Polyphasic taxonomic identification of marine cyanobacteria of the Indian Sundarbans and their biotechnological potential

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This Thesis is dedicated to my Family and Teachers

DETAILS OF THESIS

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CERTIFICATE FROM THE SUPERVISOR

This is to certify that the thesis entitled **"Polyphasic taxonomic identification of marine cyanobacteria of the Indian Sundarbans and their biotechnological potential"** submitted by **Shri, Arup Ratan Roy,** who got registered (Registration no. **D-7/ISLM/95/19**, dated on **November 1st , 2019)** his name under the **Faculty of Interdisciplinary Studies, Law & Management** for the award of **PhD (Science)** degree of **Jadavpur University**, is absolutely based upon his own work under the supervision of **Prof. Joydeep Mukherjee** and that neither this thesis nor any part of it has been submitted for either any degree/diploma or any other academic award anywhere before.

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STATEMENT OF ORIGINALITY

I, **Arup Ratan Roy** (Reg. No. **D-7/ISLM/95/19**), registered on **November 1st, 2019** do hereby declare that this thesis entitled "**Polyphasic taxonomic identification of marine cyanobacteria of the Indian Sundarbans and their biotechnological potential**" contains a literature survey and original research work done by the undersigned candidate as part of doctoral studies.

All information in this thesis has been obtained and presented in accordance with existing academic rules and ethical conduct. I declare that, as required by thesis rules and conduct, I have fully cited and referred to all materials and results that are not original to this work.

I also declare that I have checked this thesis as per the "Policy on Anti-Plagiarism, Jadavpur University, 2019," and the level of similarity as checked by iThenticate software is **9** %.

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DECLARATION

I hereby declare that this thesis represents my own work and has not been previously included in my thesis or any other institution for a degree, diploma, or other academic qualifications. Further, I have acknowledged all sources used and have cited these in the reference section.

I hereby submit the record of my observations for evaluation for the award of the degree of Doctor of Philosophy in Science.

Date: 8/1/25

Place: Jadavpur

Arup Ratan Roy

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LIST OF ABBREVIATIONS

%	Percentage
&	And
~	Approximately
µl	Microliter
µmol	Micromolar
µm	Micrometer
ASN	Artificial Seawater Nutrient
BLAST	Basic Local Alignment Search Tool
Bp	Base pair
BI	Bayesian Inference
Cat. No.	Catalogue number
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleic acid
ESS	Effective Sample Size
G	Gram
hrs.	Hours
IPTG	Isopropyl β-D-1-thiogalactopyranoside
ITS	Internal Transcribed Spacer
Kb	Kilo bases
L	Liter
LB	Luria Broth
M	Meter
M	Molar
mM	Millimolar
MCMC	Markov chain Monte Carlo
Mg	Milligram
$MgCl_2$	Magnesium Chloride
Min	Minute
ML	Maximum Likelihood
Ml	Milliliter
MP	Maximum Parsimony
NaCl	Sodium Chloride
NCBI	National Centre for Biotechnology Information
Ng	Nanogram
Nm	Nanometer
OUT	Operational Taxonomic Unit
PAUP	Phylogenetic Analysis Using Parsimony
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
pmol	Picomolar
rpm	Revolutions per minute

rRNA	Ribosomal Ribonucleic acid
s	Second
SD	Standard Deviation
TBE	Tris-Borate-EDTA
TYG	Tryptone Yeast Genome
UV	Ultraviolet

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ABSTRACT

The Sundarbans, world's biggest estuarine mangrove forest, is characterized by poikilotrophic harsh environmental circumstances. The microbial diversity of this ecosystem is understudied. To continue of work on the documentation of cyanobacterial biodiversity, soil biofilms were collected from multiple locations of Sundarbans islands. These biofilms were cultured under controlled laboratory conditions and subsequently processed for purification. Five samples were established as an unicyanobacterial cultures, identified through the modern polyphasic taxonomic approach including morphological, ultrastructural, and molecular examinations. Each of the five specimens exhibited a simple, thin, thread-like homocytous filaments. Among the five specimens analyzed, S1, S6, and S10 exhibited a 94.4% resemblance of the 16S rRNA gene with the reference strain, resulting in the formation of a distinct clade in the phylogenetic tree which was separate from the reference cyanobacteria genus. Furthermore, morphological, and ultrastructural analyses revealed that the three strains displayed distinct characteristics compared to the reference genus. In addition to being distinct from the reference genus, the secondary structure and sequence sizes of the various domains of the ITS were also distinct from those of the other members of the Nodosilineaceae family. The strains S19 and S23 exhibited 99% identity in 16S rRNA similarity to *Thainema salinarum*, which was recently discovered. Although there are similarities observed, the analyses of morphology and ultrastructure revealed that the distinguishing features, for example the form of the apical cell, the presence of a mucilaginous sheath, and the types of cellular inclusions, differed from those of the reference genus. The phylogenetic tree revealed that the two isolates formed clade with *Thainema salinarum*, which should be categorized under the Nodosilineaceae family according to the latest classification scheme of cyanobacteria published by Strunecky *et al*. (2023). The comprehensive evaluation of the five strains revealed that the S1, S6, and S10 strains should be identified as a new genus and species, designated as *Almyronema*

epifaneia gen. nov. sp. nov., within the Nodosilineaceae family, as per the recent classification system of cyanobacteria proposed by Strunecký et al. (2023). S19 and S23 are identical to *Thainema salinarum* genus within the Nodosilineaceae family, not the Leptolyngbyaceae family of *Thainema* genus as was previously known. This study revised family positions S19 and S23 in accordance with the monophyletic genus concept, alongside a new biodiversity report from India. Moreover, using Next-Generation Sequencing (NGS) technology, a draft genome study of *Almyronema epifaneia* (S1) was carried out, which revealed the characteristics of the genome. The genome is 4.6 MB and consists of 144 contigs, with a G+C content of 50.6% and including 4,595 protein-coding genes. Spectrophotometric, HPLC, and ESI-MS investigations confirmed that *Thainema salinarum* (S19) produces shinorine, a mycosporine-like amino acid that has application in the therapeutic and cosmetic industries, making it a possible resource of anti-UV chemicals. In addition, the *Almyronerma epifaneia* (S1) strain was able to produce 19% of lipid in the ASN-III medium that was supplemented with 1.0 g/l of $NaNO_3$, which classified it as a high-yield variant of cyanobacteria. The lipid content extracted from *A*. *epifaneia* strongly suggested that it may be used as a biodiesel source.

Chapter 1.
INTRODUCTION

1.1. Conception: Cyanobacteria

According to phycologists, any organism that has chlorophyll a (Chl a) but lacks a root and shoot should be classified as an alga (Lee, 1989). They are categorized into two distinct types: i) prokaryotic algae and ii) eukaryotic algae. Cyanobacteria are a kind of prokaryotic algae that perform oxygenic photosynthesis, using chlorophyll a as their primary photosynthetic pigment (Whitton & Potts, 2007). The lack of distinct membrane-bound organelles such as mitochondria, chloroplasts, and nuclei, as well as the ability to photosynthesize, describe cyanobacteria as primitive organisms with an intermediate structure between bacteria and plants. There is debate among taxonomists about whether Cyanobacteria, a group of prokaryotic blue-green algae, should be called bacteria or algae (Chapman, 1973; Palinska, 2014). They were the earliest oxygenic photosynthetic organisms that could produce oxygen as a consequence of photosynthesis (Olson, 2006). The oldest microfossils thought to be cyanobacteria were found in the early Archean period, but their origin is still being discussed. Cyanobacteria likely originated around 3.5–3.3 billion years ago, as suggested by Schopf (1987) and Brocks (1999). According to Demoline (2019), cyanobacteria emerged approximately 2.4 to 2.7 billion years ago and played a crucial part in the Great Oxygenation Event, which considerably elevated oxygen levels in the Earth's atmosphere and oceans.

Cyanobacteria exhibit a range of morphological shapes, including basic unicellular forms as well as multicellular filamentous structures that may be either unbranched or branched (Rippka, 1979). The cells exhibit variations in both size and shape. Filament branching might be either true or false. False branching occurs in *Scytonema* when a sheathed filament is divided into two unrelated ones, each of which continues to develop independently (Komarek, 1999). True branching is seen in filamentous cells, such as Nostocales order, where the cells have the ability to

divide on many planes (Komarek, 2005). Cyanobacteria primarily use chlorophyll-a as their major photosynthetic pigment (Blankenship, 2021). Additionally, they may also possess xanthophylls, carotenoids and phycobiliproteins which consist of phycoerythrin and phycocyanin (Glazer, 1985 & 1989). They appear blue-green due to the presence of allophycocyanin and c-phycocyanin pigment. Chlorophyll-b is present in some cyanobacteria, such as *Prochlorococcus* and *Prochloron* that lives in symbiotic relationships with sea squirts (Partensky *et al*. 1999). A unicellular cyanobacterium *Acaryochloris marina*, isolated from the shoreline of Palau, which has a simple morphology. It is distinguished by a distinctive metabolic process and a unique pigment for photosynthesis, Chlorophyll-d (Miyashita *et al*. 1996, 2004). It was discovered recently in some cyanobacteria situated within microbial biofilms and stromatolites, which are habitats with severe low light or shadow. *Halomicronema hongdechloris* is a specific kind of cyanobacterium that has chlorophyll f. (Chen *et al*. 2012).

Cyanobacteria have many specialized cells that have roles in both reproduction and physiological functions. A few single-celled cyanobacteria have the ability to form baeocytes and exocytes. These structures are distinguished from their mother cells based on their form and size, along with the process of multiple divisions followed by discharge into the surroundings. (Komárek and Anagnostidis, 1998). Hormogonia are short, often moving filaments that are produced by filamentous cyanobacteria (Rippka, 1979). Nostocales have the ability to generate two distinct kinds of specialized cells, which are heterocytes and akinetes. The primary function of heterocytes is the anaerobic fixation of nitrogen from the environment utilizing the enzyme nitrogenase (Meeks & Elhai, 2002), which is also present in certain soil bacteria. Consequently, they lack a functional photosynthetic system. Heterocytes are different compared to vegetative cells because their contents are more uniform and they have polar holes. Heterocytes can be found

between filaments or at the tips of the cells (Komárek *et al*. 2014). Akinetes are often bigger than vegetative cells and contain huge quantities of preserved food, which are observed like grains. Akinetes are dormant propagative units that may remain inactive under favorable situation and active under unfavorable circumstances (Kaplan-Levy *et al*. 2010). *Anabaena cylindrica* akinetes may be able to live on Mars, according to Olsson-Francis *et al*. (2009).

Cyanobacteria possess cell walls which are identical to common to gram-negative bacteria, but they are also different in some ways that make them separate from prokaryotes. The peptidoglycan layer in gram-negative bacteria has a thickness ranging from 2-6 nm, while for cyanobacteria it ranges from 10 nm in unicellular form to 35 nm in filamentous species (Golecki, 1977; Hoiczyk and Baumeister, 1995). According to Hoiczyk and Baumeister (1995), the peptidoglycan width in *Oscillatoria princeps* varies between 300 nm and 700 nm. In addition, the level of cross-linkage between the peptidoglycan chains in cyanobacteria is like that seen in gram-positive bacteria (Hoiczyk, 2000). The type of lipopolysaccharide (LPS) found in cyanobacteria does not include keto-deoxyoctonate, a component often seen inside the LPS of gram-ve bacteria (Mikheyskaya *et al*. 1977). Additionally, cyanobacteria possess distinctive components that are typically not noticed in gram-ve bacteria, as well as the outer membrane. As mentioned by Schrader *et al*. (1981); Hansel *et al*. (1998), those components are porins, carotenoids, and β-hydroxypalmitic acid. According to Castenholz *et al*. (2015), cells are often surrounded by mucilaginous sheaths which are made of exopolysaccharides, the thickness may vary from less than 1 μm to more than the thickness of the filament. Sheaths may be responsive to environmental signals and perform several important functions, including shielding against UV radiation and preventing dehydration (Ehling-Schulz and Scherer, 1999). Additionally, cyanobacteria have membrane structures known as thylakoids, which perform a critical function in photosynthesis

process. The positioning and organization of thylakoids play a crucial role in taxonomy classification. According to Komárek *et al*. (2014), thylakoids may be situated in the cell in a parietal, radial, or irregular manner, with their position being most significant at the lineage or order level.

Cyanobacteria have diverse cellular inclusions that play essential roles in their survival, adaptability, and metabolic activities (Stainer, 1977). Carboxysomes are a kind of cellular structure that may be found in several genera, including *Synechococcus*, *Prochlorococcus*, and *Anabaena*. According to Price *et al*. (2008), these microcompartments are home to important carbon fixation enzymes such carbonic anhydrase and RuBisCO, which are essential for effective photosynthesis in low CO_2 situations. Cyanobacteria, such as *Spirulina* and *Oscillatoria*, have been discovered to preserve both energy and carbon in poly-β-hydroxybutyrate (PHB) granules, especially when they are under nutrient-limited situations such nitrogen depletion (Schlebusch & Forchhammer, 2010). Cyanophycin granules, which consist of arginine and aspartate, serve as a nitrogen storage mechanism, particularly in nitrogen-fixing cyanobacteria like *Anabaena* and *Nostoc*. They are reserve sources to cope during periods of nitrogen shortage (Simon, 1987). Polyphosphate granules, which are present in genera such as *Synechococcus* and *Microcystis*, play a crucial role in storing phosphorus and are particularly important when there is a shortage of phosphate (Allen *et al*. 1984). Cyanobacteria possess lipid droplets that serve as storage sites for neutral lipids, especially during periods of stress, as seen in *Nostoc punctiforme* (Peramuna *et al*. 2014). These examples demonstrate the impressive capacity of cyanobacteria to adapt to different environmental circumstances, which helps them survive and flourish in a varied type of environments.

1.2. Diversity of cyanobacteria

Cyanobacteria can be found in nearly all ecological habitats on Earth, owing to their remarkable adaptability, which allows them to exist throughout diverse environments for example boiling geothermal springs, frozen desserts, coast and freshwater ecosystems (Whitton and Pots, 2000). The widespread distribution of cyanobacteria could possibly be connected to their distinctive morphological and physiological characteristics. They possess significant physiological adaptability and a high degree of elasticity, allowing them to thrive in almost every location on Earth (Castenholz, 2015, Gaysina *et al*. 2019). Cyanobacteria has remarkable physiological adaptability, enabling them to readily create beneficial endophytic and symbiotic partnerships (Adams, 2008; Thajuddin, 2005). They are found on beaches, surfaces of stones, plants, and manmade objects, particularly in harsh environments. Cyanobacteria is ubiquitous in diverse environments, including rivers and lakes, seepages, rocky cracks bark of trees, agricultural land, sandstone, salt-subjugated land, marine habitats, arid regions, cold habitats, biological soil crusts and soil surfaces (Stal, 2000, Gaysina, 2019). This illustrates their capacity to easily and effectively exist and thrive in these environments.

Freshwater environments including streams, rivers, lakes, wetlands, and lagoons have been the subject of several reports containing cyanobacteria. Freshwater cyanobacteria are classified as planktonic or benthic (Fogg *et al*. 1973). Planktonic cyanobacterial taxa that are commonly observed include species from the genera *Coelosphaerium*, *Chroococcus*, *Snowella*, *Cyanobium*, *Synechococcus*, Microcystis, *Planktothrix*, *Limnothrix*, *Anabaena*, *Cyanodictyon*, *Aphanizomenon*, and *Nostoc* (Gaysina, 2019; Komarek, 2003; Komarek and Anagnostidis, 1999; Komarek *et al*. 2014). Typically, uniform mats are formed by benthic cyanobacteria that reside in the lowest remains of water ecosystems. The often-seen benthic freshwater cyanobacteria include

Aphanocapsa, *Phormidium*, *Leptolyngbya*, *Schizothrix*, *Cylindrospermopsis*, *Aphanothece*, *Lyngbya*, *Anabaena*, and *Tychonema* (Gugger, 2005; Gaysina, 2019; Komarek and Anagnostidis, 1999; Komarek, 2003).

Cyanobacteria are found in estuaries, coastlines, and open seas, among other niches in marine environments. The marine cyanobacteria frequently observed include *Prochlorococcus*, *Trichodesmium*, *Crocosphaera*, *Nodularia*, *Romeria*, *Spirulina*, *Hydrocoleum*, *Microcoleus*, *Moorea*, *Lyngbya* (Flombaum, 2013; Gaysina, 2019; Saito, 2011; O'Neil, 2012; Whitton and Potts, 2007). The presence of diverse groups of cyanobacteria in terrestrial ecosystems such as desert, agricultural field, salt pane, moist soils, rocks, and epiphytic cyanobacteria have been recorded: *Sypmplocastrum*, *Microcoleus*, *Desmonostoc*, *Phormidium*, *Leptolyngbya*, *Nostoc*, *Cyanothece*, *Calothrix*, *Roholtiella*, *Scytonema*, *Fischerella*, *Gloeocapsa*, *Kamptonema*, *Stigonema* (Gaysina, 2019; Komarek *et al*. 2014). Mangrove cyanobacteria are found in the unique intertidal environments of mangrove ecosystems, where they play vital roles in nutrient cycling and primary production. Using polyphasic approach of taxonomic identification several cyanobacteria were reported from mangrove ecosystem worldwide such as *Dapisostemon*, *Halotia*, *Kryptousia*, *Phyllonema*, *Foliisarcina*, *Leptochromothrix*, *Vermifilum*, *Ophiophycus*, *Okeania*, *Euryhalinema*, *Leptoelongatus*, *Aerofilum* (Alvarenga, 2015; Hentschke *et al*. 2016; Genuario *et al*. 2015; Alvarenga *et al*. 2017; Berthold *et al*. 2020; Chakraborty *et al*. 2019, 2021).

1.3. Cyanobacterial systematics

For almost a century, the cyanobacterial taxonomic classification system has been continuously evolving (Palinska, 2014). The identification of cyanobacteria formerly relied only on their morphological traits; however, the development of molecular biology has given researchers novel approaches to explore their biosystematics and evolutionary history (Iteman *et al*. 2000; Johansen

et al. 2011, Chakraborty *et al*. 2018; Mai *et al*. 2018; Chakraborty *et al*. 2021; Johansen *et al*. 2017). This led to the transition of taxonomy from phenetic to new phylogenetic methodologies for classification (Dvorak, 2017). Nevertheless, it is crucial to underscore the high significance of morphological characteristics. Hence, a mix of morphological, ecology, and genetic data was utilized (Komarek, 2016). This created the idea of the "polyphasic taxonomic approach," which is now recognized as the most effective method for identifying and describing a cyanobacteria (Komarek, 2006). Woese *et al*. (1990) established the 16S rRNA gene sequences as a genetic marker for genus and species identification. It has now been extensively used for the taxonomical analysis. Nevertheless, Johansen & Casamatta (2005) noted that additional molecular markers were required to carry out evolutionary reconstruction since the highly conserved 16S rRNA domains were insufficient to determine the inter-specific connection. Later, secondary structures of the 16S–23S internal transcribed spacer regions were analyzed for decode the intra-species relationship along with the morphology, ecology, and ultrastructural characteristics (Berthold *et al*. 2022, Saraf *et al*. 2019, Johansen *et al*. 2017). Historically, cyanobacterial species were classified based on the International Code of Nomenclature for algae, fungi, and plants (ICN), since they were first considered to be plants (Oren & Ventura, 2017). Subsequently, the International Code of Nomenclature of Prokaryotes (ICNP) was implemented, emphasizing the phylogenetic connections between cyanobacteria and bacteria (Oren, 2017). The distinctive ecological, physiological, and evolutionary characteristics of cyanobacteria make it difficult to map their taxonomy from the botanical to the bacteriological code (Komárek, 2010). In recent years, the use of whole genome sequencing (WGS) has started in the field of cyanobacterial systematics (Skoupý *et al*. 2022). It is progressively becoming the new standard for describing cyanobacterial species. Whole genome sequencing and phylogenomic analysis offer

crucial insights into the evolutionary origins and interrelationships of species. Nevertheless, the use of 16S rRNA phylogenetic analysis and ITS rRNA region analysis will continue to be crucial in species identification (Villanueva *et al*. 2024). As the field of cyanobacterial systematics moves towards phylogenomic study, it will be crucial to incorporate current and future taxonomy into a genomics-driven system by carefully analyzing ITS rRNA secondary structure analysis, ecological character, and morphology.

1.4. Modern polyphasic taxonomic approach

The most effective technique now available for identifying cyanobacteria is the polyphasic technique, which comprises of molecular, ecological, biochemical, morphological, and ultrastructural analysis (Komarek *et al*. 2006, 2014 & 2016). Given their widespread presence, cyanobacteria are extremely intricate organisms which cannot be accurately identified utilizing a single approach based on either morphological characteristics or genetic analysis. Here are the main characteristics of each approach that are discussed:

Morphological traits are mostly the outcome of genetic foundation. Genetic factors control important morphological features such cell size, shape, arrangement, presence of specialized cells, and development of colonies or filaments (Flore *et al*. 2005, 2010; Schirrmeister, 2011; Zhang, 2006). Apomorphic characteristics include filament structure, trichome types (uniseriate or multiseriate), trichome polarity, apical cell shape (narrower, broader, or elongated), mucilaginous sheath (single or multi-layer), and cellular constrictions (Komarek *et al*. 2014). Despite the fact that genomic data (16S rRNA) is the primary basis for the contemporary classification of cyanobacteria, it is impossible to disregard these morphological characteristics, as they evolve over an extended period. In addition to having an evolutionary past, cyanobacteria are composed of a number of specialized cells that are essential to their activities. Structures that include akinetes,

hormogonia, heterocytes, baeocytes, necridic cells, and others are considered significant for the classification of cyanobacteria.

Through the use of electron microscopy, cyanobacterial cells have been shown to have cellular inclusions that are crucial for differentiating between different species of the cell. There are different kinds of thylakoid arrangements in cyanobacterial cells, including parietal, radial, fascicular, parallel, cyanothece type, and some other unusual forms (Mareš, 2019). Although the 16S rRNA gene is stable, widely accepted, and has a huge collection of genomes that can be used for comparison, there were some issues with figuring out the evolutionary tree. An analysis may be conducted using a partial sequence of cyanobacterial 16S rRNA that is about 1400 base pairs in length. In order to facilitate grouping during phylogenetic study, Yarza *et al*. (2014) established a taxonomic cutoff range for every level of rank. The threshold limitations are as follows: 98.7% for species separation, 94.5% for genus separation, 86.5% for family separation, and 82% for order separation. Mai *et al*. (2018) suggested applying these cutoff limitations as the main principles for the taxonomic investigation of new genera and species using the 16S rRNA gene sequencing information.

FIGURE 1.1. A schematic illustration of the 16S-23S Internal Transcribed Spacer (ITS) region showing different conserved and variable regions. Arrows are denoted as different domains and dots as spacer regions.

Due to the widespread belief among cyanobacteria researchers that 16S-23S ITS regions provide more information compared to the 16S rRNA gene when it comes to detecting variations for the species distinction, numerous new genera and species were identified over the past ten years, including Hasler *et al*. (2017), Mai *et al*. (2018), Soares *et al*. (2019), Tawong *et al*. (2019),

Johansen *et al*. (2011), Shalygin *et al*. (2018), Strunecky *et al*. (2011, 2013, 2014), and multiple others. The ITS region spans 450–600 bp in length (Fig. 1.1). Boyer *et al*. (2001) discovered the existence of several distinct operons in the ITS portion. The spacer consists of a total of 14 parts, as shown by Boyer *et al*. (2001), Johansen *et al*. (2011) and Iteman *et al*. (2000),. The length and secondary structure of those regions differ across different species (Fig. 1.2). While many cyanobacterial species share highly conserved sequences, these areas are mostly associated with the regulatory role in the transcription process.

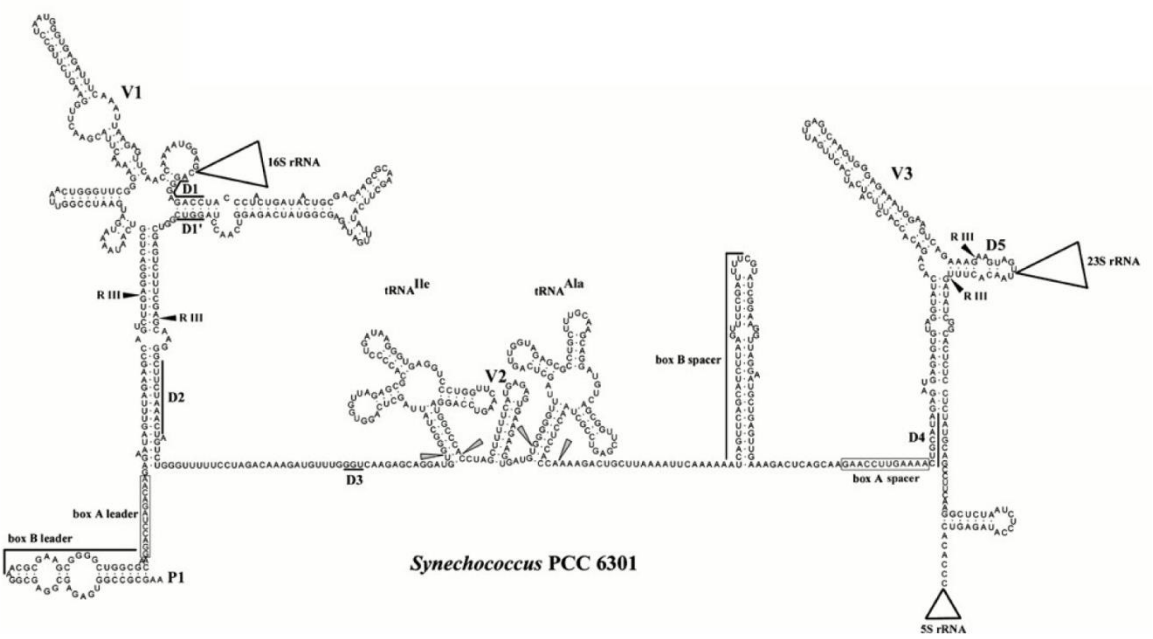

FIGURE 1.2. Secondary structure of 16S and 16S-23S ITS parts of Synechococcus PCC 6301 reveals distinct domains within the region. The image reproduced from Iteman *et al*. (2000) with license id 1533673-1.

The sequence of the 14 sections comprises the leader sequence, D1-D1' helix, D2 with spacer, D3 with spacer, $tRNA^{ile}$, V2 spacer, $tRNA^{Ala}$, pre-Box B spacer, Box B, post-Box B spacer, Box A, D4, V3, and D5. Among these 14 regions, the D1-D1' helix, V2, V3, and Box B helix, are often used for comparative analysis across different cyanobacterial taxa (Fig. 1.2). In addition, the

dissimilarity value (as percentage) obtained from the p-distance analysis of the ITS regions is considered to be crucial in determining inter-specific relationships. This value is believed to be a consistent characteristic according to studies conducted by Erwin & Thacker (2008), Berthold *et al*. (2021), Bohunicka *et al*. (2015), Shalygin *et al*. (2020), Casanatta *et al*. (2020), Chakraborty *et al*. (2021), and numerous other researchers.

1.4.1. Genus concept

In order to properly classify cyanobacteria, a polyphasic strategy is required, the most important of which is the analysis of their 16S rRNA gene sequence. Approximately 95% or less genetic similarities to the reference strains are required for a novel genus to be recognized (Komarek *et al*. 2016). Moreover, in accordance with Yarza *et al*. (2014), the 16S rRNA gene arrangement must show a genetic individuality of 94.5% or less for a new genus to be recommended. Nevertheless, these threshold values are not seen as absolute standards (Kastovsky *et al*. 2024). The monophyletic classification should be maintained by the taxa forming a singular phylogenetic branch. Unique scientific names must be given to each new genus. In addition to genetic data, the new genus must contain cytomorphological and ecological traits that may aid in the identification and differentiation of natural populations (Komarek *et al*. 2018).

1.4.2. Species concept

A polyphasic assessment is used as well to investigate novel species applying comprehensive data. On the basis of 16S rRNA, the genetic similarity must be at least 98.7% or lower for establishment as new species (Yarza *et al*. 2014). The integration of morphospecies and ecospecies theories are necessary for the thorough identification of novel cyanobacterial taxa. In the beginning, researchers depended on specific morphological characteristics such as cell form, dimensions,

filament structure, and the existence of specialized cells like heterocyst before coming to the conclusion that a prospective newly discovered species belongs to a morphospecies. Nevertheless, given that influences from the environment might impact morphology on its own, the ecospecies concept is used to examine the adaptations of these organisms to distinct ecological niches, such as distinct freshwater, marine, or extreme environments. The ecological viewpoint is useful for differentiating between species that look identical on the exterior but have evolved to live in quite distinct habitats. To ensure accuracy, both methods use molecular techniques like 16S rRNA sequencing, which confirms genetic uniqueness with ecological and morphological data. This polyphasic technique enables more accurate identification of novel cyanobacteria species, taking into account both morphological and ecological specialties.

1.4.3. Challenges in polyphasic approach of taxonomy

The simple thallus structure of numerous cyanobacterial species makes them morphologically indistinct. Synechococcales is one of the groups of cyanobacteria that exhibit both basic unicellular and filamentous structures (Komarek *et al*. 2014). In terms of phylogenetic position, this group does not belong to a monophyletic group. In recent years, many changes have been done to attain the monophyletic positions of the members of this group, although more are required in the future (Mai *et al*. 2018). One of the key challenges of current taxonomy is bridging the gap between a taxa's natural and cultural conditions. After being maintained in lab settings for an extended amount of time, cyanobacterial strains lose their morphological characteristics. The issue might be overcome by carefully studying natural samples. This should be clarified in the species description if the specimen was only investigated in culture condition (Kastovsky *et al*. 2024). The conventional classification system mainly depended on morphological and ecological features, with an emphasis on visible characteristics such as shape, structure, and habitat. However, modern

classification integrates genetic and morphological data, therefore minimizing the significance of many conventional defining characteristics, such as morphological features. In contrast, molecular features such as DNA sequences, which were previously unnoticed, now have a vital function in modern taxonomy, fundamentally altering the classification and understanding of species. Another major challenge is, the nomenclature of cyanobacteria, currently which follows both ICN and ICNP. The ICN follows nomenclatural rules based on botanical aspects, which priorities morphological characters rather than molecular data. Whereas ICNP adheres to bacteriological aspects focusing on molecular data (16S rRNA sequencing) rather than purely morphological character. For type specimens of cyanobacteria under ICN, are often physical collections of the organism, such as herbarium samples, but ICNP typically requires a designated type strain must be deposited in an accredited culture repoaitory (Komarek *et al*. 2018).

1.5. Biotechnological potential of mangrove cyanobacteria

Thorough investigation of underexplored ecosystems conducted to identify innovative and environmentally friendly sources of medicines, agricultural chemicals, clean energy, and colors has shown the promise of cyanobacteria, or blue-green algae, being producers of these materials. Mangroves constitute an area of significant biodiversity which is severely challenged by abiotic influences and susceptible, located in the intertidal zones of tropical and subtropical climates, supporting various microorganisms. Nevertheless, compared with other prokaryotic organism there is presently a lack of information on the taxonomic categorization of cyanobacteria and the investigation of secondary metabolites that are comprised of new compounds for mangrove cyanobacteria. Comprehensive study conducted for many years has revealed that cyanobacteria, particularly those sourced from intertidal and marine habitats, are high producers of both established as well asnew bioactive molecules and natural products (NPs) of commercial

significance, with broad applicability throughout multiple industries. Despite their significance for environmental benefits as well as the industrial production of several products, there are presently very few articles on cyanobacterial NPs, particularly from mangrove and coastal environments. Only few organisms which described below have been the subject of in-depth studies on bioactive chemicals obtained from mangrove cyanobacteria. This is likely because of the disordered nature of the conventional method of screening naturally existing cyanobacterial populations in mangrove habitats for desired compounds, which does not use polyphasic taxonomy. The polyphasic characterization of cyanobacteria in mangroves improves our knowledge of the cyanobacterial diversity structure in these ecosystems, which in turn helps us to know their potential uses in various biotechnological industries.

Antimicrobial compounds from cyanobacteria

Water-soluble extracts from *Synechococcus* sp. as well as *Gloeocapsa* sp. demonstrated inhibitory effects upon the growth of clinical strains, including *Proteus vulgaris* and *Pseudomonas aeruginosa* (Anburaj *et al*. 2020). Additionally, Shishido *et al*. (2019) found that *Staphylococcus aureus* and *Candida albicans* were inhibited by methanolic intracellular extracts from various isolates of *Fischerella* sp. as well as *Aliinostoc* sp. Carmabin A, dragomabin, dragonamide A, and dragonamide B. functional linear alkynoic lipopeptides were identified from a Panamanian sample of the marine cyanobacterium *Lyngbya majuscule* and have shown a strong activity against malaria (McPhail *et al*. 2007). According to Pramanik *et al*. (2011), eight halophilic, euryhaline cyanobacteria that were isolated from the Sundarbans mangrove forest in India shown antibacterial properties.

Antitumor and anticancer activity

According to Shishido *et al*. (2019), methanol extracts of *Nodosilinea* sp., *Chlorogloea* sp., *Cyanobium* sp., *Halotia wernerae*, and *Fischerella* sp. were shown to be cytotoxic to the human leukemia MOLM-13 cell line.

Mangrove cyanobacteria as functional food

A popular cyanobacterium species called *Spirulina subsalsa* is commonly identified throughout three mangrove areas through the southeast coast of India. It is an excellent supplier of amino acids, carbohydrates, fats, and several essential vitamins and nutrients (Sakthivel *et al*. 2013). The species *Synechocystis*, which is found in Brazilian mangrove habitats, was shown to generate large quantities of fatty acids such as linoleic and linolenic acid, which have become important dietary additives (Matsudo *et al*. 2020). An acidulant and leavening agent, adipic acid is produced by a member of the *Cyanobium* genus that is found in Brazilian mangroves (Armstrong *et al*. 2019).

Mangrove cyanobacteria as a biofuel source

Oxynema, *Nostoc*, *Synechocystis*, and *Cyanobium* are genera that inhabit mangroves which have most recently been identified to generate compounds that are crucial for the production of biofuel (Armstrong *et al*. 2019). Da Ró *et al*. (2013) investigated both the amount as well as quality of five cyanobacterial strains belonging to the genera *Chlorogloea, Synechococcus*, *Leptolyngbya*, *Trichormus* and *Microcystis* as lipid source for biodiesel production. Notably, none of these strains produced toxins.

Photoprotective compounds from cyanobacteria

Cyanobacteria, particularly those inhabiting mangrove ecosystems, such as *Lyngbya* cf. *aestuarii*

and *Microcoleus chthonoplastes*, experience significant exposure to sunlight due to their geographical positioning in tropical and subtropical coastline intertidal zones. *Lyngbya* belongs to the Oscillatoriaceae family and may produce photoprotective chemicals (Karsten *et al*. 1998).

Others

Gloeocapsa sp. and *Synechococcus elongatus*, which were isolated from mangroves, efficiently eliminated cadmium and lead (Anburaj *et al*. 2017, 2020). Cyanobacteria that inhabit mangroves were studied as possible dye degrading agents; for example, a Brazilian strain belonging to the *Phormidium* genus was shown to efficiently bleach indigo blue (Silva-Stenico *et al*. 2012). It was shown that when exposed to intense light, species belonging to the genus *Synechocystis* that were obtained from the Brazilian mangrove habitat generated and accumulate enormous amounts of PHAs (Gracioso *et al*. 2021). Some of the additional benefits of mangrove cyanobacteria include: the release of indole-3-acetic acid (IAA) by mangrove-derived *Phormidium* sp. (Boopathi *et al*. 2013) along with additional metabolites which promoted healthy crop growth; and the fixation of nitrogen from the atmosphere in the soil, particularly by *Microcoleus chthonoplastes*, which increased the nitrogen level of farmland (Bashan *et al*. 1998).

