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Polyphasic identification of Cyanobacteria from Muthukuda mangrove, South East coast of India

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ABSTRACT

Mangrove ecosystems are highly productive areas significantly impacted by tides, environmental conditions, and human activity. Cyanobacteria, a photoautotrophic phylum, holds significant importance due to its long evolutionary history. Cyanobacteria is known for its wide range of morphological, physiological, and genetic diversity. The traditional method of identification, which depends on the morphological characteristics may not provide accurate resolution due to closely related species. Hence a polyphasic approach is carried out in this study which involves the combination of methods including morphological, physiological and molecular techniques. Cyanobacterial strains from Muthukuda mangrove ecosystem on the southeastern coast of India, when examined under a microscope, had numerous unicellular and filamentous cyanobacteria from the orders *Chroococcales* sp, *Synechococcales* sp., *Chroococcales* sp, and *Oscillatoriales* sp, according to the observations. To ensure the purity of the cyanobacterial strain under the microscope, meticulous examination of cellular morphology and the absence of any contaminating organisms were conducted. DNA was extracted from the purified cyanobacteria strains and amplified with cyanobacteria-specific 16S rRNA genes, sequencedand submitted in NCBI. The relationship between the cyanobacterial isolates was monitored by constructing a phylogenetic tree. Both morphological and molecular analysis was compared and the results complimented each other. Using the polyphasic approach, our four cyanobacterial strains were identified: *P. mucicola, P. foetida, Gloeocapsea* sp., and *S. elongatus*. This study highlights and insists on the polyphasic identification of cyanobacterial species, as accurate identification is essential for isolating and characterizing cyanobacterial strains with desired traits for understanding diversity and distribution. This comprehensive approach not only enhances our understanding of cyanobacterial ecology but also provides valuable insights for the sustainable management of aquatic environments and the development of biotechnological solutions leveraging cyanobacterial capabilities.

Key words: *Mangrove ecosystem, Muthukuda mangrove, Cyanobacteria, 16s rRNA sequences, Polyphasic identification.*

Introduction

Among prokaryotes, oxygenic photosynthesis is carried out only by Cyanobacteria. As primary producers, cyanobacteria are considered necessary in aquatic ecosystems due to their ability to fix CO_2 using solar energy (Vermaas, 2001) with high photosynthetic efficiency, maintaining CO_2 – O_2 equilib-

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rium, and be capable of inhabiting extreme environmental conditions (Lin *et al.,* 2014). Cyanobacteria can grow in non-arable, wastewater environments with minimum competition for nutritional requirements and a short lifecycle, which attracts many biotechnological applications (Nozzi *et al.,* 2013; Zhou and Li, 2010). Cyanobacteria surviving in extreme environments are differently adapted and explored towards biodiesel production, capable of producing different substances that are widely applied in the food and pharmaceutical industry (Hussain *et al*., 2020b, Singh *et al*., 2016; Mourelle *et al.,* 2017, Hussain *et al.,* 2020a). Morphologically, cyanobacteria exist in various forms, including unicellular, trachomatous, filaments, multicellular, and perennation types (Pham *et al.,* 2017). Although visually similar cyanobacterial morphotypes may appear virtually identical genetically, the opposite can also be true: morphologically disparate colonies can share much of the same genetic makeup. Moreover, laboratory-cultivated strains can display anomalous characteristics that defy easy identification. To address these issues, scientists have increasingly turned to a polyphasic approach, which involves analyzing organisms based on their physical traits, physiology, ecology, and genetic profile, including the sequence of specific molecular markers. This comprehensive approach enables researchers to pinpoint the key features that distinguish between seemingly identical organisms, as well as to differentiate between genetic and environmental factors affecting physical traits. Over the past decade, the polyphasic approach has become widely embraced, as it offers a more robust method than relying solely on physical characteristics or a limited set of molecular markers. The cellular nature of cyanobacteria, which resembles Gram-negative bacteria, was previously known as blue-green algae; later, they were grouped with bacteria and are called cyanobacteria. The morphological traits of these groups that were found to be elastic or malleable under various environmental and cultural conditions have mainly contributed to the taxonomy of this group (Anand, 1998; Hoffmann and Demonlin, 1985). This group of organisms' unsatisfactory status has existed for a long time, and several authors have assigned the strain *Anacystisnidulas* to four different genera (Komarck, 1970). Though currently, Cyanobacterial taxonomy is based mainly on molecular sequencing and various genetic approaches (Laamanen *et al.,* 2002; Turner *et al*., 2001), in many instances, molecular sequencing methods do not agree with the morphology-based classification system, making them not absolute methods. Hence, the gene sequence analysis was performed in conjugation with the predominantly accepted 16sr RNA gene sequence method (Lee *et al.,* 2014). This work reports on identifying four cyanobacteria and comparing the morphological and 16S rRNA gene similarities.

