Indonesian Journal of Limnology
Vol. 05 No. 01, 2024 (39–48)
E-ISSN :2774-2571

Environmental DNA Application to Identify Protozoan Community in the Sediment of Balekambang Lake, Dieng, Central Java

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Received 16 November 2023 Accepted 25 June 2024 Published 29 August 2024

DOI: 10.51264/inajl.v5i1.48

Abstract

Lake Balekambang, Dieng, has a high level of sedimentation that can affect water quality, so it is necessary to identify organisms to assess the lake's environmental conditions. The use of eDNA methods in Indonesia is minimal, especially in the Dieng area. This research aims to identify the protozoan communities in sediment samples from Lake Balekambang using the Environmental DNA (eDNA) method to assess the lake ecosystem. This study targeting the 18S rRNA gene for single cell eukaryotes. Data analysis was performed using GALAXY and RStudio. Bioinformatics analysis obtained 51,172 reads divided into 48 Amplicon Sequence Variants (ASVs). All specimens were identified as eukaryotes. Protozoa taxa that can be identified are Tritrichomonadidae, Ophryoscolecidae, Gregarinidae, Cyrtolophosididae, Hexamitidae, Isotrichidae, Oxytrichidae, Vannellidae, Vermamoebidae, and other unidentified Eukaryota taxa**.** Using the eDNA method this study able to identify the protozoan community found in the sediments of Lake Balekambang, Dieng. Protozoa taxa that are identified are taxa that generally inhabit the rumen of ruminants and the gastrointestinal tract of rodents, and are generally pathogens that can cause disease in humans and animals. New knowledge about Environmental DNA Metabarcoding (eDNA) can support research in identifying organisms. This study shows that the eDNA method utilizing the Next Generation Sequencing (NGS) approach are able to identify the protist more effectively, massively, and quickly compared to conventional methods based on morphological characteristics.

Keywords: Protozoa, *Next Generation Sequencing* (*NGS), Environmental DNA, 18S rRNA, Balekambang Dieng*

1. Introduction

Balekambang Lake is one of the small lakes in Dieng, Central Java, that is threatened by high sedimentation and runoff rates. This lake is located near the Arjuna Temple area, which functions as a reservoir and drainage (Pamrayoga *et al.*, 2017). The very high sedimentation rate of Balekambang Lake causes the lake surface to be covered by herbaceous plants (Soeprobowati *et al.*, 2019). In addition, the surrounding community is utilized to irrigate agricultural land, resulting in potential eutrophication problems.

In assessing water quality, organisms (bioindicators) such as Protozoa can be used. Protozoa are a group of protists found in waters and the bodies of other organisms (Astuti, 2017). The presence of protozoa in the environment is used to determine environmental health. A polluted

environment allows pathogenic microbes (viruses, bacteria, and protozoa) to grow and develop (Winarni 2016).

Currently, identification techniques are still done conventionally. This method is based on morphological characteristics visible using a microscope (Opat *et al.*, 2016; Indraswari *et al.*, 2017). Identification of microalgae and Protozoa was carried out using identification reference books from Edmonson (1959), Basmi (1999), Prescott (1964), Wickstead (1965), Davis (1995), Greeson (1982), Levine (1985), Hall (1953) (Opat *et al.*, 2016). In addition, conventional methods are carried out by culturing on artificial media, microscopic examination, and biochemical tests (Wulandari *et al.*, 2021). However, microorganisms are difficult to show clear morphological differences and are difficult to culture (Annenkova *et al.*, 2020). This method is considered ineffective because it requires extra costs and labor (Rota *et al.*, 2020) and a proficient and experienced taxonomist is needed (Zhao *et al.*, 2021).

Currently, identification techniques for organisms are carried out molecularly by DNA sequencing. DNA sequencing is used to determine the sequence of nitrogenous bases (adenine, guanine, cytosine, and thymine) in DNA samples. The first sequencing method developed was Sanger sequencing (Ip *et al.*, 2019). In its work, the Sanger method uses a DNA barcoding approach. The DNA barcoding method can only identify single organisms and is based on short pieces of DNA (Achmad *et al.* 2019). Next Generation Sequencing (NGS) is the second generation in DNA sequencing, so the sequencing process is faster, more specific, and can read longer DNA nucleotide sequences at one time (Pavan-Kumar *et al.* 2015). This method uses a DNA metabarcoding approach so that DNA reading can be done in large quantities simultaneously. Metabarcoding in identification uses existing and recognized molecular markers such as 16S rRNA, 18S rRNA or Cytochrome Oxidase I (COX/COI) (Pavan-Kumar *et al.*, 2015). The protozoan identification method uses 18S rRNA (ribosomal RNA) molecular markers. The 18S rRNA gene encodes a ribosomal subunit that makes up a small portion of eukaryotic ribosomes (Roslim *et al.*, 2018).

An identification method that uses a DNA metabarcoding approach is Environmental DNA (eDNA) (Collins *et al.*, 2019). Environmental DNA is the development of methods used in monitoring biodiversity that experience limitations in conventional methods using morphological approaches. The eDNA approach is an efficient method because organisms are complex to identify using conventional methods (Taberlet *et al.*, 2012; Ruppert *et al.*, 2019). This method is based on genetic material left in the environment (soil, sediment, and water) because organisms that live in water leave genetic material through feces, urine, or exfoliated tissue (Djalil *et al.*, 2018). Sediment samples contain more sources of genetic material, are durable, and provide good results (Kang *et al.*, 2021).

