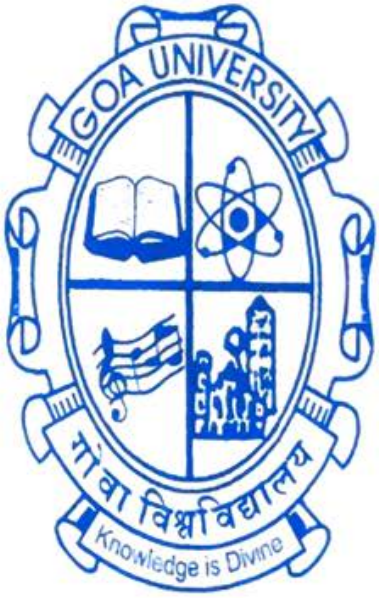
Evaluating the eDNA from freshwater lakes for barcoding

Dissertation thesis submitted to Goa University

In partial fulfilment of the requirement for the degree of

Master of Science in Biotechnology

In the academic year 2022-23



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Declaration

I hereby declare that the dissertation work entitled "Evaluating the eDNA from freshwater lakes for barcoding" submitted in partial fulfilment of the M.Sc. Biotechnology degree in the School of Biological Science and Biotechnology, Goa University in accordance with results obtained by me in the time period of 2022-2023, is completely my original work and not submitted in part or full for any other degree to any other university. The literature pertinent to this study has also been cited. Due acknowledgement has been made wherever facilities and suggestions have been availed of.

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Certificate

This is to certify that the dissertation entitled "Evaluating the eDNA from freshwater lakes for barcoding" submitted by Ms. Samradny Narayan Phadte is a bona fide piece of work carried out in partial fulfilment of the degree of Master of Science in Biotechnology at the School of Biological Science and Biotechnology, Goa University during 2022-23 in accordance with the results obtained under my guidance and supervision. It is further certified that the dissertation or any part of the work has not been submitted elsewhere for any other degree or diploma.

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List of tables

List of figures

List of abbreviations

µL	Microliter
mL	Milliliter
L	Liter
ng	Nano gram
°C	Degrees Centigrade
DNA	Deoxyribonucleic acid
eDNA	Environmental Deoxyribonucleic acid
g	Gram
HTS	High-throughput sequencing
h	Hour
PCR	Polymerase Chain Reaction
TAE	Tris-acetate-EDTA
HCl	Hydrochloric acid
rpm	Revolutions per minute
FP	Forward Primer
RP	Reverse Primer
GF/F	Glass fiber filter
bp	Base pair
km	Kilo meter

Table of Contents

CHAPTER 1

Introduction and Literature Review

Introduction

In nature, DNA shed by organisms during their lifespan is termed as environmental DNA or eDNA (Banerjee *et al*, 2021). eDNA is a possibly degraded low concentrate complex mixture of different genomic DNAs from various organisms found in an environmental sample such as soil, water, air, snow etc (Taberlet *et al*.2013). It consists of intracellular as well as extracellular DNA (Banerjee *et al*, 2021). The environmental samples contain living cells and organisms which are the source of intracellular DNA. On the other hand, extracellular DNA originates from cell death and/or destruction of cell structures which can be degraded by physical, biological or chemical activities (Tsuji *et al*, 2019). It is more difficult to analyse as compared to the DNA that comes from fresh tissue of a singular organism (Taberlet *et al*, 2018). eDNA can include microbial DNA, plants DNA as well as DNA from vertebrates (Tsuji *et al*, 2019). A huge part of the ancient flora and fauna does not fossilize, but tends to shed extracellular DNA traces in the sediments. eDNA can be deposited through urine, skin flakes, hair, faeces, eggshells, feathers, saliva, insect exuviae, regurgitation pellets, pollen, leaves, root cap cells etc (Banerjee *et al*, 2021). In prokaryotes it can be comprising of plasmid and/or chromosomal DNA (Taberlet *et al*, 2013).

There can be three possible fates of DNA that is released into the environment. These include, metabolism of vulnerable DNA by bacterial and fungal exonucleases; persistence in the environment facilitated by binding of environmental compounds like clay minerals, larger organic compounds as well as other charged particles that protect the DNA from nuclease activity and natural transformation where in the surrounding cells take up the environmental DNA and integrate it into their own genome (Pedersen et al, 2015).

Long exposures of a particular organism to a specific environmental condition will have more abundance of eDNA from that particular organism when samples are taken from that environmental site and will have lower concentration if the organism has been removed (Takahara *et al*, 2012). Similarly, as for low exposure of another organism will have to make use of complex procedures for the detection of less abundant DNA from the same sampling site (Takahara *et al*, 2012). The presence of eDNA in the environment is dependent on the body size, life history, behaviour, seasonal and reproductive activity. (Banerjee *et al*, 2021) Aquatic environments are especially suited for eDNA analysis because the DNA dispersion

is rapid in water columns with more homogeneous distribution as compared to the soil and other solid substrates (Hänfling *et al*, 2016). The capture, extraction, amplification, and analysis of eDNA of the organisms from many trophic levels provides a practical and comprehensive means for monitoring aquatic biodiversity over huge spatial domains as well as time (Djurhuus et al, 2017).

Harrison *et al*, 2019 state that horizontal dispersion of eDNA is usually limited in lake ecosystems, inferred from different studies reporting a decrease in the concentration of eDNA beyond 100 m of the organisms. The gravitational force primarily influences the vertical transport in lentic ecosystems because the thermal stratification limits transport from water flow, therefore, the faecal particles along with the associated eDNA is concentrated more in the lower layers of the water column. Thus, sampling is recommended from the upper water layers for the determination of viable populations from eDNA (Takahara *et al*, 2012). Additionally, there is a possibility of huge vertical and horizontal transports occurring during lake overturns, when temperature-driven stratification is lost with the whole lake water mixing taking place especially in the areas where significant seasonal temperature variations are seen. This is expected to have unpredictable impacts on eDNA transport. Therefore, collecting eDNA samples during lake overturns should be avoided (Harrison et al, 2019).