Materials and Methods

Sample collection

The study's water samples were obtained from the Muthukuda mangrove environment, situated near Mimisal town on the southeast coast of India in the Palk Bay region of Tamil Nadu's Pudukkotai district. The coordinates of the location are Lat. 9° 51' 48'' N and Long 79° 7' 15'' E. A diverse range of mangrove plants and seagrass beds identifies the location. The water samples were collected using sterile polyethylene bottles and were transported with ice to the laboratory for further analysis and processing.

Isolation of Cyanobacteria

Cyanobacteria were isolated from water samples collected from the Muthukuda mangrove area using solid MN media (Table 1). The cultures were incubated at 25 ± 2 ºC under a light of 2500lux, 12:12 light and dark cycles for 7- 14 days. The inoculated plates were periodically observed for growth until pure cultures were obtained, which were confirmed under a light microscope.

Morphological Identification

The cyanobacterial strains isolated were observed for its morphological features of were carried out using light microscope at 40x and 100x magnifications. Morphological identification based on key characters like cell shape, size, sheath morphology, and trichome were considered, compared and identified by using literature of Desikachary (1959); Rippka *et al*.(1979); Waterbury (2006); Castenholz (2001) and Komarek (2003).

Molecular identification

DNA extractions were conducted using the E.Z.N.A. Tissue DNA Kit from 50-100 mg wet weight of the cyanobacterial samples, following the

manufacturer's instructions. The isolated DNA were amplified with universal primers for the 16s gene region specific for all the cyanobacteria samples. The primer sets CYA106F: 5'-CGGACGGGTGAGTAACGCGTGA-3' and 1492R: 5'-GGTTACCTTGTTACGACTT-3' were used for amplification. The 16S rRNA gene regionswere sequenced. The sequences of NCT65, NCT 372, NCT 373, NCT 374 and those retrieved were aligned with ClustalW using MEGA11. Phylogenetic analysis was performed to enlighten the evolutionary connection of isolates using NJ method (Saitou and Nei, 1987). The final sets comprise 1150 positions, and the taxa cluster from the bootstrap test with 500 replicates shows that the associated taxa cluster together where the maximum composite likelihood method is used to compute the evolutionary distance. (Felsenstein, 1985; Tamura *et al.*, 2004).

Results and Discussion

Sampling and Physiological parameters of the study site

A study conducted at a site characterized by a tropical climate and dominated by mangrove trees found that environmental factors significantly influenced the abundance of cyanobacterial populations. Several physiological parameters were measured to understand the impact of these factors. The study revealed that the pH level of the site was 7.7, while the salinity level was 22. Additionally, the temperature of the site was found to be 27 °C. These findings suggest that these environmental factors influence the abundance of cyanobacteria in the area.