The existence of Balekambang Lake is currently endangered (Pudjoarinto & Cushing, 2001; Soeprobowati *et al.*, 2018; Harriyadi, 2020). However, its existence is very important because it is used as irrigation, a source of spring water, and storage flooding and drainage for Arjuna Temple (Pudjoarinto & Cushing, 2001; Harriyadi, 2020; Soeprobowati *et al.*, 2018). Therefore, it is necessary to assess environmental quality with protozoan bioindicators. Research on protozoa in water sources has been done but is still limited (Wahyuni *et al.*, 2022), especially in Balekambang lake has never been done. According to Stat *et al.* (2017), research using the eDNA method in identifying a community is still very limited. Therefore, this research was conducted in order to assess the protist community in Balekambang Lake to determine its condition.

2. Material and Methods

2.1. Sample Collection

Sampling was carried out in the sediment waters of Balekambang Lake, Dieng, at one point (-7.2076195S, 109.9082364E) by taking approximately 10 grams of surface sediment using a sterile spatula and then putting it into a 15 ml falcon tube containing 10 ml of 96% ethanol. The samples

were then stored in a coolbox at 4^{o}C. We choose this location since the point was close to the inlet from agricultural area surrounding the lake (Figure 1)

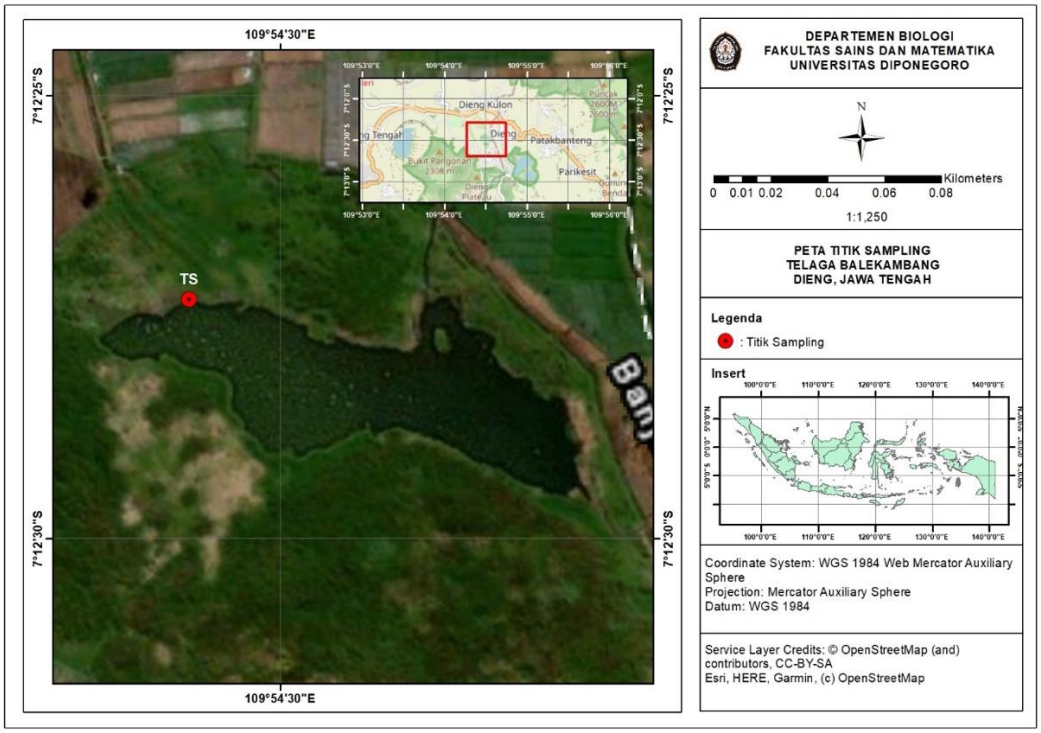

Figure 1. Research Site for Coring Sediment Sample in Lake Balekambang

2.2. DNA Extraction

DNA extraction was performed using the Zymo Quick-DNA^{TM} Fecal/Soil Microbe MiniPrep Kit. The Quick-DNA^{TM} Fecal/Soil Microbe MiniPrep Kit is a commercial DNA extraction kit that uses column filtration in DNA separation (Wardoyo *et al.*, 2020). The extraction procedure follows the available guidelines (Sani *et al.*, 2021).

2.3. DNA Quantitative test

Quantitative tests were performed using NanoDrop with a wavelength of 260/280 nm. DNA purity can be measured by the ratio of absorbance at wavelengths of 260 nm and 280 nm, good DNA purity is with the ratio of 1.8 - 2.0 (Dewanata and Mushlih, 2021). Good DNA purity measurement results are around 1.8 - 2.0 with concentrations above 100 ng/µl.

