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Genetic monitoring of zooplankton from Manado Bay, northern Sulawesi, Indonesia, by cytochrome oxydase I sequence

Joice R.T.S.L. Rimper[1]*, Markus T. Lasut[1], Natalie D C Rumampuk[1], Veibe Warouw[1], Robert A. Bara[1], Silvana D. Harikedua[2], Joudy R.R. Sangari[3], Beivy J. Kolondam[4]

[1] *Marine Science Study Program, Department of Aquatic Resources Management, Faculty of Fisheries and Marine Sciences, Sam Ratulangi University, Indonesia*
[2] *Fishery Product Technology Study Program, Department of Fishery Product Processing, Faculty of Fisheries and Marine Science, Sam Ratulangi University*
[3] *Aquatic Resources Management Study Program, Faculty of Fisheries and Marine Sciences,Sam Ratulangi University, Indonesia*
[4] *Department of Biological Sciences, Faculty of Mathematics and Natural Science, Sam Ratulangi University, Indonesia*
*Corresponding author: joice.rimper@unsrat.ac.id

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ABSTRACT: Plankton are essential components of aquatic ecosystems, serving as the base of the marine food web and playing a crucial role in global biogeochemical cycles. Understanding plankton community dynamics is vital for predicting changes in marine ecosystems, especially amid the challenges of climate change and human activities. Traditional plankton identification using microscopy requires extensive taxonomic expertise and is time-consuming, often limited by morphological complexity. To overcome these limitations, molecular methods such as DNA barcoding have been developed, allowing for rapid and accurate species identification. In this study, phytoplankton samples were collected and preserved in 95% ethanol, then processed through centrifugation and DNA extraction using the Qiaprep Miniprep Kit. The Cytochrome oxidase subunit 1 (COI) gene was amplified using universal PCR primers, and the PCR products were visualized on an agarose gel. Successful amplification products were then sequenced at First-Base Co., Selangor, Malaysia, and analyzed using Geneious software and the GenBank database via BLAST. PCR results from samples JR1-JR6 showed a band length of 725 bp. BLAST analysis for sample JR6 revealed a 90% similarity to *Paracalanus aculeatus* and *Calocalanus styliremis*, but this level of similarity is considered too low for definitive identification. This suggests that the species from sample JR6 may be a new species not yet recorded in GenBank and different from the genera *Paracalanus* and *Calocalanus*.

Keywords: species identification; marine copepods; COI sequence, manado bay; sulawesi; Indonesia

INTRODUCTION

The presence of plankton is an essential component in aquatic environments, playing a crucial role as the foundation of marine food webs and in the global biogeochemical cycle. Plankton consists of various microscopic organisms drifting in the water column, including phytoplankton (photosynthetic organisms) and zooplankton (heterotrophic organisms). The diversity and distribution of plankton are influenced by environmental factors such as temperature, salinity, nutrients, and ocean currents. Understanding plankton community dynamics is crucial for predicting changes in marine ecosystems amidst challenges posed by climate change and human activities (Romimohtarto and Juwana, 2009; Rumengan and Rimper, 2016; Hertika et al., 2021).

Manado Bay is a vital area for local fishermen since it has long been utilized for harvesting fisheries resources to meet food demands in nearby area. Additionally, Manado Bay serves as a shipping route due to tourism activities. Several studies have documented that this region is a potential resource for zooplankton and phytoplankton (Rimper et al., 2008; Rumengan and Rimper, 2016), the bioactive content of rotifers (Rimper, 2014), sponges (Nowin et al., 2018), and marine ascidians (Sumilat et al., 2019; Angkouw et al., 2023). Specifically for plankton identification, it has traditionally been based solely on phenotypic characteristics, observing only morphology, while molecular-based identification has not been widely implemented. Accurate and timely identification of plankton communities is crucial for understanding ecosystem dynamics, responses to environmental changes, and fisheries resource management. Traditional plankton identification relies on microscopy, which requires deep taxonomic expertise and extensive time. However, this approach is often limited by morphological complexity, small size, and morphological variations within species. To address these limitations, molecular methods have emerged as powerful tools for the identification and characterization of plankton, enabling more detailed genetic identification and analysis. One well-known molecular identification technique is DNA barcoding, which uses standardized gene regions as species markers to swiftly and accurately identify species. Each DNA strand can serve as

Figure 1. Map of study area at Manado Bay, northern Sulawesi, Indonesia

either coding or non-coding regions, providing comprehensive genetic information. Molecular identification techniques can resolve identification challenges posed by cryptic species and taxonomic similarities due to phenotypic plasticity, sexual dimorphism, and complex life histories, which are difficult or impossible to distinguish using traditional morphology-based methods. Furthermore, molecular identity allows determination of the origin of plankton resources, which is crucial for ensuring that all species, including rare or unidentified ones, are included in ecosystem studies (Blanco-Bercial et al., 2014; Glacio et al., 2022).