Macrobial eDNA, i.e the eDNA generated from larger organisms is being studied since 1991 in various fields including agricultural transgenics, human forensics, paleogenetics and faecal pollution source tracking (Turner *et al*, 2014). Later, in 2008 eDNA was first used for the detection of aquatic macrofauna. Since then, aqueous macrobial eDNA has proved itself as an alternative to directly observing rare aquatic macrofauna for external morphology (Turner et al, 2014). These techniques, reduce field survey time with little to no impact on ecosystems (Lodge et al., 2012). In addition, eDNA can help in detecting a big range of aquatic species from a single water sample (Takahara et al 2015). eDNA analysis is an important and impactful tool in water quality monitoring, the early detection of invasive and other harmful species and the surveillance of imperilled species (Staehr *et al*, 2022).

To summarise, eDNA is applicable across broad taxonomic boundaries; indicating the recent presence of organisms; in learning more about population abundance with qPCR; and next-generation sequencing to estimate species richness. A cup of water can give a lot of information with eDNA analysis (Lodge *et al*, 2012). Genome analysis of complex environmental samples has become an important tool to understand evolutionary history as well as functional and ecological biodiversity. It avoids the individual specimen laboratory cultivation and isolation (Shokralla *et al*, 2012). eDNA provides with the opportunity to analyse species, populations, communities and also map their geographical distribution over large scales and for long periods (Hinz *et al*, 2022). In case of the rapid diffusion of DNA from its source, the presence of the organism can be detected anywhere within the sample source (Ex- Water, air, soil, snow etc). UV light and microbial activity eventually breaks down the DNA released in the environment (Rees *et al*, 2014). eDNA can only last for about 7–21 days in the aquatic environments (Keskin E, 2014). Thus, availability of eDNA from a target species allows detection of its very recent presence, excluding compulsory direct observation or trapping and it's especially useful for the detection of species that are hard to find using conventional methods (Rees *et al*, 2014).

Analysis of eDNA requires a combination of different skills or fields such as classical ecology, bioinformatics and molecular biology (Shokralla *et al*, 2012). DNA barcoding uses short sequences from a unique region that is constant in organisms for identification (Gu *et al*, 2013). DNA barcoding helps to give species specific regions in the genome which can be used to prepare genomic libraries and identification. eDNA barcoding can also contribute greatly to this field (Shokralla *et al*, 2012). eDNA metabarcoding is a sensitive, cost effective, time effective tool that has broad application for biodiversity research and environmental monitoring (Deiner *et al*, 2017). Species identity by eDNA detects the short DNA fragments. Thus, despite wide interests, uncertainty persists surrounding the physical processes that influence eDNA persistence and its fate within the environment. (Harrison et al, 2019) In case of rare and evasive species, it might increase the probability of detection of a particular rare/evasive species. Although this method is also said to have some risk of errors in case of improper analysis (Bista *et al*, 2017). These DNA particles are extracted and amplified with designed primer sets targeting specific taxa, sequenced and compared to a reference database for identification. This process eliminates the need for multiple taxonomic experts for processing samples (Bessey *et al*, 2021).

Polymerase chain reaction (PCR) is extensively used in DNA analysis to amplify specific sequences because of its sensitivity, specificity, speed, and simplicity of the reaction. It uses everyday molecular biology reagents to make multiple copies of a target sequences giving it the name 'DNA photocopier'. This concept is simple however the process is complex which takes the low concentration of template and amplifies it which can be later subjected to a wide range of analysis methods. (Kelly et al, 2019)

PCR has become an irreplaceable and important tool in science allowing scientists to analyse genome, alter genome, biodiversity studies etc. However, it depends on the scientist how he can amplify this power (Lorenz T *et al*, 2012). It is very important to work in nuclease free environment when performing a PCR reaction for the best results (Maddocks & Jenkins, 2016).

In the present study includes, environmental DNA extraction from freshwater lake and it's PCR analysis for barcoding.

Review of literature

2.1eDNA and metabarcoding

There is no standard or simple procedure that can analyse all types of eDNA. The procedure is heterogenous and a wide range of problems can be encountered while assessing eDNA. These include DNA samples that are degraded and highly diluted, high quality with no inhibitors, with inhibitors etc (Taberlet *et al*, 2018). The rate of biodiversity loss is increasing with the need for measuring the changes in biodiversity; eDNA metabarcoding is an efficient and fast method (Deiner *et al*, 2017). eDNA metabarcoding allows simultaneous detection of multiple species and hence is helpful in filling the gaps of taxonomic studies (Miya M, 2022). Various methods from sample collection to data analysis are summarized in this section.