2.4. Sequencing

The extraction results then underwent PCR (Polymerase Chain Reaction) and sequencing processes performed by the sequencing facility. Primer 18S gene V4 region is one of the primers used to amplify the 18S rRNA gene in eukaryotes. This primer amplifies about 270-387 bp (Choi and Park 2020). The uses in this study are forward primer TAReuk454FWD1 (5′-CCAGCASCYGCGGTAATTCC-3′) and reverse primer TAReukREV3 (5′-ACTTTCGTTCTTGATYRA-3′). Sequencing was performed by NGS using an amplicons-based method. The targeted gene was 18S rRNA. Sequencing using Illumina MiSeq sequencing machine. The results obtained were raw data in FASTQ format.

2.5. Bioinformatics analysis

Sequencing data were processed using the GALAXY (https://usegalaxy.org/) and Rstudio programs. In the GALAXY program, the data were checked for quality. Furthermore, cutting adapters, primers and unwanted sequences (*chimera*) and merging sequences with DADA2 were carried out to get clean data. DADA2 gives sample sequences exactly (Callahan *et al*., 2016). Taxonomic comparisons were made with the database. PR2 was downloaded from https://pr2-database.org (Batut *et al*., 2021; Escudié *et al*., 2017; Vaulot *et al*., 2021). Furthermore, data visualization was done with Rstudio. This program produces graphs in the form of barplots (Li *et al*., 2022). In addition, Rstudio also calculates the Shanon-Wiener and Simpson indices.

3. Results and Discussion

3.1. DNA Quantitative Test Results and Sequencing

The results of quantitative tests on samples have DNA purity which is obtained 2.31. while the resulting DNA concentration shows 0.8 ng/µL, less than 100 ng/µL. The quantitative test results on the sample have DNA purity obtained of 2.31. DNA extraction kits in DNA isolation can influence the DNA purity value in these samples. DNA extraction kits are designed for DNA isolation with quality results (Sophian and Syukur 2021).

The resulting DNA concentration shows less than 100 ng/µL, which is 0.8 ng/µL. However, the DNA can still be used at the amplification stage by adding amounts to increase its concentration. The DNA concentration and purity can be affected by extraction, damage, and contaminants (Mustafa *et al*., 2016). The level of purity and concentration of DNA produced can still be used for further analysis in the Library Preparation process at Genetics Science because the minimum required is 2 ng/uL. At the library preparation stage, the DNA sample is carried out a quality control process which aims to ensure that the DNA sample used is of good quality so that accurate results are obtained (Zuhdi *et al*., 2021).

Based on bioinformatics analysis using GALAXY and Rstudio, 51,172 reads were obtained, divided into 48 Amplicon Sequence Variants (ASVs). Based on the data obtained from the sample, all ASVs belong to the Eukaryota Domain, Taxa identified were 12 classes, 12 orders, 13 families, 14 genera, and 13 species. Based on the 13 taxa identified, most of them were Protozoan taxa, with 9 taxa found in the samples, namely Tritrichomonadidae, Ophryoscolecidae, Gregarinidae, Cyrtolophosididae, Hexamitidae, Isotrichidae, Oxytrichidae, Vannellidae, Vermamoebidae. Other Eukaryota taxa are Rhabditidae, Insecta, and Saccharomycetidae. Dominance percentage based on the ASV results shown in Figure 1.

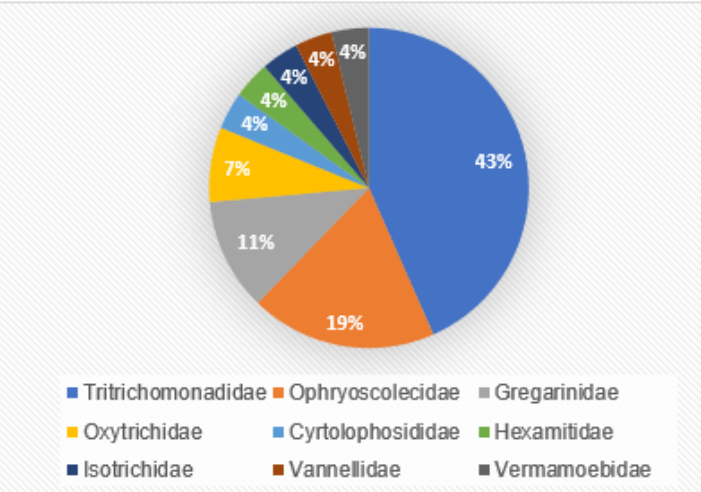

Figure 2. Percentage of Identified Protozoan Taxa from Balekambang Lake, Dieng based on the number of Amplicon Sequence Variants (ASVs).

The 18S rRNA primers are markers that can detect microscopic eukaryotic organisms in the ecosystem (Wang *et al*., 2014). Using 18S rRNA in PCR is a marker that can determine the phylogeny of random species in eukaryote biodiversity (Prakoso *et al.*, 2016). Metabarcoding research with eukaryote objects uses the 18S rRNA gene (Vaulot *et al*., 2016). The 18S rRNA gene is a commonly used marker for protists (Pawlowski *et al.*, 2012). eDNA study in Sanmenxia Reservoir, China using 18S rRNA and COI markers in Zooplankton identification with DNA Metabarcoding showed a difference in the number of Zooplankton taxa identified. The use of 18S rRNA resulted in 319 OTUs divided into 12 phyla, 23 classes, 54 orders, 83 families, and 110 genera. While the use of COI obtained 395 OTU which is divided into 4 phyla, 7 classes, 19 orders, 42 families, and 80 genera. Identification of Protozoa on 18S rRNA produces more taxa than the use of COI (Zhao *et al*., 2021).