The waters of Manado Bay are rich in biodiversity but are potentially under environmental pressure from anthropogenic activities. The development of tourism and residential areas in the region has impacted these waters. Integrating DNA sequencing technology and bioinformatics analysis is expected to provide a more comprehensive understanding of plankton diversity in Manado Bay. That is why the objective of the study is to determine the plankton community of Manado Bay using molecular identification. The outcomes of this study will not only enhance knowledge of local plankton ecology but also contribute to global understanding of aquatic ecosystems and sustainable environmental management strategies. The long-term goal of this research is to obtain detailed and accurate information about the current and future conditions of plankton resources in Manado Bay.

MATERIALS AND METHODS

This study was conducted in the waters of Manado Bay, North Sulawesi, Indonesia. Three plankton sampling stations were designated in this study: the port area (MB1), the fishing area (MB2), and the estuary (MB3) (Figure 1). Plankton samples were collected using a plankton net placed at the water surface and towed slowly for a distance of 10 meters with the assistance of a boat, with three replicates per station. The filtered water was then transferred into sample bottles containing 4% formalin for preservation (Rimper et al., 2008). Subsequently, the samples were morphologically identified using a microscope in the laboratory. Meanwhile, for molecular identification purposes, samples were preserved in 95% ethanol, followed by DNA extraction and amplification of the target Cytochrome Oxidase I (COI) gene.

Molecular identification techniques, such as DNA sequence analysis, enable faster and more accurate species recognition. One of the most used genetic markers is the Cytochrome Oxidase I (COI) gene, often referred to as the 'barcode of life.' This gene has proven effective in distinguishing between various plankton species, including those with very similar morphologies. The use of COI sequences in plankton identification offers several advantages: it can be applied to a wide range of environmental samples, including complex and diverse ones. This technique allows for the detection of rare or cryptic species that might be overlooked by traditional methods. Molecular analysis can be integrated with global genetic databases, such as GenBank, for validation and comparison of identification results.

