Isolation of eDNA From Estuarine Water

Dissertation Thesis Presented to Goa University

As a Partial Fulfilment of the Requirements for Obtaining the degree of

Master of Science in Marine Biotechnology

In the academic year 2022-23

By

Abhranil Doloi

With the guidance from

Prof. Sanjeev Ghadi

School of Biological Science and Biotechnology

Goa University,

Goa-403206, India

Declaration

I affirm that the dissertation titled **"Isolation of eDNA From Estuarine Water"** which I have submitted as partial fulfilment of my M.Sc. Marine Biotechnology degree in the School of Biological Science and Biotechnology at Goa University, is entirely my own work. The research was conducted between October 2022 and April 2023, and no part of it has been previously submitted for another degree at any other university. I have appropriately acknowledged any assistance or guidance received and have provided appropriate citations for all relevant literature.

Abhranil Doloi

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Date:

Prof. Sanjeev Ghadi

Biotechnology,

School of Biological Sciences and Biotechnology

sanitaherkar 12/5/23

Dean of School of Biological Sciences & Biotechnology
Goa University, Goa-403206
Office Ph. 8669609246

Prof. Savita S. Kerkar

Dean,

School of Biological Sciences and Biotechnology

Goa University

Date:

School Stamp

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_____

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Dean,

School of Biological Sciences and Biotechnology School Stamp

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Once again, thank you to all!

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List of abbreviation

uL	Microliter
mL	Millilitre
°C	Degrees Centigrade
DNA	Deoxyribonucleic acid
g	Gram
HTS	High-throughput sequencing
h	Hour
PCR	Polymerase Chain Reaction
rpm	Revolutions per minute

Abstract

The preservation of biodiversity in the face of increasing human pressure is hindered by our limited knowledge of species occurrence, distribution, abundance, habitat requirements, and threats. Efficient and sensitive methods are needed to obtain this information, particularly for rare, cryptic, and elusive species. One promising technique for the above is environmental DNA (eDNA), which can improve our ability to detect and quantify biodiversity, while overcoming some of the challenges of labour-intensive traditional surveys. The use of eDNA in ecology and conservation has increased rapidly without a corresponding increase in understanding its strengths and limitations. Imperfect detection, abundance quantification, taxonomic assignment, eDNA temporal and spatial dynamics, data analysis and interpretation, and assessing ecological status are all potential issues. Careful evaluation of the technical complexities and challenges involved in eDNA is essential. Therefore, it is important to assess the scope and relevance of eDNA-based studies and identify critical considerations before using this approach.

Contents

CHAPTER 1

Introduction and Literature Review

Introduction

1.1 Environmental DNA

Environmental DNA (eDNA) refers to the genetic signature that living organisms leave in aquatic or sediment environments. This genetic material can include whole cells, extracellular DNA, or even entire organisms (Barnes et al., 2021; Ficetola et al., 2008). eDNA is retrievable from an environment, where a small portion can be amplified and sequenced. The resulting sequence can then be utilized to identify various species existing in that environment (Deiner et al., 2017). eDNA can be isolated from various sources such as including roots, leaves, pollen, fruit, cells, skin, blood, mucus, saliva, sperm, eggs, feces, urine, and the decaying remains of larger animals (Barnes & Turner, 2016; Bohmann et al., 2014; Taberlet, Coissac, Pompanon, et al., 2012).

Despite being a relatively new surveying method, eDNA analysis has demonstrated significant promise in environmental monitoring. Unlike traditional taxonomic identification procedures that often involve capturing live or dead animals, eDNA only requires the genetic traces left behind by an organism, thereby minimizing habitat destruction and disruption. While eDNA may not provide population quality information, such as sex ratios, it can effectively detect endangered, invasive, elusive, and rare species (Deiner et al., 2017; Goldberg et al., 2016).

Recent advances in eDNA research methods now allow for the examination of entire communities from a single sample. While metabarcoding has a long history in microbiology, it is only beginning to gain momentum in the evaluation of macro-organisms (Deiner et al., 2017; Coissac et al., 2012; Creer et al., 2016) Contamination during metabarcoding of an ecosystem may lead to inaccurate results such as false positives or false negatives, despite the method's ability to detect both macro and microorganisms on a large scale (Ficetola et al., 2016; Hering et al., 2018). In general, eDNA metabarcoding offers several benefits compared to traditional methods, such as improved accuracy, faster identification, and lower costs. Nevertheless, to fully utilize its potential in ecological research, standardization and unification are necessary, which should include both taxonomy and molecular approaches (Coissac et al., 2012; Cristescu, 2014; Yu et al., 2012).