In bioinformatics analysis using RStudio, the calculation of the Shanon-Wiener index on Protozoa taxa that can be identified shows a value of 3.26. The Shanon-Wiener index with a value >3 indicates that the diversity of taxa is high (Baderan *et al.*, 2014). The diversity of Protozoa taxa in sediment samples in Lake Balekambang is high, indicates that based on Protozoa community, the ecosystem of Lake Balekambang is relatively stable. Simpson's index shows that Protozoa taxa that can be identified in the sample obtained a value of 0.96. Simpson's index, with a value close to one, indicates that the dominance of taxa is high (Wahyuningsih *et al.*, 2019). The diversity index value increases when the number of species increases. The Shannon-Wiener Index (H) and Simpson's Index measure species richness and abundance. High values of the Shanon-Wiener index and Simpson index indicate that individuals are more evenly distributed in an area (Sharashy, 2022).

Tritrichomonadidae dominates the Protozoa community in Lake Balekambang (Figure 2). Most of these protozoans are found in the animal's intestines as parasites. This finding could be related to the conditions around Balekambang Lake, an area of agriculture and animal husbandry (Bandeira & De Souza, 2021). The abundance of Protozoa relates to the warming, eutrophication, and pesticide pollution. Warm and eutrophic lake considerably promote an increase in protozoan whereas warming and pesticides can remarkably reduce the abundance and diversity of protozoa (Yuan *et al.*, 20224). Lake Balekambang was eutrophic due to potatoes plantation and high sedimentation (Harriyadi, 2020).

3.2. Identified Taxa

Based on the 13 Eukaryote taxa identified, there are 9 Protozoan taxa with 3 dominating taxa based on the number of ASVs in the sediment samples of Balekambang Lake (Fig. 2), namely Tritrichomonadidae, Ophryoscolecidae, and Gregarinidae.

Unidentified taxa dominated the sample in Balekambang Lake, accounting for 29% of the total sample, with approximately 14 Unidentified ASVs obtained. Unidentified taxa in the samples could only be identified in Domain and Phylum taxa, that is Eukaryote Domain and Metazoan Phylum. This is due to the lack of data in the PR2 database, so further molecular research is needed to collect tropical databases. Unidentified sequences of taxa that have not been recorded in the global database (Li *et al*., 2022).

Tritrichomonadidae can generally be found in the digestive tract of animals such as rodents (rats and hamsters) (Al-Masaudi *et al*., 2020) and ruminants. Tritrichomonadidae taxa, which were very dominant, were found in Balekambang Lake, namely 23% of the total samples with 11 ASVs produced, with the identified species being *Tritrichomonas muris*. *T. muris* is a protozoan found in the digestive tract of mice and other rodents (Da Costa *et al.*, 2019) and feeds on white blood cells and bacteria (Shahata, 2022). *T. muris* is found in mice's colon and small intestine and can exacerbate disease in mice (Escalante *et al.* 2016).

Ophryoscolecidae is a group of protozoa belonging to the ciliate class that could generally be found in the gastrointestinal tract of ruminant animals such as cattle and sheep (Ito and Tokiwa 2018). Ophryoscolecidae taxa was found in Balekambang Lake is 10% of the samples with a total of 5 ASVs identified, including the genus *Entodinium* and *Ophryoscolex*. The first taxonomy of Ophryoscolecid ciliates in the rumen was from *Entodinium* Stein and *Ophryoscolex* Stein (Cedrola *et al*., 2022).

Gregarinidae are unicellular parasites that generally infect the digestive tract of invertebrates and insects (Devetak *et al*., 2013). The Gregarinidae taxa were found in Balekambang Lake in 6% of the total samples, with 3 ASVs produced, consisting of *Gregarina cloptoni. G. cloptoni* is one of the species in *Gregarina* found in the digestive tract of insects (Nocciolini *et al*., 2018).

Oxytrichidae is a protozoan of the ciliate group that belongs to the phylum Ciliophora. Oxytrichidae are usually found in freshwater and seawater. The identified Oxytrichidae found in Balekambang Lake obtained 4% in the total sample, namely 2 ASVs. The Oxytrichidae could only be identified at the family level due to the lack of data in the database used. Oxytrichidae taxa protozoa are generally non-parasitic intestinal protozoa. In another study in the rehabilitation center Yiari Ciapus, Bogor, Oxytrichidae taxa was found, namely the species *Oxytricha granulifera* in the feces of the Sumatran slow loris (*Nycticebus coucang*) (Rukmana *et al*., 2016).

Cyrtolophosididae taxa is a protozoan that belongs to the phylum Ciliophora. This taxa can be found in marine, freshwater and land-based environments. Cyrtolophosididae taxa found in Balekambang Lake is 2% of the samples with 1 ASVs. This taxon was also only identified in the family taxa due to the lack of database used.

