IDENTIFICATION OF FILAMENTOUS CYANOBACTERIA OF THE INDIAN SUNDARBANS THROUGH POLYPHASIC TAXONOMY

Thesis

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In

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By

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LIST OF ABBREVIATION

% - Percentage

& - And

µl - Microlitre

µmol - Micromolar

µm - Micrometre

ASN - Artificial Seawater Nutrient

BLAST - Basic Local Alignment Search Tool

bp - Base pair

BI - Bayesian Inference

Cat. No. - Catalogue number

CBD - Convention on Biological diversity

DNA - Deoxyribonucleic acid

dNTP - Deoxyribonucleotide triphosphate

g - Gram

hrs - Hours

IPTG - Isopropyl β-D-1-thiogalactopyranoside

ITS - Internal Transcribed Spacer

kb - Kilo bases

l - Litre

LB - Luria Broth m metre

M - Molar

mg - Milligram

MgCl2 - Magnesium Chloride ii

min - Minute

ML - Maximum Likelihood

ml - Millilitre

MP - Maximum Parsimony

NaCl - Sodium Chloride

NCBI - National Centre for Biotechnology Information

NJ - Neighbor-Joining nm Nanometre

OTU - Operational Taxonomic Unit

PBS - Phosphate Buffer Saline

PCR - Polymerase Chain Reaction

rpm - Revolutions per minute

RNA - Ribonucleic acid

rRNA - Ribosomal Ribonucleic acid

s - Second

TBE - Tris-Borate-EDTA

TYG - Tryptone Yeast Glucose

UV - Ultraviolet

V - Volt

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Abstract

The Indian Sundarbans, world's largest tidal mangrove region, comprises of various unique and unpublished biological species. Cyanobacteria were one of those species that can identify according to their characteristics based on morphological, physiological, and genetic taxon studies. They were collected and maintained in an axenic culture under suitable condition in the laboratory. The cyanobacterial species were isolated and purified for polyphasic taxonomical studies.

The two distinctive purified strains were named as S9 and S15. The morphological study of S9 strain and S15 strain revealed the similarities with simple filament structure, predominantly constricted cell wall, and presence of mucilaginous sheath. The ultrastructural study revealed the presence of homologous thylakoid arrangement in each strain as compared to their respective families. The physiological study revealed the amount of chlorophyll a content, carotenoid content, and phycobiliprotein content among the two distinctive strains.

The S9 strain and S15 strain were subjected to the identification through a polyphasic taxonomic study. The 16S rRNA gene sequencing revealed the similarity values with their reference strains and assumes to be assigned as a novel taxon by consensus value of phylogenetic tree with high bootstrap values. The representation of the phylogenetic tree topology of S9 strain and S15 strain showed the closest relation and well-supported clade with the reference strain *Euryhalinema mangrovii* AP9F belongs to the Nodosilineaceae family. The secondary structure of the 16S-23S ITS region of S9 strain and S15 strain revealed the dissimilarities values with their respective strains in terms of basal sequence length as well as terminal loop and bulges.

The overall analytic investigation leads to the S9 strain and S15 strain to be considered as two species under a novel genus.

1. Introduction and Review of Literature

1.1 Introduction about Cyanobacteria

Cyanobacteria were one of the most ancient morphologically diverse prokaryotic phyla on the earth. Cyanobacteria are oxygenic phototrophic prokaryotes are having chloroplast which absorbs light for photosynthesis. This process is considered as ubiquitous. Cyanobacteria were known as Cyanophyta, a phylum of Gram-negative bacteria that obtained energy via. photosynthesis. Cyanobacteria have an asexual mode of reproduction.

The cyanoprokaryote constitutes as a major share of the primary producers in the ecosystem. Cyanobacteria were the first autotroph to colonize bare areas of rock and soil. They were the microscopic organisms found naturally in all types of water bodies. They are single-celled organisms live in fresh, brackish (combined salt and fresh) water, and marine water.

The cyanobacteria were the oldest known fossils, more than 3.5 billion year old. Most Proterozoic oil deposits are attributed to the activity of cyanobacteria. Cyanobacteria were important in shaping the course of evolution and ecological changes in aquatic ecosystem. The atmospheric oxygen was generated by numerous cyanobacteria during the Archean and Proterozoic Eras. Cyanobacteria have contributed to the origin of plant. In the late Proterozoic, or in the Cambrian era, cyanobacteria began to take up residence within certain eukaryotic cells, made food for the host eukaryotic species in return. This event known as endosymbiosis, took place in the origin of the eukaryotic mitochondria.

According to Komarek (2006), the static evolution of the cyanobacterial species were associated with their genetical and morphological flexibility leads to wide range of adaptability at extreme conditions. Gradually, this evolutionary event gives rise to the newly formed morphotypes and ecotypes.

According to Oren (2011) explained the morphological analysis of the cyanobacterial filament formation ranges from the unicellular unit of filament to multicellular filamentous with branched or unbranched forms. The cyanobacterial cells varied in shape and size. The cyanobacterial species have green pigment consists of chlorophyll a and carotenoid. The blue pigment consists of c-phycocyanin, and allophycocyanin as the blue-green pigment of the thallus. Several cyanobacterial strain consists of c-phycoerythrin, a water soluble light harvesting accessory pigment and responsible for absorption of wavelength. Generally, the red cells are contained phycoerythrin in a structure called phycobilisomes.

According to Lewin (1976) the taxonomic divisions such as the members of Prochlorophyta contains chlorophyll b than chlorophyll a while other accessory pigments are absent. The primitive type of cyanobacterial cells plays a crucial role by performing various cellular metabolite functions. The cellular inclusion included carboxysomes stored ribulose 1,5 bis-phosphate carboxygenase or, oxygenase enzyme, cyanophycean granules stored amino acid like arginine, aspartic acid and other storage compartment for glycogen reserve, polyphosphate granules etc. This cellular compartment helps to provide essential nutrient and accumulated energy to sustain at unfavorable condition.

Komárek, J. *et al*.(2013) coined the presence of aerotope in planktonic species as hollow vacuole-like compartments made up of protein subunits that keep the cells floating on the surface with the suitable light requirement.

According to Perkerson *et al*. (2011) the physiological as well as ecological analysis introduced many cellular formations of cyanobacterial species. Many unicellular filamentous cyanobacteria formed a special cellular structure known as heterocytes (found in the members of Nostocales and Stigonematales). The heterocytes are the reservoir of nitrogenous enzyme which helps in nitrogen fixation. Many non-heterocytous members exhibit the phenomenon of nitrogen fixation; it revealed that heterocytes was not only pre-condition for the phenomenon.

Reproductive features implemented another specialized structure called akinete. Akinete helps the cyanobacterial cell to survive in unfavorable conditions as a dormant phase. A common feature was the hormogonium which was a short, 2-4 celled, motile filament was formed fragmentation as a result. Baeocytes were reproduced as hundreds of minute cells, formed as coccoid.

According to Popall *et al*. (2020) an ecological background explained the occurrence of the cyanobacterial species in three forms; mat-forming species, bloom-forming species, and picocyanobacteria. Mat-forming cyanobacterial species serve as a biofilm on the surface of soil, rock, plants, and substrata. According to the records of Pre-Cambrian period, some of the mat-forming species were reported as fossils, where they exist in the form of layered entities, and solidified as stromatolite. In the past assumption, stromatolite structures were considered to be the primary cause of oxygenation in the atmosphere. Mat-forming species were common in wetland, swampy habitats like rice fields, mangroves, and other habitats including the common genera such as *Leptolyngbya, Lyngbya, Oscillatoria, Chroococcus, Phormidium* and heterocytous genera such as *Anabaena, Nostoc, Anabaenopsis, Calothrix* etc. proposed by Debnath *et al*. (2017), Chakraborty *et al*. (2018). The cyanobacterial cells produced mucilaginous sheath and secreted exopolymeric substances having cohesive force in the formation of biofilms. The extracellular substances (polysaccharides) can help the cells prevent desiccation and exhibit under unfavorable conditions. They can able to remove the heavy metals and other hazardous chemicals from the wastewater as suggested by Barros *et al*. (2020). Bloom-forming species inhibited the stable nutrient-rich aquatic habitat with major occupied genera such as *Anabaena*, *Aphanizomenon* and *Microcystis*. Rastogi *et al*. (2015) explained the function of primary production; the bloom-forming cyanobacteria produced several secondary metabolites such as toxin which was known as cyanotoxins. Picocyanobacteria are another type of cyanobacterial species that are possesses a relatively small diameter. *Synechococcus* and *Prochlorococcus* are the main genera of this group, which was characterized as the high surface-to-volume ratio and they can adapt to survive in the nutrient-scare environment.

1.1.1 History of Cyanobacterial Systematics

The taxonomists tried to categorize various group of species based on their relative resemblance. According to an alpha-level biodiversity analysis include the separation of one group of species from other group of species was performed by a taxonomist. The term 'taxonomic impediment' introduced in the Convention on Biological diversity (CBD) concerned about crisis in the research oriented on the species identification faced by the taxonomist. The studies of cyanobacteria as a primary factor made by taxonomist were concerned on the study of cyanobacterial diversity in alpha-level. On the other aspects, the present cyanobacterial study includes the Sundarbans delta region. Various unique and unpublished species were still undefined in the Indian Sundarbans, world largest tidal mangrove region. It constituted as vegetation which act as transition between the sea and mainland. It comprises cyanobacterial flora with optimal growth in moderate saline condition. Therefore, the cyanobacterial diversity was investigated in mangrove region, various novel taxa was introduced from mangrove ecosystem. The cyanobacterial flora has complex niches with a periodic variation as its physio-chemical parameters. They are more likely to exhibit as a morpho-ecological selection that leads to speciation.

According to the traditional system, the identification of cyanobacteria was based on morphological characterization, whereas an advanced study of molecular biology provides a set of tools for investigating their biosystematics and evolution. The classification of taxonomy moved from the phenetic method to the modern phylogenetic methods. The 16S rRNA gene sequences, a set of genetic markers applied for the identification of genus and species. Johansen & Casamatta (2005) coined the necessity of molecular markers for the reconstruction of the phylogenetic tree, as highly conserved domains of the 16S rRNA was not significantly resolve the inter-specific relationship. Iteman *et al*. (2000), Boyer *et al*. (2002), Johansen *et al*. (2011), Perkerson *et al*. (2011), Johansen *et al*. (2017) demonstrated the concept on the analysis of secondary structures of the ITS region (16S - 23S internal transcribed spacer) which was utilized for the taxonomical evaluation of cyanobacteria according to the morphology, ecology, and ultrastructural aspects.

The study of cyanobacterial taxonomy was investigated by the following rules of the *International Code of Nomenclature for Algae, Fungi, and Plants (ICN)* rather than the *Bacteriological Code* because of certain criteria for their taxonomy not yet standardized, as it was for Eubacteria. Komarek (2006) coined that the numbers of strains were wrongly submitted to the worldwide database which created delusional thought about the placement of taxa according to their respective clusters. An appreciable database for the deposition of whole genome sequence data for various cyanobacterial species was recorded. Dvorak *et al*. (2017) proposed the concept of the bioinformation used in the taxonomic investigation of species resolves the phylogenetic approach associated with cyanobacteria.

The classification system of the cyanobacterial species was not uniform, rather based on the morphological features. During late 19th century, the first classification of the cyanobacterial species according to Palinska & Surosz (2014) and the phenotypic features based on monograph published by Bornet & Flahault (1887-1888). This classification system was discarded due to the artificially selectable characters.

The hierarchy of current classification system of high-level cyanobacterial species as follows:

- A classification system of whole cyanobacterial species divided the group into seven orders such as - *Chroococcales, Entophysalidales, Pleurocapsales, Dermocarpales, Siphononematales, Nostocales,* and *Stignonematales*; was proposed by Geitler (1925).
- After seven years, the modification in the system of cyanobacterial species divided into three orders such as - *Chroococcales, Chaemaesiphonales* and *Hormogonales;* was proposed by Geitler (1932).
- The order of *Stignometales* and categorized all the unbranched filmentous cyanobacterial species into one group; was studied by Desikachary (1959).
- The cyanobacterial species classified into five sections of order - *Chroococcales, Pleurocapsales, Oscillatoriales, Nostocales* and *Stignometales;* was proposed by Rippka *et al*. (1979).
- Many taxonomic system based on the molecular methods combined with morphological marker proposed by Anagnostidis *et al*. (1985), Komarek & Anagnostidis (1989), (2005).

- The importance of 16S-23S ITS spacer regions as a potent taxonomic marker mostly for the inter-specific relationships described by Iteman *et al*. (2000). The inter-specific relationships between the species were currently more important than 16S rRNA for species delineation proclaimed by Johansen *et al*. (2011).
- After the year 2000, a combined approach includes molecular evidence collaborated with other stable morphological markers, ecological, ultrastructural, and biochemical parameters considered for the taxonomic evaluation of the cyanobacterial species known as polyphasic assessment.
- Hoffmann *et al*. (2005) proposed a comparative system and a modern higher-level system for cyanobacterial species classification, Class - *Cyanophyceae* classified into four subclasses - *Gloeobacteriophycidae, Synechococcophycidae, Oscillatoriophycidae*, and *Nostocophycidae.* Out of these four subclasses, the first member and the last member are the monophyletic groups. This classification system implied the proper phylogeny among the member which differs and superior to the earlier proposed classification system. On the basis of the phylogeny system showed the coccoid and filamentous members were similar and kept both members under a common subclass.
- The revised version of the cyanobacterial classification system proposed by Komarek *et al*. (2014) accepted one till date instead of the classification system proposed by Hoffmann *et al*. (2005). The Class *Cyanophyceae* was divided into eight orders such as - *Gleobacterales, Synechococcales, Spirulinales, Chroococcales, Pleurocapsales, Oscillatoriales, Chroococcidiopsidales,* and *Nostocales.*

- Komarek & Anagnostidis (1998, 2005) introduced a series of sweet water flora (Suβwasserflora) of cyanoprokaryotes. A well-supported taxonomic keys comparison based on the morphology, as well as molecular parameters, was served by Komarek (2013).
- Mai *et al*. (2018) proposed that in the last two decades, a traditional classification system of cyanobacterial species artificially based on the morphological characters was replaced by the polyphasic assessment, and introduced more than 50 new species. Many members are organized based on phylogenetic affinities. Reconstruction of the order of *Cyanophyta* had been evolving vigorously till date; some disputes still exist to be resolved.
- Komarek (2010), (2014) conducted serious revision of the classification of genera as polyphyletic in nature. Predominantly, the morphological features were polyphyletic in nature except for few genera such as the heterocytes present in *Nostocales.* Cryptogenera is used for two evolutionarily unrelated and distant genera which are morphologically indistinguishable described by Komarek *et al*. (2014).
- Osorio - Santos et al. (2014) made critical observations in the case of the simple filamentous genus, *Leptolynbya*, and the unicellular, *Synechococcus.*
- Komarek (2018) described the experimental strains investigated under axenic culture in the laboratory condition simultaneously for several years developed or lose any morphological features due to its phenotypically plastic in nature. The investigators highlight the feature of discrimination which was not stable, and resultant created ambiguity in the taxonomic affiliation of the particular strains.

