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Molecular Identification and Phylogenetic Relationship of *Chlorella* species from Pallikaranai Marshland using rbcL Gene

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ABSTRACT: The Pallikaranai Marshland has spread over 1,247.5 ha of land and serves as an aquatic buffer of the flood-prone Chennai and Chengalpattu districts. *Chlorella* is a common green alga and widely distributed in Indian freshwater habitats. Identifying *Chlorella* species based on morphological characteristics can be challenging and there is a need for a reliable and accurate method to assess genetic variations. DNA barcoding is an effective method to identify diverse algal species across various taxa and habitats. This study aims to apply molecular approaches for the identification of algae isolated from using rbcL gene. Molecular identification of the isolates 23SPPCA01, 23SPPCA02, 23SPPCA03, 23SPPCA05 and 23SPPCA06 from the study area has been done. The rbcL gene, which encodes the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), is widely recognized for its role in DNA barcoding of plants and has also been developed for molecular identification of microalgae. The results revealed that isolates 23SPPCA01 and 23SPPCA05 were found to be *Chlorella variabilis* and *Chlorella vulgaris* respectively with a genetic similarity of 99% toward identified species in the NCBI database using BLAST program. Similarly, the isolates 23SPPCA02, 23SPPCA03, and 23SPPCA06 were reported as *Chlorella sorokiniana.* Further, the DNA sequences were submitted to NCBI database and received accession numbers *viz.*, PP316335, PP316336, PP316334, PP196469 and PP110481. The phylogenetic analysis indicated that the isolates of *Chlorella* belong to both clades and clearly distinguished. This research provides the valuable perceptions into the molecular identification and phylogeny of *Chlorella sp.* from an ecologically significant spot and allow us to enhance conservation efforts.

Keywords: Molecular identification, *Chlorella*, *rbcL* gene and Pallikaranai.

INTRODUCTION

The genus *Chlorella* M.W.Beijerinck is a unicellular microalga belong to Chlorophyta. Species of the genus are known for their diverse physiological and ecological characteristics. They are commonly found in various aquatic environments, including freshwater and marine habitats (Fatemeh and Mohsen 2016). These microalgae play a significant role in aquatic ecosystems, contributing to primary production and serving as a fundamental component of the food web (Medlin and Kooistra 2010). They are characterized by their green color, which is due to the presence of chlorophyll, a pigment involved in photosynthesis. Species of *Chlorella* are notable for their simple, unicellular structure, which enables efficient nutrient uptake and rapid reproduction. Their cellular simplicity does not undermine their ecological significance; rather, it underscores their adaptability and resilience in diverse environmental conditions. They can accumulate substantial amounts of lipids, proteins, and carbohydrates, which are essential for their survival and reproduction (Sharma *et al.*, 2013).

The physiological traits of Chlorella include a high growth rate and the ability to photosynthesize efficiently, even under suboptimal light conditions. These attributes make them highly competitive in natural environments and valuable for various biotechnological applications. Genetic engineering holds potential for further improving the yield and quality of *Chlorella* biomass, making it even more viable for industrial applications (Ratnasingham and Hebert 2007) and contribute to sustainable development goals (De Vargas *et al.*, 2015). *Chlorella variabilis, Chlorella vulgaris* and *Chlorella sorokiniana* are three species within this genus that have gained significant attention due to their potential applications in various fields such as biofuels, bioremediation, and nutraceutical production. These unicellular species have unique physiological characteristics. Hence, they are suitable for a range of industrial applications.

The study and identification of algae have traditionally relied on morphological characteristics such as cell shape, size, and the structure of reproductive organs. However, morphological approaches need to be reviewed due to few species are morphologically similar but genetically distinct. This lacuna can be filled by molecular identification methods that have gained importance for the accuracy and reliability of algae

identification. Molecular identification provides a suitable solution for more accurate and fast identification of microalgae at species level (Yanuhar *et al*., 2019). Molecular identification involves the use of genetic markers to distinguish between different algal species. Molecular approaches, such as DNA sequencing, polymerase chain reaction (PCR), and DNA barcoding, help us to analyse specific regions of the algal genome. The most targeted regions include the ribosomal RNA genes (18S rRNA, 28S rRNA), the internal transcribed spacer (ITS) regions, and the plastid-encoded genes such as rbcL and tufA (Medlin and Kooistra 2010; Paul, 2001).