Literature Review

1.2 eDNA Extraction and Analysis

The methods for studying eDNA vary significantly among published studies, posing a challenge for comparing results between them. The uniqueness of each species being studied and the location of the research make it challenging to establish a standardized approach (Hering et al., 2018) The widespread use of metabarcoding in biodiversity research and assessment has the potential to establish some level of standardization in methodologies (Cristescu, 2014).

Following workflow summarizes various methods, from sample collection up to data study.

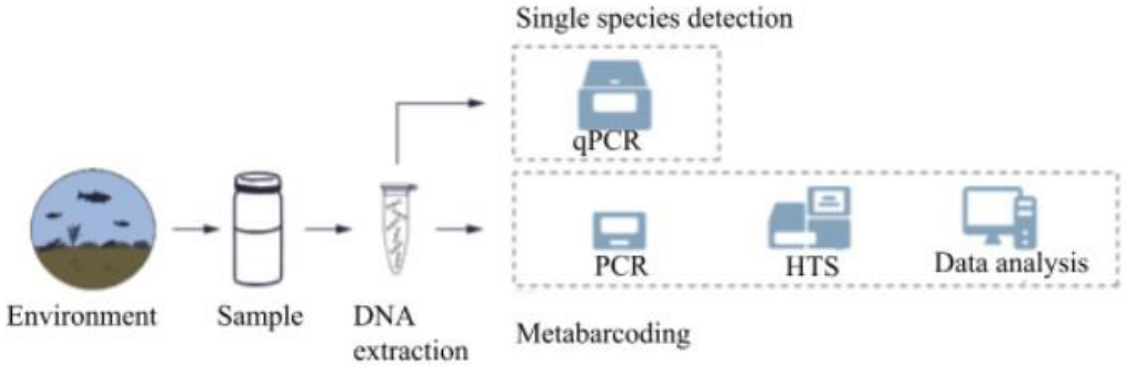

Fig. 1.2 Workflow of eDNA study

1.2.1 Collection Methods

To extract eDNA from water samples, the most used methods are filtration, ethanol precipitation, and centrifugation. Each approach has its own advantages and disadvantages, and the optimal method should be selected based on the research objectives and the properties of the samples being analysed. Filtering is the most employed technique for enriching eDNA in water samples. It holds great potential for obtaining a higher yield of eDNA as it can process large volumes of water, usually between 0.1 to 1 litre, as compared to other methods. The choice of filter material and pore size can significantly impact eDNA collection, with pore diameters ranging from 0.02 to 0.4 μm appearing to be the most effective at capturing macro-organism eDNA (Barnes et al., 2014) If the water sample has an excessive number of suspended particles, small pore filter papers can become

blocked easily. Filtering is the most widely utilized method for concentrating eDNA in water samples, as it has the potential to process large volumes of water, generally ranging from 0.1 to 1 litre, compared to other approaches. The selection of filter material and pore size can significantly influence eDNA yield, with pore diameters ranging from 0.02 to 0.4 μm being most effective in capturing macro-organism eDNA.

Ethanol precipitation is a commonly used method for extracting eDNA from small water samples, typically around 15 ml in volume (Deiner et al., 2015; Doi et al., 2015) This method is particularly advantageous for research conducted in challenging environments such as high altitudes or jungles, where access to electricity and specialized equipment may be limited. Unlike other methods, it requires fewer instruments and is capable of instantly recovering any eDNA present in water samples. However, it is important to note that the yield of eDNA recovery through this method may be limited by sample volume, in contrast to the ethanol precipitation method (Yamamoto et al., 2016). If the eDNA level of the desired species is elevated, then the use of ethanol precipitation might be a suitable option (Doi et al., 2015)

Centrifugation is a viable method for directly extracting eDNA from water samples as well (Klymus et al., 2015; Takahara et al., 2013) Although not commonly used, this method's simplicity could be beneficial when testing many samples. Nevertheless, the amount of sample water that can be centrifuged depends on the centrifuge device, which may only allow small quantities.