- Komerek (2018) suggested improving the taxonomic system that leads to the complication to build the phylogenetic tree. Earlier the traditional method, various strains were designated on the basis of morphological data without nomenclature revision data. By applying the modern concept of classification was published in Komarek *et al*. (2014), it rectified the mistake continually to the construction of modern phylogenetic tree with the proper selection of an introductory material and identification nomenclature.
- Strunecký, O., Ivanova, A. P., & Mareš, J. (2023) described the evolution of the cyanobacterial taxonomy faced with the period that ease of 16S rRNA gene sequencing and established the advanced workflow for the description of new taxa based on the comprehensive modern approach of the cyanobacterial system in 2014 until 2021. A total of 273 species in 140 genera were described. The taxa were placed into previously defined order and families although introduced several new families. The classification of the taxa described on the hierarchical relationships inherited based on the classical morphological taxonomy and convergence of the phylogenetic distant taxa.

1.2 Modern Polyphasic Approach to Taxonomy

The polyphasic approach to taxonomical study was a concept that included a combined aspect which resolves the taxonomy of cyanobacteria including morphological, ecological, and ultrastructural markers congruent to the well-supported phylogenetical studies.

1.2.1 Morphological Approach

During the late 19th century for the first time, cyanobacterial classification was proposed based on morphometry. This system is not accepted today, but it was an important criterion for the identification of cyanobacteria.

Komarek (2010) explained the morphological features should collaborate with the phylogenetic results. The morphological feature analysis depends upon the presence or absence of sheaths, cellular dimensions, branching patterns, etc., that do not appear to be congruent with the natural molecular clusters. Due to this reason they have an ecotypic value that varies with an unknown genetic basis. Only stable morphological markers should be used to support phylogenetic data. Phenotypic features are considered according to filament morphology, polarity, branching, cell division patterns, reproductive behaviour, apical cell morphology, and cell wall constrictions. Other features such as necridic cells, hormogonia, baeocytes, heterocysts, etc., are studied and considered to be an important taxonomic marker for comparative analysis.

Traditional method in Komarek & Anagnostidis, (2005), the genera of Oscillatoriales were simple filamentous, non-heterocytic in nature, and distinguished by the presence or absence of mucilaginous sheath. The colour of the cells was considered as generic feature.

Chatchawan et al. (2012) separated the genus *Oxynema* from the polyphyletic genus *Phormidium* on the basis of comparative analysis with morphology along with molecular data. In this morphological study, the mucilaginous sheath and the apical cell morphology was used as diacritical marker. Cellular motility was an essential point of comparison. On the basis of cell motility, *Geitlerinema* and *Jaaginema* were differentiated by Anagnostidis & Komarek, (1988).

There are two kinds of branching patterns such as - true branching and false branching, both of which took place according to the different physiological phenomenon. In the case of false branching, cell division occurs within the mucilaginous sheath with the help of necridic cells. The trichome is divided and grows outside the mucilaginous sheath at one or both ends. In the case of true branching, cell division occurs in more complex forms in cyanobacteria where cells grow longitudinally. The branching pattern was considered a consistent generic feature by Desikachary (1959), Bourelly (1970).

The reproductive features usually perpendicular to the trichomes, axial end of the filamentous forms as well as coccoid forms were similar as described by Komarek & Johansen (2015). Solitary cells took part in reproduction (*Aetokthonos, Rexia)* while others undergo fragmentation. Two kinds of fragmentation were shown such as hormogonia and necridic cells. The simple filamentous cyanobacterial species belongs to Synechococcales and fragmentation by hormogonia.

1.2.2 Physiological Approach

Estimation of the chlorophyll a content, carotenoid content, and phycobiliprotein content in the cells measured in spectrophotometer. Jensen (1978) determined the amount of carotenoid. Patel *et al*. (2005) demonstrated that phycobiliprotein extracted by 0.1M sodium phosphate buffer (pH 7.0) and then quantified the concentration of the cell. The absorbance value was recorded by using spectrophotometer (model Lambda 25; PerkinElmer Inc., Waltham, MA, USA).

1.2.3 Ultrastructural Approach

The electron microscopic analysis visualizes the ultrastructure of cyanobacterial cells and provides various cellular features encoded by their genotype. The thylakoid arrangement showed a stable character in the evolutionary time scale. The thylakoid membrane arrangement ranges from simple parietal to radial, parallel, and coiled such as:

- The Gloeobacteriaceae family belongs to the genus *Gloeobacter violaceus* lacks thylakoid in its cellular ultrastructure, and contains electron-dense regions along some phosphate granules explained by Mares *et al*. (2019).
- The parietal thylakoid arrangement showed several layers of membrane arrangement uninterruptedly and concentrically observed in *Cyanobium gracile*. The coccoid and filamentous form of Synechococcales e.g., *Leptolyngbyaceae* was reported as two or more layers of thylakoid surrounding the periphery of the cell-like bundle explained by Mares *et al*. (2019).
- Some modifications in the parietal thylakoid arrangement includes the genus *Kamptonema* showing fascicles of thylakoids at the lining of the periphery towards the cell wall or protruding towards the centre of the cell proposed by Strunecky *et al*. (2014).
- The genus *Crinum magnum* showed the thylakoid arrangement as a circular or helical twisted form situated in the cytoplasm, it was a rare configuration of parietal thylakoid arrangement reported by Mikhailyuk *et al*. (2019).
- The radial thylakoid arrangement showed a radial membrane arrangement perpendicular to the cell wall. It can be visualized in a longitudinal section as parallel lines radiate outrage towards the periphery from the centre. The pattern

was documented by the members of the Oscillatoriales family *Microcoleaceae*, *Phormidiaceae*, etc. as reported by Komarek *et al*. (2014), Mares *et al*. (2019).

- The fascicular thylakoid arrangements were found in some strains such as *Cyanothece sp.*, *Chroococcidiopsis thermalis*, and *Arthrospira.* Komarek (1976) explained that these ultrastructural features of the cyanobacterial strains exhibit as fascicular, looped, spiral, and wavy border.
- An irregular thylakoid arrangement was a different and rare type from the fascicular thylakoid arrangement. In this case, irregular thylakoid arrangements were distributed in the cytoplasm but does not form fascicles instead of single-layered irregular coiled. Bornet & Flahault (1886-88) reported that the single-layered irregular coiled thylakoid arrangements were present in the genus *Stigonema ocellatum.*
- The parallel thylakoid arrangements were a unique pattern of the thylakoid membrane found in *Cyanobacterium* and *Geminocystis*. Mares *et al*. (2019) reported that the thylakoid membrane arrangement in these species was found as parallel lamella covering a whole surface area in transverse and longitudinal cross-sections.

1.2.4 Molecular Approach

1.2.4.1 16S rRNA Sequence Data (Small subunit of ribosomal RNA)

The variable regions of the 16S rRNA gene sequence are primarily targeted for the identification of prokaryotes. The partial sequence of the cyanobacterial 16S rRNA approximately length 1200bp -1400 bp was sufficiently applicable for the analysis.

Wang & Qian (2009) reported that the primers applied for amplification of the 16S rRNA gene are critically chosen because any kind of error selection can fail to amplify the target sequence. The conserved region of the 16S rRNA gene sequences template was fixed, and the length of the primer was around 15-20 nucleotides suitable for the 16S rRNA gene sequence. Fitzsimons & Smith (1984) and Castenholz & Waterbury (1989) reported that cyanobacterial cultures were difficult to purify, so they were cultivated as a unialgal culture but in the presence of some other bacterial strain growth, which is known as non-axenic culture.

Nubel *et al*.(1997), and Boyer *et al*. (2001) designed a set of primers specified for cyanobacterial strains such as CYA106F, CYA350F, CYA781R (a), and CYA781R (b). The set of primers was applied for amplification of the 16S rRNA gene of cyanobacteria when the cultures are kept in non-axenic condition. The 16S rRNA data utilized in two ways for cyanobacterial taxonomic evaluation -

- The sequence was aligned and analyzed in BLAST through the NCBI server for searching the closest genetic similarities followed the construction of the consensus phylogenetic tree to establish a relationship among organisms.
- The 16S rRNA sequences obtained to fold them into secondary structure.

The secondary structures of the 16S rRNA gene contained various helices such as stems and loops. Wang & Qian (2009) reported that some helices contain conserved sequences that are similar to all bacterial species, whereas other part remains conserved and specific to a particular class.

Johansen *et al*. (2017) reported the overall folded structure of the genus *Scytonema hyalinum* (Nostocaceae), that the structures folded out of five ribosomal operons included highly divergent regions were helpful for the generic taxonomic assignment. There are 44 helices with different conformations depicting similarities and variations among the particular family or class. Some structures are conserved throughout the cyanobacterial species including common functions related to transcription.

Smith & Bond (2003) reported the difference in function bears a variation in their primary structure and consequently changes in secondary structure were more evolutionarily significant than the changes in the primary sequence. Smith *et al*. (2007) reported an evolutionary rate depends on the rRNA structure described in the case of prokaryotes; a helix conformation constitutes stem, loop, and bulges. The bulging helical structure showed the evolution of the stem region faster than the loop or bulge region because the compensatory mutation in the paired region was responsible for the bulk changes to the 16S rRNA evolution. Rehakova *et al*. (2014) described that the structure of the 16S rRNA of *Nostoc commune* is the highly informative.

The primary sequence of the 16S rRNA data was used for phylogenetic tree inferences. The numerous sequences of partial 16S rRNA were submitted to the database of GenBank, and NCBI. The molecular data involved in the cyanobacterial taxonomy based on the 16S rRNA gene sequences before the year 2000. Komarek (2006) reported that 16S rRNA region was not sufficient for the study of taxonomy. Iteman *et al*. (2000) and Boyer *et al*. (2001) demonstrated the study of species discrimination according to the critical region of the 16S-23S ITS spacer.

Yarza *et al*. (2014) reported the sets of taxonomical threshold limits according to each hierarchy well-supported to the cluster in an analysis of the phylogenetic tree. The threshold limits were 98.7% determined for the separation of two species, 94.5%

determined for the separation of the genus, 86.5% determined for the separation of two families, and 82% determined for the order. Mai *et al*. (2018) reported that these threshold limits are recommended as the primary criterion for the study of biosystematics of novel genera and species based on the 16S rRNA gene sequencing data.

1.2.4.2 16S-23S Internal Transcribed Spacer (ITS) region

Over the last decade, researchers like Johansen *et al*. (2011), Dadheech *et al*. (2012), Osorio-Santos *et al*. (2014), Strunecky *et al*. (2011), (2013), (2014), (2017), Hasler *et al*. (2017), Mai *et al*. (2018), Gonzalez-Resendiz *et al*. (2018), (2019) explained that the ITS region was more informative in detection for variability in species discrimination of 16S rRNA gene based on many novel genera and species.

Earlier reports by Iteman *et al*. (2000) compared the ITS spacer regions among various representation of the cyanobacterial species and families. Based on these comparisons, the authors demonstrated structural details of conserved and variable regions of the spacer. Boyer *et al*. (2001) investigated many multiple unidentical operons present in the rRNA region. The length of the ITS region varied from 450-600 bp. One should be cautious while applying the primers for amplifying the desired operons. Nelissen *et al*. (1994), Wilmotte (1994), Nubel *et al*. (1997), and Boyer *et al*. (2001) developed the ITS regions for cyanobacterial species amplified by the suitable primers.

Iteman *et al*. (2000), Boyer *et al*. (2001), and Johansen *et al*. (2011) explained that there are total 14 regions in the spacers whose sequence length and secondary structure vary. Some regions are highly conserved in most cyanobacterial species denoted in the conservative function related to transcription. The 14 regions were - leader sequences, 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.

The D1-D1' helix, V2, V3 and Box B helix were widely used to compare among various genera and species. The ITS region constitute two operons, one having both the tRNA genes and another without tRNA genes. Primarily the operons were detected in the gel electrophoresis by the presence of two close bands near the expected amplicon size. Iteman *et al*. (2000), Boyer *et al*. (2001),(2002) demonstrated the structural details performed the ITS comparative analysis in various species of *Microcoleus* and *Scytonema*. Erwin & Thacker (2008), Johansen *et al*. (2017), Mai *et al*. (2018) and many others believed to be a stable feature and the dissimilarity percentage value calculated by the p-distance analysis for the ITS region became a pre-requisite to deduce the inter-specific relationship. Since, last two decades, the 16S-23S ITS structural analysis was a mandatory criterion of the polyphasic assessment of cyanobacterial species.

1.3 Challenges in modern polyphasic taxonomic approach

The introduction of ultrastructural electron microscope, molecular and genetics methods evaluated for the cyanobacterial taxonomical study. The taxonomists were resolved many problems regarding the artificial system.

- The problem in cyanobacterial biosystematics is a prokaryote; it does not follow *International Code of Nomenclature for Prokaryotes (ICNP)*. Oren (2011b) described the strictness of the rules of ICNP required an axenic culture for its evaluation, whereas the maintenance of bacterial-free cyanobacterial culture was unachievable.