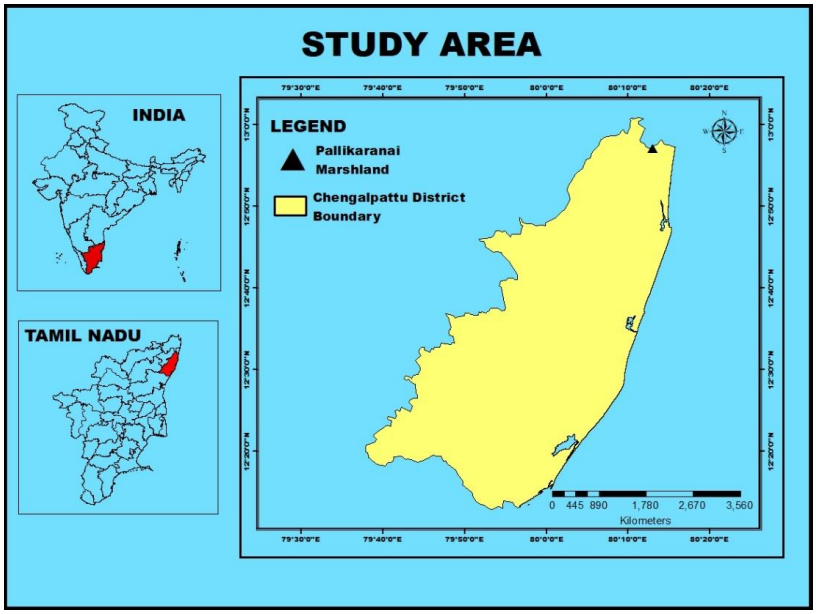
The ribulose-bisphosphate carboxylase (rbcL) sequence method has been extensively utilized in various fields such as evolution, phylogeny, biogeography, population genetics, and systematics due to its ease of amplification and relatively conserved nature among related species (Sheng-Guo *et al.,* 2008; Doyle *et al.*, 1997). Several studies have documented the sequence of rbcL is highlighting its significant potential and utility in documenting genetic variations within natural populations (Hamdan *et al.*, 2013). Despite the lack of a universal PCR marker for DNA barcoding, the rbcL marker is considered a reliable option for green algae (Hadi *et al.*, 2016). Moreover, the rbcL gene can be used as a DNA marker for molecular identification of species in the genus *Chlorella* mainly (Fitriyah *et al*., 2021).

Wetlands are the most important of life-supporting ecosystems that have sustained human lives and communities over the millennia. Postel & Carpenter (1997) have lucidly said that "it is no coincidence that early human civilizations sprang from river valleys and floodplains. Sufficient quantities of freshwater have underpinned the advancement of human societies since their beginning. Today, we rely on the solar-powered hydrological cycle not only for water supplies, but also for a wide range of goods and services, many of which are hidden and easy to take for granted". The Pallikaranai Marsh is amongst the few and last remaining natural wetlands of south India. The Marsh that was till about 30 years ago spread over an area of more than 5000ha (50 km^2) has been reduced to around one-tenth of its original extent due to the non-recognition of the area as a wetland, and urbanization. Hence an attempt was made to apply molecular approaches for the identification of algae isolated from this ecologically significant spot using rbcL gene. The study is also aimed to documentation and submission of DNA sequences to NCBI database and phylogenetic analysis.

MATERIALS AND METHODS

A. Collection, Observation & Isolation of Algae

Algal samples 23SPPCA01, 23SPPCA02, 23SPPCA03, 23SPPCA05 and 23SPPCA06 were collected from the Pallikaranai Marshland during November 2022. Pallikaranai marshland is found within Chengalpattu district of Tamil Nadu, India and spread over 1,247.5 ha of land and serves as an aquatic buffer of the flood-prone Chennai and Chengalpattu districts (Map 1). This lentic ecosystem is freshwater marsh and partly saline wetland situated located about 20 km from the state's capital Chennai and falls the geo-coordinates of 12.949371° (North) latitude and 80.218184° (East) longitude. Large portions of the Pallikaranai Marsh have been lost due to reduction of wetland area, fragmentation and adhoc manipulation, destroying 90% of the Marsh. The remnant 10%, which is announced as protected area is the last hope for the city of Chennai that supports the biodiversity including prolific growth of algae (Plate I). Fresh specimens were examined as wet mounts under a light microscope and they were isolated by using Bold's Basal Medium as prescribed by Stein (1973).