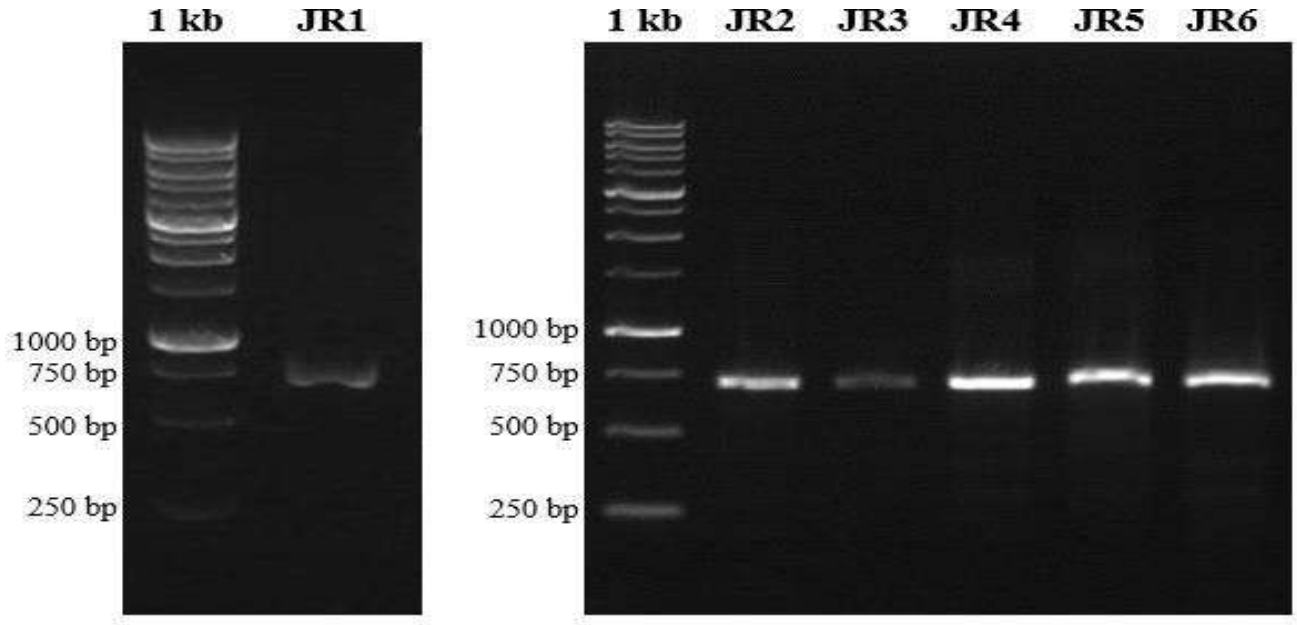

Figure 2. Agarose gel (0.8%) electrophoresis result of PCR

	Name	HQ%	Sequence Le...
	JR1_HCO.ab1	11.5%	711
	JR1_LCO.ab1	15.4%	723
	JR2_HCO.ab1	22.8%	710
	JR2_LCO.ab1	0.4%	687
	JR4_HCO.ab1	34.3%	694
	JR4_LCO.ab1	1.9%	698
	JR5_HCO.ab1	18.0%	696
	JR5_LCO.ab1	10.5%	706
	JR6_HCO.ab1 ...	93.4%	686
	JR6_LCO.ab1	93.9%	685

Figure 3. Chromatogram quality based on Geneious v5.6.4 software readings

Sample Preparation of Plankton

Phytoplankton samples were collected from Manado Bay waters, North Sulawesi, Indonesia, and preserved in 95% ethanol. The samples were then transferred into 1.5 mL Eppendorf tubes and centrifuged at 10,000 rpm for 1 minute, followed by removal of the supernatant (Taylor et al., 2007; Sano et al., 2020).

Molecular Identification

Genomic DNA from plankton samples was extracted using the Qiaprep Miniprep Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions (Kolondam, 2015). The cytochrome oxidase subunit 1 (COI) gene was amplified using the universal PCR primer pair LCO1490: 5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3' (forward primer) and HCO2198: 5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3' (reverse primer) (Folmer et al., 1994). The amplification reaction was carried out in a total volume of 40 µl using 20 µl MyTaq HS Red Mix (Bioline), 1.5 µl of each primer (10 µM), 2 µl DNA template, and 14 µl ddH2O. The PCR settings followed (Kolondam, 2015). The PCR reaction used the MyTaq HS Red Mix kit (Bioline). Each 40 µl reaction contained 15 pmol of each primer and DNA template. The PCR reaction conditions were as follows: initial denaturation at 95°C for 3 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 30 seconds.

PCR products were electrophoresed on 0.8% agarose gel and visualized with ethidium bromide staining to check the success of the PCR amplification. For DNA sequencing, the amplicons and primers (forward and reverse) were sent to First-Base Co., Selangor, Malaysia. The obtained DNA sequences were edited using Geneious v5.6 software and aligned using the ClustalW algorithm. Identification was performed using the GenBank database (www.ncbi.nlm.nih.gov) for comparison via the BLAST (Basic Local Alignment Search Tool). BLAST results were limited to percent identity values between 97-100% and query coverage between 98-100% (Folmer et al., 1994; Kolondam, 2015).

RESULTS AND DISCUSSION

The types of plankton found in the waters of Manado Bay based on morphological identification include *Calanus* sp., *Acartia* sp., *Oithona* sp. However, some plankton species could not be accurately identified using morphological methods, necessitating molecular identification as a solution to these limitations. Molecular identification provides the capability to identify species quickly and accurately, particularly for taxa that are difficult to classify morphologically. This approach also supports a deeper understanding of biodiversity and the composition of plankton communities in aquatic ecosystems.