Hexamitidae taxa includes protozoa, a group of Flagellates that can generally be found in the digestive tract of rodents. Hexamitidae taxa were found in Balekambang Lake 2% of the total samples, namely the species *Spironucleus muris. S. muris* (previously known as *Hexamita muris*) is a type of pathogenic protozoan that is pear-shaped, has many flagellates, namely 6 anterior and 2 posteriors (Voros *et al*., 2021). *S. muris* is a type of protozoan that generally colonizes the intestinal tract in rodent species (Rodentia). *S. muris* is found in rodents such as rats and hamsters (Heidari *et al*., 2018).

Isotrichidae is a group of ciliate protozoa that can generally be found in the digestive tract of ruminants. According to Yanuartono *et al*., (2019) Isotrichidae is included in the type of ciliate protozoa found in the rumen, the cilia on Isotrichidae grow around its body so that it is included in the Holotricha group with a simple morphological form. Family Isotrichidae was found in Balekambang Lake only 2% from the total sample, which is only 1 ASVs, namely *Isotricha prostoma*. Three species in the type of holotrichia found in the gastrointestinal tract of ruminants are mainly *I. intestinalis, I. prostoma,* and *Dasytricha ruminatum* (Gurelli *et al*., 2016).

Vannellidae taxa was found in Balekambang Lake after analysis is 2% of the total sample, which is 1 ASVs. The species in Vannellidae taxa that can be identified is *Ripella* sp. Vannellidae taxa is a group generally of protozoa that has characteristics such as a membrane sheath on its body, and in its movement, there are vibrating bristles (cilia) (Cavalier *et al*., 2016).

Vermamoebidae taxa is a protozoan that belongs to the phylum Amoebozoa. This taxon consists of various species that can generally be found in water and soil environments. This taxon was identified in Balekambang Lake as 2% of the total sample with 1 ASV obtained, was *Vermamoeba vermiformis* (previously *Hartmannella vermiformis*), which can generally live freely in the aquatic environment (Delafont *et al*., 2018).

The large number of unidentified taxa suggests that molecular research using the NGS approach needs to be developed to add databases from tropical lakes. The eDNA method is very helpful in molecular identification more quickly, briefly, and accurately (Taberlet *et al*., 2012).

Based on this result, the new finding from this research is the use of eDNA approach to determine the lake's health. Balekambang Lake is mostly contaminated with pathogenic protozoa, which may infect animals and humans

4. Conclusion

Molecular identification with the eDNA method through the NGS approach using 18S rRNA gene can be used in identifying Protozoan communities in Balekambang Lake, Dieng. Data analysis conducted obtained 13 taxa. The dominant Protozoan taxa indicates eutrophic condition of the lakes due to high nutrients from agriculture and husbandry,, which indicate the influence of agriculture and husbandry.

5. Acknowledgements

We thanks to Barra, Zahrah, Melvie who helped during field work. This research is part of the Research Grant of RPI (Riset Publikasi Internasional) funded by Diponegoro University with the contract number 569-57/UN7.D2/PP/IV/2022.

6. References

Achmad, M. J., Djamhur, M., Fabanyo, M. A., & Akbar, N. 2019. DNA Barcoding Application of Garfish (*Hemirhampus* sp.) in North Maluku Sea. *Jurnal Iktiologi Indonesia*, *19*(3), 463-473. https://doi.org/10.32491/jii.v19i3.506

Al-Masaudi, H. K., Mhaisen, F. T., & Al-Zubaidy, A. B. 2020. In vivo Effect of Water and Alcohol Extracts of Garlic, *Allium sativum* on the Murid Intestinal Flagellate *Tritrichomonas muris*. *Aalborg Acad. Journal of Pure Science*, 1(2), 14-20.

Astuti, D. S. 2017. Inventarisasi Protozoa Di Objek Wisata Umbu Cokro Tulung Klaten. *In Prosiding SNPBS (Seminar Nasional Pendidikan Biologi dan Saintek)*. http://hdl.handle.net/11617/9314

Bandeira, P. T., & De Souza, W. 2021. Costain 1 (ARM19800.1) - The first identified protein of the costa of the pathogenic protozoan Tritrichomonas foetus. Experimental Parasitology, 232, 108177. https://doi.org/10.1016/j.exppara.2021.108177

Batut, B., van den Beek, M., Doyle, M. A., & Soranzo, N. 2021. RNA-Seq Data Analysis in Galaxy. *RNA Bioinformatics*, 367-392. https://doi.org/10.1007/978-1-0716-1307-8_20

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. 2016. DADA2: High-resolution Sample Inference from Illumina Amplicon Data. *Nature Methods*, 13(7), 581-583. https://doi.org/10.1038/nmeth.3869

Cavalier-Smith, T., Chao, E. E., & Lewis, R. 2016. 187-Gene Phylogeny of Protozoan Phylum Amoebozoa Reveals a New Class (Cutosea) of Deep Branching, Ultrastructurally Unique, Enveloped Marine Lobosa and Clarifies Amoeba Evolution. *Molecular Phylogenetics and Evolution*, 99, 275-296. https://doi.org/10.1016/j.ympev.2016.03.023

Cedrola, F., Senra, M. V. X., Fregulia, P., D'Agosto, M., & Dias, R. J. P. 2022. Insights Into the Systematics of the Family Ophryoscolecidae (Ciliophora, Entodiniomorphida). *Journal of Eukaryotic Microbiology*, 69(4), e12915. https://doi.org/10.1111/jeu.12915

Choi, J., & Park, J. S. 2020. Comparative Analyses of the V4 and V9 Regions of 18S rDNA for The Extant Eukaryotic Community using The Illumina Platform. *Scientific Reports*, 10(1), 6519. https//doi.org/10.1038/s41598-020-63561-z

Collins, R.A., J. Bakker, O.S. Wangensteen, A.Z. Soto, L. Corrigan, D.W. Sims, M.J. Genner & S. Mariani. 2019. Non-Specific Amplification Compromises Environmental DNA Metabarcoding with COI. *Methods in Ecology and Evolution*, 10(11). https://doi.org/10.1111/2041-210x.13276.