Map 1. Study Area.

B. Isolation of DNA

DNA isolation and molecular analysis were conducted at Rajiv Gandhi Centre for Biotechnology (RGCB), Trivandrum by using NucleoSpin® Plant II Kit (Macherey-Nagel). Isolation of DNA was done by the steps includes homogenizing the sample, Cell lysis using Buffer PL1 and Buffer PL2, Filtration, DNA binding, Wash and dry silica membrane and DNA Elute. The eluted DNA was stored at 4°C.

C. Amplification of the rbcL gene and Sequencing

The oligonucleotide primers rbcL were amplified using modified forward primer 5'-AAAGATGATGAAAACGTGAACT-3' and reverse primer 5'-CTTTCCAWAYTTCACAAGCAGCAG-3' as described by Ghosh and Love (2011). This set of primers amplified DNA from plants including green algae, fungi, and certain bacteria, like Cyanobacteria with a product size ~615 bp. The PCR protocol included an initial denaturation of 30 sec at 98 °C and externed to 40 cycles of 5 sec at 98°C, 10 sec at 54 °C and 1 min 15 sec at 72 °C, and 4°C in ∞ of final extension step.

The PCR products were visualized using 1.2% agarose gel stained with EtBr under UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad). The purified DNA was sequenced by using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) with the same forward and reverse primer used in the PCR process.

D. Molecular data analysis

The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1 (Drummond *et al.,* 2010). Each consensus sequence measured in this study was Basic Local Alignment Search Tool (BLAST) against the sequence in the Genbank database in NCBI. Based on BLAST results the sequences of the samples were submitted to the NCBI database with accession number. The phylogenetic analysis was carried out by constructing the neighbor-joining tree by using software Mega Molecular Genetic Analysis - M11 MEGA Version 11.0.13 1993-2024.

RESULTS AND DISCUSSION

The identification and classification of algae species based on cell morphology dates back to the 19th century. However, the morphological traits used for identifying many species are often not sufficiently precise, leading to the need for major revisions in the genera level. Though the identification of algae species through morphological examination remains important, molecular studies are expected to provide beneficial data for species identification (Alshehri, 2020). The molecular investigation showed that genomic DNA of five algal isolates collected from the study area was extracted and sequenced using rbcL gene as primer. The molecular analysis of the algae isolates using rbcL gene separated 23SPPCA01, 23SPPCA02, 23SPPCA03, 23SPPCA05 and 23SPPCA06 shows PCR amplification (Fig. 1). The nucleotide amplification of rbcL revealed about 615bp fragments in each algal sample. Further the molecular analysis extended to obtained nucleotide sequences of algal samples were trimmed to provide an equivalence sequence among each morphological triad was tabulated in Table 1.

Then the specific DNA fragment of rbcL of the samples were analyzed using the BLAST (Basic Local Alignment Search Tool) program of the NCBI (National Center for Biotechnology Information) database. BLAST search is a fundamental tool in molecular biology, providing critical support for research and applications in genomics, bioinformatics, and evolutionary biology (Syngai *et al*., 2013). The BLAST results revealed that the sequence data of 23SPPCA01 and 23SPPCA05 were found to be *Chlorella variabilis* and *Chlorella vulgaris* respectively with a genetic similarity of 99% toward identified species in the NCBI database using BLAST program. Similarly, the isolates 23SPPCA02, 23SPPCA03, and 23SPPCA06 were reported as *Chlorella sorokiniana.* This conformed the molecular identification of the algal samples 23SPPCA01, 23SPPCA02 and 23SPPCA05 isolated from Pallikaranai Marshland was *Chlorella variabilis Chlorella sorokiniana* and *Chlorella vulgaris* respectively. Further it also conforms the molecular identification of the algal samples 23SPPCA03 and 23SPPCA06 as *Chlorella sorokiniana.* A homology level of 99-100% with an E-value smaller than e-0.4 in a BLAST search is defined as identical and is used to identify the species (Yanuhar *et al*., 2019).