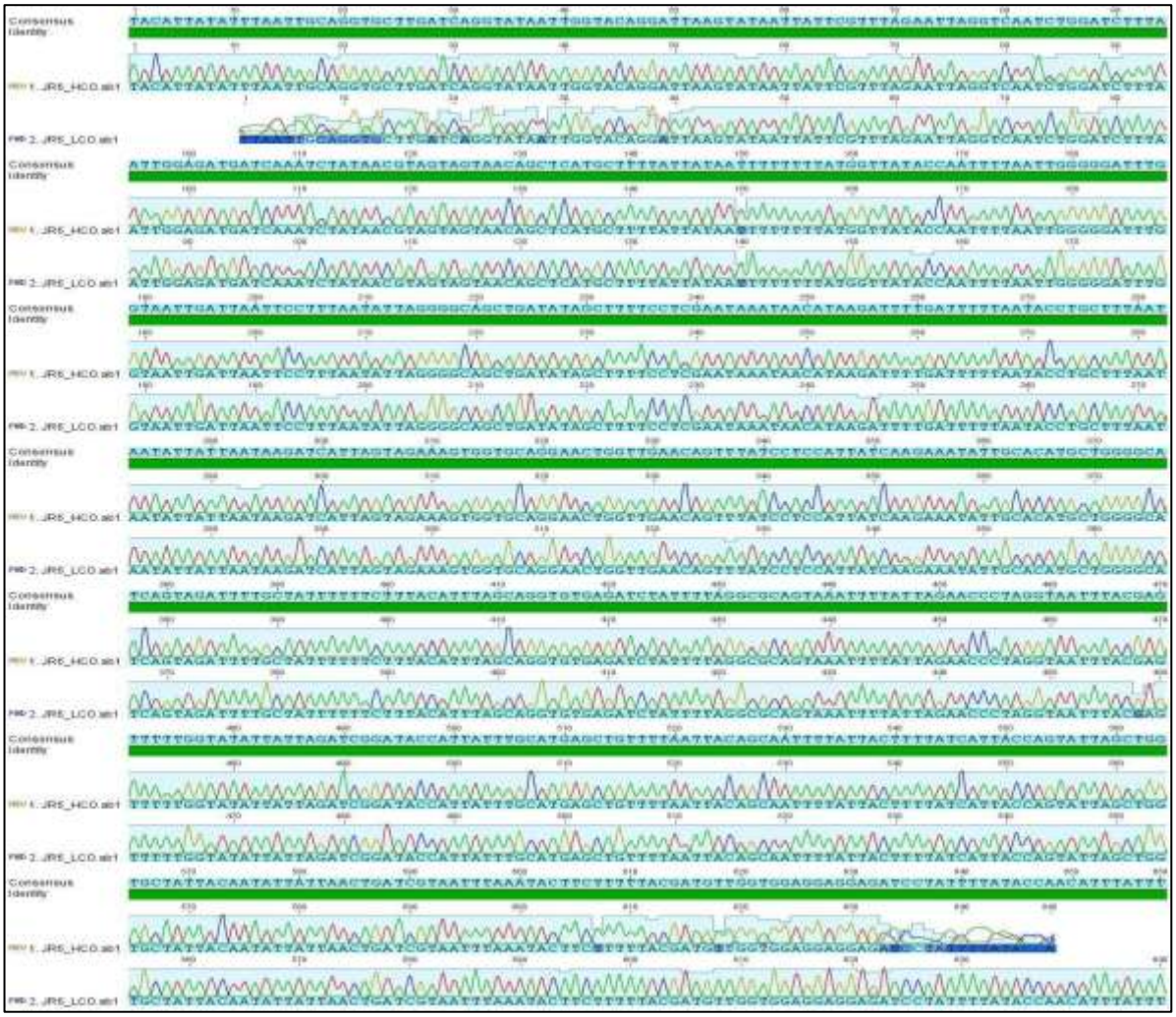

Figure 4. Alignment of chromatograms for sample JR6

	Description	Max score	Total score	Query cover	E value	Ident	Accession
	Paracalanus aculeatus haplotype paac25 cytochrome c oxidase subunit I (cox1) gene, partial cds	861	861	100%	0.0	90%	KC784344.1
	Paracalanus aculeatus voucher PaAc1 cytochrome c oxidase subunit I (COI) gene, partial cds; mit	856	856	99%	0.0	90%	KC287770.1
	Calocalanus styliremis voucher P0122 cytochrome oxidase subunit I (COI) gene, partial cds; mitoc	843	843	99%	0.0	90%	KP861439.1
	Undinula vulgaris voucher Co63.6.1 cytochrome c oxidase subunit I (COI) gene, partial cds; mitoch	828	828	100%	0.0	89%	KC287909.1
	Bestolina sp. AC-2013 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	815	815	93%	0.0	90%	JQ911969.1
	Paracalanus aculeatus voucher PaAc4 cytochrome c oxidase subunit I (COI) gene, partial cds; mit	811	811	99%	0.0	89%	KC287796.1
	Paracalanus aculeatus voucher PaAc7 cytochrome c oxidase subunit I (COI) gene, partial cds; mit	808	808	98%	0.0	89%	KC287804.1
	Undinula vulgaris voucher M3084 cytochrome oxidase subunit I (COI) gene, partial cds; mitochonc	806	806	100%	0.0	89%	KF985239.1
