Utilizing Environmental DNA to Identify Eukaryotic Diversity in Mangrove Sediments at Demak, Central Java, Indonesia

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Abstract

The mangrove ecosystem, found along tropical and subtropical coasts, adapts to extreme conditions like rapid tidal changes, high salinity, anthropogenic influences, and anoxic environments. Mangrove sediments host diverse organisms, particularly invertebrates and bacteria, which significantly influence sediment structure and biochemical processes by enhancing permeability and water flow. Modern molecular approaches, notably Next-Generation Sequencing (NGS), are increasingly used to identify macro and microorganism communities in sediments. NGS, a powerful tool for DNA and RNA sequencing, allows for parallel sequencing of numerous DNA fragments, providing comprehensive insights into genome structure, genetic variants, gene expression, and epigenetic modifications. Its efficiency and cost-effectiveness make NGS vital for both basic biological research and clinical diagnostics. Recent NGS studies on mangrove sediments have focused on bacterial, archaeal, and fungal diversity. The study examines eukaryotic diversity in mangrove sediments at two locations, targeting the Cytochrome Oxidase Subunit I (COI) gene, a universal marker for eukaryotes. Results indicate distinct taxa at each site with minimal overlap, demonstrating eDNA's potential for assessing both macro and microorganism diversity in mangrove sediments. This preliminary study underscores the utility of molecular techniques in biodiversity research and also dynamic ecosystem changes in the mangrove sediment ecosystem. The high influence of the environment around the mangrove ecosystem will affect the quality of the mangrove itself. eDNA here provides a fast method for recording possible changes to be able to carry out better management in the future.

Keywords: *Environmental DNA, sediment, Mangrove Ecosystem, COI*

Introduction

The mangrove ecosystem is an ecosystem that is generally found along coasts in tropical and subtropical regions. This ecosystem is able to adapt to extreme environments, such as rapid changes in tides, temperature, high salinity, and anoxic conditions (Alongi, 2022). These driving factors force structural and functional limits, promote physiological processes for adaptation, and consequently render the ability to survive in a high-saline and low-oxygen environment (Alongi *et al*., 2016). Mangrove ecosystems play a vital role in the environment, particularly coastal environments, and provide various ecosystem services that benefit the environment globally and communities' livelihoods (Getzner and Islam, 2020). These services include preventing coastal abrasion (Indarsih and Masruri, 2019), protecting coastal communities from climate disasters (Nur and Hilmi, 2021), providing habitat for a variety of organisms (Widayanti *et al*., 2023), regulating nutrients (Constance *et al*., 2022), and serving as a natural carbon sink that is important for nature-based approach to climate mitigation (Arifanti *et al*., 2022). This ecosystem's ability to absorb and store long-term carbon has made the mangrove ecosystem recognized as part of the "blue carbon" ecosystem (Donato *et al*., 2011; Lovelock and Duarte, 2019).

Furthermore, mangrove sediments also provide a habitat for many organisms, especially invertebrates and bacteria. The presence of benthic invertebrates within the mangrove ecosystem imposes a significant impact on sediment structure and biochemical processes, primarily through enhancing sediment permeability and water flow (Pravinkumar, 2013). A study conducted by Santos *et al*. (2010) observed that the *Metazoa* group was the most prevalent eukaryote in mangrove sediments,

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followed by *Stramenopiles* and *Alveolata* from SAR group. In recent years, several techniques have been developed for assessing microorganism communities in mangrove sediments. These techniques include the conventional cultivation approach, DNA fingerprinting techniques, clone libraries, and sequencing technologies (Zhang *et al*., 2009; Dias *et al*., 2010; Santos *et al*., 2010; Andreote *et al*., 2012; Mendes and Tsai, 2014; Sá *et al*., 2014)

Demak, Central Java, Indonesia has a mangrove area of 13,960.5 ha. However, pressures such as abrasion, population growth, land conversion and industrialization have caused a decline in the quality of the mangrove ecosystem in Demak (Sihombing *et al*., 2017). This can certainly affect the function of the mangrove ecosystem at large. Apart from that, the entry of pollutants including rubbish and other organic substances will affect the condition of the mangrove sediment ecosystem which will threaten the diversity of its original biodiversity.

Presently, molecular approaches are increasingly used to identify macro and microorganism communities within sediment due to their perceived ability to produce massive results in a short time. Over the last two decades, sequencing technology has developed rapidly and entered third-generation or next-generation sequencing (NGS), which is a new approach that overcomes the limitations of the two previous generations of sequencing technology (Salk *et al*., 2018; Kumar *et al*., 2019; Goto *et al*., 2020). NGS is an emerging technological advancement used in genetic research for DNA and RNA sequencing, as well as the identification of variants and mutations (Qin, 2019). NGS also enables the efficient sequencing of many DNA fragments in parallel, facilitating an extensive knowledge of genome structure, genetic variants, gene expression patterns, and epigenetic alterations (Satam *et al*., 2023). Due to its rapid data processing and attractive cost-performance ratio, NGS has become an essential tool for basic biological research and clinical diagnostics (Goodwin *et al*., 2016). Several recent studies based on NGS on mangrove sediments have been conducted and focused on bacterial composition and diversity (Liu *et al*., 2019; Jeyanny *et al*., 2020; Iturbe-Espinoza *et al*., 2022; Nimnoi and Pongsilp, 2022), prokaryotic diversity (Zhang *et al*., 2019; Lemoinne *et al*., 2023) and fungal diversity (Haldar and Nazareth, 2019).