Data submission to NCBI is essential for several reasons, extending beyond promoting scientific collaboration, ensuring data quality, meeting publication and funding requirements, and advancing research across various scientific disciplines. The study further carried based on the BLAST results the nucleotide sequences of algal samples 23SPPCA01, 23SPPCA02, 23SPPCA03, 23SPPCA05 and 23SPPCA06 were submitted in Genbank NCBI database. The submissions were approved and accession numbers were assigned for the same by the NCBI database (Table 2). Currently, NCBI hosts more than 2.5 million BioSample IDs and over 33,500 entries in the Sequence Read Archive (SRA). This significant growth in SRA data volume, expanded from 5 TB in 2009 to over 20,000 TB in 2018, with approximately 8,000 TB of this data publicly accessible (Feindt *et al.,* 2018).

Phylogenetic analysis allows us to integrate our understanding of biological diversity into structural classifications and provides insights into the diversity and distribution of organisms (Hall, 2013). Constructing a phylogenetic tree aids in understanding the genetic relationships both within and between species populations. In the present investigation construction of a phylogenetic tree was performed on rbcL gene sequences of five *Chlorella* isolates from the study area with other sequences from literature and NCBI Genbank (Table 3). A total of 15*Chlorella*

isolates from various counties including China, India, Japan and USA were aligned with rbcL gene of 23SPPCA01, 23SPPCA02, 23SPPCA03, 23SPPCA05 and 23SPPCA06 sequences.

Alignment of 15 sequences was then used to construct phylogenetic tree with the Neighbor-Joining Tree method. In this phylogenetic tree the root splits into two major clades with bootstrap support (Fig. 2). Clade 1 shows 94% bootstrap support which includes sequences EU038282, EU038283, AB260909 and AB260911. Clade 2 shows variations in bootstrap support that includes sequences PP316335, KF975596, KM514866, PP196469, PP572474, KC917289, KY629617, PP316336, PP316334, PP575825 and PP110481. Further sequence data of rbcl can be utilized to explore the phylogenetic relationships of *Chlorella*. The phylogenetic analysis aid in classification, taxonomy, and conservation, enhancing comprehension of species origins and distributions (Wiens *et al.*, 2009). These studies provide a framework for interpreting biological data and addressing fundamental inquiries regarding the history and interconnectedness of organisms. Additionally, the phylogenetic tree enhances our understanding of taxonomy and elucidates the evolutionary lineage of species, organisms, or a specific ancestor.

(a)

(b)

(c)

Figure a&c– Collection of algae from Pallikaranai Marshland – Study area; **b**– Satellite Image of the Study area (Source: Google Earth)

Table 1: Nucleotide sequences of *Chlorella* species obtained using rbcL gene.