Da Costa, A. S., Graham, T. M., Duncan, J. A., Pillai, S. P., & Lund, J. M. 2019. Detection and Treatment Strategy for *Tritrichomonas muris* in the Common Laboratory Mouse. *bioRxiv*, 827055. https://doi.org/10.1101/827055

Delafont, V., Rodier, M. H., Maisonneuve, E., & Cateau, E. 2018. *Vermamoeba vermiformis*: a Free-living Amoeba of Interest. *Microbial ecology*, 76(4), 991-1001. https//doi.org/10.1007/s00248-018-1199-8

Devetak, D., Omerzu, M., & Clopton, R. E. 2013. Notes on The Gregarines (Protozoa: Apicomplexa: Eugregarinorida) of Insects in Slovenia. *In Annales: Series Historia Naturalis*. 23(1), 73-89.

Dewanata, P. A., & Mushlih, M. 2021. Differences in DNA Purity Test Using UV Vis Spectrophotometer and Nanodrop Spectrophotometer in Type 2 Diabetes Mellitus Patients. *Indonesian Journal of Innovation Studies*, 15, 10.21070. https://doi.org/10.21070/ijins.v15i.553

Djalil, V. N., Farajallah, A., & Wardiatno, Y. 2018. Aplikasi Teknik Environmental DNA (eDNA) untuk Deteksi Spesies *Cheraxquadricarinatus* (Von Martens 1868) Menggunakan Sampel Air. *Jurnal Biologi Tropis*, 18(2), 134-140. https://doi.org/10.29303/jbt.v18i2.878

Escalante, N. K., Lemire, P., Cruz Tleugabulova, M., Prescott, D., Mortha, A., Streutker, C. J., ... & Mallevaey, T. 2016. The Common Mouse Protozoa *Tritrichomonas muris* Alters Mucosal T cell Homeostasis and Colitis Susceptibility. *Journal of Experimental Medicine*, 213(13): 2841-2850. https://doi.org/10.1084/jem.20161776

Escudié, F., Auer, L., Bernard, M., Mariadassou, M., Cauquil, L., Vidal, K., ... & Pascal, G. 2017. FROGS: Find, Rapidly, OTUs with Galaxy Solution. *Bioinformatics*, 34(8): 1287-1294. https://doi.org/10.1093/bioinformatics/btx791

Gürelli, G., Canbulat, S., Aldayarov, N., & Dehority, B. A. 2016. Rumen Ciliate Protozoa of Domestic Sheep (*Ovis aries*) and Goat (*Capra aegagrus hircus*) in Kyrgyzstan. *FEMS Microbiology Letters*, 363(6): 1–7. https://doi.org/10.1093/femsle/fnw028

Harriyadi, H. 2020. Pertimbangan Pemilihan Lokasi Kompleks Candi Dieng. Amerta, 37(2), 123–138. https://doi.org/10.24832/amt.v37i2.123-138

Heidari, S., Mohebali, M., Zarei, Z., Nateghpour, M., & Motevalli-Haghi, A. 2018. *Spironucleus muris* and *Eperythrozoon coccoides* in Rodents from Northwestern Iran: Rare Infections. *Journal of Arthropod-Borne Diseases*, 12(4): 334-340.

Indraswari, A. A. S., Suwiti, N. K., & Apsari, I. A. P. 2017. Protozoa Gastrointestinal: *Eimeria auburnensis* dan *Eimeria bovis* Menginfeksi Sapi Bali Betina di Nusa Penida. *Buletin Veteriner Udayana*, 9(1): 112-116. https://doi.org/10.21531/bulvet.2017.9.1.112

Ip, Y.C.A., Tay, Y.C., Gan, S.X., Ang, H.P., Tun, K., Chou, L.M., Huang, D., Meier, R. 2019. From Marine Park to Future Genomic Observatory? Enhancing Marine Biodiversity Assessments Using a Biocode Approach. *Biodiversity Data Journal*, 7:e46833. https://doi.org/10.3897/BDJ.7.e46833

Ito, A., & Tokiwa, T. 2018. Infraciliature of *Opisthotrichum janus*, *Epidinium ecaudatum*, and *Ophryoscolex purkynjei* (Ciliophora, Entodiniomorphida). *European Journal of Protistology*, 62: 1-10. https://doi.org/10.1016/j.ejop.2017.10.001