Therefore, this research aims to utilize the NGS method (Environmental DNA or eDNA Metabarcoding) to examine the structure and composition of eukaryotes communities in mangrove sediments at Demak, Central Java, Indonesia. Environmental DNA is a technique for identifying DNA from organisms that has been released into the environment, such as in soil, water, or air. One of the molecular approaches for eDNA study is DNA Metabarcoding. DNA Metabarcoding is a fast and rapid molecular technique that identifies many species in a single sample by analyzing DNA sequences from short regions of genes. This research will use Cytochrome Oxidase Subunit I (COI) genetic markers and wants to see the advantages and disadvantages of this marker. It is hoped that there will be a protocol ready to be used to see the diversity of the mangrove sediment ecosystem in a short time and with massive data. This research will contribute to becoming a scientific reference in developing research related to the application of NGS technology, especially its relevance in efforts to develop sustainable mangrove ecosystem management strategies

Materials and Methods

The sampling was conducted in two locations, Bedono and Morosari village, Sayung district, Demak Regency, Central Java (Figure 1.) in June, 2022. These two locations were chosen because they have different characteristics. Bedono situated in the Mangrove Forest Conservation areas in the Demak region, close with the beach with high level of abrasion every year. On the other hand, Morosari situated in the estuaries of the Sayung river which is a fishing area and is affected by settlements. Two sediment samples were collected from each sampling point. 10 grams of mangrove sediment were collected using sterile spatula and preserved in 96% ethanol in new and sterile 15 ml falcon tubes. Samples were stored in the close cool box and then transported to Integrated Laboratory, Universitas Diponegoro for further analysis.

DNA extraction and molecular analysis

Around 0.25 g of sediment samples were extracted using ZymoBIOMICS Quick-DNA™ Fecal/Soil Microbe Miniprep Kit as described by the manufacturer. DNA was amplified using an amplicon-based approach utilizing the Next Generation Sequencing (NGS) methods. Cytochrome Oxidase Subunit 1 (COI) genes were targeted using the mlCOIintF (5'-GGW ACW GGW TGA ACW GTW TAY CCY CC-3') and dgHCO2198 (5'-TAA ACT TCA GGG TGA CCA AAR AAY CA-3') primers (Meyer, 2003; Leray *et al*., 2013). Library preparation for COI amplicons followed a single indexing approach where barcodes were incorporated into the forward primer to facilitate multiplexing of up to 96 samples per run. Sequencing was performed using the Illumina MiSeq sequencing platform at PT. Genetika Science Indonesia.

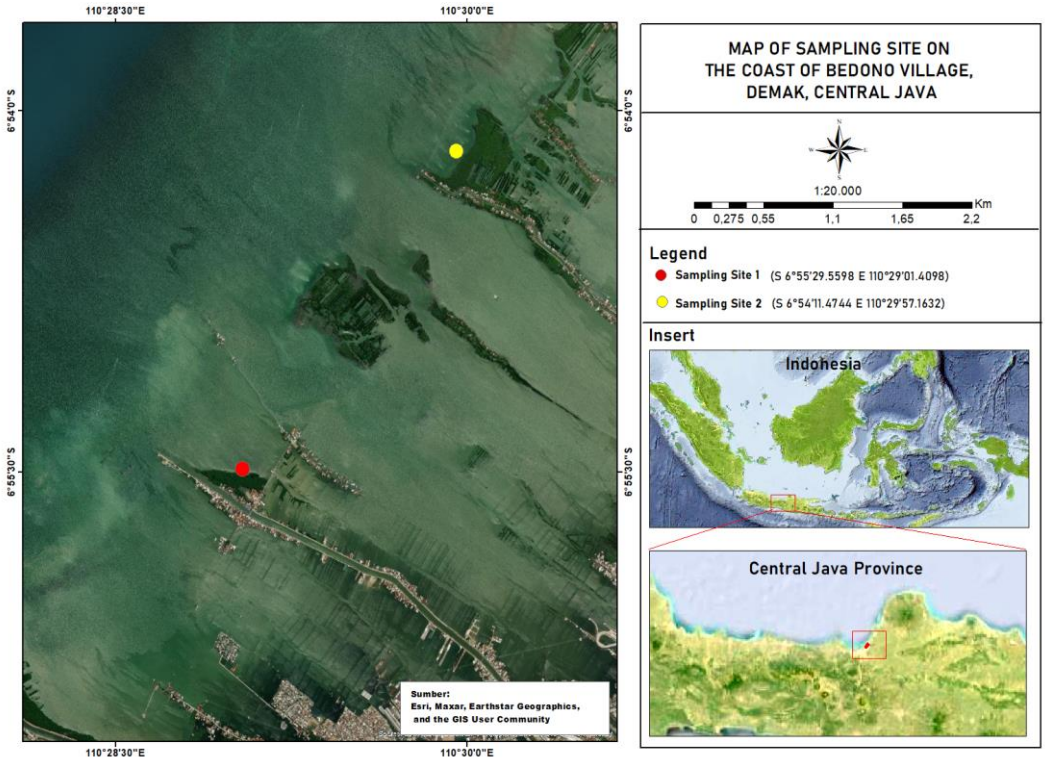

Figure 1. The map of sampling locations in Desa Bedono, Demak, Jawa Tengah, Indonesia. The red dot is the sampling site 1 in Morosari and the yellow dot is sampling site 2 in Bedono.