	Paracalanus aculeatus voucher PaAc5 cytochrome c oxidase subunit I (COI) gene, partial cds; mit	806	806	98%	0.0	89%	KC287802.1
	Paracalanus aculeatus voucher PaAc3 cytochrome c oxidase subunit I (COI) gene, partial cds; mit	806	806	99%	0.0	89%	KC287792.1
	Undinula vulgaris voucher Co20963.1.3 cytochrome oxidase subunit I (COI) gene, partial cds; mitc	806	806	100%	0.0	89%	GU171333.1
	Paracalanus parvus haplotype paap22-1 cytochrome c oxidase subunit I (COI) gene, partial cds; n	804	804	93%	0.0	90%	EU856801.1
	Undinula vulgaris cytochrome oxidase subunit I gene, partial cds; mitochondrial gene for mitochon	804	804	97%	0.0	89%	AF462318.1
	Paracalanus aculeatus voucher PaAc6 cytochrome c oxidase subunit I (COI) gene, partial cds; mit	802	802	98%	0.0	89%	KC287803.1
	Paracalanus aculeatus voucher PaAc2 cytochrome c oxidase subunit I (COI) gene, partial cds; mit	802	802	97%	0.0	89%	KC287791.1
	Paracalanus aculeatus haplotype paap22-2 cytochrome c oxidase subunit I (COI) gene, partial cds	798	798	93%	0.0	90%	EU856807.1
	Delibus sp. V128.D1b cytochrome oxidase subunit I gene, partial cds; mitochondrial	763	763	96%	0.0	88%	KF715874.1