- As per the onset practice of the modern taxonomic method till date, numerous 16S rRNA sequence data for strains have been submitted to NCBI. Many of them are wrongly submitted in the database. Komarek (2006) faced this serious problem of the incorrect naming of the submitted strain continuously appearing in the phylogenetic analysis. This situation leads to the misinterpretation of the phylogenetic data and an erroneous identification.
- Komarek (2016) explained the concept of cryptotaxa in which the phylogenetic tree, clearly built with different and separated clades often occur which are almost impossible to separate morphologically or are detectable by very unclear morphological markers. The particular problem of cryptotaxa is relevant when they are not yet able to find clear distinguishing features apart from molecular separation. The cryptogenera and cryptospecies should characterize taxonomically but the form of their nomenclature division is not clear corresponding methods. The cryptotaxa are clearly defined and delimited taxonomic units according to molecular criteria, morphotaxa according to cytomorphological markers, and ecotaxa according to ecological differences, while sharp limits without transitions do not exist for other types of features.
- Komarek (2018) mentioned an incorrect input of naming of nomenclature type that leads to several complications in the revision of the cyanobacterial study of the modern approach. In the earlier method, various strains existed in the database are often designated by the traditional method according to morphological studies without revision, or without the concept of nomenclature were often outdated and might be incorrect; which leads to difficulty to build the phylogenetic tree. In the

construction of the phylogenetic tree, sometime the species belongs from the same genera or same family, but forms a different clade due to the wrong input of data.

1.4 Current status of cyanobacterial taxonomy of mangrove ecosystem

Kathiresan & Bingham (2001) explained how the mangrove covers the coastal regions of 112 countries in the tropical and sub-tropical zones. In the interface between land and sea, the mangroves encounter variations and periodic adaptations in environmental factors such as - temperature, salinity, tidal current, light intensity, etc. Alongi (2009) explained on the fluctuations result in the deprived growth of many flora and fauna. A substantial microbial diversity is found in these regions. Salinity in mangroves varies greatly in response to tide and time. Joset *et al*. (1996) proposed that the cyanobacterial cells exposed to the salinity fluctuations caused a loss of water and an influx of ions to the intracellular areas. These losses are compensated by the active antiport of non-toxic organic molecules and the ions inside and outside the cytoplasm respectively prevent the cyanobacterial cells from desiccation.

Alvarenga *et al*. (2015) demonstrated the extremities of the mangrove ecosystem subject the cyanobacterial species to selection pressures leading to the development of new ecotypes and morphotypes and enlisting around 80 genera reported from the mangroves of different countries.

Pramanik *et al*. (2011) reported eight obligately halophilic cyanobacterial strains and screened their antimicrobial potentiality. These eight strains were temporarily proposed to the ‘LPP-group’ (Lyngbya-Plectonema-Phormidium) based on the partial 16S rRNA gene sequencing. The current work was based on these isolates and was aimed to follow a thorough taxonomic assessment based on the polyphasic approach.

In the study of Debnath et al. (2017) based on the simple trichal non-heterocytous morphotypes was found to be unique. The **novel morphotype species**, named as ***Leptolyngbya indica sp.nov.*** isolated from lower Gangetic plains, the Sagar Island of the Sundarbans region. It provided vital information on morphotypic diversity of Cyanobacteria from specific biotopes which can contribute information on their biogeography and potential application in green remediation.

In the study of Chakraborty et al. (2018) based on the taxonomic characterization by a polyphasic approach carried out by cyanobacterial strain isolated from the soil biofilms of the Indian Sundarbans. The strain was studied morphologically by light microscopy, scanning and transmission electron microscopy. Growth responses to various salinities were recorded. Molecular data included sequencing and phylogenetic study of the 16S rRNA gene as well as analysis of the 16S-23S ITS regions. This taxonomic study was based on morphological analysis, ecological and molecular differences in comparison, isolated strain considered as a **second novel species** in the ***Oxynema*** genus, for which the name ***Oxynema aestuarii sp. nov***. was proposed.

In the study of Chakraborty et al.(2019) based on the molecular data based revision on ***Leptolyngbya***, the largest polyphyletic genus of the family **Leptolyngbyaceae** (**Synechococcales**) was imperative. 16S rRNA gene sequences showed the non-redundant nucleotide sequences of their closest relatives of the ***Leptolyngbya*** genus. A test strain was located in the phylogenetic tree in a clade (a group of organisms believed to comprise all the evolutionary descendants of a common ancestor) different from one type of species. A single operon having both tRNA^{ile} and tRNA^{ala} genes were present in the ITS regions which are compared to two operons in the ITS region of the genera ***Leptolyngbya*** and ***Nodosilinea*****:** one having both tRNAile and tRNAala genes and another lacking both the genes. Molecular phylogenetic and morphological data suggested to be monophyletic taxa which introduced **two different novel species**, named as ***Euryhalinema mangrovii gen. nov., sp. nov.*** and ***Leptoelongatus litoralis gen. nov., sp. nov.*** are proposed respectively. In the study of Chakraborty et al.(2021) based on the two novel cyanobacterial strains with thin cells and simple morphology which was isolated from the Indian Sundarbans. The 16S rRNA phylogeny data revealed the distinct lineage which was nearest to the clade incorporating the genus ***Oculatella*** and ***Tildeniel*****.** The 16S-23S ITS region for both strains displayed significant variations and uniqueness when compared with the respective reference strain (***Euryhalinema mangrovii*** and all the genera of **Oculatellaceae**). **One novel mono-specific genus species,** named as ***Aerofilum fasciculate*** and **one novel species,** named as ***Euryhalinema pallustris*** isolated respectively.

In the study of Iteman et al. (2000) based on the PCR amplification of the internal transcribed spacer (ITS) between 16S- 23S rRNA genes of the cyanobacterium *Nostoc* PCC 7120. The ITS region of cyanobacteria was varied by the length from 283-545 nucleotides. It contained either both tRNA^{Ile} and tRNA^{Ala} genes, only the tRNA^{Ile} gene, or there is no correlation between ITS size and coding capacity for tRNAs. It described about highly conserved motifs which were important for folding and maturation of the rRNA transcripts was identified. The box B – box A regions were homologous to bacterial antitermination.

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2. Aims and Objective

2.1 Aims of the study

Banerjee & Santra (2001), and Debnath *et al*. (2017) opined that the Indian Sundarbans, world's largest mangrove forest situated on the shore of the Bay of Bengal lacks documentation on the cyanobacterial diversity except a few till date. Pramanik *et al*. (2011) discussed according to the literature review revealed that cyanobacterial diversity in the Bay of Bengal was not exploited to that extent concerning other oceans surrounding the Indian sub-continent.

Alvarenga et al. (2015) explained the periodic variations in the physical parameters of the mangrove regions of the Indian Sundarbans like tidal current, salinity gradients, etc. created a dynamic environment for the microbes to inhabit. The environment created a selection pressure that leads to speciation and hence, undoubtedly countless new species to be recorded.

Based on the background, the principal aim of the study was:

- **To focus on the biosystematics of the cyanobacterial populations of the Indian Sundarbans leading to the enrichment of the alpha-diversity of these species.**
- **To undergo taxonomical study of the polyphyletic family, assuming to which most of our isolated belonging strains.**

2.2 Objectives of the study

To achieve the aims, the following specific objectives were fulfilled:

1. Isolation of the samples from different islands of the Indian Sundarbans, the purification to obtain a unialgal condition for each strain, and deposition of holotypes for each isolate to a culture collection for maintenance and valid description.
2. Identification of the isolated strains based on morphological and genetical variability (polyphasic approach).
3. Establishment of valid description for each of the isolated strain characterized by apomorphic features were following the proper rules of the *International Code of Nomenclature for Algae, Fungi and Plants* (ICN).
4. Investigating the effect of environmental variables of mangrove ecosystem over the evolutionary patterns of the isolated cyanobacteria.
5. Understanding the overall phylogenetic relationship within the isolated cyanobacterial strains.

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3. Materials and Methods

3.1 Sample Collection Study Site

The sample study site included in the research consisted of two different islands of the Indian Sundarbans.

The geographical sites were as follows:

- Maipith of the Indian Sundarbans, West Bengal, India (21º47’28”N 88º22’31”E) in November 2018.
- Namkhana of the Indian Sundarbans, West Bengal, India (21º55’06”N 88º15’54”E) in November 2018.

Pramanik *et al*. (2011) explained that the collected samples were treated in the laboratory according to the parameters of the sampling site like water conductivity, pH, temperature, salinity was recorded.

3.2 Isolation and sample processing

The samples collected from the sample-collecting site were processed by dissolving 1.0g of soil sample into 10.0 ml of PBS. The dilution was serially followed up to ten to thousand folds. According to Rippka *et al*. (1979) explained that 1.0mL of the suspension of the diluted sample was inoculated into 100ml of ASN III medium.

Light with the irradiance of 50 µmol photons m^2 s^{-1} along with 12 hrs light: 12 hrs dark period had been illuminated in the culture racks where the samples was placed.

A temperature of about 25±1 ºC was fixed for their growth. Sufficient growths were observed after 40 days and the biomass checked under the light microscope for the presence of the viable cyanobacterial filament.

3.3 Purification and maintenance of the cyanobacterial culture

The culture with viable cyanobacterial filaments was observed and found to be mixed with multiple cyanobacterial strains along the bacterial load. Purification to obtain the unialgal culture was performed by utilizing various procedures.

3.3.1 Serial Dilution

Serial dilution of 1.0ml suspension containing the mixed cyanobacterial cultures was done in 10.0 ml of ASN III medium and then plated in the ASN III medium petri dishes. The inoculated petri plates were then incubated at 25±1 ºC along with sufficient light. The plates were checked under the light microscope for growth and the whole procedure was repeated till the plates showed diffused growth of the filaments separated apart from the other strains under the microscope.

3.3.2 Cycloheximide treatment

Urmeneta *et al*. (2003) explained to prevent the growth of eukaryotic algae in the culture of 1.0 ml of cycloheximide from a stock solution (50 mg/ml in methanol at 75%) was added to 1000ml of ASN III medium.

3.3.3 Triple Antibiotic Solution

Following Guillard (2005), the mixture of 100 mg Penicillin G (Na salt), 50 mg Streptomycin sulfate dissolved in 10 ml distilled water, and 10 mg Chloramphenicol dissolved in 1 ml 95% ethanol was prepared and filter sterilization (Acrodisc syringe filter; Pall Corporation, Port Washington, New York, USA). The mixture was then treated as triple antibiotic solution. 1 ml of a suspension of non-axenic cyanobacterial culture inoculated into 50 ml of ASN III medium in a 250 ml Erlenmeyer flask was treated with different volumes (0.125, 0.25, 0.5, 1.0, 2.0, 3.0 ml) of triple antibiotic solution and were inoculated for 24 hrs. to 48 hrs. to get rid of the bacterial load allowed the contamination-free cyanobacterial growth.

According to Vaara *et al*. (1979), 500 µl of the suspension, after incubation, was inoculated into 10.0 ml of fresh ASN III medium and incubated for 20-25 days. After incubation, the purity of the cultures was determined by the absence of bacterial growth in the culture when it was inoculated in TYG broth and incubated for 7 days at 25±1 ºC and 37±1 ºC. The inoculated tubes have shown a healthy growth of cyanobacterial cultures as well as no bacterial growth was further selected for sub-culturing into large volumes.

3.3.4 Purification by the phenomenon of phototaxis

The gliding movement of the filamentous cyanobacterial species helps to purify them as a unialgal culture very efficiently. After serial dilution, 1 ml of non-axenic culture suspension was spread into a petri plate having agar ASN III medium. The non-axenic culture was inoculated within the covered part of the petri plate and was incubated for 5-6 days in an illuminated condition of 50 µmol photon m^{-2} s^{-1} for 12:12 hrs light: dark photoperiod at 25±1 ºC.

After incubation, the plate was observed under the light microscope and the filaments were found to be moved towards the light and hence diffused towards the other half of the petri dish. The single unialgal filament were then isolated axenically by the help of sterile needle and freshly inoculated further into plates as well as liquid ASN III medium in test tubes.

3.3.5 Stock Cultures

The purified unialgal culture was maintained as a pure culture in 100 ml ASN III medium in an appropriate illumination and temperature for further analysis. This was known as stock culture. The morphology of the culture was checked at regular intervals of time by observing under light microscope. Sub-culturing was repeated every 20 days intervals.

3.4 Methodology for morphometric analysis

3.4.1 Light Microscopy

Morphological characteristics were observed with the help of a light microscope. The ten days-old log phase culture of cyanobacterial culture was isolated and a temporary mount of the specimen was prepared on a glass slide aseptically.

The slide was observed under a light microscope (Model DM750; Leica Microsystems, Buffalo Grove, USA) under 400-1000 times magnification. Photomicrographs of the unialgal filaments for each of the six isolates were obtained by using the camera attached to the microscope (ICC50 HD). The cellular length and width were observed and marked in photomicrograph with the help of auxiliary software (LAS-EZ, Leica Microsystems) were associated with the microscope.

3.4.2 Scanning Electron Microscopy

The cell surface features were resolved and studied by using scanning electron microscopy. The sample preparation involved the centrifugation process for the isolation of the biomass at 8000 rpm (Eppendorf 5810R, rotor F-34-6-38, Hamburg, Germany) to make it more concentrated. These steps followed the fixation of the cell concentrate by adding 3% glutaraldehyde and incubating it for 2 hrs., and then successively washed in the distilled water. Dehydrating of the cells was done by passing the cells through enhancing the gradation of ethanol from 30% for 15 min to 100% for 60 min.

After the dehydration process, samples were dried at the critical point and the grids containing the samples were scanned under the scanning electron microscope (Jeol JSM-6700F, Jeol, Tokyo, Japan).

3.5 Methodology for Ultrastructural analysis

3.5.1 Transmission Electron Microscopy

Ultrastructural properties of a cyanobacterial cell contributed to a critical evaluation of a kind of synapomorphic marker used for their taxonomic delineation.