Bioinformatic analysis

Forward and reverse raw fast sequence files were obtained from the Illumina machine. Raw sequences were checked for quality, cleaned, de-noised, and merged with QIIME2 (the *Quantitative Insights Into Microbial Ecology 2 program*, https://qiime2.org/) (Bolyen *et al*., 2018) and Divisive Amplicon Denoising Algorithm 2 (*DADA2*) software (Callahan *et al*., 2016), wrapped in *QIIME2*. To assign taxonomy to the resulting ASVs (Amplicon Sequence Variants), we use "Creating Reference libraries Using eXisting tools" (CRUX), which generates comprehensive reference databases for specific user-defined metabarcoding loci from database such as NCBI (https://www.ncbi.nlm.nih.gov/) (Curd *et al*., 2018) for COI data. The *phyloseq* package (McMurdie & Holmes, 2013) in R Studio (R development core team) was used to summarize the taxonomic composition of each sample. Stacked bar plots summarizing taxonomic composition and sequence abundance were generated using *ggplot2* (Wickham, 2009) in R Studio (R development core team). Alpha and beta diversity were analysed using Vegan package (Oksanen, 2007; Oksanen *et al*., 2018) in R Studio (R development core team). For each sample and location, we used http://bioinformatics.psb.ugent.be/webtools/Venn/ to create a set of Venn diagrams to determine how many ASVs were shared among samples and sampling locations. The rarefaction curves were created with the Ranacapa package using the *grade* command (Kandlikar, *et al*., 2018).

Result and Discussion

Environmental DNA (eDNA) is an approach that is widely used to assess environmental conditions and also collect biodiversity data. The High Throughput Sequencing approach used in eDNA research enables the massive identification of DNA in the environment. One of the benefits of eDNA is looking at eukaryote communities in sediments, such as mangrove sediments in the Bedono area, Central Java. Biodiversity disclosures at the two locations used as sampling locations show that eukaryotic communities vary and are influenced by environmental conditions and factors at each sampling location.

There are only three samples that were successfully amplified using a COI marker (ID M20708 from Morosari and S0706 and S0709 from Bedono). The total reads obtained from this study is 96,419 reads and 828 ASVs range from 23,688 - 9,132 reads per sample. Eukaryote dominated the samples with 305 ASVs and 47,627 reads, followed by unidentified (47,893 reads and 492 ASVs), and

Bacteria (899 reads and 31 ASVs). The downstream analysis will exclude Bacteria and Unidentified ASVs. The Unidentified ASVs are sequences that did not assigned at a specific taxonomic level based on the database. The reads from each sample were rarefied to 6,824 reads to get an equal number of reads and avoid bias. A total of 20,472 reads and 303 ASVs were observed from the result. The rarefaction curve of COI amplicons reaches a plateau, indicating sufficient depth of sequencing to account for most of the amplified taxa (Figure 2.).

Overall, there are 16 Phylum (Chordata, Arthropoda, Bacillariophyta, Mollusca, Ascomycota, Phaeophyceae, Gastrotricha, Annelida, Nematoda, Nemertea, Basidiomycota, Cnidaria, Chlorophyta, Mucoromycota, Eustigmatophyceae, Kinorhyncha) and an unidentified group of taxa (Figure 3.). The communities were dominated by Chordata (45% of total reads), Arthropoda (17% of total reads) and also Bacillariophyta (11% of total reads). The analysis also reports 9% reads of unidentified taxa in both sampling areas.

Both sampling locations show a different taxa composition. The first location, Bedono is one of the Mangrove Forest Conservation areas in the Demak region (Wijaya *et al*., 2022) but has a high level of abrasion every year. Meanwhile, the location in Morosari was characterized by one of the estuaries of the Sayung river which is a fishing area (Maisaroh *et al*., 2020) and is affected by settlements and fishing activities. The differences between both locations lead to a difference in taxa composition, where Bedono was dominated by Chordata, Bacillariophyta, Ascomycota, and Arthropoda. Meanwhile, Morosari was dominated by Chordata and Arthropoda. In Bedono we can see the dominance of unidentified reads at species level is 75% and in Morosari it is 32% compared to total reads in each location. The results also show the dominance of *Homo sapiens* reads in Morosari (46%) and Bedono (13%) compared to total reads in each location.