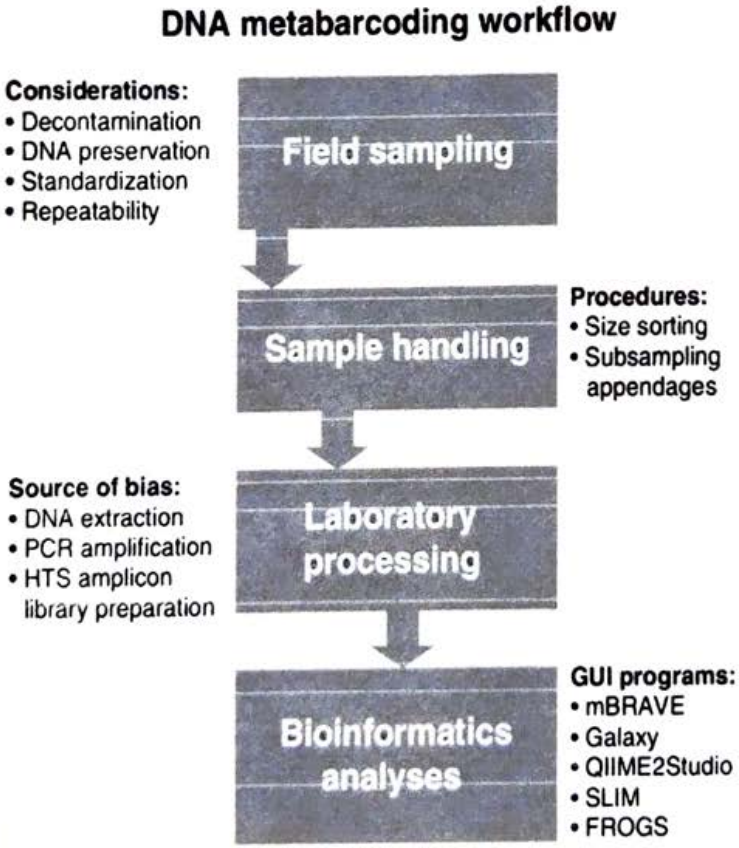


Fig 2.1: Workflow (Liu *et al*, 2020)

2.2 Extraction of eDNA from water samples:

2.2.1 Methods of extraction of eDNA

For eDNA extraction from water samples, three methods are widely used-ethanol precipitation (for extraction from small volumes), filtration (for extraction from larger volumes), centrifugation and ultrafiltration (Tsuji *et al*, 2019). The sampling design should be adjusted as per the requirements of the type of sample by taking into consideration various parameters for higher eDNA yield and concentration. (Goldberg *et al*, 2015)

2.2.1.1 Filtration

Filtration is most simple and commonly employed method. The advantage of this method is that it can be used to collect eDNA from larger volumes of water sample. However, there's also a disadvantage that there may be loss of dissolved eDNA which can led to false results stating higher yield/concentration of the extracted eDNA. (Goldberg et al, 2015) Various types of filters are used with pore sizes of 0.45 µm (cellulose nitrate), 0.7 µm (glass microfiber, GF/F- Glassfiber filter), and 1.2 µm (glass microfiber), for investigating taxa. Some studies indicate that filter material and pore size can greatly impact extraction of eDNA (Tsuji et al, 2019). Majority of microbial eDNA is most effectively extracted using a filter of pore size ranging from 1–10 µm (Turner et al., 2014). However, the greatest problem with small pore size filter papers is that they can be clogged easily in presence of large amounts of suspended solids. Clogging of filters can be reduced by passing the sample through a filter with larger pore size as a pre-processing step (Takahara *et al*, 2012). The most frequently used filtering material is glass microfiber having chemical characteristics of adsorbing DNA as well as protein onto its surface. This may lead to an increase the efficiency of eDNA collection. (Tsuji et al, 2019)

2.2.2.2. Ethanol Precipitation

Ethanol precipitation is frequently used when small volumes of water sample is used for eDNA collection (Tsuji *et al*, 2019). This method is ideal for studies performed in rainforests or at higher altitudes where water access is difficult with no electricity, as it doesn't require a lot of equipment as compared to other methods (Tsuji et al, 2019). as Another advantage is of this method is that sample fixation is immediate. However, due to the sample volume limitation the detection power for eDNA is restricted. Alternatively, isopropanol precipitation can also be used for the collection of eDNA where lower volume of isopropanol

is required thereby increasing processable volume of the sample. However, this method best works when a high eDNA concentration is present in small volumes of water sample (Chen *et al*. 2010).

2.2.2.3 Centrifugation

Centrifugation can be used to collect eDNA from water without the addition of reagents (Takahara et al., 2013). This method is rarely used but can be advantageous in case of testing multiple samples (Takahara *et al*, 2013). Whereas, the disadvantage include, restriction of sample volume due to the size of the centrifuge (Tsuji *et al*, 2019). There are some variants on extraction methods, such as chloroform-based extraction (Renshaw et al., 2014), physical disruption of cells; and silica-based extractions (Sahu *et al*. 2012). Detection of eDNA is hugely affected by the type of sample used and also by the type of extraction methods. (Deiner et al, 2015)

Regardless of the methods used, careful handling of water samples is needed until eDNA purification. eDNA in the water samples is degrades over time (Dejean et al., 2011). eDNA degradation is accelerated at higher temperatures (Tsuji et al, 2019). Therefore, collected water samples with eDNA need to be quickly fixed or kept under low-temperature conditions in the laboratory until processed. However, it is advisable to immediately extract and purify eDNA after sampling. (Deiner et al., 2015).

2.2.2 Methods of purification of eDNA:

The eDNA extraction and purification with commercial DNA extraction kits has been most commonly used method (Chen *et al*, 2010). It may be because of the simplicity of experimental designing of the commercially available DNA extraction kit having greater eDNA recovery efficiency. A variety of commercially available eDNA extraction kits are available giving higher yields such as DNeasy Blood and Tissue DNA extraction kit (hereafter DNeasy, Qiagen, Hilden, Germany), PowerWater DNA Extraction Kit (PowerWater, Qiagen), PowerSoil DNA Extraction Kit (PowerSoil, Qiagen), PowerMax Soil (Qiagen), QIAamp DNAStool Mini Kit (Qiagen), etc (Tsuji *et al*, 2019). Each of the commercial eDNA extraction kits vary in depending on the combination of sample collection and sample type (including the type of inhibitors and their concentration). (Tsuji et al, 2019)

The main techniques that have been used for liquid phase separation methods making the use of organic solvents (Turner *et al.*, 2014) include, cetyltrimethylammonium bromide (CTAB) method and the phenol–chloroform–isoamyl alcohol (PCI) method (Renshaw *et al.* 2015).