One milliliter of suspension from the fresh log-phase culture was taken and it was centrifuged at 8000 rpm (Eppendorf 5810R, rotor F-34-6-38, Hamburg, Germany). The cell pellet was washed carefully in distilled water and then successively 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1M phosphate buffer (pH 7.8) were added to the cell pellet for pre-fixation for 5-6 hrs. at 4 ºC. After the incubation process, the surplus fixation was washed off by rinsing the sample in 0.1M phosphate buffer. A 1% solution of osmium tetroxide was added to the cells for post-fixation and incubated for 60 min. Subsequently, dehydration of the cells was carried out by passing through the increasing concentration of ethanol with proper incubation. Dehydrated cells were then infiltrated and embedded in Araldite CY212 (Agar Scientific, Stansted, UK) for cutting the sections. The polymerization of the resin blocks was done by heat treatment at 50 ºC overnight and the second treatment was undergone at 60 ºC for 2 days. The thin sections of the samples were incised with the help of an ultramicrotome. After the ultrathin section was obtained, uranyl acetate and lead citrate used for the contrast of sections were followed by the observation under TECNAI G20 transmission electron microscope (FEL, Eindhoven, Netherland).

3.6 Growth analysis based on different pigment estimation

3.6.1 Estimation of Chlorophyll *a* (Chl *a*)

Cyanobacterial strains have Chlorophyll *a*, and the quantitative estimation is important to the study of growth and photosynthetic rates. Chlorophyll *a* extracted completely in solvents like chilled methanol/ethanol, and characteristics were exhibited under absorption at 632nm, 652nm, 665nm, 696nm, and 750nm.

- The known volume of homogenous cyanobacterial suspension was centrifuged at 8000 rpm for 10 min.
- Cyanobacterial pellets were washed twice with sterile ASN III media.
- The pellets were suspended with 4ml of chilled methanol/ethanol and vortexed thoroughly and covered the falcon tube with aluminum foil.
- Then the falcon tube was incubated at -20 ºC refrigerator for 1 hr.
- After cooling the falcon tube, the total content was centrifuged at 8000 rpm for 5 min, and the supernatant was transferred to another falcon tube. Again the solvent extraction process was repeated as described in earlier steps.
- To ensure the complete extraction, 2ml solvent were added and the total supernatant volumes were made up to 10ml.
- Finally, the readings were taken at 632nm, 652nm, 665nm, 696nm, and 750nm in a spectrophotometer (Labtronics model LT-291, Microprocessor UV-Vis Spectrophotometer) against methanol/ethanol as a blank.

Equation for Chlorophyll *a* Estimation

Chl a (µg/ml)

$= -2.0780*(A_{632} – A_{750}) – 6.5079*(A_{652} - A_{750}) + 16.2127*(A_{665} – A_{750}) – 2.1372*(A_{696} - A_{750})*(\pm 0.0070)$

A = Absorbance at 632nm, 652nm, 665nm, 696nm, 750nm

3.6.2 Estimation of Carotenoids

Carotenoids included pigments like carotenes and xanthophylls. They were soluble in a solvent like methanol/ethanol, and the characteristics were exhibited under absorption at 480nm and 750nm.

- The known volume of homogenous cyanobacterial suspension was centrifuged at 8000 rpm for 10 min.
- Cyanobacterial pellets were washed twice with sterile ASN III media.
- The pellets were suspended with 4ml of chilled methanol/ethanol, and vortexed thoroughly.
- The total content was centrifuged at 8000 rpm for 5 mins, and the supernatant was stored in a -20 C refrigerator.
- The extraction procedure by chilled methanol/ethanol was repeated still the methanol/ethanol was colorless.
- Finally, the supernatant was measured at 480nm and 750nm in a spectrophotometer (Labtronics model LT-291, Microprocessor UV-Vis Spectrophotometer) against methanol/ethanol as a blank.

Equation for Carotenoids Estimation

Carotenoids (µg/ml) = $4*(A_{480} – A_{750})$

A = Absorbance at 480nm, 750nm

3.6.3 Estimation of Phycobiliproteins

Phycobiliproteins are generally 24% dry weights of the soluble proteins of cyanobacterial cell strains were important for physiological, biochemical, and ecological studies. The C-phycocyanin (PC), C-phycoerythrin (PE), and allophycocyanin (APC) are water soluble pigments were extracted in phosphate buffer (pH 6.8). The characteristics were exhibited under absorption at 562nm, 615nm, and 652nm.

- The known volume of homogenous cyanobacterial suspension was centrifuged at 8000 rpm for 10 min.
- Cyanobacterial pellets were washed twice with sterile ASN III media.
- The cyanobacterial pellets were suspended in 3ml of sodium-phosphate buffer (0.1M, pH 7.0, containing 1 mM sodium azide)
- The process of freezing was done at -20 ºC repeatedly and thawing at room temperature in the dark followed the further process of extraction of phycobiliproteins.
- The total content was centrifuged at 10000 rpm for 30 min at 4 ºC.
- The extracted supernatant was stored in the refrigerator and the process was repeated for complete extraction.
- Finally, the supernatant was measured at 562nm, 615nm, and 652nm in a spectrophotometer (Labtronics model LT-291, Microprocessor UV-Vis Spectrophotometer) against sodium phosphate buffer as a blank.

Equation for C-Phycocyanin Estimation

C-Phycocyanin (µg/ml) = $[A_{615} - 0.474*(A_{652})]/5.34$

A = Absorbance at 615nm, 652nm

Equation for C-Allophycocyanin Estimation

C-Allophycocyanin (µg/ml) = $[A_{652} - 0.208 *(A_{615})]/5.09$

A = Absorbance at 652nm, 615nm

Equation for C-Phycoerythrin Estimation

C-Phycoerythrin (µg/ml) = $[A_{562} – 2.41(PC)-0.849(APC)]/9.62$

A = Absorbance at 562nm, 615nm, 652nm

3.7 Molecular analysis

3.7.1 Genomic DNA Extraction

For the isolation process, Genomic DNA was extracted by collecting 1ml of suspension of cells from the pure culture at its exponential growth phase. The cells homogenized in a pre-autoclaved homogenizer to get a uniform suspension. Homogenized cells were centrifuged at 8000 rpm (Eppendorf 5810R, rotor F-34-6-38, Hamburg, Germany) for 10 min at room temperature. Then the supernatant was discarded, and the cell pellet was used for further extraction process. GeneJET^{TM} Genomic DNA Purification Kit (Cat. No. K0721, Thermo Scientific, Waltham, USA) was used for the genomic DNA extraction followed by the manufacturer's protocol.

After the completion of the extraction process, the purified genomic DNA was checked by running gel electrophoresis respectively. The gel was stained with ethidium bromide and the DNA bands were visualized under UV-transilluminator. The genomic DNA extracts were stored at -20 ºC for future experiments.

3.7.2 PCR Amplification for the 16S gene sequence and the 16S – 23S Internal Transcribed Spacer (ITS) region

PCR amplification of the 16S rRNA gene sequence and the associated ITS space region was performed.

The set of primers that were utilized for the amplification process of 16S rRNA gene sequence:

(Nubel *et al*. 1997)

CYA 106F - 5' -CGGACGGGTGAGTAACGCGTGA- 3' (Forward)

(Lane 1991)

1492R - 5' -ACCTTGTTACGACTT- 3' (Reverse)

The set of primers that were utilized for the amplification process of the 16S - 23S ITS spacer region:

(Iteman *et al*. 2000)

16SF - 5' -TGTACACACCGGCCCGTC- 3' (Forward)

23SR - 5' -CTCTGTGCCTAGGTATCC- 3' (Reverse)

After several trials of standardized programs, it can fix to run the cycles of PCR amplification of both genes.

The program for the PCR amplification of the 16S rRNA gene sequence is as follows:

Chakraborty *et al*. (2018)

Initial denaturation at 94 ºC for 5 min

30 cycles at 94 ºC for 1 min,

50 ºC for 1 min and 72 ºC for 2 min

Final elongation at 72 ºC for 10 min

The program for the PCR amplification of the 16S - 23S ITS spacer gene sequence is as follows:

Chakraborty *et al*. (2018)

Initial denaturation at 95 ºC for 5 min

30 cycles at 95 ºC for 30s,

58 ºC for 15s and 72 ºC for 40s

Final elongation at 72 ºC for 5 min

The PCR master mix reaction volume 20 µl were performed in a Mastercycler Nexus Gradient PCR machine (Eppendorf, Hamburg, Germany).

The components of PCR mixture as followed:

Chakraborty *et al*. (2018)

	BIO-BHARATI Kit	**THERMO SCIENTIFIC Kit**
Template DNA	2µl	2µl
10X Taq polymerase buffer (with 15 mM $MgCl_2$)	2.5µl	2.5µl
dNTP	2µl	0.5µl
Each primer • **Forward primer** • **Reverse primer**	0.5µl	0.5µl
Taq polymerase (Invitrogen, Waltham, USA)	0.2µl	0.5µl

The amplification process was followed by the purified PCR products using PCR Purification Kit (Cat. No. K0701, Thermo Scientific, Waltham, USA). The products were checked for acceptable amplicon by running them in a horizontal gel electrophoresis system.

3 µl of the purified PCR product were mixed with the 1 µl of 6X loading buffer and dispensed in a well on an agarose gel (1.5% w/v) dissolved in 1 X TBE buffer. A DNA ladder of 500kb and 100kb were loaded in the gel, the reference determines the size of the amplicon. A voltage of 80 V for 2 hrs. was applied to perform the gel electrophoresis.

The gel was stained with ethidium bromide and the DNA bands were visualized under UV-transilluminator.

The appropriate bands for amplicon were incised from the agarose gel under UV light with the help of a sharp razor. The purification of PCR product was extracted by GeneJET^{TM} Gel Extraction Kit (Cat. No. K0691, Thermo Scientific, Waltham, USA) followed by manufacturer's instruction. Then, the purified PCR product extracts were stored at -20 ºC. Later, it was used for cloning process.

3.7.3 Amplicon Cloning and Sequencing

The purified PCR product for the 16S rRNA and 16S – 23S ITS gene sequences was cloned by InsTAclone^{TM} PCR Cloning Kit (Cat. No. K1213, K1214, Thermo Scientific, Waltham, USA). The commercial vector used for cloning was pCR 2.1 (Life Technologies, Invitrogen, USA). The PCR product were inserted, and ligated to the vector, according to the pCR 2.1 manufacturer’s protocol followed by its transformation into the competent cells of *E.coli* DH5α. The transformation mixtures were spread gently onto the LB plates supplemented with ampicillin, IPTG, and X-gal as supplement. The plates were incubated at 37°C for overnight. The protocol was followed according to the manufacturer’s instruction.

After overnight incubation, plates are screened for the positive transformants by blue-white screening method. For plasmid DNA isolation, white colonies was picked aseptically and inoculated into a fresh LB media (supplemented with ampicillin). The isolation of plasmid DNA was done by using Thermo Scientific GeneJET Plasmid Miniprep Kit (Cat. No. K0502, K0503, Waltham, USA). The cloned PCR product was digested by using restriction enzymes, Eco RI and Hind III. Then, the cloned PCR product was run on the gel electrophoresis to estimate the insert size. The samples were sequenced by using an automated DNA sequences, (Genetic Analyzer 3500xL, Applied Biosystems, Waltham, USA) as reported by Chakraborty *et al*. (2018).

3.8 Submission of Gene Sequence in the GenBank

After obtaining the sequences, the submission of those sequences followed into NCBI GenBank (http:/www.ncbi.nlm.nih.gov/genbank/) prior to their phylogenetic analysis. So, all the sequences for two strains namely S9 and S15 were submitted and their accession numbers represented as tabular form.

3.9 Analysis of 16S rRNA

3.9.1 Construction of Phylogenetic tree

The BLAST server performed to differentiate the gene sequences of 16S rRNA and ITS spacer regions were obtained from the isolate strains and compared to the other member sequences with their respective families to determine the similarity percentages. Based on the sequence similarity values compared to other genera and species available in the database of NCBI, many sequences closer to the test strains were selected for phylogenetic tree construction. Other sequences were considered for the robustness of the phylogenetic tree.

3.9.1.1 Analysis and Construction of Phylogenetic tree for strain S9 and S15

In the case of purified strains of S9 and S15, morphological and partial 16S rRNA data inferred that the reference strain close to the studied isolate was *Euryhalinema mangrovii* which belongs to the Nodosilineaceae family proposed by Strunecký *et al*. (2023). The sequences of reference organisms along with other related Oscillatorean members, the strains were under investigation and the outgroup taxon, *Gloeobacter violaceus* were obtained from the database of NCBI and were aligned using the program CLUSTAL W (Larkin et al. 2007). The aligned sequences were used to construct the phylogenetic tree based on ML and NJ analysis.

The gaps were excluded from the phylogeny. Confidence values on the phylogenetic branch were based on the bootstrap calculation of 1000 replications. The output of the phylogenetic tree was visualized using Tree Graph 2 represented as Stover & Muller (2010). The bootstrap values of the tree constructed by NJ analysis were represented in the ML tree and highly supported values in both analysis was marked by asterisks.

3.10 Analysis of 16S – 23S Internal Transcribed Spacer (ITS) secondary structure

The sequences of the ITS spacer regions for all the two purified strains were aligned with the reference organisms according to the variable and conserved domains had been identified.

Johansen *et al*. (2011) explained that there are 14 regions in the ITS spacer region which consist of highly conserved as well as highly variable regions. The nucleotide length of 14 regions was identified for each strain along with their references and compared their differences. According to Iteman *et al.* (2011) reported that the regions were identified with the help of provided knowledge about the conserved basal sequence for each helix.

There are some regions like D1-D1’ helix, Box B helix, V2 region, and V3 helix folded with the help of M-fold web server, version 2.3 adapted from Zucker (2000) into secondary structures which were then compared with their references to find the inter-relationship among them.