Morphological Identification

The water samples collected from the Muthukuda mangrove environment underwent a meticulous examination under a microscope before they were inoculated in MN media plates to confirm the presence of a cyanobacterial population. The examination revealed the existence of various unicellular and filamentous cyanobacteria belonging to the orders *Chroococcales*, *Synechococcales*, *Chroococcales*, and *Oscillatoriales*. Further study was conducted on four isolates taken from the streaking pure cultures to identify the characteristics of the cells. The examination was carried out in a more detailed manner, taking into account factors such as trichomes (vegetative cells located at the terminal of filaments), size, arrangement of the cell filaments, extracellular sheath material, shape, and the presence or absence of heterocyst and akinet (thick-walled resting cells). This thorough examination aimed better to understand the cyanobacterial population in the Muthukuda mangrove environment. In accordance with the micromorphological characters, observed with the light microscope, following the identification keys of Desikachary, 1959; Rippka *et al*., 1979; Waterbury, 2006 and Komarek, 2003, the isolates were identified as *Synechococcus elongatus* (NCT 65), *Pseudanabaena mucicola* (NCT 372), *Pseudanabaena foetida* (NCT 374), and *Gloeocapsa* sp. (NCT 374) (Figs. 1-4).

The morphological characters of the isolates are described below:

Synechococcus elongatus

Strain:NCT 65(Family: Choroococcaceae; order: Choroococales).

Cells appeared light to deep green when dispersed in the culture flask. Cells, cylindrical 1.4-2µm broad, 1.5-3 times as long as broad, and often found in pairs. Cells were motile (Fig. 1). The 16S rRNA gene sequence of the isolate NCT 65 shared maximum identity with the sequences of three strains of *Synechococcus elongatus*. The isolate revealed 99.21% average nucleotide identity (ANI) with reference sp. CP033061, *Synechococcus elongatus* (Yang *et al.*, 2018). Based on the 16S rRNA sequences, ANI >95%, and morphological characteristics, the isolate was identified as *Synechococcus elongate*. Gene Bank Accession No: OP673540.

Pseudanabaena mucicola

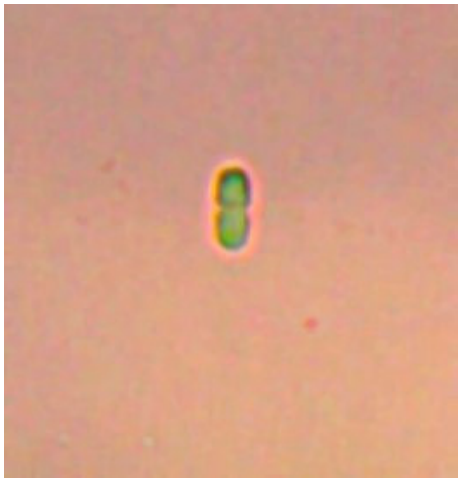

Fig. 1. *Synechoccus elongates* (NCT 61)

Strain: NCT372 (Family: Pseudanabaenaceae: Order: Pseudanabaenales)

The cultures were blue green, pale blue green. Free floating in culture flask. Filamentous, straight or slightly bend, short, arranged in two to six rows, cells were cylindrical, trichomes present, constricted at cross-walls 0.7 to 20 µm, pale blue-green or tending to be colorless, colourless mucilage (Fig. 2). The isolate showed 99.32% similarity to *Phormidium mucicola* equivalent to *Pseudanabaena mucicola* IAM M-221 NCBI: txid 454134. Gene bank accession no: ABB039019. The polyphasic observation of NCT372 allowed the identification of this strain as *Pseudanabaena mucicola* Gene Bank Accession No: OP673538