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Chapter 2.
REVIEW OF LITERATURE

2.1. Traditional classification of cyanobacteria

Cyanobacteria were traditionally characterized mainly on their morphological and physiological features, categorizing them as blue-green algae because of their photosynthetic activity. The traditional approach categorizes cyanobacteria according to their cell morphology (filamentous, coccoid, or unicellular), the existence of specialized cells, such as heterocyst (for nitrogen fixation) and akinetes (resting cells), and their reproductive mechanisms, such as binary fission or fragmentation. Moreover, they were classified according to ecological aspects as well, such as the kind of habitat they lived in (marine or freshwater, for example).

The initial works on cyanobacteria, which were published by Lyngbye (1819), Agardh (1824), Kutzing (1843), and Nageli (1849), exclusively use morphological characteristics. The first complete taxonomic monographs were published later by Thuret (1875), Bornet & Flahaut (1886, 1887, 1888), and Gomont (1892). These publications were considered by phycologists to be the starting point of the classification of cyanobacteria. Following this, Geitler (1925) classified cyanobacteria into seven distinct orders upon the basis of their morphological characteristics. These orders were as follows: Pleurocapsales, Siphononematales, Stigonematales, Entophysalidales, Nostocales, Chroococcales, and Dermocarpales. Shortly thereafter, Fremy (1929) classified cyanobacteria into only three orders: Chroococcales, Hormogonales, and Chamaesiphonales. Following Fremy's classification, Geitler (1932) presented an improved systematic classification of cyanobacteria. This classification included three orders, twenty families, 145 genera, and 1300 species. The study of Geitler was entirely focused on morphological characteristics, and it is considered by phycologists and microbiologists to be one of the foundational stones of the classification system for cyanobacteria. Approximately ten years later, in 1942, Geitler reorganized his classification system by dividing cyanobacteria into four

distinct categories. These groupings were referred to as Choococcales, Hormogonales, Pleurocapsales, and Dermocarpales. Numerous taxonomists have used the classification system over an extended period of time, with some minor revisions made by Elenkin *et al*. (1938, 1949), Desikachary *et al*. (1959), Fritsch *et al*. (1945), Starmach *et al*. (1966), Bourelly *et al*. (1970), and Golubic *et al*. (1976). Consequently, all of these classification schemes used identical approaches based on conventional botanical criteria. Alternatively, a bacteriologically based taxonomy of cyanobacteria was put forward by Krumbein (1979), Rippka (1979), and Waterbury and Stainer (1977). Instead of dividing cyanobacteria into orders, Rippka (1979) classified them into five subsections. Unicellular cyanobacteria are described in Sections I and II under the names Chroococcales and Pleurocapsales, respectively. Furthermore, filamentous cyanobacteria are described in sections III, IV, and V, which are respectively referred to as Oscillatoriales, Nostocales, and Stigonematales.

2.2. Modern classification of cyanobacteria

The emergence of electron microscopy and molecular biology methods greatly reshaped the study of cyanobacterial classification. Through a set of publications, Anagnostidis and Komarek presented the very first significant modern taxonomic classification of cyanobacteria (Anagnostidis and Komarek 1985, 1988, 1990; Komarek and Anagnostidis 1986, 1989). Chroococcales, Nostocales, Stigonematales, and Oscillatoriales are the four orders that were established for the classification of cyanobacteria, which were based on that classification. The taxonomic groups Chroococcales consist of single-celled cyanobacteria, Oscillatoriales consist of filamentous non-heterocytous cyanobacteria, filamentous heterocytous cyanobacteria multiplied in a single plane are classified under Nostocales, and filamentous cyanobacteria multiplied in more than one plane are classified under Stigonematales. A modified classification scheme relying on

the polyphasic method was first presented by Hoffmann *et al*. in 2005. This classification approach provided a more precise representation of the phylogenetic affiliations among the cyanobacteria in comparison to the conventional classification systems that relied only on morphological features. The class Cyanophyceae has been partitioned into four subclasses, namely Gloeobacteriophycidae, Synechococcophycidae, Oscillatoriophycidae, and Nostochopycidae, as well as six orders, namely Gloeobacterales, Synechococcales, Pseudanabaenales, Chroococcales, Oscillatoriales, and Nostocales, according to Hoffmann *et al*. (2005). In 2014, Komárek published a new classification scheme which was also based on polyphasic analyses and made numerous modifications at the upper taxonomic level, dependent upon a variety of freshly published data. A total of six new families and two new orders were reported by Komárek *et al*. (2014). Additionally, four sub-families were promoted to the family level in terms of their taxonomic classification. The cyanobacteria were classified into eight orders by Komárek *et al*. (2014). They are the Gloeobacterales, Synechococcales, Spirulinales (new family), Chroococcales, Pleurocapsales, Chroococcidiopsidales (new family), Oscillatoriales, and Nostocales. The newly developed classification scheme discussed the problems of morphogenera, which are defined by morphological differences but lack adequate molecular data, and cryptogenera, which are characterized by morphological similarities but phylogenetically dissimilar and provided answers for the taxonomic handling of these two types of problems. Similarly, the classification method of Hoffmann *et al*. (2005) addressed problems with invalid cyanobacterial strains and strains with little or no genetic information. Additionally, Komárek *et al*. (2014) organized cyanobacteria under five distinct groups. Category I included the genera that were confirmed by phylogenetic analysis and the sequencing of the 16S rRNA gene of the type species represented like *Brasilonema*, *Coleofasciculus*, *Phormidesmis*, *Cyanothece*, *Gloeobacter*, *Microcoleus*, and *Starria*

etc. Category II consisted of genera of which the molecular information of the type species was not available so only a small number of additional species of these genera were examined using phylogenetic analysis (e.g. *Hyella*, *Schizothrix*, *Trichocoleus*, *Dermocarpella*, *Aulosira*, and *Solentia* etc). Category III referred to taxa that were investigated employing genetic techniques and were proven to be either polyphyletic or the paraphyletic like *Anabaena*, *Nodularia*, *Synechococcus*, *Trichormus*, *Calothrix*, *Nostoc*, *Synechocyctis*, and *Oscillatoria* etc. Category IV comprised of the genera that were never investigated employing DNA sequencing e.g. *Cyanosarcina*, *Geitleribactron*, *Homoeothrix*, *Leibleinia*, *Porphyrosiphon*, *Rhabdoderma*, *Asterocapsa*, and *Coelomoron*. In Category V, there were genera that were not taxonomically legitimate (*Xeronema*, *Crocosphaera*, *Thermosynechococcus* and *Euhalothece* etc). Komárek *et al*. (2014) classification system endorses the concept of gaining monophyly in multiple tiers rather than establishing large groups with weakly characterized polyphyletic genera that contained unrelated species. In comparison to previous systems, the new system more accurately portrayed the evolutionary history of cyanobacteria, which offered some hope for its eventual dominance.

In very recent years, Strunecky *et al*. (2023) reported a new classification system of cyanobacteria which is constructed on phylogenetic and phylogenomic tree. The classification is based on available genomic data and better sampled 16S rRNA data. Though, the proposed classification was based on phylogenomic data, but morphological apomorphic characters were used for correct assignments. In this classification Strunecky classified Cyanophyta into 20 orders named, Gloeobacterales, Aegeococcales, Nostocales, Pseudanabaenales, Gloeomargaritales, Acaryochloridales, Prochlorotrichales, Thermostichales, Synechococcales, Nodosilineales, Spirulinales, Leptolyngbyales, Oculatellales, Geitlerinematales, Desertifilales, Oscillatoriales, Coleofasciculales, Chroococcales, Gomontiellales, Chroococcidiopsidales.

2.3. Current status of cyanobacterial taxonomy

In recent years cyanobacterial taxonomy has undergone a rapid change. The modern polyphasic taxonomic approach now makes it possible to achieve monophyletic position, a rare feat in traditional taxonomy. Development of several databases, such as CyanoDB (CyanoDB 2), AlgaeBase (AlgaeBase : Listing the World's Algae), and Cyanoseq (Lefler *et al*. 2023), can help build a robust phylogenetic or phylogenomic tree to identify a cyanobacterial strain. Advancements in various techniques, such as sequencing platforms and metabolomics techniques, open up new ways to carry out cyanobacterial taxonomy. Whole genome sequencing will be a game changer in the upcoming days. Whole genome analysis can decipher many evolutionary questions that arise in cyanobacterial taxonomy, like cryptic taxa and morphotaxa (Devorak *et al*. 2023). While whole genome analysis alone may not be sufficient to bridge the gaps in cyanobacterial taxonomy, it can address several taxonomic gaps. Using LC-MS and GC-MS, cyanometabolite profiling can solve the ecological plasticity of different cyanobacterial taxa.

2.4. Problems in cyanobacterial taxonomy

Incongruence between molecular and morphological-ultrastructural data

Cyanobacteria were traditionally categorized according to their external and ecological characteristics. Based on the findings of a molecular investigation, it was discovered that some features are required, while others are the result of a choice. It is often difficult to classify cyanobacteria because of their changeable nature, which may lead to confusion. Certain species of the group Microcyctaceae and Aphanizomenonaceae and maybe even each member of the family can possess genes that are associated with gas vesicles or aerotopes. During the whole period of their vegetative life, each member of the family displayed gas vesicles and aerotopes. However,

Komarek (2016) noted that sometimes, individuals of the Scytonemataceae family display aerotopes even though they do not normally possess these genes.

Cryptotaxa, morphotaxa and ecotaxa

A wide variety of adaptive strategies may be possessed by cyanobacteria due to their extensive evolutionary record. They have the potential of altering the biochemical properties as well as cell architecture of their cells with response to their surroundings wherever they exist (Stanier, 1978). According to Herrero *et al*. (2001), the potential of such cells undergoes morphological changes, which allows individuals to develop into specialized cells such as heterocytes, permits individuals to grow and thrive in a broad variety of situations. These factors account behind the phylogenetically unique positions of several cyanobacterial taxa, despite morphological similarity or near indistinguishability. Cryptotaxa are the terms that are used to describe these kinds of cyanobacterial strains (Komarek *et al*. 2016). Although no principles or guidelines for identifying cryptotaxa have been developed, but electron microscopy data or physiological information may be employed for taxonomic classification. Unlike cryptotaxa, strains in the morphotaxa and ecotaxa situation vary significantly in their morphological and physiological characteristics, even if they are phylogenetically found within an identical lineage. In this instance, although displaying comparable genetic characteristics, the isolates need to be recognized as distinct taxa.

Nomenclatural types

The contemporary cyanobacterial identification is mostly created on the 16S rRNA phylogeny. Nonetheless, the molecular data of a variety of isolates are occasionally presented incorrectly or without a correct set of nomenclatural types (Komarek *et al*. 2018). As a result, issues occur while constructing the phylogenetic tree. It is considered to be polyphyletic in nature when a single taxon

is seen at many places on a phylogenetic tree. Cyanobacteria that have been described are required to provide a correct nomenclatural type alongside a name that is distinct in order to continue a monophyletic classification.

Nomenclature rules

The "Cyanophytes" had been categorized as "blue-green algae" according by the regulations of the International Code of Nomenclature for algae, fungi, and plants (ICN; previously ICBN). This classification was based solely on their phenotypic variety and shape in comparison with comparable microalgae, while also considering how they function in the surrounding natural ecosystems. As a result, they were considered to be tiny plant as cryptogams for a considerable amount of time, which led to their inclusion as a subject of study among ecological scientists and botanists specially by phycologists. The reclassification of cyanobacteria by microbiologists as bacteria was initiated by developments in molecular biology, including the identification of these organisms as prokaryotes by technologies like 16S rRNA sequencing. Because of this, cyanobacteria started to be defined according to the International Code of Nomenclature of Prokaryotes (ICNP) beginning during the end of the 1970s and continuing into the beginning of 1980s (Stanier 1977, 1978). There are now cyanobacterial classification systems incorporating both plant and bacterial codes (Oren and Ventura 2017; Oren, 2014; Oren & Garrity, 2014). The presence of two separate rules, namely the Botanical Code (which has been in effect since 2012 and is known as the International Code of Nomenclature for algae, fungi, and plants; Oren, 2014) and the Bacteriological Code, both of which are applicable to an identical particular group of organisms creates a significant dilemma. Regulations of the Botanical Code and the Bacteriological Code are extremely divergent to one another, it is rather difficult to bring together the naming schemes used in the botanical and bacteriological fields.

When examining the phylogenetics using 16S rRNA gene or different genes, the polyphasic method may be employed along with with the strictly monophyletic speciation principle. A species is defined as the most compact distinct group that has distinct autapomorphy, describing a characteristic found to be exclusive of an assigned taxonomic category (Johansen and Casamatta 2005). In addition, authors developed an actual species description considered acceptable to cyanobacteria that can potentially be utilized in accordance with the Botanical Code. This definition of cyanobacteria is likely the most universally acknowledged as evidenced by the volume of taxonomic literature adhering to the Botanical Code, albeit occasionally with imprecision (Siegesmund *et al*. 2008; Perkerson *et al*. 2011; Dvořák *et al*. 2014; Hašler *et al*. 2012, 2014 and Osorio-Santos *et al*. 2014). The main benefit regarding the monophyletic individual idea is its broad application towards asexual organisms. Furthermore, monophyletic organism idea is not acknowledged by the Bacteriological code. The ICN principles serves as the fundamental framework for phycological analysis involving populations of natural origin, having preserved-type samples serving for taxonomic standards and implementing the principle of priority for nomenclature. Conversely, microbiologists utilize pure cultures for the fundamental taxonomic unit (Palinska *et al*. 2014). However, it is not feasible to execute a comprehensive evaluation of every cyanobacterial variety that occurs in environment, especially at a level of species, since the guidelines for the bacteriological regulations prohibit such a study. The botanical method states that species names are legitimate for publication in any scientific journal and that the body of literature on botanical nomenclature is dispersed broadly. The material that has to be reviewed to determine whether or not the species seems new to science goes back more than century. Conversely, to report a novel bacterium species IJSM/IJSB is the only journal (Palinska *et al*. 2014).

Polyphyletic orders

According to Komarek *et al*. (2014), cyanobacteria were classified under eight orders through the polyphasic taxonomic approach: Gloeobacterales, Pleurocapsales, Nostocales, Spirulinales, Oscillatoriales, Chroococcidiopsidales, and Chroococcales. Having eleven families including single-celled as well as filamentous cyanobacteria, Synechococcales constitutes one of especially troublesome order within all. Leptolyngbyaceae was one among of the families that has undergone multiple revisions because of their existence of an abundance of polyphyletic genera whose characteristics are visually indistinguishable from one another. Since *Leptolyngbya boryana* was chosen being the type taxa for this family, a number of genera were formed within the family. These genera include *Nodosilinea*, *Elainella*, *Plectolyngbya*, *Stenomitos*, *Halomicronema*, *Prochlorothrix*, *Phormidesmis*, *Kovacikia*, *Picocchia*, *Timaviella*, *Trichocoleus*, *Thermoleptolyngbya*, and many others. Although the revision efforts have been implemented, the family continues to need substantial taxonomic amendment to achieve monophyly. Utilizing the polyphasic taxonomic strategy, Mai *et al*. (2018) conducted extensive family level research to separate the different genus of the Leptolyngbyaceae between four families.

2.5. Cyanobacteria from mangrove ecosystem

The mangrove environment, which lies between the ocean and coastline, represents one amongst the most distinctive habitats on the planet. Because of their location on the land-sea border, mangrove forests experience constant changes in daylight, temperature, gaseous availability, saltiness, tidewater patterns, and moisture (Neogi *et al*. 2016). Very few organisms have evolved to thrive in this harsh environment caused by constantly changing weather conditions. Salinity, a key component of estuarine ecosystems, changes substantially with the course of time, as well as with changes in site and the strength of the tides. The cyanobacterial populations in mangrove

ecosystems are also affected by this water saltiness gradient. As a result, salinity, pH, temperature, as well as sunlight all affect mangrove cyanobacteria, that could result in the development of novel strains that are better able to survive and the formation of newer cyanobacterial species (Komarek and Anagnostidis 2005). Compared to studies conducted in various ecosystems, taxonomic evaluation of cyanobacterial biodiversity in estuaries has the potential to uncover a higher number of new genus and species (Alvarenga, 2015). In tropical and subtropical areas, mangroves grow along the coasts of 112 nations. Regardless, research on the variety of cyanobacteria which dwell in mangroves has only been reported from a small number of regions in India, Saudi Arabia as well as Brazil, while globally mangrove ecosystem covers a huge span of land.

A comprehensive review that Alvarenga (2015) published was centred on the variety of cyanobacteria that are found in mangrove ecosystems. The article provides a comprehensive documentation of 277 cyanobacterial taxa that have been discovered across multiple mangrove habitats throughout the world. It highlights the ecological importance of these organisms, as well as their distribution throughout the world and their possible uses in biotechnology. In addition to highlighting the global significance on cyanobacteria within estuarine ecosystems, this article provides an invaluable resource for gaining knowledge of the essential function that cyanobacteria play throughout mangrove ecosystems. Despite the existence of numerous reports pertaining to cyanobacteria found in mangrove ecosystems, little research has been carried out on these organisms using the current polyphasic taxonomic technique.

According to Ellison *et al*. (2001), the Sundarbans are the biggest estuarine mangrove forest of the globe and a significant estuarine habitat in Asia. A process of alpha-level systematic identification has recently initiated for the cyanobacterial variety throughout this region. There have been reports of new cyanobacterial genera as well as species that belong to different families

and orders, which suggests that this estuarine habitat has the potential for identification of additional novel cyanobacteria. The following table contains a tabulation of every genus and species of cyanobacteria that has been reported from Indian Sundarbans (Table 2.1).

Table 2.1. Cyanobacteria isolated from Indian Sundarbans and classified using modern polyphasic taxonomic approach.

No	Genus	Species	Family	Order	References
1	*Leptolyngbya*	*Indica*	Leptolyngbyaceae	Synechococcales	Debnath (2017)
2	*Oxynema*	*aestuarii*	Microcoleaceae	Oscillatoriales	Chakraborty (2018)
3	*Euryhalinema*	*mangrovii*	Nodosilineaceae	Nodosilineales	Chakraborty (2019)
4	*Leptoelongatus*	*litoralis*	Leptolyngbyaceae	Leptolyngbyales	Chakraborty (2019)
5	*Synechococcus*	*moorigange*	Synechococcaceaae	Chroococcales	Singh (2019)
6	*Euryhalinema*	*pallustre*	Nodosilineaceae	Nodosilineales	Chakraborty (2021)
7	*Aerofilum*	*fasciculatum*	Oculatellaceae	Synechococcales	Chakraborty (2021)

2.6. Advantage and applications of mangrove cyanobacteria

Marine cyanobacteria may be found in a variety of environments along the coastline and in the ocean. According to Kreitlow *et al*. (1999) and Biondi *et al*. (2008), marine cyanobacteria are the most abundant producer of biologically therapeutic compounds that are used extensively in toxicological and pharmacological fields, including antimicrobial, antiviral, antifungal, and antitumor. Several important factors make marine and mangrove cyanobacteria biotechnologically more effective compared to freshwater cyanobacteria. Those are as follows:

- The environmental conditions found in mangroves are very diverse and often harsh (e.g., excessive salinity, poor nutrients, and considerable UV radiation). Cyanobacteria from

these habitats are a treasure trove of bioactive chemicals due to their distinct metabolic capacities that have developed for survival.

- The distinctive genes and metabolic processes seen in mangrove cyanobacteria are the result of natural selection processes that have produced distinct genotypes.
- Because they can thrive in saltwater rather than freshwater, mangrove cyanobacteria are more cost-effective and eco-friendly options for producing biofuel and bioethanol.
- Marine cyanobacteria such as *Prochlorococcus* and *Synechococcus* make significant contributions to world carbon sequestration and may be modified to improve carbon sequestration technology.

Mangrove cyanobacteria have been identified as suppliers of many different bioactive molecules, leading to their utilization as a source of diverse bioactive substances in pharmaceutical companies. Mangrove cyanobacteria generate a variety of antibiotic, anticancer, and photoprotective compounds that may be used in a variety of therapeutic uses (Nandagopal *et al*. 2021). Mangrove cyanobacteria have potential applications in food processing for the production of organic dyes as well as superfoods due to their rich content of beneficial substances. Salinity stress represents an environmental variable which may be effectively controlled in the cultivation of microalgae to promote the formation of lipids. Numerous findings previously shown the efficacy of inducing salt stress on varied microalgae genera, for example freshwater genus such as *Chlorella* sp. and oceanic ones like *Nannochloropsis* sp., *Tetraselmis* sp., and *Dunaliella* sp. (Falfushynska *et al*. 2024). As a result of their preexisting presence in saltwater conditions, mangrove cyanobacteria are capable of withstanding extreme salinity stress to generate oils for the manufacture of biodiesel in various biofuel sectors.

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Chapter 3.

AIMS AND OBJECTIVES

3.1. Aims of the study

In accordance with the fossil evidence, the initial evolution and diversification of cyanobacteria occurred in estuarine and intertidal ecosystems (Demoulin *et al*. 2019). The ancient records of cyanobacteria are more common in estuaries and shallow marine locations than on terrestrial environments. Sundarbans, the world's most significant estuarine mangrove ecosystem, is located on the estuary of the Indian river Ganga, Brahmaputra, and Bangladeshi river Meghna (Ellison *et al*. 2001). The changing conditions in estuaries, caused by tidal flow, create new biologically active compounds, and help cyanobacteria separate into several distinct species. The emergence of new species is significantly influenced by external factors (Hoffmann *et al*. 2020). Latest studies have shown the existence of new cyanobacteria in mangrove ecosystems worldwide (Alvarenga *et al*. 2015). Since the cyanobacterial population of the Sundarbans has not been thoroughly investigated, there might be a possibility that the region is habitat to cyanobacteria that have not been documented before (Pramanik *et al*. 2011). In light of this scenario, the following are the particular aims of these research:

- **Evaluation of the alpha-diversity of cyanobacteria of the Indian Sundarbans following the modern polyphasic taxonomic methodology**
- **Establishing monophyletic taxonomy of cyanobacteria and revising the classification of simple filamentous cyanobacteria.**
- **Extraction and characterization of bioactive compounds from the identified strains for biotechnological applications.**

3.2. Objectives of the study

To accomplish the aforementioned aims, this research proposes to achieve these particular objectives:

I. Collection of samples from various parts of the Indian Sundarbans.

II. Purification, development of axenic cyanobacterial strains, maintaining under laboratory conditions, and placing of the holotypes in a publicly available culture collection.

III. Characterization of all strains utilizing the modern polyphasic taxonomic approach.

IV. Appropriate description of each isolate based on its apomorphic features according to the principles established by ICN.

V. Comprehending the broad evolutionary links between all the strains.

VI. Record the complete genome information of the strains that were studied for future investigations

VII. Examining the presence of anti-ultraviolet compounds in the identified cyanobacterial strains.

VIII. Lipid extraction from selected strains for use as biodiesel feedstock.

3.3. References

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Chapter 4.

MATERIALS AND METHODS

4.1. Study area and sample collection

The cyanobacterial samples utilized in this investigation were collected from distinct locations of the Indian Sundarbans (Fig. 4.1). All the samples were collected during low tide in November 2018. Green or brownish-green coloured phototrophic biofilms were collected from the upper surface of the soil using a trowel. They were transferred into plastic sample collection bags and carried to the laboratory for further investigation.

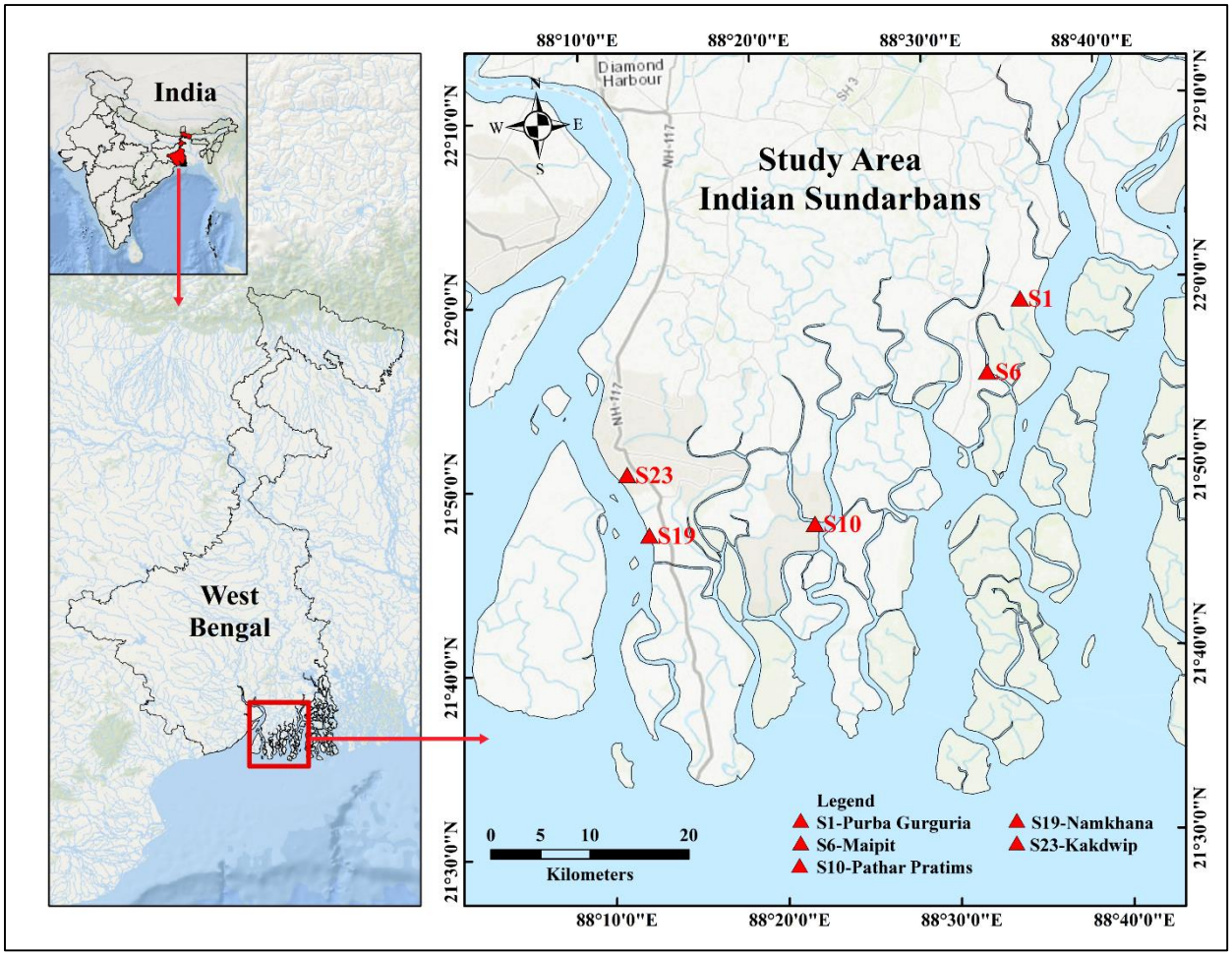

FIGURE 4.1. Map (drawn in ArcGIS 10.8) showing the different sample collection areas in the Indian Sundarbans in West Bengal, India.

The sampling sites were as follows:

a. Six different locations of Purba Gurguria (21°59.16′N, 88°35.08′E), West Bengal, India.

b. Three locations of Maipit (21°55.28′N, 88°32.93′E) of the Indian Sundarbans, West Bengal, India.
c. Five locations of Pathar Pratima island (21°47.47′N, 88°22.524′E) of the Sundarbans mangrove forest, West Bengal, India.
d. Six locations of Namkhana, West Bengal, India.
e. Five locations of Kakdwip, West Bengal, India.

4.2. Sample processing and isolation of the cyanobacterial strains

A small portion of the biofilm samples (about 1.0 g) was put into a 100ml Erlenmeyer flask containing 30ml of ASN-III solution media (Rippka *et al*. 1979). The bottles were lightened for 12h a day using white tube light under room temperature (Chakraborty *et al*. 2019). Afterwards, tenfold dilutions of these cultures were made. Later, a small portion of diluted sample was inoculated again into 30ml of ASN-III medium and kept undisturbed under illumination. After 30 days sufficient growth was observed and the greenish biofilms was checked using the light microscope to confirm the abundance of filamentous cyanobacteria.

4.3. Purification of the cyanobacterial strains

Cyanobacteria cultures were seen mixed with other phototrophs as well as heterotrophs under light microscopes. To obtain the pure, axenic filamentous cyanobacterial strain, several strategies taken. In the first step, to prevent eukaryotic growth, 1.0 mL cycloheximide from a stock solution (50 $mg\cdot L^{-1}$ in methanol at 75%) was added to 1000 mL of ASN-III liquid medium (Urmeneta *et al*. 2003) and to prevent the growth of other heterotrophs, a triple antibiotic solution was used. 100 mg of penicillin G (Na salt), 50 mg of streptomycin sulphate dissolved in 10 mL distilled water, and 10 mg of chloramphenicol dissolved in 1 ml of 95% ethanol were mixed and filtered by syringe

filtration (Acrodisc syringe filter; Pall Corporation, NY, USA) following Guillard (2005). A small amount of biofilm, about 1 ml was added to 100ml Erlenmeyer flask containing 30 ml of ASN-III media, to which separate amounts (0.125, 0.250, 0.500, 1.0, 1.5, 2.0, 2.5, 3.0 ml) of the triple antibiotic mixture supplemented and were inoculated for 48 to 72 hrs to achieve the ideal level that permissible unicyanobacterial growth (Pramanik *et al*.2011).

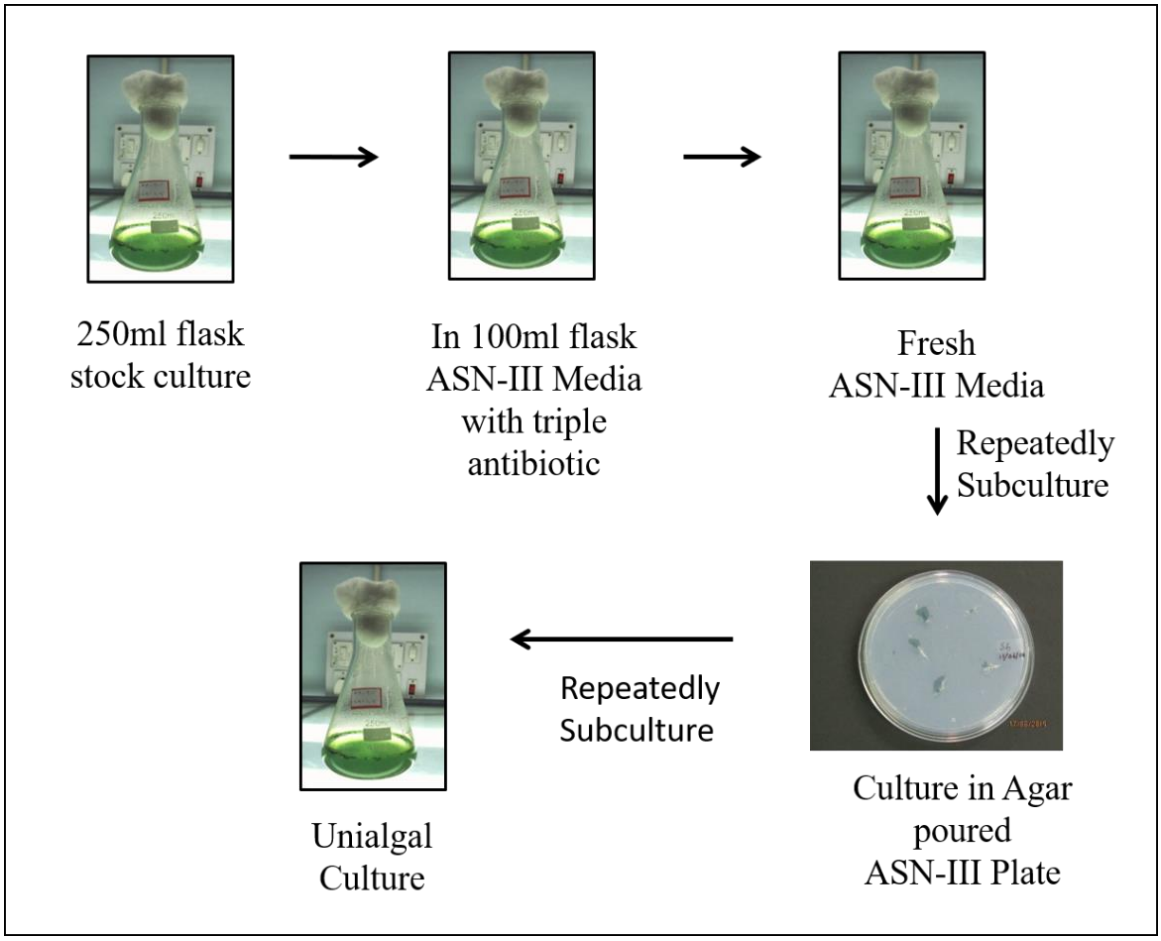

FIGURE 4.2. Illustrative depiction outlining the purification procedures for the unialgal culture employed in the current study.

After incubation, 500 µl of the cyanobacterial cell suspension was inoculated into fresh 50 ml ASN-III medium and raised for 25-30 days. The pureness of the cultures was checked by the lack

of microbial growth in tryptone-yeast-glucose (TYG) broth (Vaara *et al*. 1979) after incubation for 7 days at 25 ± 1°C and 37 ± 2°C. The pureness was again further confirmed by the absence of bacterial growth in ASN-III medium supplemented with 5.0 gL^{-1} glucose and 0.5 gL^{-1} yeast extract, incubated in the dark for 5 days at 25 ± 1°C and 37 ± 2°C. The flask that exhibited

FIGURE 4.3. Image showing the isolated unialgal stock cultures of this study, maintained under controlled laboratory conditions.

healthy cyanobacterial growth and no bacterial impurity was present afterward chosen for further experiments. The second step of isolation was done by repeated transfers between liquid culture and solid agar plate culture (Fig. 4.2). The method is called spread plate method. A small amount of cyanobacterial biomass was spread on agar solidified ASN-III medium petri plate. Alternating transfers between solid and liquid media were carried out until uni-cyanobacterial strains were

obtained. It is achievable to lift up single monofilaments of cyanobacteria from solid agar plate culture using inoculating loop and then transferring them into a 100 ml Erlenmeyer flask having 30 ml of ASN-III media (Fig. 4.3). After 7-10 days, axenic cyanobacterial strains were obtained from the flask by confirming under microscopic analysis.

4.4. Maintenance of the cyanobacterial culture

Following the successful purification of the cyanobacterial cultures, five strains namely S1, S6, S10, S19 and S23 (Table 4.1) were obtained for further maintenance and future taxonomic study.

Table 4.1. List of the strains that were purified and chosen for taxonomic assessment, along with the accession numbers that were obtained by submitting specimens to the National Centre for Microbial Resource (NCMR), Pune, India.

No.	Sample name	Collection location	Culture centre accession number
1	S1	Purba Gurguria	MCC5400
2	S6	Maipit	MCC5313
3	S10	Pathar Pratima	MCC5312
4	S19	Namkhana	MCC5402
5	S23	Kakdip	MCC5403

4.4.1. Parent cultures

The purified axenic cyanobacterial cultures were retained in 250 ml Erlenmeyer bottles, each having 100 ml of ASN-III liquid medium. The flasks were illuminated by fluorescent lamps for 12 hours daily (50 µmol photons $m^{-2}s^{-1}$) incubated at room temperature (Chakraborty *et al*.2018).

These parent cyanobacterial cultures were subcultured every 30 days of intervals. Bacterial contamination was monitored using TYG broth after 60 days of interval.

