MOLECULAR SYSTEMATICS OF THE BLUE-GREEN ALGAE *NOSTOC* SPP. AND *ANABAENA* SPP. (NOSTOCALES) OF KERALA

Thesis submitted to the
University of Calicut in partial fulfilment of
the requirements for the degree of

Doctor of Philosophy in Botany

by

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CERTIFICATE

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Certified that no corrections/ suggestions were made by the adjudicators for the Ph D thesis titled ' Molecular Systematics of the Blue-green algae *Nostoc* spp. and *Anabaena* spp. (Nostocales) of Kerala', submitted by Ms . Swetha Thilak T, to the University of Calicut.

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DECLARATION

I hereby declare that the thesis entitled **'Molecular Systematics of the Blue-green Algae *Nostoc* spp. and *Anabaena* spp. (Nostocales) of Kerala'** submitted by me in partial fulfilment of the requirements for the Degree of **Doctor of Philosophy** in **Botany**, **University of Calicut** is the bona fide work carried out by me in the Malabar Botanical Garden and Institute for Plant Sciences, Post Box No. 1, Kozhikode-673014, Kerala. No part of the work has formed the basis for the award of any other degree or diploma previously.

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Swetha Thilak T.

ABBREVIATIONS

Absorbance	A
Amplified Fragment Length Polymorphism	AFLP
Blue Green Algae	BGA
Basic Local Alignment Search Tool	BLAST
base pair	bp
Calcium nitrate dehydrate	$Ca(NO_3)2.4H_2O$
Calcium chloride decahydrate	$CaCl_2.10H_2O$
Carbon dioxide	CO_2
Cobalt nitrate	$CoNO_3$
Cetyl trimethyl ammonium	CTAB
Copper sulphate	$CuSO_4$
DNA Data Bank of Japan	DDBJ
Deoxy Ribo Nucleic acid	DNA
Escherichia coli	E. coli
Ethylene diamine tetra acetic acid	EDTA
European Molecular Biology Laboratory	EMBL
Farm Yard Manure	FYM
Gram per Litre	gm/ L
Glutamine oxaloglutarate amino transferace	GOGAT
Glutamine synthase	GS
Boric acid	H_3BO_3
Hydrochloric acid	HCl
Highly Iterated Palindromes	HIP
di Potassium hydrogen phosphate	K_2HPO_4
di Potassium molybdate	K_2MoO_4
Kilo base pair	kb
Picomolar	pM
Potassium chloride	KCl
Potassium nitrate	KNO_3
Long Term random repeating sequences	LTRR
Acronym of Malabar Botanical Garden Herbarium	MBGH
Magnesium chloride hexahydrate	$MgCl_2.6H_2O$
Magnesium sulphate heptahydrate	$MgSO_4.7H_2O$
Maximum Likelihood	ML
Manganese chloride tetrahydrate	$MnCl_2.4H_2O$
Normality	N
Sodium carbonate	Na_2CO_3
Sodium chloride	NaCl

Sodium hydrogen carbonate	$NaHCO_3$
Sodium nitrate	$NaNO_3$
Sodium hydroxide	NaOH
Nano gram	ng
Neighbour joining	NJ
Nitrogen, Phosphorous, Potassium	NPK
Polymerase Chain Reaction	PCR
Poly hydroxyl butyrate	PHB
Pentose phosphate pathway	PPP
Photo System I	PS I
Photo System II	PS II
Random Amplified Polymorphic DNA	RAPD
Ribosomal DNA	rDNA
Ribosomal Database Project	RDP
Restricted Fragment Length Polymorphism	RFLP
Ribo nucleic acid	RNA
Ribosomal RNA	rRNA
Ribulose, bis phosphate	RuBP
Sodium dodecyl sulphate	SDS
SDS- Polyacrylamide gel electrophoresis	SDS-PAGE
Second	sec
Tris acetic acid EDTA	TAE
Tris EDTA	TE buffer
Tris hydrochloric acid	Tris HCL
Ultra violet	UV
Zinc sulphate heptahydrate	$ZnSO_4.7H_2O$
Micro litre	µL

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INTRODUCTION

1. INTRODUCTION

1.1 BLUE-GREEN ALGAE

Blue-green algae have an ancient history of almost 3.5 billion years and diversified extensively to become one of the most successful and ecologically significant organisms on earth, with respect to longevity of lineage and impact on earth's early environment. Their origin in the early Pre-Cambrian era is considered to be one of the most important events in the organic evolution (Schopf, 1970).The advent of the electron microscopy revealed that blue-green algae are bacteria, presenting a well defined sub microscopic organization bereft of membrane-bound organelles. Their cells are surrounded by a more or less defined exo-polysaccharide (peptidoglycan) covering and a cell envelope similar to that of Gram negative bacteria.

They differ from other prokaryotes in the ability to perform oxygenic photosynthesis, localized in the thylakoid membranes. Unlike bacteria, they possess chlorophyll a and chlorophyll b which distinguishes them from other bacteria. Cyanobacteria contain a characteristic phycobilin pigment, phycocyanin, which gives the cells a bluish-green colour and is responsible for the popular name 'Blue-green algae'. They are considered to be the first organism to evolve elemental oxygen as a by-product of photosynthesis and thus make the earth's environment suitable for life. Much of the earth's oxygen is being contributed by them. They are unique since they are the only prokaryotes that perform oxygenic photosynthesis like vascular plants where both the photosystems (PS I and PS II) are present (Stal, 1995). They stand morphologically and physiologically a diverse group; morphology ranges from unicellular to multicellular organisms.

Cyanobacteria (BGA) are ubiquitous microorganisms capable of both photosynthesis and nitrogen fixation simultaneously and thus help in augmenting the fertility of soil. Biological nitrogen fixation is done only by certain microorganisms, which makes them even more special since N_2 is a

vital element in the amino acids, nucleic acids, etc and their availability in the soil is essential for the crop production as such these organisms serve as biofertilizers. Certain highly defined cells called the heterocysts are responsible for the nitrogen fixation. They possess a special enzyme called, nitrogenase which convert nitrogen to ammonia. The enzyme nitrogenase is oxygen sensitive. The cyanobacteria, that are oxygenic microorganism, maintains anoxygenic condition in the heterocyst in such a way that the heterocyst lacks PS I.

Apart from the oxygen evolving property, the ability for nitrogen fixation makes this group economically important one. Nostocalean strains of heterocystous cyanobacteria are considered to be the most important organisms of the N_2-fixing community in nutrient poor, arid and semiarid soils worldwide (Dodds *et al.,* 1995; Wynn-Williams, 2000; Bhatnagar and Bhatnagar, 2005). *Nostoc* and *Anabaena* are the two important genera of heterocystous cyanobacteria, and hence they are economically important as biofertilizers. They are seen to grow in association with the paddy crops in the field, all over the world.

Moreover, some species of Blue-green algae are used as food supplement in different parts of the world due to their high nutritional value. Certain delicacies are prepared from them in China and Japan.

The 'Blue-green algae', or the Cyanophytes, were in the field of interest of botanists (Phycologists) and ecologists, as they were treated as microscopic plant organisms for a long period of time. Traditionally BGA have been included in the algae as a major class classified under the provisions of International Code of Botanical Nomenclature (Stafleu *et al.,* 1972), but there exists much confusion such as the concept of type specimen in the botanical nomenclature is not applied in the taxonomy of BGA. Even now descriptions of new species are published without specimen being deposited. Such problems

could be overcome by the BGA cultured in axenic state (Desikachary, 1970; Stainer *et al.,* 1971). But the Botanical code does not recognise cultures as valid type materials, so they have to be dealt under Bacteriological Code which recognises cultured strains as holotype. Stainer *et al.* (1971) coined the name cyanobacteria for BGA, based on their resemblances to bacteria.

However, the suggestion to establish the nomenclature of blue-green under the rules of International Code of Nomenclature of Bacteria was made (Stainer *et al.,* 1978); the concept of cyanobacteria was not universally accepted (Bourrelly, 1979; Kondrat'eva, 1981). A solution to this problem was evolved in International Botanical Code, Sydney (1981) and International Microbial Congress, Boston (1982) that both the codes may co-exist and recognise each other for the nomenclature of cyanobacteria or Blue-green algae.

Traditionally, the blue-green algae (cyanobacteria) have been classified on the basis of their morphological and physiological characteristics in nature (Gietler, 1932; Desikachary, 1959). Despite the availability of various monographs based on morphological and ecological variants, the identification and classification of cyanobacteria remain a cumbersome and confusing task leading to uncertain identifications. Early taxonomic classifications of cyanobacteria were elaborated based on distinct morphological characters. The introduction of modern methods in last decades of 20^{th} century substantially changed our understanding of these organisms. Not mere morphological or physiological or biochemical or molecular attribute prove to generate sufficient data to provide true diversity and phylogeny of the cyanobacteria at the generic level, for which a polyphasic approach would be appropriate and reliable (Mishra *et al.*, 2013).

One difficulty in the use of previous literatures of nomenclature on experimental research is that there is no agreed policy for giving names to the strains of blue-green algae used. Some names refer to the morphology of the

organism when it was first isolated, but, in other cases as an organism changes in culture its name also changes (Whitton, 1992). However, morphology of the cyanobacterial strains may change depending on environmental conditions thus the selective culture conditions can alter the diversity of the strains (Palinska *et al.,* 1996). The morphology of cyanobacteria in laboratory cultures is known to be modified and the variation between different species become reduced due to the controlled culture conditions (Dores and Parker, 1988).

Classical classification of Blue-green algae frequently proves to be unsatisfactory, so a solution is suggested by the use of different approaches (Whitton, 1969). Molecular techniques are known to resolve many of the issues and problems in cyanobacterial taxonomy (Giovannoni *et al.,* 1988; Wilmotte and Golubic, 1991).Molecular (phylogenetic) data provide a basic criterion for taxonomic classification (Komarek, 2006). Therefore, molecular approaches based on PCR techniques and DNA fingerprinting has been adopted for taxonomical studies nowadays. Molecular methods such as RAPD, RFLP and AFLP are used for the PCR techniques (Oinam *et al.,* 2011). Appropriate combination of morphological attributes and physiological parameters can aid in clarifying phylogenetic relationships to some extent (Narayan *et al.,* 2006). To overcome the limitations of classical methods in taxonomy, molecular method based on the use of different molecular markers like small subunit rRNA gene has been proposed (Woese, 1987).

Since early 1990s, DNA sequencing technology and molecular-based phylogenetic analyses has been used to assist algal taxonomic studies (Draisma *et al.,* 2001). The unique properties of 16S rRNA gene such as, the conservative nature, its universal distribution and the vast availability of sequence information in public databases (GenBank, EMBL, DDBJ and RDP) make it a user friendly choice for systematic studies (Weisburg *et al.,* 1991).

Genomic sequences helps in the identification, study, modification and comparison of cyanobacterial genes and facilitates analysis of evolutionary relationships. Moreover, changes in genetic expression at transcriptional and translational levels in response to variations in environmental conditions could be monitored by the genomic sequence data (Koksharova and Wolk, 2002).

The biological sequence information including Protein, DNA and RNA sequence data are used as a subdiscipline of molecular phylogenetics to infer phylogenetic relationships. However, sequence data offers a number of key advantages: first, since every nucleotide position is a character with four different states (A, G, C or T), a single sequence can provide information on many different characters; second, molecular character states are unambiguous and third, molecular data are easily converted to numerical form and hence are amenable to mathematical and statistical analysis (Brown, 2002).

1.1.1 *Nostoc* and *Anabaena*

The scientist, philosopher and alchemist Aureolu Philippus Theophrastus Bombastus von Hohenheim on the 15nth century coined the word “Nostoch” to describe the gelatinous colonies of the ubiquitous terrestrial cyanobacterium *Nostoc commune* (Potts, 1997). The genus *Nostoc* is one of the earliest described cyanobacterial genera (Bornet & Flahault, 1888). It is one of the five genera in the family Nostocaceae of subgroup IV, section A, of phototrophic bacteria (Holt *et al.*, 1994). The *Nostoc* remains desiccated for months or years in the soil till the availability of water and can also withstand repeated cycles of freezing and thawing thus forms an important component of the extreme terrestrial habitats. *Nostoc* are found both as free living forms in the soil and they also form symbiotic with other plant groups like Bryophyte (*Anthoceros*), Pteridophyte (*Azolla*), Gymnosperm (*Cycas*) and Angiosperm (*Gunnera*), thus helping them in fixing the atmospheric nitrogen. The free living forms are also heterocystous and fix atmospheric nitrogen, almost all the free living forms are with mucilage. *Nostoc flagelliforme* is known to be used by the Chinese as a

food delicacy and for its herbal values for more than a century (Gao, 1998). In Japan also the colonies of *Nostoc* is being used for making soups.

Anabaena on the other hand forms planktonic community; in fresh water habitatsand can withstand a wide range of salinity. The word *Anabaena*is derived from the Greek word *anabainein* meaning to shoot up so called because; they rise to the surface at intervals. Majority are found in an alkaline environment. Biometric characters of vegetative cells, heterocysts and spores are the traditionally used morphological criteria for the identification of *Anabaena* spp. An important feature for species identity of the taxa is the proximity of the akinetes to heterocysts (Anand, 1976; 1979). *Anabaena* species are devoid of the mucilaginous envelope and they mostly form a mat on the soil. The major difference that differentiate *Anabaena* from *Nostoc* is that, the trichome of the *Nostoc* are highly coiled where as that of *Anabaena* are wavy in nature.