Bedono has higher diversity compared with Morosari. Bedono has 218 ASVs, meanwhile Morosari has 91 ASVs. There are 87 genus and 78 species were identified in this study. This is supported by the Shannon-Wiener diversity index in Morosari of 2.97 and in Bedono of 4.27 and 2.84. In addition, Bray-Curtis dissimilarity analysis shows high taxa compositional differences between Morosari and Bedono. (Table 1). By subtracting the *Homo sapiens*, we can see many other species composed of both locations. In total, Bedono consists of 53 species, including mammalian, fish, and protists. On the other hand, Morosari get a smaller number of species (27 species), consisting of fish, arthropoda, and algae. The This study report 212 unique ASVs in Bedono, 85 unique ASVs in Morosari and 6 ASVs shared on both locations (Figure 4.).

Table 1. Bray-Curtis dissimilarity matrix between samples from Morosari (M20708) and Bedono (S0706 and S0709)

Sample ID	M20708	S0706
S0706	0.9838804	
S0709	0.8716295	0.7482415

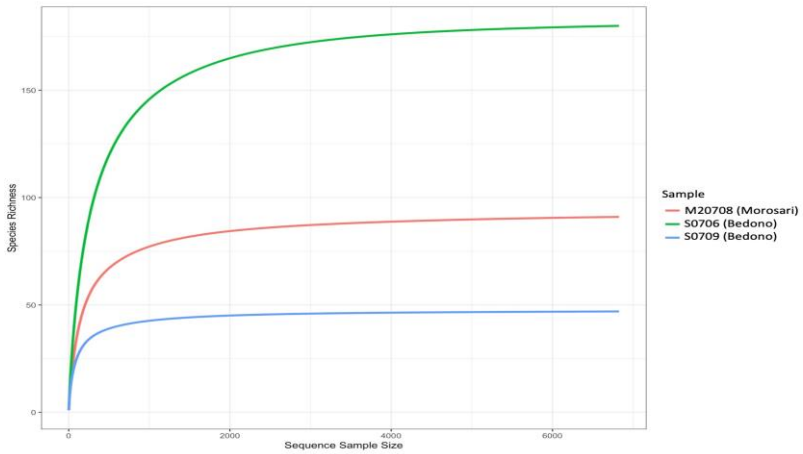

Figure 2. Rarefaction plot between two locations examined in this study. Species richness (left axis) plotted against sequencing depth (bottom axis).

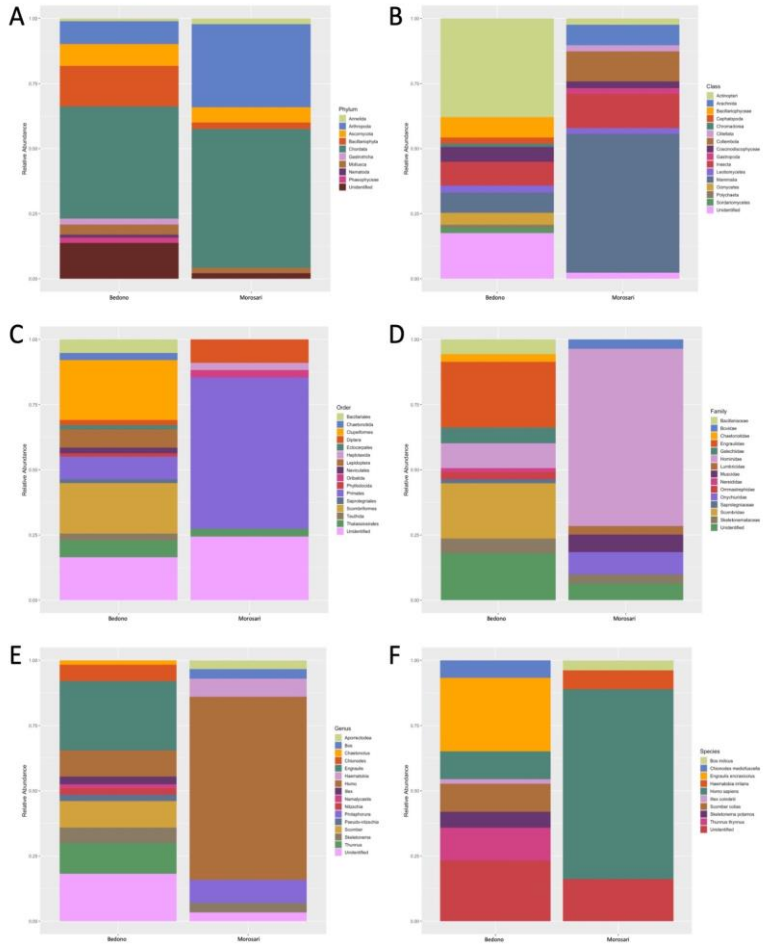

Figure 3. Bar plot showing the taxonomy composition based on the read abundance of eukaryotic taxa from each sample. The bar plot shows the taxonomic hierarchy of (A) Phylum, (B) Class, (C) Order, (D) Family, (E) Genus, and (F) Species. The plot only shows the taxa groups that represented more than 2% of total abundance in the sample.