PCI method has been also used for the macrobial DNA extraction which is captured on polycarbonate track-etch filters, nylon filters and glass fibre filters (Barnes et al. 2014; Turner *et al.* 2014). PCI extraction protocol has promising future in eDNA research to make it affordable (Tsuji *et al*, 2019). However, the disadvantage of PCI is that careful handling of reagents and the proper waste disposal is needed because it uses harmful substances such as phenol and chloroform which can lead to genetic defects, skin/eye irritation respectively (Renshaw *et al*, 2015). Therefore, commercial kits are preferred due to standard safe protocols and high yield/concentration of eDNA (Tsuji et al, 2019).

2.2.3 Detection and analysis of purified eDNA

For detection and analysis of purified eDNA, molecular biology techniques such as PCR, mass spectrometry, and sequencing have been optimized (Vingataramin & Frost, 2015). eDNA detection methods can be divided into two main types (a) species-specific detection and (b) eDNA metabarcoding (Thomsen & Willerslev, 2015; Tsuji *et al.*, 2018). Species-specific detection is one of the oldest methods that has been extensively used eDNA studies. It is well suited for estimation of the distribution of endangered or invasive species as it has high sensitivity for detection (Tsuji *et al*, 2019). On the contrary, use of eDNA metabarcoding with high-throughput sequencing (HTS) has increased for last few years to be used for the monitoring of the aquatic biota as well for taxonomy (Jerde & Mahon, 2015). The probability of detection of eDNA is dependent on the species density as well as the ratio between the release of DNA by the organism and the degradation of DNA by environmental factors. (Dejean *et al*, 2011)

Determination of the DNA quality can be done by agarose gel analysis followed by PCR amplification where in the absence smear with high molecular weight indicates high quality DNA and amplification indicates absence of amplification inhibitors (Wu *et al*, 2009). Alternatively, Nanodrop spectrophotometer (Chen *et al*, 2010) and Qubit can be used for DNA quantification. DNA is tightly bound by proteins which is difficult to remove during isolation therefore the difference in the UV absorbance is used to identify and quantify DNA

(Chen *et al*, 2010). Qubit is highly sensitive, fluorescence-based quantitation system and it is preferred over nanodrop due to the tendency of nanodrop to show higher values than usual (Masago *et al*, 2021). Species-specific detection includes amplification and detection of short fragments of DNA from a target species (typically 80–200 bp) using PCR with species-specific primers (Bohmann *et al*., 2014). Due to high mutation rates, abundant copies, high level of coverage in genetic databases mitochondrial DNA (mtDNA) is used as a genetic marker. (Goldberg *et al*, 2016). The major target regions of mtDNA are as follows: cytochrome b (Cytb) (33%), cytochrome c oxidase subunit 1 (COI) (30%), D-loop (11%), 12S ribosomal RNA (12S) (6%), 16S ribosomal RNA (16S) (6%), and ITS of nuclear DNA (3%). For the development species-specific primers for eDNA analysis, there is a need to collect the sequence information of target species as well as non-targeted related species (Tsuji *et al*, 2019). Thus, regions which have a sufficient amount of sequence data in genetic databases would be advantageous in developing species-specific primers (Tsuji *et al*, 2019).

The target DNA is primarily detected using: gel electrophoresis of PCR amplicons; real-time quantitative PCR (qPCR); or digital PCR (Tsuji *et al*, 2019).

PCR is widely used in eDNA experimentation as an exponential increase is observed for even very low DNA concentrations. This means that small biases in the PCR process can compound into large differences in the abundance of each species' amplicons as compared to the DNA concentrations. Also, DNA usually amplifies at different rates from different species. It is such that each PCR cycle preferably amplifies templates with greater affinity for the used primers and this is called amplification bias. (Kelly *et al*, 2019) The electrophoresis of PCR amplicons has traditionally been used for analysis. It reduces the cost of analysis as it does not require a real-time PCR system and expensive reagents. The positive detection is observed with the appearance of PCR gel bands of the correct length (Evans & Lamberti, 2018). The concentration of eDNA can be estimated semi quantitatively by the colour density of the bands or the number of positives obtained out of PCR replicates (Tsuji *et al*, 2019).

qPCR assay has been used for the detection of freshwater animals' eDNA in environmental samples rapidly (Takahara *et al*, 2015). It has high specificity, quantification ability and sensitivity (Wilcox *et al*, 2013). Probe based qPCR is more efficient than dye-based qPCR because it is specific (Farrington *et al*, 2015).

In DNA barcoding, using a short piece of DNA differences between species is found out. The progress of DNA barcoding has developed various approaches and increased use of molecular markers for detection (Kazi *et al*. 2013). Ribulose- 1,5-bisphosphate carboxylase oxygenase (RuBisCO) enzyme is responsible for the fixation of primary CO2 in the Calvin cycle. The plastid encoded larger subunit (rbcL) of the 8 larger subunits from the quaternary structure of this enzyme marker has been popular for studying taxonomic position of unknown species to obtain clear phylogenetic relationships between different species. (Alshehri et al, 2019)

The Internal Transcribed Spacer (ITS) regions of fungal ribosomal DNA (rDNA) is used to distinguish fungal species by PCR analysis due to the presence of highly variable sequences. PCR primers are available for amplifying these sequences from environmental samples and degree of success at distinguishing against plant DNA is varying along with maintaining a broad range of compatibility (Martin *et al*, 2005). ITS region contains ITS1 separated from ITS2 by 5.8s gene is used for environmental barcoding due to large copies of this per cell (Bellemain *et al*, 2010).