The distinguishing characters for the identification of these genera are so narrow that there are greater chances for misidentification.

1.2 SYSTEMATICS

It is the urge of human beings to classify and to group organisms that lead to the understanding of the diversity present in the world. Naturalists have tried to detect, describe and explain diversity in the biological world; this endeavour is known as “Systematics”. Taxonomy forms the root for all the researches since the knowledge about the lineage of the particular organism has a marked influence on its character. The first study on the hierarchical system of nomenclature is that of Linnaeus (1758), who is regarded as the ‘Father of Taxonomy’. This formed the basis of categorising biological diversity. The Linnaean system does not have any evolutionary basis which was the most debated drawback of this system. External morphological features of the

organisms were used to discriminate them. This become practical where there is a consistent number of differences in characters.

Molecular systematics is a comparatively new field of systematics where the evolutionary basis is also taken into consideration. The need for an evolution based systematics became very essential during the first half of the twentieth century where there were controversies on the species concept and the speciation. This new method use molecular data such as DNA or Proteins to deduce the phylogenetics relationships.

Phylogenetic method enables to understand the microevolution that has taken place in the genomic level and also helps in interpreting all kinds of biological processes and their patterns. Earlier, the phylogenetic method was based on the shared attributes of extant and fossil organisms. Algorithms based on these criteria were implemented in computer programs thus allowing the analysis of large and complex data sets.

In 1960s the methods for examining the molecular structure of proteins and nucleic acids were developed which helped in the further estimation of phylogeny. DNA is the basic units of information that resides in an individual as the three letter codon. DNA sequencing is the determination of nucleotide sequence in the DNA. Each character of the organism is controlled by the three letter codon. Two methods are there for sequencing the DNA *i.e.*; Maxam Gilbert or Chemical sequencing method and Sanger's di-deoxy nucleotide sequencing method. The most widely used and popular among these is the di-deoxy nucleotide sequencing method. The scope and applications of molecular systematics increased with the development of new applications of Polymerase Chain Reaction or PCR, which helped in getting enough amount of DNA thus helping in DNA sequencing.

Taxonomy is one of the major disciplines in biology; it is regarded as mother of all other disciplines of science. Without the correct identification of a taxon

their utilization for other purposes will be meaningless. Even though it is one of the oldest sciences, it derives information from other related disciplines such as embryology, cytology, chemotaxonomy, etc. Even though molecular techniques are comparatively new aspect; taxonomy derives information from this also. Every branch has helped taxonomy to evolve. It is the need of the century to evolve according to the changing scenario and all the good changes are to be accepted. Taxonomy has been transformed from a system that simply placed morphologically similar taxa into a hierarchical system of classification to that ideally reflects evolutionary relationships and creates a network of hypotheses about evolutionary history (Komarek *et al.*, 2014).

Systematics is a never ending synthesis. Being a synthetic discipline without data of its own, taxonomy and systematics have to rely on data produced by other disciplines. So, whenever newer and newer data are supplied, the mode and mood of systematics change. Consequently systematics undergoes vicissitudes from time to time making it a never ending synthesis.

1.3 IMPORTANCE OF THE STUDY

Nostocales are the common BGA of Kerala, of which *Nostoc* and *Anabaena* are the commonest genera. The species distinction in these genera, based on morphological feature is cumbersome due to morphological plasticity in nature as well as in culture and also due to their simple morphology. Much confusion exists in the distinction of these genera and species owing to morphological plasticity.Genetic relationships sometimes conflict with the morphological classification. It is expected that Molecular Systematics using nucleotide sequences can solve these problems. Comparison of morphological and genetic data is hindered by the lack of adequate isolated germplasm and inadequate morphological data.

These organisms contribute much in soil fertility, antimicrobial and antifungal, antioxidant properties; provide immense prospects in pharmaceuticals and

neutraceuticals etc. Correct identification of the organism is mandatory for the further use for mass production in industrial purposes. The information on the native species of *Nostoc* and *Anabaena* is essential. These organisms could be used as a cost effective and eco-friendly biofertilizers. The taxonomic problems existing due to the lack of considerable number of distinguishing characters could be overcome by the use of molecular methods. The pure cultures of all the strains are well maintained in the laboratory which could be used for the further references and other studies in future.

1.4 OBJECTIVES

- Standardise a protocol for isolation of the *Nostoc* spp. and *Anabaena* spp. occurring in Kerala.
- To attempt a taxonomic key based on the morphological features under light microscope.
- To analyse the molecular data using nucleotide sequences and elucidate phylogenetics relations.
- To develop the phylogeny of the *Nostoc* and *Anabaena* in Kerala.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 BLUE-GREEN ALGAE

The Blue-green algae characterised by the oxygenic photosynthesis and the occurrence with eukaryotic algae, prompted their treatment in the phycological circles since recent years, their prokaryotic nature, similarity to bacteria has been recognised for a century from where onwards they are being called as the Cyanophytes or Cyanobacteria. The Blue-green algae or the Cyanobacteria form one of the important groups both ecologically and economically. The taxonomic account of the cyanobacterial community in the country began in the early 90s.

Earlier contributions on the Cyanobacterial (Blue-green algae) flora of different states of the country were those of Ghose (1919, 1930, and 1931) who studied the Blue-green algae of Burma and Punjab. Bharaddwaja (1933) gave an account on the myxophyceae of India. The cyanobacteria of various regions of India were studied by different workers such as; Ghose *et al.* (1923) studied the cyanobacterial diversity of British India (Lahore and Shimla). Banerjee *et al.* (1938) studied the lower group of myxophyceae of Bengal, fresh water algae of Manipur were studied by Bharaddwaja (1963). Bendre and Kumar (1975) surveyed the cyanophyceae of Meerut, Grover and Pandol (1975) studied the algal flora of paddy fields of Ludhiana and its adjacent areas.

The Nostocales of Maratwada, Maharashtra were investigated by Asthekar and Kamat (1980), Blue-green algae of the paddy fields of Uttar Pradesh was surveyed by Prasad and Mehrotra (1980), Goyal *et al.* (1984) studied the algal flora of rice fields of Jammu and Kashmir. Blue-green algae of Bhubaneswar and its adjoining regions were surveyed by Mohanty (1984). Cyanophyceae from Andaman and Nicobar Islands were studied by Prasad and Srivastava (1986), Kolte and Goyal (1985) investigated the distribution of Blue-green algae in rice field soils of Vidarbha region of Maharashtra. Cyanobacteria of rice field soils of Pusa were studied by Jha *et al.* (1986).

During the past 10 years also the Cyanobacterial flora of the rice fields of the country were investigated by many workers (Nayak *et al*., 2001, Kaushik and Prasanna, 2002, Choudhury and Kennedy, 2005, Nayak and Prasanna, 2007, Rao *et al*., 2008). Studies conducted on the distribution of cyanobacteria from arid zones of Rajasthan by Tiwari *et al.* (2005) indicated their ubiquitous occurrence. Cyanobacterial diversity from different freshwater ponds of Thanjavur, Tamil Nadu was recorded by Muthukumar *et al.* (2007) which includes 39 species belonging to 20 genera. By surveying the cyanobacterial diversity in some local rice fields of Orissa, Dey (2010) documented 58 taxa of which majority were non-heterocystous and also highest abundance of cyanobacteria was of the order Nostocales. Selvi and Sivakumar (2011) studied the diversity of cyanobacteria in paddy fields of Cuddallor district of Tamil Nadu and collected 30 heterocystous forms.

Ghadai *et al.* (2010) surveyed the occurrence and abundance of cyanobacteria in various paddy fields of Gunupur district, Orissa and their study indicated the occurrence and abundance of *Oscillatoriaceae* and *Nostocaceae* in all sites. Cyanobacterial diversity in Ahmednagar, Pune and Satara district of Maharashtra was studied from screening of 627 soil samples by Nikam *et al*. (2013). They isolated 94 cyanobacterial spp. belonging to 38 genera, 14 families and 5 orders, among which *Westiellopsis prolifica* was abundant followed by *Nostoc calcicola*. The most densely populated genera were *Nostoc*, *Chroococcus* and *Anabaena.*

Cyanobacterial diversity and distribution of Chhattisgarh has been investigated by Singh *et al.* (2014) and recorded 29 strains of cyanobacteria, which included unicellular, colonial, un-branched, heterocystous, etc. They also found that environmental factors and physiochemical properties decide the structure and distribution of cyanobacteria. Priyadarshani *et al.* (2014) evaluated the blue-green algal biodiversity of Satara district, Maharashtra and collected 127

species from 36 genera, and also found that the filamentous forms were dominant over unicellular forms.

Chandra and Rajashekhar (2015) when studied the distribution pattern of the freshwater cyanobacteria in Kaiga region, Karnataka found that cyanobacterial diversity is more in the monsoon season compared to that of post-monsoon, and also water plays an important role in their abundance. Cyanobacterial diversity of River Ganga was perceived by Rishi and Aswathi (2015) and recorded a total of 114 species in which the most dominating genera were *Anabaena, Aphanizomenon, Chroococcus, Cylindrospermum, Lyngbya, Microsystis, Nodularia, Nostoc, Oscillatoria*, *Phormidium* and *Spirulina.*

Diversity of nitrogen fixing cyanobacteria in the forest soils of Upper Assam were studied by Adhikari and Baruah (2015). They isolated 18 taxa of which *Nostoc* and *Anabaena* were abundant. Nostocales of Eastern Himalayas were surveyed and collected 25 taxa which include 3 new reports from India, one new combination (*Macrospermum iyengarii* var. *unispora*) and a new taxon, *Diplocolon indicum* Keshri et Das (Das and Keshri, 2017).

The pioneers who worked on the Blue-green algal diversity occurring in Kerala include, Parukutty (1940) who studied the Blue-green algae of Travancore. John (1963) and Aiyer (1965) are the first to study the algal flora of Kerala. Amma *et al.* (1966) conducted studies on the occurrence of Blue-green algae in acid soils of Kerala. Anand and Hooper (1995) was the first to carry out the survey of the blue-green algae occurring in the rice fields of Kerala.

Recent studies on the occurrence of cyanobacteria were those of Madhusoodanan and Dominic (1995) who studied the variations in the diversity of blue-green algae in an acidic paddy field near Calicut University and encountered 39 species. They (1996) also isolated and characterised acid tolerant cyanobacteria from paddy fields of Kerala. Dominic and Madhusoodanan (1999) surveyed the blue-green algae from extreme acidic

environments of Kerala, Umamaheswari (2005) isolated and characterised the Nostocales of paddy fields of Kerala and reported eleven species of *Nostoc* and nine species of *Anabaena.*

Vijayan and Ray (2015) studied the ecology and diversity of cyanobacteria in Kuttanadu paddy wetlands. Sivakamasundari and Rajendran (2015) documented the cyanobacterial diversity of the mangrove vegetation of the Paravanar Estuary; of which majority belong to the non heterocystous cyanobacteria. The only heterocystous type documented is the *Nostoc microscopium.* Cyanobacteria biodiversity of the mangrove environments were studied by Ram and Shamina (2015) and recorded a total of 31 species coming under 10 genera and 4 families. *Oscillatoria* was the genus with maximum distribution, *Nostoc* ranks the second position.

2.2 ECONOMIC IMPORTANCE AS BIOFERTILIZERS

The most important property of the Blue-green algae is their ability to photosynthesise and they are the first primary producers in many environments. The nitrogen fixing capacity of cyanobacteria makes these organisms economically important. During the 19th century cyanobacteria (then considered as blue-green algae) were first suspected to fix atmospheric nitrogen due to their ability to grow well under nitrogen deficient conditions (Frank, 1889; Schloesing & Laurent, 1892), but the observations were not based upon axenic cultures. Conclusive proof was provided by Drewes (1928) and this work was supported by De (1939), who showed that nitrogen fixing cyanobacteria were abundant in paddy fields of India. This showed the economic importance of these organisms as contributors for sustaining the natural fertility of rice soils (Singh, 1961). The algal growth in paddy fields promotes the paddy crops by nitrogen fixation and secretion of growth promoting substances (Gupta, 1966). Direct application of algal suspensions helps in the increased growth and yield of crop plants (Rodgers *et al.*, 1979). This proves the proximity of the use of nitrogen fixing genera as biofertilizers.

The presence of the specialised cells called “heterocysts” are responsible for the nitrogen fixing capacity where the atmospheric nitrogen is fixed and converted to the form that can be directly utilised by the plant, the enzyme that helps in the nitrogen fixation is nitrogenase and the enzyme responsible for its assimilation is the glutamine synthetase. Dharmawardene *et al.* (1973) reported high level of enzyme Glutamine synthetase from *Anabaena cylindrica.* Stacey *et al.* (1977) found that N_2 assimilatory enzyme glutamine synthetase isolated from a marine *Anabaena* sp. closely resembles the enzyme obtained from other prokaryotes. Nitrogen is the essential nutrient limiting the production of agricultural crops and their yield, which is often met with the help of chemical fertilizers. The problems caused by the excessive use of chemical fertilizers could be tackled by substituting biofertilizers (Choudhury and Kennedy, 2005). Biofertilizers are eco-friendly, effective and economical alternate to chemical fertilisers (Sahu *et al.*, 2012), all these properties are met with BGA, thus the heterocystous BGA will be a substitute for chemical fertilizers.