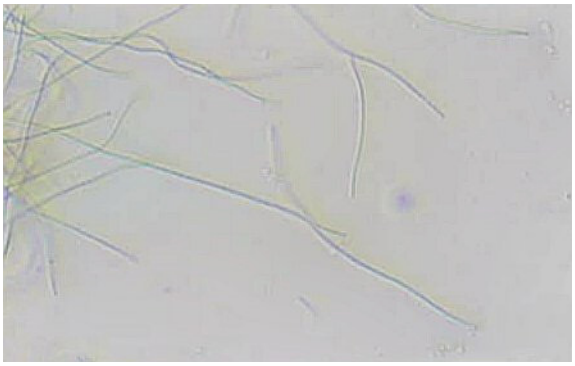

Fig. 2. *Pseudanabaena mucicola* (NCT 372)

Pseudanabaena foetide

Strain: NCT 373(Family: Pseudanabaenaceae; Order: Pseudanabaenales)
The cultures were deep green and found to be floating as well as sedimented at the bottom. Filamentous, straight or slightly curved, cells cylindrical with rounded ends, trichome present, 3- 5µm long. (Fig. 3) from the blast search highly similar sequence with more than 98% similarity was retrieved. Of the different sequences, the sequence of the isolate *Pseudanabaena foetida* LCO16779 which shared 98.95% similarity. The morphological characteristics also supported the 16S r RNA sequence similarity and the isolate were identified as *Pseudanabaena foetida* Gene Bank Accession No: OP673539.

***Gloeocapsa* sp.**

Strain: NCT 374(Family: Chroococcaceae; Order: *Chroococcales*) Chroococcales

In the media the cultures were deep green found as free floating at the top. Cells surrounded by mucus, cells small oval, found in pairs or in small clusters, arranged within a thin film of colourless sheath. Cells 2.5-4 µm long (Fig. 4). The sequence of the isolate were highly similar to *Gleocapsa* sp. AB039000 which was 99% similar. The morphology and 16S rRNA analysis of the strain NCT374 was compared. The isolate was identified as *Gleocapsa* sp. Gene Bank Accession No: OM677385.

Fig. 3. *Pseudanabaena foetida* (NCT 373)

All four sequences of the 16S rRNA gene synthesized were >1319 bp (Table 1). The sequences were subjected to BLASTn analysis, and the percentage of similarity were between 98.9% and 99.47% (Table 1). Based on BLASTn analysis the strains NCT65, NCT372, NCT373 and NCT374 were identified as explained. The phylogenetic tree constructed based on the 16s rRNA gene sequences also confirmed the species identity by clustering into clades of same species (Fig. 5).

Table 1. MN Media Composition.

MN MEDIUM	g /l
Sodium nitrate	0.75
Dipotassium hydrogen phosphate	0.02
Magnesium sulphate	0.038
Calcium chloride	0.018
Ferric ammonium citrate	0.003
EDTA	0.0005
Sodium carbonate	0.02
Trace metal mix	1 ml
Sea water	750 ml
Deionized water	250 ml
TRACE METAL MIX	g /l
Boirc acid	2.86
Manganese chloride	1.81
Zinc sulphate	0.22
Sodium molybate	0.39
Copper sulphate	0.079
Cobalt nitrate	0.0494

Cyanobacteria are the only group of prokaryotes that use light energy for photosynthesis and are found to be the oldest organisms on Earth (Malik *et al.*, 2001; Gahlout *et al.*, 2017). Though they are widely distributed in soil and aquatic environments where moisture and sunlight are available, certain groups grow in specific environments like marine, freshwater, and terrestrial soil. Hence, a thorough investigation must be made on environmental parameters in the isolation region. Physiological parameters like pH, temperature, and salinity play a vital role in the isolation when designing the media to mimic the environmental conditions (Fitri *et al.*, 2021). MN media with a seawater base with additional nutrients and required trace metals are used to isolate and culture the intertidal marine cyanobacteria (Waterbury and Stanier, 1978; Waterbury, 2006). Cyanobacteria are mostly mesophilic and have a growth optimum between 20 and 35 °C. They are generally tolerant to different salt concentrations except those isolated from marine environments, possibly due to the relatively constant salinity. They are present in environments of neutral to alkaline pH. Cyanobacteria are photoautotrophic and use simple organic compounds for growth and light as the source of energy in the case of cultured cyanobacteria (Waterbury, 2006), which occur in a wide range of light. Generally, during laboratory conditions, light intensities above 500 lux, preferably 1200 lux, are considered optimal growth; however, high light intensities are avoided as they inhibit the growth of certain phycoerythrin-producing strains (Ripka *et al.*, 1979). Though many can grow under continuous illumination by light, certain filamentous and non-heterocystous nitrogen fixers require a light-dark cycle (Waterbury, 2006). The culturable

