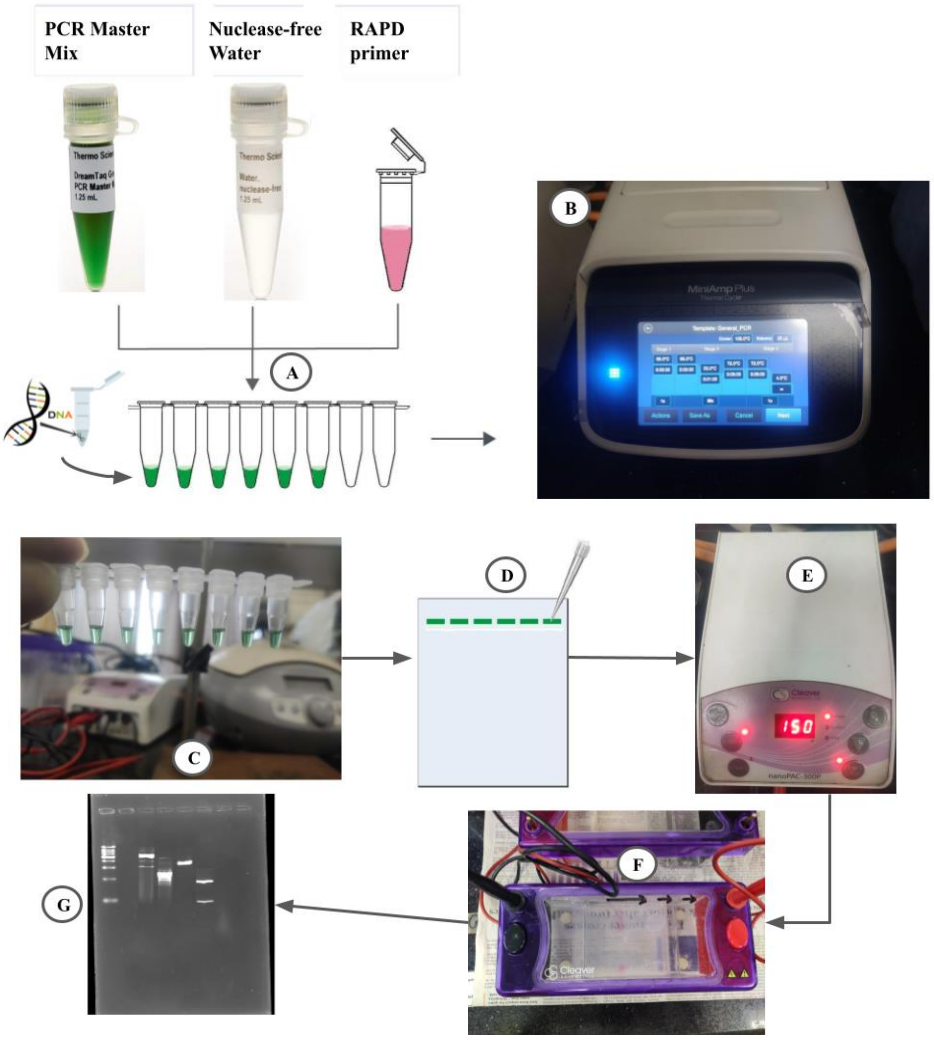


Figure 6: A) Take NF water, DNA, RAPD primer and Master mix into the PCR tubes **B)** Place the tubes in the MiniAmp™ Thermal Cycler and intimate the RAPD program **C)** PCR products D) Loading gel **E)** Set voltage for electrophoresis **F)** Gel run **G)** Visualization of RAPD band patterns

Data Analysis

All statistical analyses were conducted using Python (v3.6.0), for data visualization, Seaborn and Matplotlib libraries were used to generate high-quality bar plots illustrating DNA purity and concentration across tissues and extraction protocols. Shaded green areas on the graphs indicate optimal absorbance ranges (A260/A280: 1.7–2.1; A260/A230: 1.8–2.2), facilitating intuitive assessment of DNA quality across samples. Custom color schemes were applied to distinguish between the four methods and ensure clarity in visual interpretation.