Apart from nitrogen, these organisms possess attributes that directly or indirectly facilitate plants in the absorption of Phosphorous (P), Potassium (K), Iron (Fe) and other mineral contents (Kumar *et al.*, 2015). From a study conducted in the rice field soils of Philippines, India, Malaysia and Portugal, it was noted that heterocystous BGA were present in all the samples (Roger *et al.*, 1987) their study suggested the prospects of the utilisation of the indigenous strains of cyanobacteria adapted to local environment as biofertilizers. Faster germination of the rice seeds and the increased physiological features of the plants were noted in the pot rice experiment by Saadatnia and Riahi (2009). Such a result was also attained by Gomes *et al.* (2011) in rice plants grown in Rabi season. Cyanobacteria (BGA) inoculum along with NPK and FYM found to be most effective agronomic treatment for the high rice production (Alam *et al.*, 2014).

The enzyme nitrogenase is very sensitive to oxygen, so it is very essential that the cells performing nitrogen fixation to be devoid of oxygen. Tel-Or (1980) found that the nitrogen fixation in cyanobacteria was hindered by the salt concentration than that of photosynthesis. Heterocyst of cyanobacteria sequesters oxygen by compartmenting both nitrogen fixation and photosynthesis in different cells, where they possess only PSI thus there is no oxygen evolution. Spiller and Shanmugam (1987) examined the physiological conditions for the nitrogen fixation in a marine cyanobacterium *Synechococcus* sp. The importance of nitrogen fixing Blue-green algae is well known, the symbiosis of the cyanobacteria with *Azolla* makes this plant also economically important as biofertilizers.

Apart from supplying nitrogen to rice fields, BGA liberate extracellular organic compounds and photosynthetic O_2 thus helping in improving the productivity (Mandal *et al.,* 1999), certain growth promoting substances such as Gibberilic acid are liberated which help in the growth and stress tolerance by the rice (Rodriguez *et al.,* 2006). Cyanobacterial inoculums improves the physico-chemical properties of soil, helps in gradual build up of residual soil nitrogen and carbon, improvement of soil pH and electrical conductivity (Kaushik, 2014). Cyanobacterial population in the soil is affected by micro and macro elements in the soil such as P, S and negatively by total N content of the soil (Begum *et al.,* 2008).

The rhizosphere of *Withania somnifera* when replenished with Blue-green algae culture filtrates (*Anabaena ambigua* and *Oscillatoria foreaui*), it was noted that the growth of plants was enhanced and the yield potential of the roots also increased with the culture filtrate of *Oscillatoria foreaui* (Lakshmi and Annamalai, 2008). The nitrogen fixing capacity of the Blue-green algae is affected by many factors, such as the chemical fertilizers, this was evident by comparing the nitrogen fixation of blue-green algae noted by the grain yield of rice grown in pots and in paddy fields (Huang, 1978). Shalini *et al.* (2007)

investigated the difference in N2 assimilatory enzymes among the *Anabaena, Nostoc* and *Calothrix* using RAPD-PCR method and a genetic relatedness dendrogram was proposed.

2.3 GENETICS OF NITROGEN FIXATION

The most important characteristic of the heterocystous cyanobacteria is their ability to fix atmospheric nitrogen. As mentioned earlier, they do this with the help of specialised cells called the heterocysts. The vegetative cells are differentiated to heterocysts when they are deprived of any nitrogen source (Fay *et al.,* 1968). Within the heterocyst they contain the enzyme complex needed for the nitrogen fixation. The nitrogenise enzyme complex contains two separate components; Mo-Fe protein or complex I and Fe protein or complex II. They are highly sensitive to oxygen. Heterocyst makes the environment inside them anoxygenic and permits the entry of nitrogen into the cell.

Nitrogen fixation is very energy sensitive and is genetically controlled process which is under the control of proteins encoded by *nif* genes. The best studied organism of heterocystous cyanobacteria regarding the N2 fixation is the *Anabaena* sp strain PCC 7120. 14 *nif* genes associated with nitrogen fixation have been identified that include *nif*K, D, H operon (Mazur *et al.,* 1980, Rice *et al.,* 1982), *nif*B, *fdx*N, *nif*S, *nif*U operon (Mulligan and Haselkorn, 1989).

The primary enzyme encoded by the *nif* genes is the nitrogenise complex which is in charge of converting atmospheric nitrogen to other nitrogen forms. The nitrogenise enzyme combines the nitrogen and the hydrogen present in the cell to form two molecules of Ammonia (NH_3). NH4+ ions are toxic to the plants as they inhibit dinitrogenase activity and also metabolism of cells (especially ATP production). So the ammonia formed is assimilated through the sequential operation of glutamine synthase (GS) and glutamate synthase (GOGAT) commonly known as the GS-GOGAT pathway (Meeks *et al.,* 1978). The ammonia is readily incorporated to an amino acid.

The nitrogen fixation in *Anabaena variabilis* was extensively studied by Wolk (1973). He studied the enzymological comparison between heterocyst and vegetative cell. According to him the various enzymes involved in the nitrogen fixation is compartmentalised in both heterocyst and the vegetative cell. The enzymes found in the heterocyst are; Nitrogenase, Glutamine synthase, Glucose-6-phosphate dehydrogenase (PPP pathway), uptake hydrogenase (encoded by *hup gene*), Bidirectional or reversible hydrogenase (*hox* gene), oxidase in high concentration. In the vegetative cells the major enzymes are; Glitamine Synthase (GOGAT- Glutamine OxaloGlutarate Amino Transferase Pathway), RuBP Carboxylase, Oxidase in low concentration.

The expression of *nif* genes is induced as a response to low concentration of fixed nitrogen and O_2 concentration. The fixed nitrogen factor available for the plants acts as a repressor preventing the transcription.

2.4 INDUSTRIAL APPLICATIONS

Apart from the taxonomic and nitrogen fixation, Blue-green algae are rich sources of pigments and other components. Curtis and Clegg (1984) reviewed a comparative data on the evolution of the chloroplast genes and found that the chloroplast genome of the plant taxa has maintained a similar structural organisation over the past and the comparison of cyanobacterial and chloroplast *rbcL* nucleotide sequence divergence reflect high levels of similarity at the amino acid sequence level such that it is clear that the coding region of *rbcL* is conserved between cyanobacteria and plants. The analysis of the pigment gives information about the cellular state of the organism. An easy protocol for the measurement of chlorophyll a and total carotinoids were proposed (Zavrel *et al.,* 2015). Tiwari *et al.* (2005) investigated the distribution and physiological characters like chlorophyll content, soluble proteins of cyanobacteria in arid zones of Rajasthan and found that chlorophyll content was high in non-heterocystous type. Tabita *et al.* (1976) studied the structure of Ribulose 1, 5-

Bis -Phosphate Carboxylase for the first time and was found to be closely related to the composition of the large subunit from eukaryotic photosynthetic organisms.

Furthermore, cyanobacteria are nowadays finding industrial application because of its rich source of biologically active compound. Cyanobacteria produce an array of secondary metabolites, which has antiviral, antibacterial, antifungal and anti-cancerous activities. So these organisms are a promising future for the chemical industries as a raw material for pharmaceuticals, neutraceuticals and other bio-molecules. Antioxidants scavenge the free radicals thus inhibit the oxidation of other molecules, synthetic antioxidants possess many harmful effects thus an alternative with natural antioxidants will be advantageous, cyanobacteria such as *Spirulina platensis*, possess highest radical scavenging activity and *Nostoc linckia* has the highest chelating activity among different fresh water cyanobacteria studied (Ismaiel *et al.*, 2014).

It is found that the extracts of cyanobacterial species contained high quantity of total phenol and total flavonoid, which are supposed to have antioxidant properties (Singh *et al.*, 2017). Phytonutrients and pigments present in cyanobacteria act as antioxidants, which helps in body's defence mechanism against free radical damage (Hossain *et al.*, 2016). Fresh water cyanobacteria such as *Lyngbia* sp., *Oscillatoria* sp., *Anabaena* sp., etc are found to be potent source of pharmaceutical and other source of industrial uses. Hexane fractions of marine cyanobacteria *Leptolyngbya* sp. and *Limnothrix* sp. are found to possess antioxidant and anti cancerous properties respectively (Anas *et al.*, 2016).

Cyanobacteria are found in all possible environments making them ubiquitous in distribution. For thriving in extreme environmental conditions and to compete with other microbes, they must have anti bacterial and anti fungal properties. Extracts from cyanobacteria isolated from the soil when examined

for antifungal properties against pathogenic fungi, it was found that 6.34% of the cyanobacteria exhibited antifungal effects (Kim, 2006). They also noted that growth temperature plays an important role in antifungal activity. Apart from plant pathogens, the cyanobacterial bioactive compounds showed weak to strong antimicrobial activity towards one or more human pathogenic microorganisms (Al-Wathnani *et al.,* 2012).

The potentiality of the use of marine microalgae and cyanobacteria in cosmetics and pharmaceuticals were studied by Mourelle *et al.* (2017). Polysaccharides, carotenes, lipids and proteins derived from cyanobacteria and microalgae are recognised to have positive effect on health. The pigments from cyanobacteria could be used as natural colorants (lipsticks, eye shadows etc). Rastogi and Sinha (2009) studied the biochemical and industrial significance of cyanobacterial secondary metabolites and found that certain mycosporine like Amino Acids have the capacity to overcome the UV radiation toxicity, which could be exploited by the cosmetic industry.

The most recent break through to the application of Blue-green algae is their ability to produce PHB, which is used as a biodegradable plastic. The highest PHB production was confirmed in *Nostoc muscorum* than other heterocystous and non-heterocystous cyanobacteria used for the study by Ansari and Fatma (2016). From all these it is clear that Blue-green algae are potential source of many important products that are useful to mankind. Basic research is needed to identify new cyanobacterial strains of high value products (Thajuddin and Subramanian, 2005), which can be potentially exploited by the industries.

2.5 ISOLATION AND PURE CULTURE

The pre requisite for every study is the axenic or unialgal cultures, which require a great deal of time and patience. It can take from few days to even some months to achieve an axenic culture and in some cases it even seemed to be impossible. The first report on the culture of algae was that of Beijerick who

achieved a pure culture of *Chlorella.* The methods of isolation include streaking of the field material in agar plates. Allen (1952) and Kratz & Myers (1955) were those who first attempted and attained the pure culture of Blue-green algae (Cyanobacteria).

Unlike other micro organisms that require minimum nutrient, blue-green algae that are photosynthetic like green plants require both macro and micro nutrients, these nutrients are provided by the medium in which they are grown. The essential nutrients like C, N, P, S, K, Fe etc are utilised along with photosynthesis to increase their biomass and multiplication (Markou *et al.,* 2014). Carbon, nitrogen and phosphorous is among the major elements contributing towards the biomass. Magnesium which forms the major element involved in chlorophyll thus helping in the photosynthetic activity, Phosphorus; the key substrate involved in the synthesis of nucleic acids and membranes.

The role of trace metals in the cyanobacterial growth and metabolism was studied by Rueter and Peterson (1987), and found that trace metals play an important role in carbon and nitrogen metabolism, photosynthesis (Fe, Mo). Allen and Stainer (1968) noted that blue-green algal growth was greater at temperature 35^0C and heterocyst forming filamentous type was obtained when nitrogen source was omitted. Stainer *et al.* (1971) used BG-11 medium for the first time, which was a modification of G-11 medium. Later on this medium became popular and is now used widely for the culture. Later on Rippka *et al.* (1979) modified the BG-11 medium with the omission of nitrogen source for the heterocystous group; they also found that the cyanobacteria from marine sources cannot grow in BG-11 medium, even when supplemented with NaCl. An optimum condition for the growth of cyanobacteria on solid media was investigated by Thiel *et al.* (1989), for which the solidifying agent used was agar. All the strains used were found growing well in autoclaved medium and the addition of thiosulphate increased the plating efficiency.

Othman and Wollum (1987) evaluated the effect of shaking time and medium composition (pH) and it was noted that shaking time had no influence on algal population, pH 6.0 produced more algal count and Bold's Basal medium resulted in greatest number of algae. Bano and Siddiqui (2004) investigated the optimum condition for the culture of marine cyanobacteria and found that all the cyanobacterial species preferred neutral to alkaline pH. Nagle *et al.* (2010) attempted the isolation of ten species of non-heterocystous marine cyanobacteria; they were subjected to different concentrations of NaCl, pH, light and nutrients and found that 5-25% of NaCl concentration, pH 7.5 and increasing concentration of NO_3-N were found to be ideal for the growth.

The main hindrance for the production of axenic culture is the contamination by both bacteria and also other unwanted cyanophycean members. Parikh and Madamwar (2006) stated that the carbohydrates synthesised by BAG such as glucose, mannose, xylose and ribose can be used as a carbon source for the contaminants. Various methods have been employed by different workers in order to purify the contaminated cyanobacterial cultures. Fitzsimons and Smith (1984) reported a method to produce high algal filaments to the bacterial ratio and also found that optimum condition for the growth of axenic cultures is under a light: dark regime than in continuous light.