The secondary structures were generated under the ideal conditions with a default temperature of 37 ⁰C and the structure was assigned to untangle loop fix. The analysis of secondary structures and the source of the sequence r reference from each group of strains were described under the following sub-sections such as:

3.10.1 Comparative Analysis of 16S – 23S Internal Transcribed Spacer (ITS) secondary structure for strain S9 and S15

In the case of purified strains S9 and S15, the reference strain *Euryhalinema mangrovii* which belongs to the family Nodosilineaceae (Strunecky *et al*. 2023) was sequenced for the ITS spacer region since the reference article only presented the partial sequence information of its 16S rRNA gene. After the sequencing, the variable and conserved regions of the ITS were identified and compared in Table 5.2. Box B, D1-D1’ and V3 helices are folded into secondary structures.

3.10.2 Uncorrected p-distance for 16S rRNA and 16S – 23S ITS secondary structure strain S9 and S15

The divergence rates of 16S rRNA and ITS were calculated by uncorrected p-distance using MEGA 6.0 and the output was calculated for 16S rRNA using similarity percentage matrix [100 × (1- p-distance)] and for ITS using dissimilarity percentage (100 × p-distance).

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4. Taxonomy study on Cyanobacterial Strain S8 and S15

4.1 Result and Discussion

4.1.1 Morphology Analysis

The two strains S9 and S15 were examined under the microscope. According to the key information by Komarek & Anagnostidis (2005), the preliminarily morphological comparison study was done.

The two strains S9 and S15 are morphologically similar and assumed to belong to the Nodosilineaceae family with morphologically similar affinity to *Euryhalinema* and *Nodosilinea* genus. The S9 strain and S15 strain morphologically revealed similar properties and compared with their respective reference strains *Euryhalinema mangrovii* (MK402979), Euryhalinema *pallustris* AP3 from Chakraborty *et al*. (2018), (2019), (2021), *and Nodosilinea nodulosa* UTEX 2910 from Perkerson *et al*. (2011). (Table-1)

Both strains show gliding movement in the growth phase visible under light microscope. The cell division of S9 strain and S15 strain were seriated and unbranched. The S9 strain and S15 strain, both are having the presence of the mucilage sheath visible under the scanning electron microscope. The cell length-to-width ratio for both strains was smaller in comparison to their respective reference strains. The cell length of S9 strain is approx. 0.542 µm and S15 strain is approx. 0.522 µm, whereas, the cell width of S9 strain is approx. 0.570 µm and S15 strain is approx. 0.588 µm. Terminal cell morphology was studied and compared with the reference strains. The apical cell end S9 strain bear a rounded corner and S15 strain bear cylindrical with a slight rounded end, observed according to Komarek (2014) whereas in the reference strains, apical cell ends were rounded in all cases according to Johansen *et al*. (2011).

4.1.2 Ultrastructural Analysis

The parietal arrangement of thylakoids and ultrastructural properties revealed under transmission electron microscopy which typical isolate feature of the Nodosilineaceae family. The contrasting similarities were found in the inter-thylakoidal space which was distant in the isolated two strains of the S9 and the S15 as compared to the reference strain *Nodosilinea nodulosa*. Cellular structures like sheath, cell wall, constrictions in the cross walls, thylakoids arrangements and presence of different cell inclusions were clearly visible. The feature was considered a diacritical marker for species or genus-level discrimination by Bruno *et al*. (2009). The S9 strain and S15 strain belong to the upper surface of estuarine soil and the intertidal area shows slight similarities to the reference strain belonging to the lentic systems of habitat according to Johansen *et al*. (2011).

Table 1. The morphological features of test strains S9, and S15 comparative analysis with other genera of Nodosilineaceae family. Previously the characteristics were described the genera *Euryhalinema mangrovii* and *Euryhalinema pallustris* were obtained from Chakraborty *et al*. (2019 & 2021); *Euryhalinema epiphytium* from De Araujo *et al*. (2022); *Nodosilinea nodulosa* from Perkerson *et al*. (2011) and *Nodosilinea chupiquarensis* from Vazquez-Martinez *et al*. (2018).

Feature	***S9***	***S15***	***Euryhalinema mangrovii***	***Euryhalinema Pallustris***	***Euryhalinema epiphytcum***	***Nodosilinea nodulosa***	***Nodosilinea chupiquarensis***
Thallus	**Bluish green in color, fine mat**	**Bluish green in color, fine mat**	**Pale bluish green color mats**	**Thin biofilm of light greenish color**	**Pale bluish green color mats**	**Greenish mat**	**Blue-green patina, embedded in mucilage**
Mucilage sheath	**Thin, colorless mucilage sheath present**	**Thin, colorless mucilage sheath present**	**Absent**	**Absent**	**Absent**	**Occasionally present**	**Present**
Motility	**Yes**	**Yes**	**No**	**No**	**No**	**No**	**No**
Constrictions at cross walls	**Prominently constricted**	**Prominently constricted**	**Prominent constrictions**	**Prominent constrictions**	**Slightly constricted**	**Distinctly constricted**	**Prominent constrictions**
Cell length(µm)	**0.54**	**0.52**	**1.1-1.6**	**1.25-2.6**	**1.6-4.3**	**1.1-1.25**	**1.1-1.3**
Cell width (µm)	**0.57**	**0.58**	**0.4-0.6**	**0.4-0.5**	**0.8-1.0**	**0.7-0.9**	**0.9-1.2**
False branching	**Absent**	**Absent**	**Absent**	**Absent**	**Absent**	**Occasionally**	**Absent**
Cell form	**Distinctly much longer than wide**	**Distinctly much longer than wide**	**longer than wide**	**longer than wide**	**Cylindrical, longer than wide**	**Mostly isodiametric**	**Barrel shape, occasionally disc or isodiametric**
Trichome apex/ Apical Cell Shape	**Rounded**	**Cylindrical with a slight rounded**	**Rounded, not attenuated**	**Rounded not attenuated**	**Rounded not attenuated**	**Barrel Shaped**	**Dome shaped, sometimes elongated**

Habitat	**Upper surface of estuarine soil and intertidal area salinity ranging from 1.6-1.8%**	**Upper surface of estuarine soil and intertidal area salinity ranging from 1.7-1.8%**	**Intertidal area (estuarine) with salinity ranging from 1.7-1.8%**	**Intertidal area (estuarine) with salinity ranging from 1.7-1.8%**	**Marine, epiphyte on benthic red macroalgae *Bostrychia mantagne***	**Marine (planktonic tow) from depth 10 m**	**Stone surface, epilithic**

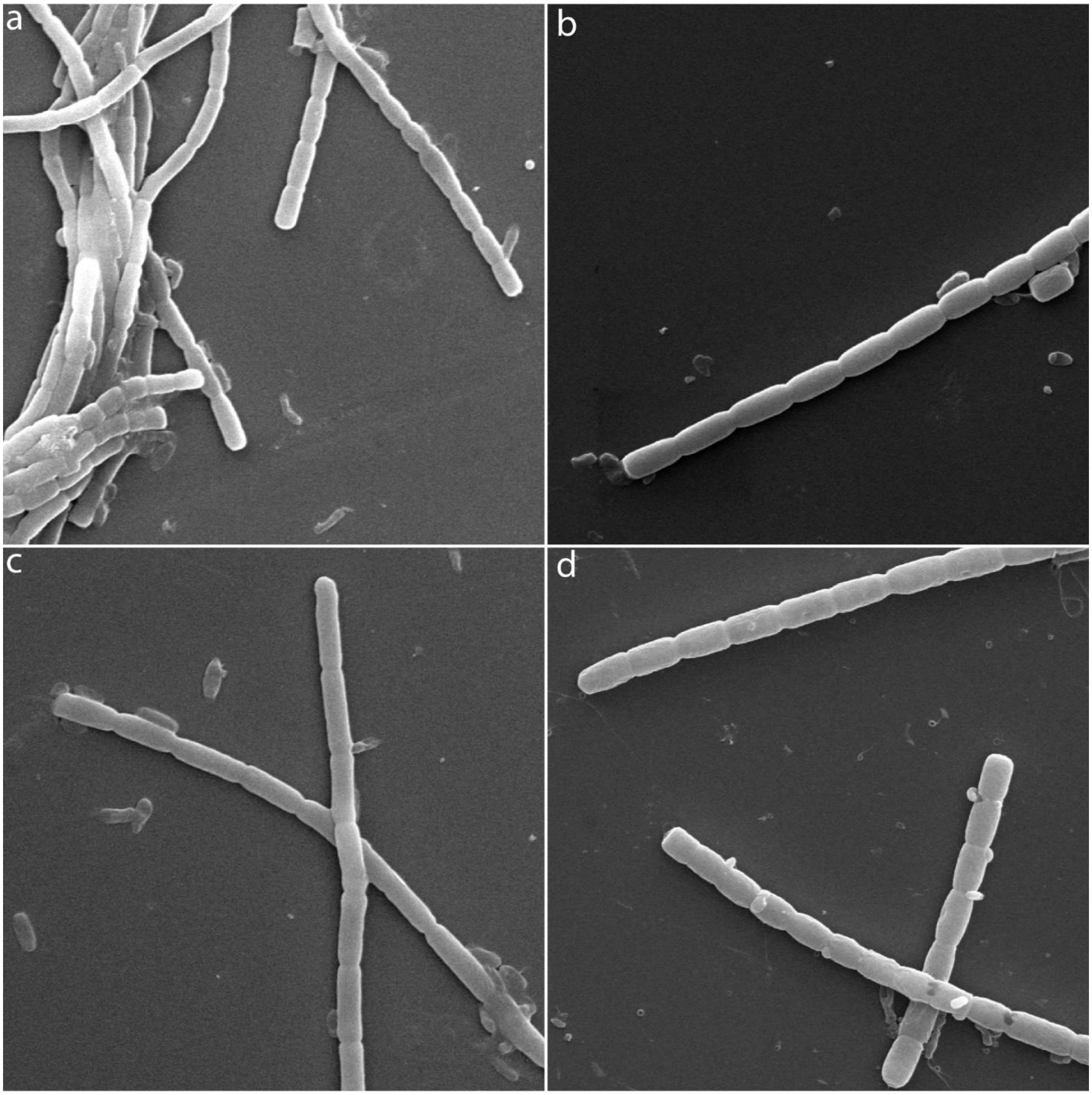


FIGURE 1. Scanning electron microscopy of two strains S9 and S15. **a) & b)** The apical part of the S9 strain showed presence of mucilage sheath predominantly constricted and rounded apex, and **c) & d).** The apical part in the S15 strain showed presence of mucilage sheath, predominantly constricted and cylindrical with slightly rounded apex.

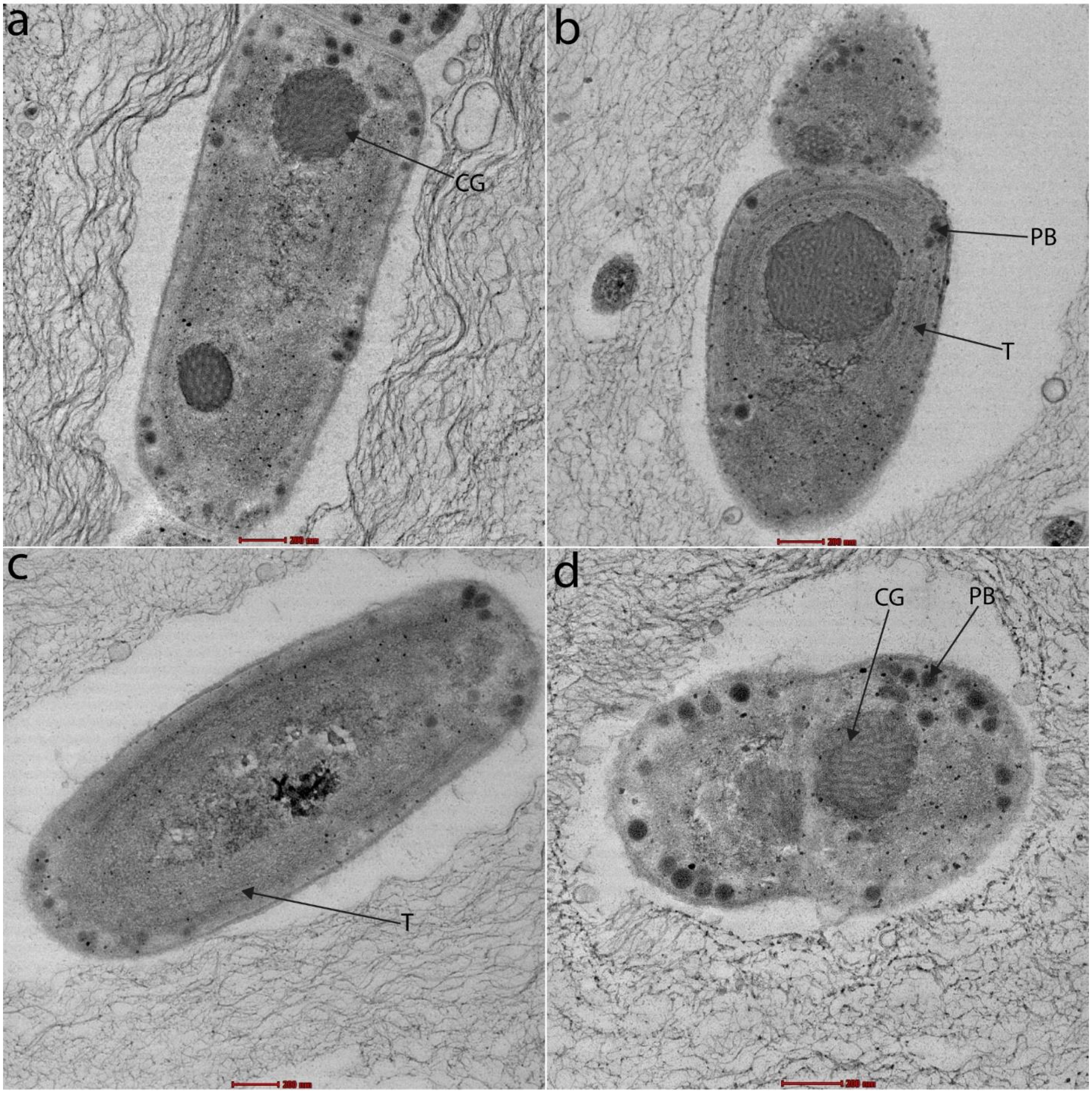


FIGURE 2. Transmission electron microscopy of two strains S9 and S15. **a) & b)** showed the transverse section of S9 strain, and **c) & d)** showed the transverse section of S15 strain. **CG = Cyanophycin Granules, T = Thylakoid arrangement, and PB = Phosphate Body.**

4.1.3 Growth Analysis based on different pigment estimation

In the growth analysis based on the different pigment composition showed slight changes in both S9 and S15 cyanobacterial strains. In general information, the carotenoid content was either higher or equal to the chlorophyll *a*, whereas the phycocyanin (PC) content was higher than the phycoerythrin (PE) content. There are slight variations in the chlorophyll *a* content and carotenoid content of S15 strain is slightly higher value than S9 strain. The phycocyanin (PC) content and allophycocyanin (APC) content of S15 strain is higher value than the S9 strain whereas phycoerythrin (PE) content of S9 strain is greater value than S15 strain. According to the absorption spectra value of the extracted phycobilins of S9 strain contains lowest phycocyanin and highest phycoerythrin and S15 strain contains highest phycocyanin and lowest phycoerythrin. Whether S9 strain and S15 strain belongs from the Nodosilineaceae family and shows the similar morphological feature but slightly varies in the physiological growth factor due to changes in location and adaptative feature.