Kang, W., Anslan, S., Börner, N., Schwarz, A., Schmidt, R., Künzel, S., Rioual, P., Echeverría-Galindo, P., Vences, M., Wang, J & Schwalb, A. 2021. Diatom Metabarcoding and Microscopic Analyses from Sediment Samples at Lake Nam Co, Tibet: The effect of Sample-Size and Bioinformatics on the Identified Communities. *Ecological Indicators*, *121*, 107070: 1-13. https://doi.org/10.1016/j.ecolind.2020.107070

Li, C., Long, H., Yang, S., Zhang, Y., Tang, F., Jin, W., Jiang, K. 2022. eDNA Assessment of Pelagic Fish Diversity, Distribution, and Abundance in the Central Pacific Ocean. Regional Studies in Marine Science. 56,102661. https://doi.org/10.1016/j.rsma.2022.102661

Mustafa, H., Rachmawati, I., & Udin, Y. 2016. Pengukuran Konsentrasi dan Kemurnian DNA Genom Nyamuk. *Jurnal Vektor Penyakit*, 10(1), 7-10.

Nocciolini, C., Cucini, C., Leo, C., Francardi, V., Dreassi, E., & Carapelli, A. 2018. Assessing the Efficiency of Molecular Markers for the Species Identification of Gregarines Isolated from

the Mealworm and Super Worm Midgut. *Microorganisms*, 6(4): 119. https://doi.org/10.3390/microorganisms6040119

Opat, M. O., Setyawati, T. R., & Yanti, A. H. 2016. Inventarisasi Mikroalga dan Protozoa pada Instalansi Pengolahan Air Limbah Karet Sistem Biofilter Skala Laboratorium. *Jurnal Protobiont*, 4(3): 19-25. http://doi.org/10.26418/protobiont.v4i3.13241

Pamrayoga, G., T. R. Soeprobowati, S. P. Putro. 2017. Diatom Epipelik di Telaga Balekambang, Dieng, Jawa Tengah. *Prosiding Seminar Nasional Hasil-Hasil Penelitian Pascasarjana UNDIP*, 220-224

Pavan-Kumar, A., Gireesh-Babu, P., & Lakra, W. S. 2015. DNA Metabarcoding: a New Approach for Rapid Biodiversity Assessment. *Journal Cell Science and Molecular Biology*, 2(1): 111.

Pawlowski, J., Audic, S., Adl, S., Bass, D., Belbahri, L., Berney, C., ... & de Vargas, C. 2012. CBOL Protist Working Group: Barcoding Eukaryotic Richness Beyond the Animal, Plant, and Fungal Kingdoms. *PLoS biology*, 10(11): e1001419

Prakoso, S. P., Wirajana, I. N., & Suarsa, I. W. 2016. Amplifikasi Fragmen Gen 18S rRNA pada DNA Metagenomik Madu dengan Teknik PCR (Polymerase Chain Reaction). *Indonesian Journal of Legal and Forensic Sciences*, 7:, 1-7.

Pudjoarinto, A. & Cushing, EJ. 2001. Palaeogeography, Palaeoclimatology, Palaeoecology, 171(3–4), 329–340 (2001).

Roslim, D. I., Nuryani, N., & Herman, H. 2018. Sekuen Penyandi 18S Ribosomal RNA dan Ubiquitin pada *Pandanus* sp. Asal Riau. *Jurnal Bios Logos*, 8(1). https://doi.org/10.35799/jbl.8.1.2018.20590

Rukmana, N., Rosa, E., & Prameswari, W. 2016. Prevalensi Protozoa Usus pada Kukang Sumatera (*Nycticebus coucang*) Melalui Penggunaan Berbagai Macam Media Pengawet dan Konsentrasi Berbeda di Pusat Rehabilitasi Yiari Ciapus, Bogor. *Jurnal Ilmiah Biologi Eksperimen dan Keanekaragaman Hayati (J-BEKH)*, 3(2), 45-51. https://doi.org/10.23960/jbekh.v3i2.84

Ruppert, K. M., Kline, R. J., & Rahman, M. S. 2019. Past, Present, and Future Perspectives of Environmental DNA (eDNA) Metabarcoding: A Systematic Review in Methods, Monitoring, and Applications of Global eDNA. Global Ecology and Conservation, 17, e00547. https://doi.org/10.1016/j.gecco.2019.e00547

Sani, L. M. I., Benyamin, A., Husna, A. K., Arafat, D., Subhan, B., Sunuddin, A., ... & Madduppa, H. 2021. A Contrast Pattern of Reef Fish Species Diversity and Distribution Using Environmental DNA (eDNA) Metabarcoding in Longitudinal Distance From Jakarta Bay. *Jurnal Ilmu dan Teknologi Kelautan Tropis*, 13(3), 469-484. https://doi.org/10.29244/jitkt.v13i3.37971

Setiaputri, A. A., Barokah, G. R., Sahaba, M. A. B., Arbajayanti, R. D., Fabella, N., Pertiwi, R. M., ... & Abdullah, A. 2020. Perbandingan Metode Isolasi DNA pada Produk Perikanan Segar dan Olahan. *Jurnal Pengolahan Hasil Perikanan Indonesia*, 23(3): 447-458. https://doi.org/10.17844/jphpi.v23i3.32314