The environmental samples when collected contain many organisms that are contaminants, being microscopic picking or the separation of single filaments or colonies are near to impossible. The advancement of technology has lead to various new techniques such as capillary pipette (Pringsheim, 1964) that enables to pick single cells or filaments thus reducing the contamination rate. Ferris and Hirsch (1991) employed nutrient saturated glass fibre filters for the isolation of cyanobacteria from freshwater, with a reduction in contamination by heterophilic bacteria using imipenem, a broad spectrum antibiotic. They used BG-13 medium for the isolation.

Tel-Or (1980) studied the response of N_2-fixing cyanobacteria to salt in two species of cyanobacteria, *Nostoc muscorum* and *Calothrix scopulorum*. From their studies they came to a conclusion that photosynthesis was more resistant to high salt concentration than was N_2 fixation. Elango *et al.* (2008) overcome the task of isolation of pure cyanobacterial culture from contaminated culture employing double distilled and tap water as medium having pH of 7.12 and 8.65, and found that tap water was less efficient than double distilled water.

Das and Sarma (2015) conducted experiments to determine the optimized culture media for the growth and biomass production of *Anabaena spiroides* and *Nostoc punctiforme.* They found that better growth was exhibited by modified BG-11 ($NaNO_3$-2.5 and $MgSO_4$- 0.095) and the optimum growth of these two strains occurred in between 14-28th days of culture, thereafter the growth declined. And also chlorophyll-a maximum value was also shown on the 28th day. Nehul (2014) also obtained good growth with highest biomass production and more carotenoids production in *Lyngbia punctata* in BG-11 medium from a variety of media tried.

2.5.1 Different Media Compositions for Axenic Culture

De (1939) stated about the difficulty in obtaining bacterial free cultures of Blue-green algae in order to study the physiological processes. He used a modified Beneck's solution that consisted of; Potassium Monohydrogen Orthophosphate, $MgSO_4.7H_2O$, $CaCl_2.10H_2O$, Ferric Chloride and K_2MoO_4.

Modified Chu-10 medium was used by Gerloff *et al.* (1950) and the composition of which were Ca $(NO_3)_2$, K_2HPO_4, $MgSO_4.7H_2O$, Na_2CO_3, Na_2SiO_3, Ferric Citrate and Citric Acid. Another medium tried for the culture of cyanobacteria was; Z8 medium (Kotai, 1972) that consisted of $NaNO_3$, Ca $(NO_3)_2$. $4H_2O$, K_2HPO_4, $MgSO_4.7H_2O$, Na_2CO_3 and trace metal solution Gromov no.6 nutrient medium (Gromov and Titova, 1983) that consisted of KNO_3, K_2HPO_4, $MgSO_4.7H_2O$, NaCl, and NaHCO3.

For the culturing of halophytic cyanobacteria a new medium was tried *i.e.*, ASN III medium (Rippka *et al.,* 1981) which consisted of; $NaNO_3$, K_2HPO_4, $MgSO_4.7H_2O$, $MgCl_2.6H_2O$, $CaCl_2.2H_2O$, NaCl, KCl, Na_2CO_3, Citric acid, Ferric Ammonium Citrate and trace metal solution. This medium was altered by excluding the NaCl originally used in this medium so that the fresh water species also could be successfully grown in this medium.

Ferris and Hirsch (1991) used BG-13 medium which consisted of $NaNO_3$, $NaHCO_3$, K_2HPO_4, $MgSO_4.7H_2O$, $CaCl_2.H_2O$, $NaNO_3$, Citric acid, ferric ammonium citrate, Disodium magnesium EDTA, H_3BO_3, $MnCl_2.4H_2O$, $ZnSO_4.7H_2O$, $Na_2MoO_4.2H_2O$, $CuSO_4.5H_2O$ and $CoCl_2.6H_2O$ and the pH was adjusted to 7.5 to 7.6 and incubated under an atmosphere of 5% (v/v) CO_2. BG-11 medium is the most commonly used medium that gave better results of axenic cultures.

In case of the nitrogen fixing Blue-green algae (cyanobacteria), the medium was modified such that the nitrogen source was omitted.

2.6 DNA ISOLATION

By the increasing use of molecular techniques, the need for the DNA isolation became mandatory and during the last decade, many methods for the extraction of DNA from microbes, plants and animals have been proposed. Of the different methods, the popular one among them is CTAB method (widely used for plants), protease hydrolysis, cell lysis using liquid nitrogen, etc. Readymade kits suitable for different organisms are readily available nowadays. These kits make the extraction much easier but a little costly. Several methods for extracting cyanobacterial DNA have already been reported, majority of which are often time consuming and differ according to species or genera. The major hindrance in the DNA isolation of Blue-green algae is the lysis of the tough cell walls made up of polysaccharides which requires enzymatic and mechanical disruption of the cell walls.

DNA extraction from desert cyanobacterium *Chroococcidiopsis* was attained by Billi *et al.* (1998). This method consisted of lysis with glass beads in presence of hot phenol and a lyzozyme treatment followed by osmotic shock which could possibly overcome obstacles by bacterial contamination and the thick cyanobacterial cell envelop. Tillet and Neilan, (2000) proposed a novel method for the isolation of high molecular weight DNA and RNA from both cultured and environmental cyanobacteria that require no enzymatic or mechanical cell disruption. The method follows xanthogenate-SDS (XS) DNA isolation protocol, this method was further improved by Yilmaz *et al.* (2009) which yielded high quality DNA that could be amplified in PCR and digested with restriction enzyme. In majority of the protocols the DNA is precipitated with ethanol, Wu *et al.* (2000) incorporated a high salt concentration for the precipitation of DNA in the presence of isopropanol. Fiore *et al.* (2000) developed a miniprep method for the isolation of DNA from unicellular and filamentous cyanobacteria. The method was devoid of phenol extraction and the product could be directly used for the PCR amplification. Fawley and Fawley and Fawely (2004) proposed a method for isolating DNA suitable for PCR with minimum quantity of algal culture and less time.

Magana-Arachchi and Wanigatunge (2011) developed a simple and rapid DNA extraction for the cyanobacteria and monocots where the sample grinding using liquid nitrogen, further purification by phenol chloroform isoamyl mixture and treatment with RNase for obtaining RNA free DNA were all not necessary. The DNA isolation involves the phenol treatment and enzymes like proteinase K and RNAse. Phenol extraction- based procedure was employed for the isolation and purification of genomic DNA from filamentous cyanobacteria *Anabaena, Nodularia* and *Nostoc* by Kaczynskaand Wegrzyn (2013) and they obtained a high yield of pure genomic DNA.

A soft mechanical lysis with enzymatic disruption of the cell wall of *Arthrospira,* an important cyanobacterium, this method resulted in a high

molecular weight DNA, with reduced degradation (Morin *et al.*, 2009). A new method which was phenol freefor the isolation of genomic DNA from filamentous cyanobacteria was proposed by Singh *et al.* (2012) which resulted in a high quality DNA suitable for PCR and restriction digestion. Motham *et al.* (2014) evaluated different methods for the effective isolation of DNA from cyanobacteria *Nostochopsis* spp. and found that crushing with liquid nitrogen and addition of polyvinylpolypyrrolidone yielded the highest quality genomic DNA, devoid of protein contaminants and polysaccharides.

An efficient and reproducible genomic DNA extraction protocol for Archaea, Bacteria, Cyanobacteria, Diatoms, Fungi and Green algae which included a heat shock with multiple freeze-thaws and an increased concentration of detergents (SDS) to yield Sufficient amount of DNA for PCR amplification of single or multiple fragments was proposed by Saba *et al.* (2016). This method was found to be highly reproducible and can be used in diverse microbial samples. Ma *et al.* (2016) compared the DNA isolation using conventional method, without using lysozyme and phenol extraction, and bacterial DNA extraction kit. It was noted that the quality of DNA thus obtained was high and the yield was also high and the DNA could be directly used for PCR amplification.

Gaget *et al*. (2016) compared the efficiency DNA extraction using different commercial kits and the conventional Phenol-chloroform extraction method for benthic cyanobacteria and found that the commercial kits allow greater recovery in terms of yield and quality of DNA.

2.7 PHYLOGENETIC ANALYSIS

The success of phylogenetic analysis relies on the choice of the molecular markers and the sequence used for the study. One should make the right choice of sequence for the better and accurate result. One of the most widely used genes for the phylogenetic analysis of bacteria and cyanobacteria is the 16S

rRNA sequence (Lane *et al.*, 1985, Woese, 1987, Ludwig and Klenk, 2001). The proximity of the use of 16S rRNA sequence for the deduction of phylogenetics relations were proved by Giovannoni *et al.* (1988) and Wilmotte *et al.* (1994). Nellissen *et al.* (1996) attained phylogenetics relationships of non-axenic cultures of filamentous cyanobacteria, for which they used the 16S rRNA gene sequences that have divergent sequences from non target organisms thus the non target DNA such as bacterial DNA could be prevented from amplification.

Other than the 16S rRNA sequences, the nitrogen fixing gene *nif* genes, the sequence encoding the RUBISCO, *rbcL*, etc are also used for deducing the phylogenies and molecular characterisation. The conserved regions of the genes encoding β and α-phycocyanin subunits of phycobilisomes (cpcB and cpcA) were used for the genetic characterization of cyanobacterial strains (Bolch *et al.*, 1996). Lu *et al.* (1997) proved that PCR relatedness of heterocystous cyanobacteria to the non- heterocystous was on the order of 10-20%.

The applicability of RAPD in cyanobacterial taxonomy was revealed by the study of Shalini *et al*. (2007). They conducted molecular polymorphism analysis of selected *Calothrix* strains using single and multiplex randomly amplified polymorphic DNA which helped in the differentiation of the strains as well as understanding their genetic relatedness.

Ishida *et al.* (1997) used 16S rRNA to determine the phylogenetic position of eight genera of *Oscillatoriae*. Their study revealed the heterogenous nature of Oscillatoriae, thus their current taxonomic position is obscure. (Otsuka *et al.*, 1999) investigated the phylogenetic relationship between toxic and non-toxic strains of *Microcystis* based on 16S to 23S ribosomal internal transcribed spacer sequences. Their study revealed that these intergenic spacer sequences were effective in understanding relationships between closely related species

and strains. Iteman *et al*. (2000) found that cyaobacterial ITS are potential markers for detection and identification of cyanobacteria at different taxonomic levels, Boyer *et al.* (2001) also analysed the potential of 16S- 23S rRNA ITS region for molecular systematics and population genetics.

Han (2009) from his studies based on different markers like rpoC1, hetR, rbcLX and 16S rRNA-tRNA (Ile)-tRNA (Ala)–23S rRNA Internal Transcribed Spacer suggested that all the trees were congruent with the 16S rRNA tree and the randomised variations and recombination results in congruence of gene trees obtained from different molecular markers. The efficiency of 16S rRNA gene sequence to evaluate relationship of certain taxa of cyanobacteria was studied by Shariatmadari *et al.* (2017) and found to be inefficient in certain intermixed taxa.

Narayan *et al.* (2006) from their studies on the physiological characters of the selected cyanobacterial isolates found out that cyanobacterial genus cannot be differentiated based on physiological parameters alone. In this respect studies using phylogenetic methods gain importance.

Phylogenetic analysis of sponge-associated cyanobacteria using 16S rRNA sequences showed them to be polyphyletic *i.e.;* they are derived from multiple independent symbiotic events (Steindler *et al.,* 2005).

Gugger and Hoffmann (2004) confirmed the monophyletic origin of heterocyst forming cyanobacteria and also the polyphyly of true branching cyanobacteria from the 16S rDNA data. Monophyletic origin of the heterocystous cyanobacteria were further evident from the *Nif*H sequence analysis of the 41 heterocystous cyanobacterial strains collected from all over India (Singh *et al.,* 2013).

Iteman *et al.* (2002) examined the taxonomic coherence and phylogenetic relationships of 11 planktonic heterocystous cyanobacterial isolates using 16S

rRNA gene and ITS located between 16S rRNA and 23S rRNA gene, the data obtained showed that all the planktonic strains form a distinct sub-clade within monophyletic clade of heterocystous cyanobacteria except the genus *Cylindrospermopsis.*

Phylogenetic analysis of heterocystous cyanobacteria of subsections IV and V (true branching and false branching) were conducted by Henson *et al.* (2004) using *nifD* gene and the maximum likelihood criteria. They found that hetrocystous cyanobacteria are monophyletic but neither subsection IV nor subsection V is monophyletic; since both subsection are intermixed in two sister clades. On the other hand Mishra *et al.* (2013), done the phylogenetic comparison of some heterocystous cyanobacteria (Subsections IV and V) using a polyphasic approach such as antibiotic resistance, morphological, physiological and biochemical analysis, molecular analysis based on 16S rRNA analysis and their results suggested that true phylogeny could be achieved by a polyphasic approach that is more appropriate and reliable.

Wanigatunge *et al.* (2014) studied the diversity of cyanobacteria in Sri Lanka; phylogenetic analysis was done with 16S rRNA sequences. From their results they confirmed the monophyletic origin of the Order Nostocales and Pleurocarpales where as the order Chroococcales, Oscillatorials and Stiogonematales are polyphyletic. They also recommended a polyphasic approach for ascertaining the diversity of cyanobacteria.