After the HTS, bioinformatic tools are used to assign the resulting DNA sequences to a previously described, known, taxon. This can be done by subjecting the sequence to NCBI BLAST analysis followed by the construction of phylogenetic trees (Kazi *et al*, 2013). These are computer programs where large sequencing datasets of HTS are analysed. For the purpose of sorting, filtering, and clustering sequences into operational taxonomic units (OTU), expertise of a bioinformatician proficient in computer programming is needed. (Evans & Lamberti, 2018) Further the obtained DNA sequences from rbcL and ITS2 can be aligned using ClustalW (Asahina *et al*, 2010).

CHAPTER 2

Aims and Objectives

2.1 Aim of the project

To isolate environmental DNA from freshwater lakes for barcoding

2.2 Objectives

- To isolate Environmental DNA
- To find the concentration of the extracted DNA
- To PCR amplify for barcoding

CHAPTER 3

Materials and Methods

Materials and methods

3.1 Equipment used

Microcentrifuge, Centrifuge, Water bath, Thermal cycler (Agilent SureCycler 8800), NanoDropSpectrophotometer-2000, Gel electrophoresis unit, Qubit 2.0, UV transilluminator, Refrigerator 4°C, Freezer -20°C, Laminar airflow cabinet, weighing balance etc.

3.2 Reagents used

Sodium hydroxide, Sodium hypochlorite, Ethanol, Taq polymerase (supplied with 10x buffer containing MgCl2), dNTP mix, Triton-x-100, Tween20, Tris-HCl, EDTA, Agarose, TAE buffer, Hydrochloric acid, Primers to be added for ITS2 and rbcL

3.3 Sample Collection

A. Na-EDTA method

   In October 2022, a total of 5L of water sample was collected at the surface level of Carambolim Lake (15.4864604, 73.9300050) in North Goa, using a plastic can.

B. Nucleospin eDNA water kit

   In February 2023, a total of 5L of water sample was collected from various locations of Mayem Lake (15.5761945, 73.9388607) in Bicholim, Goa.

All supplies were washed with detergent and rinsed with distilled water before sample collection. The samples were taken to the lab and immediately filtered. 1 L aliquots from the 3 L samples were vacuum filtered using a membrane filter.

3.4 Sample Filtration

A. Na-EDTA method (Vingataramin & Frost, 2015)

   Triplicate 1 L seawater samples were filtered through nitrocellulose mixed ester membrane filters (pore size 0.2 µm, diameter 47 mm). The filter was mounted onto a filtration cup and secured to a filtration unit connected to a vacuum pump. All

supplies were sterilized with 70% before filtration. The membranes were immediately used to extract eDNA.

B. Nucleospin eDNA water kit (Takara Bio USA.Inc. ; 740402.10)

1 L seawater sample was filtered through glass fibre membrane filters (pore size 0.2 µm, diameter 47 mm). The filter was mounted onto a filtration cup and secured to a filtration unit connected to a vacuum pump. All supplies were autoclaved before filtration. The membrane was wetted with absolute ethanol and stored in -20C.

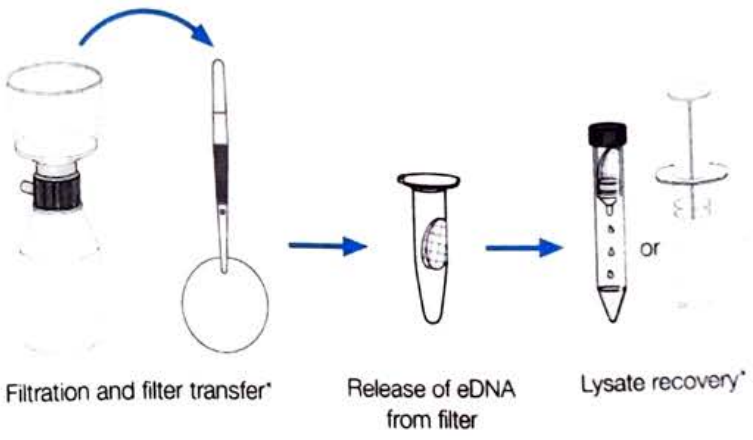


Fig 3.4 Sample filtration workflow (TaKaRa Bio)

3.5 eDNA Extraction

3.5.1 NaOH extraction method

After the filtration process, the filter paper was rolled using flame-sterilized forceps, and placed in a 50mL centrifuge tube filled with 5 mL solution of NaOH-ethanol (Table 3.5.1.1). The tube was heated to 80°C in a water bath for 10 minutes. With a sterilized forceps, the membrane filter was carefully removed and placed in a 2 mL sterile syringe. The membrane filter was compressed to squeeze out any leftover liquid, which was added to the 50-mL tube. Then, the tube was centrifuged for ten minutes at 10,000 RPM. The supernatant was removed, 100 µL of elution buffer (Table 3.5.1.2) was added to solubilize the denatured DNA, and stored at 4°C. This was done in triplicates (Vingataramin & Frost, 2015).

Table 3.5.1.1 NaOH-ethanol based extraction solution (Vingataramin & Frost, 2015)

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

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

Component	Volume (mL)	Final concentration
5M Tris HCl (pH 8)	5	50mM
0.5M EDTA	0.01	0.1mM
Triton-x-100	0.5	1%
Tween20	0.25	0.5%
Distilled water	44.25	
Final Volume	50.01	

3.5.2 Nucleospin eDNA water kit (740402.10)

eDNA was isolated using the kit using the simplified protocol and the precipitation methods mentioned in Nucleospin eDNA water kit manufactured by TaKaRa Bio USA Inc.