Irrespective of the methodology employed, handling of water samples should be cautious until eDNA is extracted. The decay of eDNA takes place gradually, and its breakdown speed amplifies with elevated temperatures (Tsuji et al., 2017). Therefore, it is essential to extract or preserve the eDNA from the collected water sample promptly, preferably under conditions of low temperature.

Table 1.2.1 Procedures for extracting eDNA from varying volumes of aquatic samples.

For 15ml of Sample	For 1-2 litre of Sample
1.5ml of $CH_3COOHNa$ + 33ml EtOH absolute ↓ Centrifuge at 5500g, 35 min, 6°C ↓ Discard the supernatant Pellet is subjected to Classical DNA extraction (Ficetola et al., 2008)	Membrane Filtration ↓ 2ml EtOH absolute ↓ Centrifuge ↓ Discard the supernatant Pellet is subjected to classical DNA extraction (Laramie et al., 2015)

1.2.2 Commonly Employed Extraction Methods

Standardization of eDNA extraction methods is yet to be achieved, which leads to a significant influence of the filter-based eDNA extraction method on eDNA detection (Deiner et al., 2015; Yamamoto et al., 2016) For the extraction and purification of eDNA, commercial DNA extraction kits or liquid phase separation techniques are commonly utilized.

Ficetola et al. (2008) popularized the use of commercial DNA extraction kits for the extraction and purification of eDNA. Since then, the method has been adopted by many researchers due to its experimental simplicity and higher recovery efficiency.

However, it is important to note that the choice of DNA extraction kit should be carefully considered for each study based on the conditions and/or purpose of the research. This is because

the kit's performance is determined by the combination of eDNA collecting method and the inhibitory content of the sample.

Different commercial kits have varying protocols and efficiencies for extracting and purifying eDNA, and therefore, the selection of the appropriate kit is crucial for obtaining reliable and accurate results. It is recommended that researchers evaluate the efficacy of different kits and choose the most suitable one based on their specific experimental conditions.

In 2013, liquid phase separation techniques were initially employed, utilizing cetyltrimethylammonium bromide (CTAB) and phenol-chloroform-isoamyl alcohol (PCI) (Turner et al., 2014). These techniques showed that PCI recovered a higher amount of DNA from membrane filters in comparison to commercial DNA extraction kits (Deiner et al., 2015; Renshaw et al., 2015).

Despite the higher yield, the use of hazardous compounds in PCI necessitates careful reagent handling and proper waste disposal. As such, caution should be exercised when using this method.

1.2.3 Widely Used Detection Method

There exist two main categories of eDNA detection: (1) species-targeted detection, and (2) eDNA metabarcoding (Thomsen & Willerslev, 2015; Tsuji et al., 2019a) The most frequently utilized technique is species-specific detection, which can detect endangered or invasive species with great accuracy. Meanwhile, eDNA metabarcoding is employed to monitor aquatic and sediment biota since it can detect all organisms in the sample (Jerde & Mahon, 2015).

The detection of a specific species is typically accomplished through PCR, which utilizes primers that are specific to that species to amplify and identify small fragments of DNA (usually 80-200 base pairs) (Bohmann et al., 2014). Mitochondrial DNA (mtDNA) is frequently utilized as a genetic marker in these studies because of its high mutation rate, large number of copies per cell, and extensive representation in genetic databases (Goldberg et al., 2016; Handley, 2015). The main target regions of mtDNA include cytochrome b (Cytb), cytochrome c oxidase subunit 1 (COI), D-

loop, 12S ribosomal RNA (12S), 16S ribosomal RNA (16S), as well as the internal transcribed spacer (ITS) region of nuclear DNA.

The detection of the DNA of the target species is primarily accomplished through one of three methods: (1) electrophoresis of PCR amplicons on a gel; (2) real-time quantitative PCR; or (3) digital PCR.

eDNA metabarcoding involves the use of universal primers that amplify a small section of DNA with sufficient sequence diversity to accurately identify species within a specific population (Miya et al., 2015). These amplified DNA fragments are subsequently analysed through high-throughput sequencing (HTS) of the target barcoding region from eDNA samples of different species. Bioinformatics methods are then employed to match each obtained DNA sequence to a recognized taxonomic group.