Additionally, RAPD-PCR banding patterns were manually scored using GelAnalyzer 23.1.1, and the number of amplified bands, polymorphic bands, and polymorphism percentage were calculated according to standard protocols (Sahabuddin *et al*., 2019; Mahdi *et al*., 2018).

3. RESULTS AND DISCUSSION

3.1. Impact of DNA Extraction Method on Yield and Purity

This study demonstrates that DNA extraction protocols employing laboratory-prepared reagents, rather than commercial kits, can yield genomic DNA of sufficient quality for downstream molecular applications such as RAPD-PCR. DNA yield was markedly influenced by both the extraction technique and the tissue type utilized. Among all tested variables, the optimized in-house protocol developed in this study consistently produced the highest mean DNA concentrations across all tissue types. Notably, it also yielded significantly superior absorbance purity ratios (A260/A280 and A260/A230), indicative of minimal protein and organic contaminant interference, compared to the other evaluated methods. A detailed summary of the influence of individual variables on the final DNA yield and quality is provided in Table 4.

Table 4: Effect of Different Variables on the Total Yield of Extracted DNA from *H. nehereus*

Variable	Optimal	Neutral	Suboptimal
Specimens Conservation	-20 °C	-10 °C	-4 °C
Maceration	Lysis with Liquid Nitrogen (LN_2)	-	Chemical lysis
Target-Tissue	Ocular tissue	Muscle	Gill and fin
Time of Incubation	1 hour at 55 °C	24 hours at 27 °C	-
RNase	Presence	-	Absence
Proteinase K	Presence in high concentration (20 mg/ml)	-	-
Storage After Extraction	-40 °C	-20 °C.	-

3.2. Evaluation of concentration and purity of DNA

This study evaluated four DNA extraction protocols for *Harpadon nehereus* to optimize yield, purity, and suitability for RAPD-PCR analysis. DNA yield and purity were assessed across muscle, gill, fin, and ocular tissues using NanoDrop spectrophotometry and agarose gel electrophoresis. Among the methods tested, Method 4 (liquid nitrogen lysis with RNase) consistently produced the highest DNA concentrations, with a mean of 2546.68675 ± 546.6009533 ng/µL (Mean total amount ± standard deviation) across tissues, significantly outperforming other protocols ($p < 0.05$). Purity metrics for Method 4 were also superior, with A260/A280 ratios ranging from 1.82 to 1.95 and A260/A230 ratios from 1.75 to 1.93, indicating minimal protein and organic contamination. Muscle and ocular tissues yielded the highest DNA concentrations and purity, while gill and fin tissues showed lower yields and higher variability.

Table 5: Comparison of concentrations and purities of DNA extracted from 4 extraction methods. **[**MC = Muscle, OT = Ocular tissue, GL = Gills, FN = Fin.**]**

Method	DNA concentration (ng/µL)	Tissue type	A_{260}/A_{280} ratio	A_{260}/A_{230} ratio
1	17.88656	MC	1.43	0.86
	31.72136	OT	1.79	1.13
	21.31525	GL	1.8	1.24
	5.57903	FN	1.59	0.49
2	500.7423	MC	1.88	1.55
	1011.755	OT	1.93	1.7
	256.21153	GL	1.85	1.63
	111.28073	FN	1.51	0.56
3	666.69798	MC	1.9	1.69
	948.61227	OT	1.83	1.79
	424.37487	GL	1.57	1.11
	220.50704	FN	1.49	1.61
4	2685.921	MC	1.85	1.84
	3205.106	OT	1.95	1.86
	2398.15	GL	1.82	1.75
	1897.57	FN	1.86	1.93