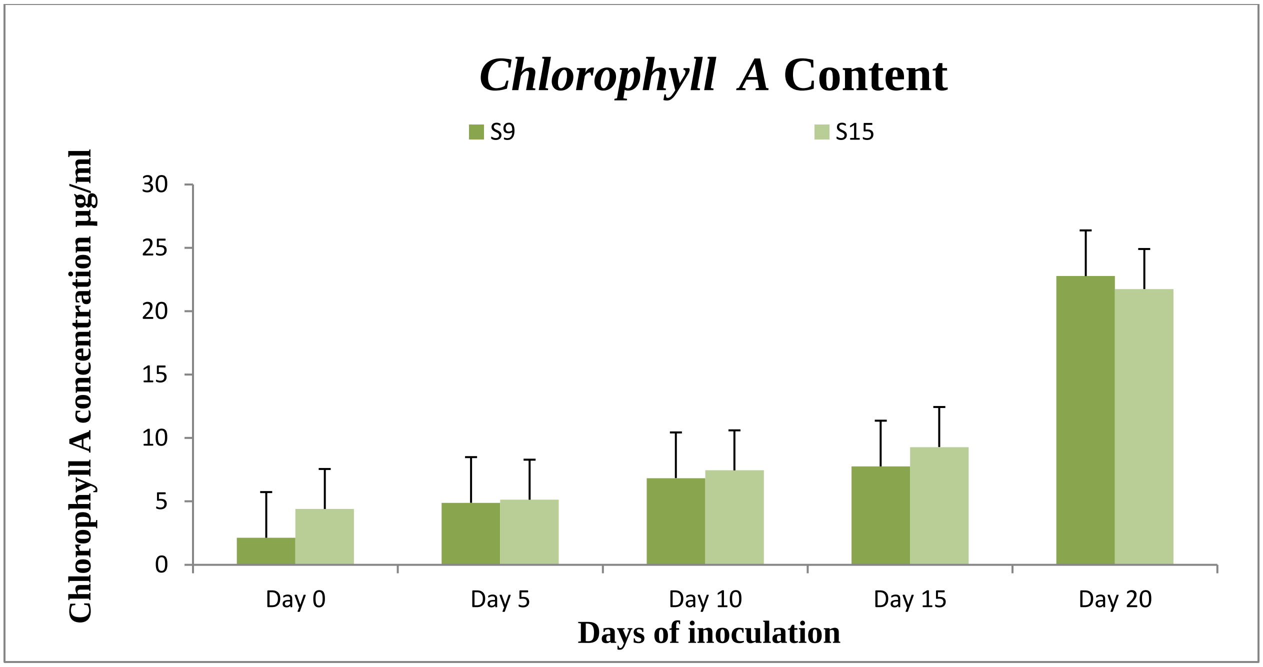


FIGURE 3. Estimation of Chlorophyll A content in the two distinctive purified S9 strain and S15 strain

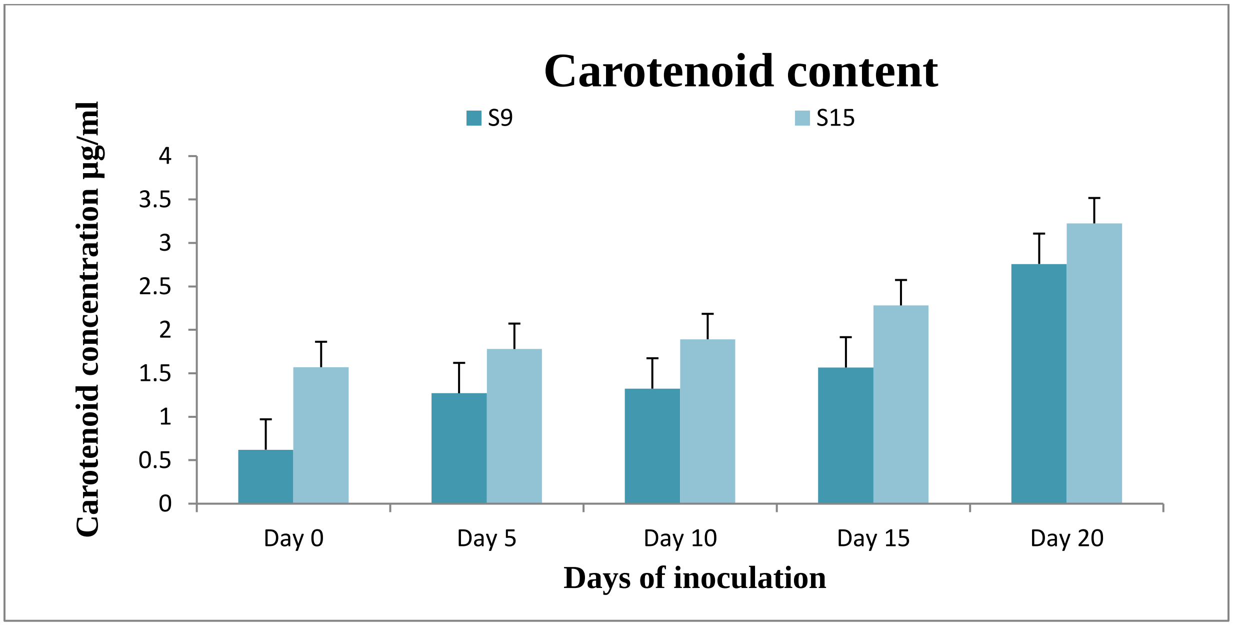
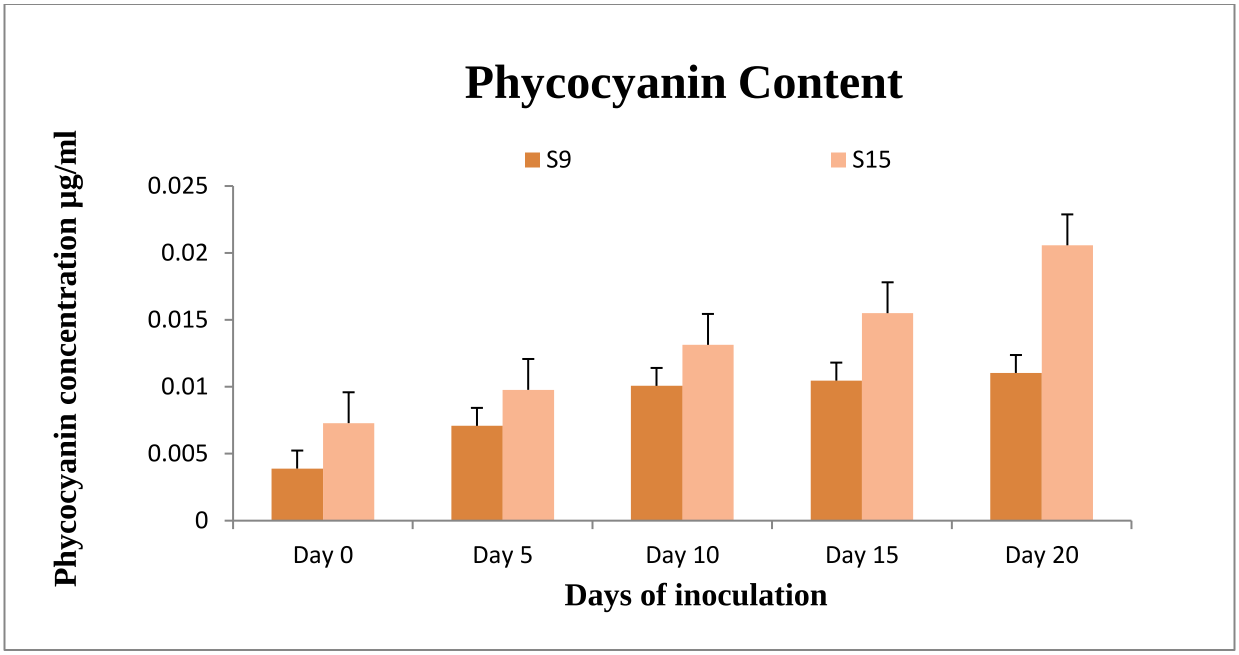
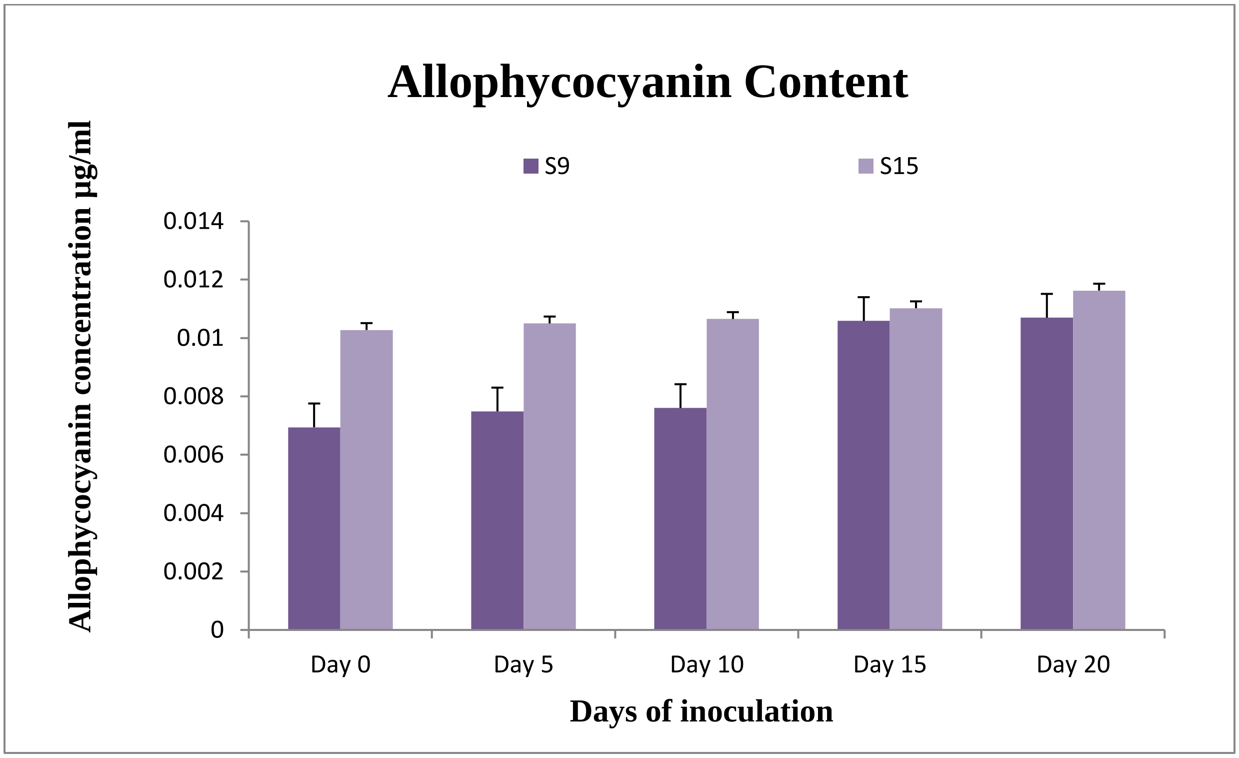


FIGURE 4. Estimation of Carotenoid content in the two distinctive purified S9 strain and S15 strain


Phycocyanin Content
S9
S15
Phycocyanin concentration µg/ml
0.025
0.02
0.015
0.01
0.005
0
Day 0
Day 5
Day 10
Day 15
Day 20
Days of inoculation


Allophycocyanin Content
S9
S15
Allophycocyanin concentration µg/ml
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
Day 0
Day 5
Day 10
Day 15
Day 20
Days of inoculation

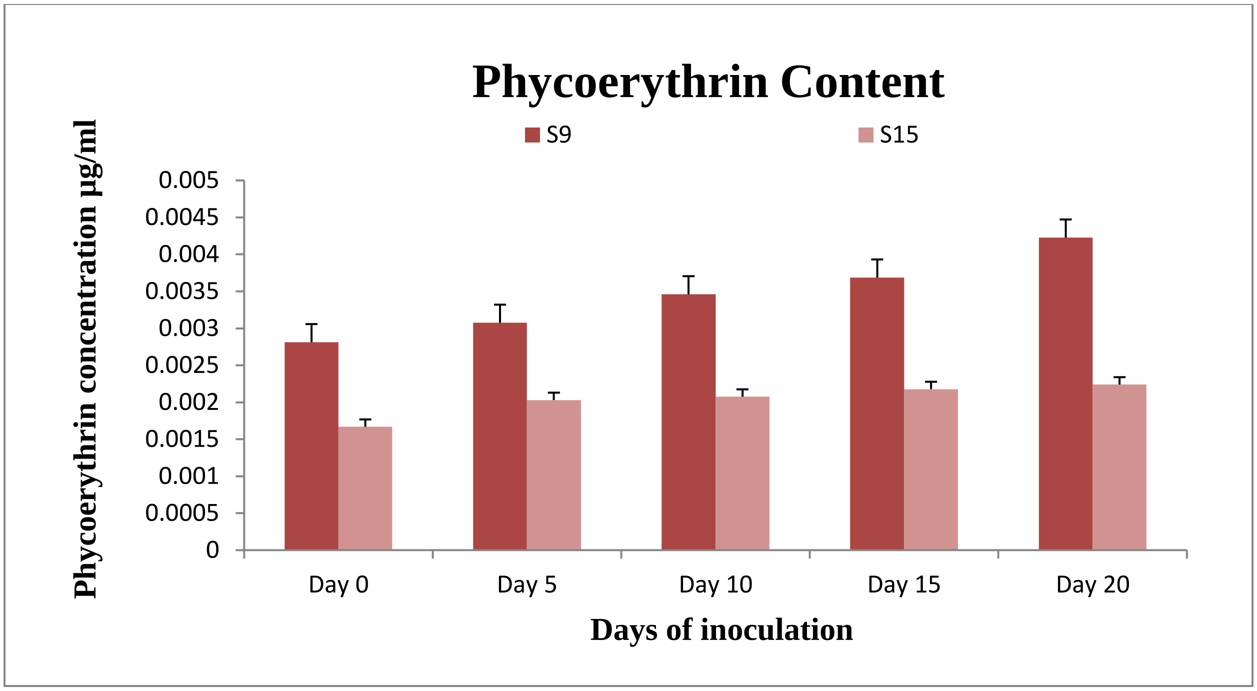


FIGURE 5. Estimation of Phycobiliprotein consist of Phycocyanin, Allophycocyanin and Phycoerythrin content in the two distinctive purified S9 strain and S15 strain

4.1.4 Analysis of 16S rRNA, and construction of phylogenetic tree

In the PCR purification and amplification process, the composition of PCR master mix of ThermoScientific Kit showed the positive result whereas, Bio Bharati Kit showed the blank result. The purified PCR - cloning strains of the 16S rRNA phylogenetic tree analysis such as - S9 with the length of 1136 bp and S15 with the length of 1066 bp respectively.