1.2.4 Data Analysis Methods

A series of software are used to transform raw sequence data into an OTU-table, which contains Operational Taxonomic Units as rows and samples as columns. This process, called the bioinformatics component of the metabarcoding method, involves five phases: (1) Demultiplexing samples, (2) Reads joining, (3) Quality filtering, (4) OTU clustering, and (5) Taxonomic assignment. Different software can handle one or more of these steps. They can be called successively via command-line or bash scripts to form an analytic pipeline. MOTHUR, USEARCH, QIIME, OBI Tools, and VSEARCH are toolkits designed for eDNA metabarcoding data analysis. Additionally, SLIM, an open-source web application, can be utilized to process metabarcoding data starting from raw sequences and producing an annotated OTU table (Dufresne et al., 2019)

1.3 Direct PCR System

Direct PCR is a technique that enables the amplification of DNA without the need for prior purification or quantification. This approach involves directly amplifying crude DNA samples, which can save time and resources in the preparation of DNA templates for PCR analysis (Bergkessel & Guthrie, 2013). The reduction of pre-PCR procedures from days to hours enables efficient processing of large-scale samples and lowers extraction expenses. Nevertheless, the limited success rate of the method is attributed to inhibitory compounds and low DNA yield. As a result, there are only a few direct PCR techniques developed, despite their advantages (Rogers & Parkes, 1999). While liquid phase separation methods have been utilized for eDNA extraction in some reports (Tsuji et al., 2019), a direct PCR method for eDNA has not been documented yet.

CHAPTER 2

Aims & Objectives

2.1. Aim of The Project

The objective of this project is rapid extraction of eDNA from Estuarian sample by both NaOH based and kit based eDNA methodology.

2.2. Objectives

- Implications of two different types of eDNA extraction methodology (NaOH based and kit based) and to avoid hazardous chemicals while extraction.
- Performing PCR amplification using various PCR primers to amplify eDNA.

CHAPTER 3

Materials & Methods

3.1 Equipment Used

3.1.1 NaOH Based Method

HDPE bottle, Filtration Unit, Filter Paper, Syringe, Micropipettes, Tips, Centrifuge Tubes, Centrifuge, Water bath, Qubit Fluorometric Quantification, Thermal Cycler, Gel Electrophoresis Unit.

3.1.2 Kit Based Method

NucleoSpin eDNA Water commercial kit (MACHEREY-NAGEL, n.d.), Water collection tank, Filtration Unit, Filter Paper, Syringe, Micropipettes, Tips, Microcentrifuge Tubes, centrifuge, Qubit Fluorometric Quantification, Thermal Cycler, Gel Electrophoresis unit.

3.2 Reagents Used

Sodium Hydroxide (NaOH), Ethanol, EDTA, Tris HCl, Triton-X-100, Tween20, Distilled Water, Taq Polymerase (supplied with 10x buffer containing $MgCl_2$), dNTPs mix, Agarose, TAE buffer.

3.3 Sample Collection

3.3.1 For NaOH based Method

In November 2022, 3 Litre of Estuarian water subsamples were collected from the Mandovi River, (15.501737, 73.875426) Panjim, Goa, using a 100 mL measuring cylinder. Subsamples were homogenized by compiling them in a 5 L HDPE bottle. Before collecting samples all the equipment were washed with 10% of HCl. After being collected, the samples were promptly transported to the laboratory where they underwent filtration. To filter the samples, 1 litre aliquots were extracted from the 3-liter samples and vacuum filtered through a membrane filter.

3.3.2 For Kit based Method

In March 2023, 3 litre of water subsamples were collected from Zuari River, (15.403304, 73.926831sss) Ponda, Goa, by using 100 mL of measuring cylinder. Subsamples were homogenized by compiling them in a 5 L HDPE bottle. Before collecting samples all the equipment were washed with 10% of HCl. After being collected, the samples were promptly transported to the laboratory where they underwent filtration. To filter the samples, 1 litre aliquots were extracted from the 3-liter samples and vacuum filtered through a membrane filter.