The two research locations have different regional characteristics, so the composition of the organisms that make up the sediment ecosystem will also be different. In general, the taxa that dominate the two locations in this study are Phylum Arthropoda, Ascomycota, Bacillariophyta, Chordata. These taxa have different relative read abundances in each location. For example, at the Morosari sampling location, almost 46% of the reads in Morosari were identified as human (*Homo sapiens*) DNA. Although human DNA was also found at the Bedono location, the percentage was very small (7% of the total sequences at Bedono). Humans certainly contribute most of their DNA which escapes from residential areas and enters mangrove areas. This could be because the presence of a river is one of the factors that influences whether there is a lot of human DNA in the sediment, considering that the sampling location in Morosari is close to the river.

The Bedono sampling location, which is in an area with high mangrove cover, has several taxa closely related to marine ecosystems that have been identified, including marine fish, several types of insects, fungi and Bacillariophyta. If you pay attention, the types of vertebrates and invertebrates found in this area are dominated by taxa that are commonly found in the sea, such as molluscs, cnidaria, and fish. Meanwhile, the Morosari location is more influenced by land, so that some of the taxa identified (insecta and chordata) are taxa that are commonly found on land.

The chordates identified in mangrove sediments consist of several types of fish (*Auxis rochei, Engraulis encrasicolus, Gnathodentex aureolineatus, Pomadasys hasta, Scomber colias, Takifugu obscurus*, and *Thunnus thynnus*), mammals (*Bos indicus, Rattus norvegicus*, and *Homo sapiens*), and aves (*Gallus gallus*). *Bos indicus* and *Homo sapiens* are the two taxa shared by both locations, while *Homo sapiens* is the dominant taxa identified from Morosari (15% of the total reads) (Figure 5.).

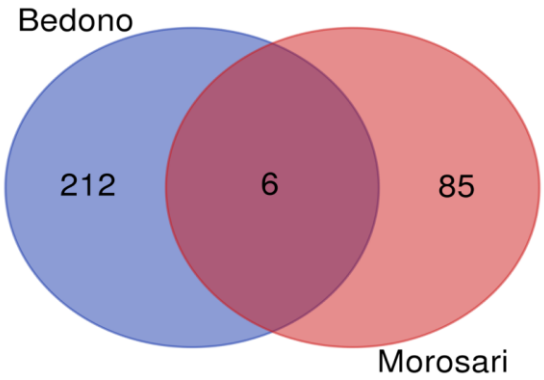

Figure 4. The Venn diagram shows the number of unique and shared ASVs (Amplicon Sequence Variance) from two different study locations.

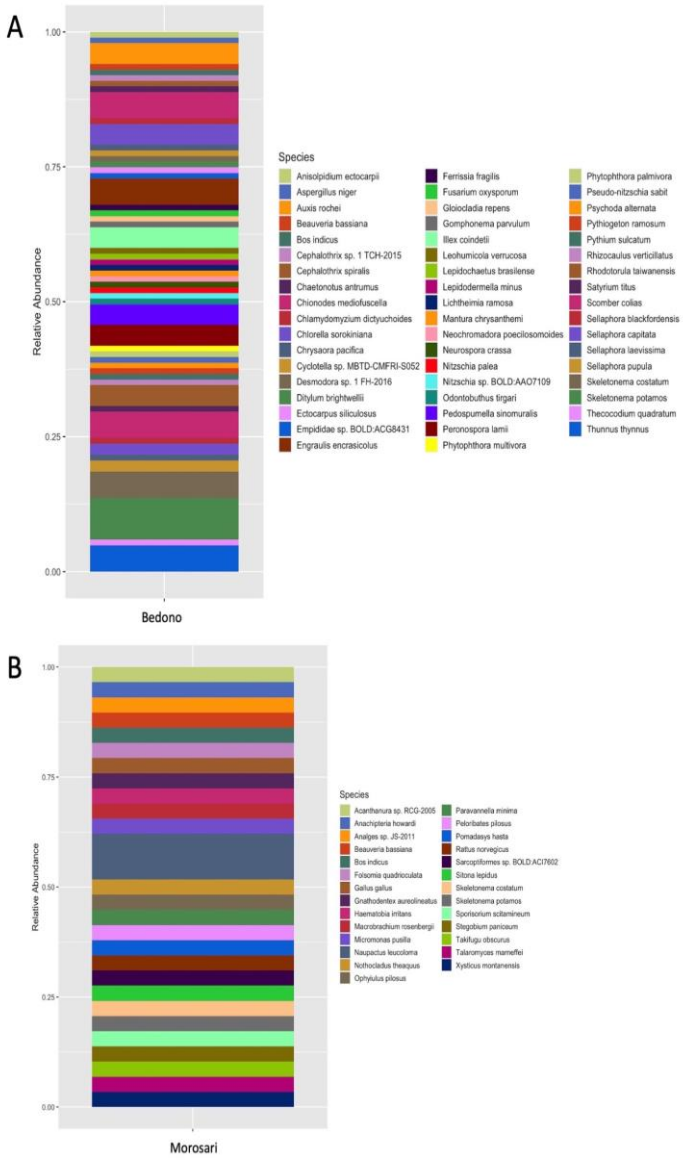

Figure 5. Bar plot showing the taxonomy composition based on the read abundance of eukaryotic taxa from each sample after removing the *Homo sapiens* and unidentified group. The bar plot shows the taxonomic hierarchy of (A) Sample from Bedono and (B) Sample from Morosari.