Figure 5. BLAST results of sample JR6 in GenBank

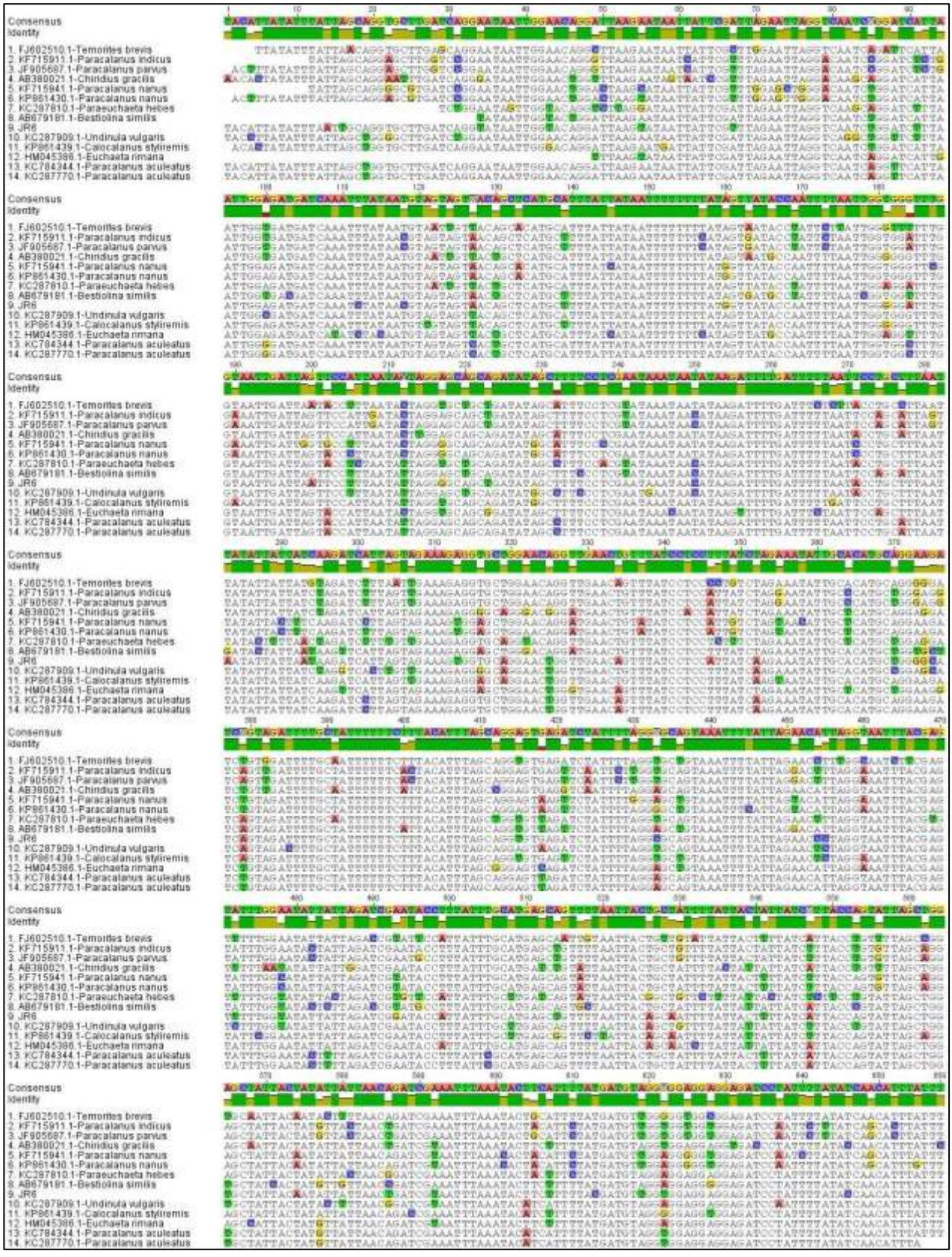

Figure 6. Alignment of sample JR6 with closest related specimens

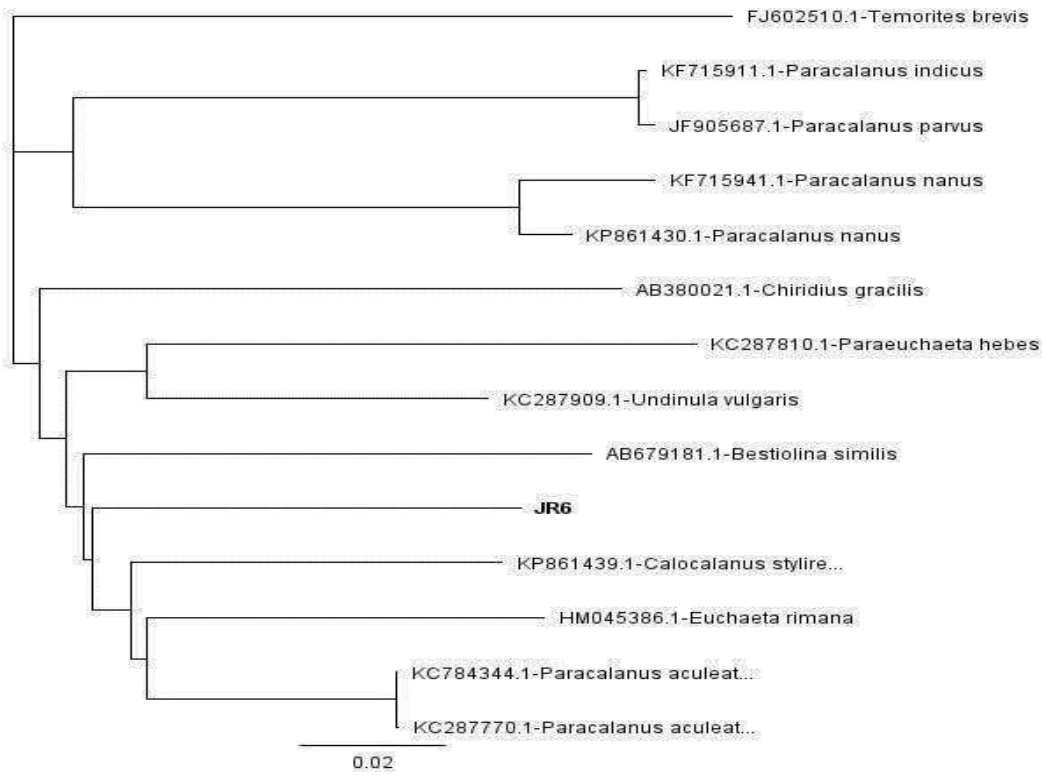

Figure 7. Phylogenetic tree of COI sequences of sample JR6 compared to closely related specimens