4.4.2. Cryopreservation

Maintaining cyanobacterial pure culture for long-term purposes, cryopreservation is among the most efficient approaches, as defined by Rastoll *et al*. (2013). Following Esteves-Ferreira *et al*. (2013) with slight modification, the cyanobacterial biofilms were lightly homogenized and centrifuged at 15000 ×g for 10 minutes. After that, supernatant was discarded, and the pellet was rinsed with distilled water until the residual media was removed. This procedure was repeated 2-3 times. The final cyanobacterial pellet was resuspended in fresh ASN-III medium. Then, dimethyl sulfoxide (DMSO) and glycerol were added to the medium at 5% and 10% v/v concentrations, respectively, as cryoprotective agents. The cryovials containing cyanobacteria strains were vortexed to uniformly spread the cryoprotectant to all the filaments of cyanobacteria and were left undisturbed under low light at room temperature for 15 mins to ensure adequate penetration. Freezing was performed in triplicate by gradually exposing the cryovials to different temperature. The vials were first cooled from room temperature to 4℃, then 4℃ to -20℃ and finally from -20℃ to -80℃. The freezing rate was maintained at 1℃ per minute up to 80℃. Cultures were revived by gradually increasing the exposure temperature to 30 ℃ until the ice crystal had melted. After that, the cryovials were centrifuged at 10000 ×g for 10 mins then the supernatant was removed. The pellets were rinsed twice with distilled water and resuspended in fresh cryoprotectant-free ASN-III medium. The cyanobacterial cultures were illuminated under fluorescent lamps with a 12hrs: 12 hrs. light and dark period under specific lab conditions (Chakraborty *et al*. 2018).

4.4.3. Culture collection deposition

After obtaining the five cyanobacterial pure culture, they were deposited into an IDA-approved culture collection centre, the National Centre for Microbial Resource (NCMR) in Pune, India. After rechecking the purity and thorough evaluation, accession numbers and certificates were provided for each of the submitted cyanobacterial cultures (Table 4.1).

4.5. Morphological analysis

The morphological features of the five isolated strains were examined by two methods. They were as follows:

4.5.1. Light Microscopy

The morphological features of all the purified strains were examined using a light microscope. A small amount of 8–10 day-old cyanobacterial isolates was positioned on a glass slide and temporarily mounted by cover slip aseptically. The slides were then observed under 400-1000X magnification using DM750 light microscope (Leica Microsystems, Buffalo Grove, Illinois, USA). Images of the five cyanobacterial strains were captured using an ICC5HD camera attached to the microscope. Morphological characteristics such as trichome length, branching pattern, apical cell shape, cellular dimensions, mucilaginous sheaths, motility, and other phenotypic traits were recorded using microscope associated software (LAS-EZ, Leica Microsystems).

4.5.2. Scanning electron microscopy

SEM was employed to achieve a clearer resolution and detailed study of cell surface features. Fresh 7-day old cyanobacterial filaments were taken in a centrifuge tube and centrifuged at 8000×g for 10 mins (Eppendorf 5810R, Rotor F-34-6-38, Hamburg, Germany). The supernatant was

discarded and the cell pellet was rinsed by 0.1 M phosphate buffer solution (pH 7.8) by vortexing. The cell suspension was then centrifuged again at 8000×g for 10 min, and the pellet was rinsed with distilled water by vortexing and pipetting. For fixing the cells, 3% glutaraldehyde was used as fixative in 0.1 M phosphate buffer saline. The biomass was incubated for 16 hours at room temperature, followed by washing with distilled water. Cyanobacterial cells were dehydrated by passing them through different gradients of ethanol, starting from 30% for15 mins and increasing to 100% for 60 minutes (Roy *et al*. 2024). After dehydration, the samples were placed on the glass grids and subjected to critical point drying. Then the specimen-holding grids were taken for observation under a scanning electron microscope (ZEISS EVO 18, Carl Zeiss AG, Oberkochen, Germany).

4.6. Ultrastructural analysis

Ultrastructural analysis was used for critical evaluation of synapomorphic characters of cyanobacterial strains for species level identification. This was analysed using transmission electron microscopy.

4.6.1. Transmission electron microscopy

Fresh 7-day old cyanobacteria culture was taken in a centrifuge tube and centrifuged at 8000 ×g for 5 minutes. The supernatant was gently removed, and the pellet was cleansed with 0.1 M phosphate buffer (pH 7.8) using a vortex. The cell suspension was then centrifuged again at 8000 ×g for 5 minutes, and the supernatant was removed. The pellet was washed with distilled water. Pre-fixation was done using 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.8). The cells were incubated at 4 ℃ for 5-6 hrs. and rinsed with 0.1 M phosphate buffer (pH 7.8) to wash off the extra fixative. Post-fixation of the samples was performed using osmium tetroxide (1%) for 60 minutes

(Roy *et al*. 2024). The cells were dehydrated by gradually increasing the concentration of ethanol, starting from 30% for 15 mins and increasing to 100% for 60 minutes. After dehydration, the cells were infiltrated and inserted in Araldite CY212 CY 212 (Agar Scientific, Stansted, UK) for section cutting. The resin chunks underwent polymerization through heat treatment, initially at 50 ℃ overnight and subsequently at 60 ℃ for 2 days. Following polymerization, the blocks were thin-sectioned. The sections were contrasted with uranyl acetate and lead citrate and viewed using a Tecnai G20 transmission electron microscope (FEI, Eindhoven, Netherlands).

4.7. Molecular analysis

4.7.1. Genomic DNA extraction

Genomic DNA was isolated from 8-10 days old axenic cyanobacterial isolates. About 1 ml of the axenic unicyanobacterial culture was taken, and the cells were homogenized using an autoclaved glass homogenizer. The homogenized cell suspension was used for further experimentation. GeneJET^{TM} Genomic DNA Purification Kit (Catalog number K0721, Thermo Fisher Scientific, Waltham, Massachusetts, USA) and NucleoSpin Microbial DNA Kit (REF Code 740235.50, MACHEREY-NAGEL, Düren Germany) were used for genomic DNA extraction following the manufacturers' protocols. The pureness of the extracted genomic DNA was verified by running a gel electrophoresis in 1.5% agarose gel. The extracted genomic DNA of all the strains was stored at -20 ℃ for future experiments.

4.7.2. PCR amplification of 16S rRNA and 16S-23S ITS genes

PCR amplification experiments were achieved in a Master Cycler Nexus Gradient PCR (Eppendrof, Hamburg, Germany) to amplify the 16S rRNA and 16S-23S ITS genes. The following primers are employed to amplify the marker genes (Table 4.2):

Table 4.2. List of PCR Primers used during the present investigation.

Primer Direction	Primer Sequence	Reference	Marker gene
Forward	CYA106F (5′-CGGACGGGTGAGTAACGCGTGA-3′)	(Nübel *et al*. 1997)	16S rRNA
Reverse	1492R (5′-ACCTTGTTACGACTT-3′)	(Lane *et al*. 1991)	
Forward	16SF (5′-TGTACACACCGGCCCGTC-3′)	(Iteman *et al*. 2000)	16S-23S ITS
Reverse	23SR (5′-CTCTGTGCCTAGGTATCC-3′)	(Iteman *et al*. 2000)	

Each PCR master mixture contains 25 µl of reaction mixture. The final concentration of the components used in the PCR master mixture contains 2.5 µl of 10X buffer excluding $MgCl_2$, 0.5 µl of mM $MgCl_2$, 0.5 µl of 10mM dNTPs mix, 0.5 µl of 10 pmol of each forward and reverse primer, 2 µl of template DNA in 18 µl of RNase-free water (Catalog No. 10977-015, Invitrogen), and after mixing, finally 0.5 µl of 5U/l Taq DNA polymerase (Catalog No. 10342-053, Invitrogen, Waltham, Massachusetts, USA) was added. The PCR tubes were gently tapped 2-4 times before being placed in the PCR machine. The PCR reaction for the 16S rRNA gene was carried out as follows: primary denaturation at 94 ℃ for 5 minutes, 30 cycles (denaturation at 94 ℃ for 1-minute, primer annealing at 50 ℃ for 1 minute, extension at 72 ℃ for 2 minutes) and finishing extension at 72 for 10 minutes. For the amplification of the 16S-23S ITS region, initial denaturation was at 95 ℃ for 5 minutes, followed by 30 cycles (denaturation at 95 ℃ for 30 seconds, primer annealing at 58 ℃ for 15 seconds, extension at 72 ℃ for 40 seconds) and final extension at 72 ℃ for 5 minutes. Three microliters of amplified PCR products were imagined by running in 1.5% agarose gel electrophoresis with 1 µl of 6X loading buffer. In order to estimate the amplicon's size, a 5 Kb ladder of DNA was added to the gel as a reference. Moreover, after the after completion of gel

electrophoresis, the gel was stained with ETBR and the bands were visualized under UV light. After confirmation, all the amplified PCR products were subjected to purification.

4.7.3. Purification of amplified PCR products

After confirming the PCR products with agarose gel electrophoresis, the rest of the amplified PCR products were filtered using the Gene JET Gel extraction kit (Catalogue No. K0691, Thermo Scientific). In brief, approximately 20 µl of PCR amplified products were loaded on 1.5% agarose gel with 5 µl of 6X loading buffer dissolved in 1X TBE buffer. To determine the size of the amplicon, 2 µl of a reference 5Kb ladder was added to the gel. The selected bands were cut out using a sterile blade and promptly placed into an equal amount of binding buffer as per the manufacturer's protocol. Then the gel mixture was incubated at 60 ℃ for 10 minutes. After that, the mixture was transferred to the GeneJET purification column, and followed by two successive washing steps. At the end the PCR product was eluted with 50 µl of distilled water.

4.7.4. Cloning of the amplified PCR products and Sanger sequencing

The purified PCR products of 16S rRNA and 16-23S ITS regions were cloned using pGEM®-T Easy Vector plasmid and pJET 1.2/blunt cloning vector plasmid, which were provided by pGEM®-T Easy Vector System I (Cat No. A1360, Promega Corp., Madison, Wisconsin, USA) and Clone JET PCR Cloning Kit (REF No. K1231, Thermo Fisher Scientific, Waltham, MA, USA) cloning kits, respectively. JM109 competent cells were used for transformation using the heat shock method. After that, 100µl of each transformation culture was spread onto a duplicate set of LB-Agar-Amp-IPTG-Xgal plates. After the overnight incubation at 37 ℃, recombinant colonies were selected using the blue-white selection method. The white colonies were picked by sterile toothpicks aseptically and inoculated in a fresh liquid nutrient broth added with ampicillin. After

that, plasmids were isolated from the liquid culture using the GeneJET Plasmid Miniprep Kit (Cat No. K0502, Thermo Fisher Scientific). Recombinant plasmids were digested using the Not I restriction enzyme (Cat No. ER0591, Thermo Fisher Scientific), and the insert size was confirmed using 1.5% agarose gel electrophoresis. Purified recombinant plasmids were used for 16S and 16S-23S ITS gene sequencing. The mentioned genes were sequenced using the primers pUC/M13 forward (5′-CGCCAGGGTTTTCCCAG TCACGAC-3′) and pUC/M13 reverse (5′-CAGGAAAC AGCTATGAC-3′). The automated Genetic Analyzer 3730Xl DNA sequencer (Applied Biosystems, Waltham, Massachusetts, USA) was utilized to perform the sequencing process. In the case of cloning by using the Clone JET PCR Cloning Kit, there was no requirement for blue-white screening. The recombinant white colonies were taken for plasmid DNA isolation. Following that, recombinant plasmids were digested using Fast Digest BglII (Cat No. FD0084, Thermo Fisher Scientific, Waltham, MA, USA), and 1.5% agarose gel electrophoresis were carried out to confirm the desired size. Recombinant plasmids were used for sequencing using forward sequencing primer (5'-CGACTCACTATAGGGAGAGCGGC-3') and pJET1.2 reverse sequencing primer (5'-AAGAACATCGATTTTCCATGGCAG-3'). The sequencing procedure was achieved utilizing a programmed Genetic Analyzer DNA sequencer (Applied Biosystems, Waltham, Massachusetts, USA).

4.8. GenBank submission

After obtaining the forward and reverse primer sequences, the chromatograms of the sequences were checked visually. Then the sequences were evaluated against one another by generating the “reverse” sequence of the reverse complement employing BioEdit v7.0.5.3 (Hall *et al*. 1999). Contigs were generated by using ClustalW to align the sequences from the 5’ end sequencing reaction. The generated consensus sequences were checked in NCBI BLAST to confirm their

cyanobacterial origin. So, all the sequences of pure cyanobacterial strains named S1, S6, S10, S19, and S23 were submitted to NCBI GenBank before their phylogenetic tree analysis. The accession numbers of all the sequences were as follows in the table (Table 4.3).

Table 4.3. A list of accession numbers of 16S and 16S-23S ITS gene sequences received from NCBI GenBank, along with corresponding strains utilized in this study.

No	Strain	16S rRNA	16S-23S ITS
1	S1	OP740688	OP740693
2	S6	OP740689	OP740694
3	S10	OP740690	OP740695
4	S19	OP740691	OP740696
5	S23	OP740692	OP740697

4.9. Phylogenetic analysis

Each of the 16S RNA gene sequences of all the isolated strains, named S1, S6, S10, S19, and S23, was examined using the Basic Local Alignment Search Tool (BLAST) and contrasted with other 16S rRNA sequences accessible in the extensive National Center for Biotechnology Information database (NCBI GenBank). For each of the five strains, the sequences corresponding to the closest hit taxon were retrieved from the NCBI database. Many sequences that were closest to the strains under consideration were chosen for phylogenetic tree construction based on the sequence similarity values related to the other genera and species available in the database. Furthermore, additional sequences have been considered to ensure robust phylogenetic tree construction. The methods for constructing the phylogenetic tree were divided into two separate subsections

considering the five strains that were investigated: group (i) S1, S6, and S10 and group (ii) S19 and S23.

4.9.1. Phylogenetic tree for strain S1, S6 and S10

About 146 OTUs were used to construct a 16S rRNA phylogenetic tree, with *Gloeobacter violaceus* as the outgroup. Families such as Leptolyngbyaceae, Prochlorotrichaceae, Nodosilineaceae, and Oculatellaceae contributed to the additional 16S rRNA sequences of numerous newly described genera. All the OTUs were then used to make a multiple sequence alignment (MSA) utilizing Clustal W software (Larkin *et al*. 2007). By employing a highest of 1,789 characters, containing nucleotides and gaps, the MSA of the 16S rRNA genes of the 146 OTUs was subjected to Bayesian Inference (BI), Maximum Likelihood (ML), and Maximum Parsimony (MP) analysis. According to Posada *et al*. (2008), the model test was conducted using jModel Test 0.1.1. GTR+I+G was the best-fit model applied to ML and BI analysis. PhyML 3.0 (Guindon *et al*. 2010), W-IQ-TREE webserver (Trifinopoulos et al. 2016), and RAxML T-REX (Boc *et al*. 2012) were used to create the ML tree with 1000 replications. To calculate the relative support of the branches, an MP tree with 1,000 replications was created on the MEGA program package v6.0 (Tamura *et al*. 2013). The multiple sequence alignment was uploaded to MrBayes v3.2.7 (Ronquist *et al*. 2012) for a BI analysis using the GTR+G+I model of nucleotide substitutions. Two separate runs, each consisting of four chains, were carried out in parallel for the 50 million Markov Chain Monte Carlo (MCMC) generation. The first 25% of samples were removed as burn-in. Split frequencies of the final average standard deviation were 0.00119 less than 1. The statistical accomplishment of the convergence of the MCMC chains was indicated by the potential scale reduction factor (PSRF) value of 1.00 for all parameters (Gelman & Rubin 1992). Moreover, two runs of four Markov chains were used for the BI analysis on the

NGPhylogeny.fr website (Lemoine *et al*. 2019). Each run comprised of 50 million generations. The iTOL website presented the resulting tree (Letunic *et al*. 2006). Using Adobe Illustrator, the bootstrap values for the ML and MP analyses were plotted onto the BI analysis tree.

4.9.2. Phylogenetic tree for strain S19 and S23

The 16S rRNA phylogenetic tree of S19 and S23 was constructed using the methodology described in section 4.10.1. Due to the strains belonging to a distinct genus, certain modifications were implemented in the methodology. The sequence data of 16S rRNA genes was checked and modified using the BioEdit software version 7.0.5.3 (Hall *et al*. 1999). The sequences were compared with other 16S rRNA gene sequences obtained from the comprehensive database of the National Center for Biotechnological Information (NCBI) and examined using the Basic Local Alignment Search Tool (BLAST). Based on the similarities between the two test strains and other cyanobacterial 16S rRNA gene sequences in the NCBI database, a consensus phylogenetic tree was made. A total of 84 OTUs were included in the sequences that were utilized for constructing the phylogenetic tree and *Gloeobacter violaceus* was chosen as the outgroup taxon. Moreover, sequences from numerous recently discovered taxa in families like Oculatellaceae, Nodosilineaceae and Leptolyngbyaceae were also included. Using the CyanoDB2 website and the CyanoSeq v1.2 database (Lefler *et al*. 2023) and Strunecky et al. 2023, all 16S r-RNA gene sequences were gathered. Sequences exceeding 1,500 bp were trimmed from the 3' end using BioEdit software "TCACCTCCT". These 84 OTUs were subjected to multiple sequence alignment (MSA) using the Clustal software (Larkin *et al*. 2007). The MSA of 16S r-RNA gene sequences from 84 OTUs was analyzed using Bayesian Inference (BI) and Maximum Likelihood (ML), with a maximum of 1,496 characters, including nucleotides and gaps. The jModel Test 0.1.1 model test run was conducted in accordance with Posada *et al*. (2008). For ML and BI analysis, GTR+I+G

was the most well-suited model used. The BI tree was made by running the MSA within MrBayes v3.2.7 (Ronquist *et al*. 2012) and using the GTR+G+I model of nucleotide changes with two separate runs of four chains. Two runs of the 50 million Markov Chain Monte Carlo (MCMC) generation were carried out consecutively. The usual standard deviation for split frequencies was less than 0.002 percent. The convergence of MCMC chains was statistically fulfilled, as shown by the potential scale reduction factor (PSRF) values of 1.00 for all parameters (Gelman and Rubin *et al*. 1992). The BI analyses were also conducted on the NGPhylogeny.fr webservers (Lemoine *et al*. 2019) with two cycles of four Markov chains, each of which was implemented over 50 million generations. The generated tree was visualized on the iTOL web server (Letunic *et al*. 2019). The ML tree was generated with 1000 replications using MEGA software version 6.0 (Tamura *et al*. 2013), as well as Kimura's two parameter model for sequence evolution, which included gamma-circulated evolutionary rates and a projected proportion of invariable sites. The bootstrap values for ML analysis were diagrammed into the BI tree using Adobe Illustrator.

4.10. 16S-23S ITS secondary structure analysis

The ITS spacer regions of all five cyanobacterial strains were compared with their corresponding reference genus and both the variable and conserved domains were identified. According to Johansen *et al*. (2011), the ITS spacer region comprises 14 regions that are both highly conserved and highly variable. The nucleotide length of all 14 distinct regions was identified for each experimental strains (S1, S6, S10, S19, and S23) along with their reference strains and compared for the significant differences. The conserved basal sequence for each helix was acquired to identify the regions (Iteman *et al*. 2000). In addition, the M-fold web server, version 2.3 (Zucker *et al*. 2003), was used to fold the specific regions, such as D1-D1'helix, Box-B helix, V2 spacer region and V3 helix, into secondary structures. The resulting secondary structures were then

compared to their reference strains in order to determine their inter-species relationship. The secondary structures were produced under ideal circumstances, with a default temperature of 37 °C and the structure was chosen for an untangle loop fix. The examination of secondary structures and the origin of the reference sequences for each strain group under this study were detailed in the subsequent subsections:

4.10.1. Comparative analysis of ITS for strain S1, S6 and S10

16S rRNA phylogenetic analysis confirmed that the S1, S6, and S10 cyanobacterial strains were placed under the Nodosilineaceae family, according to Strunecky et al. 2023. So, the generated ITS secondary structures of these three strains were compared with the members of the Nodosilineaceae family. Sequences of ITS and the secondary structure of reference strains were obtained from published research articles: *Euryhalinema* genus by Chakraborty *et al*. (2019, 2021). *Marileptolyngbya sina* and *Salileptolyngbya diazotrophicum* Zhou *et al*. (2018), *Nodosilinea* from Perkerson III *et al*. (2011), and *Nodosilinea chupicuarensis* from Vázquez-Martínez *et al*. (2018).

4.10.2. Comparative analysis of ITS for strain S19 and S23

Based on the reference genus *Thainema salinarum* (Rasouli-Dogaheh *et al*. 2022), the secondary structures of 16S-23S ITS regions were studied. The structures were made in the M-fold web server version 2.3 (Zuker *et al*. 2003). The secondary structures were generated using an untangled loop fix and the temperature was set to the default value of 37 °C. The structures were edited using Adobe Illustrator 2020, and a comparison was made between the specific ITS areas, such as D1-D1' and Box-B helices.

4.11. Uncorrected p-distance analysis for 16S and 16S-23S ITS

4.11.1. p-distance analysis for 16S and 16S-23S ITS for strain S1, S6 and S10

The uncorrected p-distance approach was used to calculate the divergence rates of 16S r-RNA and ITS using MEGA 6.0. A similarity matrix [100×(1-p-distance)] was used to obtain the results for 16S r-RNA, while dissimilarity values (100× p-distance) was determined for ITS. The final results of the examination were displayed in Table 5.2 and Table 5.3.

4.11.2. p-distance analysis for 16S for strain S19 and S23

According to phylogenetic analysis, strains S19 and S23 were identical with *Thainema salinarum*, so the only similarity value of 16S rRNA was calculated to confirm the sample position. Methods were followed as per Section 4.12.1. The result was displayed in Table 6.2.

4.12. Whole genome analysis of S1 strain

4.12.1. Genomic DNA extraction and draft genome sequencing

Small amount of biofilm from a 6-7-day-old unicyanobacterial culture of the S1 sample was subjected to centrifugation and then rinsed with nuclease-free water. Following the manufacturer's guidelines, the genomic DNA was obtained from the rinsed sample employing the DNeasy plant mini kit (Catalog No. 69104, Qiagen, Hilden, Germany). The qualitative and quantitative assessment of the isolated genomic DNA was conducted utilising a Nanodrop 1000 spectrophotometer. The concentration of isolated genomic DNA was quantified as 79.8 ng/µl, having an optical density of 1.8 at A260/280. The library was prepared utilizing the KAPA HyperPlus Kit (Catalog No. 07962428001, Roche, Basel, Switzerland). The Qubit 4.0 fluorometer (Catalog No. Q33238, Thermofisher, Waltham, Massachusetts, USA) was used to quantify the

final libraries. The DNA HS assay kit (Catalog No. Q32851, Thermofisher, Waltham, Massachusetts, USA) was utilized in accordance with the manufacturer's guidelines. Following the manufacturer's instructions, the very sensitive D1000 screentapes (Part No. 5067-5582, Agilent, Santa Clara, California, USA) used with the Tapestation 4150 (Agilent) were used to estimate the insert size of the library. In order to verify overall quality, extracted DNA was loaded onto a TBE 2% agarose gel, stained with a two-fold loading dye (Catalog No. R0641, Thermofisher, Waltham, Massachusetts, USA), and the presence or absence of smearing—a sign of potential contamination—was documented. Puregene NEX-GEN DNA Ladder (Catalog No. PG010-500DII-NV, Genetix Biotech Asia, New Delhi, India) was loaded alongside the DNA. Sequencing achieved employing Illumina MiSeq platform (Illumina, CA, USA).

4.12.2. Draft genome preparation and bioinformatics analyses

The quality assessment of the raw FASTQ readings from the sample was conducted using FastQC v.0.11.9 with default settings (Andrews, 2017). The raw FASTQ readings underwent pre-processing using Fastp v.0.23.4 (Chen *et al*. 2018). Processed data were re-evaluated with FastQC. The processed readings were assembled de novo via Unicycler v.0.4.4 (Wick *et al*. 2017) with default settings. The final genome was derived employing the metagenomic binning approach, which ensured that it was free of any microbial and viral contaminants, as genomic DNA was obtained from a mono-specific non-axenic culture of isolate S1. The draft genomes were subsequently binned using MaxBin2 version 2.2.7 to retrieve the genomes from the draft assemblies. (Wu *et al*. 2016). The completeness and contamination rates of the recovered genome bins were evaluated using CheckM2 v.1.0.1 (Chklovski *et al*. 2023). The 16s rRNA gene fragments from the assembled bins were retrieved employing the ContEST16S program from EzBioCloud. The assembled draft genome was evaluated for quality using QUAST (Gurevich *et*

al. 2013). The annotation of the chosen genome bin (S1_bin1) was conducted via BAKTA (Schwengers *et al*. 2021) with reference to the Bakta database release of February 20, 2023, accessible at https://zenodo.org/record/7669534. Annotation was conducted utilizing the following databases: AMRFinderPlus (release 2022-12-19.1), COG (release 2020), DoriC (release 12), ISFinder (release 2019-09-25), Mob-Suite (release 2.0), Pfam (release 35), RefSeq (release r216), Rfam (release 14.9), UniProtKB/Swiss-Prot (release 2022_05), and VFDB (release 2023-02-10). Functional annotation of the protein sequences, acquired from Bakta, was performed using PANNZER2 (Törönen *et al*. 2018). The whole genomic sequence of S1 has been submitted to DDBJ/ENA/GenBank with the accession number JBHZOL000000000. The version referenced in this document is JBHZOL010000000.

4.13. Anti-UV compound production by strain S19

4.13.1. Culture condition

Biofilms of growing cyanobacteria (S19) from a culture that was 6 days old were put on clear, germ-free Petri dishes and placed in an incubation chamber. The incubation chamber included two types of light: one is ultraviolet (UV) lamp (Philips, Netherlands), and the other is a white fluorescent light (Philips, Netherlands). Exposure to UV light at approximately 280-315 nm was achieved using the UV-B lamp, while photosynthetically active radiation (PAR) at 400-700 nm was exposed using the white light. In order to mitigate thermal negative impact, the ambient temperature was maintained at 25 ± 2℃. To make sure that every part of the biofilm was exposed, the cultures were stirred every several hours using a sterile toothpick. Control samples were subjected to white, fluorescent light only. Continuous exposure was given to the cyanobacterial biofilms for 12 hrs.: 12hrs light: dark photoperiod for 5 days.

4.13.2. Extraction and partial purification of MAAs

MAAs were isolated then partly purified following the procedures reported by Ahmed *et al*. 2021, with minor changes. After UV treatment, cyanobacterial cells were collected by centrifugation at 8000 × g for 10 minutes (Eppendorf 5810R, Rotor F-34-6-38, Hamburg, Germany). A total of about 0.5 grams of dry powder cells were dissolved in 5.0 ml of 100% methanol (HPLC grade), and the mixture was then incubated at a temperature of 4 ℃ uninterrupted overnight. Following the incubation period, the cells were homogenized employing a glass homogenizer, and then it was subjected to sonication for 5 minutes. After that, the solution was centrifuged at room temperature for 10 minutes at 8000 × g. Following centrifugation, the supernatants were put in to a fresh centrifuge tube, and the resulting pellet was removed. The supernatants underwent spectroscopic investigation within the wavelength range of 250-700 nm with a UV-VIS spectrophotometer (Perkin Elmer, Lamda 25, Shelton, Connecticut, USA) (Fig. 7.1). Software connected to the spectrophotometer (Lamda 25) was used to analyze the raw spectra (peaks). A rotary evaporator was then used to evaporate the methanolic extracts at 35 °C (Eyela, model no. N-1200A, Tokyo, Japan). The residual substances were dissolved in 0.5 µl of distlled water. This solution was mixed with 75 µl of chloroform, centrifuged at 8000 × g for 10 minutes, and then gently vortexed. Following centrifugation the aqueous phase (uppermost layer) was delicately moved into new centrifuge tube to exclude contamination with lipophilic photosynthetic pigments in the MAAs (water-soluble). Absorption spectra were once again captured (Fig. 7.2). The MAAs were partially purified by passing the materials through microcentrifuge filters with pore sizes of 0.2 mm. The HPLC analysis was conducted on the partially purified MAAs.

4.13.3. HPLC and ESI-MS analysis

The MAAs that were partially filtered, were subjected to additional analysis and purification utilizing a high-performance liquid chromatography (HPLC) system (Shimadzu Corp, Serial no. L203054, 61426, Kyoto, Japan). This system consisted of with a reverse-phase semi-preparative C18 column made by Shimadzu Corp., which was linked to a guard column that had a photodiode array detector (SPD-M20A). Approximately 50 µl of partly purified MAA sample was injected using a Hamilton syringe (Switzerland) onto the HPLC column. The mobile phase consisted of 0.2% acetic acid, and the recognition wavelength was 334 nm and the flow rate was 1.0 ml/min The PDA check wavelength ranges from 280 nm to 370 nm. The distinct peak, exhibiting a retention duration (RT) of around 2.9 minutes, was eluted and obtained using a fraction collector connected to the HPLC machine (Fig. 7.3). Additionally, the purified MAA was categorized using liquid chromatography–electrospray ionization mass spectrometry in the positive electrospray ionization (ESI) mode, with a scan range of m/z 200–600. The specific MAA were determined using UV/Vis and mass spectrometry analysis.

Electrospray ionization mass spectrometry (ESI–MS) (Waters Xevo G2-XS QTOF, USA) was used to create protonated molecules from the fraction of MAAs that was purified using high-performance liquid chromatography (HPLC) from *Thainema* sp. strain. Mass spectra were acquired utilizing a Waters Xevo G2-XS-QTOF (Waters Corporation, Milford, MA, USA) ESI-MS spectrometer. A cone voltage of 3 kV was shown to facilitate the synthesis of $(M + H)^{1+}$ within a mass range of 200–600 m/z. The program Mass Lynx version 4.0 was used to examine the data.

4.13.4. Photoprotective activity analyses of MAAs

The function of MAAs in UV-protection was evaluated by applying on the bacteria *Escherichia*

coli (MTCC 1195). The MAAs-mixed (1 mg/ml) and pure *E. coli* cells were subjected to UV-B radiation for about 30 minutes and then inoculated into sterile Petri plates with solid LB medium. Following a 12-hour incubation period at 37 °C, the amount of bacterial colonies on every Petri plate was determined, and the formula was used to calculate the percentage survival rates following Rastogi *et al*. 2013.

The percent survival rate (SR%) = $\frac{N \times 100}{NC}$

N represents the quantity of colonies subjected treated (without MAAs, only UV-B without MAAs and MAAs with UV-B) cells and NC represents the quantity of control colonies (without MAA mixture).

4.14. Biodiesel production by strain S1

4.14.1. Optimization of culture conditions

Nutrient deprivation is a popular growing method for improving lipid synthesis as well as fatty acid content in microalgae because of its low cost and easy modification (Ördög *et al*., 2016). For many microalgae species, nitrogen is the most identified factor for boosting lipid synthesis in both open as well as closed culture methods. In the present study, cyanobacterial biofilm cells that were initially developing exponentially were obtained from the pure axenic culture. The biofilms were then rinsed twice with distilled water to remove excessive media before being reinoculated into ASN-III medium with varying nitrogen contents. As a nitrogen source, sodium nitrate ($NaNO_3$) was used in ASN-III medium. In order to maximize lipid synthesis, we used five distinct concentrations of $NaNO_3$ (0.25, 0.5, 0.75, 1.0, and 1.25 g/l) in ASN-III medium. The control medium for growth conditions was 0.75 g/l $NaNO_3$ in ASN III media.

4.14.2. Lipid extraction and estimation

After 15 days of continuous growth in ASN-III medium with different nitrogen condition, the cyanobacterial biomass was harvested and cleaned with distilled water. Lyophilization of the biomass was carried out in a freeze dryer (Eylea Pte. Ltd.) subsequent to its cleaning. Approximately 0.5 grams of biomass was ground in a mortar and pestle with 5.0 ml of a chloroform and methanol mix in a 2:1 ratio. To lyse the cyanobacteria cell walls, liquid nitrogen was applied simultaneously. Following the extraction process, the liquid solution was maintained at 25 ℃ under shaking conditions for a duration of 5 hours. Subsequently, the mixture was centrifuged at 7000 g for 10 minutes. Moreover, the pellet was disposed of after centrifugation, and the greenish supernatant was taken in a glass vial that had been previously weighed and incubated at 30 °C for the whole night. The glass vial was weighed once again to determine the lipid content after incubation.

$$\text{Lipid content (\%)} = \frac{\text{Final Lipid}}{\text{Biomass}} \times 100$$

4.15. References

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Chapter 5.

TAXONOMY OF STRAINS S1, S6 AND S10

5.1. Results and Discussion

5.1.1. Morphological and Ultrastructural analysis

After establishing the axenic culture, the experimental three strains showed significant growth in ASN-III media under laboratory-controlled conditions. The three strains exhibited the formation of a blue-green biofilm mat-like structure that adhered to the bottom layer of the Erlenmeyer flask. Initial morphological characteristics were identified according to the key by Komarek *et al*. (2005), which confirm the strains belonged to the Leptolyngbyaceae family. Moreover, the 16S rRNA NCBI blast results of the three strains showed that the strains had the closest similarity with the members of the Nodosilineaceae family and *Euryhalinema mangrovii* was the closest taxa (Strunecky *et al*. 2020, Chakraborty *et al*. 2019). Therefore, the morphological features of all the strains were compared with the reference genus *Euryhalinema mangrovii* and the family characters of Nodosilineaceae. Microscopic analysis of the 10-day-old strain exhibited that the filaments were thin, long, unbranched, straight or wavy. The trichomes of the three strains were uniseriate, non-heterocytous, cells longer than width, and cylindrical in shape (Fig. 5.1). The cell length of all the strains ranges from 1.6 µm to 2.5 µm in length and 0.7 µm to 1.1 µm in width. Most of the members of the Nodosilineales are reported as filamentous, with a 2 µm width of the cell (Strunecky *et al*. 2023). The shape and size of the cells may be induced by different ecological as well as environmental factors of the sampling area (Zapomelova *et al*. 2008). The width of the members of the Nodosilineaceae was commonly 1.0-3.5 µm (Strunecky *et al*. 2023). The experimental three strains showed thinner cells than the reference genus, which was *Euryhalinema mangrovii* which showed 0.4-0.6 µm cell width, 0.8-1.0 µm cell width for *E. epiphyticum* and 0.4-0.5 µm cell width for *E. pallustre.* Moreover, the cells were also thinner than the phylogenetically two closest cyanobacteria, *Salileptolyngbya diazotrophica* and *Gibliniella alaskaensis* which were reported to

have 0.93-1.44 µm of cell width and 0.7-1.2 µm of cell width, respectively (Zhou *et al*. 2018, Strunecký *et al*. 2020). The apical cell shape of all three taxa showed a conical shape which is unique as

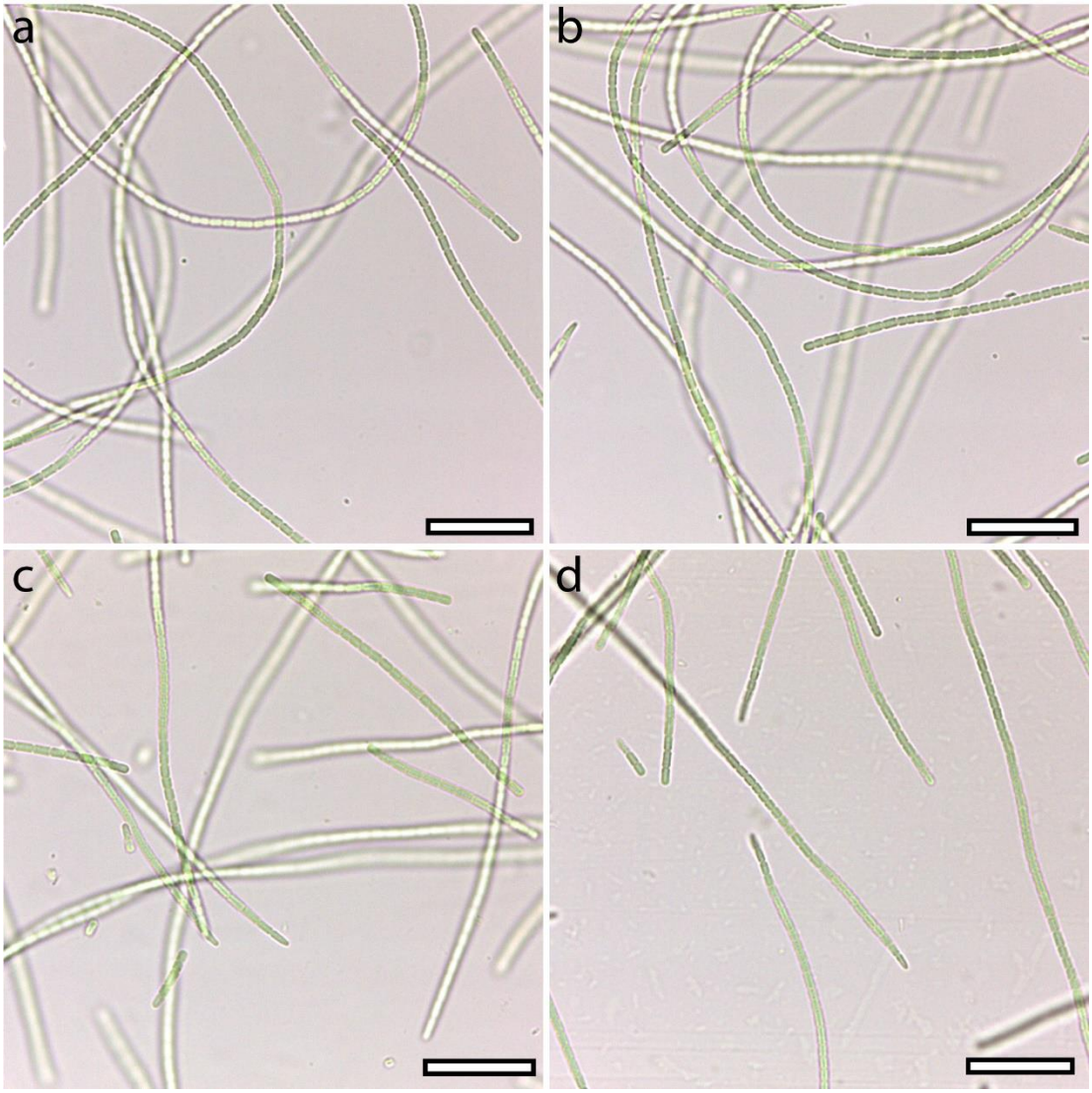

FIGURE 5.1. Light microscopic images of *Almyronema epifaneia gen*. *nov*. *sp*. *nov*. Image **a** and **b** show filaments of S1 and S6 strains, respectively. Image **c** and **d** show the straight and waving filaments of strain S10. Scale bar for all the images is 5 µm.

compared with the other reference strains. *Euryhalinema* sp. has a cylindrical apical cell shape, whereas *Salileptolyngbya diazotrophica* and *Gibliniella* both possess roundish apical cells. Moreover, the other members of Nodosilineaceae, such as *Haloleptolyngbya*, *Halomicronema*,

Marileptolyngbya, and *Nodosilinea* also exhibit rounded apical trichomes (Dadheech *et al*. 2012; Abed *et al*. 2002; Zhou *et al*. 2018; Perkerson III *et al*. 2011). According to the traditional classification of cyanobacteria (Komárek & Anagnostidis 2005; Komárek *et al*. 2014), apical cell morphology is one of the primary criteria for cyanobacteria identification. All three cyanobacterial strains exhibited gliding movement, which was absent in the reference strains. There was no presence of akinetes, hormogonia, and aerotopes reported in the three strains.