The invertebrates identified in this study were from the phylum Arthropoda, Mollusca, Cnidaria, Nematoda, Nemertea and Gastrotricha. The taxa found at the Bedono sampling location were dominated by Mollusca, Cnidaria, Nematoda, Nemertea and Gastrotricha and several arthropods including moths, butterflies and beetles. Meanwhile, from the Morosari area, only types of land arthropods such as collembola, millipede, arachnids, beetles and spiders were identified (Appendix 1.).

This study also identified several types of fungi, both from the phylum Ascomycota, Basidiomycota and the class Oomycetes. Most types of these fungi were identified from the Bedono area (14 of 16 total species), and only 3 species were identified from Morosari (*Beauveria bassiana*, *Talaromyces marneffei*, and *Sporisorium scitamineum)*.

There are three classes of Phylum Bacillariophyta, 5 orders, 6 families, and 7 genus. There are 12 species identified, *Cyclotella* sp. MBTD-CMFRI-S052, *Ditylum brightwellii, Gomphonema parvulum, Nitzschia palea, Nitzschia* sp. BOLD:AA007109, *Pseudo-nitzschia sabit, Sellaphora blackfordensis, Sellaphora capitata, Sellaphora laevissima, Sellaphora pupula, Skeletonema costatum,* and *Skeletonema potamos* (Appendix 1).

Apart from that, the taxa identified from the mangrove sediments in Bedono and Morosari are groups of algae from the phyla Chlorophyta and Phaeophyceae and the class Florideophyceae. These algae are types that are commonly found in marine waters, for example filamentous brown algae (*Ectocarpus siliculosus*), red algae (*Gloiocladia repens*)

The eDNA identification results, especially for vertebrates, must also be interpreted with caution. In the results of this analysis, several other vertebrates found, such as several types of fish found (*Engraulis encrasicolus, Thunnus thynnus, Scomber colias, Gnathodentex aureolineatus, Pomadasys hasta, Auxis rochei,* and *Takifugu obscurus*) are indeed types that might be found in sediment because DNA that escapes from the organism and settles in sediment. However, several organisms such as *Homo sapiens, Bos indicus, Rattus norvegicus*, and *Gallus gallus* are often considered contaminants if found in marine or fresh waters (Klymus *et al*., 2017; Dass *et al*., 2022). However, in this study, these taxa are still counted as part of the community, because of the close relationship between the mangrove community and land, so it is still possible for DNA from these taxa to enter the mangrove ecosystem. The use of the eDNA method in conducting environmental assessments is very effective (Juhel *et al*., 2020; Madduppa *et al*., 2021; Marwayana *et al*., 2022). Not only as a tool to identify species in an ecosystem, but it can also be used as a tool to determine the introduction of alien species (Mohd Dali *et al*., 2023; Rahmi *et al*., 2023) or pathogens (Sato *et al*., 2019; Brannelly *et al*., 2020). For sampling locations in remote areas, several research teams have also developed portable labs (Chang *et al*., 2020; Ames *et al*., 2021) which allow all activities to be carried out directly in the field.

However, this method also has weaknesses. Some of the weaknesses that often occur are poor sampling and sample transportation processes (Wee *et al*., 2023). Apart from that, choosing the right marker and sequencing platform according to the target organism is also very challenging. For example, the selection of COI and 18S rRNA markers in sediment samples must be chosen based on the target organism (Giebner *et al*., 2020). COI will capture more metazoans (Clarke *et al*., 2021) while 18S rRNA will capture more single cell eukaryotes (rRNA) (Guo *et al*., 2015; Pratomo *et al*., 2022).

In the future, by paying attention to all the advantages and disadvantages of this method, it is hoped that we can carry out better sampling and carry out a sampling plan according to the research question, so that our interpretation of the data can be close to what we expect. The development of platforms for sequencing and bioinformatics should also be a strength of eDNA researchers in the field considering its rapid development. The hope is that the eDNA approach can be used as an important tool in collecting biodiversity data in Indonesia.

Conclusion

Molecular approaches such as eDNA metabarcoding are a complementary method for collecting biodiversity data, especially mangrove biodiversity data collection in Indonesia. Mangroves are a vital part of the ecosystem, providing many functions, including food security, nesting and migration, and soil stabilization. Mangrove sediment plays a vital role in the mangrove ecosystem and the environment as a whole, by accumulating carbon, supporting biodiversity, and providing nurseries. The research is a preliminary study to see the diversity of eukaryotes in mangrove sediments at two research locations. The gene targeted is the COI gene which is a universal marker for recording eukaryotes. In this study, it can be seen that the two research locations have different types of taxa with little overlap. This shows that eDNA can be used to assess diversity in mangrove sediments, both macro and microorganism.