> 23SPPCA01_*Chlorella* agatcgtttcttatttgttgctgaagcaatttataaatctcaatctgaaacaggtgagattaaaggtcactatttaaacgcaacagcagctacagctgaagaaatgctgaaacgt gctgaatgtgcaaaggatttaggtgtgcctattatcatgcacgattacttaactggtggtttcacagcaaacactagtttagctcattactgtcgtgataatggtcttcttcttcac attcaccgtgcaatgcacgctgtaattgaccgtcaaagaaatcacggtatccacttccgtgttttagcaaaagctcttcgtctatctggtggtgaccatttacactctggtacag ttgtaggtaaattagagggtgaacgtgaggtaactctaggtttcgttgacttaatgcgtgatgattacatcgagaaagatcgtagccgtggtatttacttcactcaagattgggt ttctttaccgggtacaatgccagtagcttctggtggtattcacgtatggcatatgccagctcttgttgagatttttggtgatgatgcttgtttacaattcggtggtggtactttaggt caccattgggggaacgctccaggagctgctgctaaccatg
>23SPPCA02_ *Chlorella* caaccaatcacgcgtgggagagatcgtttcttattcgtagctgaagcgatttacaaatctcaagcagaaacaggtgaaattaaaggtcactatttaaatgctactgcagctac tgctgaagaaatgttaaaacgtgcagaatgtgcgaaagatttaggtgtacctattatcatgcacgattacttaactggtggtttcactgcaaacacaagtttagctcactactgc cgtgacaatggtcttcttttacacattcaccgtgcaatgcacgcggttattgaccgtcaaagaaaccacggtattcacttccgtgttttagcaaaagctcttcgtttatcaggtg gtgaccacttacactcaggtactgttgtaggtaaattagaaggtgaacgtgaagtaacattaggtttcgttgacttaatgcgtgatgactacattgaaaaagatcgtagccgtg gtatttacttcactcaagactgggtttctttaccaggtacaatgccaatagcttctggtggtattc
>23SPPCA03_ *Chlorella* caaaacaagcgtgggagagatcgtttcttattcgtagctgaagcgatttacaaatctcaagcagaaacaggtgaaattaaaggtcactatttaaatgctactgcagctactgc tgaagaaatgttaaaacgtgcagaatgtgcgaaagatttaggtgtacctattatcatgcacgattacttaactggtggtttcactgcaaacacaagtttagctcactactgccgt gacaatggtcttcttttacacattcaccgtgcaatgcacgcggttattgaccgtcaaagaaaccacggtattcacttccgtgttttagcaaaagctcttcgtttatcaggtggtg accacttacactcaggtactgttgtaggtaaattagaaggtgaacgtgaagtaacattaggtttcgttgacttaatgcgtgatgactacattgaaaaagatcgtagccgtggta tttacttcactcaagactgggtttctttaccaggtacaatgccagtagcttctggtggtattcacgtatggcacatgccagctcttgttgaaattttcggtgatgatgcttgcttac aatttggtggtggtactttaggtcacccttggggtaacgctccaggtgctgctgcaaaccgtgtagctttagaagcatgtacacaagctcgtaacgaaggtcgtgaccttgc tcgtgaaggcggtgatgtaatccgtgcagcgtgcaaatggagtcccgaattag
>23SPPCA05_ *Chlorella* atgcttaagcgtgctgaatttgcaaaagatttaggtgtacctattatcatgcacgactacttaactggtggtttcacagcaaacacaagcttagctcactactgtcgtgataatg gtcttcttctacacattcaccgtgcgatgcacgctgtaattgaccgtcaaagaaaccatggtattcacttccgtgttttagctaaagctcttcgtttatctggtggtgaccacttac actctggtactgttgtaggtaaactagaaggtgaacgtgaagtaacattaggtttcgttgacttaatgcgtgatgactacattgaaaaagatcgtagtcgtggtatttacttcact caagactgggtttctttaccaggtacaatgccagtagcttctggtggtattcacgtatggcatgccagctcttgttgagattttcggtgatgatgcttgtttacaattcggtgg tggtactttaggtcacccttggggtaacgctccaggtgcggctgctaaccgtgttgctttagaagcatgtacacaagctcgtaacgaaggtcgtgaccttgctcgtgaaggt ggtgatgtaatccgtgctgcttgcaagtggagtcctgagttagctgctgcttgt
>23SPPCA06_ *Chlorella* aaagatgatgaaaacgtaaactctcaaccattcatgcgttggagagatcgtttcttatttgtagctgaagctatttacaaatctcaagcagaaactggtgaaattaaaggtcact atttaaacgctactgcagctactgctgaagaaatgttaaaacgtgctgaatgtgctaaagatttaggtgtacctattatcatgcacgactacttaactggtggtttcacagcaaa cacaagtttagctcattactgccgtgataatggtcttcttttacacattcaccgtgcgatgcacgcggttattgaccgtcaaagaaaccatggtattcacttccgtgttttagcaa aagctcttcgtttatcaggtggtgaccacttacactcaggtactgttgtaggtaaattagaaggtgaacgtgaagtaacattaggtttcgttgacttaatgcgtgatgactacatt gaaaaagatcgtagtcgtggtatttacttcactcaagactgggtttctttaccaggtacgatgccagtagcttctggtggtattcacgtatggcacatgccagctcttgttgaaa ttttcggtgatgatgcttgcttacaatttggtggtggtactttaggtcacccttggggtaacgctccaggtgctgctgcaaaccgtgtagctttagaagcatgtacacaagctc gtaacgaaggtcgtgaccttgctcgtgaaggcggtgatgtaatccgtgcagc

Table 2: Sequences of *Chlorella* species published in NCBI database.