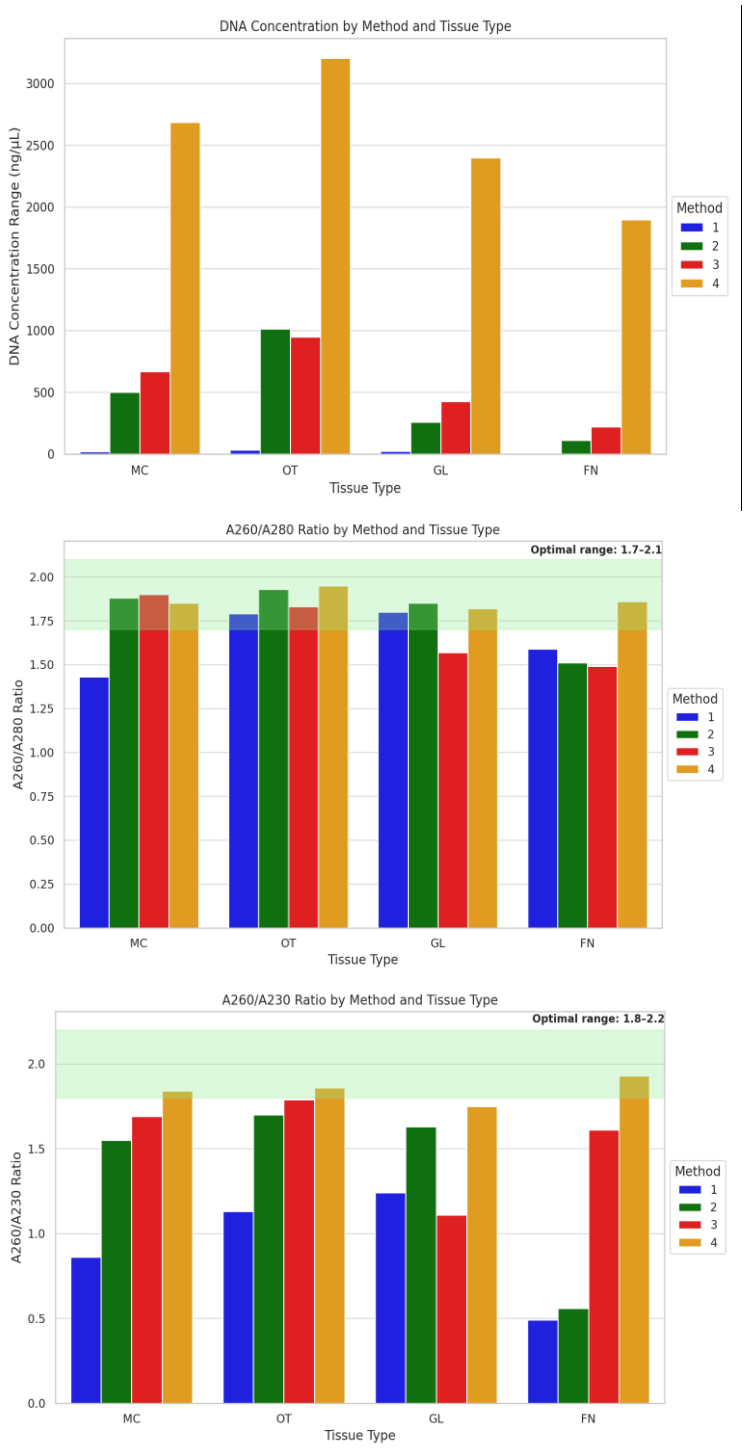


Figure 7: Genomic DNA extraction efficiency of *H. nehereus* varies dependent on the extraction method and target tissue

3.3 PCR-RAPD Amplification

RAPD-PCR analysis using primer OPA-03 (5′-AGTCAGCCAC-3′) generated reproducible banding patterns, with an average of 10 amplified bands per sample and 70% polymorphism, confirming the suitability of the extracted DNA for genetic fingerprinting. Methodological enhancements, including β-mercaptoethanol addition and extended incubation, were critical in reducing inhibitors and improving DNA integrity, particularly for challenging tissues like gills and fins. These findings demonstrate that Method 4 provides a reliable, high-quality DNA extraction protocol for downstream molecular applications in *H. nehereus*.