The investigation sequences of the 16S rRNA genes of the purified strains of S9 and S15 were examined in the Basic Local Alignment Search Tool (BLAST) within the robust database of the National Center of Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/). The pairwise similarities were determined with other members available in the database. The closest relationship of S9 strain was revealed by BLAST with the species *Euryhalinema mangrovii* (MK402979) which showed genetic similarities of about 94.10% and S15 strain was revealed by BLAST with the species Euryhalinema *mangrovii* (MK402979) which showed genetic similarities of about 92.30%.

According to the taxonomic description of the S9 strains were *Halomicronema hongdechloris*, *Nodosilinea chupicuarensis*, and *Nodosilinea nodulosa* with 93.09%, 93.27%, and 93.27% genetic similarities respectively with the experimental strains and the S15 strains were *Halomicronema hongdechloris*, *Nodosilinea chupicuarensis* and *Nodosilinea nodulosa* with 91.15%, 91.52%, and 92.84% genetic similarities respectively with the experimental strains. The species of the genus *Euryhalinema* were selected as reference strains which were the closest match with the two strains - S9 and S15 in the NCBI BLAST analysis i.e., *Euryhalinema* *mangrovii* proposed by Chakraborty *et al*. (2019), *Euryhalinema pallustris* proposed by Chakraborty *et al*. (2021) and *Euryhalinema epiphyticum* proposed by Araujo *et al*. (2022) are belonging to the genera of the Nodosilineaceae family was considered in the 16S rRNA phylogenetic analysis explained by Strunecký *et al*. (2023)

The consensus phylogenetic tree construction includes all the data studied of the two strains S9, and S15 compared with the closest relation between the species of the family Nodosilineaceae, Oculatellaceae, and Leptolyngbyaceae to justify the evolutionary relationship. The generated phylogenetic tree confirmed that S9 strain, and S15 strain clustered together and formed the well-supported clade along with S1 strain, S6 strain and S10 strain under the Nodosilineaceae family. The clade was separated from the nearest relative genus *Euryhalinema* genus as well as the clades of other genera formed a distinct novel phylogenetic lineage can be depicted according to Maximum likelihood (ML), Neighbor-joining (NJ), Maximum parsimony (MP) analyses and Bayesian Inference (BI).

The establishment of p-distance analysis was undertaken between the inter-species relationship of the test strains S9 and S15 with the closest Nodosilineaceae family. The similarities percentage varied from 92.9% to 94.1% for 16S rRNA data, and the dissimilarities percentage of the 16S-23S ITS sequences were as certain from 26.3% to 32.6%.

TABLE 2. List of gene sequences of the purified experimental cyanobacterial strains submitted in NCBI GenBank with their accession numbers.

Strain name	**S9**	**S15**
NCBI GenBank Accession numbers for 16S rDNA	OR026460	OR026461
NCBI GenBank Accession numbers for 16S-23S ITS rDNA	OR039042	OR039043
Length of 16S rDNA (bp)	1136	1066
Length of 16S-ITS (bp)	636	636

FIGURE 6. Phylogenetic tree based on 16S rRNA gene sequences and *Gloeobacter violaceus* as outgroup. Bootstrapping with 1000 resampling was performed. The support values ML bootstrap. The investigated two strains S9 and S15 are shown in bold.

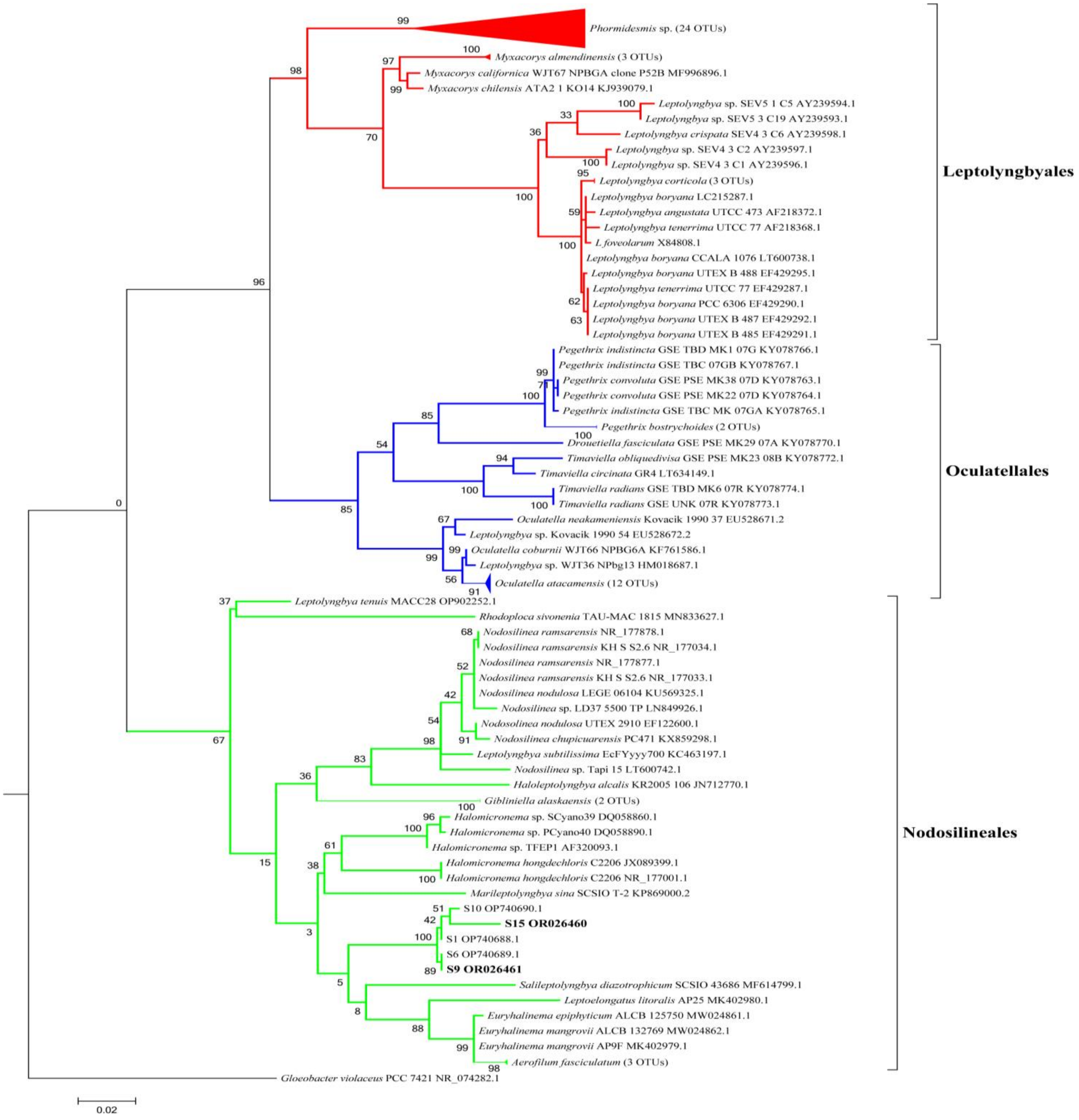


TABLE 3. Similarities percentage of the p-distance analysis of the 16S rRNA gene sequence data for the two strains S9, and S15 based on BLAST results.

	Strains	**1**	**2**	**3**	4	5	6	7	8	9	10	11	12
1	**S9**												
2	**S15**	**96.73**											
3	*Euryhalinema mangrovii* AP9F	94.1	94.6	94.3									
4	*Euryhalinema pallustris* AP3	92.9	93.3	93	98.2								
5	*Halomicronema hongdechloris* C2206	93.2	93.4	93.1	93.4	92	93.3	92.4	93				
6	*Nodosilinea chupicuarensis* PC471	93	93.4	93.1	94	93	93.9	98.7	92.8	92.3			
7	*Nodosilinea nodulosa* UTEX 2910	92.9	93.3	93	94.3	93.2	94.1	98.3	92.8	92.2	98.8		
8	*Nodosilinema ramsarensis* KH-S S2.6	92.9	93.3	93	94.1	93	93.9	98.4	92.8	92.3	98.6	99.7	

TABLE 4. Dissimilarities percentage of the p-distance analysis of 16S-23S ITS gene sequence data for the two strains S9, and S15 based on the closest relative strains.

	Strains	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	**S9**										
2	**S15**	**0.3**									
3	*Euryhalinema mangrovii* AP9F	26.3	26.6	26.3							
4	*Euryhalinema pallustris* AP3	28.1	28.3	28.1	9.3						
5	*Halomicronema hongdechloris* C2206	32.6	32.8	32.6	33.3	33.6	36.8				
6	*Nodosilinea chupicuarensis* PC471	28.8	29.1	28.8	29.6	31.1	33.8	31.6			
7	*Nodosilinea nodulosa* UTEX 2910	28.1	28.3	28.1	29.6	30.1	33.3	31.3	2.8		
8	*Nodosilinea ramsarensis* KH-S S2.6	27.3	27.6	27.3	28.1	28.8	32.6	32.6	6	4.8	

4.1.5 Analysis of 16S-23S Internal Transcribed Spacer (ITS) region

The PCR amplification of the analysis of the 16S-23S ITS regions of S9 strain and S15 strain are about 636 bp long sequences was generated respectively. According to Johansen et al. (2011) demonstrated the ITS comprising of the two tRNA genes can be apportioned into 14 regions: (i) leader (ii) D1-D1' helix (iii) D2 helix and spacer (iv) D3 spacer (v) $tRNA^{Ile}$ gene (vi) spacer between tRNA genes, often including the V2 helix (vii) $tRNA^{Ala}$ gene (viii) spacer preceding Box B (ix) Box B helix (x) spacer following Box B (xi) Box A (xii) D4 (xiii) V3 and (xiv) D5.

The ITS sequence similarity among the two strains S9 and S15 was about 99.84% with respect to other members of the Nodosilineaceae family. The most conspicuous feature differentiating the isolates under investigation with reference was the presence of only a single operon containing the genes for $tRNA^{Ile}$ and $tRNA^{Ala}$ while the other operon lacking those genes was absent. Experimental strains possess similar ITS sequence with <1% similarity with a single operon having both tRNA genes ($tRNA^{Ile}$ and $tRNA^{Ala}$).

The sequences of 16S-23S internal transcribed spacer (ITS) regions of the experimental strains were used for the taxonomic identification of the strains at the species level.

The complete ITS sequences data studied of S9 strain and S15 strain were aligned with the six respective reference strains such as *Euryhalinema mangrovii, Euryhalinema pallustris, Halomicronema hondechloris, Nodosilinea chupiqurensis, Nodosilinea nodulosa,* and *Nodosilinea ramsarenis*. All the conserved regions as well as variable regions were identified. The ITS sequence region of the reference strains was obtained from NCBI GenBank.

The fundamental ITS regions like D1-D’ helix, Box-B, and V3 helices were evaluated with the equivalent secondary structures of the reference strains. According to Iteman *et al.* (2000) explained that the basal helix sequence near the 5' - end of the D1-D1' helix is highly conserved in cyanobacterial genera and found as 5' - GACCU. The basal sequences of D1-D1' helices of the ITS region of S9 strain and S15 strain were identical to the six reference strains: 5'- GACCU - AGGUC - 3'. The length of the D1-D1' helices of the ITS gene sequences of S9 strain and S15 strain is about 59 nucleotides respectively; under investigation which are distinct from the six reference strain *Euryhalinema mangrovii* about 63 nucleotides, *Euryhalinema pallustris* about 63 nucleotides, *Halomicronema hondechloris* about 60 nucleotides, *Nodosilinea chupiqurensis* about 63 nucleotides, *Nodosilinea nodulosa* about 62 nucleotides, and *Nodosilinea ramsarenis* about 62 nucleotides. The D1-D1' helix of the ITS gene sequence of S9 strain and S15 strain consists of a small terminal loop of 9 nucleotides, two bilateral bulges, and a side bulge with respect to reference strains. According to Iteman *et al.* (2000) explained that the basal helix sequence near the 5' - end of the Box-B helix was found as 5' - AGCA. The basal sequences of Box-B helices of the ITS region of S9 strain and S15 strain were identical to six reference strains: 5'- AGCA - UGCU - 3'.