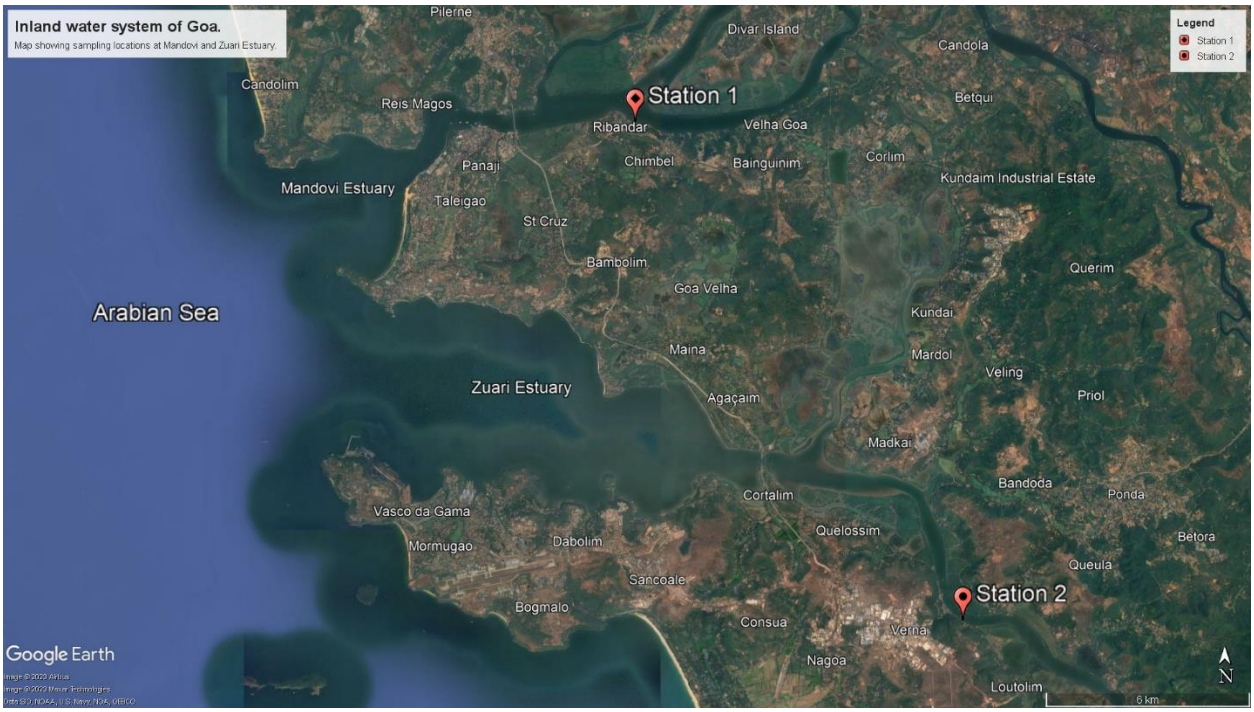

Fig3.3.2.1 Sampling Site. Station 1: Mandovi Estuary, Station 2: Zuari Estuary

3.4 Sample Filtration

3.4.1 For NaOH Based Method

Nitrocellulose mixed ester membrane filters with a pore size of 0.2 μm and a diameter of 47 mm wer used to filter 1 L estuarian samples in triplicate. The filtration cup was mounted with the filter and secured to a filtration unit that was connected to a vacuum pump. Prior to filtration, all supplies were sterilized with 10% bleach. The isolated eDNA was immediately obtained from the membranes. As a negative control, 1 litre of Milli Q water was also filtered.

3.4.2 For Kit Based Method

Glass Fibre Filter papers (diameter of 47 mm) were used to filter 1 L estuarian water samples in duplicate. The filtration cup was mounted with the filter and secured to a filtration unit that was connected to a vacuum pump. Prior to filtration, all supplies were sterilized with 10% bleach. The isolated eDNA was immediately obtained from the membranes.

3.5 eDNA Extraction Protocol

3.5.1 NaOH Based Extraction Protocol

After completing the filtration process, the filter paper was carefully rolled with flame-sterilized forceps and placed into a 15-mL microcentrifuge tube containing 5 mL of NaOH-ethanol solution (Table 3.5.1.1). The tube was then heated to 80°C for 10 minutes in a water bath. Using sterile forceps, the membrane filter was gently extracted and transferred into a 1 mL sterile syringe. Any remaining liquid was expelled by compressing the membrane filter, and the extracted liquid was added to the 15-mL tube. The tube was subsequently centrifuged for 10 minutes at 10,000 RPM and the supernatant was removed. To solubilize the denatured DNA, 100 μL of elution buffer (Table 3.5.1.2) was added and the sample was stored at -20°C.