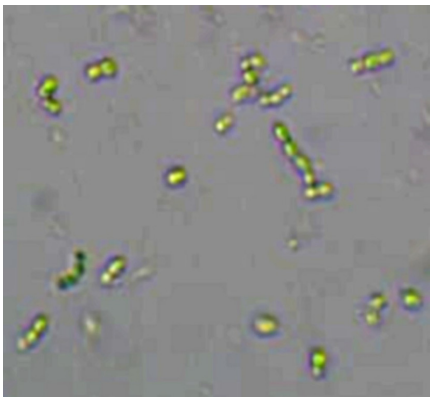

Fig. 4. *Gloeocapsa sp.* (NCT 374)

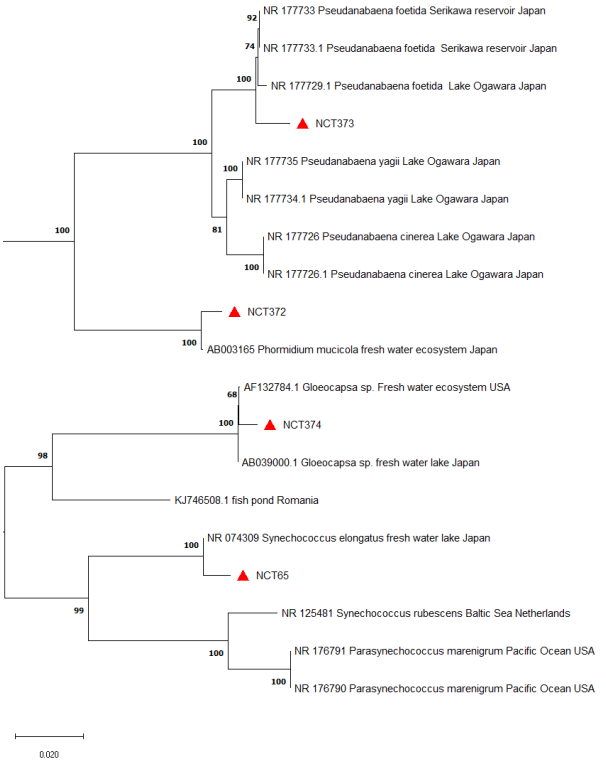

Fig. 5. The sequences produced in this study are indicated by red triangles at the branch tips. Reference sequences from GenBank are indicated by their GenBank accession numbers, followed by the binomial name of the species, the source, and the country of isolation. This analysis included 19 nucleotide sequences. All positions with gaps and missing data were excluded (complete deletion option), resulting in a final dataset of 1305 positions. The sequences produced in this study closely cluster with their respective similar species.

Table 2. GenBank accession numbers of Cyanobacteria species in this study

Strain name	Sequence length (bp)	GenBank acc. No.	Similarity (%)	Reference species	GenBank acc. No. of reference sp.
NCT65	1387	OP673540	99.21	*Synechococcus elongatus*	CP033061
NCT372	1319	OP673538	99.32	*Pseudanabaena mucicola*	AB003165
NCT373	1328	OP673539	98.95	*Pseudanabaena foetida*	LC016779
NCT374	1325	OM677385	99.47	*Gloeocapsa sp.*	AB039000