The genetic variability and phylogenetic relatedness of hetercystous cyanobacteria of 12 genera collected from different geographical areas of India were assessed with Highly Iterated Palindromes (HIP) by Singh *et al.* (2014), and a dendrogram was constructed suggesting the monophyletic origin of the heterocystous clade. Ribosomal conserved sequences were used for the molecular phylogeny of both marine and fresh water cyanobacteria by Baldev

et al. (2015). According to them molecular characterization is more reliable technique for better identification of cyanobacteria.

Cyanobacterial isolates from North East India were studied by Keithellakpam *et al.* (2015). They isolated 5 strains of cyanobacteria belonging to different genera and investigated for their morphological and phylogenetic relationships using 16S rRNA gene. The polyphyletic origin of *Nostoc* and *Anabaena*, taxonomic coherence *Nostoc* and *Calothrix* were inferred from this. Oinam *et al.* (2015) did the molecular characterisation of diazotropic cyanobacteria of North east India.

Tropical cyanobacterial genera; *Nostoc, Calothrix, Tolypothrix* and *Scytonema,* of the tropics were investigated by Morales *et al.* (2017) for the molecular identification PCR sequencing of the rpoC1 and16S rRNA gene primers were done and found that both morphological and molecular analysis yielded consistent taxonomic grouping.

Polyphasic study using morphological, genetic and biological methods of cultures of cyanobacteria from Brazilian mangrove by Silva *et al.* (2014), found to be helpful in deducing the phylogeny of the 50 strains isolated. High variability of 16S rRNA gene sequences among the genotypes that was not associated with morphology was observed and several branches formed by these gene sequences were also observed in the phylogenetics analysis.

Blue-green algae collected from freshwater sites in Tokat province were isolated by Karan *et al.* (2017). These were purified and extracted the DNA and PCR amplified using 16S rRNA and phycocyanine gene regions. The maximum likelihood tree obtained from 16S rRNA sequences showed distinction of genus at high frequency where as the tree from phycocyanin gene region does not show a fully separated clade.

2.8 *NOSTOC* AND *ANABAENA*

Wide distributions of Nostoc and Anabaena have been reported by many workers (Sinha and Mukherjee, 1975 a & b; Paul and Santra, 1982). Abundance of heterocystous BGA, especially *Nostoc*, followed by *Anabaena* and then *Calothrix* was reported by Roger *et al.* (1987) in the rice fields of Philippines, India, Malaysia and Portugal. Goyal *et al.* (1984) reported that *Nostoc* and *Anabaena* were widely distributed in the rice field soil samples collected from Jammu division. Jaiswal (2017) did a systematic study on the occurrence of Nostocales of Sakri and Navapur, Maharashtra and reported ten species and one variety of *Nostoc* and nine species, five varieties and one form of *Anabaena.*

Among the heterocystous forms *Nostoc* has the most widespread distribution (Dodds *et al*., 1995). They are found as free living forms (Hoffmann, 1996; Lyra *et al*., 2001) but most of them are found in symbiosis with other plant groups and fungi, thus helping in nitrogen fixation (Guevara *et al*., 2002). Symbiotic associations are observed with bryophytes (*Anthoceros*), Gymnosperms (cycads), Pteridophytes (*Azolla* fern), an angiosperm (genus *Gunnera*), and Fungi (lichens) (Baker *et al*., 2003; Bergman *et al*., 1992; Meeks and Elhai, 2002; Rikkinen *et al*., 2002; West and Adams, 1997; Adams and Duggan, 2008). According to Papaefthimiou *et al*, (2008), distinct pattern of evolution lead to symbiotic behaviour on Nostocacean Cyanobacteria; one leading to symbiosis (e.g.: Bryophyte and Gymnosperm) and other leading to association (e.g.: *Azolla*).

The abundance of *Nostoc* in the arid zones of Rajasthan was reported by Tiwari *et al.* (2005). By assessing the cyanobacterial population and nitrogen fixation of heterocystous cyanobacteria isolated from the rice fields of Bangladesh it was noted that *Nostoc* and *Anabaena* were the predominating genera (Begum *et al.,* 2008). Recent studies have shown that *Nostoc* and *Anabaena* are capable of forming associations with wheat roots grown in liquid culture, which may

contribute nitrogen and other growth promoting substances to the growing plant (Kaushik, 2014).

Anabaena species are found in a wide variety of habitats from brackish, freshwater, and marine to extreme environments (Sheath *et al.,* 1996; Sheath and Muller, 1997; Spaulding *et al*., 1994). It is reported from the paddy fields of Kerala. The morphological plasticity of *Anabaena* in different environmental or culture conditions were investigated by treating the strain with liquid mixotrophe by Deylami *et al.* (2014) and found that the biometric characters have changed in different mixotrophe treatments. Abundance of *Anabaena* in the rice fields of Bundi District in Rajasthan was reported by Maheshwari (2013). From the physico-chemical characterization of 14 species of *Anabaena* spp. collected from five districts of Chattisgarh, Sharma and Jain (2016) noted that lesser nitrogenous fertilizer application benefits in the growth of the genus. This further pointed to the nitrogen fixing capability of these species.

Even though the nitrogen fixation is the major property of these genera, they are found to have some other properties also that are useful for the human kind. Nath *et al.* (2015) evaluated the biopigments that are nowadays gaining economic value as natural colourants from the genera *Nostoc* and *Anabaena* strains isolated from Loktak Lake. They isolated 25 strains each of *Nostoc* sp. and *Anabaena* sp. respectively from fresh water habitat. Their study revealed the need for a morpho-physiological and molecular approach for characterisation of cyanobacteria, and these organisms could be utilized in agriculture and other related industry. *Nostoc* and *Anabaena* belonging to Nostocacean taxa are primarily important because of its nitrogen fixing ability, but their identification and taxonomy are cumbersome based on mere morphology. There are conflicting opinions on the distinction of these two genera. Numeric taxonomic data of *Nostoc* and *Anabaena* strains by Mcguire (1984) found that these genera are separate and distinct.

When the nucleotide sequence of the entire genome of a filamentous cyanobacterium, *Anabaena* sp. strain PCC 7120, was determined, only 37% of the *Anabaena* genes showed significant sequence similarity to those of *Synechocystis*, indicating a high degree of divergence of the gene information between the two cyanobacterial strains (Kaneko, 2001).

Free living *Nostoc* and *Anabaena* were attempted to differentiate with the cellular fatty acids by Caudales and Wells (1992). 10 strains each of *Nostoc* and *Anabaena* were selected and their fatty acid composition determined by gas chromatography-mass spectroscopy. From their study they concluded some species should be reconsidered based on their fatty acids such as *Anabaena variabilis* should be reclassified as *Nostoc variabilis* and *Anabaenopsis circularis* should be retained in the genus *Nostoc*. Lachance (1981) found that genus *Nostoc* was heterogenous and some strains previously placed in *Anabaena* belonged to *Nostoc*.

Molecular and chemotaxonomic approaches were used to study the phylogeny of terrestrial cyanobacterium *Nostoc commune* and its neighbouring *Nostoc* spp. (Arima *et al.*, 2012), for which they used the differences among 16S rRNA gene sequences, *pet*H gene and RAPD analysis. *Nostoc and Anabaena* species were compared based on their carotenoid composition. In the phylogenetics tree constructed it was found that *Nostoc commune* and *Nostoc punctiforme* were a monophyletic group.

30 *Anabaena* strains isolated from diverse geographical regions of India were characterised based on their morphological, physiochemical and molecular markers by Prasanna *et al.* (2006). The strains showed significant differences in their morphological and physiochemical characters. They showed that differentiation of strains and the understanding of genetic relations were easy with the analysis of molecular polymorphism.

Rajaniemi *et al.* (2005) conducted the morphological and phylogenetic analysis of the genera *Anabaena, Aphanizomenon, Trichormus* and *Nostoc*. The phylogenetic analysis was done with 16S rRNA gene, *rpo* and *rbcLX* sequence. The data showed that the benthic and planktic *Anabaena* strains were intermixed, *Nostoc* strains were heterogenous and form a monophyletic cluster, *Trichormus* strains were heterogenous morphologically and phylogenetically and did not form monophyletic cluster.

Shariatmadari *et al.* (2014) collected isolates of *Anabaena* from paddy field soils of Iran. Morphological characters and numerical taxonomic methods were used to classify species and the phylogenetic relationships determined by constructing 16S rRNA gene tree using neighbour joining algorithm which showed the populations of each species were placed close to each other and the morphological characters separate them. Their study also revealed that 16S rRNA gene site cannot separate genera like *Anabaena, Trichormus* and *Wollea.* The phylogeny of symbiotic *Nostoc* strain sequences and database 16S rDNA sequences of both symbiotic and free-living cyanobacteria by maximum likelihood and Bayesian inference techniques were analysed by Svenning *et al.* (2005). Their results showed that the symbiotic strains form separate clades and also the *Azolla* isolates were assigned to genus *Anabaena.* They also noted that the *Anabaena* isolates produced hormogonia indicating this criterion cannot be used to distinguish *Anabaena* from *Nostoc.* On the other hand Baker *et al.* (2003) based on the comparison of the phycocyanin intergeneric spacer sequences and 16S rRNA found that the symbiont from *Azolla* belongs to neither *Anabaena* nor *Nostoc.*

Teneva *et al.* (2012) stated the heterogeneity of *Nostoc* by analysing the molecular and phylogenetic data of two *Nostoc* species (*N. linckia* and *N. muscorum*) based on *cpcB-IGS-cpcA* locus of the phycocyanin operon. Their study also revealed that the IGS region between the *cpcB* and *cpcA* was useful to solve the taxonomic problem between *Nostoc* and *Anabaena.*

10 species of *Anabaena* were used to test the compatibility between the conventional morphological classification and current molecular system by Ezhilarasi and Anand (2009). Genetic diversity was assessed using the banding patterns of repetitive DNA sequences. The dendrograms based on the LTRR sequences did not show a clear correlation with the dendrogram based on morphology. Phylogenetic study of Japanese planktonic *Anabaena* species were carried out by Tuji and Niiyama (2010), they collected planktonic *Anabaena* strains from lakes and ponds and cultured. Based on the information from16S rDNA and *rbcLX* analyses the strains were divided into four clusters.

Gugger *et al.* (2002) conducted morphological analysis of *Anabaena* and *Aphanizomenon* strains isolated from Denmark, Finland and France, which differed in their morphological characters. Their phylogenetics tree based on 16S rDNA, ITS1 and *rbcLX* regions revealed that the *Anabaena* strains cannot be distinguished from *Aphanizomenon* strains. From their studies they came to a conclusion that the *Anabaena* and *Aphanizomenon* strains belong to same genus.

Henson *et al*. (2002) differentiated *Nostoc* and *Anabaena* by complete sequencing of nitrogen fixation gene *nif*D and their phylogenetic analysis. They suggested that both the genera should remain as separate genera. This result was supporting the classical taxonomy. Glhano *et al.* (2011) used a polyphasic approach based on morphological, biochemical and genotypic characters to characterise *Anabaena*, *Aphanizomenon* and *Nostoc* strains and found that morphological and 16S rRNA sequence clearly distinct these genera.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 STUDY AREA

The Kerala State, situated in the southern part of the West coast of South India, is rich in flora and fauna including several endemics. Among them, the algae are one of the least known and less documented groups of lower plants (Easa, 2004). Kerala lies between $8^0 18^{'}$-$12^0 48^{'}$N and $74^0 52^{'}$-$77^0 22^{'}$ E. The state is a small strip of land in the west coast of India. It has a length of 560 km and maximum width of 132 km and a geographical area of 38,863 km^2, which constitutes 1.8% of the total geographical area of India. Kerala has 3 distinct geographical formations like low land, mid land and high land.

The state is bounded on the north and northeast by Karnataka, east by Western Ghats and South by Tamil Nadu, and west by the Arabian Sea. The state has about 590 km of coastal belt. Kerala which lies in the tropic region has a humid tropical wet climate. Average temperature varies from 27 to 32^0 C. The state receives an average annual rainfall of 3107 mm- some 7,030 crore m^3 of water. Kerala's rains are mostly the result of seasonal monsoon, which experiences two monsoons *i.e.*, South west monsoon starting from June and North east monsoon starting from mid October. Though small in size, Kerala is a land effluent in water sources. It is criss-crossed by 41 west flowing rivers and 4 east flowing rivers and a network of interconnected brackish canals, lakes, estuaries and rivers. The soil is mainly lateritic in the mid and high lands, alluvial in the lowlands.

Rice being the staple food, the culture and practices of Kerala is related to the paddy cultivation. It was once the major occupation of majority of the population. Alappuzha, Palakkad and Trissur are the major places where large scale paddy cultivation is practiced. Kuttanad of Alappuzha is known as the "Rice Bowl" of Kerala. The nitrogen fixing cyanobacterial community plays an important role in augmenting the soil fertility and thus helps in the crop yield.