3.6 Concentration of eDNA

The concentration of environmental DNA was determined using a Qubit 2.0.

3.7 PCR amplification

Thermal cycler (Agilent SureCycler 8800) was used for the amplification of the desired bands. A PCR reaction was performed to check for amplification of rbcL and ITS2 genes

from cDNA. PCR reaction mixture was prepared as Table 3.7.1 and the parameters were set as Table 3.7.2. The resultant PCR amplicons were electrophoresed and visualized on UV transilluminator.

Table 3.7.1: Reaction mixture components

Components	Volume (µL)	
	rbcL	ITS
Sterile miliQ water	36.8	37.8
10X Taq buffer	5	5
dNTPs mix	4	4
Forward primer	1.3	1.1
Reverse primer	1.9	1.1
Taq Polymerase	1	1
Total	50	50

Table 3.7.2: PCR parameters

	RbcL &ITS		
Parameters	Temp. (°C)	Time (Minutes)	No. of Cycles
Initial denaturation	94	5	1
Denaturation	94	0.30	40
Annealing	47	0.30	
Extension	72	0.30	
Final extension	72	7	1

Table 3.7.3. Primers used in the present study

Gene	Primer	Sequence	Length (bp)	Reference
rbCL	FP	5'-ATGTCACCACAAACAGAGACTAAAGC-3'	26	(Asahina *et al.* 2010)
	RP	5'-GCAGCAGCTAGTTCCGGGCTCCA-3'	23	
ITS2	FP	5'-ATGCGATACTTGGTGTGAAT-3'	20	(Gu *et al.* 2013)
	RP	5'-GACGCTTCTCCAGACTACAAT-3'	21	

3.8 Gel electrophoresis

Agarose gel electrophoresis was used to check the quality of PCR amplicons. 0.7% agarose gel was prepared by dissolving 0.35 g of agarose in a 50 ml 1x Tris-acetate-EDTA (TAE) buffer. 10 µL of amplicons with 2 µL of dye were loaded and the samples were run on the gel. 1kb ladder was loaded to check the obtained amplicon size. Electrophoresis was performed for an hour at room temperature with a constant voltage of 100 V. The bands obtained were visualized on UV transilluminator.

CHAPTER 4

Results and Discussion

Results and discussion

4.1 eDNA extraction

DNA was extracted from two samples. One sample was collected from Carambolim Lake and the other was collected from Mayem Lake. Mayem lake is located about 1.5km from a closed down mining area in Bicholim taluka, north Goa with the coordinates, 15.5760° N, 73.9400° E. However, Chaulya *et al* (2000), stated that mining did not affect the water and the main source of the water is rainfall. The Carambolim Lake is a popular birding location (73055'N, 15030'E) in north Goa that is also a big irrigation tank near Carambolim railway station (Shanbhag *et al*, 2001).

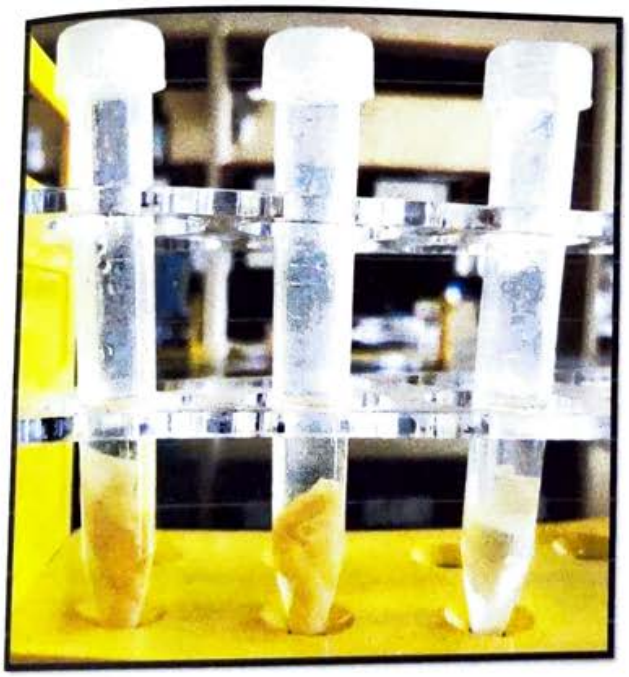
Both methods recovered eDNA. However, Na-EDTA method gave a brown precipitate indicating contamination by phenolic compounds (Sahu *et al*, 2012) Lentic environments, contain a lot of suspended solids that can clog the pores with small size and so it is suggested to use larger pore size (Takahara *et al*, 2012). In case of the kit procedure, a white DNA precipitate was observed. Staehr *et al* (2022), used Nucleospin eDNA water kit for monitoring of biodiversity hotspots because it prevents cross sample contamination.