cyanobacterial population is generally underestimated, as it is believed that culturing axenic cyanobacteria is difficult as some physiological factors may affect the growth of the isolates during cultivation. Though achieving pure culture is considered tedious (Fitri *et al*., 2021), the isolates were purified using several streakings on the mineral medium agar surface. Waterbury(2006) reported the purification of cyanobacteria by streaking them several times on solid media and transferring them to liquid media. Extensive studies have been carried out on the diversity of cyanobacteria and their potential applications in different regions. Cyanobacteria are potential wonders, as they are considered indicators of nature due to their production of toxins in water bodies that cause ill effects in humans. They also play a role in sustainable agricultural and environmental development by acting as indicators of productive ecosystems, nitrogen fixation, CO_2 sequestration, soil fertility improvement, and as a source of food (SCP) (Kumar Kol *et al.,* 2023). The identification of cyanobacteria by morphological methods has been well-studied since the 19th century. Taxonomic classification is considered essential for comprehending and investigating knowledge regarding organismal variety. It has proven difficult and troublesome to classify cultured cyanobacteria. However, there were differences and misinterpretations of results, and numerous revisions are required in the case of new genera and species. Hence, molecular approaches have been carried out in recent years for appropriate results (Wehr and Heath, 2003; Hasler *et al*., 2012). Recently, a multidisciplinary approach to identification using a combination of morphological observations and molecular characterization has been emphasized. Various filamentous and unicellular cyanobacteria are being identified using morphological and molecular methods, i.e., polyphasic identification; when the morphology is compared to the molecular technique, particular clarity is achieved.

Future outlook

More research is needed to understand the genetic characters of various species of Cyanobacteria and how they relate with their physical features. Furthermore, it is essential to know whether or not these strains can complement the 16S rRNA sequence whose relatively constant nature is already known for efficacy. These findings further indicate that a polyphasic approach will be more potent in distinguishing strains than using only 16S rRNA method alone. However, fundamental biodiversity information should be established by implementing polyphasic approaches to obtain precise ecological data on taxa. In situations where all these parameters show some definable differences, taxonomic identification of cyanobacteria entities is necessary, especially in cases involving crypto taxa, morphology or ecospecies. Correctly identifying cyanobacteria development can help science grasp its diversity, ecology as well as laboratory practice. Nevertheless, formal possibilities and implementation of this variation still need clarity in order to progress further into cyanobacteria science by establishing them and choosing them before implementing them. Whereupon any differential characteristics that define anything can become clear cut manifestations then phylogenetic classifications which morphologically and ecologically defined stable units are always used when natural ecosystems are considered must also identify those organisms as part of the same unit after another study was published on principles of 'polyphasic evaluation' up to genera level about Cyanobacteria. However, for future developments in research on cyanobacteria such an attitude needs a prescription even if recording different stable crypto taxa, morphology or ecospecies would be very much desirable (future studies should prescribe this). We would suggest revisiting the nomenclature committee results on cyanobacteria using special committees' reports and assessing current proposals; hence proposing guidelines across morphological ecology and laboratory studies for isolating difficult cases that we must know about cyanobacteria diversity. The main goal of this particular study using a polyphasic approach was therefore geared towards optimizing how best strains within their natural habitat could be identified based on morphological and genotypic criteria even in determining their ecological significance.

Conclusion

A recent study has emphasized the significance of polyphasic identification when dealing with cyanobacteria. The study suggests that a comprehensive approach that considers phenotypic traits, ecology, and genetic profile (16S rRNA gene sequences) is necessary when faced with ambiguous identifications. While both molecular analysis and morphological characteristics were used in this

study to identify cyanobacteria, the results of these analyses only occasionally corroborated each other. However, the study found that four isolates from Palk Bay, a region located on the southeastern coast of India, consistently produced precise results for both molecular and morphological analyses. This discovery highlights the importance of using a polyphasic approach to identify cyanobacteria accurately.

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Conflict of Interest- None

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