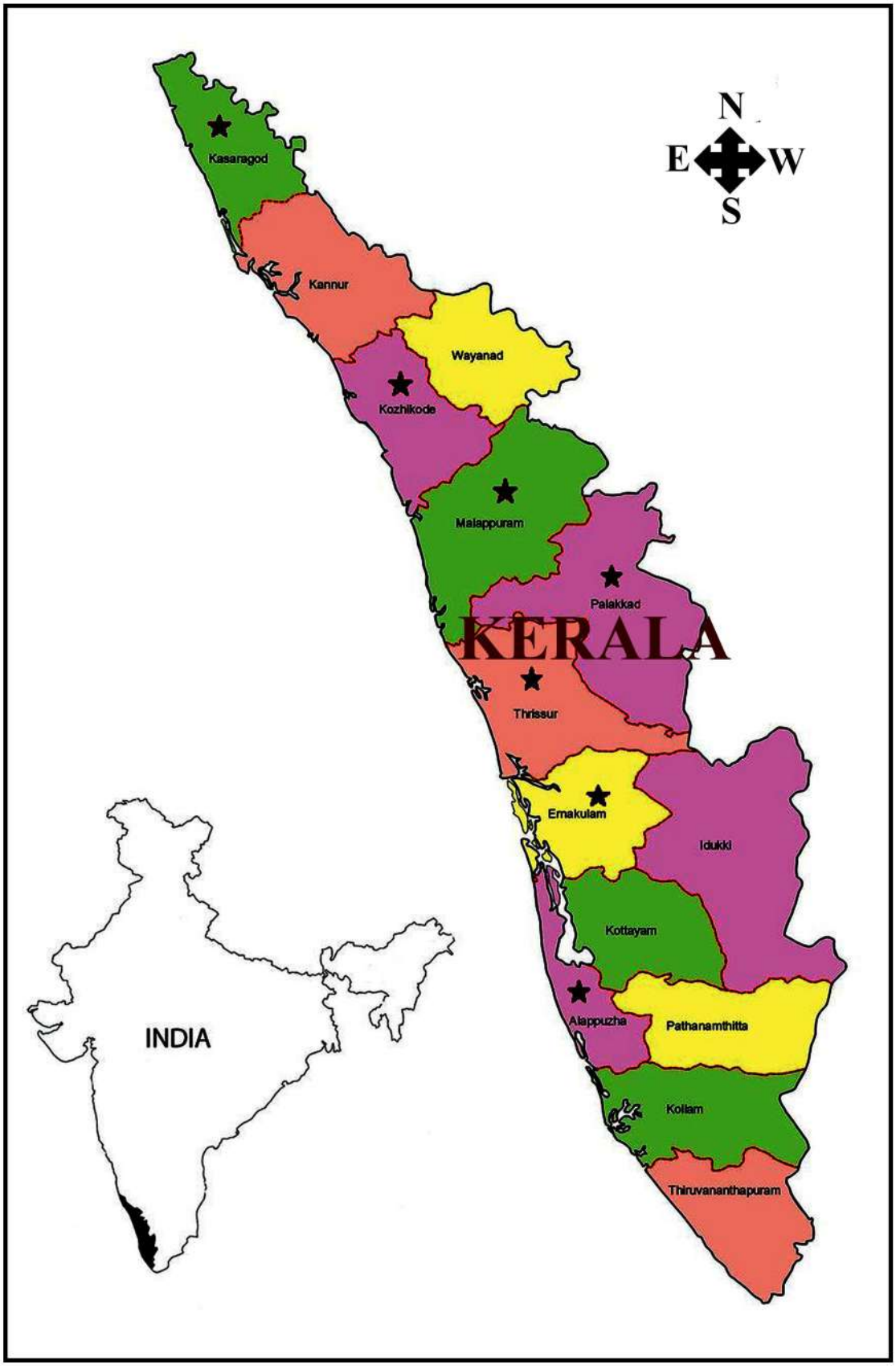


Fig. 1. Map of Kerala State showing districts from where the isolations of Blue-green algae were made.

Cyanobacteria contribute about 25-30 kg/ha nitrogen to rice crops (Kaushik, 1994). Some of the common genera of rice field cyanobacteria are *Anabaena, Aulosira, Calothrix, Nostoc, Scytonema*, etc. Of the different species of cyanobacteria, *Nostoc* was found to be prominent (Kaushik, 1999). pH of the soil plays an important role in the abundance and occurrence of cyanobacteria (Kannaiyan and Kumar, 2004). Application of cyanobacteria in paddy fields as the alternative to nitrogen fertilizers has been practised by many Asian countries like China, Vietnam, India, etc (Venkataraman, 1972).

Kerala although being blessed with water bodies, the fresh water habitats are among the most threatened and vulnerable ecosystems. The micro algae of Kerala are one of the least understood and studied organisms of which the nitrogen fixing blue-green algae play an important role in augmenting the fertility of the soil especially paddy fields as nitrogen fixing biofertilizers.

3.2 MATERIALS

3.2.1 Chemicals

All the media compositions, agar, acids and the solvents used for the study were of analytical and molecular biology grade purchased from Hi Media (Mumbai), SRL (Mumbai), and Merck (Mumbai). Agarose was purchased from Invitrogen, fisher scientific, Bangaluru. The primers were purchased from eurofins, Eurofins Genomics India Pvt., Ltd, Bangaluru. Master mix for PCR was purchased from Takara, Japan

All the reagents and buffers were prepared prior to the study and autoclaved at 15lb pressure for 20 minutes and stored in a screw cap bottle until used.
Double glass distilled water was used for the preparation of media, buffers and other reagents.

Table 1. Details of the Primers used in the PCR amplification.

Name of the primers	Sequence of the primers
8-27 F	5'- AGA GTT TGA TCC TGG CTC AG-3'
1495 R	5'- CTA CGG CTA CCT TGT TAC GA-3'

3.2.2 Glassware

All the glassware used was purchased from Borosil (India). Prior to the use the glassware were cleaned with detergent, rinsed with double distilled water and dried in hot air oven for 6 hrs.

3.2.3 Instruments

Table 2. List of Instruments used for the study.

Generic Name	Make/Model
Weighing Balance	Shimadzu, Japan
Digital pH meter	Systronics, Ahmedabad
Double Distillation Unit	Riviera, Mumbai
Hot Air Oven	ROTEK, Mumbai
Vortex Mixture	REMI, Mumbai
Autoclave	ROTEK, Mumbai
Laminar Air Flow Chamber	Frendz Inc., India
Culture Rack	LABLINE, Ernakulam
Microscope	Labomed, India
Camera attached microscope	Labomed, India
Camera	Nikon
Table Top Centrifuge	REMI, Mimbai
Ref. Centrifuge	Eppendorf, Germany
Ultrasonic Probe Sonicator	PCi Analytics, Mumbai
Pipette	Eppendorf, Germany
Water Bath	ROTEK, Mumbai
-20ºC Deep Freezer	Celfrost, India
Gel Electrophoresis	BIO-RAD, USA
Gel Documentation	Life technologies, USA
Transilluminator	E-Gel Imager, USA
Spectrophotometer	JASCO, Japan
Double Beam Spectrophotometer	Systronics, India
Spin Win	TARSONS, India
PCR Thermal Cycler	BIO-RAD, USA
-20º C Mini Cooler	TARSONS, India

3.3 METHODS

In this study, *Nostoc* and *Anabaena* distributed all over the Kerala were selected to deduce the relationships between them. The aim of the study was to deduce the relationship between these taxa by using molecular techniques. Before going for the morphological study, morphological analysis was done as a pre requisite for understanding the morphological affinities.

3.3.1 Collection of Blue-green algae

Samples were collected from different districts of Kerala, through extensive field trips. Visible and planktonic samples and soils from the paddy fields were collected in pre sterilised sample collection bottles (ETO Sterile). Samples were brought to the laboratory and stored at 25 ± 2^0 C until used. A portion of the collected samples were used for morphological study.

The Blue-green algae that grow in the soil are often found as a mixture growing along with other organisms. This forms one of the major sources of contamination.

3.3.2 Enrichment of cultures

Collected samples were enriched in BG_0-11 medium (devoid of nitrogen source, $NaNO_3$), (Rippka *et al.*, 1979) in presence of light. The composition of the medium is given in Table 2.

pH of the medium was adjusted to 7.2±2 with 1N HCl or NaOH using a digital pH meter. The medium was autoclaved at 121^0 C for 15 minutes. To plate 0.4% w/v (4 g/L) of agar is used. The collected planktonic samples were directly placed in the BG_0-11 agar poured in petri plates; dilution and pour plate method is adopted for soil samples. The plates were well sealed, placed in culture racks provided with cool white fluorescent lamps and incubated in the growth chamber with temperature 25 ± 2^0 C and alternate light and dark cycles of 12/12 h (3000 lux). The growth of cyanobacteria was noted on the visual observations after 2-3 weeks.

Table 3. Composition of $BG_0$11 medium used for the culture of Blue-green algae.

Composition	**gm/L**
$MgSO_4.7H_2O$	0.075
$K_2HPO_4.3H_2O$	0.04
$CaCl_2.2H_2O$	0.036
Na_2CO_3	0.02
Citric Acid	0.006
Ferric Ammonium Citrate	0.006
Disodium EDTA	0.001
Trace Metal Solution	1 ml/L

Composition of Trace Metal Solution

Chemical	gm/L
H_3BO_4	2.86
$MnCl_2.4H_2O$	1.81
$ZnSO_4.7H_2O$	0.22
$CuSO4.5H_2O$	0.079
$Co(NO3).6H_2O$	0.049
$Na_2MoO4.2H_2O$	0.39

3.3.3 Raising of Axenic Cultures

Once the growth is noticed, filaments or colonies were isolated from the periphery of the agar plates under aseptic conditions in a laminar air flow chamber and transferred to similar medium; incubated under similar conditions for additional 1-2 weeks. The isolated culture purified by number of transfers to the liquid medium, 100ml of the liquid medium taken in 250ml conical flask, sterilised. The cultures were maintained in the medium with periodic sub culture.

3.3.4 Morphological identification

Identification of the isolates to the genus and species level was based on the morphology of the colony and individual cells revealed using microscopic examinations. 1ml of the cultured isolate was pipetted out and vortexed. Small amount of the vortexed mixture was placed on a clean and dry glass slide, covered with a cover slip and observed under compound light microscope. The

microphotographs were taken with the help of camera attached to the microscope and documented. The observations were made in the high powers (40X and 100X). Oil emulsion was used for 100X power, where the dimensions of the cells were well studied in this. Identification was made using the taxonomic literature of Desikachary (1959). The morphological characters used for the identifications were; nature of the trichome, cell shape and dimensions, heterocyst position, position of akinetes with respect to heterocyst, dimensions of the akinetes.

The axenic cultures of 17 isolates collected from different districts of Kerala were used for constructing the phylogenetic tree, the details of which are given in Table 4.

Table 4. Details of taxa used for the DNA isolation and 16S rDNA gene amplification.

Sl. No.	Name of the Species	Specimen Number	Place of Collection
1.	*Nostoc muscorum*	MBGH-8894	Malampuzha canal, Palakkad
2.	*Anabaena aphanizomenoides*	MBGH-14843	Anthikad, Trissur
3.	*Nostoc humifusum*	MBGH-14839	Kothamangalam, Ernakulam
4.	*Nostoc paludosum*	MBGH-14841	Anthikad, Trissur
5.	*Anabaena sphaerica*	MBGH-14837	Thalappara, Malappuram
6.	*Anabaena torulosa*	MBGH-14846	Kuruvattoor, Kozhikode
7.	*Nostoc entophytum*	MBGH-14847	Kuruvattoor, Kozhikode
8.	*Nostoc* sp.	MBGH-14849	Kuruvattoor, Kozhikode
9.	*Nostoc muscorum*	MBGH-8885	Ambenkunne, Alappuzha
10.	*Nostoc muscorum*	MBGH-14850	Chakkulath, Alappuzha
11.	*Nostoc ellipsosporum*	MBGH-8882	Palakkad
12.	*Nostoc spongiaeforme*	MBGH-14852	Nellikatte, Kasargod
13.	*Nostoc* sp.	MBGH-14848	Kuruvattoor, Kozhikode
14.	*Anabaena aphanizomenoides*	MBGH-14829	Vazhakkad, Kozhikode
15.	*Nostoc punctiforme*	MBGH-14860	Jhandamukk, Trissur
16.	*Anabaena orientalis*	MBGH-14807	Bidunampallam,Palakkad
17.	*Nostoc linckia*	MBGH-14809	Kakathara, Palakkad

3.4 MOLECULAR CHARACTERISATION

3.4.1 DNA Isolation

Freshly grown cultures of cyanobacteria were harvested at their growth phase (14-21 days old cultures) by centrifugation at 12000 rpm for 5 minutes, and then washed with washing buffer (50mM Tris HCl pH 8.0, 50mM EDTA). The pellets thus obtained were suspended in 200µl of the same solution. The cells were disrupted by sonication in an ultra probe sonicator for 2 minutes, 500 µl lysis buffer added and incubated at 60^0C water bath for 30 minutes. The mixture was cooled to room temperature and added 700 µl Chloroform: Isoamyl alcohol mixture (24:1) and mixed thoroughly to form an emulsion. The emulsion was centrifuged at 12,000 rpm for 15 minutes at 25^0 C.

After centrifugation the upper layer was transferred to a fresh 2 ml centrifuge tube and added equal amount of chloroform, mixed and centrifuged at 12,000 rpm for 10 minutes at 4^0 C. After centrifugation the upper layer (supernatant) was transferred to a fresh 2 ml tube and added 500 µl 100% chilled ethanol through the sides and kept at -20^0 C for 30 minutes. After 30 minutes, centrifuged at 10,000 rpm for 10 minutes at 4^0C and collected the pellets. The pellets were washed with 70% ethanol twice and dried under vacuum. The pellets were suspended in 50 µl TE buffer.