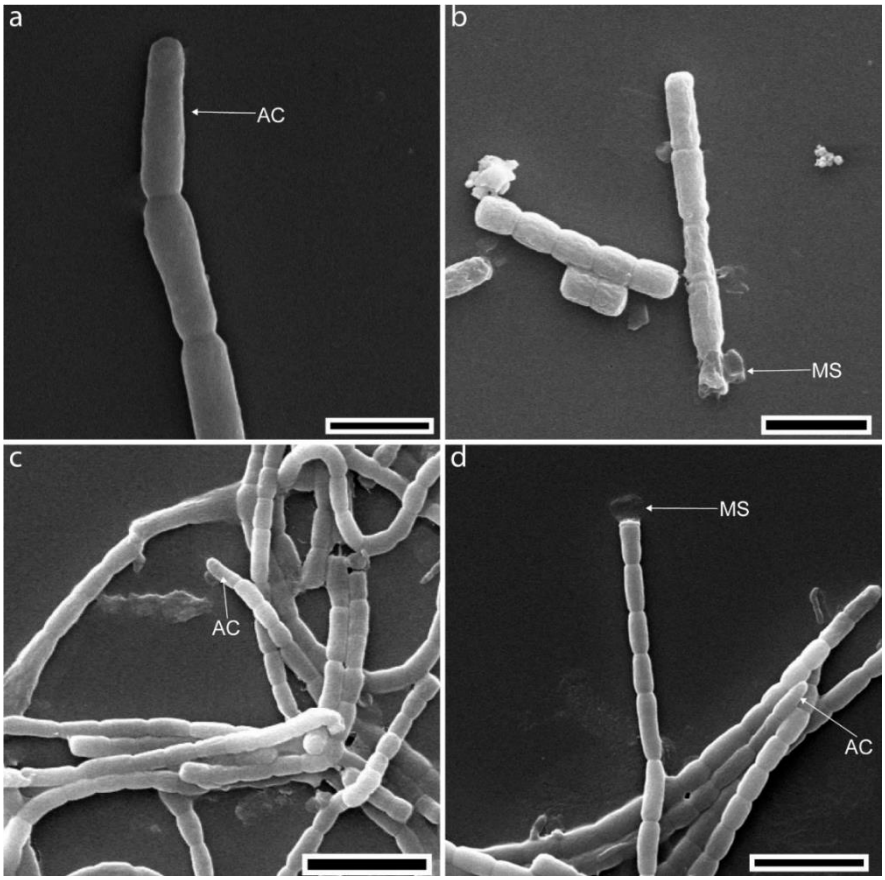

FIGURE 5.2. Scanning electron microscopic images of *Almyronema epifaneia gen*. *nov*. *sp*. *nov*. Image **a** and **b** show filaments of S1 and S6 strains, respectively. Image **c** and **d** show the filaments of strain S10. AC= Apical cell, MS= Mucilaginous sheath. Scale bar for image **a**, **b**, **c**, **d** is 1 µm, 3 µm, 5 µm, 5µm respectively.

Moreover, a thin, colourless, mucilaginous sheath was present in the surroundings of the trichomes.

The scanning electron microscopy study verified the morphological characteristics seen using light

microscopy (Fig. 5.2). Considering all the morphological evidence, it can be concluded that the three strains exhibited different morphological features as compared to the reference strains such as *Euryhalinema*, *Salileptolyngbya* and *Gibliniella*, which supported the claim of a novel taxa. Phylogenetic analysis is the primary criteria for polyphasic taxonomic analysis; however, morphological traits can be utilized as well to establish lineage separation at the family or genus level. Based on morphological characteristics, several cryptic genera were identified with minor morphological differences compared to their reference strains. These include *Cyanomargarita*, *Trichotorquatus*, *Limnolyngbya*, *Pinocchia*, *Alkalinema*, and *Pantanalinema* (Shalygin *et al*. 2017; Pietrasiak *et al*. 2021; Li *et al*. 2016; Dvořák *et al*. 2015; Vaz *et al*. 2015). Furthermore, morphological features like terminal cell dimenssion, mucilaginous sheath, and cellular inclusion were used to distinguish *Salileptolyngbya diazotrophicum* and *Marileptolyngbya sina* from one another. Ultrastructural analysis of the three strains using transmission electron microscopy showed that the ultrastructural characters were similar to the family characters of Nodosilineaceae (Fig. 5.3). Parietal thylakoid arrangement in cyanobacterial cells is one of the primary characteristics of the Nodosilineaceae family (Strunecký *et al*. 2023). The organization of thylakoid bands in cyanobacteria cells is an essential characteristic for classifying them at the family level (Rippka *et al*. 1979, Komarek *et al*. 2006). However, because of their varied evolutionary histories, the thylakoid of cyanobacterial cells is very unstable, making it unsuitable for use as the only characteristic to distinguish cyanobacteria at the genus or family level (Mares *et al*. 2019). Three studied strains were distinguished from *Salileptolyngbya* and *Euryhalinema* based on their ultrastructural characteristics like apical cell shape, trichome size, and mucilaginous sheath, including the presence of a large cyanophycin granule in each cell of the trichome. In our strains, there was a much higher abundance of glycogen α granules and lipid β granules compared

to *Salileptolyngbya*. However, these granules were not found in *Euryhalinema* and other genera of Nodosilineaceae. Based on phylogenetic examinations, *Ammassolinea* was distinguished from *Phormidium* with very slight ultrastructural differences (Hašler, 2014). The unique evolutionary position of *Aerofilum fasciculatum* as a new genus was determined, and the presence of an aerotopes distinguished it from the genus *Oculatella* ultrastructurally (Chakraborty *et al*. 2021). *Almyronema* (S1, S6, and S10) exhibited unique morphological features, including the shape and size of its apical cell, several cellular inclusions, and a distinctive physiological trait of gliding movement. These morphological and ultrastructural characters served to differentiate *Almyronema* (S1, S6, and S10) from the genus *Euryhalinema* and other species in the Nodosilineaceae family (Table 5.1).

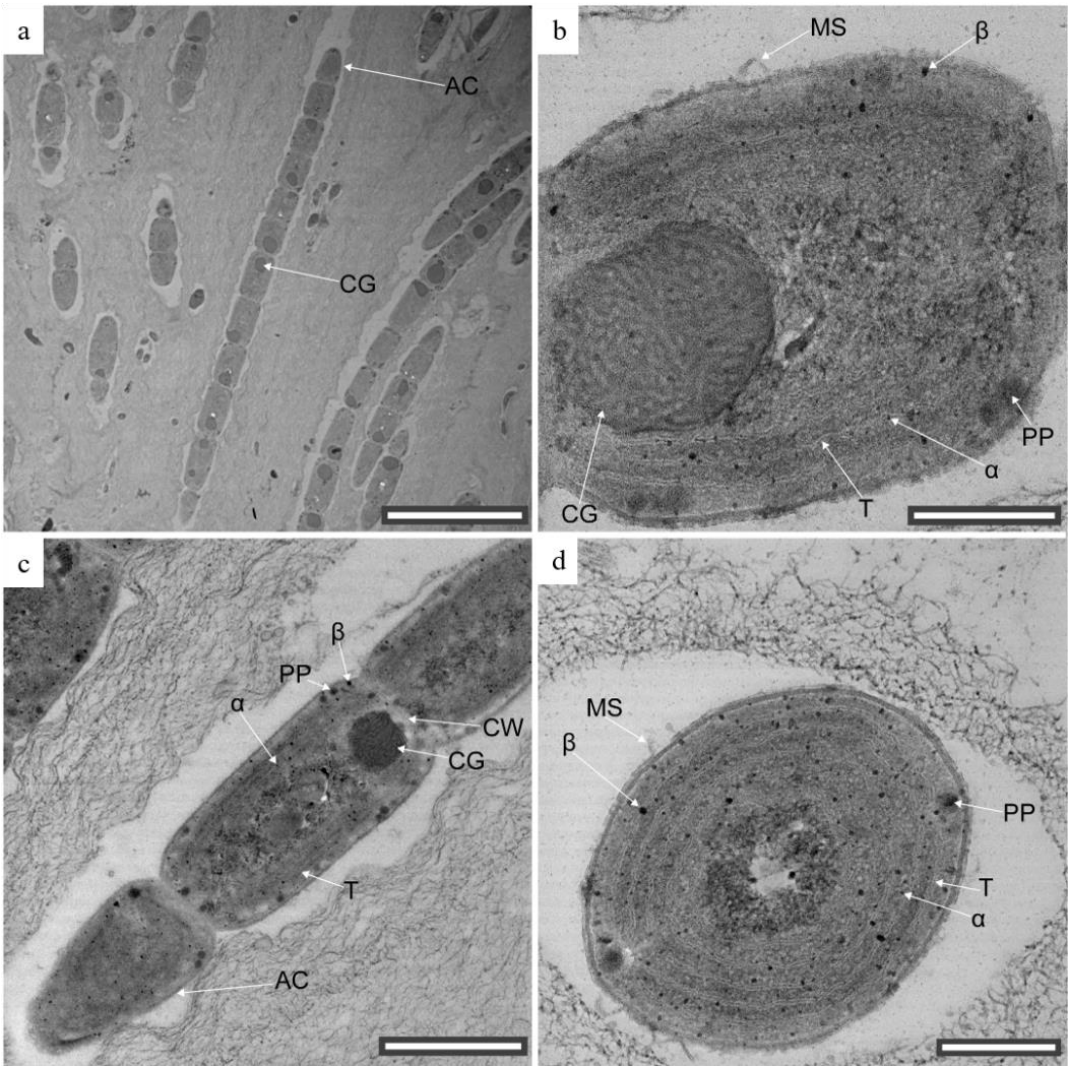

FIGURE 5.3. Scanning electron microscopic images of *Almyronema epifaneia gen. nov. sp. nov.* **a)** Image showing the longitudinal section of a full filament of Strain S1. **b)** Cross section of S6 strain showing different cell inclusions. **c)** Longitudinal section of apical cell of Strain S10. **d)** Cross section of Strain S10 showing different cell inclusions. AC= Apical cell, Glycogen granules, lipid granules, CG= Cyanophysin granules, CW= Cross wall, MS= Mucilaginous sheath, PP= Polyphosphate granules, T= thylakoid bands. Scale bar for image **a**, **b**, **c**, **d** is 5 µm, 250 nm, 700 nm, 300nm respectively.

Table 5.1. Morphological comparison of *Almyronema epifaneia* (S1, S6 and S10) with other phylogenetically related species.

Strains/Features	Thallus	Mucilage sheath	Motility	Cell length(µm)	Cell width (µm)	Cell form	Trichome apex	Habitat
***Almyronema epifaneia* (S1)**	Bluish green in color, fine mat	Thin, colorless sheath present	Yes	1.6-2.5	0.7-1.1	Distinctly much longer than wide	Conical	Upper surface of estuarine soil salinity ranging from 1.6-1.8%
***Almyronema epifaneia* (S6)**	Bluish green in color, fine mat	Thin, colorless sheath present	Yes	1.4-2.3	0.6-1.0	Distinctly much longer than wide	Conical	Upper surface of estuarine soil salinity ranging from 1.6-1.8%
***Almyronema epifaneia* (S10)**	Bluish green in color, fine mat	Thin, colorless sheath present	Yes	1.3-2.5	0.6-1.1	Distinctly much longer than wide	Pointed	Upper surface of estuarine soil salinity ranging from 1.6-1.8%
Euryhalinema mangrovii	Pale bluish green color mats	Absent	No	1.1-1.6	0.4-0.6	longer than wide	Rounded, not attenuated	Intertidal area (estuarine) with salinity ranging from 1.7-1.8%
Salileptolyngbya diazotrophica	Blue green mat forming	Firm, multilayer	No	1.53-2.37	0.93-1.44	Cylindrical	Rounded	Isolated from marine plantonic organism
Gibliniella alaskaensis	Thin yellow-green to green	Thin, firm clear	Intensely motile	0.9-3.6	0.7-1.0	Cylindrical, long, wavy	Rounded without calyptra	Freshwater, crust on stone

5.1.2. 16S rRNA gene sequences and Phylogenetic tree analysis

Utilizing the PCR-cloning techniques explained in Section 4.8, partial 16S rRNA gene sequences of the experimental strains S1, S6, and S10 were obtained, with the lengths of the sequences were 1377, 1385, and 1408 base pairs, respectively. The sequences were analyzed through BLAST in GenBank, and the closest six sequences of the three strains were identified as four anonymous and two uncultured cyanobacterial sequences. The closest relative of the three strains as revealed by BLAST was *Euryhalinema mangrovii* (MK402979) which showed about 94.30 %, 94.33%, and 92.71% genetic similarities with the S1, S6 and S10 strains respectively. The cut-off value for claiming a novel genus is 94.5% similarity of the 16S rDNA sequence and 98.7% for novel species (Yarza *et al*. 2014). Therefore, morphological features and 16S rRNA gene sequence comparison confirmed that the strains under examination were members of the Nodosilineaceae family. The lineage relationship was established by constructing a unified phylogenetic tree that included all the nearest relatives, as well as other members of the families such as Oculatellaceae, Leptolyngbyaceae, Nodosilineaceae, and Prochlorotrichaceae (Mai *et al*. 2018; Strunecký *et al*. 2023). The phylogenetic tree revealed that strains S1, S6, and S10 formed a well-supported clade inside the Nodosilineaceae family (Fig. 5.4). According to the BI, ML, and MP analyses, this clade was isolated from its closest relative genus *Euryhalinema* and the other clades associated with different genera by forming a unique and new phylogenetic lineage. Although the 16S rRNA gene sequence of *Almyronema* was most identical to *Euryhalinema* (94.1%-94.4%), genera *Salileptolyngbya* and *Gibliniella* were found to be the nearest relatives of the *Almyronema* clade in the phylogenetic tree, with 16S rRNA gene sequence similarities of 91.9%-92.1% and 92.2%-92.7% respectively (Chakraborty *et al*. 2019; Zhou *et al*. 2018; Strunecký *et al*. 2020). *Euryhalinema*, on the other hand, was significantly distant from *Almyronema*.

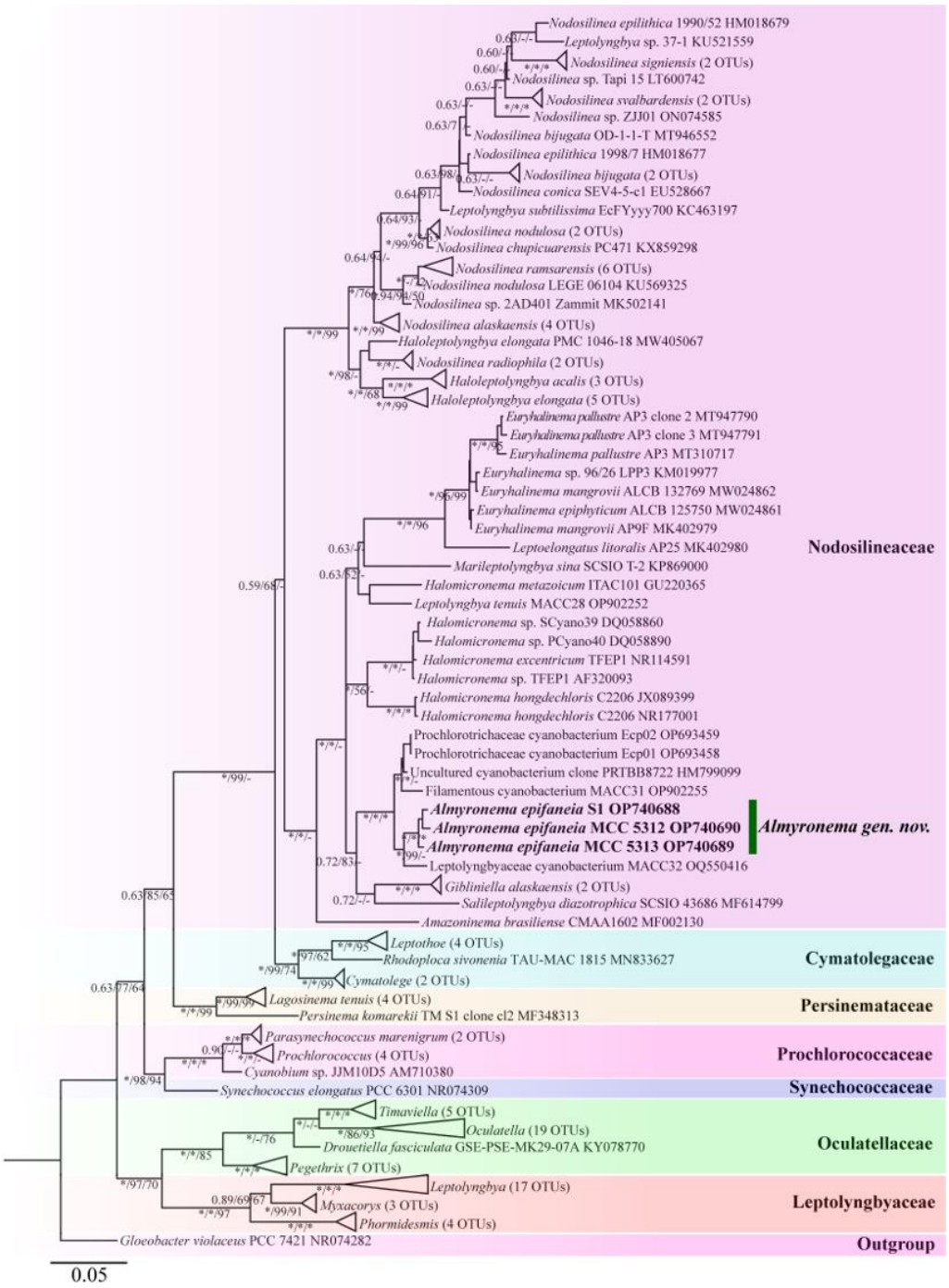

FIGURE 5.4. A phylogenetic tree was created using 16S rRNA gene sequences of 146 OTUs, containing *Gloeobacter violaceus* as the outgroup. The structure of the tree for Bayesian analysis. Scores for ML and MP trees were plotted in the tree. Bootstrap values based on 1000 resampling are given as for BI/ML/MP analysis at the nodes. Nodes indicated with an asterisk (*) were supported by both posterior probability and bootstrapping, achieving values 1.0 and 100%, respectively. Scores indicated by a dash (-) for any node demonstrated support of less than 50% for that branch.

All three isolates of *Almyronema* are grouped closely together, forming a unique lineage that is clearly different from *Euryhalinema*. According to the 16S rRNA phylogenetic tree, *Salileptolyngbya diazotrophica* and *Gibliniella alaskaensis* were identified as the most closely related species to *Almyronema*. The BI analysis confirmed a node supported by high posterior probability, while the ML and MP examinations were supported by bootstrap values. All three trees had quite similar topologies. However, only the Bayesian Inference (BI) tree is shown in Fig. 5.4. which contains support values obtained from all three studies. Each tree produced by several platforms showed an excellent bootstrap score for the *Almyronema epifaneia* cluster inside the Nodosilineaceae family. This supports the claim that *Almyronema epifaneia* should be recognized as a distinct genus from *Euryhalinema*. The p-distance calculation was conducted to determine the inter-species relationship between the three isolates and the nearest members of the Nodosilineaceae family. The similarities for 16S rRNA data ranged from 94.1% to 94.4%, whereas the dissimilarities (as percentage) of the 16S-23S ITS sequences ranged from 22.2% to 24.5% (Table 5.3). According to the analysis of p-distance using 16S rRNA, *Almyronema epifaneia* showed highest similarity with *Euryhalinema*, ranging from 94.1% to 94.4% (Table 5.2). Based on the 16S rRNA phylogenetic tree, *Salileptolyngbya diazotrophica* and *Gibliniella alaskaensis* were the most closely related genera of *Almyronema*. Previous literature shows when combination of phylogenetic, morphological, ultrastructural, and ecological features are considered, higher levels of 16S rRNA gene sequence resemblance may be regarded for describing new genera. Soares *et al*. (2020) identified *Parakomarekiella sesnandensis* as a new genus, even though it had a 98% resemblance to *Komarekiella atlantica* (Johansen *et al*. 2017). In a similar approach, *Purpureonostoc* established as a distinct branch in the phylogenetic tree and was classified as a new genus while sharing 94.4%-94.72% similarities with *Aliinostoc* (Cai, 2020; Bagchi, 2017).

Table 5.2. Similarities (as percentage) for *Almyronema epifaneia* (S1, S6 and S10) with the nearest BLAST findings derived from p-distance evaluation of the 16S rRNA gene sequence data.

	Strains	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1	***Almyronema epifaneia* S1**																							
2	***Almyronema epifaneia* S6**	**99.3**																						
3	***Almyronema epifaneia* S10**	**99.3**	**99.2**																					
4	Prochlorotrichaceae cyanobacterium Ecp02	98.9	99.2	98.8																				
5	Prochlorotrichaceae cyanobacterium Ecp01	98.9	99.2	98.8	0																			
6	Leptolyngbyaceae cyanobacterium MACC32	98.1	98.4	98	98.6	98.6																		
7	Uncultured bacterium clone ZZ16S2	98.8	99.1	98.7	99.7	99.7	98.7																	
8	Uncultured cyanobacterium clone PRTBB8722	98.9	99.2	98.8	99.8	99.8	98.6	99.7																
9	Filamentous cyanobacterium MACC31	95.4	95.8	95.3	96.4	96.4	95.6	96.5	96.4															
10	*Euryhalinema mangrovii* AP9F	94.1	94.4	94.1	94.3	94.3	94.7	94.3	94.3	91.4														
11	*Euryhalinema pallustre* AP3	93.3	93.6	93.3	93.5	93.5	93.9	93.5	93.5	90.6	99.3													
12	*Euryhalinema epiphyticum* ALCB 125750	93.9	94.2	93.8	94.1	94.1	94.4	94.1	94.1	91.1	99.8	99.1												
13	*Leptolyngbya tenuis* MACC28	92.9	93.3	92.8	93.4	93.4	93.2	93.5	93.4	91.5	93.3	92.5	93.1											
14	*Halomicronema hongdechloris* C2206	92.5	92.5	92.1	92.6	92.6	92.6	92.7	92.7	90.1	93.4	92.7	93.2	92.7										
15	*Amazoninema brasiliense* CMAA1602	92.2	92.6	92.2	92.7	92.7	92.5	92.5	92.7	90	94.3	94.2	94.1	94.7	92.4									
16	*Leptolyngbya subtilissima* EcFYyyy700	92.1	92.3	91.9	92.3	92.3	92.2	92.4	92.3	89.9	93.6	92.8	93.4	98.9	92.1	92.2								
17	*Salileptolyngbya diazotrophica* SCSIO 43686	91.9	92.1	91.6	92.5	92.5	92.6	92.6	92.5	90.5	93	92.2	92.8	93	93.8	92.3	91.5							
18	*Marileptolyngbya sina* SCSIO T2	91.7	91.9	91.5	91.6	91.6	91.6	91.5	91.6	89	92.7	92.6	92.5	93.2	94.3	94	93.6	91.8						
19	*Leptoelongatus litoralis* AP25	91.2	91.6	91.2	91.5	91.5	91.2	91.5	91.5	89.3	95.5	94.7	95.3	92	92.1	91.8	91.4	92.7	91.6					
20	*Nodosilinea chupicuarensis* PC471	91.5	91.7	91.3	91.7	91.7	91.6	91.8	91.7	89.2	93.2	93.4	93	91.6	91.7	92.9	98.7	90.6	93.4	91				
21	*Nodosilinea nodulosa* LEGE 06104	91.3	91.6	91.2	91.5	91.5	91.6	91.7	91.6	88.9	93.3	93.5	93.1	91.1	91.5	92.2	98.4	91.1	92.8	91.4	98.9			
22	*Nodosilinea ramsarensis* KH-S S2.6	91.3	91.6	91.2	91.5	91.5	91.6	91.7	91.6	88.9	93.3	93.5	93.1	91.1	91.5	92.2	98.4	91.1	92.8	91.4	98.9	99.8		
23	*Gibliniella alaskaensis* L31	92.3	92.7	92.2	92.9	92.9	92.8	92.7	93	90.5	92.9	92.8	92.7	92.7	91.7	91.8	98.8	91.7	92.5	91.4	93.1	92.3	92.3	
24	*Haloleptolyngbya alcalis* KR2005/106	89.5	89.5	89.1	89.9	89.9	90	90	90.1	88.6	90.6	90.3	90.4	92.2	93.4	90.5	94	91.4	92.9	90.1	94.4	93.9	93.9	93.1

5.1.3. 16S-23S ITS sequences and secondary structure analysis

All three tested strains had almost identical ITS sequences, having just over one percent of variation and contained only one operon comprising the tRNA gene $tRNA^{ile}$ and $tRNA^{Ala}$. The folded secondary structures of the D1-D1' helices, Box-B helices, and V3 regions of strains S1, S6, and S10 were retrieved and contrasted with the phylogenetically nearest strains identified using the 16S rRNA gene sequences. The 16S rRNA phylogenetic tree indicated that *Gibliniella* and *Salileptolyngbya* were the most closely related genera. Compared with the experimental strains, the ITS sequences from different Nodosilineaceae taxa revealed unique, varied secondary structures along with conserved domains. Table 5.4 presents the detailed findings. The folded secondary structure of the 16S-23S ITS region is crucial to differentiate between species. The secondary structures of the D1-D1' helix, the Box-B helix, and the V3 region were also folded and described for all the strains. These can be seen in Fig. 5.5. and Fig. 5.6. The D1-D1' helix structure of the ITS portions of each of the three isolates under the study has lengths of 59 nucleotides, making them the shortest of all the genera in the designated Nodosilineaceae family. The structure consisted of a 9-nucleotide terminal loop, with one bulge on both sides and two bulges on one side. All the experimental strains exhibited identical basal sequences 5′-GACC::CUGG-3′ within the D1-D1' helix. Moreover, the three strains had a distinct 5′-CAUCUC-3′ sequence in their unilateral basal bulge, contrasting them from other Nodosilineaceae taxa. The terminal loop of helix D1-D1' exhibited notable distinctions when contrasted to other genera of the same family. The Box-B helix region of ITS in all three isolates consisted of 41 nucleotides. which was greater than the sequence size of *Salileptolyngbya diazotrophica* and other Nodosilineaceae species, such as *Euryhalinema mangrovii*, *E. pallustre*, *Nodosilinea nodulosa*, and *N. chupicuarensis*, but shorter than that of *N. ramsarensis* (Zhou, 2018; Chakraborty, 2019; Pekerson III 2011; Vazquez-Martinez, 2018). Since

the whole ITS sequence of *Gibliniella alaskaensis* is not included in GenBank, it is not possible to identify its Box-B helix structure (Strunecký *et al*. 2020).

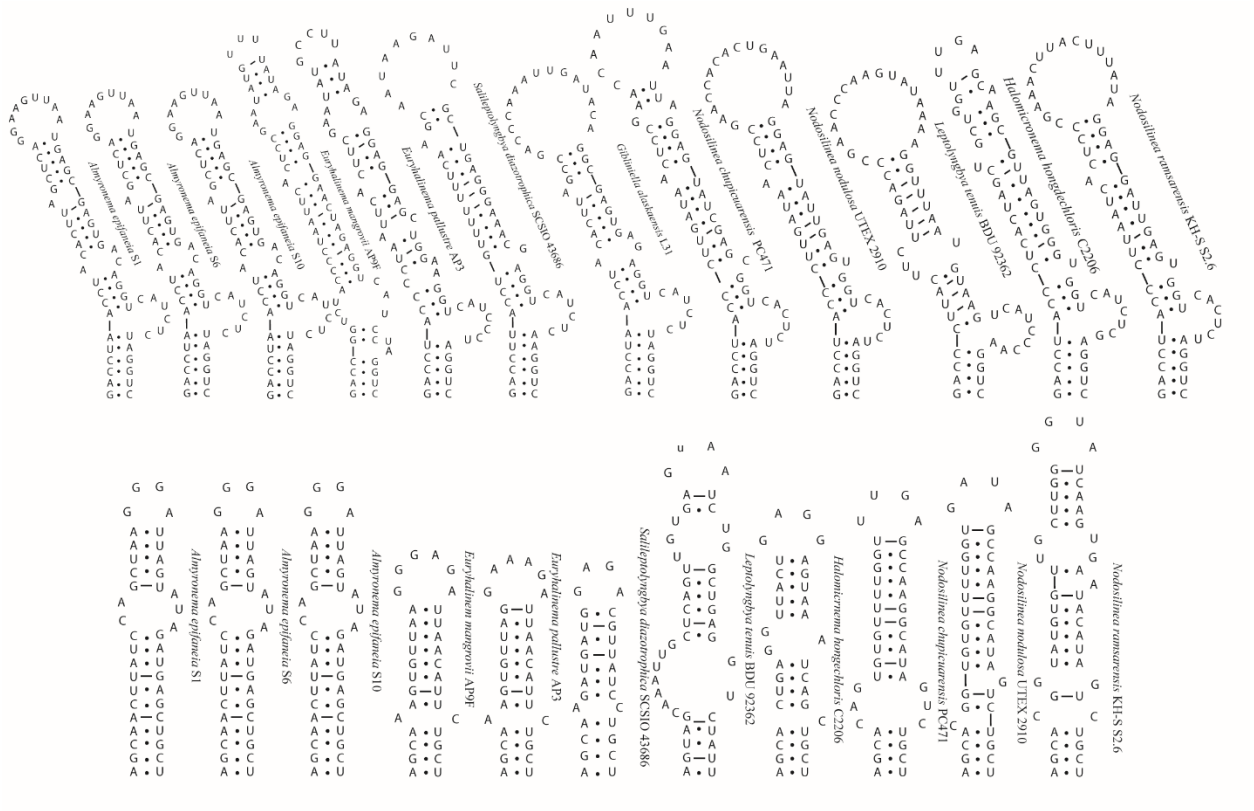

FIGURE 5.5. Comparative analysis of helix D1-D1′ (Upper row) and Box-B (Lower row) of the 16S-23S ITS domain of *Almyronema epifaneia gen*. *nov*. *sp*. *nov*., alongside closely related phylogenetic members of the family Nodosilineaceae.

It had a small terminal loop of four nucleotides, including a bilateral bulging in the three isolates. The basal nucleotide sequence of the Box-B domain was 5′-AGCA::UCGU-3′, which remained consistent across all strains examined. In addition, the terminal loop consisting of the sequence 5′-GGAA-3′ was distinct compared to the Box-B terminal loop pattern seen in all the remaining

members of this family. The sequence's length (41nt) The overall findings of Box-B ITS Helix validated its distinct structure in the Nodosilineaceae family.

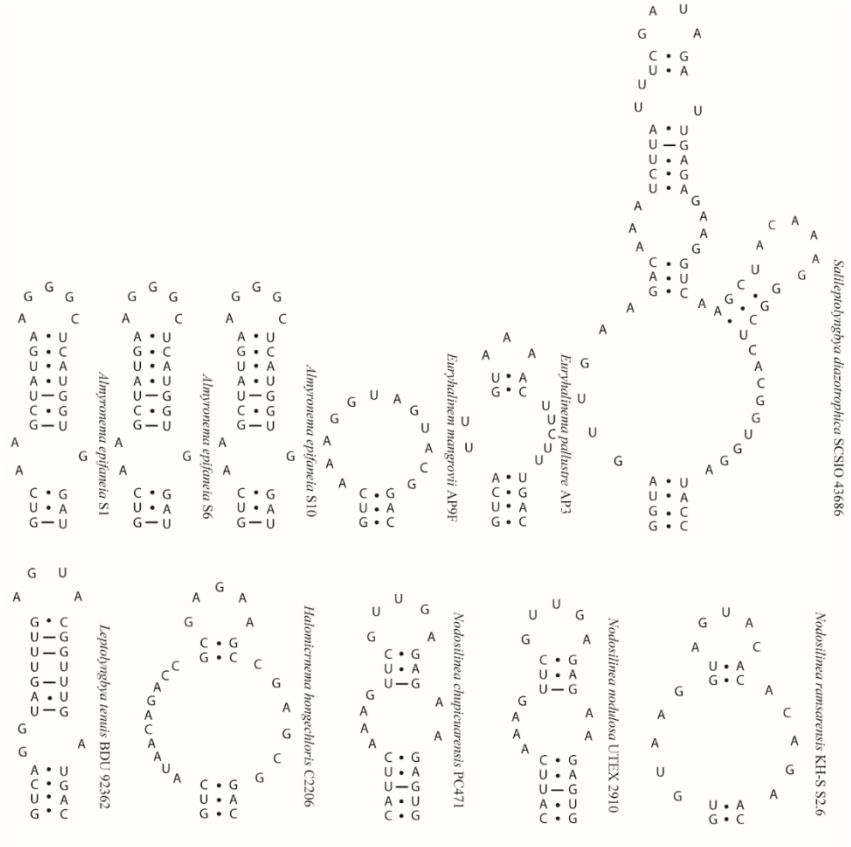

FIGURE 5.6. Characterization of the V3 helices of the 16S–23S ITS sections of *Almyronema epifaneia* gen. nov. sp. nov. strains S1, S6, and S10, alongside closely related phylogenetic relatives within the family Nodosilineaceae.