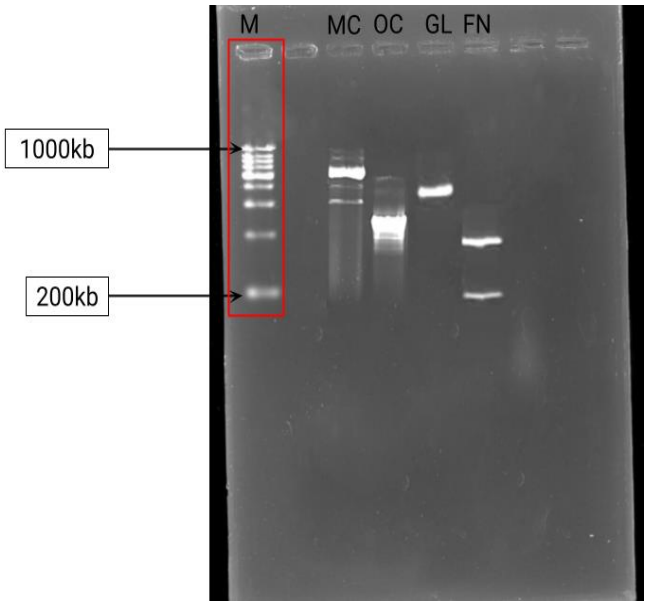


Figure 8: Randomly amplified polymorphic DNA (RAPD) fragmention patterns generated with Primer OPA-03 in fish *H. nehereus*; Lane 1 represents patterns of marker DNA; Lane 3-6 represent DNA patterns of fish of different tissue types; [M = Marker, MC = Muscle, OT = Ocular tissue, GL = Gills, FN = Fin.]

Table 6: Nucleotide sequence of RAPD primer from Operon Technologies, USA used in this experiment

Primer	Oligonucleotide primer sequence (5' → 3')	Length of Oligonucleotide	GC content (%)	Tm value
OPA-03	5' AGT CAG CCA C 3'	10-mer	60	26.8

Table 7: Total number of amplified fragments, number of polymorphic bands and percentage polymorphism generated by PCR primer

Fragmentation Number	10 bands
Polymorphic Bands	7 bands
% Polymorphism	70%

4. CONCLUSION AND RECOMMENDATION

This study optimized DNA extraction for *Harpadon nehereus*, a vital fishery resource in the Bay of Bengal, improving RAPD-PCR reliability for genetic diversity assessment. Method 4, employing liquid nitrogen-assisted lysis, Proteinase K, and RNase, delivered high-quality DNA with optimal purity and concentration, as verified by NanoDrop and electrophoresis, while RAPD-PCR with primer OPA-03 generated consistent banding patterns, confirming its effectiveness for genetic fingerprinting. These results highlight the value of standardized extraction techniques in reducing variability in molecular studies of non-model marine species. We recommend adopting Method 4 as a standard protocol, prioritizing muscle and ocular tissues, and standardizing RAPD-PCR conditions for reproducibility, while expanding population genetic research across the Bay of Bengal to detect genetic bottlenecks and guide conservation efforts. Integrating this approach with eDNA metabarcoding could facilitate non-invasive monitoring, and building local capacity in molecular ecology will support sustainable fisheries management.

Author Contribution

M. R. Washikur—Conceptualization, provider for the acquisition of data and writing, investigation, finalization of the methodology and analysis and interpretation of data, software analysis, development of study area map, writing of manuscript content, drafting of the manuscript, review of the manuscript and provided input in writing, development of citations and references**,** language correction, draft review.

Md. Abdullah Al Masud - Assisted with laboratory work.

Hussain Abbas Uddin - Assisted with laboratory work.

Madia Manjum Mohona - Assisted with laboratory work.

Shomaya Akhter - Assisted with laboratory work, contributed to research design and study conception, provided critical review, participated in investigation, and suggested solutions for analysis and comparison.

Corresponding Author: Professor Dr. Mohammad Nazir Hossain – Conceptualization and Supervision. Develop an original idea for this research. Monitor and guide, provide the necessary reagents and instruments, give a critical review, and finalize the full manuscript.

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Data Availability: The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest: The authors declare that they have no conflicts of interest.

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