3-5 µl RNase were added and incubated at 37^0 C for 1 hr. After incubation 500 µl Phenol: Chloroform: Isoamyl alcohol (24:25:1) mixture were added, mixed well and centrifuged at 10,000 rpm for 5 minutes at 4^0 C. After centrifugation the supernatant was collected in 1.5 ml tube and 500µl chloroform was added mixed and centrifuged. The supernatant was collected and double volume alcohol and $1/1^{10}$ (50 µl) 3M sodium acetate was added and kept overnight. The pellets wee collected by centrifugation at 10,000 rpm for 15 minutes. The pellets were washed in 70% ethanol and re-suspended in TE buffer (50/100 µl).

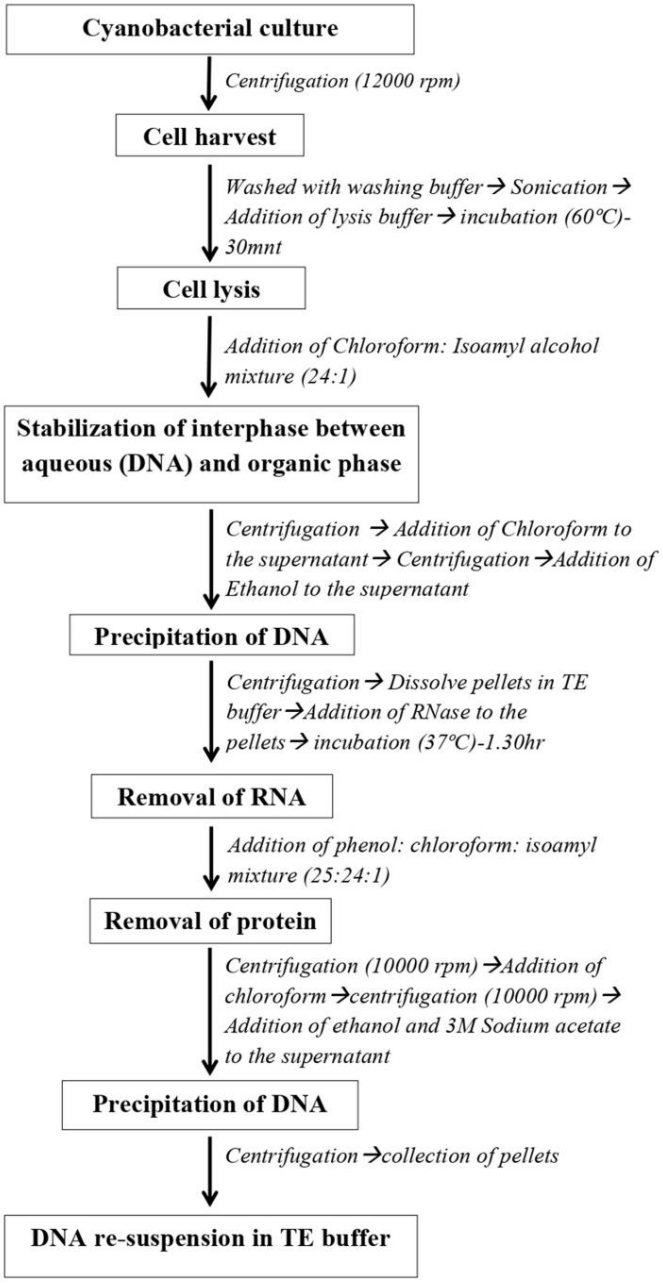


Table 2. Schematic represetation of the DNA isolation protocol.

3.4.1.1 *Quantification of DNA*

Genomic DNA was quantified by measuring Optical Density (OD) at 260 and 280 nm in UV Spectrophotometer. The ratio of A_{260nm} to A_{280nm} was calculated to check the purity level.

3.4.1.2 *Agarose Gel Electrophoresis of Isolated DNA*

The quantity of the DNA isolated was checked using agarose gel electrophoresis. 1µl of gel-loading dye (Xylene cyanogen) mixed in 5µl of the DNA. The samples were loaded to 0.8% agarose gel prepared in 1X TAE (Tris-Acetate-EDTA) buffer containing 0.5µg/ml ethidium bromide. Electrophoresis was performed with 1X TAE as electrophoresis buffer at 90 V until bromophenol dye front has migrated to the bottom of the gel. The gels were visualised in a UV transilluminator and the image was captured under UV light using Gel documentation system.

3.4.2 PCR Amplification

PCR amplification of approximately 1.5kb of 16S ribosomal DNA (rDNA) of the selected isolate was performed using EmeraldAmp® GT PCR master mix (Takara, Japan) in PCR thermal cycler with eubacterial primers 8-27F (*E. coli* positions 8 to 27), and 1495R (*E. coli* positions 1495 to 1476) that were modified from primers fD1 and rP2 respectively of Weisburg *et al.* (1991). The reaction mixture contained 50 ng of DNA, 1xEmeraldAmp® GT PCR master mix and1µl of 10 µM of each primer. PCR conditions consisted of an initial denaturation at 98ºC for 1min;30 cycles at 98ºC for 10 sec, annealing 58ºC for 30 sec and 72ºC for 1.30 sec; and final 10 mins extension at 72ºC. The amplification products were examined by agarose gel electrophoresis. The complete 16S rDNA gene was sequenced by using the PCR products directly as sequencing template with above mentioned primers.

Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR system 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) following manufactures protocol.

The PCR mix consisted of the following components:

PCR Product (purified)	10-20 ng
Primer(either Forward or	3.2 pM

Reverse)	
Sequencing Mix	0.28 µl
5x Reaction buffer	1.86 µl
Sterile distilled water	up to 10µl

The sequencing PCR temperature profile consisted of a 1st cycle at 96^{o}C for 2 minutes followed by 30 cycles at 96^{o}C for 30 sec, 50^{o}C for 40 sec and 60^{o}C for 4 minutes for all the primers.

Agarose gel electrophoresis of PCR products- The PCR products were checked in 1% agarose gel prepared in 1X TAE buffer containing 0.5µg/ml ethidium bromide. 1µl of 6X loading dye was mixed with 5µl of PCR products and was loaded and electrophoresis was performed at 90 V power supply with 1 X TAE as electrophoresis buffer for about 1-2 hours, until the bromophenol blue front migrated to almost the bottom of the gel. The molecular standard used was a 100 bp DNA ladder. The gels were visualised in a UV transilluminator and the image was captured under UV light using Gel documentation system.

Post Sequencing PCR Clean up

1. Prepared master mix I of 10µl milli Q and 2 µl 125mM EDTA per reaction
2. Added 12µl of master mix I to each reaction containing 10µl of reaction contents and are properly mixed.
3. Prepared master mix II of 2 µl of 3M sodium acetate pH 4.6 and 50 µl of ethanol per reaction.
4. Added 52 µl of master mix II to each reaction.
5. Contents were mixed by inverting.
6. Incubated at room temperature for 30 minutes
7. Spun at 14,000 rpm for 30 minutes
8. Decanted the supernatant and add 100 µl of 70% ethanol
9. Spun at 14,000 rpm for 20 minutes.
10. Decanted the supernatant and repeat 70% ethanol wash
11. Decanted the supernatant and air dry the pellet.

The cleaned up air dried product were sequenced in ABI 3500 DNA Analyzer (Applied Biosystems).The qualities of the sequences were checked using Sequence Scanner Software v1 (Applied Biosystems).

3.4.3 Sequence Analysis

Sequences were assembled using BioEdit (Hall, 1999) Program. Homology search was performed using BLAST search algorithm. Alignment of similar sequences was done using CLUSTAL MUSCLE software and the phylogenetic tree was constructed using MEGA7 (Kumar *et al.*, 2016) software. The evolutionary history was inferred using the Neighbour-Joining method following Saitou and Nei (1987) and the evolutionary distances were computed using the Maximum Composite Likelihood method Tamura *et al.* (2004). The stability of relationship was assessed from bootstrap analysis of the neighbour-joining data based on 1000 re-sampling.

RESULTS

4. RESULTS

4.1 MORPHOLOGICAL STUDIES

Morphological identification of the isolates was done by microscopic observation. The structural simplicity provided with only few distinguishing characters. The identifying characters were the nature of colony, colour, trichome, shape and size of the cells and the position and presence of spores or the akinetes. But these characters are prone to environmental changes *i.e.;* they show different morphology in environmental and cultural conditions. Morphological plasticity is observed within the same species collected from different localities making the identification difficult. The morphological data along with the molecular techniques are used for the identification. The species identity is confirmed by comparing the nucleotide sequences with those available in the GenBank. BLAST tool is used for retrieving the data from GenBank.

Based on the morphological observations and the data retrieved from the GenBank, a taxonomic Key for the identification of *Nostoc* and *Anabaena* occurring in Kerala was attempted and detailed description of the taxa is also given below;

4.2 KEY TO THE GENERA

1. Trichome highly coiled and in a definite colony.....2
   Trichome not so coiled and seen as planktonic mass.....*Anabaena*
2. Colonies usually spherical and filaments in a definite mucilaginous mass.....*Nostoc*

Nostoc Vaucher ex Bornet & Flahault, 1888. Ann. Sci. Nat. Bot., Ser. VII, 7: 181.

Type species**.** *Nostoc commune* Vaucher ex Bornet & Flahault

4.2.1 Key to the identification of *Nostoc* species

1a. Cells barrel in shape.....2
1b. Cells not barrel shaped.....9

2a. Colonies spherical to globose.....3
2b. Colonies not spherical.....5

3a. Colonies brownish in colour.....4
3b. Colonies blue-green.....5

4a. Akinetes barrel shaped.....*N. linckia*
4b. Akinete oblong..... *N. punctiforme*

5a. Trichomes pale blue-green, loosely arranged.....*N. paludosum*
5b. Trichomes dark green tightly entangled..... *N.* sp. (14848)

6a. Spores compressed; epispore brown and smooth..... *N. entophytum*
6b. Spores not compressed..... 7

7a. Spores seen away from heterocyst.....*N. spongiaeforme*
7b. Spores seen adjacent to heterocyst..... 8

8a. Spores spherical- barrel shaped..... *N. muscorum*
8b. Spores elongate and curved.....*N.* sp. (14849)

9a.Akinete ellipsoidal.....*N. ellipsosporum*
9b. Akinete spherical to barrel.....*N. humifusum*

Anabaena Bory
Type species *Anabaena oscillarioides* Bory

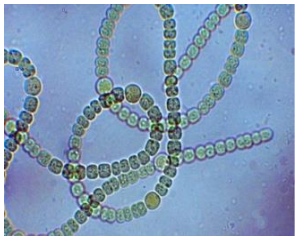
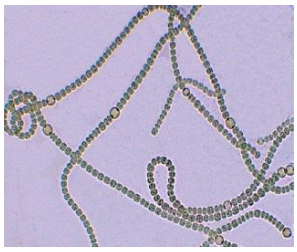
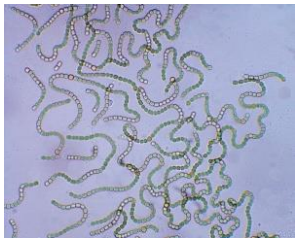
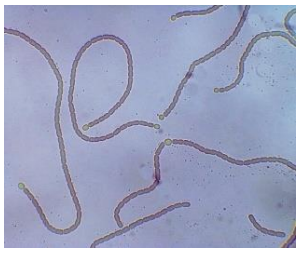
4.2.2 Key to the identification of *Anabaena* species

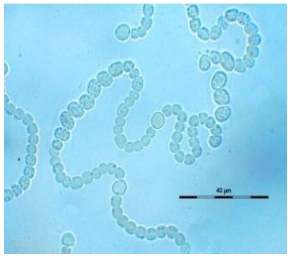
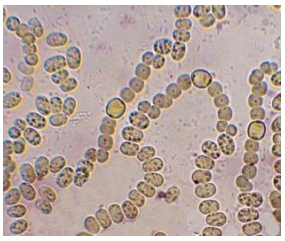
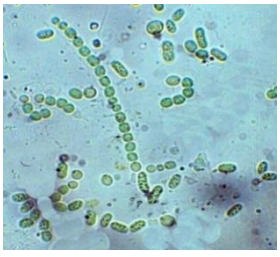
1a. Akinetes on both sides of the heterocyst.....2
1b. Akinetes formed on one side.....*A. aphanizomenoides*

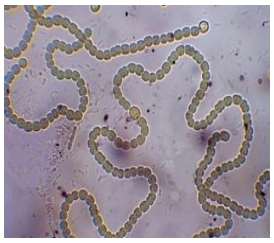
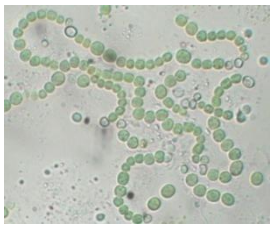
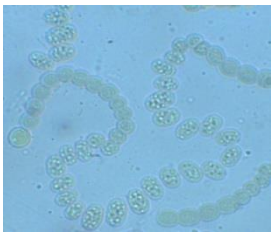
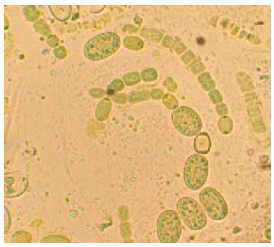
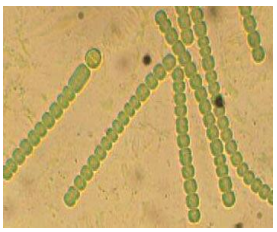
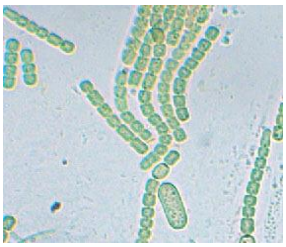
2a. Apical cell rounded or dome shaped.....*A. sphaerica*
2a. Apical cell conical.....*3*

3a. Vegetative cells cylindrical.....*A. orientalis*
3b. Vegetative cells barrel shaped.....*A. torulosa*

Table 5. Comparison of morphological characters of the cultured Blue-green algae.