The V3 sections in the ITS sequences consisted of 28 nucleotides, including a apical loop of 5 nucleotides and a single unilateral loop. The basal sequence of the V3 region was 5′-GUC::UAG-3′, and this sequence was the same in S1, S6, and S10. The folded secondary structure of region V3 in *Almyronema* strains showed variations not just in structure but also in nucleotide sequence length compared to other strains.

Table 5.3. Dissimilarities (as percentage) for *Almyronema epifaneia* strains (S1, S6, S10) compared to their nearest relatives, derived from p-distance examination of 16S-23S ITS gene sequencing data.

	Strains	1	2	3	4	5	6	7	8	9	10	11
1	***Almyronema epifaneia* S1**											
2	***Almyronema epifaneia* S6**	**0.0**										
3	***Almyronema epifaneia* S10**	**0.0**	**0.0**									
4	*Euryhalinema mangrovii* AP9F	22.2	22.2	22.2								
5	*Euryhalinema pallustre* AP3	24.5	24.5	24.5	3.9							
6	*Salileptolyngbya diazotrophica SCSIO 43686*	22.7	22.7	22.7	20.5	21.6						
7	*Gibliniella alaskaensis L31*	8.8	8.8	8.8	18.9	21.7	22.1					
8	*Leptolyngbya tenuis* MACC28	25.2	25.2	25.2	19.4	21.1	23.9	23.9				
9	*Halomicronema hongdechloris* C2206	21	21	21	21.6	21.6	16.8	25.6	26.4			
10	*Nodosilinea chupicuarensis* PC471	19.4	19.4	19.4	11.8	14.9	17.8	17.8	22.2	21		
11	*Nodosilinea nodulosa* UTEX 2910	19.4	19.4	19.4	13.7	14.3	17.3	19.4	21.7	18.8	2.5	
12	*Nodosilinea ramsarensis* KH-S S2.6	20.6	20.6	20.6	11.7	12.2	17.8	19.4	22.2	18.9	5.6	3.4

Table 5.4. Study comparing the nucleotide sequences of the ITS sections of the strains of *Almyronema epifaneia* (S1, S6, and S10) to those of *Euryhalinema mangrovii* and other closely related Nodosilineaceae family members.

Strains	Leader	D1-D1'helix	Spacer + D2 with spacer	D3 with spacer	$tRNA^{Ile}$ gene	V2 spacer	$tRNA^{Ala}$ gene	Pre-Box B spacer	Box B	Post Box B spacer	Box A	D4	V3	D5
***Almyronema epifaneia* S1**	8	**59**	**35**	**12**	74	**5**	73	**36**	**41**	19	11	**7**	**28**	**15**
***Almyronema epifaneia* S6**	8	**59**	**35**	**12**	74	**5**	73	**36**	**41**	19	11	**7**	**28**	**15**
***Almyronema epifaneia* S10**	8	**59**	**35**	**12**	74	**5**	73	**36**	**41**	19	11	**7**	**28**	**15**
Euryhalinema mangrovii AP9F	8	**63**	**36**	**12**	74	**7**	73	**34**	**32**	19	11	**7**	**20**	**16**
Euryhalinema pallustre AP3	8	**63**	**39**	**9**	74	**15**	73	**37**	**32**	24	11	**11**	**24**	**18**
Salileptolyngbya diazotrophica SCSIO 43686	8	**62**	**40**	**7**	74	**6**	73	**24**	**28**	24	12	**7**	**72**	**17**
Gibliniella alaskaensis L31	8	**62**	**38**	**7**	74	**6**	73	-	-	-	-	-	-	-
Leptolyngbya tenuis MACC28	8	**64**	**34**	**12**	74	**5**	73	**34**	**45**	11	11	**7**	**29**	**19**
Halomicronema hondechloris C2206	8	**60**	**34**	**7**	74	**72**	73	**17**	**36**	18	11	**7**	**23**	**6**
Nodosilinea nodulosa UTEX 2910	8	**62**	**33**	**71**	74	**6**	73	**24**	**40**	18	11	**38**	**27**	**34**

5.2. Species Description

The taxonomic classification and species details for strains S1, S6 and S10 are described in Roy et al. (2024) as follows:

Phylum: Cyanophyta

Class: Cyanophyceae Schaffner

Order: Nodosilineales Strunecky & Mares

Family: Nodosilineaceae Strunecky & Mares

Genus: ***Almyronema*** **A.R. Roy & J. Mukherjee** ***gen. nov.***

Description: A mat-like biofilm that is pale green in color and is found on the top surface of the soil. Uniseriate, unbranched filament; mucilaginous sheath delicate, colorless; trichomes oscillating. Cells elongated relative to width, conical shaped apical cell, shrinkage at septa. Heterocyst and akinetes are missing. Thylakoids pattern parietal, presence of phosphate granules close to cell walls. Trichomes exhibit motility by gliding action. Reproduction using hormogonia.

Type species: ***Almyronema epifaneia*** **A.R. Roy & J. Mukherjee.**

Etymology: The generic name originates from the Greek word “Almyros” meaning salty, which pertains to the saltwater coastal habitat from where experimental strains originated, and the Greek word “nema” meaning string.

Species: ***Almyronema epifaneia*** **A.R. Roy & J. Mukherjee sp. nov.**

Description: Unbranched filament with narrow trichomes, having cells measuring 1.5-2.5 µm in length and 0.6-1.1 µm in width. A translucent mucilaginous sheath was observed. Filaments are

pale green in color. Motile filaments. Thylakoids parietal. A single significant cyanophycin granule was seen in nearly each cell. A significant quantity of polyphosphate granules is located throughout the cell wall.

Habitat: Intertidal sediment of mangrove ecosystems.

Etymology: The specific name derives from the Greek term "epifaneia" meaning "visible surface," referencing the species presence on the estuarine soil surface of the mangrove ecosystem.

Holotype: Strain S6 (accession number MCC 5313) maintained in a metabolically idle condition by cryopreservation, stored at the National Centre for Microbial Resource (NCMR), Pune, India.

Paratype: Strain S10 (accession number MCC 5312) is stored in a metabolically idle condition by cryopreservation and placed at the National Centre for Microbial Resource (NCMR), Pune, India.

Type locality: Maipit island (22°55.28′N, 88°32.93′E) of Indian Sundarbans, Dakshin 24 Parganas, West Bengal, India.

5.3. Genome characteristics of *Almyronema epifaneia* (S1)

The de novo assembly of the S1 raw genomic sequence reads resulted in an optimal assembly approximately 4.6 Mb genome size had a completeness of 94.05% with low contamination 0.43%. The genome comprising of 144 contigs, with lengths ranging from a minimum of 1,510 bp to a maximum of 265,967 bp, and a N50 value of 78,042 bp (Table No. 5.5). The experimental *Almyronema* sp. (S1) was found to have a G+C concentration of 50.6%. The RAST server utilized for genome annotations identified approximately 40 genes encoding RNA, which includes one copy of the 16S rRNA gene measuring 1,494 bp. The S1 genome annotation report indicates the presence of 4,595 protein-coding genes distributed across 209 subsystems. The major number of

genes of different subsystems are shown in a Pie chart with number of genes. (Fig. 5.8). The circular map of the genome shows the position of the different genes such as tRNA, rRNA, tmRNA and CRISPR-Cas genes (Fig. 5.7).

Table 5.5. Characteristics and summary of the assembled draft genome obtained of S1 sample.

Size of the Genome (Mb)	Genome completeness (%)	Contamination (%)	Length of the genome (bp)	Contig number	G+C (%)	N50 (bp)	L50	CDS
4.6	94.05	0.43	4,661,909	144	50.6	78042	19	4595

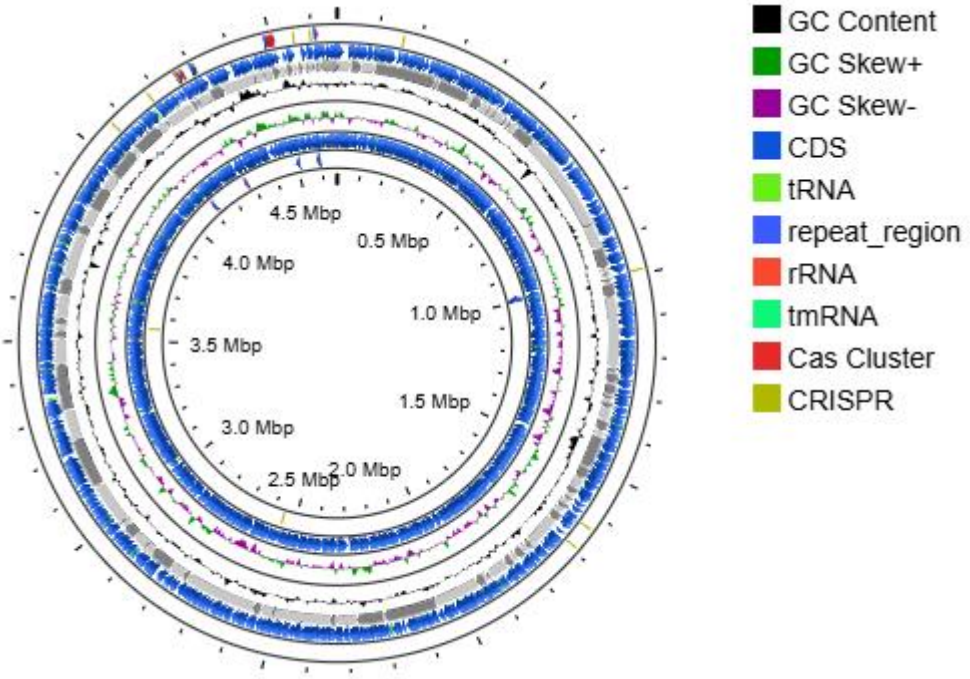

FIGURE 5.7. Circular chromosome map of *Almyronema epifaneia* (S1) showing the distribution of CDS, tRNAs, rRNAs, GC content skew and CRISPER/Cas genes. The map was generated using Proksee server.

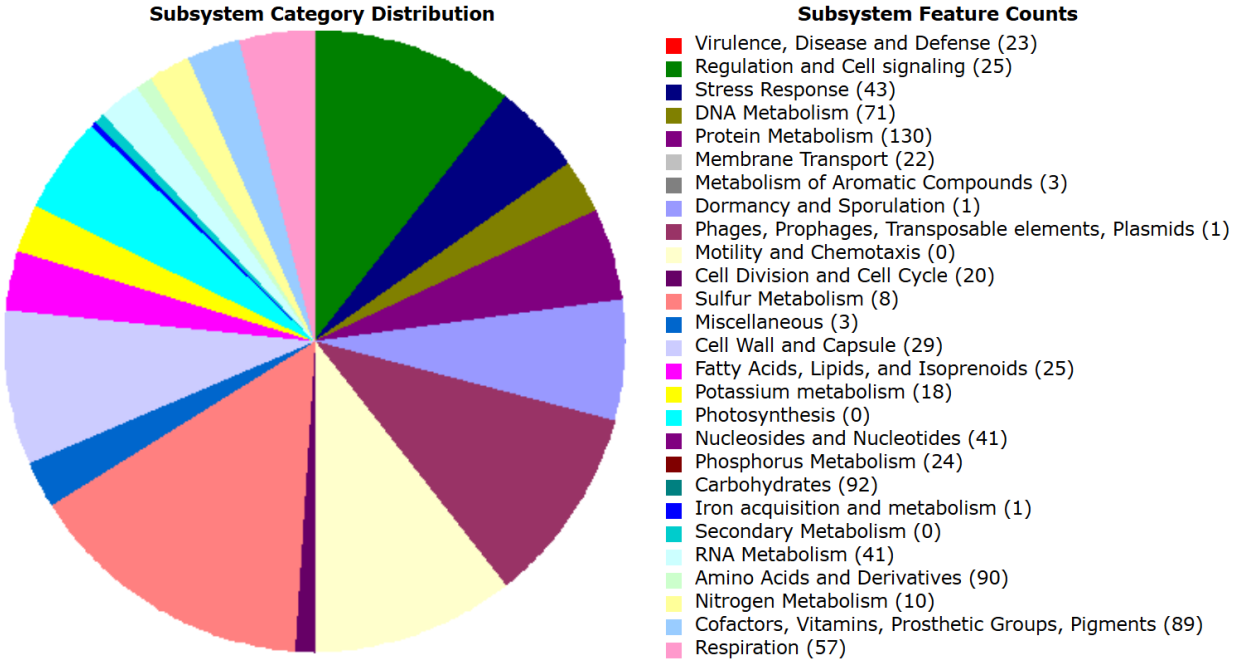

FIGURE 5.8. Number of genes present on the total CDS of the annotated *Almyronema epifaneia* (S1) genome.

5.4. References

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Chapter 6.

TAXONOMY OF STRAINS S19 AND S23

6.1. Results and Discussion

6.1.1. Morphological and Ultrastructural analysis

A comparison was made between the two cyanobacteria strains S19 and S23 and the reference genus *Thainema salinarum* (Rasouli-Dogaheh *et al.* 2022). The examined strains exhibited comparable morphological characteristics to the reference *Thainema* sp. Under light microscopy, both strains exhibited similar characteristics. The filaments were thin, homocytous, and had a straight pattern (Fig. 6.1). They appeared bluish green in color. The cells were

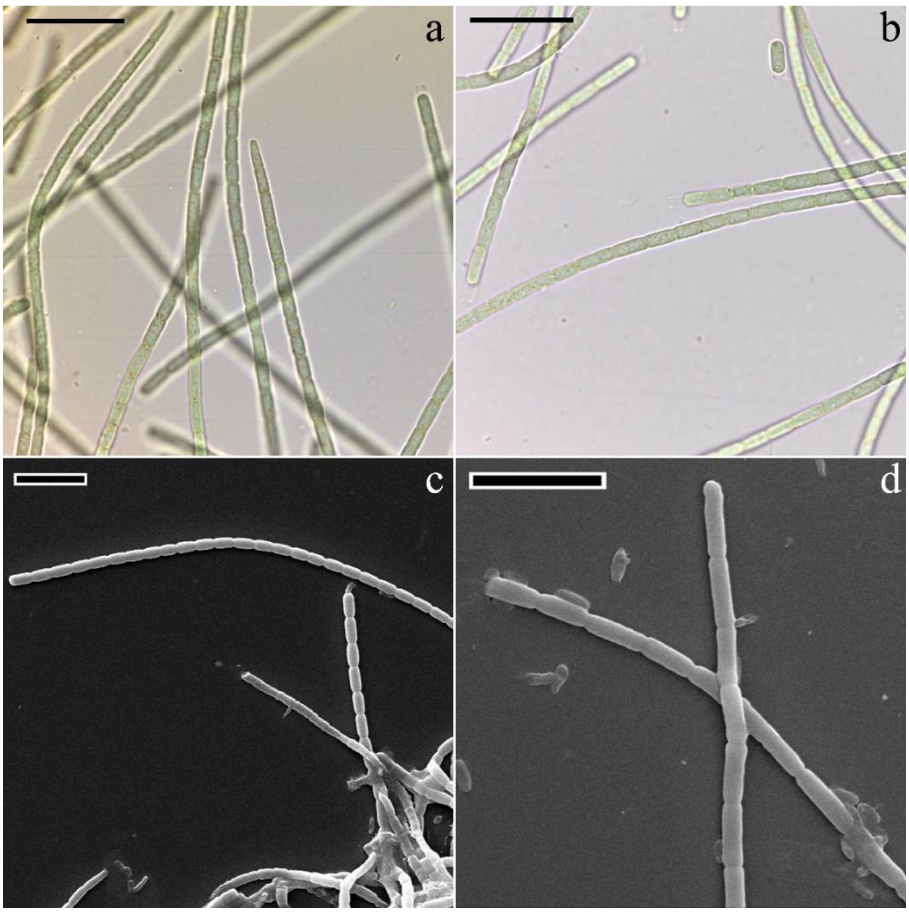

FIGURE 6.1. Image **a** and **b** showing light microscopic photograph of S19 and S23 strain respectively. Image **c** and **d** showing SEM photograph of S19 and S23 strain respectively. Scale bar for light microscopic and SEM images are 5 µm and 300 nm respectively.

cylindrical in form, capable of movement, and surrounded by a thin, transparent, mucilaginous sheath. The length of the cells varied between 2.0-2.19 µm, while the width was measured to be between 0.55-0.69 µm. On the other hand, *Thainema salinarum* CCALA 10287 had a 1-3 µm cell width, which could have resulted from its isolation from a very salty environment (Rasouli-Dogaheh *et al*. 2022). The cells exhibited a greater length-to-width ratio in comparison to the isodiametric

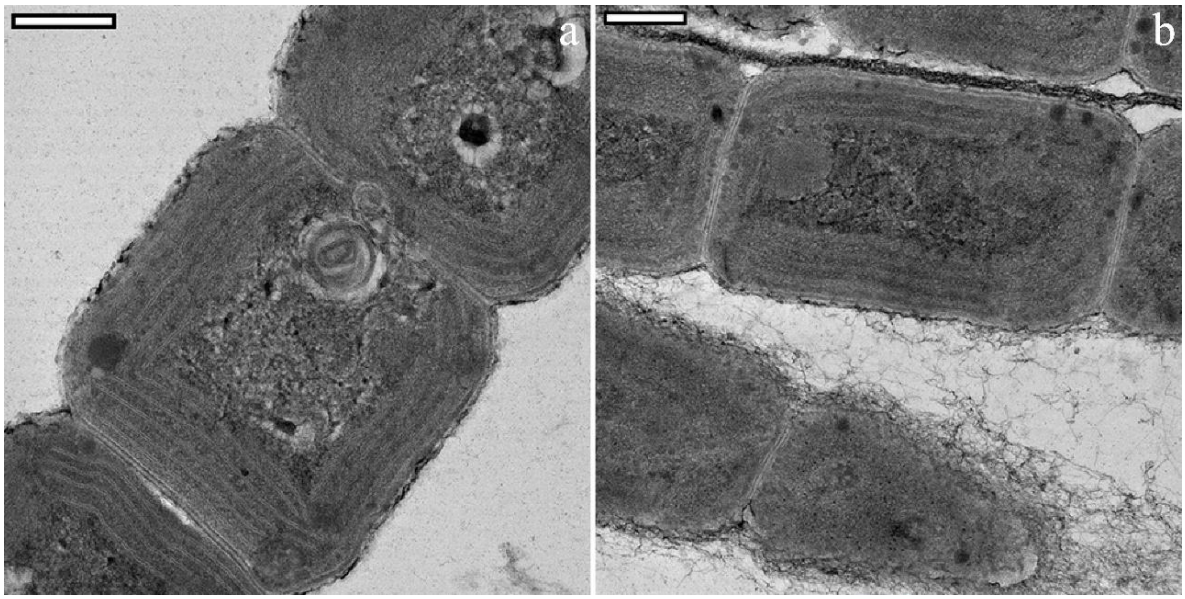

FIGURE 6.2. Image **a** and **b** showing cross section of S19 and S23 strain under transmission electron microscopy. Scale bar for both images were 700 nm.

shaped reference cells. No gas vacuoles or akinetes were detected in the test strains. The terminal cells of the filaments were pointed and constricted, which was distinct from the previously reported *Thainema salinarum*. The morphological differences and similarities of S19 and S23 in contrast with the reference organism, *Thainema salinarum,* are displayed in Table 6.1. The morphological characteristics of the two test strains as shown in light microscopic investigations were confirmed by scanning electron microscopy (SEM) observations (Fig. 6.1). The characteristics that were seen by transmission electron microscopy (TEM) were similar to the features of the Nodosilineaceae

family. The presence of a thin mucilaginous sheath, distinct constrictions in the cross walls and various cellular inclusions were easily seen in samples S19 and S23 (Fig 6.2). According to Strunecky *et al*. (2023), the arrangement of thylakoids was a parietal type, a characteristic unique to the Nodosilineaceae family. The TEM study also showed the presence of polyphosphate granules and cyanophycin granules in both strains.

Table 6.1. The comparative examination of the morphological characteristics of the two examined isolates (S19 and S23) alongside *Thainema salinarum* CCALA (Rasouli-Dogaheh et al. 2022).

Features/Strains	***Thainema salinarum* (S19)**	***Thainema salinarum* (S23)**	***Thainema salinarum* CCALA 10287**
Thallus	Yellowish green, fine mat	Yellowish green, fine mat	Bright blue-green
Mucilage sheath	Colourless, thin, sheath present	Colourless, thin, sheath present	Colourless, Sheath present
Constrictions at cross walls	Prominently constricted	Prominently constricted	Slightly constricted
Cell length (µm)	2.0-2.19	2.2-2.4	2-3.5
Cell wideness (µm)	0.55-0.81	0.51-0.91	1-3
Pseudobranching	Not present	Absent	Absent
Cell shape	Barrel shaped	Greater in length than in width	Isodiametric
Motility	Yes	Yes	No
Trichome apex	Conical shaped	Conical shaped	Round
Habitat	Upper layer of coastal sediment, with salt content between 1.6% and 1.8%	The uppermost layer of the estuarine soil; saltiness that varies between 1.6% to 1.8%	Extremely saline environment

6.1.2. 16S rRNA gene sequences and Phylogenetic tree analysis

The partial sequences of the 16S rRNA genes of S19 and S23 isolates, which are 1121 bp and 1368 bp in length were used to create the phylogenetic tree. The 16S rRNA gene sequences of strain S19 and S23 exhibited sequence resemblances of 98.22% and 99.27% respectively contrasted to the closest genus *Thainema salinarum* CCALA 10287 as reported by Rasouli-Dogaheh et al. in 2022. According to Yarza *et al*. (2014), the level of similarity exceeded the cutoff threshold (more than

94.5%) to differentiate between the two genera. Due to the presence of the common clade in the phylogenetic tree and the minimal genetic dissimilarity between *Thainema* sp. and the strains under investigation (S19 and S23), it seemed that the experimental strains were closely related to both *Thainema salinarum* and the morphotaxa or ecotaxa of *T*. *salinarum* CCALA 10287 (Fig 6.3). According to the p-distance study, the closest strains were found to be 98% to 100% similar to *Thainema salinarum* CCALA 10287 (Table 6.2). This showed that they are related to each other within species. To establish the evolutionary relationship, a consensus tree was created using members of the Leptolyngbyaceae, Nodosilineaceae, Oculatellaceae, and other families (Strunecký *et al*. 2023). The results of the Bayesian inference analysis (BI) and maximum likelihood (ML) studies indicated that the experimental strains under study, S19 and S23, were closely related to *Thainema salinarum* CCALA 10287. This relationship was supported by high posterior probability and bootstrap score of 100/100. The strains were found to belong to the newly described Nodosilineaceae family, as published by Strunecký *et al*. in 2023. Previously, *Thainema salinarum* CCALA 10287 was classified as a member of the Leptolyngbyaceae family (Rasouli-Dogaheh *et al*. 2022). However, the authors expressed ambiguity about the placement of the taxa due to the polyphyletic character of the Leptolyngbyaceae family. In their study, Rasouli-Dogaheh *et al*. (2022) proposed the reevaluation of *Thainema salinarum* CCALA 10287 in the future and identified it as a new genus within the Leptolyngbyaceae family. According to a 16S rRNA evolutionary tree study by Li *et al*. 2024, *Thainema salinarum* CCALA 10287 is also a member of the Nodosilineales family. Thus, our research determined that *Thainema salinarum* should be

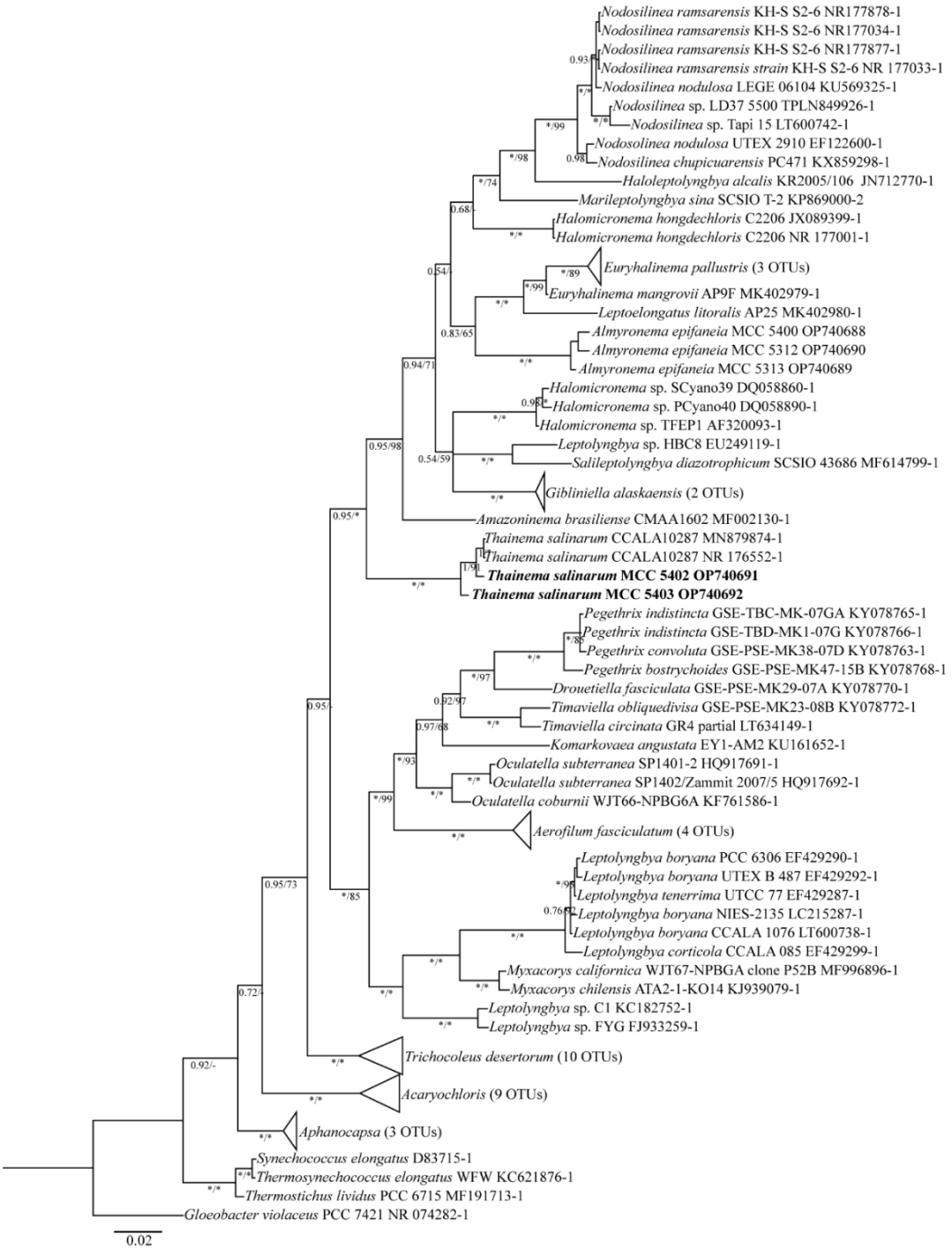

FIGURE 6.3. Phylogenetic tree of *Thainema salinarum* MCC 5402 generated from 84 OTUs of 16S rRNA gene sequences, having *Gloeobacter violaceus* serving as the outgroup. Supported values were BI/ML values given to the BI tree. The nodes shown by a star (*) obtained confirmation from both the posterior probability and the bootstrap, achieving values of 1.0 and 100%, accordingly. Scores indicated by a dash (-) for each node reflected less than 50% support for that branch.

reclassified as a part of the Nodosilineaceae family according to an examination of the 16S rRNA phylogenetic tree. According to the updated taxonomy, several distinct genera have undergone taxonomic revisions. *Elainella* and *Thermoleptolyngbya* were first identified as belonging to the Leptolyngbyaceae family by Jahodarova *et al*. (2018) and Sciuto *et al*. (2016) respectively. However, Mai *et al*. (2018) revised the position of both genera and incorporated them into the newly established Oculatellaceae family. *Haloleptolyngbya alcalis*, *Halomicronema excentricum*, and *Nodosilinea* sp. were first categorized as members of the Phormidiaceae, Peudanabaenaceae, and Pseudanabaenalean families, respectively (Dadheech *et al*. 2012; Abed *et al*. 2002; Perkerson III *et al*. 2011). According to a detailed investigation of many taxonomic characteristics, the classification of *Haloleptolyngbya alcalis*, *Halomicronema excentricum*, and *Nodosilinea* sp. was changed. These taxa are now placed into the Prochlorotrichaceae family, according to analysis by Mai *et al*. (2018). Moreover, their positions were further adjusted and eventually they were classified within the newly established Nodosilineaceae family by Strunecky *et al*. (2023).

Table 6.2. Similarities (as percentage) of *Thainema salinarum* (S19 and S23) with the nearest cyanobacterial relatives, determined using p-distance analysis of the 16S rRNA gene sequence.

	Strains	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
1	***Thainema salinarum* S19**											
2	***Thainema salinarum* S23**	**99.3**										
3	*Leptolyngbya* sp. L-32	100	99.3									
4	*Thainema salinarum* CCALA 10287 clone 1	99.2	99.1	99.2								
5	*Thainema salinarum* CCALA 10287	99.2	99.1	99.2	100							
6	*Pseudanabaena galeata* UTEX B SP44	99.3	99.2	99.3	99.1	99.1						
7	*Pseudanabaena galeata* UTEX B SP44	99.3	99.2	99.3	99.1	99.1	100					
8	*Thainema* sp. N15-MA6B	98.2	98	98.2	97.9	97.9	98.6	98.6				
9	*Thainema salinarum* CCALA 10287 clone2	99.3	99.2	99.3	99.1	99.1	99.2	99.2	98			
10	*Thainema salinarum* CCALA 10287	99.3	99.2	99.3	99.1	99.1	99.2	99.2	98	100		
11	*Leptolyngbya* sp. UMPCCC 1239	99.7	99.6	99.7	99.5	99.5	99.6	99.6	99.01	99.6	99.6	

6.1.3. 16S-23S ITS sequences and secondary structure analysis

S19 and S23, two strains under examination exhibited 99.17% and 99.49% sequence similarity to *Thainema salinarum* CCALA 10287 respectively when compared using NCBI BLAST. All 16S-23S ITS sequences are composed of a single operon including $tRNA^{Ile}$ and $tRNA^{Ala}$. The D1-D1´ helices and Box-B helix regions of both isolates exhibited a similar type of structure formation to that of *Thainema* sp. in their folded secondary structures (Fig. 6.4). The D1-D1´ helices of both strains were 61 nt in length and comprised a 13 nt-long large terminal loop, two unilateral bulges, and one bilateral bulge. For both strains, GACC:: CUGG was the basal sequence in the D1-D1´ helices. With two bilateral, one unilateral and a terminal loop (6 nt), the secondary structure analysis of the Box-B helix in both strains was 55 nt in length. AGC::UCGU was the basal sequence of the Box-B helix. One of the primary distinguishing factors for species-level identification is the folded secondary structural analysis of the conserved and variable regions of 16S-23S ITS.

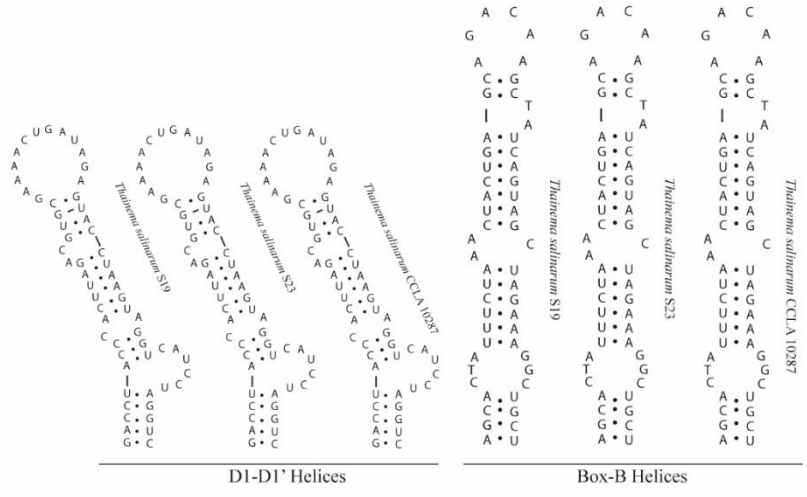

FIGURE 6.4. A comparative analysis examining the folded secondary structures of D1-D1′ and Box-B helices of 16S-23S ITS of *Thainema salinarum* strains S19 and S23 in relation to *Thainema salinarum* CCLA 10287 (Rasouli-Dogaheh et al. 2022).

6.2. Species Description

In accordance with the latest Strunecký (2023) cyanobacterial classification system, *Thainema salinarum* is classified taxonomically as follows.

Phylum: Cyanophyta

Class: Cyanophyceae Schaffner

Order: Nodosilineales Strunecky & Mares

Family: Nodosilineaceae Strunecky & Mares

Genus and Species: ***Thainema salinarum***

Description: The thallus is blue-green, with straight, frequently wavy filaments. The cells are elongated, exhibiting a notable constriction at the junction of the walls. Existence of a transparent, narrow mucilaginous coating around the trichome, with the trichome apex exhibiting a conical morphology. Parietal thylakoid type. Cells exhibiting movement. Absence of heterocyst and akinetes.

Habitat: Intertidal sediment of mangrove ecosystems.

Holotype: Strain S19 (accession number MCC 5402) maintained in a metabolically idle condition by cryopreservation, stored at the National Centre for Microbial Resource (NCMR), Pune, India.

Type locality: Namkhana (21° 47.15′ N, 88° 12.52′ E) of Indian Sundarbans, Dakshin 24 Parganas district, West Bengal, India.

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Chapter 7.

BIOTECHNOLOGICAL POTENTIALS FROM THE IDENTIFIED STRAINS

7.1. Anti-UV compound production by *Thainema salinarum* (S19)

7.1.1. UV-Vis spectroscopic analysis

To identify if *Thainema salinarum* (S19) contains UV-absorbing MAAs, spectrophotometric examination was first performed on the raw methanolic extract. After 7 days of incubation, the cyanobacterial extract that was mixed with 100% methanol showed absorption peaks at various wavelengths. These wavelengths indicated various photosynthesis pigments and other molecules in the S19 sample (Fig. 7.1). Chlorophyll a absorbs 420 and 665 nm, carotenoids absorb 470 nm, phycobiliproteins absorb 616 nm, and Mycosporine-like amino acids absorb 280-400 nm. It was clear from the absorption spectra that *Thainema salinarum* (S19) contains MAAs because it absorbs light with a wavelength of about 334 nm. Additionally, after the partial purification of MAAs, the absorption spectra validated the existence of UV-absorbing molecules, MAAs (Fig. 7.2).

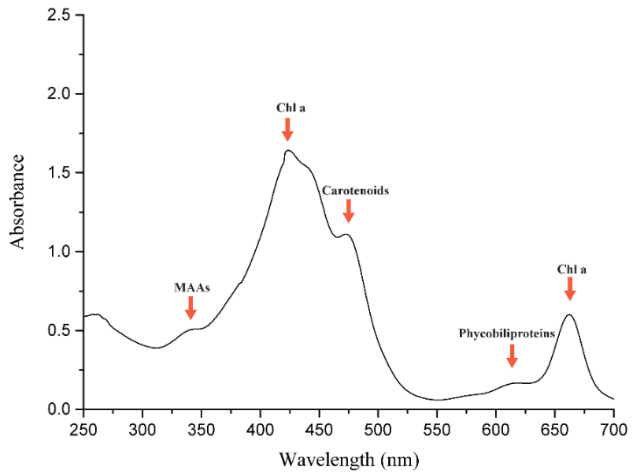

FIGURE 7.1. Ultraviolet-visible spectrum of a 100% methanolic solution of *Thainema salinarum* (S19) showing peaks for MAAs (332 nm), Chlorophyll a (420 nm and 665 nm), carotenoids (470 nm) and phycobiliproteins (616 nm).