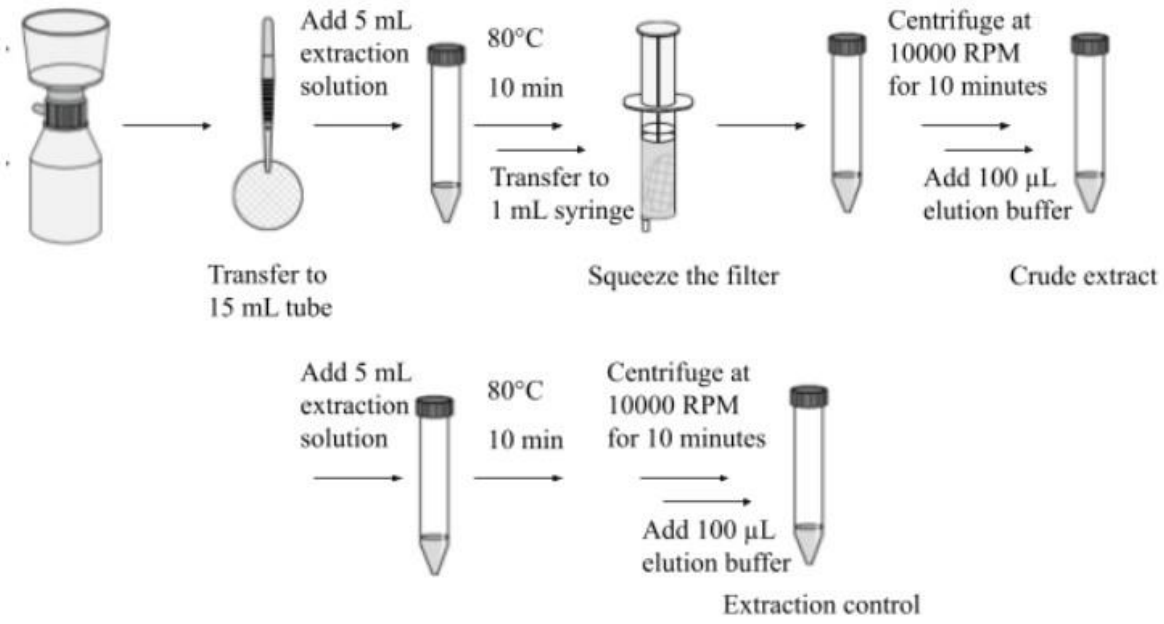

Fig 3.5.1 Schematic illustration of extraction technique.

Table 3.5.1.1 NaOH-EtOH based eDNA extraction reagents (Vingataramin & Frost, 2015).

Reagents	**Volume (mL)**	**Concentration**
NaOH 2 M	5.5	240 mM
Ethanol 96%	35	74%
EDTA 0.025 M	5	2.7 mM
Final Volume	45.5	

Table 3.5.1.2 Tris-EDTA based elution buffer (Vingataramin & Frost, 2015).

Reagents	Volume (mL)	Concentration
Tris-HCl, 0.5 M, pH 8	5	50 mM
EDTA 0.5 M	0.01	0.1 mM
Tritin-x-100	0.5	1%
sTween20	0.25	0.5%
Mili Q Water	44.25	
Final Volume	50.01	

3.5.2 Kit Based Extraction Protocol

eDNA was extracted using Nucleospin kit by following the instruction mentioned in the manual.

3.6 PCR Amplification

The extracted eDNA was amplified by conducting a PCR reaction using a thermal cycler to amplify the 16S, ITS2, and rbcL genes.

The 16S rRNA gene encodes the RNA of the small subunit of the ribosome in bacteria, and is present in all bacterial cells as well as in eukaryotes. Studies on 16S rRNA sequences from multiple organisms indicate that certain regions of the molecule undergo rapid genetic changes, allowing for differentiation between different species within the same genus (16s rRNA - n.d.).

The ITS2 region within the nuclear ribosomal DNA is considered a promising DNA barcode option due to its advantageous traits. These include the presence of conserved areas that allow for

the creation of universal primers, straightforward amplification, and ample variability that enables the differentiation of closely related species (Yao et al., 2010).

The rbcL gene serves as a DNA barcode for plant species, as it is a universal and easily amplified coding region located in chloroplast DNA (cpDNA). Compared to other barcodes in cpDNA, the rbcL gene has a lower mutation rate and high similarity between species, making it a superior option. This gene allows for detailed examination of intraspecies genetic and phylogenetic variations due to its advantageous characteristics (Nurhasanah et al., 2019).

Reaction mixture of PCR was prepared as Table 3.6.1 and the used parameters were in Table 3.6.2. For ensuring accurate amplification and appropriate fragment size, all PCR amplicons were subjected to electrophoresis and then observed under UV light.