A B

Fig 4.1 Vacuum filtration using nitrocellulose membrane (A) and Glass fiber filter (B)

A B

Fig 4.2 Filters used: Nitrocellulose (A) and Glass fibre filter (B)



Filter paper in Na-EDTA before boiling (A)

Filter paper in Na-EDTA after boiling in waterbath

(B)

Fig 4.3 Nitrocellulose membrane filter in Na-ethanol before (A) and after (B) boiling for Na-EDTA

Carambolim 1

Carambolim 2

Fig 4.4 Precipitate obtained after DNA isolation by Na-EDTA method

Fig 4.5 Precipitate obtained by Nucleospin eDNA water kit

4.2 Concentration

NaOH based DNA extraction yields were low ranging from 1-3 ng/µl (Fig. 4.2.1), because eDNA concentration is generally low (Takahara *et al*, 2012). DNA can well withstand the heating temperature of 80°C for 10 minutes, but DNase I and the RNA are denatured completely. Although the crude DNA extract contains residue of denatured enzymes and RNA, it is reported to have no effect on PCR amplification when Tris-EDTA concentration is high in the elution buffer (Vingataramin & Frost, 2015). As crude DNA extract contains denatured enzymes, it cannot degrade DNA over time. If maintained in a TE buffer, DNA can be preserved at 4°C for 6 to 12 months (Wu *et al*., 2009). Thus, the crude extract of this study was stored at 4°C.

The yields from Nucleospin eDNA kit varied in the two methods used. Using simplified extraction protocol, the yield was found to be 13.6 ng/µl and with the precipitation method it was measured to be 1.75ng/µl. This could be because the precipitation method uses small volume of water sample for DNA extraction and eDNA concentration is usually low and well dispersed in water environment (Takahara *et al*, 2012). As for simplified protocol, the yield was more due to the usage of 3L of water sample and glass fibre filter to avoid any kind of impurities (Staehr *et al*, 2022). Piaggio *et al*, compared eDNA isolation kits with

traditional method and got effective results with the kit, however concluded there maybe clogging of filter and possible accumulation of inhibitors by the kit protocol (2013)

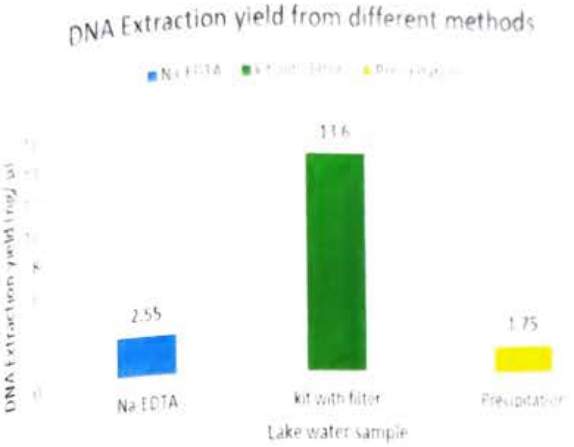


Fig 4.2.1 eDNA extraction yield by NaOH method (Blue), Kit with filtration (green) and Precipitation (Orange)

4.3 PCR amplification and Gel electrophoresis

The concentration of NaOH method samples and Nucleospin precipitation method were too low to be analysed. The Mayem lake sample isolated from Nucleospin simplified protocol were subjected to PCR amplification by primers- ITS2, and rbcL.

ITS2 PCR amplification was carried out with 50ng as well as 100ng of DNA but no bands were obtained when the amplified product were subjected to gel electrophoresis. The possible reasons for this include (Lorenz T, 2012), presence of PCR inhibitors such as humic acid, bad quality of the template (Maddocks, S. & Jenkins, R. 2016). Bellemain *et al* (2010), used different ITS primers and found that some primers hampered the reaction, others introduced PCR bias and only ITS1 avoided bias, suggesting the use of different primer combinations in parallel to amplify different regions of ITS gene. ITS2 primers were used to identify medicinal plants *Selaginellaceae* in Gu *et al* (2013). In a study comparing the efficacy of metabarcoding markers, rbcL provided more informative barcode as compared to ITS2 (Coghlan *et al*, 2021).

rbcL PCR amplification was carried out using 50ng of DNA and a light band was obtained with smear with annealing temperature 47 and not 50 or 52(Fig 4.3.1). The smear could be because of low quality of the template, too much DNA was loaded, too many cycles (Maddocks, S. & Jenkins, R. 2016; Wu *et al*, 2009). The band size obtained is 573.984bp by graph method. Alshehri *et al* (2019), performed PCR amplification for species identification and DNA barcoding for Saudi Arabia Seaweeds using rbcL gene and obtained bands of average size ~600bp, sequencing and NCBI-BLAST of which identified them to be *Padina pavonica, Turbinaria gracilis, Carpomitra costata, Pterocladiella capillacea, Cladostephus spongiosus, Ulva lactuca, Sporochnus comosus* and *Sargassum muticum*. Thus, the band obtained in this study could belong to one of these.

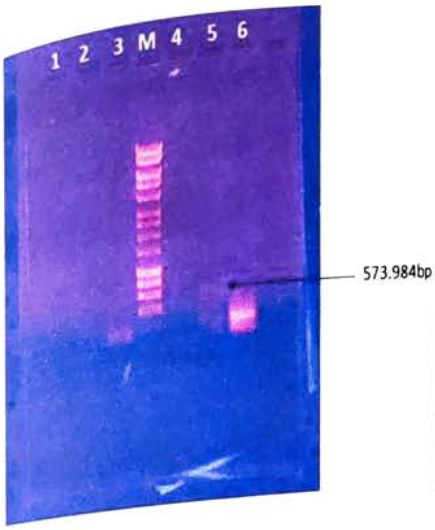


Fig 4.3.1 Gel electrophoresis for ITS2 and rbcL

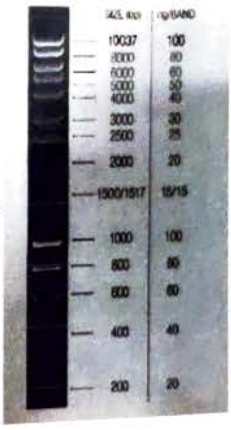


Fig 4.3.2 DNA ladder

Well Number	
2	ITS amplified eDNA
3	Positive control for ITS
M	Ladder
4	rbcL amplified eDNA
5	Positive control for rbcL