S. No	Isolates	Accession number	Species Name
1	23SPPCA01	PP316335	*Chlorella variabilis*
2	23SPPCA02	PP316336	*Chlorella sorokiniana*
3	23SPPCA03	PP316334	*Chlorella sorokiniana*
4	23SPPCA05	PP196469	*Chlorella vulgaris*
5	23SPPCA06	PP110481	*Chlorella sorokiniana*

Table 3: Sequences of *Chlorella* species used for phylogenetic tree construction.

Species	Accession Number	Country	Reference
Chlorella vulgaris	AB260909	Japan	NCBI Genbank
Chlorella sp.	AB260911	Japan	NCBI Genbank
Chlorella pyrenoidosa	EU038282	China	NCBI Genbank
Chlorella pyrenoidosa	EU038283	China	NCBI Genbank
Chlorella sp.	KC917289	China	NCBI Genbank
Chlorella sp.	KF975596	India	NCBI Genbank
Chlorella sp.	KM514866	China	NCBI Genbank
Chlorella sp.	KY629617	USA	NCBI Genbank
Chlorella variabilis	PP572474	India	NCBI Genbank
Chlorella sorokiniana	PP575825	India	NCBI Genbank(This study)
Chlorella variabilis	PP316335	India	NCBI Genbank(This study)
Chlorella sorokiniana	PP316336	India	NCBI Genbank(This study)
Chlorella sorokiniana	PP316334	India	NCBI Genbank(This study)
Chlorella vulgaris	PP196469	India	NCBI Genbank(This study)
Chlorella sorokiniana	PP110481	India	NCBI Genbank(This study)

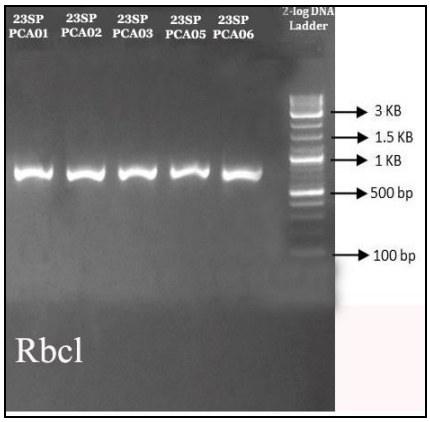


Fig. 1. PCR amplification of two *Chlorella* isolates using the rbcL gene.

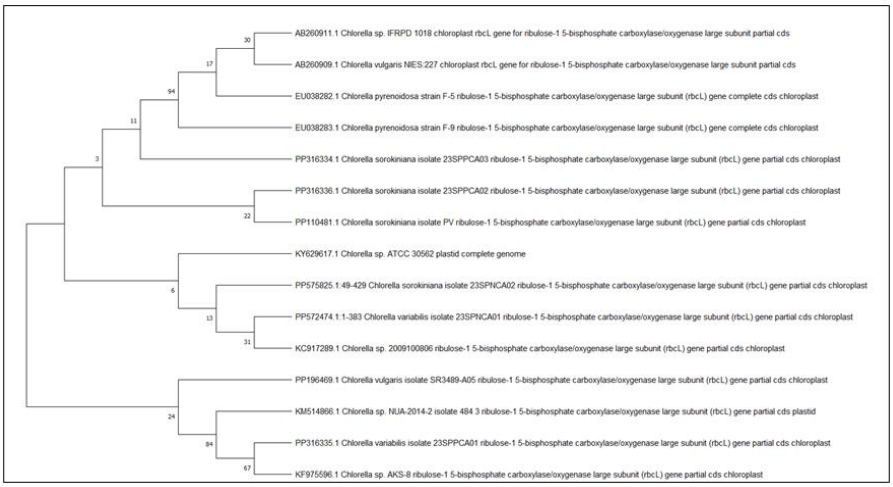


Fig. 2. Neighbour-Joining (NJ) phylogenetic tree based on rbcL gene of *Chlorella* species from the study area.

CONCLUSIONS

The current study reveals that rbcL could be used as one of the molecular markers for species-level identification in *Chlorella* species through DNA barcoding. Although this molecular marker shows promising results, it is important to consider morphological examination as a complementary approach. This research provides the valuable perceptions into the molecular identification and phylogeny of *Chlorella* sp. From Pallikaranai Marshland. The paper emphasis the documentation of authentic diversity data in ecologically significant locations may helpful us to enhance conservation efforts.

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Conflict of Interest. None.

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