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Appendix 1. The species list identified from the eDNA samples from Bedono and Morosari, Demak, Central Java

Phylum	Class	Order	Family	Genus	Species	Location	
						Bedono	Morosari
Chordata	Actinopteri	Scombriformes	Scombridae	*Auxis*	*Auxis rochei*	1	
Chordata	Mammalia	Unidentified	Bovidae	*Bos*	*Bos indicus*	1	1
Chordata	Actinopteri	Clupeiformes	Engraulidae	*Engraulis*	*Engraulis encrasicolus*	1	
Chordata	Aves	Galliformes	Phasianidae	*Gallus*	*Gallus gallus*		1
Chordata	Actinopteri	Spariformes	Lethrinidae	*Gnathodentex*	*Gnathodentex aureolineatus*		1
Chordata	Mammalia	Primates	Hominidae	*Homo*	*Homo sapiens*	1	1
Chordata	Actinopteri	Lutjaniformes	Haemulidae	*Pomadasys*	*Pomadasys hasta*		1
Chordata	Mammalia	Rodentia	Muridae	*Rattus*	*Rattus norvegicus*		1
Chordata	Actinopteri	Scombriformes	Scombridae	*Scomber*	*Scomber colias*	1	
Chordata	Actinopteri	Tetraodontiformes	Tetraodontidae	*Takifugu*	*Takifugu obscurus*		1
Chordata	Actinopteri	Scombriformes	Scombridae	*Thunnus*	*Thunnus thynnus*	1	
Arthropoda	Collembola	Unidentified	Neanuridae	*Acanthanura*	*Acanthanura sp. RCG-2005*		1
Arthropoda	Arachnida	Oribatida	Achipteriidae	*Anachipteria*	*Anachipteria howardi*		1
Arthropoda	Arachnida	Astigmata	Analgidae	*Analges*	*Analges sp. JS-2011*		1
Arthropoda	Insecta	Lepidoptera	Gelechiidae	*Chionodes*	*Chionodes mediofuscella*	1	
Arthropoda	Insecta	Diptera	Empididae	*Empididae*	*Empididae sp. BOLD:ACG8431*	1	
Arthropoda	Collembola	Unidentified	Isotomidae	*Folsomia*	*Folsomia quadrioculata*		1
Arthropoda	Insecta	Diptera	Muscidae	*Haematobia*	*Haematobia irritans*		1
Arthropoda	Malacostraca	Decapoda	Palaemonidae	*Macrobrachium*	*Macrobrachium rosenbergii*		1
Arthropoda	Insecta	Coleoptera	Chrysomelidae	*Mantura*	*Mantura chrysanthemi*	1	
Arthropoda	Insecta	Coleoptera	Curculionidae	*Naupactus*	*Naupactus leucoloma*		1
Arthropoda	Arachnida	Scorpiones	Buthidae	*Odontobuthus*	*Odontobuthus tirgari*	1	
Arthropoda	Diplopoda	Julida	Julidae	*Ophyiulus*	*Ophyiulus pilosus*		1
Arthropoda	Arachnida	Oribatida	Haplozetidae	*Peloribates*	*Peloribates pilosus*		1
Arthropoda	Insecta	Diptera	Psychodidae	*Psychoda*	*Psychoda alternata*	1	
Arthropoda	Arachnida	Unidentified	Unidentified	*Sarcoptiformes*	*Sarcoptiformes sp. BOLD:ACI7602*		1
Arthropoda	Insecta	Lepidoptera	Lycaenidae	*Satyrium*	*Satyrium titus*	1	
Arthropoda	Insecta	Coleoptera	Curculionidae	*Sitona*	*Sitona lepidus*		1
Arthropoda	Insecta	Coleoptera	Anobiidae	*Stegobium*	*Stegobium paniceum*		1
Arthropoda	Arachnida	Araneae	Thomisidae	*Xysticus*	*Xysticus montanensis*		1
Mollusca	Gastropoda	Unidentified	Ancylidae	*Ferrissia*	*Ferrissia fragilis*	1	
Mollusca	Cephalopoda	Teuthida	Ommastrephidae	*Illex*	*Illex coindetii*	1	
Cnidaria	Scyphozoa	Semaeostomeae	Pelagiidae	*Chrysaora*	*Chrysaora pacifica*	1	
Cnidaria	Hydrozoa	Leptothecata	Campanulariidae	*Rhizocaulus*	*Rhizocaulus verticillatus*	1	
Cnidaria	Hydrozoa	Anthoathecata	Ptilocodiidae	*Thecocodium*	*Thecocodium quadratum*	1	
Nematoda	Chromadorea	Desmodorida	Desmodoridae	*Desmodora*	*Desmodora sp. 1 FH-2016*	1	
Nematoda	Chromadorea	Chromadorida	Chromadoridae	*Neochromadora*	*Neochromadora poecilosomoides*	1	
Nemertea	Palaeonemertea	Unidentified	Cephalothricidae	*Cephalothrix*	*Cephalothrix sp. 1 TCH-2015*	1	

Appendix 1. The species list identified from the eDNA samples from Bedono and Morosari, Demak, Central Java (continue)