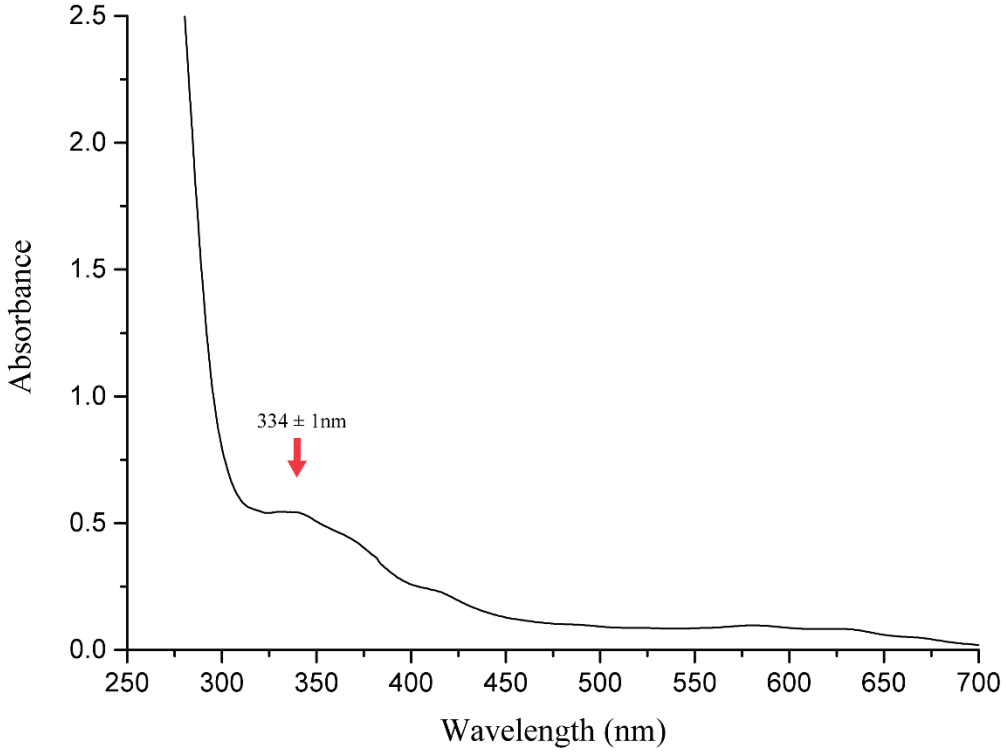

FIGURE 7.2. Ultraviolet light absorption spectra of *Thainema salinarum* (S19) MAAs in water after partial purification.

7.1.2. HPLC and ESI-MS analysis

The HPLC examination of partly purified MAAs revealed a distinct single peak with a retention period of 2.9 minutes, with a λmax of 334 nm. The fraction was collected and then subjected to ESI-MS analysis for characterization (Fig. 7.3). The ESI-MS examination of *Thainema salinarum* (S19) produced a significant ion peak of protonated molecules $[M+H]^+$ at m/z 333.1 (Fig. 7.4). UV-VIS spectrophotometric study, HPLC analysis, and ESI-MS analysis indicated Shinorine to be the only MAA present in the sample. Shinorine is an UV absorbing chemical produced by a variety of cyanobacteria. Shinorine is produced by *Aphanizomenon* sp. filamentous cyanobacteria that were isolated from eutrophic lakes in Wuhan, China. This was validated by a UV-Vis adsorption

spectrum at 334 nm with UPLC-MS-MS analysis, which showed the largest peak of a fragment of m/z=333 (Zhang *et al*. 2022). Shinorine is produced by *Anabaenopsis* sp., which was isolated from a Sambhar Lake in Rajasthan, India, and had an absorption maximum at 334 nm. Similar to our findings, the HPLC analysis in that work revealed a noticeable peak in the HPLC chromatogram at 2.8 min retention time (Bairwa *et al*. 2021). Furthermore, Shinorine was discovered in Antarctica-isolated pteropods and phytoplankton (Whitehead *et al*. 2001). HPLC examination revealed a peak for Shinorine with a retention time of 3.05 minutes, with a maximum wavelength of 334 nm. LC-MS analysis of the extract indicated a mass-to-charge ratio of 333, with significant fragments of Shinorine at m/z 255, 241, 230, and others. Carignan *et al*. (2013) also reported that *Alexandrium*, a toxic dinoflagellate, is a producer of Shinorine and other MAAs. Different forms of Shinorine are produced by the cyanobacteria *Scytonema crispum*, which was identified from a freshwater lake in New Zealand (D' Agostino et al. 2016). The several fragment types with varying masses (333, 318, 274, 265, 256, 287, 318) were verified to be Shinorine by reverse-phase liquid chromatography-mass spectrometry (RPLC-MS) and hydrophilic interaction liquid chromatography (HILIC) analysis. Shinorine production was reported by *Sphaerospermopsis torques-reginae*, which was isolated from the Tapacura Reservior in Brazil (Geraldes *et al*. 2020). With a retention period of 4.34 minutes in HPLC analysis and ESI-MS analysis displaying m/z 333, the cyanobacteria's synthesis of MAAs is confirmed.

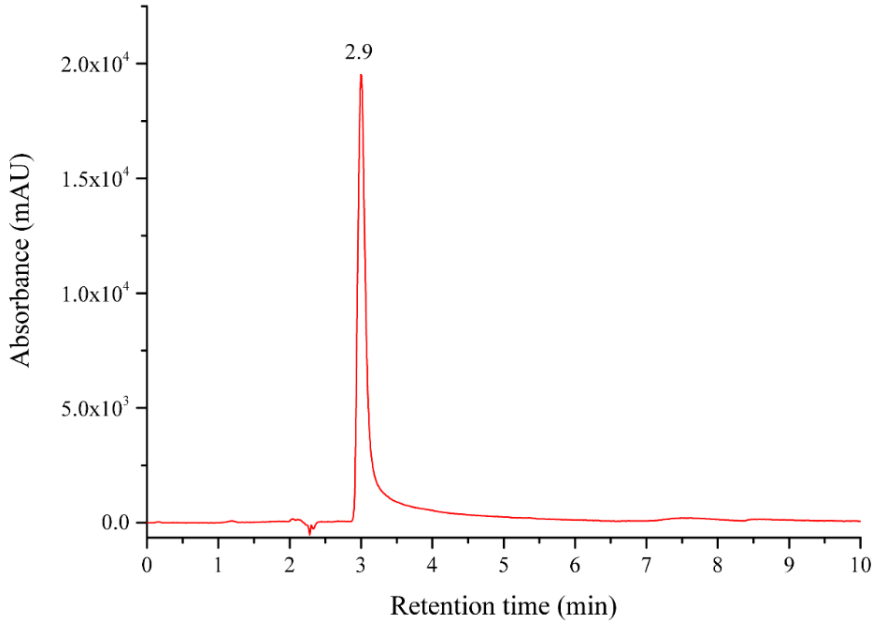

FIGURE 7.3. HPLC chromatogram of *Thainema salinarum* (S19) partially purified MAAs displays the characteristic peaks at retention time of 2.9 minute.

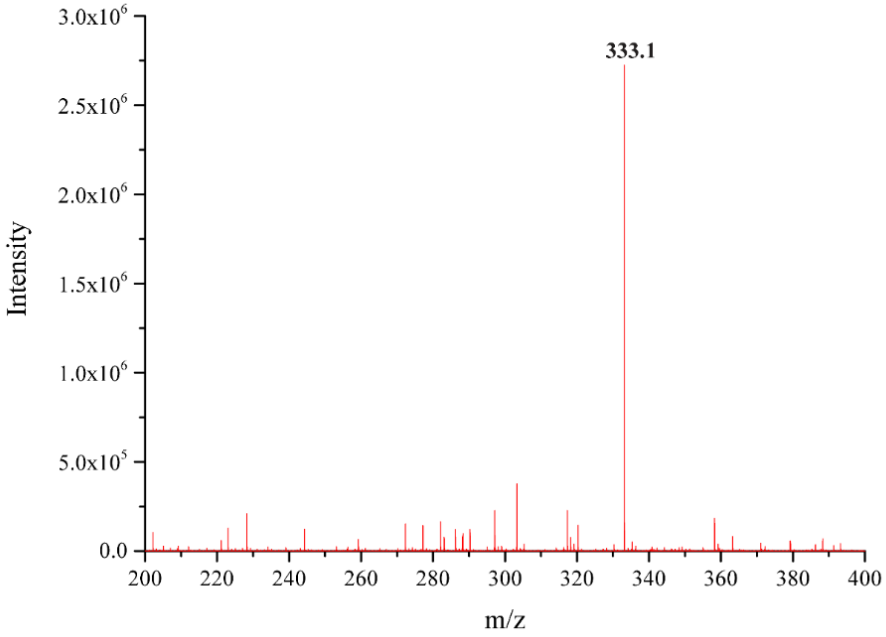

FIGURE 7.4. Mass spectrum of purified MAAs shows a noticeable peak at m/z 333.1 in the ESI-MS study, which indicates Shinorine.

7.1.3. Photoprotective activity analyses

To validate the photoprotective effect of MAA extracted from *Thainema salinarum* (S19), *E. coli* cells were tested. The *E. coli* cultures was cultivated in LB broth at 37°C until an optical density of 0.6 was reached. Subsequently, the cells were diluted to $10^{\wedge-4}$ and streaked onto LB agar plates, with or without the addition of MAAs. Under UV-B light emission, the survival rate of *E. coli* cells decreased to 13.88%, but in with the addition of MAAs, the mortality rate increased to 59.74% (Fig. 7.5). This investigation validates the photoprotective role of Shinorine extracted from *Thainema* sp. against UV-B radiation. According to Bairwa *et al*. (2021), *E. Coli* cells treated with Shinorine had an 83.5% survival rate. MAAs such as Mycosporine-2-2Glycine and Euhalothece-362 have been shown to increase the survival rate of *E. coli* cells by 56% (Rastogi *et al*. 2023). Furthermore, varying concentrations of MAAs demonstrated distinct survival rates of 26%, 29.8%, 38.8%, and 59.7% in *E. coli* cells (Rastogi *et al*. 2013).

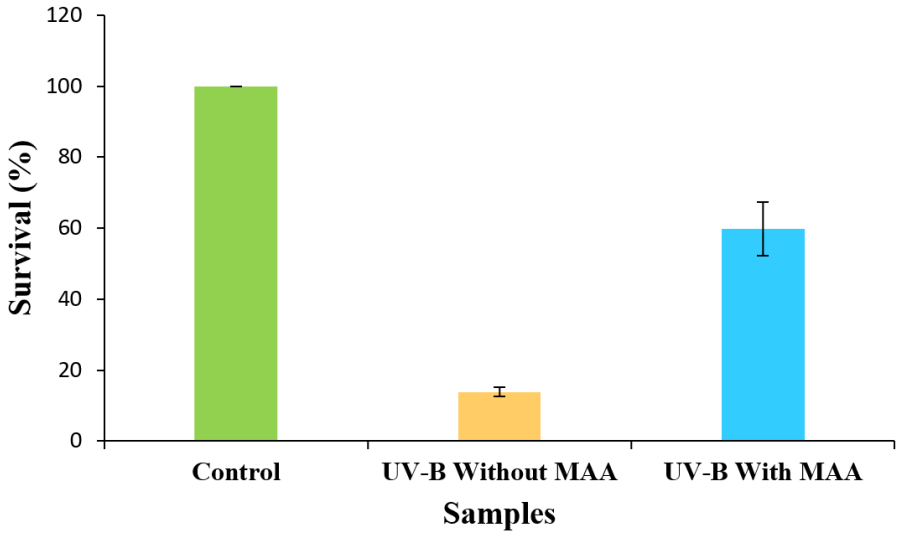

FIGURE 7.5. Photoprotective role of mycosporine-like amino acids (MAAs) in *Escherichia coli* cells exposed to UV-B light radiation.

7.2. Biodiesel compound production by *Almyronema epifaneia* (S1)

7.2.1. Effect of Nitrogen on lipid production

Using the procedure outlined in section 4.14.2, lipid was extracted in order to measure the amount of lipid produced by *Almyronema epifaneia* (S1). *Almyronema epifaneia* exhibited a lipid content of 11±2% when cultured in standard ASN-III media. It was regarded as a cyanobacteria that produced lipids with an average yield (5–15%) (Uma *et al*. 2020). In order to boost lipid production, the S1 strain was cultivated under four distinct nitrogen concentration ASN-III media conditions with control. Remarkably, as compared to 0.75 g/l $NaNO_3$ mixed controlled ASN-III media, the lipid content rose when the strain grown in 0.5, 1.0, and 1.25 g/l $NaNO_3$ mixed ASN-III medium. The Almyronema strain was cultivated in 0.5, 1.0, and 1.25 g/l $NaNO_3$ mixed ASN-III medium, yielding lipid contents of 13±2%, 19±2%, and 15±2%, respectively (Fig. 7.6). However, in 0.25 g/l mixed ASN-III medium, the lipid percentage dropped by 9.2±2%. In 1.0% $NaNO_3$ mixed ASN-III medium, *Almyronema epifaneia* generated 19±2% of the lipid content, making it a high lipid yielder (above 15%) cyanobacteria (Uma et al. 2020). A study of the lipid content of 55 marine cyanobacterial strains was carried out by Uma *et al*. 2020. Six of them generate above 15% of the lipid content, fourteen are moderate lipid yielders (10%–15%), and the remaining thirty-five are low yielders (0–10%). *Spirulina subsalsa* BDU51591, three strains of *Oscillatoria* sp., *Phormidium angustissimum* BDU41401, and *Plectonema terebrans* BDU41401 are among the highest lipid yielders, generating 18.56%, 16%, 15%, and 16.67%, respectively. Griffiths and Harrison (2009) evaluated five strains of cyanobacteria for lipid production study. *Spirulina platensis* exhibited the maximum lipid content of all, at 22%. A lipid content of 18.8% has been recorded by the coccoid cyanobacteria *Myxosarcina* sp. KIOST-1, which was isolated from Micronesia's mangrove forest (Kim *et al*. 2017).

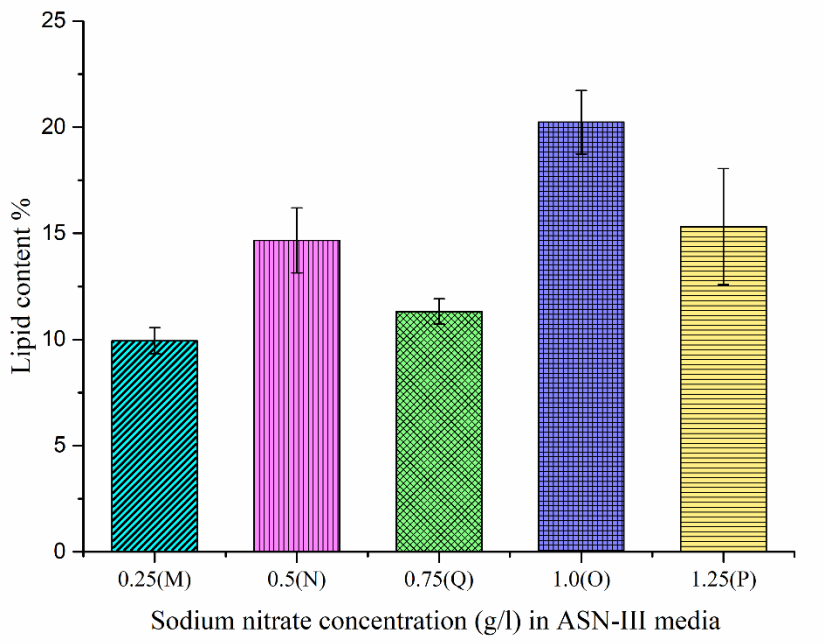

FIGURE 7.6. Lipid content (as percentage) of *Almyronema epifaneia* grown at various nitrogen concentrations.

7.3. References

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Chapter 8.

CONCLUSIONS AND FUTURE PROSPECT

8.1. Conclusions

The objective of this research was to use a thorough polyphasic approach to conduct a systematic evaluation of filamentous cyanobacteria isolated from different locations within the Indian Sundarbans and determine if they had any biotechnological potential. Thirty specimens were taken for the present research from Maipit, Purba Gurguria, Namkhana, Pathar Pratima, and Kakdwip. These specimens were soil surface biofilms. Five among the thirty specimens were purified; these samples had different morphologies and the strains were taken for further investigation. The samples were designated as S1, S6, S10, S19, and S23 and were chosen for the taxonomic study employing a polyphasic approach. All the purified isolates had simple, unbranched filaments devoid of heterocyst in their morphology. Out of five strains, S1, S6 and S10 were established as a novel genus under the newly established family Nodosilineaceae namely *Almyronema epifaneia* (Roy *et al*. 2024). The three isolates of *Almyronema* sp. created a novel cluster within the phylogenetic tree that was made using the nucleotide sequences of 16S rRNA gene. The newly formed cluster appeared different from the clade that included the genus *Euryhalinema* which was considered the most similar in terms of both ecology and morphology. The folded secondary structure of the different helices of ITS regions such as D1-D1', Box-B, and V3 were distinct in comparison to those observed in *Euryhalinema* along with other species in the family Nodosilineaceae. The unique morphological and ultrastructural features, such as the presence of different cellular inclusions (including large cyanophycin granules, glycogen α granules, lipid β granules, and a mucilaginous sheath), a cone-shaped apical cell, and changes in cell width, supported the discovery of a new taxon.

The remaining two isolates, strain S19 and S23 showed almost 100% similarity and a high affinity towards the genus *Thainema salinarum* (Rasouli-Dogaheh *et al*. 2022). Despite

these similarities, two strains showed distinct morphological characters like cell dimensions, apical cell shapes and ecological characters which are more aligned with the characteristics of Nodosilineales order. Additionally, S19 and S23 formed a unique clade under Nodosilineales order in the 16S rRNA phylogenetic tree. We emphasize the necessity of taxonomic revision of *Thainema salinarum* to address the polyphyletic problem. Therefore, considering physical, ultrastructural, ecological, and molecular information, we propose strains S19 and S23 as *Thainema salinarum* and positional amendment of *Thainema* genus within Nodosilineaceae family under Nodosilineales order and removed from Leptolyngbyaceae family following the latest cyanobacterial classification of Strunecký *et al*. 2023.

The draft genome analysis of *Almyronema epifaneia* (S1) was conducted using the latest Next Generation Sequencing technology (NGS). The de novo assembly of the genomic sequence represented the second genome within the Nodosilineaceae family, after the Nodosilinea genus. The genome annotation reveals several genes that may be utilized for subsequent comparative studies on evolutionary biology and biotechnology.

Thainema salinarum (S19), is a potential source of anti-UV compounds identified from mangrove cyanobacteria. Spectrophotometric, HPLC, and ESI-MS analyses verify that *T. salinarum* produces Shinorine, a mycosporine-like amino acid that which can use in the pharmaceutical and cosmetic industries. In addition, *Almyronerma epifaneia* (S1) strain generated 19% of lipid in 1.0 g/l $NaNO_3$-supplemented ASN-III medium, making it high-yielding cyanobacterium. The lipid content isolated from *A. epifaneia* clearly shows that it may be utilized as biodiesel feedstock.

8.2. Future prospect

The Sundarbans mangrove environment is a distinctive and vulnerable ecosystem on Earth, as previously mentioned (Hazra *et al*. 2002). This environment is among the least studied in terms of cyanobacterial diversity on Earth. This research identifies one unique genus and one existing recently described genus from several regions of the Sundarbans. These studies have the potential to increase the cyanobacterial diversity of ecosystems on earth that have received the least amount of attention. The polyphasic approach to taxonomy enhances our understanding of biodiversity at the alpha-level, which is critical for the under-investigated biodiversity hotspots (Stackebrandt and Goebel 1994). By using these standardized approaches, we should explore further regions of the Sundarbans forest to enhance our understanding of biological diversity and contribute to worldwide knowledge of cyanobacteria.

Taxonomy constitutes the first phase of a biodiversity research, after which we may examine the many physiological, ecological, and biotechnological aspects of the isolated organism (Martiny *et al*. 2006). Cyanobacteria serve as primary producers within a food chain; hence, using these isolates allows us to get insights into the ecosystem's food chain via various experimental applications of these strains. Since cyanobacteria are among the oldest living things on Earth and the fossil record indicates that they originated in the intertidal regions, these taxonomic studies allow us to examine the evolution of cyanobacteria on the earth. Exposure to a highly stressful environment renders them resilient to many environmental conditions; hence, using these isolates allows for the examination of the ecological interactions among numerous elements that govern metabolism in biological organisms. Organisms often generate new metabolites with biotechnological promise when subjected to stress. Utilizing these isolates enables the production of many biotechnologically significant metabolites such as MAAs, lipids, and c-phycocyanin.

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Chapter 9.

PUBLICATIONS AND CONFERENCE

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Almyronema epifaneia gen. & sp. nov. (Cyanobacteria, Nodosilineaceae) isolated from an Indian mangrove forest

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Almyronema epifaneia gen. & sp. nov. (Cyanobacteria, Nodosilineaceae) isolated from an Indian mangrove forest

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ABSTRACT

Three simple filamentous cyanobacteria (strains S1, S6 and S10) were isolated from the upper surface of intertidal soil of an Indian mangrove forest, the Sundarbans. The three cyanobacterial strains formed a new cluster within the phylogenetic tree calculated based on 16S rRNA gene sequences, which was distinctive from the clade of the ecologically and morphologically most similar genus, *Euryhalinema*. Moreover, the sequence similarity of 16S rRNA gene was less than 94.4% compared with the reference strain of *Euryhalinema*, which confirmed that the new strains belonged to a novel genus. The folded secondary structure of D1-D1′, Box-B and V3 helices of the internal transcribed spacer (ITS) region were different from those of *Euryhalinema* and other members of family Nodosilineaceae. P-distance analysis of the ITS region of the test strains showed 22.2% dissimilarity with the reference genus *Euryhalinema*, which exceeded the cut off (7%) and strongly supported that it is a novel species within a novel genus. Differential morphological and ultrastructural characters, such as the presence of various cellular inclusions (prominent large cyanophycin granule, glycogen α granules, lipid β granules and mucilaginous sheath), conical-shaped apical cell as well as cell width, supported the description of a new taxon. Therefore, based on morphological, ultrastructural and molecular analysis we propose that cyanobacterial strains S1, S6 and S10 belong to a new monophyletic genus and species *Almyronema epifaneia gen. & sp. nov.*, in the recently established family Nodosilineaceae.

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Intertidal; Nodosilineales; Polyphasic approach; Sundarbans

INTRODUCTION

Cyanobacteria are currently classified into eight orders based on the polyphasic approach of taxonomic identification (Komárek *et al.* 2014). Amongst them, order Synechococcales *sensu lato* is challenging to consolidate due to the presence of numerous polyphyletic cyanobacterial members which are phylogenetically amalgamated (Mai *et al.* 2018). Most of the filamentous taxa in the Synechococcales are cryptic in nature: they share a similar set of morphological features but are genetically very different. Leptolyngbyaceae is the largest family of Synechococcales, and it has been examined more intensively than any other synechococcalean families in recent times, in an attempt to establish monophyletic taxa (Casamatta *et al.* 2005; Johansen *et al.* 2008; Zammit *et al.* 2012; Komárek *et al.* 2014). Family-level taxonomic evaluation was performed and four monophyletic families, consisting of the two previously described families Leptolyngbyaceae and Prochlorotrichaceae, and the two new families Oculatellaceae and Trichocoleaceae, were proposed based on the polyphasic approach of taxonomic studies (Mai *et al.* 2018).

A very recent major revision of cyanobacterial classification was carried out by Strunecký *et al.* (2023) to solve the polyphyletic problems within the different orders. Based on whole genome analysis and 16S rDNA phylogenetic analyses, 10 new orders (Aegeococcocales, Acaryochloridales, Coleofasciculales, Desertifilales, Geitlerinematales, Gomontiellales, Leptolyngbyales, Nodosilineales, Oculatellales and Prochlorotrichales) and fifteen new families were proposed to achieve monophyly of all the cyanobacterial orders. As Synechococcales contains several polyphyletic taxa, Strunecký *et al.* (2023) split this order into four new orders: Prochlorotrichales, Nodosilineales, Oculatellales and Leptolyngbyales to define monophyletic taxa. Among the four orders, Nodosilineales primarily comprises simple filamentous cyanobacterial taxa having parietal thylakoid arrangement. Additionally, Strunecký *et al.* (2023) proposed three families within the order Nodosilineales: Nodosilineaceae, Cymatolegaceae and Persinemataceae. Nodosilineaceae mainly consists of halotolerant cyanobacteria living in habitats ranging from fresh water to marine. Nine genera were described in Nodosilineaceae: (i) *Halomicronema* Abed, Garcia-Pichel & Hernández-Mariné was reported from a salty lake in Israel (Abed *et al.* 2002); (ii) *Nodosilinea* Perkerson & Casamatta was isolated from South China Sea (Perkerson *et al.* 2011); (iii) *Haloleptolyngbya* Dadheech, Mahmoud, Kotut & Krienitz was described from saline-alkaline Lake

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Nakuru in Kenya (Dadheech *et al.* 2012); (iv) *Marileptolyngbya* W.G. Zhou & J. Ling and (v) *Salileptolyngbya* W.G. Zhou were isolated from marine ecosystems of Xincun Bay and South China Sea, respectively (Zhou *et al.* 2018); (vi) '*Amazoninema* Genuário *et al.*' (name invalid) was isolated from Amazon river in Brazil (Genuário *et al.* 2018); (vii) *Euryhalinema* Sandeep Chakraborty & J. Mukherjee and (viii) *Leptoelongatus* Sandeep Chakraborty & J. Mukherjee were identified from estuarine mangrove forest, Indian Sundarbans (Chakraborty *et al.* 2019); and (ix) *Gibliniella* Strunecký & Raabova was reported from freshwater ecosystem of Toolik Lake in Alaska (Strunecký *et al.* 2020). All nine genera of this family possess similar morphological characters: trichomes homocytous, flexuous, short or long, solitary or entangled (some genera exhibit nodules), straight, bent or spirally coiled, uniseriate, constricted at cross walls, with very fine or facultative mucilage sheath, absence of false branching, cells longer than wide, and apical cells conical to cylindrical with rounded apices.

Mangroves are pristine but highly threatened ecosystems present in the coastal regions of several tropical and subtropical countries around the world (Holguin *et al.* 2001). The continuous change in estuarine environmental parameters due to periodic inundation and desiccation is the driver for cyanobacterial metabolic diversity, which can generate novel bioactive compounds and stimulate cyanobacterial speciation. Physical parameters such as temperature, pH, salinity and carbon dioxide levels fluctuate extensively in this intertidal mangrove forest (Neogi *et al.* 2016). The transient environmental conditions create physiological stress on the resident cyanobacteria. The organisms attempt to overcome this stress by altering their physiological functions leading to changed genetic compositions, and hence stimulating evolutionary processes. Environmental factors play a major role in the evolution of new lineages (Hoffmann *et al.* 2020). The diverse morphological variations and ecophysiological characteristics corroborate this evolutionary process (Komárek & Anagnostidis *et al.* 2005). Understandably, fossil records confirm cyanobacterial diversification in intertidal regions worldwide (Demoulin *et al.* 2019). In modern times, novel cyanobacteria have been described from mangrove ecosystems around the world (Alvarenga *et al.* 2015). Following the contemporary approach of polyphasic taxonomy, *Dapisostemon apicaliramis* Hentschke, Sant'Anna & J.R. Johansen (Hentschke *et al.* 2016), *Halotia wernerae, H. longispora* and *H. branconii* (all described in Genuário *et al.* 2015), *Kryptousia macronema* and *K. microlepis* (both described in Alvarenga *et al.* 2017), *Phyllonema aviceniicola* and *Foliisarcina bertiogensis* (both described in Alvarenga *et al.* 2016) were reported from the Brazillian mangrove ecosystems. Furthermore, *Leptochromothrix engenei, Vermifilum ionodolium* and *Ophiophycus aerugineus* (all described in Berthold *et al.* 2020) were also discovered from the mangrove forest of South Florida, USA, and *Okeania hirsuta* Engene, V. J. Paul, Byrum, Gerwick, A. Thor & Ellisman (Engene *et al.* 2013) was identified from Panama. The Sundarbans is the world's largest tidal mangrove forest and a major estuarine ecosystem in Asia (Ellison *et al.* 2001). Alpha-level taxonomic identification of the cyanobacterial diversity of the Indian Sundarbans has just begun. Reports of novel cyanobacterial genera and species like *Leptolyngbya indica* M. Debnath & Bhadury (Debnath *et al.* 2017), *Oxynema aestuarii* Sandeep Chakraborty & J. Mukherjee (Chakraborty *et al.* 2018), *Euryhalinema mangrovii* Sandeep Chakraborty & J. Mukherjee (Chakraborty *et al.* 2019), *Leptoelongatus litoralis* Sandeep Chakraborty & J. Mukherjee (Chakraborty *et al.* 2019), *Synechococcus moorigangae* T. Singh & Bhadury (Singh *et al.* 2019), *Euryhalinema pallustre* Sandeep Chakraborty & J. Mukherjee (Chakraborty *et al.* 2021) and *Aerofilum fasciculatum* Sandeep Chakraborty & J. Mukherjee (Chakraborty *et al.* 2021) indicate the potential of this estuarine ecosystem for further discovery of novel cyanobacteria. In the current investigation, three cyanobacterial strains (S1, S6 and S10) were isolated from the intertidal soil of different regions of the Indian Sundarbans. The strains displayed morphological dissimilarities and resolved as a distinct and separate clade in the 16S rDNA phylogenetic tree from the closest reference genus *Euryhalinema*. So, based on a combination of morphological, ultrastructural, physiological and molecular methods, following the polyphasic approach to identification and in accordance with the International Code of Nomenclature for Algae, Fungi and Plants (ICN), the three test strains are herein proposed as *Almyronema epifaneia gen.* & *sp. nov.*, a new member of the family Nodosilineaceae following the recent classification of cyanobacteria by Strunecký *et al.* (2023).

MATERIAL AND METHODS

Sample collection

Phototrophic biofilms found in Purba Gurguria (21°59.16′N, 88°35.08′E), Maipit (21°55.28′N, 88°32.93′E) and Pathar Pratima (21°47.47′N, 88°22.524′E) islands of the Indian Sundarbans were scraped off from the upper soil surface (Fig. 1). All samples were collected during low tide in November 2018.

Isolation and maintenance

A small portion of each biofilm sample was transferred to a 100-ml Erlenmeyer flask containing 30 ml of ASN-III liquid medium (Rippka *et al.* 1979). Fluorescent lamps (50 µmol photons m^{-2} s^{-1}) were employed to illuminate at a 12:12 h light:dark photoperiod. The flasks were bubbled with 0.1% CO_2 in air and incubated at 25 ± 2°C (Chakraborty *et al.* 2018). After repeated subculture in liquid ASN-III medium, as well as solid (1.5% agar) medium, the unialgal cyanobacterial culture obtained was confirmed by microscopic observation. The cultures were maintained in liquid ASN-III medium in 12:12 h light:dark photoperiod in a culture room at 25 ± 2°C. No significant phenotypic changes were noticed during the continuous subculturing of the strains. Cyanobacterial strains designated S1, S6 and S10 were deposited in the National Collection of Microbial Resource (NCMR), Pune, India.

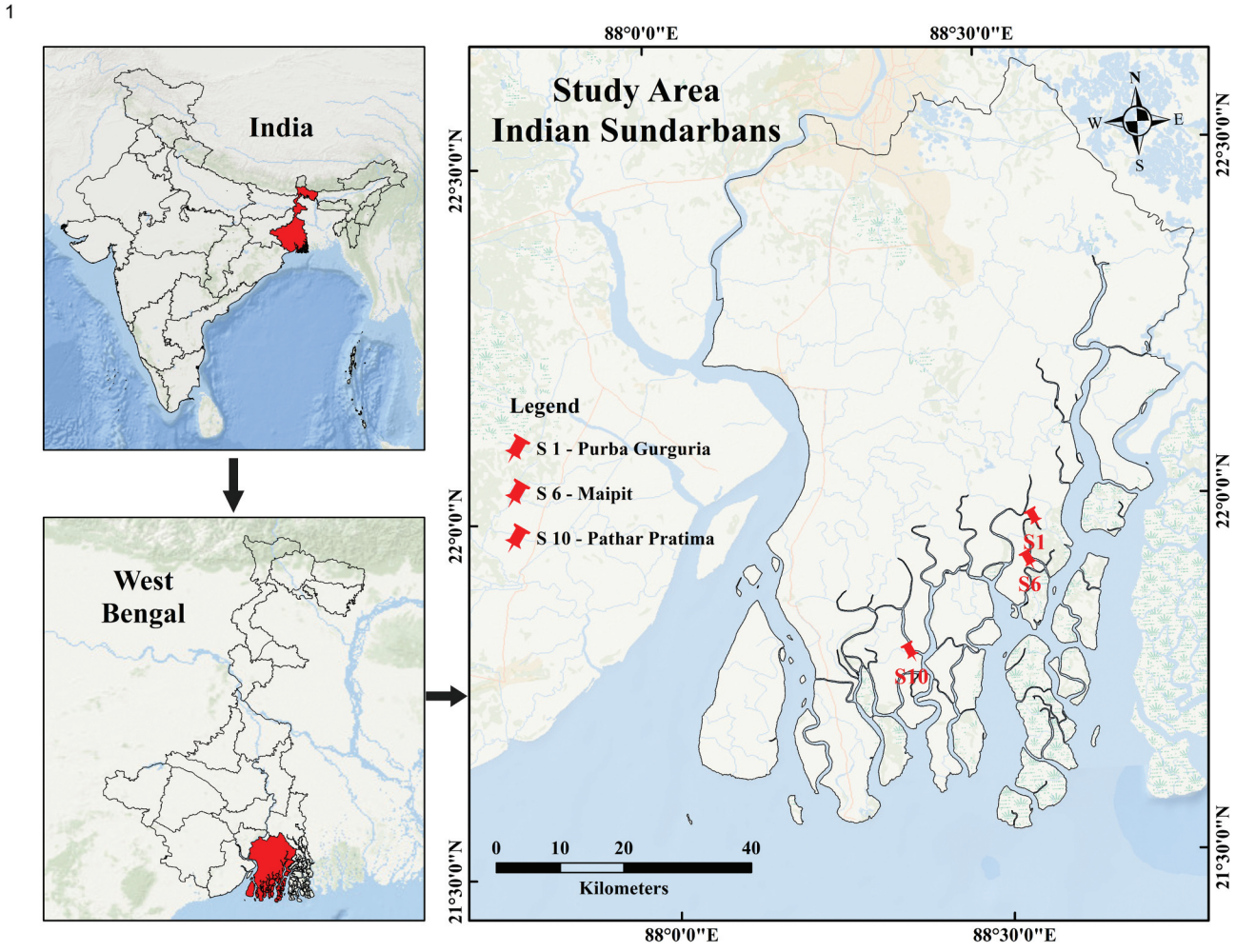


Fig. 1. Map (drawn in ArcGIS 10.8) showing field collection stations in the Sundarbans mangrove forest ecosystem in West Bengal State, India.

Light microscopy

To investigate strain morphology, 8–10-d old pure cultures were examined under 400–1,000× magnification with a DM750 light microscope (Leica Microsystems, Buffalo Grove, Illinois, USA). Photomicrographs of the studied cyanobacterial strains were obtained with an ICC5HD camera attached to the microscope. Measurements of filaments, cell dimensions and apical cell sizes, along with other phenotypic characteristics such as mucilaginous sheaths and motility, were registered by the microscope-associated software (LAS-EZ, Leica Microsystems).

Scanning electron microscopy

About 1 ml of suspension of fresh filaments of 7-d old culture of the three strains was taken in a 1.5-ml centrifugation tube. After microscopic examination the suspension was centrifuged at 8,000× *g* for 5 min (Eppendorf 5810R, Rotor F-34-6-38, Hamburg, Germany) and the cell pellet was washed by vortexing with 0.1 M phosphate buffer (pH 7.8). The tube contents were centrifuged at 8,000× *g* for 5 min and the pellet was further washed with distilled water by pipetting and vortexing. Three percent glutaraldehyde in 0.1 M phosphate buffer saline was applied for fixing the cells for 16 h followed by washing in distilled water. Increasing concentrations of ethanol from 30% for 10 min to 100% for 30 min was used for dehydrating the cells. Grids holding the specimens were taken for observation under a ZEISS EVO 18 scanning electron microscope (Carl Zeiss AG, Oberkochen, Germany) after critical-point-drying.