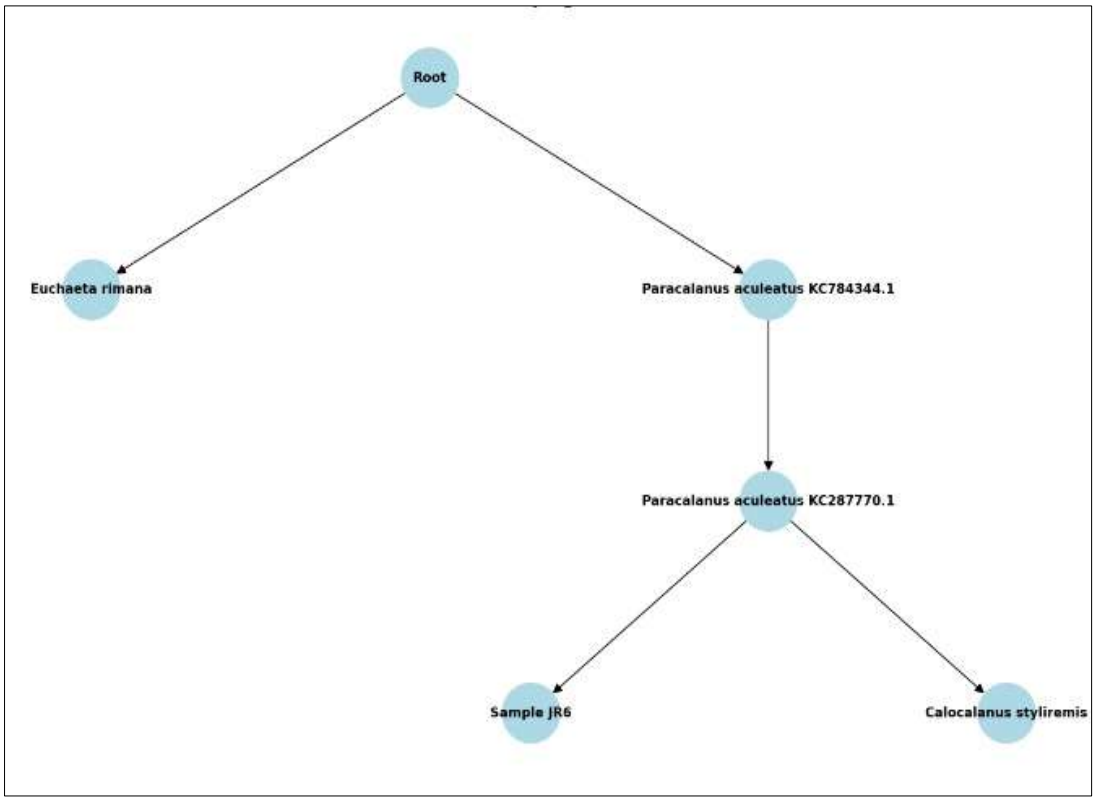

Figure 8. The relationships among the organisms, *Euchaeta rimana*, *Paracalanus aculeatus* (with KC784344.1 and KC287770.1), sample JR6, and *Calocalanus styliremis*

PCR Success and Agarose Gel Electrophoresis

The research results showed that PCR reactions from samples JR1-JR6 yielded good results, with a band length of 725 bp (Figure 2). These bands were single bands successfully separated using 0.8% agarose gel electrophoresis. The primer pairs from Folmer (Folmer et al., 1994), were able to bind to the COI gene region (referred to as the Folmer Region) in plankton mitochondria, and through PCR amplification, this region produced more than one billion copies, considered sufficient for sequencing purposes.

COI Gene Sequencing

Sequencing was conducted on the six samples, producing chromatograms of the COI gene sequences. The high-quality nucleotide reading percentage (HQ%) of the chromatograms obtained was less than 35% (poor quality) for all samples (Figure 3), except for Sample JR6, which had a value of 93% (very good quality). Sample JR6 was deemed suitable to proceed to the chromatogram editing stage.