Table 3.6.1 Reagents of PCR reaction mixture

Reagents	**Volume (uL)**		
	16s	**rbcL**	**ITS2**
Sterile Mili Q Water	32.7	33.8	34.8
10X Taq buffer	5.0	5.0	5.0
dNTPs mix	4.0	4.0	4.0
Forward primer	0.7	1.3	1.1
Reverse primer	0.7	1.9	1.1
Template DNA	6	3	3
Taq polymerase	1.0	1.0	1.0
Total	50	50	50

Table 3.6.2 PCR parameters used for amplification

Parameters	16s		rbcL, ITS2	
	Temp, Time	No. of cycle	Temp, Time	No. of cycle
Initial denaturation	95°C, 5 min	1	94°C, 5 min	1
Denaturation	95°C, 1 min	35	94°C, 30 sec	40
Annealing	55°C, 1 min		56°C, 30 sec	
Extension	72°C, 1 min		72°C, 45 sec	
Final extension	72°C, 10 min	1	72°C, 10 min	1

3.7 Gel Electrophoresis

To assess the quantity and quality of PCR amplicons, agarose gel electrophoresis was employed. A 0.7% gel was made by dissolving 0.35 g of agarose in 50 mL of 1x Tris-acetate-EDTA (TAE) buffer. 5-10 uL of the amplicons were loaded onto the gel and electrophoresed for 0.5 hours at room temperature, under a constant voltage of 100 V.

CHAPTER 4

Results and Discussion

4.1 Extracted eDNA

For further studies eDNA quantification is required. One of the best methods to detect the eDNA is the Qubit Fluorometer. The triplicate eDNA was stored in three different tubes. For measuring the eDNA a mixture of buffer and dye were needed.

Table 4.1.1 Reagents mixture for eDNA quantification.

Reagents	Volume (uL) 1X
Buffer	199
Dye	1
Total	200

Qubit 2.0 was used for analysis of the extracted DNA samples. It has a specific Qubit tube which should have the reaction mixture and can be inserted inside the Fluorometer. Each of these tubes required 200 uL of reaction mixture. So, for 5 uL of sample 195 uL of reagent mixture is needed.

Table 4.1.2 Reaction mixture for eDNA quantification.

Reagents	Volume (uL)
Reagent mixture	195
Sample (eDNA)	5

4.2 Readings of Qubit Fluorometer

4.2.1 In NaOH based Method

The samples are labelled as Sample 1, Sample 2, Sample 3. The concentration of eDNA is provided in Table 4.2.1.1.

Table 4.2.1.1 eDNA concentration in triplicate samples.

Sample	eDNA concentration (ng/uL)
Sample 1	19.10
Sample 2	7.09
Sample 3	1.25

4.2.2 In Kit based Method

The samples are labelled as Sample 1, Sample 2. The concentration of eDNA is provided in Table 4.2.1.2. Kit based method provides lower concentration of eDNA as compared to NaOH based method due to too many purification stages each of which may have led to the loss of some amount of eDNA.

Table 4.2.2.1 eDNA concentration in duplicate samples.