Transmission electron microscopy

Fresh filaments of 7-d old cultures were taken and centrifuged at 8,000× *g* in 5 min. Phosphate buffer (0.1 M, pH 7.8) was used to wash the obtained pellet by vortexing. After centrifugation at 8,000× *g* for 5 min, the pellet was washed again with distilled water. Pre-fixation was done with 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.8) at 4°C for 5–6 h. Phosphate buffer (0.1 M) was used for rinsing the cyanobacterial cells to wash off the extra fixative. Post fixation of the samples was carried out with osmium tetroxide (1%) for 60 min. Cells were dehydrated with a graded ethanol series. Infiltration and embedding were done in Araldite CY 212 (Agar Scientific, Stansted, UK) for section cutting. The resin blocks were polymerized using heat treatment overnight at 50°C and subsequently 2 d at 60°C, followed by thin sectioning. Uranyl acetate and lead citrate were applied for contrasting the sections, which were finally

observed with a Tecnai G20 transmission electron microscope (FEI, Eindhoven, Netherlands).

DNA extraction and PCR amplification

Cells of 10-d old unialgal cyanobacterial cultures were mechanically broken using a glass homogenizer. GeneJET™ Genomic DNA purification Kit (REF Code K0721, Thermo Fisher Scientific, Waltham, Massachusetts, USA) was used for genomic DNA extraction. The extracted DNA samples were stored at –20°C and purity of the DNA was checked by gel electrophoresis in 1.5% agarose gel. The 16S rRNA gene and the 16S–23S ITS rRNA gene region were amplified by the PCR using a Master cycler Nexus Gradient PCR (Eppendorf, Hamburg, Germany). For amplification of 16S rRNA gene, cyanobacterial specific forward primer CYA106F (5′-CGGACGGGTGAGTAAC GCGTGA-3′; Nübel *et al.* 1997) and universal reverse primer 1492R (5′-ACCTTGTTACGACTT-3′; Lane *et al.* 1991) were used, while 16S–23S ITS regions were amplified using forward primer 16SF (5′-TGTACACACCGGCCCGTC-3′) and reverse primer 23SR (5′-CTCTGTGCCTAGGTATCC-3′; Iteman *et al.* 2000). PCR reaction volumes were 25 µl containing 2 µl of DNA, 2.5 µl of 10X PCR buffer, 0.75 µl of $MgCl_2$ (50 mM), 0.5 µl of 10 mM dNTP mix, 1.25 µl of each primer (10 µM), 0.5 µl of 5 U/l Taq DNA polymerase (Catalog No. 10342-053, Invitrogen, Waltham, Massachusetts, USA) in 16.25 µl of RNase free water (Catalog No. 10977-015, Invitrogen). The reaction conditions applied for amplification of the 16S rRNA gene were: initial denaturation for 5 min at 94°C followed by 30 cycles of 1 min at 94°C; 1 min at 50°C, 2 min at 72°C and final extension for 10 min at 72°C. The 16S–23S ITS region was amplified applying the reaction conditions: initial denaturation for 5 min at 95°C followed by 30 cycles of 30s at 95°C; 15s at 58°C; 40s at 72°C and a final elongation step for 5 min at 72°C. Agarose gel (1.5%) was used for separation of the amplified PCR products. The gel was stained with ethidium bromide, and PCR fragments of suitable size were acquired from the agarose gel and stored at 4°C. PCR fragments were purified by Gene JET Extraction Kit (Cat. No. K0691, Thermo Scientific). Purified PCR products obtained from 16S rDNA and ITS regions were inserted into pGEM®-T Easy Vector plasmid using pGEM®-T Easy Vector System I (Cat No. A1360, Promega Corp., Madison, Wisconsin, USA) cloning kit. The plasmid vector used for insertion of the sequence was available with the cloning kit. Competent JM109 cells were used for the transformation. Recombinant colonies were selected by blue-white screening method and plasmids were recovered from the recombinant white colonies with GeneJET Plasmid Miniprep Kit (Cat No. K0502, Thermo Fisher Scientific). Clones containing recombinant plasmids were digested with Not I restriction enzyme (Cat No. ER0591, Thermo Fisher Scientific) and run on 1.5% agarose gel electrophoresis to estimate the size of the inserts. The 16S rRNA and the 16S-23S ITS rRNA genes of the recombinant plasmids were sequenced. Primers pUC/M13 forward (5′-CGCCAGGGTTTTCCCAG TCACGAC-3′) and pUC/M13 reverse (5′-CAGGAAAC AGCTATGAC-3′) were applied for sequencing of the mentioned genes. Sequencing was carried out using the automated Genetic Analyzer 3730Xl DNA sequencer (Applied Biosystems, Waltham, Massachusetts, USA). The resulting sequences of the 16S rRNA gene and the 16S–23S ITS rRNA regions of the three test strains were deposited in GenBank and their accession numbers are provided in Table 1.

16S rRNA gene sequence analysis and construction of phylogenetic trees

All 16S rDNA sequences shorter than 1,500 bp were gathered from different databases and articles. The 16S rDNA sequences of all nine genera of family Nodosilineaceae, namely *Nodosilinea, Euryhalinema, Haloleptolyngbya, Halomicronema, Amazoninema, Leptoelongatus, Marileptolyngbya, Salileptolyngbya* and *Gibliniella*, as well as genera *Cymatolege* Konstantinou & Gkelis, *Rhodoploca* Konstantinou & Gkelis and *Leptothoe* Konstantinou & Gkelis, of family Cymatolegaceae, and additionally genera *Persinema* Heidari & Hauer and *Lagosinema* Akagha & J.R. Johansen, of family Persinemataceae, were collected from CyanoSeq v1.2 following Lefler *et al.* (2022), Strunecký *et al.* (2023) and various research articles present in the CyanoDB2 website. Sequences greater than 1,500 bp were trimmed in BioEdit v7.0.5.3 (Hall *et al.* 1999). Larger sequences were trimmed from the 3' end by identifying the marker sequence 'TCACCTCCT'. All 16S rDNA sequences used in construction of the phylogenetic tree are available as a spreadsheet (Table S1). The longest 16S rRNA gene sequence was 1,487 bp, of *Synechococcus elongatus* (Nägeli) Nägeli, and the shortest sequence was 1,009 bp, of *Leptolyngbya* sp. All collated sequences were manually trimmed in BioEdit software, following which 146 sequences were aligned in Clustal W 1.5 (Larkin *et al.* 2007) in the MEGA 6 software (Tamura *et al.* 2013). The aligned 16S rDNA sequence matrix was 1,789 bp long and formed the basis on which the phylogenetic tree was constructed (Table S2). Another phylogenetic tree was constructed with the smaller aligned 16S rDNA sequence matrix of 1,496 bp length (Table S4). However, for proper placement of all the families of Nodosilineales in the 16S rRNA phylogenetic tree, the final tree generated with 1,789 bp long nucleotide matrix was chosen.

The 16S rRNA gene sequences of the isolates under investigation (S1, S6, S10) were examined by the Basic Local

Table 1. List of gene sequences of the experimental cyanobacterial strains submitted in NCBI GenBank with their accession numbers.

Strain name	*Almyronema epifaneia*	*Almyronema epifaneia*	*Almyronema epifaneia*
Strain number	S1	S6	S10
NCBI GenBank Accession numbers for 16S rDNA	OP740688	OP740689	OP740690
NCBI GenBank Accession numbers for 16S-23S ITS rDNA	OP740693	OP740694	OP740695
Length of 16S rDNA (bp)	1377	1385	1408
Length of 16S-ITS (bp)	628	636	628

Alignment Search Tool (BLAST) and contrasted with the other sequences available in the robust database of the National Center for Biotechnology Information (NCBI GenBank). A consensus phylogenetic tree was constructed based on the similarities of the 16S rRNA gene sequences of the three test strains with sequences of other cyanobacterial taxa. A phylogenetic tree was constructed with 146 OTUs, with *Gloeobacter violaceus* Rippka, Waterbury & Cohen-Bazire as outgroup. Additional sequences of many recently described genera of the families Nodosilineaceae, Leptolyngbyaceae, Oculatellaceae and Prochlorotrichaceae were also obtained. The multiple sequence alignment (MSA) of these OTUs was carried out in the Clustal W program (Larkin *et al.* 2007). Bayesian Inference (BI), Maximum Likelihood (ML) and Maximum parsimony (MP) analyses were performed for 16S rRNA gene sequences of the 146 OTUs with a maximum of 1,789 characters, including nucleotides and gaps. Model test was run on jModel Test 0.1.1 following Posada *et al.* (2008). The best fit model applied for ML and BI analyses was GTR+I+G. A ML tree was constructed using the W-IQ-TREE webserver (Trifinopoulos *et al.* 2016) as well as PhyML 3.0 (Guindon *et al.* 2010) and RAxML T-REX (Boc *et al.* 2012) with 1,000 replications. A MP tree was run on MEGA program package v6.0 (Tamura *et al.* 2013) with 1,000 replications to evaluate the relative support of the branches. A BI analysis was executed by submitting the alignment to MrBayes v3.2.7 (Ronquist *et al.* 2012) applying a GTR+G+I model of nucleotide substitutions. Two independent runs with four chains were executed and each of the two runs was concurrently conducted for 50 million Markov Chain Monte Carlo (MCMC) generations. The initial 25% samples were discarded as burn-in. The final average standard deviation of split frequencies was 0.00119. The potential scale reduction factor (PSRF) value for all the parameters was 1.00, signifying the statistical attainment of the convergence of MCMC chains (Gelman & Rubin 1992). The BI was also performed in NGPhylogeny.fr webserver (Lemoine *et al.* 2019) with two runs of four Markov chains and each of the two runs was executed for 50 million generations. The resultant tree was visualized in the iTOL webserver (Letunic *et al.* 2006). The bootstrap values for both ML and MP analysis were mapped on the BI analysis tree using Adobe Illustrator. The divergence rates among the sequences of 16S rDNA and 16S–23S ITS with other reference strains were studied by computation of the uncorrected p-distances in MEGA 6.0. The output data was used to calculate the dissimilarity matrix (as percentage) (100 × p-distance) for ITS and similarity matrix (as percentage) [100 × (1 – p)] for 16S rDNA (Chakraborty *et al.* 2021).

Analysis of 16S–23S ITS secondary structures

The sequences of 16S–23S ITS and 16S regions of the experimental strains were also used for the taxonomic identification of the strains at species level. The complete ITS sequences of the three studied strains were aligned with their respective reference strains and all the conserved regions, as well as variable regions, were identified (Table S3). The sequences of ITS region of the reference strains were obtained from GenBank. The secondary structures were generated applying the M-fold web server, v2.3 (Zuker *et al.* 2003) under ideal conditions of untangled loop fix with the fixed default temperature 37°C. Structures were redrawn in Adobe Illustrator 2020 and fundamental ITS regions like D1-D′ helix, Box-B and V3 helices were compared with equivalent secondary structures of the reference strains. Also, the sequence length of different regions of ITS were identified and compared with the reference strains.

RESULTS

Taxonomy

Order Nodosilineales Strunecký & Mareš
Family Nodosilineaceae Strunecký & Mareš

Almyronema A.R. Roy & J. Mukherjee *gen. nov.*

DESCRIPTION: Mat-like biofilm on upper surface of soil, light green in colour. Uniseriate, homocytous filament; sheath fine, colourless; trichomes flexuous, cells longer than wide, conical-shaped terminal cell, constriction at cross walls. Heterocytes and akinetes absent. Parietal thylakoids, phosphate granules near cell walls. Trichomes motile with gliding movement. Reproduction via hormogonia.

DIAGNOSIS: Filaments 0.6–1.1 μm wide, thinner than those of members of family Leptolyngbyaceae (1.5–2.5 μm). Strains occur as single filaments with fine mucilaginous sheath indistinguishable from those of Leptolyngbyaceae. However, the flexuous trichomes, cells longer than wide, presence of constriction at cross walls, and presence of polyphosphate granules near cell walls justify the morphological association of the strain with the family Nodosilineaceae (Strunecký *et al.* 2023). The presence of conical shaped apical cell, mucilaginous sheath and gliding movements differentiate the strains morphologically from the genus *Euryhalinema*. The phylogenetic position in the 16S rDNA phylogenetic tree as a distinct clade significantly distant from the closest genus *Euryhalinema* supports the description of a new genus.

TYPE SPECIES: *Almyronema epifaneia* A.R. Roy & J. Mukherjee.

ETYMOLOGY: Generic name derived from Greek *almyros*, salty, referring to the saline intertidal environment from where strains were isolated; and Greek *nema*, thread.

Almyronema epifaneia A.R. Roy & J. Mukherjee *sp. nov.*

Figs 2–5

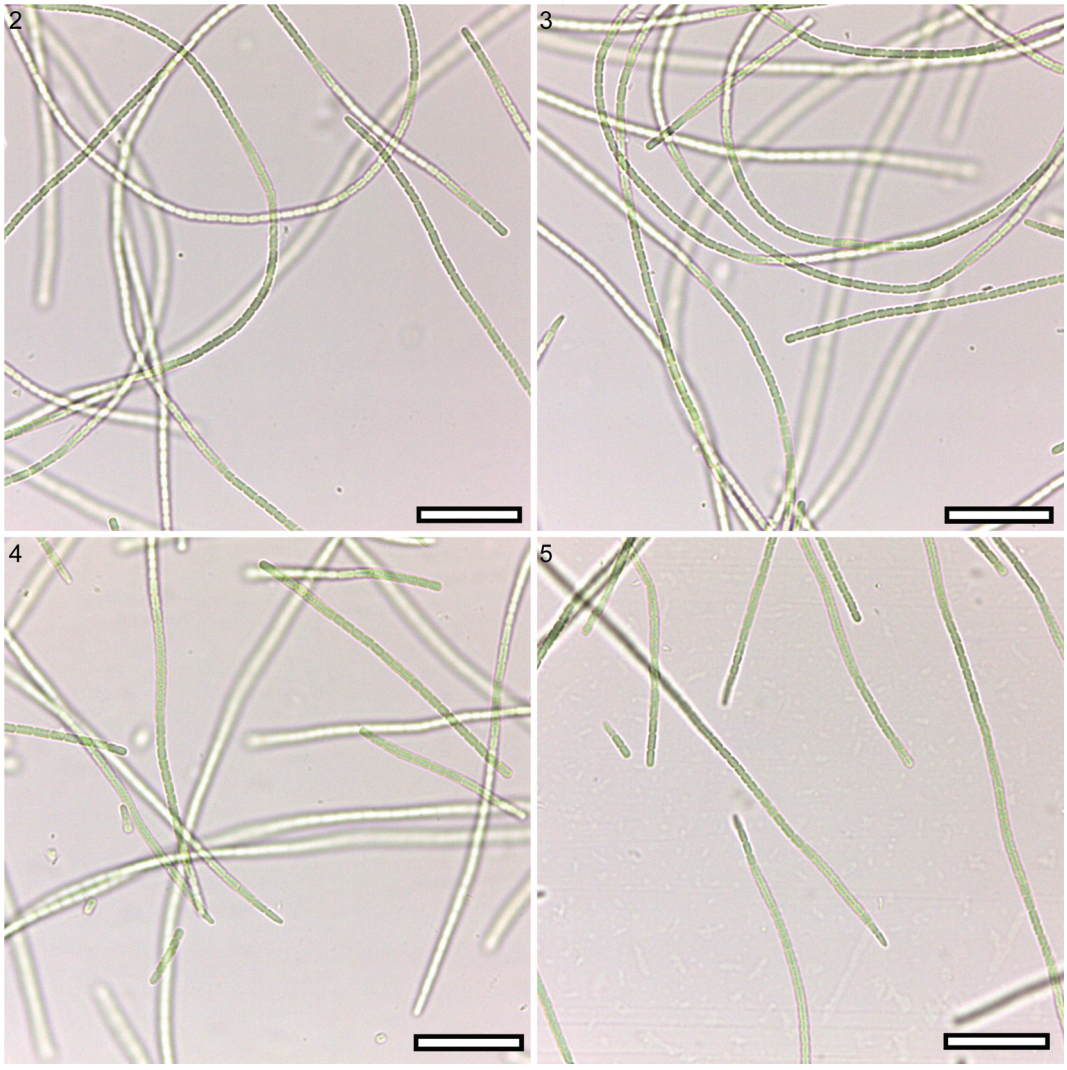
DESCRIPTION: Homocytous filament with thin trichomes, cells 1.5–2.5 μm long and 0.6–1.1 μm wide. Fine colourless mucilaginous sheath present. Filaments light green in colour. Filaments motile. Parietal thylakoids. One large cyanophycin granule present in almost every cell. Large number of polyphosphate granules present along the cell wall.

HABITAT: Intertidal soil of mangrove forest.

ETYMOLOGY: Specific epithet from Greek noun *epifaneia*, in the sense of 'visible surface', alluding to the occurrence of the species on the surface of intertidal soil of the mangrove forest.

HOLOTYPE: Portion of strain S6 (accession number MCC 5313) preserved in a metabolically inactive state by cryopreservation, deposited in National Centre for Microbial Resource (NCMR), Pune, India.

PARATYPE: Portion of strain S10 (accession number MCC 5312) preserved in a metabolically inactive state by cryopreservation, deposited in National Centre for Microbial Resource (NCMR), Pune, India.



Figs 2–5. *Almyronema epifaneia gen. & sp. nov.*, light microscopy. Scale bars = 5 µm.
Fig. 2. Trichomes of strain S1 showing conical apical cells.
Fig. 3. Straight and wavy trichomes of strain S6.
Fig. 4. Filaments of strain S10.
Fig. 5. Thin, long filaments of strain S10.

TYPE LOCALITY: Maipit Island (21°55.28′N, 88°32.93′E) of Indian Sundarbans, South 24 Parganas district, West Bengal State, India.

Morphological characterization

The strains (S1, S6 and S10) described in this paper showed adequate growth in ASN-III medium in the laboratory conditions used. The strains were compared to each other as well as individually with the most similar of the described species, *Euryhalinema mangrovii*, following the polyphasic approach. All the strains formed unbroken thin, bluish-green biofilm mats attached to the basal surface of Erlenmeyer flasks, and all showed phenotypic characters similar to *E. mangrovii*. Light microscopy of 10-d old cultures revealed common features for the three strains, like a thin filamentous thallus forming a pale to bluish-green biofilm. Filaments were unbranched, long, straight or wavy with thin mucilaginous sheath. Trichomes were uniseriate, thin, with cells much longer than wide, cell length ranging 1.6–2.5 µm in length and 0.7–1.1 µm in width. Trichomes were non-heterocytous, cylindrical and showed motility. Apical cells of the trichomes were conical (Figs 2–5). False branching was absent in the filaments of the three strains. Aerotopes and akinetes were absent. Cells showed prominent constrictions in their cross walls. Based on 16S rRNA gene sequence BLAST search in GenBank, *E. mangrovii* (a member of family Nodosilineaceae) was identified as the closest reference strain. Therefore, the morphometric analysis was performed by comparing the strains S1, S6 and S10 with *E. mangrovii* as well as with the other closest members of the family (Table 2). Scanning electron microscopic analysis confirmed the morphological features revealed by light microscopy (Figs 6–9).

Ultrastructural studies

Transmission electron microscopic analysis of the three strains showed ultrastructural features of the cells compatible

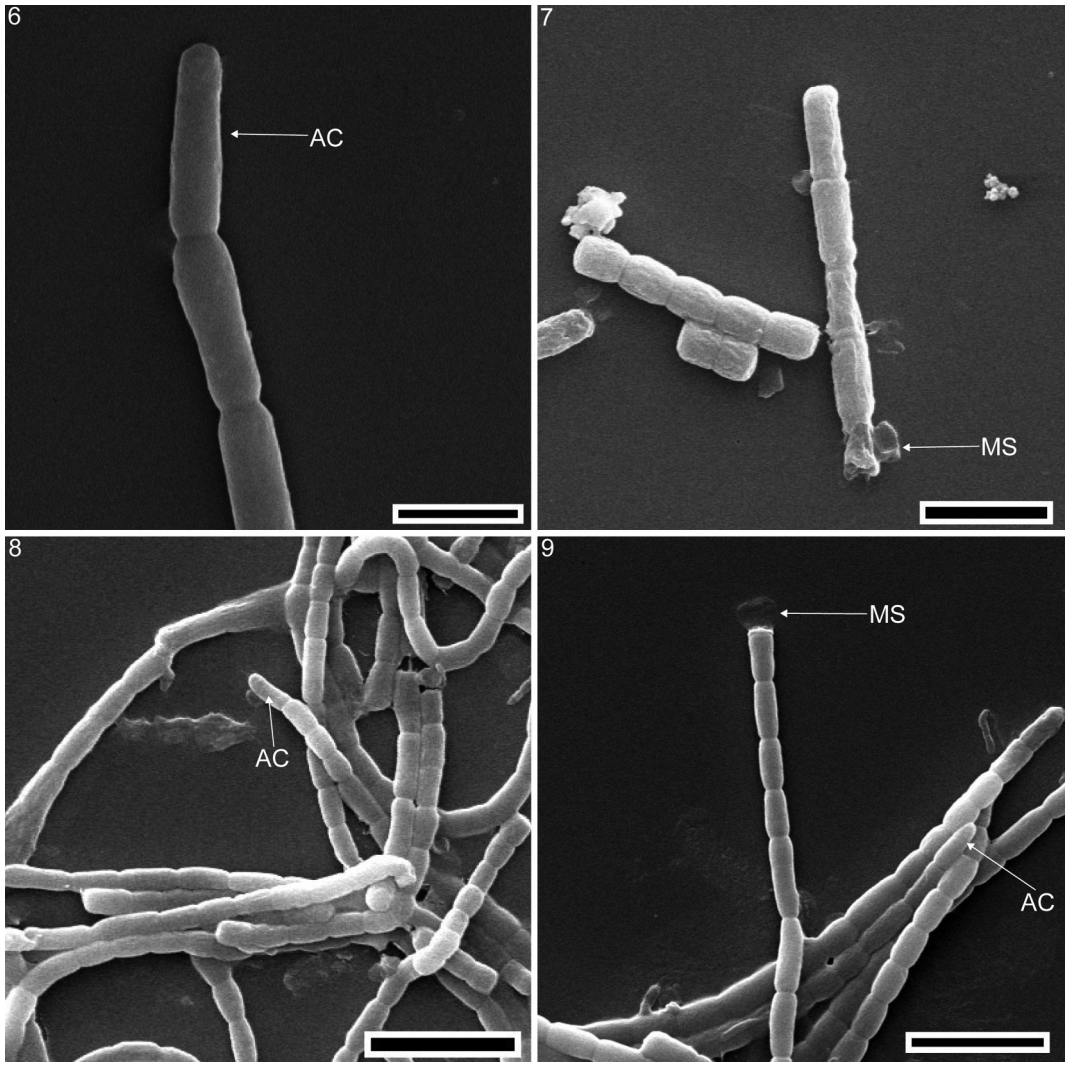
Table 2. Comparative analysis of the morphological features of test strains of *Almyronema epifaneia* (S1, S6, S10) with other different genera of Nodosilineaceae family. Characteristics of previously described genera *Euryhalinema mangrovii* and *Euryhalinema pallustre* were obtained from Chakraborty *et al.* (2019, 2021); *Euryhalinema epiphyticum* from De Araújo *et al.* (2022); *Gibliniella alaskaensis* from Strunecký *et al.* (2020); *Salileptolyngbya diazotrophica* from Zhou *et al.* (2018); *Nodosilinea nodulosa* from Perkerson *et al.* (2011) and *Nodosilinea chupicuarensis* from Vázquez-Martínez *et al.* (2018).

Strains/Features	Thallus	Mucilage sheath	Motility	Constrictions at cross walls	Cell length (μm)	Cell width (μm)	False branching	Cell form	Trichome apex	Habitat
Almyronema epifaneia (S1)	Bluish green in color, fine mat	Thin, colorless sheath present	Yes	Prominently constricted	1.6-2.5	0.7-1.1	Absent	Distinctly much longer than wide	Conical	Upper surface of estuarine soil salinity ranging from 1.6-1.8%
Almyronema epifaneia (S6)	Bluish green in color, fine mat	Thin, colorless sheath present	Yes	Prominently constricted	1.4-2.3	0.6-1.0	Absent	Distinctly much longer than wide	Conical	Upper surface of estuarine soil salinity ranging from 1.6-1.8%
Almyronema epifaneia (S10)	Bluish green in color, fine mat	Thin, colorless sheath present	Yes	Prominently constricted	1.3-2.5	0.6-1.1	Absent	Distinctly much longer than wide	Pointed	Upper surface of estuarine soil salinity ranging from 1.6-1.8%
Euryhalinema mangrovii	Pale bluish green color mats	Absent	No	Prominent constrictions	1.1-1.6	0.4-0.6	Absent	longer than wide	Rounded, not attenuated	Intertidal area (estuarine) with salinity ranging from 1.7-1.8%
Euryhalinema pallustre	Thin biofilm of light greenish color	Absent	No	Prominent constrictions	1.25-2.6	0.4-0.5	Absent	longer than wide	Rounded not attenuated	Intertidal area (estuarine) with salinity ranging from 1.7-1.8%
Euryhalinema epiphyticum	Pale bluish green color mats	Absent	No	Slightly constricted	1.6-4.3	0.8-1.0	Absent	Cylindrical, longer than wide	Rounded not attenuated	Marine, epiphyte on benthic red macroalgae *Bostrychia mantagne*
Salileptolyngbya diazotrophica	Blue green mat forming	Firm, multilayer	No	Constricted	1.53-2.37	0.93-1.44	Absent	Cylindrical	Rounded	Isolated from marine plantonic organism
Gibliniella alaskaensis	Thin yellow-green to green	Thin, firm clear	Intensely motile	Slightly constricted	0.9-3.6	0.7-1.0	Absent	Cylindrical, long, wavy	Rounded without calyptra	Freshwater, crust on stone
Nodosilinea nodulosa	Greenish mat	Occasionally present	No	Distinctly constricted	1.1-1.25	0.7-0.9	Occasionally	Mostly isodiametric	Barrel Shaped	Marine (planktonic tow) from depth 10 m
Nodosilinea chupicuarensis	Blue-green patina, embedded in mucilage	Present	No	Prominent constrictions	1.1-1.3	0.9-1.2	Absent	Barrel shape, occasionally disc or isodiametric	Dome shaped, sometimes elongated	Stone surface, epilithic

with the characteristics of the family Nodosilineaceae. Cellular structures like sheath, cell wall, constrictions at the cross walls, thylakoid arrangement and presence of different cell inclusions, were clearly visible in the ultrastructural analysis (Figs 10–15). The arrangement of thylakoids was parietal, which is compatible with the evolutionary lineage of order Nodosilineales. Ultrastructural analysis further depicted our experimental strains to possess different types of cell inclusions, such as polyphosphate granules and lipid β granules. The presence of a large cyanophycin granule in almost every cell is identical to what was reported in *Euryhalinema* (Chakraborty *et al.* 2019, 2021). Conical apical cells and mucilaginous sheaths were clearly visible.

Molecular and phylogenetic analyses

Partial 16S rRNA gene sequences of S1, S6 and S10 having lengths 1,377, 1,385 and 1,408 bp, respectively were generated by PCR-cloning methods described above. The sequences were compared by BLAST in GenBank. Four anonymous and two sequences of uncultured cyanobacteria emerged as the six closest relatives of the three strains. The nearest position was occupied by *Euryhalinema mangrovii* (MK402979), of an established genus with about 94.30%, 94.33% and 92.71% genetic similarity with strains S1, S6 and S10, respectively. This similarity was lower than the commonly applied cut off value (less than 94.5%) for differentiating two genera according to Yarza *et al.* (2014). Morphological characters, as well as 16S rDNA similarity, suggested the studied strains to be members of family Nodosilineaceae. The species *Euryhalinema mangrovii* (Chakraborty *et al.* 2019) was selected as the reference strain given its closest match in the BLAST analysis. Other species of *Euryhalinema*, i.e. *E. pallustre* (Chakraborty *et al.* 2021) and *E. epiphyticum* V. L. Araújo, Schnadelbach, J.M.C. Nunes & T.A. Caires (de Araújo *et al.* 2022), and other related genera of the Nodosilineaceae were also considered in the 16S rDNA phylogenetic analysis. A consensus phylogenetic tree was constructed including the three studied strains and their closest relatives, as well as other species of families Nodosilineaceae, Prochlorotrichaceae, Oculatellaceae and Leptolyngbyaceae to establish the evolutionary relationship. The generated

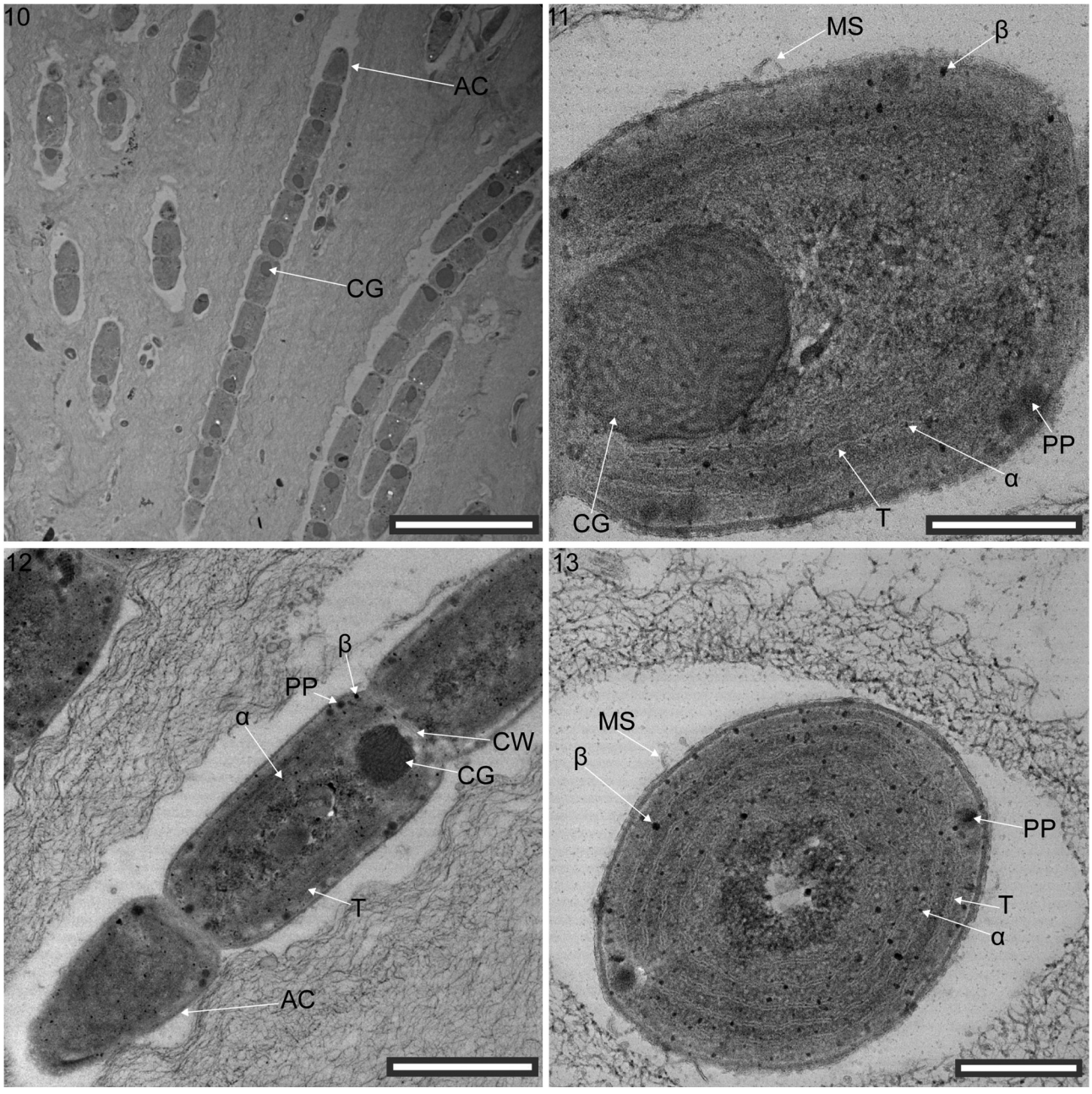


Figs 6–9. *Almyronema epifaneia gen. & sp. nov.*, SEM.
Fig. 6. Portion of filament of strain S1 showing apical cell (AC) morphology and cross wall constrictions. Scale bar = 1 µm.
Fig. 7. Portion of filament of strain S1 showing mucilaginous sheath (MS). Scale bar = 3 µm.
Fig. 8. Intertangled filaments of strain S6 showing apical cell (AC). Scale bar =5 µm.
Fig. 9. Single straight filament of strain S6 showing mucilaginous sheath (MS) and cell surface (smooth). Scale bar = 5 µm.

phylogenetic tree (Fig. 16) confirmed that strains S1, S6 and S10 clustered together in a well-supported clade within the family Nodosilineaceae. This clade was separated from its nearest relative genus *Euryhalinema* as well as from clades of other genera, and formed a distinct novel phylogenetic lineage according to BI, ML and MP analyses. In spite of the 16S rRNA gene sequence of *Almyronema* being most similar to *Euryhalinema* (94.1%–94.4%), the genera *Gibliniella* and *Salileptolyngbya* emerged as the closest relatives of the *Almyronema* clade in the phylogenetic tree of Fig. 16, displaying 92.2%–92.7% and 91.9%–92.1% gene sequence similarity, respectively (Table 3). Node support was confirmed in the BI by high posterior probability whereas ML and MP analyses were substantiated by bootstrap values. All three trees displayed close matching of topologies; only the BI tree is presented in Fig. 16, which includes support values from all three analyses. Each tree generated from different platforms demonstrated high bootstrap value (100/100) for the *Almyronema epifaneia* clade within the Nodosilineaceae, supporting the description of a genus separate from *Euryhalinema*. The p-distance analysis was undertaken for establishing the inter-species relationship between the three strains and the closest members of Nodosilineaceae. Similarities varied from 94.1% to 94.4% for 16S rDNA data (Table 3) and the dissimilarities (as percentage) of the 16S–23S ITS sequences varied from 22.2% to 24.5% (Table 4).

Analysis of 16S–23S ITS secondary structures

Experimental strains possessed similar ITS sequences with less than 1% distance from a single operon having the tRNA genes $tRNA^{ile}$ and $tRNA^{Ala}$. Folded secondary structures of the D1-D′ helix, Box-B helix and V3 helix regions of strains S1, S6 and S10 were obtained and compared with the phylogenetically closest strains evident from the 16S rRNA gene sequences. The closest relatives as revealed by the 16S rDNA phylogenetic tree were *Gibliniella* and *Salileptolyngbya*. The ITS sequences of other members of Nodosilineaceae showed



Figs 10–13. *Almyronema epifaneia gen.* & *sp. nov.*, TEM. AC, apical cell; α, glycogen α granules; β, lipid β granules; CG, cyanophycin granules; CW, cross wall; MS, mucilaginous sheath; PP, polyphosphate granules; T, thylakoid bands.

Fig. 10. Section through filaments of strain S1 showing the whole filament with different cell inclusions, and apical cell (AC). Scale bar = 5 µm.

Fig. 11. Cross section of filament of strain S1 showing single cell with different cell inclusions. Scale bar = 250 nm.

Fig. 12. Longitudinal section of filament of strain S6 showing different cell inclusions. Scale bar = 700 nm

Fig. 13. Cross section of filament of strain S6 showing different cell inclusions. Scale bar = 300 nm.

distinct variable secondary structures as well as conserved domains when contrasted with our *Almyronema* strains. Detailed results are presented in Table 5. Additionally, the secondary structures of D1-D1′ helix, Box-B helix and V3 region were folded and characterized for all the strains and are depicted in Figs 17, 18.