Shahatha, S. S., Abood, N. M., & Mousa, M. O. 2022. Effect of *Marlubium vulgare* Plant on Experimentally Infected Albino Mice with *Trichomonas muris* Parasite and Compare with Metronidazole Drug. *In IOP Conference Series: Earth and Environmental Science*. 1060(1). https://doi.org/10.1088/1755-1315/1060/1/012071

Soeprobowati, T.R., Suedy, S.W.A., & Hadiyanto. 2018. Find the future from the past: Paleolimnology in Indonesia. *E3S Web of Conferences* 31, 08002 (2018)

Soeprobowati, T. R., Hariyati, R., & Wulandari, F. 2019. Epipelic Diatom Communities in Pengilon Lake Dieng, Central Java. *E3S Web of Conferences*, 125, 01023. https://doi.org/10.1051/e3sconf/201912501023

Sophian, A., and Syukur, A. 2021. Analysis of Purity and Concentration of Isolated DNA in Making Raw DNA of Rat Species. *Eruditio: Indonesia Journal of Food and Drug Safety*, 1(2): 1-5. https://doi.org/10.54384/eruditio.v1i2.75

Stat, M., Huggett, M. J., Bernasconi, R., DiBattista, J. D., Berry, T. E., Newman, S. J., ... & Bunce, M. 2017. Ecosystem Biomonitoring with eDNA: Metabarcoding Across the Tree of Life in a Tropical Marine Environment. *Scientific reports*, 7(1): 12240. https://doi.org/10.1038/s41598-017-12501-5

Taberlet P, Coissac E, Pompanon F, Brochman C, Willerslev E. 2012. Toward Next Generation Biodiversity Assessment Using DNA Metabarcoding. *Mol Ecol*, 21(8): 2045-50. https://doi.org/10.1111/j.1365294X.2012.05470.x

Vaulot, D., Geisen, S., Mahé, F., & Bass, D. 2022. PR2-Primers: An 18S rRNA Primer Database for Protists. *Molecular Ecology Resources*, 22(1): 168-179. https://doi.org/10.1111/1755-0998.13465

Voros, G. B., Blair, R. V., Andrews, D. D., & Dobek, G. L. 2021. Evaluation of Therapeutic Approaches for the Treatment of *Spironucleus muris* in Mice. *Journal of the American Association for Laboratory Animal Science*, 60(2): 146-151. https://doi.org/10.30802/AALAS-JAALAS-20-000067

Wahyuni, S., Muin, H., & Zarkasyi, R. 2022. Identifikasi Protozoa Pada Air Kolam Renang di Kabupaten Pinrang. *Sulolipu: Media Komunikasi Sivitas Akademika dan Masyarakat*, 22(2): 379-385. https://doi.org/10.32382/sulolipu.v22i2.2935

Wang, Y., Tian, R. M., Gao, Z. M., Bougouffa, S., & Qian, P. Y. 2014. Optimal Eukaryotic 18S and Universal 16S/18S Ribosomal RNA Primers and Their Application in a Study of Symbiosis. *PloS one*, 9(3): e90053.

Wardoyo, E. H., Sabrina, Y., Suryani, D., & Indriawan, R. 2020. Optimasi Ekstraksi DNA dari Spesimen Feses Pasien Kanker Kolorektal. *The Indonesian Journal of Infectious Disease*, 6(1): 41-50. https://doi.org/10.32667/ijid.v6i1.108

Winarni, I. 2016. Peran Mikroba sebagai Biomonitoring Kualitas Perairan Tawar Pada Beberapa Situ. *Peran MST dalam Mendukung Urban Lifestle yang Berkualitas 143*.

Wulandari, K. K., Rodja, H. A., Urjiyah, U. G., Fibriani, E., & Putri, F. A. 2021. Teknik Diagnostik Konvensial dan Lanjutan untuk Infeksi Bakteri dan Resistensi Antibakteri di Indonesia. *Jurnal Widya Biologi*, 12(02): 98-116.

Yanuartono., Nururrozi, A., Indarjulianto, S., & Purnamaningsih, H. 2019. Peran Protozoa Pada Pencernaan Ruminansia dan Dampak terhadap Lingkungan. *TERNAK TROPIKA Journal of Tropical Animal Production*, 20(1): 16-28. https://doi.org/10.21776/ub.jtapro.2019.020.01.3

Yuan, G., Chen, Y., Wang, Y., Zhang, H., Wang, H., Jiang, M., Zhang, X., Gong, Y., & Yuan, S. 2024. Responses of Protozoan Communities to Multiple Environmental Stresses (Warming, Eutrophication, and Pesticide Pollution). Animals 2024, 14, 1293. https://doi.org/10.3390/ani14091293

Zhao, L., Zhang, X., Xu, M., Mao, Y., & Huang, Y. 2021. DNA Metabarcoding of Zooplankton Communities: Species Diversity and Seasonal Variation Revealed by 18S rRNA and COI. *PeerJ*, 9, e11057. https://doi.org/10.7717/peerj.11057

Zuhdi, M. F., Madduppa, H., & Zamani, N. P. 2021. Variasi Temporal Kelompok Ikan Terumbu Karang di Pulau Tidung Kecil Menggunakan eDNA Metabarkoding dan Sensus Visual. *Jurnal Kelautan Tropis*, 24(3): 283-290. https://doi.org/10.14710/jkt.v24i3.11810