Sample	eDNA concentration (ng/uL)
Sample 1	6.24
Sample 2	9.84

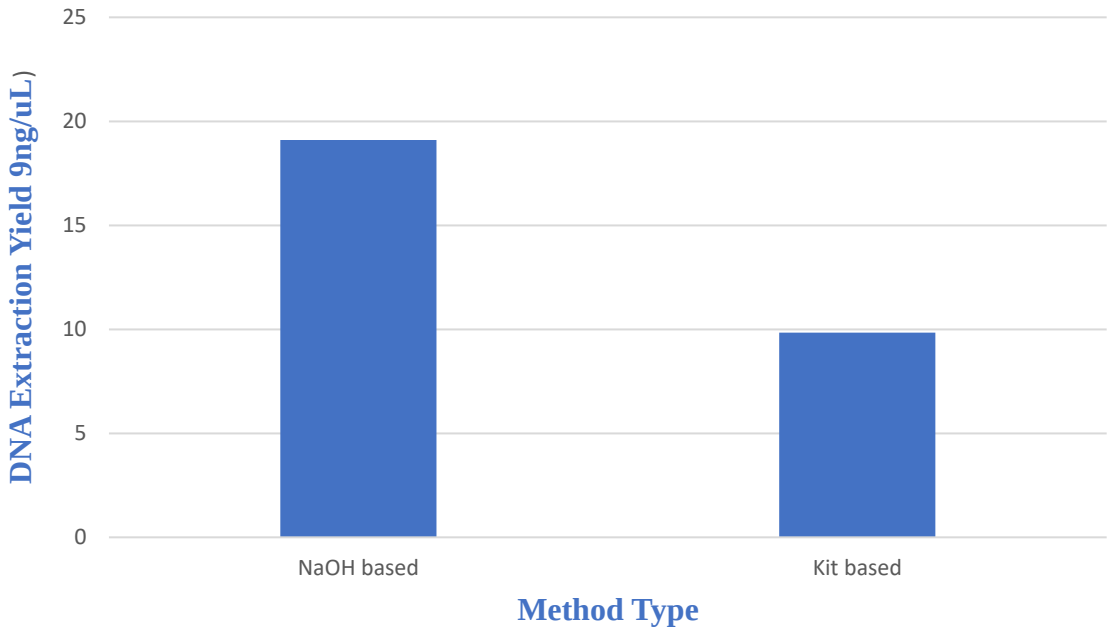

Fig 4.2.1 Comparison of NaOH based and Kit based Method of eDNA Extraction.

4.3 PCR Amplification and Gel Electrophoresis

Triplicate NaOH based Method and Duplicate Kit based Method samples were amplified separately in PCR for several times using different parameters of PCR using three different primers – 16s, ITS2, rbcL. The PCR products show no bands when run in gel electrophoresis.

4.4 Discussion

The NaOH-based eDNA extraction method resulted in a higher concentration of eDNA compared to the kit-based method. This could be attributed to the kit-based method involving multiple purification steps, which could potentially result in a loss of DNA.

The extracted eDNA concentration is quite enough for PCR amplification studies but the PCR products of the sample show no bands in the gel electrophoresis. The probable reason for that is the presence of PCR inhibitors. PCR inhibitors are a diverse group of chemical substances with varying properties. A single sample can contain multiple inhibitory substances, and the same

inhibitors can be present in different samples. Inorganic and organic substances, both in dissolved or solid forms, can act as PCR inhibitors. Calcium ions are an example of an inorganic substance that can inhibit PCR. However, organic compounds are more commonly known to have inhibitory effects on PCR, such as bile salts, urea, phenol, ethanol, polysaccharides, sodium dodecyl sulphate (SDS), humic acids, tannic acid, melanin, and various proteins, including collagen, myoglobin, haemoglobin, lactoferrin, immunoglobin G (IgG), and proteinases (Schrader et al., 2012).

CHAPTER 5

Future Prospective

5.1 Future Prospectives

The future prospectives of this analysis are

- Modification of PCR parameters to obtain bands
- Preparation of library from PCR amplicons
- Library sequencing
- Barcoding and Metabarcoding analysis

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1 CHAPTER 1 Introduction and Literature Review
2 Introduction 1.1 Environmental DNA Environmental DNA (eDNA) refers to the genetic signature that living organisms leave in aquatic or sediment environments. This genetic material can include whole cells, extracellular DNA, or even entire organisms (Barnes et al., 2021; Ficetola et al., 2008). eDNA is retrievable from an environment, where a small portion can be amplified and sequenced. The resulting sequence can then be utilized to identify various species existing in that environment (Deiner et al., 2017). eDNA can be isolated from various sources such as including roots, leaves, pollen, fruit, cells, skin, blood, mucus, saliva, sperm, eggs, feces, urine, and the decaying remains of larger animals (Barnes & Turner, 2016; Bohmann et al., 2014; Taberlet, Coissac, Pompanon, et al., 2012). Despite being a relatively new surveying method, eDNA analysis has demonstrated significant promise in environmental monitoring. Unlike traditional taxonomic identification procedures that often involve capturing live or dead animals, eDNA only requires the genetic traces left behind by an organism, thereby minimizing habitat destruction and disruption. While eDNA may not provide population quality information, such as sex ratios, it can effectively detect endangered, invasive, elusive, and rare species (Deiner et al., 2017; Goldberg et al., 2016). Recent advances in eDNA research methods now allow for the examination of entire communities from a single sample. While metabarcoding has a long history in microbiology, it is only beginning to gain momentum in the evaluation of macro-organisms