The lengths of D1-D1′ helices of the ITS regions of the three *Almyronema* strains were 59 nt long (Fig. 17) and contained a large terminal loop of 9 nt, one bilateral and two unilateral bulges. All strains possessed the same basal sequences 5′-GACC::CUGG-3′ in the D1-D1′ helix. Box-B helix domain of the ITS secondary structure of all three strains was 41 nt long with a small terminal loop (4 nt) and a bilateral bulge (Fig. 17). The basal sequence of Box-B domain was 5′-AGCA::UCGU-3′ which was similar for all *Almyronema* strains. The V3 regions of the ITS sequences were 28 nt long with a terminal loop (5 nt) and one unilateral loop. The basal sequence of V3 region was 5′-GUC::UAG-3′ which was identical in S1, S6 and S10 (Fig. 18). Overall comparison of all regions of the *Almyronema* strains with the close relatives and other species of Nodosilineaceae showed dissimilar features.

Table 3. Similarities (as percentage) for strains of *Almyronema epifaneia* (S1, S6 and S10) with closest BLAST results based on p-distance analysis of the 16S rRNA gene sequence data.

	Strains	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	***Almyronema epifaneia* S1**																								
2	***Almyronema epifaneia* S6**	**99.3**																							
3	***Almyronema epifaneia* S10**	**99.3**	**99.2**																						
4	Prochlorotrichaceae cyanobacterium Ecp02	98.9	99.2	98.8																					
5	Prochlorotrichaceae cyanobacterium Ecp01	98.9	99.2	98.8	0																				
6	Leptolyngbyaceae cyanobacterium MACC32	98.1	98.4	98	98.6	98.6																			
7	Uncultured bacterium clone ZZ16S2	98.8	99.1	98.7	99.7	99.7	98.7																		
8	Uncultured cyanobacterium clone PRTBB8722	98.9	99.2	98.8	99.8	99.8	98.6	99.7																	
9	Filamentous cyanobacterium MACC31	95.4	95.8	95.3	96.4	96.4	95.6	96.5	96.4																
10	*Euryhalinema mangrovii* AP9F	94.1	94.4	94.1	94.3	94.3	94.7	94.3	94.3	91.4															
11	*Euryhalinema pallustre* AP3	93.3	93.6	93.3	93.5	93.5	93.9	93.5	93.5	90.6	99.3														
12	*Euryhalinema epiphyticum* ALCB 125750	93.9	94.2	93.8	94.1	94.1	94.4	94.1	94.1	91.1	99.8	99.1													
13	*Leptolyngbya tenuis* MACC28	92.9	93.3	92.8	93.4	93.4	93.2	93.5	93.4	91.5	93.3	92.5	93.1												
14	*Halomicronema hongdechloris* C2206	92.5	92.5	92.1	92.6	92.6	92.6	92.7	92.7	90.1	93.4	92.7	93.2	92.7											
15	*Amazoninema brasiliense* CMAA1602	92.2	92.6	92.2	92.7	92.7	92.5	92.5	92.7	90	94.3	94.2	94.1	94.7	92.4										
16	*Leptolyngbya subtilissima* EcFYyyy700	92.1	92.3	91.9	92.3	92.3	92.2	92.4	92.3	89.9	93.6	92.8	93.4	98.9	92.1	92.2									
17	*Salileptolyngbya diazotrophica* SCSIO 43686	91.9	92.1	91.6	92.5	92.5	92.6	92.6	92.5	90.5	93	92.2	92.8	93	93.8	92.3	91.5								
18	*Marileptolyngbya sina* SCSIO T2	91.7	91.9	91.5	91.6	91.6	91.6	91.5	91.6	89	92.7	92.6	92.5	93.2	94.3	94	93.6	91.8							
19	*Leptoelongatus litoralis* AP25	91.2	91.6	91.2	91.5	91.5	91.2	91.5	91.5	89.3	95.5	94.7	95.3	92	92.1	91.8	91.4	92.7	91.6						
20	*Nodosilinea chupicuarensis* PC471	91.5	91.7	91.3	91.7	91.7	91.6	91.8	91.7	89.2	93.2	93.4	93	91.6	91.7	92.9	98.7	90.6	93.4	91					
21	*Nodosilinea nodulosa* LEGE 06104	91.3	91.6	91.2	91.5	91.5	91.6	91.7	91.6	88.9	93.3	93.5	93.1	91.1	91.5	92.2	98.4	91.1	92.8	91.4	98.9				
22	*Nodosilinea ramsarensis* KH-S S2.6	91.3	91.6	91.2	91.5	91.5	91.6	91.7	91.6	88.9	93.3	93.5	93.1	91.1	91.5	92.2	98.4	91.1	92.8	91.4	98.9	99.8			
23	*Gibliniella alaskaensis* L31	92.3	92.7	92.2	92.9	92.9	92.8	92.7	93	90.5	92.9	92.8	92.7	92.7	91.7	91.8	98.8	91.7	92.5	91.4	93.1	92.3	92.3		
24	*Haloleptolyngbya alcalis* KR2005/106	89.5	89.5	89.1	89.9	89.9	90	90	90.1	88.6	90.6	90.3	90.4	92.2	93.4	90.5	94	91.4	92.9	90.1	94.4	93.9	93.9	93.1	

Table 4. Dissimilarities (as percentage) for strains of *Almyronema epifaneia* (S1, S6, S10) with closest relative strains based on p-distance analysis of 16S-23S ITS gene sequence data.

	Strains	1	2	3	4	5	6	7	8	9	10	11	12
1	***Almyronema epifaneia* S1**												
2	***Almyronema epifaneia* S6**	**0.0**											
3	***Almyronema epifaneia* S10**	**0.0**	**0.0**										
4	*Euryhalinema mangrovii* AP9F	22.2	22.2	22.2									
5	*Euryhalinema pallustre* AP3	24.5	24.5	24.5	3.9								
6	*Salileptolyngbya diazotrophica SCSIO 43686*	22.7	22.7	22.7	20.5	21.6							
7	*Gibliniella alaskaensis L31*	8.8	8.8	8.8	18.9	21.7	22.1						
8	*Leptolyngbya tenuis* MACC28	25.2	25.2	25.2	19.4	21.1	23.9	23.9					
9	*Halomicronema hongdechloris* C2206	21	21	21	21.6	21.6	16.8	25.6	26.4				
10	*Nodosilinea chupicuarensis* PC471	19.4	19.4	19.4	11.8	14.9	17.8	17.8	22.2	21			
11	*Nodosilinea nodulosa* UTEX 2910	19.4	19.4	19.4	13.7	14.3	17.3	19.4	21.7	18.8	2.5		
12	*Nodosilinea ramsarensis* KH-S S2.6	20.6	20.6	20.6	11.7	12.2	17.8	19.4	22.2	18.9	5.6	3.4	

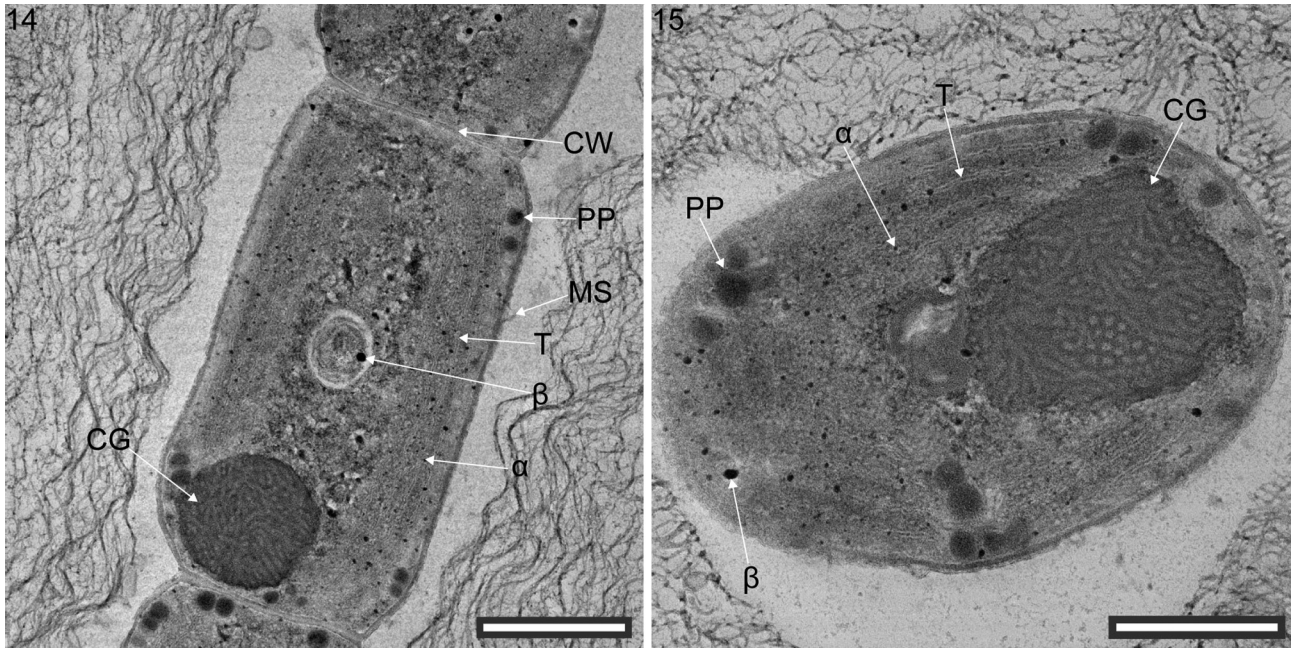
Table 5. Comparison of the nucleotide lengths of the ITS regions of *Almyronema epifaneia* strains (S1, S6 and S10) with *Euryhalinema mangrovii* and other related genera of family Nodosilineaceae. Data of reference strain *Euryhalinema mangrovii* and species of Nodosilineaceae family were obtained from Chakraborty *et al.* (2019, 2021), Strunecký *et al.* (2020), Zhou *et al.* (2018), Chen *et al.* (2012), Perkerson *et al.* (2011) and Vázquez-Martínez *et al.* (2018). Bold values indicate differences with the test strains.

Strains	Leader	D1-D1′helix	Spacer + D2 with spacer	D3 with spacer	$tRNA^{ile}$ gene	V2 spacer	$tRNA^{Ala}$ gene	Pre-Box B spacer	Box B	Post Box B spacer	Box A	D4	V3	D5
Almyronema epifaneia S1	8	**59**	**35**	**12**	74	**5**	73	**36**	**41**	19	11	**7**	**28**	**15**
Almyronema epifaneia S6	8	**59**	**35**	**12**	74	**5**	73	**36**	**41**	19	11	**7**	**28**	**15**
Almyronema epifaneia S10	8	**59**	**35**	**12**	74	**5**	73	**36**	**41**	19	11	**7**	**28**	**15**
Euryhalinema mangrovii AP9F	8	**63**	**36**	**12**	74	**7**	73	**34**	**32**	19	11	**7**	**20**	**16**
Euryhalinema pallustre AP3	8	**63**	**39**	**9**	74	**15**	73	**37**	**32**	24	11	**11**	**24**	**18**
Salileptolyngbya diazotrophica SCSIO 43686	8	**62**	**40**	**7**	74	**6**	73	**24**	**28**	24	12	**7**	**72**	**17**
Gibliniella alaskaensis L31	8	**62**	**38**	**7**	74	**6**	73	–	–	–	–	–	–	–
Leptolyngbya tenuis MACC28	8	**64**	**34**	**12**	74	**5**	73	**34**	**45**	11	11	**7**	**29**	**19**
Halomicronema hondechloris C2206	8	**60**	**34**	**7**	74	**72**	73	**17**	**36**	18	11	**7**	**23**	**6**
Nodosilinea chupicurensis PC471	8	**63**	**38**	**64**	74	**6**	73	**23**	**40**	19	11	**7**	**27**	**18**
Nodosilinea nodulosa UTEX 2910	8	**62**	**33**	**71**	74	**6**	73	**24**	**40**	18	11	**38**	**27**	**34**
Nodosilinea ramsarenis KH–S S2.6	8	**62**	**49**	**41**	74	**6**	73	**23**	**47**	19	11	**7**	**23**	**23**

DISCUSSION

Three experimental strains of *Almyronema epifaneia gen. & sp. nov.* formed a separate clade in the phylogenetic tree, sister to a clade with *Gibliniella* and *Salileptolyngbya*, and was distant from the morphologically and ecologically similar genus *Euryhalinema*. Differential features of 16S–23S ITS and 16S rRNA secondary structures between *Almyronema* and closely related genera further supported the establishment of *Almyronema* as a novel genus, distinct from *Euryhalinema*. Additionally, *Almyronema* displayed distinct morphological characteristics, such as apical cell shape, cell dimensions, different cellular inclusions, as well as a physiological character (gliding movement) that distinguished *Almyronema* from the genus *Euryhalinema* and from other taxa in Nodosilineaceae (Table 2).

The majority of Nodosilineales are filamentous structures with 2 µm cell width. Cells are slightly thinner than in Prochlorotrichales (Strunecký *et al.* 2023). However, the size and shape of the cell may be influenced by ecological and environmental factors of the collection site (Zapomělová *et al.* 2008). The width of cells of Nodosilineaceae is generally 1.0–3.5 µm, depending on the growth conditions. The three strains in this study showed 0.7–1.1 µm cell width; this was thinner than in species of the phylogenetically closest genera, viz. in *Gibliniella alaskaensis* Strunecký & Raabova and *Salileptolyngbya diazotrophica* W.G. Zhou & J. Ling, which were reported with 0.7–1.2 µm and 0.93–1.44 µm cell width, respectively (Zhou *et al.* 2018; Strunecký *et al.* 2020). Moreover, species of *Euryhalinema* showed thinner cells with 0.4–0.6 µm width for *E. mangrovii* (Chakraborty *et al.*



Figs 14, 15. *Almyronema epifaneia gen. & sp. nov.*, TEM. α, glycogen α granules; β, lipid β granules; CG, cyanophycin granules; CW, cross wall; MS, mucilaginous sheath; PP, polyphosphate granules; T, thylakoid bands.

Fig. 14. Longitudinal section of filament of strain S10 showing different cell inclusions. Scale bar = 1 μm.

Fig. 15. Cross section of filament of strain S10 showing different cell inclusions. Scale bar = 350 nm.

2019); 0.4–0.5 μm width for *E. pallustre* (Chakraborty *et al.* 2021) and 0.8–1.0 μm width for *E. epiphyticum* (de Araújo *et al.* 2022). Apical cell morphology is one of the principal criteria for traditional classification of cyanobacteria (Komárek & Anagnostidis *et al.* 2005; Komárek *et al.* 2014). The trichome of *Almyronema* possessed a conical apical cell that was different from its closest relatives, whereas *Gibliniella* and *Salileptolyngbya* both exhibited round apical cells and *Euryhalinema* presented cylindrical or flattened terminal cell morphology. Additionally, other members of Nodosilineaceae, such as *Marileptolyngbya, Nodosilinea, Haloleptolyngbya* and *Halomicronema*, possessed rounded apical cells (Abed *et al.* 2002; Perkerson *et al.* 2011; Dadheech *et al.* 2012; Zhou *et al.* 2018). *Almyronema* displayed gliding movement of trichomes (Video S1), whereas this was absent in *Salileptolyngbya* and *Euryhalinema*; in contrast, *Gibliniella* was reported as intensely motile (Strunecký *et al.* 2020). The characteristic cell dimensions, apical cell morphology and motility supported the view that our three experimental strains form a separate genus distinct from the near relative genera *Gibliniella, Salileptolyngbya* and *Euryhalinema*. Although phylogenetic analysis is the basic criterion of the polyphasic taxonomic approach, morphological features can still be applied as decisive evidence for lineage separation at family or generic level (Komárek *et al.* 2016; Casamatta *et al.* 2023). *Salileptolyngbya* and *Marileptolyngbya* were distinguished from each other as well as other *Leptolyngbya*-like strains based on morphological features: terminal cell shape, cell dimension, mucilaginous sheath as well as cellular inclusions like cyanophycin granules and lipid droplets (Zhou *et al.* 2018). In the context of morphological separation of cyanobacterial genera, it may be noted that several cryptic cyanobacterial genera such as *Cyanomargarita* Shalygin, Shalygina & J.R. Johansen, *Trichotorquatus* Pietrasiak & J.R. Johansen, *Kovacikia* Miscoe, Pietrasiak & J.R. Johansen, *Pinocchia* P. Dvořák, Jahodářová & P. Hašler, *Limnolyngbya* Xiaochuang Li & Renhui Li, *Pantanalinema* Vaz *et al.* and *Alkalinema* Vaz *et al.* were described as new genera based on minute morphological differences (Dvořák *et al.* 2015; Miscoe *et al.* 2016; Vaz *et al.* 2015; Li *et al.* 2016; Shalygin *et al.* 2017; Pietrasiak *et al.* 2021).

The arrangement of thylakoid bands in cells of cyanobacteria has been one of the basic criteria for family level classification (Rippka *et al.* 1979; Komárek *et al.* 2006). However, thylakoid arrangements cannot be used as a sole character to differentiate cyanobacteria at genus or family level as the arrangement of thylakoids in cyanobacterial cells are excessively unstable due to their diverse evolutionary history (Mareš *et al.* 2019). However, *Almyronema* was placed in family Nodosilineaceae according to thylakoid arrangement because all three experimental strains possessed parietal arrangement of thylakoids, which is one of the family level characters of Nodosilineaceae (Strunecký *et al.* 2023). Our *Almyronema* strains were distinguished from *Salileptolyngbya* and *Euryhalinema* on the basis of ultrastructural features, such as the presence of a large prominent cyanophycin granule in every cell of the trichome (Figs 10–15). Glycogen α granules and lipid β granules were also present in our strain in enormous quantity as compared with *Salileptolyngbya*, but they were absent from *Euryhalinema* and other genera of Nodosilineaceae. Although these characteristics are not genus-specific, we report them as differential features from the closely related genera *Salileptolyngbya* and *Gibliniella* (although no ultrastructural data are available for *Gibliniella*). Extensive analysis of

cyanobacterial granulations is required to establish their value as taxonomic markers for genus or species level classification (Jensen *et al.* 1985; Stanier *et al.* 1988). *Ammassolinea* P. Hašler, P. Dvořák, Poulíčková & Casamatta was separated from *Phormidium* on the basis of phylogenetic analyses, with only minor ultrastructural differences (Hašler *et al.* 2014). The distinct phylogenetic position of *Aerofilum fasciculatum* as type of a novel genus was ascertained and it was ultrastructurally differentiated from genus *Oculatella* Zammit, Billi & Albertano by the presence of aerotopes (Chakraborty *et al.* 2021).

Based on 16S rDNA p-distance analysis, *Almyronema epifaneia* demonstrated the highest similarity with *Euryhalinema*: 94.1%–94.4% (Table 3). However, phylogenetic analyses (BI, ML and MP) revealed that *Euryhalinema* was significantly distant from *Almyronema* (Fig. 16). The three strains of *Almyronema* clustered together and the clade formed a distinct lineage well separated from *Euryhalinema*. The 16S rDNA phylogenetic tree placed *Gibliniella alaskaensis* and *Salileptolyngbya diazotrophica* as the closest relatives of *Almyronema*. *Gibliniella* was first described as a member of

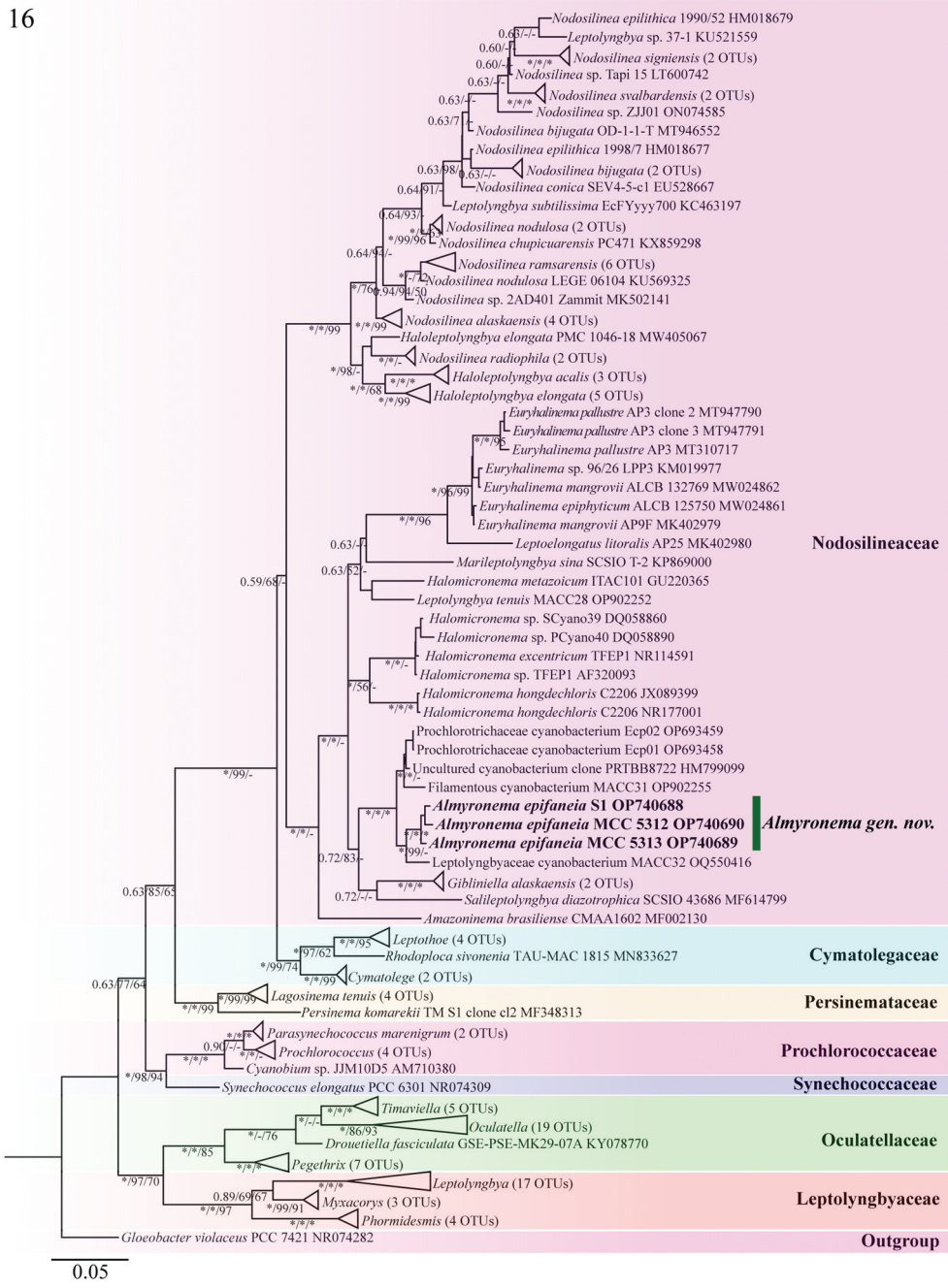


Fig. 16. Phylogenetic tree based on 16S rRNA gene sequences of 146 OTUs, with *Gloeobacter violaceus* as outgroup. Bayesian analysis topology was generated from NGPhylogeny.fr webserver (Lemoine *et al.* 2019). ML and MP tree scores were generated from W-IQ-TREE webserver (Trifinopoulos *et al.* 2016) and MEGA program package v6.0 (Tamura *et al.* 2013). Bootstrapping with 1,000× resampling was performed. Support values are BI posterior probability/ML bootstrap/MP bootstrap. Nodes marked by asterisk (*) received support by both posterior probability and bootstrap (1.0 and 100%, respectively). Scores denoted by dash (-) for any node showed less than 50% support for that branch. The investigated strains of *Almyronema epifaneia gen.* & *sp. nov.* (S1, S6 and S10) are shown in bold.

the Synechococcaceae, whereas *Salileptolyngbya* was reported as a member of the Leptolyngbyaceae. In the latest cyanobacterial classification by Strunecký *et al.* (2023), *Gibliniella* and *Salileptolyngbya* were placed in the new family Nodosilineaceae. In accordance with the most recent classification of Strunecký *et al.* (2023), we therefore classify *Almyronema* as a member of the Nodosilineaceae. The cut off value for genera is 94.5% similarity of the 16S rDNA sequence, and it is 98.7% for species (Yarza *et al.* 2014). However, higher levels of 16S rRNA gene sequence similarity have been accepted when describing new genera, when combined with definite phylogenetic, morphological, ultrastructural and ecological differences (Stackebrandt & Gobel 1994; Komárek *et al.* 2014). For example, Soares *et al.* (2020) described *Parakomarekiella sesnandensis* F. Soares, V. Ramos & Portugal as the type of a new genus despite sharing 98% similarity with *Komarekiella atlantica* Hentschke, J.R. Johansen & Sant'Anna. Likewise, *Purpureonostoc* F. Cai & Renhui Li was 94.4%–94.72% similar to *Aliinostoc* Bagchi, N. Dubey & Prashant Singh but was described as a new genus because it formed a distinct lineage in the phylogenetic tree (Cai *et al.* 2020, as '*Purpurea*').

The folded secondary structure of 16S–23S ITS region can be critical for species differentiation (Boyer *et al.* 2001; Johansen *et al.* 2011; Brito *et al.* 2017). In our study the three helix regions, namely D1-D1′, Box-B and V3 of the *Almyronema* strains were compared with those of other Nodosilineaceae. All three strains of *Almyronema* showed a unique sequence 5′-CAUCUC-3′ of the unilateral basal bulge, which was different from other genera of Nodosilineaceae (Fig. 17). The terminal loop of helix D1-D1′ also showed significant differences in comparison with other family members. Additionally, *Almyronema* showed the smallest D1-D1′ helix structure (59 nucleotides) of all genera of the family (Table 5). The Box-B helix of ITS secondary structures of all the *Almyronema* strains showed a unique arrangement, with the terminal loop composed of sequence 5′-GGAA-3′, which was different from the Box-B terminal loop structure of all other members of the family (Fig. 17). The length of the sequence (41nt) exceeded that of *Salileptolyngbya diazotrophica* and that of other Nodosilineaceae, like *Euryhalinema mangrovii, E. pallustre, Nodosilinea nodulosa* (Zhongkui Li & J. Brand) Perkerson & Casamatta, *N. chupicuarensis* J. Vázquez-Martínez, Gutierrez-Villagomez & Molina-Torres, but it was smaller than that of *N. ramsarensis* Heidari & Hauer (Table 5). Due to the unavailability of the full-length ITS sequence of *Gibliniella alaskaensis* in GenBank, its Box-B helix structure could not be determined. The overall results of Box-B ITS helix confirmed its unique structure within the Nodosilineaceae. The secondary structure of the ITS has

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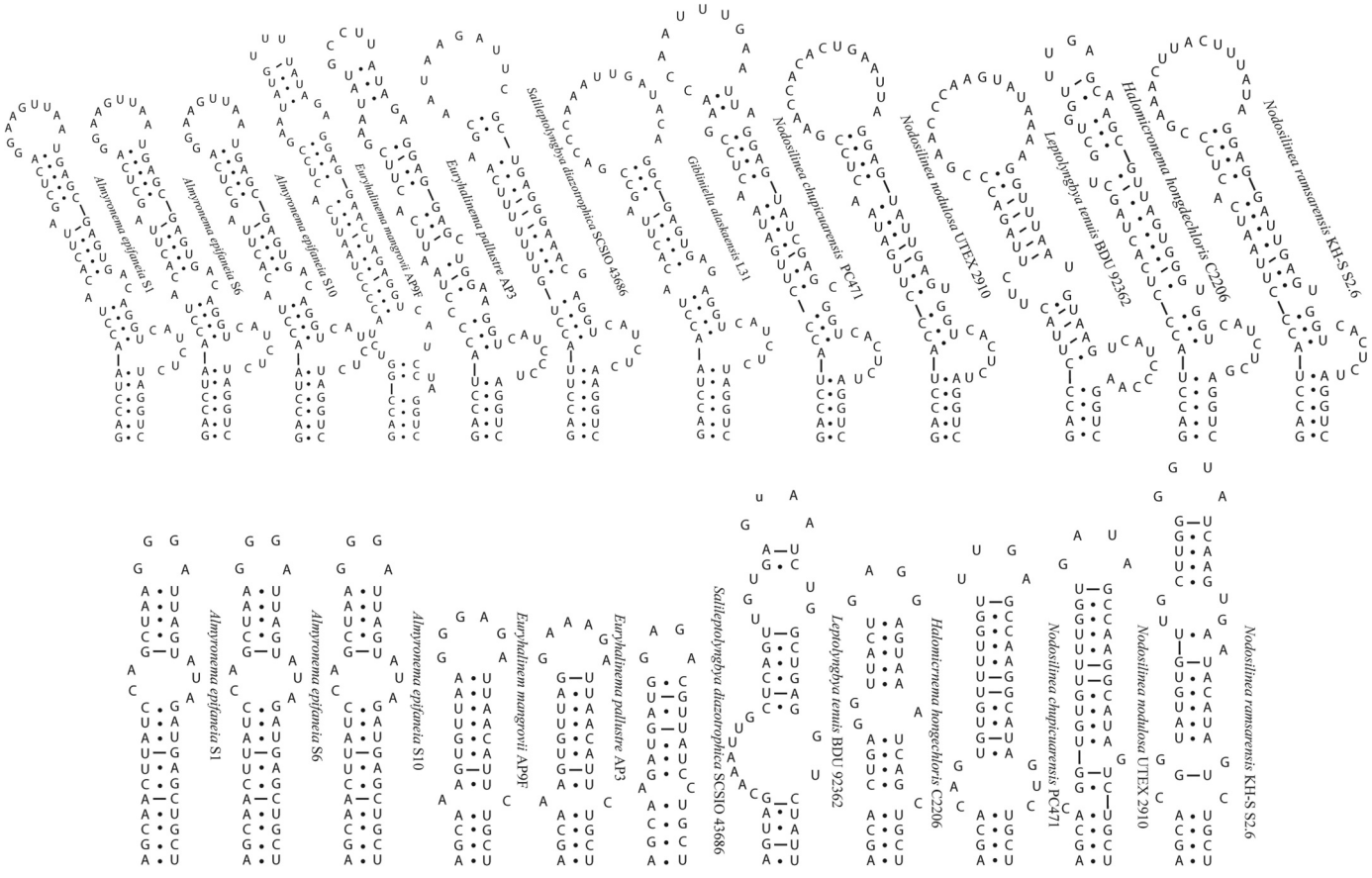

Fig. 17. Comparative analyses of helix D1-D1′ (upper row) and Box-B (lower row) of 16S–23S ITS region of *Almyronema epifaneia gen. & sp. nov.* strains S1, S6 and S10, and close phylogenetic relatives of family Nodosilineaceae.

helped fundament the establishment of several cyanobacterial genera (e.g. see Dadheech *et al.* 2012; Zammit *et al.* 2012; Zhou *et al.* 2018).

Regions V2 and V3 are the most variable of the ITS. The folded secondary structure of region V3 of *Almyronema* strains (Fig 18) differed from other strains not only in structure but also in nucleotide length (Table 5). *Almyronema* possessed the smallest V2 region of all Nodosilineaceae, with only 5 nucleotides (Table 5). To ascertain inter-species relationships, p-distance analysis is applied as an important parameter to define a new species (Osorio-Santos *et al.* 2014; Johansen *et al.* 2017; Gonzalez-Resendiz *et al.* 2018a, b; Mai *et al.* 2018; Chakraborty *et al.* 2021; Pietrasiak *et al.* 2021; Berthold *et al.* 2022; Gaysina *et al.* 2022). The p-distance analysis of the ITS region of the *Almyronema* strains showed 22.2% to 24.5% dissimilarity from the reference genus *Euryhalinema*. The cut off value of ITS sequence dissimilarity (as percentage) for species is less than 3%, and to establish a new species within a recognized genus the cut off value for ITS sequence dissimilarity is greater than 7%. When the cut-off value of ITS sequence dissimilarity falls between 3%–7%, the decision needs to be founded on other parameters, such as phylogenetic position or morphological characters (González-Resendiz *et al.* 2019; Jung *et al.* 2020; Pecundo *et al.* 2021). Three strains of *Almyronema* showed 22.7% ITS sequence dissimilarity from the sister taxon *Salileptolyngbya diazotrophica*, thereby exceeding the cut off value and strongly supporting the recognition of a novel species within a novel genus (Table 4). Moreover, the *Almyronema* strains also showed 22.2% ITS rRNA sequence dissimilarity from *Euryhalinema mangrovii*. Despite of unavailability of full length ITS rRNA sequence, *Gibliniella alaskaensis* also showed 8.8% dissimilarity from *Almyronema epifaneia*, thereby also supporting the establishment of a new taxon.

In conclusion, this research work on polyphasic taxonomy contributes to the revision of the taxonomy of cyanobacteria towards the establishment of monophyletic taxa. This study underpins the necessity of further survey of the unexplored diversity of the Indian Sundarbans for potential undiscovered cyanobacteria in this intertidal region.

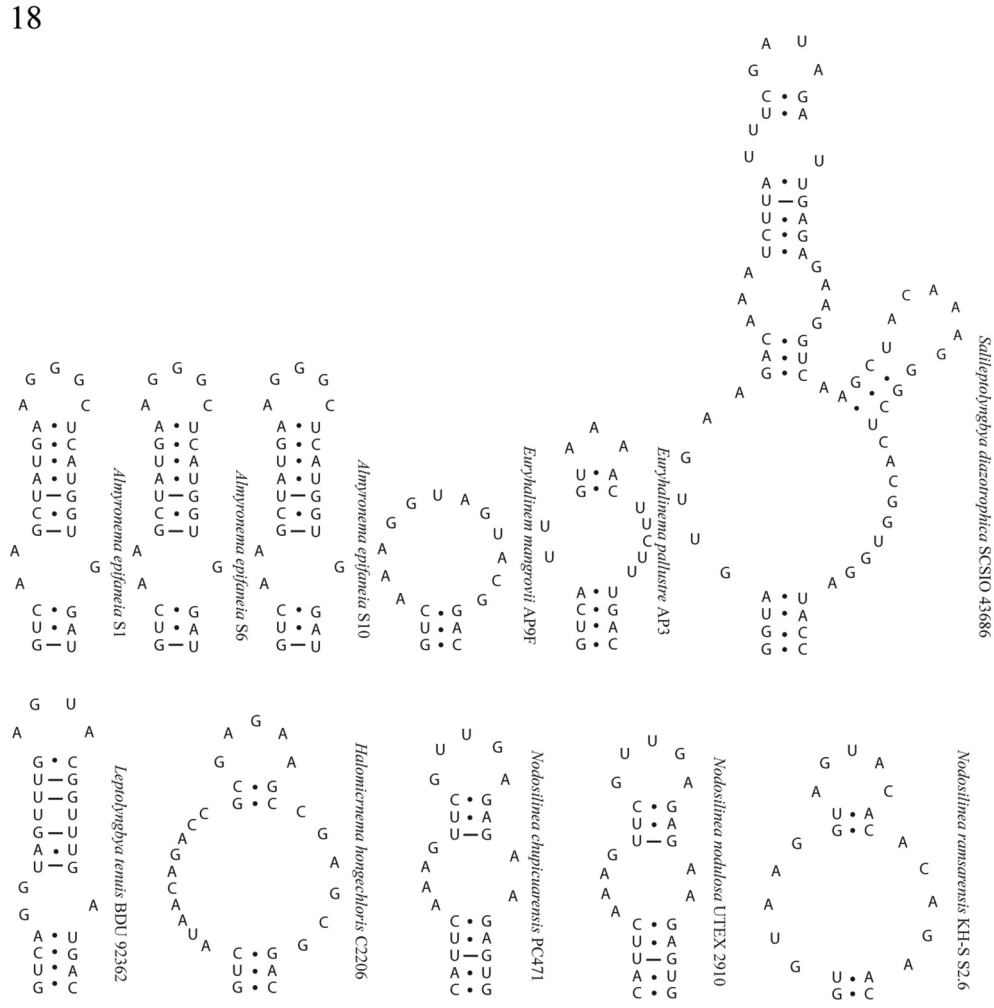

Fig. 18. Comparison of the V3 helices of 16S–23S ITS regions of *Almyronema epifaneia gen.* & *sp. nov.* strains S1, S6 and S10, and close phylogenetic relatives of family Nodosilineaceae.

DISCLOSURE STATEMENT

No potential conflicts of interest are reported by the authors(s).

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