Phylum	Class	Order	Family	Genus	Species	Location	
						Bedono	Morosari
Nemertea	Palaeonemertea	Unidentified	Cephalothricidae	*Cephalothrix*	*Cephalothrix spiralis*	1	
Gastrotricha	Unidentified	Chaetonotida	Chaetonotidae	*Chaetonotus*	*Chaetonotus antrumus*	1	
Gastrotricha	Unidentified	Chaetonotida	Chaetonotidae	*Lepidochaetus*	*Lepidochaetus brasilense*	1	
Gastrotricha	Unidentified	Chaetonotida	Chaetonotidae	*Lepidodermella*	*Lepidodermella minus*	1	
Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	*Aspergillus*	*Aspergillus niger*	1	
Ascomycota	Sordariomycetes	Hypocreales	Cordycipitaceae	*Beauveria*	*Beauveria bassiana*	1	1
Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	*Fusarium*	*Fusarium oxysporum*	1	
Ascomycota	Leotiomycetes	Unidentified	Unidentified	*Leohumicola*	*Leohumicola verrucosa*	1	
Ascomycota	Sordariomycetes	Sordariales	Sordariaceae	*Neurospora*	*Neurospora crassa*	1	
Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	*Talaromyces*	*Talaromyces marneffei*		1
Basidiomycota	Microbotryomycetes	Sporidiobolales	Sporidiobolaceae	*Rhodotorula*	*Rhodotorula taiwanensis*	1	
Basidiomycota	Ustilaginomycetes	Ustilaginales	Ustilaginaceae	*Sporisorium*	*Sporisorium scitamineum*		1
Mucoromycota	Unidentified	Mucorales	Lichtheimiaceae	*Lichtheimia*	*Lichtheimia ramosa*	1	
Unidentified	Oomycetes	Anisolpidiales	Anisolpidiaceae	*Anisolpidium*	*Anisolpidium ectocarpii*	1	
Unidentified	Oomycetes	Myzocytiopsidales	Myzocytiopsidaceae	*Chlamydomyzium*	*Chlamydomyzium dictyuchoides*	1	
Unidentified	Oomycetes	Peronosporales	Peronosporaceae	*Peronospora*	*Peronospora lamii*	1	
Unidentified	Oomycetes	Peronosporales	Unidentified	*Phytophthora*	*Phytophthora multivora*	1	
Unidentified	Oomycetes	Peronosporales	Unidentified	*Phytophthora*	*Phytophthora palmivora*	1	
Unidentified	Oomycetes	Pythiales	Pythiogetonaceae	*Pythiogeton*	*Pythiogeton ramosum*	1	
Unidentified	Oomycetes	Pythiales	Pythiaceae	*Pythium*	*Pythium sulcatum*	1	
Bacillariophyta	Coscinodiscophyceae	Thalassiosirales	Stephanodiscaceae	*Cyclotella*	*Cyclotella sp. MBTD-CMFRI-S052*	1	
Bacillariophyta	Mediophyceae	Lithodesmiales	Lithodesmiaceae	*Ditylum*	*Ditylum brightwellii*	1	
Bacillariophyta	Bacillariophyceae	Cymbellales	Gomphonemataceae	*Gomphonema*	*Gomphonema parvulum*	1	
Bacillariophyta	Bacillariophyceae	Bacillariales	Bacillariaceae	*Nitzschia*	*Nitzschia palea*	1	
Bacillariophyta	Bacillariophyceae	Bacillariales	Bacillariaceae	*Nitzschia*	*Nitzschia sp. BOLD:AAO7109*	1	
Bacillariophyta	Bacillariophyceae	Bacillariales	Bacillariaceae	*Pseudo-nitzschia*	*Pseudo-nitzschia sabit*	1	
Bacillariophyta	Bacillariophyceae	Naviculales	Sellaphoraceae	*Sellaphora*	*Sellaphora blackfordensis*	1	
Bacillariophyta	Bacillariophyceae	Naviculales	Sellaphoraceae	*Sellaphora*	*Sellaphora capitata*	1	
Bacillariophyta	Bacillariophyceae	Naviculales	Sellaphoraceae	*Sellaphora*	*Sellaphora laevissima*	1	
Bacillariophyta	Bacillariophyceae	Naviculales	Sellaphoraceae	*Sellaphora*	*Sellaphora pupula*	1	
Bacillariophyta	Coscinodiscophyceae	Thalassiosirales	Skeletonemataceae	*Skeletonema*	*Skeletonema costatum*	1	1
Bacillariophyta	Coscinodiscophyceae	Thalassiosirales	Skeletonemataceae	*Skeletonema*	*Skeletonema potamos*	1	1
Unidentified	Unidentified	Unidentified	Vannellidae	*Paravannella*	*Paravannella minima*		1
Chlorophyta	Trebouxiophyceae	Chlorellales	Chlorellaceae	*Chlorella*	*Chlorella sorokiniana*	1	
Chlorophyta	Mamiellophyceae	Mamiellales	Mamiellaceae	*Micromonas*	*Micromonas pusilla*		1
Phaeophyceae	Unidentified	Ectocarpales	Ectocarpaceae	*Ectocarpus*	*Ectocarpus siliculosus*	1	
Unidentified	Florideophyceae	Rhodymeniales	Faucheaceae	*Gloiocladia*	*Gloiocladia repens*	1	
Unidentified	Florideophyceae	Batrachospermales	Batrachospermaceae	*Nothocladus*	*Nothocladus theaquus*		1
Unidentified	Chrysophyceae	Chromulinales	Chromulinaceae	*Pedospumella*	*Pedospumella sinomuralis*	1	
					Total Species	54	28