Sample JR6 was further processed 135 to the DNA editing stage by merging the sequencing reads from the right (JR6_HCO) and left (JR6_LCO) using Geneious v5.6.4 software (Kearse et al., 2012). The merging process is shown in Figure 4. It is evident that the nucleotide reads were of high quality, allowing for the detection of signals from each base. Unreadable sections at the beginning or end were covered by other chromatogram reads, resulting in a highly accurate consensus sequence.

Sequence Alignment and Phylogenetic Tree

Sample JR6 was compared with 13 samples from GenBank to assess their similarity based on nucleotide differences. A total of 14 DNA sequences were aligned using the Muscle algorithm (Edgar, 2004) to highlight the differences (Figure 5). Based on these differences, a phylogenetic analysis was subsequently performed. Phylogenetic analysis was performed using the Neighbor-Joining algorithm (Saitou and Nei, 1987) integrated within Geneious v5.6.4 (Kearse et al., 2012). Sample JR6 appeared to be phylogenetically closer to *Calocalanus styliremis*. This relationship is illustrated in Figure 6 and Figure 7.

Based on the results obtained, the primer pair from (Folmer et al., 1994), was amplified to over one billion copies, which is considered sufficient for sequencing. The high-quality nucleotide reading percentage (HQ%) of the obtained chromatograms was less than 35% (poor quality) for all samples, except for Sample JR6, which had a value of 93% (very good quality). The poor-quality chromatograms for the other samples are likely due to the specimens being mixed with other plankton species from the natural environment. This mixing caused confusing readings for samples JR1-JR5, as the PCR occurred on non-identical DNA templates. The presence of two or more templates (non-pure) led to simultaneous amplification of the Folmer region of the COI gene. In cycle sequencing, the detection of different nucleotide signals for a single read resulted in confusing nucleotide determination.

For sample JR6, which was further processed to the DNA editing stage, DNA sequence comparison with all specimens in GenBank through the BLAST (Basic Local Alignment Search Tool) showed that this sample has a 90% similarity to *Paracalanus aculeatus* (accession number: KC784344.1) from China (Cheng et al., 2014). The same species was also identified as a species from Japan (accession number: KC287770.1) (Blanco-Bercial et al., 2014). Sample JR6 also showed COI gene similarity with *Calocalanus styliremis* (accession number: KP861439.1) found in the Mediterranean (Kasapidis et al., 2017). A similarity of 90% is considered too low for identification purposes. It is likely that the species obtained from sample JR6 is not yet recorded in GenBank and has a different species and genus name from *Paracalanus* and *Calocalanus*.

Phylogenetic analysis of sample JR6 was conducted using the Neighbor-Joining algorithm (Saitou and Nei, 1987) integrated within Geneious v5.6.4 (Kearse et al., 2012). Although JR6 showed higher similarity with *Paracalanus aculeatus*, the phylogenetic test indicated that JR6 is phylogenetically closer to *Calocalanus styliremis*. The considerable genetic distance further supports that JR6 is not the same species as either of these two species. The genetic distance between the two P. aculeatus samples indicates a close relationship between them, but JR6 remains outside the same clade, as does *C. styliremis*. Conversely, *Euchaeta rimana* shows a closer relationship with P. aculeatus. It is likely that JR6 is a different species from those mentioned, and the phylogenetic test results suggest that the relationship of JR6 is closer to other species than to *P. aculeatus* or *C. styliremis* (Figure 8).

CONCLUSIONS

Based on the research findings, the DNA sequence of sample JR6, compared with all specimens in GenBank using the BLAST (Basic Local Alignment Search Tool), shows a 90% similarity with the species *Paracalanus aculeatus* from China and Japan. Sample JR6 also exhibits COI gene similarity with *Calocalanus styliremis* obtained from the Mediterranean. A similarity value of 90% is considered very low for identification purposes. It is likely that the species obtained from sample JR6 is not yet recorded in GenBank and has a different species and genus name from *Paracalanus* and *Calocalanus*. Sample JR6 also indicates that, despite having higher similarity with *Paracalanus aculeatus* based on DNA analysis, phylogenetically, sample JR6 is actually closer to *Calocalanus styliremis*. However, the significant genetic distance between sample JR6 and both species indicates that JR6 is not the same species as *Paracalanus aculeatus* or *Calocalanus styliremis*. The genetic distance between the two P. aculeatus samples suggests a close relationship between them, but sample JR6 remains outside this clade or group. This suggests that sample JR6 may be a new species that is unidentified and not yet recorded in the GenBank database.

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