The length of the Box-B helix of the ITS gene sequences of the S9 strain and S15 strain is about 41 nucleotides respectively; under investigation which are distinct from the six reference strain *Euryhalinema mangrovii* with about 32 nucleotides, *Euryhalinema pallustris* about 32 nucleotides, *Halomicronema hondechloris* about 36 nucleotides, *Nodosilinea chupiqurensis* about 40 nucleotides, *Nodosilinea nodulosa* about 40 nucleotides, and *Nodosilinea ramsarenis* about 47 nucleotides. The Box -B helix of the ITS gene sequence of the S9 strain and S15 strain are completely different from the six reference strains. They consisted of a small terminal loop of 4 nucleotides and a bilateral bulge with 5 nucleotides with respect to the reference strains.

Iteman *et al.* (2000) explained that the basal sequence of the secondary structure of the V3 region was found as 5' - GUC. The basal sequences of the secondary structure of the V3 region of the ITS region of S9 strain and S15 strain were identical to the six reference strains: 5'- GUC - GAU- 3'. The length of the V3 regions of the ITS gene sequences of the S9 strain and S15 strain is about 28 nucleotides respectively; under investigation which is distinct from the six reference strain *Euryhalinema mangrovii* with about 20 nucleotides, *Euryhalinema pallustris* about 24 nucleotides, *Halomicronema hondechloris* about 23 nucleotides, *Nodosilinea chupiqurensis* about 27 nucleotides, *Nodosilinea nodulosa* about 27 nucleotides, and *Nodosilinea ramsarenis* about 23 nucleotides. The secondary structures of the V3 region of the ITS gene sequence of the S9 strain and S15 strain are completely different from the six reference strains concerning the changing in the one nucleotide basal sequence as S9 strain and S15 strain having 5'- GUC - GAU- 3' whereas the six reference strain having 5'- GUC - GAC- 3'.

TABLE 5. The comparison of the nucleotide lengths of the ITS sequence regions of the S9 strain and the S15 strain with *Euryhalinema mangrovii, Euryhalinema pallustris, Halomicronema hondechloris, Nodosilinea chupiqurensis, Nodosilinea nodulosa,* and *Nodosilinea ramsarenis* of the Nodosilineaceae family. The studied data of the reference strain *Euryhalinema mangrovii* and species of Nodosilineaceae family were obtained from Chakraborty *et al*. (2019), (2021),

Chen *et al*. (2012), Perkerson *et al*. (2011) and Vazquez-Martinez *et al*. (2018). Bold values indicate differences with the test strains.

Strain ID	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**
S9	8	**59**	**35**	**12**	74	**5**	73	**36**	**41**	19	11	**7**	**28**	**15**
S15	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 pallustris AP3	8	**63**	**39**	**9**	74	**15**	73	**37**	**32**	24	11	**11**	**24**	**18**
Halomicronema hondechloris C2206	8	**60**	**34**	**7**	74	**72**	73	**17**	**36**	18	11	**7**	**23**	**6**
Nodosilinea chupiqurensis 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**

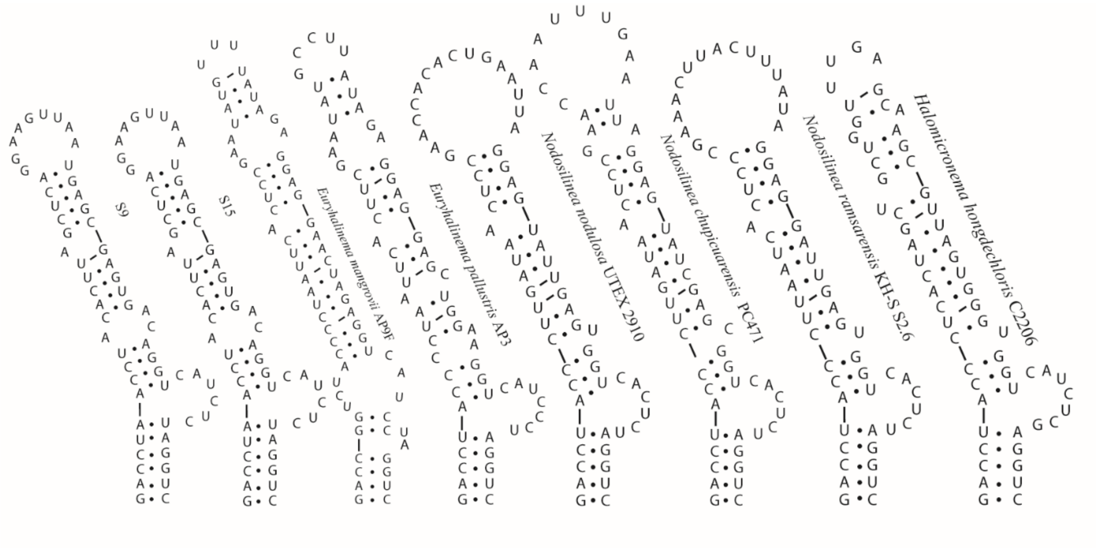


FIGURE 7. Secondary structure of D1-D1' helix. The comparative analysis of the secondary structure of S9 and S15 strain with reference strains *Euryhalinema mangrovii, Euryhalinema pallustris, Halomicronema hondechloris, Nodosilinea chupiqurensis, Nodosilinea nodulosa,* and *Nodosilinea ramsarenis* respectively. (Chakraborty *et al*. (2019), (2021))

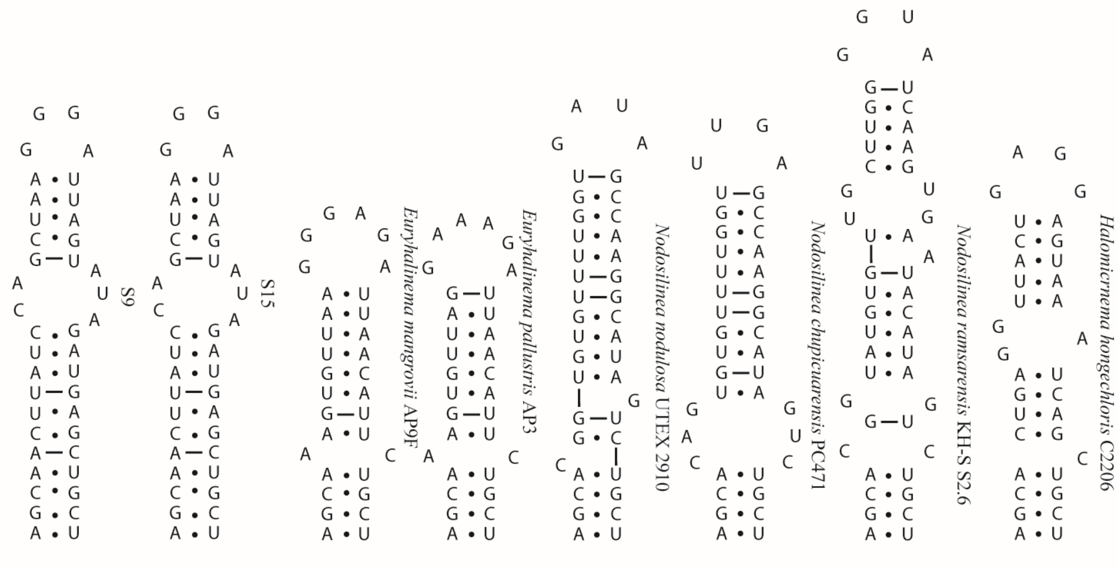


FIGURE 8. Secondary structure of BOX-B helix. The comparative analysis of the secondary structure of S9 and S15 strain with reference strains *Euryhalinema mangrovii, Euryhalinema*

pallustris, Halomicronema hondechloris, Nodosilinea chupiqurensis, Nodosilinea nodulosa, and *Nodosilinea ramsarenis* respectively. (Chakraborty *et al*. (2019), (2021))

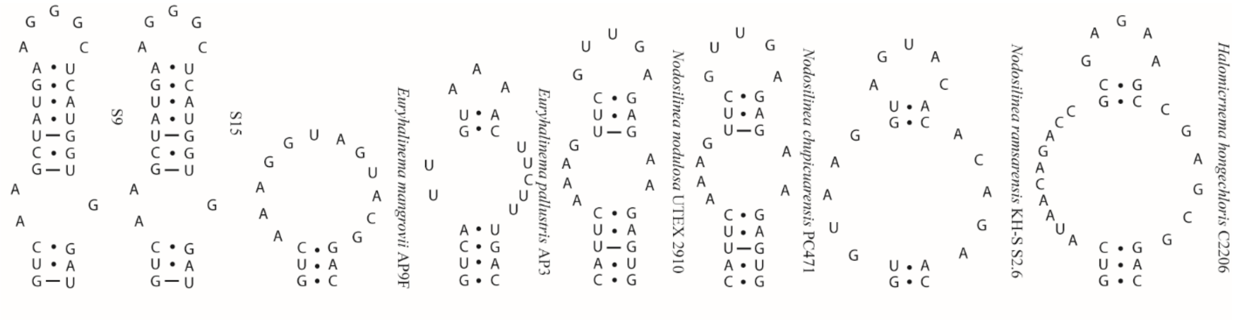


FIGURE 9. Secondary structure of V3 region. The comparative analysis of the secondary structure of S9 and S15 strain with reference strains *Euryhalinema mangrovii, Euryhalinema pallustris*, *Halomicronema hondechloris*, *Nodosilinea chupiqurensis*, *Nodosilinea nodulosa*, and *Nodosilinea ramsarenis* respectively. (Chakraborty *et al*. (2019), (2021)

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5. Concluding remarks

5.1 Conclusion

The study of the prokaryotic system depends on the inter-relationship among the organism and postulating hypothesis of identification. The studies of the taxonomic evaluation depend upon the biological membrane, photosynthetic cycle, growth factor, etc. In many cases, the identification of the species is based on their eco-geographical distribution to their physiological role. Taxonomic impediments introduced in the Convention on Biological diversity concerned about world-wide crisis of an important taxonomic information, the gaps in the taxonomic knowledge, and the crisis in the research oriented on the species. Wagele *et al*. (2011) coined that biological systematics is the oldest discipline in biology. A cyanobacterial species study was considered an alpha-biodiversity based on the habitation in the intertidal region of the Indian Sundarbans. The cyanobacterial species were collected from the soil surface biofilm and maintained in the laboratory. The cyanobacterial species were isolated from the culture and purified for the study of polyphasic taxonomic evaluation. In the laboratory, out of several strains, two distinct strains were isolates such as S9 and S15. These two strains were selected for the study of taxonomic evaluation by the concept of polyphasic approach.

According to the morphological and ultrastructural study, the two distinct S9 strain and S15 strain were observed under the microscope. The cell division of S9 strain and S15 strain is similar, both were seriated, unbranched, presence of mucilaginous sheath, the cell wall is predominantly constricted and thylakoid arrangement is visible.

According to the physiological study, the growth factor of S9 strain and S15 strain was described by the amount of chlorophyll *a* content, carotenoid content, and phycobiliprotein content. The comparison of absorption spectra value of S9 strain possess lowest phycocyanin content and highest phycoerythrin content whereas S15 strain possess highest phycocyanin content and lowest phycoerythrin content.

According to the 16S rRNA phylogenetic analysis, the two distinct S9 strain and S15 strain about 96.73% considered as novel genera. The S9 strain is about 94.10% and S15 strain is about 92.30% showed similarity and high affinity with reference strain i.e., *Euryhalinema mangrovii* (MK402979). The S9 strain and S15 strain showed the closest relation and well-supported clade with the reference strains belonging to the Nodosilineaceae family proposed by Strunecký *et al*. (2023).

According to the secondary structure 16S-23S ITS gene sequence, the basal sequence of the ITS gene sequence of S9 strain and S15 strain are supported in dissimilarity value with the reference species belonging to the Nodosilineaceae family. The basal sequence of the D1-D1' helices and Box-B helix of S9 strain and S15 strain are similar to the reference strain whereas, in the m-fold web server, the V3 region of the secondary structure of S9 strain and S15 strain generated as 5'- GUC - GAU - 3' instead of 5'- GUC - GAC - 3', which makes them an exception from the reference strain.

5.2 Future Approach

The Indian Sundarbans, consists of unique and unpublished species. This region is well known for its ideal niche to the study of biodiversity. The species composition found in mangrove regions considered as the best bioindicator of environmental condition of the ecosystem. The Indian Sundarbans was considered to be the presence of diverse population of microbial organism community which is still undiscovered and unpublished. The Indian Sundarbans regions having excessive stress of salinity and it was constantly undergoing tidal forcing and mixing of water columns that permit species habitant to flourished, resulting in considerable genetic diversity. It was noted that morphological characters of mangrove species was infrequently not in concurrence with phylogenetic relationships and had a tendency to change in response to the prevailing environmental conditions. They need to be conserved and protected for the conservation of genetically divers group of terrestrial and aquatic organisms.

The polyphasic taxonomic study is concerned with the identification of species that was diversified according to characteristics based on morphological, physiological, and genetic studies. In the case of the taxonomic studies, the identification of the cyanobacterial species, which was collected from different regions of the island established as a novel genus and species after being isolated. The study on the ability of the cyanobacterial species and their comparison leads to the establishment of a comprehensive study. The global compilation of cyanobacterial species data will improve the literature and research orientation on cyanobacterial diversity and the identification of the taxonomic system.

Komarek *et al.* (2014) required revising the arrangement of many genera as well as families. Previously, the nomenclature naming arrangement was done on the basis of morphological data which led to numerous strains being wrongly submitted in the NCBI database without underlying the polyphasic assessment which becomes difficult to construct the phylogenetic tree. This wrong input leads to the hindrance in the establishment of the monopoly in various clades. The classification of the taxa described on the hierarchical relationships inherited based on the classical morphological taxonomy and convergence of the phylogenetic distant taxa. The current taxonomic status of cyanobacteria is based on the International Code of Nomenclature for Algae, Plants, and Fungi.

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