Table 4.3.1 Legends

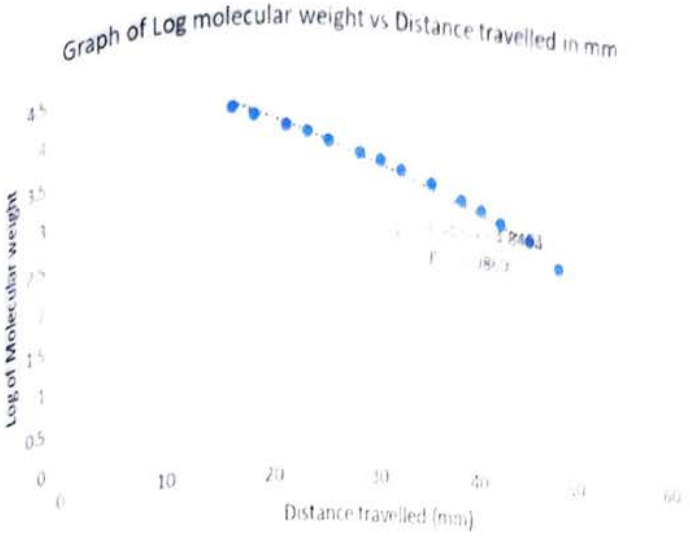


Fig 4.3.3 Estimating size of the amplicon

4.4 Summary

Environmental DNA was collected from 2 lakes, namely; Carambolim and Mayem using plastic cans. The water sample was filtered using nitrocellulose, glass fiber filter membrane so that the environmental DNA gets adsorbed on it. This filter was further subjected to treatment so as to release the bound DNA. Environmental DNA was isolated using alkaline lysis extraction method, Nucleospin precipitation and simplified protocol from the kit. The extract was quantified using qubit. Mayem lake sample (13.6 ng/µl) was used for PCR amplification with ITS2 and rbcL primers. Agarose gel electrophoresis of PCR amplicons produced band of ~600 bp for rbcL while no band was observed for ITS2.

CHAPTER 5

Future prospects

5.1 Future prospects

The future prospects of this study are:

- Library preparation from PCR amplicons
- Sequencing
- Barcoding and Metabarcoding analysis

Appendix

Appendix

1. 50X TAE buffer

242 g tris base in ddH2O

57.1ml glacial acetic acid

100ml 0.5M EDTA solution (pH 8.0)

Adjust volume to 1L.

2. 1X TAE buffer

Add 10mL 50X TAE buffer in 490mL ddH2O

3. 0.7% Agarose

0.7g Agarose powder in 100mL 1X TAE buffer

4. 2M NaOH

8g NaOH crystals in 100mL distilled water

5. 96% ethanol

96mL absolute ethanol in 4mL of distilled water

6. 0.025M EDTA

0.7306g of EDTA powder in 100 mL water

Add NaOH crystals to dissolve

7. 5M Tris HCl (pH 8)

7.88g of Tris HCl powder in distilled water.

Adjust pH with NaOH

8. 0.5M EDTA

14.61g EDTA powder in 100mL distilled water

Add NaOH for dissolving

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Introduction In nature, DNA shed by organisms during their lifespan is termed as environmental DNA or eDNA (Banerjee et al 2021). eDNA is a possibly degraded low concentrate complex mixture of different genomic DNAs from various organisms found in an environmental sample such as soil, water, air, snow etc (Taberlet et al,2013). It consists of intracellular as well as extracellular DNA (Banerjee et al, 2021). The environmental samples contain living cells and organisms which are the source of intracellular DNA. On the other hand, extracellular DNA originates from cell death and/or destruction of cell structures which can be degraded by physical, biological or chemical activities (Tsuji et al, 2019). It is more difficult to analyse as compared to the DNA that comes from fresh tissue of a singular organism (Taberlet et al. 2018). eDNA can include microbial DNA, plants DNA as well as DNA from vertebrates (Tsuji et al, 2019). A huge part of the ancient flora and fauna does not fossilize, but tends to shed extracellular DNA traces in the sediments. eDNA can be deposited through urine, skin flakes, hair, faeces, eggshells, feathers, saliva, insect exuviae, regurgitation pellets, pollen, leaves, root cap cells etc (Banerjee et al 2021). In prokaryotes it can be comprising of plasmid and/or chromosomal DNA (Taberlet et al. 2013)

There can be three possible fates of DNA that is released into the environment. These include metabolism of vulnerable DNA by bacterial and fungal exonucleases; persistence in the environment facilitated by binding of environmental compounds like clay minerals, larger organic compounds as well as other charged particles that protect the DNA from nuclease activity and natural transformation where in the surrounding cells take up the environmental DNA and integrate it into their own genome (Pedersen et al, 2015).

Long exposures of a particular organism to a specific environmental condition will have more abundance of eDNA from that particular organism when samples are taken from that environmental site and will have lower concentration if the organism has been removed (Takahara et al, 2012). Similarly, as for low exposure of another organism will have to make use of complex procedures for the detection of less abundant DNA from the same sampling site (Takahara et al. 2012). The presence of eDNA in the environment is dependent on the body size, life history, behaviour, seasonal and reproductive activity (Banerjee et al, 2021) Aquatic environments are especially suited for eDNA analysis because the DNA dispersion is rapid in water columns with more homogeneous distribution as compared to the soil and other solid substrates (Hanfling

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et al. 2016). The extraction, capture, amplification, and analysis of environmental DNA from different trophic level provides a practical means for monitoring aquatic biodiversity over huge area as well as time (Djurhuus et al, 2017)