Sl. No.	Sample code	Cell Shape	Apical Cell	Heterocyst	Akinete	Morphology
1	MBGH-8894	Short Barrel	Slightly conical	Spherical	Akinete formation not noticed in cultures	
2	MBGH-8885	Barrel	Dome shaped	Spherical to barrel	Akinete formation not noticed in cultures	
3	MBGH-14850	Barrel	Dome shaped	Spherical	Akinete formation not noticed in cultures	
4	MBGH-14848	Barrel	Dome Shaped	Spherical	Barrel shaped away from heterocyst	
5	MBGH 8882	Cylindrical	Dome shaped	Spherical	Akinetes rare in cultures	

6	MBGH 14807	Short barrel	Rounded	Spherical	Barrel to elongate adjacent to heterocyst. Very rare in cultures	
7	MBGH 14809	Barrel	Round	Sub spherical	Barrel away from heterocyst	
8	MBGH-14846	Barrel	Conical	Ovoid	Cylindrical with round ends, adjacent to heterocyst	
9	MBGH-14847	Barrel	Round	Spherical	Compressed barrel shaped adjacent to heterocyst	
10	MBGH-14849	Barrel	Round	Spherical	Elongate, freely suspended in cultures	
11	MBGH-14852	Barrel	Round	Spherical	Oblong away from heterocyst	

12	MBGH-14841	Barrel shaped	Dome shaped	Spherical	Akinete formation not noticed in cultures	
13	MBGH-14839	Barrel	Round	Spherical	Spherical away from heterocyst	
14	MBGH 14860	Barrel	Round	Spherical	Elongate later become compressed	
15	MBGH-14837	Barrel	Dome shaped	Sub-spherical	Oval, on both sides of the heterocyst	
16	MBGH-14843	Barrel	Rounded	Spherical	Oblong	
17	MBGH 14829	Short cylinder	Conical	Ellipsoidal	Oblong	

4.3 MORPHOLOGICAL DESCRIPTION

Morphological characters of 14 taxa of *Nostoc* and *Anabaena* of Kerala were recorded. The concise morphological description of each taxon along with microphotographs is given below;

Nostoc muscorum Ag.ex Born. et Flah.

Bornet et Flahault, Revision des Nostocacees heterocystees, 200, 1888; Geitler, Kryptogamen flora, 844, fig. 535, 1932; Desikachary, Cyanophyta, 385, Pl. 70, Fig. 2, 1959.

Fig. 3. A

Habitat: Moist soils of stream banks and paddy field soil.

Thallus gelatinous, membraneous, irregularly expanded. Blue-green in colour. Filaments entangled; trichome 4-5 µm broad. Cells short barrel shaped 3-5 µm long; apical cells dome shaped. Heterocyst both intercalary and terminal; spherical to barrel in shape, 5-7 µm broad. Akinetes produced adjacent to heterocysts; many in a series, 4-6 µm broad and 5-7 µm long; epispore smooth. Akiete formation not observed in cultures.

This name is currently regarded as a taxonomic synonym of *Desmonostoc muscorum* (C. Agardh ex Bornet & Flahault) Hrouzek & Ventura.

Specimens Examined: MBGH 8885 (Ambenkunnu, Palakkad), MBGH 8894 (Malampuzha, Palakkad), MBGH 14850 (Chakkulath, Alapuzha)

Nostoc paludosum Kutzing ex Born.et Flah.

Bornet et Flahault, Revision des Nostocacees heterocystees, 191, 1888; Geitler, Kryptogamen flora, 836, fig. 528a, 1932; Desikachary, Cyanophyta, 375, Pl. 69, Fig. 2, 1959.

Fig.3. B

Habitat: Paddy field soils.

Thallus microscopically small, pale blue-green colour; gelatinous colonies spherical later become lobed in nature, sheath colourless. Trichome 4 µm broad; cells barrel shaped; 3-4.5 µm long. Apical cell dome shaped; heterocysts both intercalary and terminal, spherical in shape; broader than vegetative cells, 5-6 µm broad. Akinetes oval in shape, 4 µm broad, 5-8 µm long. Akinetes not observed in cultures.

Specimens Examined: MBGH 14841 (Anthikad, Trissur),

Nostoc entophytum Born.et Flah.

Bornet et Flahault, Revision des Nostocacees heterocystees, 190, 1888; Geitler, Kryptogamen flora, 836, 1932; Desikachary, Cyanophyta, 375, 1959.

Fig. 3. C

Habitat: Isolated from paddy field soil.

Thallus macroscopic, leathery, yellowish brown in colour. Filaments densely entangled; sheath distinct, brownish. Trichome 3-4 µm broad, short barrel shaped. Heterocyst both intercalary and terminal, broader than vegetative cells (5-6 µm broad), spherical; akinetes compressed 7-8 µm long and 5-6 µm broad as a chain near the heterocyst, epispore smooth.

Specimens Examined: MBGH 14847 (Kuruvattoor, Kozhikode)

Nostoc humifusum Carmichael ex Born.et Flah.

Born. et Flahault, Revision des Nostocacees heterocystees, 201, 188; Geitler, Kryptogamen flora, 842, 1932; Desikachary, Cyanophyta, 384, 1959.

Fig. 3. D

Habitat: In stagnant water.

Thallus dark green in colour, rounded, later become expanded, attached to the periphery. Trichome 2-3.5 µm broad; cells spherical. Heterocyst both

intercalary and terminal. Akinetes spherical; away from heterocyst 5 µm broad and 6 µm long and epispore smooth.

Specimens Examined: MBGH 14839 (Kothamangalam, Ernakulam)

Nostoc linckia (Roth) Bornet ex Born.et Flah.

Born. et Flah., Revision des Nostocacees heterocystees, 192, 1888; Geitler, Kryptogamen flora, 838, fig. 528b, 1932; Desikachary, Cyanophyta, 377, Pl. 69, Fig. 4, 1959.

Fig. 3. E

Habitat: Paddy field as a gelatinous mass.

Thallus gelatinous blackish green to brown in colour, tuberculate, at first globose later irregularly expanding. Filaments densely entangled; sheath colourless; trichome 4.5-5 µm broad; cells short barrel shaped. Heterocyst sub spherical; both terminal and intercalary; akinetes sub spherical, 7-7.5 µm broad and 6-7 µm long; seen as a chain away from heterocyst.

Specimens Examined: MBGH 14809 (Kakathara, Palakkad)

Nostoc ellipsosporum (Desm.) Rabenh. ex Born. et Flah.

Bornet and Flahault, Revision des Nostocacees heterocystees, 198, 1888; Geitler, Kryptogamen flora, 841, fig. 533, 1932; Desikachary, Cyanophyta, 383, Pl. 69, Fig. 5, 1959.

Fig. 3. F

Habitat: Found as gelatinous mass on rocks.

Thallus gelatinous, irregularly expanded, attached by lower surface; reddish brown in colour. Trichomes straight or slightly curved, with the same diameter along the filament. Filaments loosely entangled; trichome 4-5 µm broad; cells cylindrical 6-10 µm long, olivaceous. Cells with dark brown granules and gas vescicles. Heterocyst both intercalary and terminal; sub spherical; 7-8 µm

broad and 10-12 µm long. Akinetes ellipsoidal 5-8 µm broad; 12-18 µm long, epispore smooth. Akinete formation very rare in cultures.

Specimens Examined: MBGH 8882 (Palakkad)

***Nostoc* sp.**

Fig. 4. G.

Habitat: From cultures of paddy field soil.

Colony at first globose later expanding; thallus pale blue-green, less coiled and microscopically small. Trichomes 3-5 µm broad; vegetative cells barrel to cylindrical in shaped, 3.5-5 µm long; end cells rounded. Heterocyst both intercalary and terminal, terminal one very rare, spherical, 5.5-7 µm broad and as long as broad. Akinetes seen as a chain and scattered, elongate, 3-4 µm broad and 6-8 µm long sometimes curved.

Specimens Examined: MBGH 14849 (Kuruvattoor, Kozhikode)

Nostoc punctiforme (Kutz.) Hariot

Geitler, Kryptogamen flora, 834, 1932; Desikachary, Cyanophyta, 374, Pl. 69, Fig. 1, 1959.

Fig. 4. H

Habitat: From the cultures of paddy field soil.

Trichome brownish green in colour, filaments slightly coiled. Vegetative cells barrel shaped 4-5 µm broad and 3-4 µm long. Apical cells rounded; heterocyst both intercalary and terminal. Spherical in shape, 5-6 µm broad and 4.5-6 µm long. Akinetes seen away from the heterocyst, compressed barrel shaped 7-8.5 µm broad and 5-6 µm long.

Specimens Examined: MBGH 14860 (Jhandamuk, Trissur)

Nostoc spongiaeforme Agardh ex Born.et Flah.

Bornet and Flahault, Revision des Nostocacees heterocystees, 197, 1888; Geitler, Kryptogamen flora, 839, fig. 531, 1932 (*non* Tilden); Desikachary, Cyanophyta, 380, Pl. 67, Fig. 3.

Fig. 4. I

Habitat: Seen as gelatinous mass on the surface of the laterite soil in the wet lands.

Thallus expanding ribbon like, gelatinous, Blue-green to brownish, filaments flexuous, loosely entangled, sheath yellowish brown; trichome 4-5 µm broad, cells barrel to cylindrical in shape, apical cell dome shaped ; heterocyst sub spherical, both intercalary and terminal, 6-7 µm broad; akinetes oblong, seen away from the heterocyst, 6-8 µm broad and 8-10 µm long, epispore yellowish.

Specimens Examined: MBGH 14852 (Barker fuel station, Nellikatte, Kasargod)

***Nostoc* sp.**

Fig. 4. J

Habitat: From cultures of paddy field soil.

Thallus dark green, leathery, highly coiled and microscopically small, trichome 2-3 µm broad; vegetative cell barrel to cylindrical, 3-3.5 µm long, end cell rounded. Heterocyst both terminal and intercalary, spherical, 3-5 µm broad and as long as broad. Akinetes away from the heterocyst, barrel to spherical in shape

Specimens Examined: 14848 (Kuruvattoor, Kozhikode)

Anabaena aphanizomenoides Forti

Geitler, Kryptogamen flora, 875, fig. 556, 1932; Desikachary, Cyanophyta, 405, Pl. 71, Fig. 4, 1959.

Fig. 4. K

Habitat: From cultures of paddy field soil.

Trichome single, straight, 4-5 µm broad; constricted at the cross walls. Cells barrel to cylindrical with gas vacuoles. Heterocyst mostly intercalary; sub spherical to barrel, 5-5.5 µm broad, 5-6 µm long. Akinetes adjacent to the heterocyst single; elongate, 5-6 µm broad and 8.5-10 µm long with smooth wall.

This name is currently regarded as a taxonomic synonym of *Sphaerospermopsis aphanizomenoides* (Forti) Zapomelova, Jezberova, Hrouzek, Hisem, Rehakova & Komarkova

Specimens Examined: MBGH 14843 (Anthikad, Trissur), 14829 (Vazhakad, Kozhikode)

Anabaena sphaerica Bornet et Flahault

Revision des Nostocacees heterocystees, 228, 1888; Geitler, Kryptogamen flora, 878, 1932; Desikachary, Cyanophyta, 393, Pl. 71, Fig. 10, 1959.

Fig. 4. L

Habitat: Found as a mat on the soil surface.

Thallus floccose, blue-green; straight. Cells spherical to barrel, 5µm broad; apical cells rounded or dome shaped. Heterocyst intercalary; sub spherical, 6-7 µm broad; akinetes on both sides of the heterocyst, oval, 8µm broad and 10-12 µm long, epispore smooth.

Specimens Examined: MBGH 14837 (Thalappara, Malappuram)

Anabaena torulosa (Carm.) Lagerh. ex Born. et Flah.

Bornet and Flahault, Revision des Nostocacees heterocystees, 236, 1888; Geitler, Kryptogamen flora, 887, fig. 567d, 1932; Desikachary, Cyanophyta, 415, Pl. 71, Fig. 6, 1959.

Fig. 4. M

Habitat: Attached to the culms of the paddy plant collected from Kozhikode.

Thalus thin, pale blue-green; trichome 3-4 µm broad, cells barrel shaped; apical cell acutely conical. Heterocyst subspherical; 5 µm broad and 6-7 µm long; akinete on both the sides of the heterocysts, cylindrical to barrel with rounded ends, 7-8 µm broad, up to twice as long as broad, epispore smooth.

Specimens Examined: MBGH 14846 (Kuruvattoor, Kozhikode)

Anabaena orientalis Dixit

Desikachary, Cyanophyta, 405, Pl. 77, Fig. 6, 1959.

Fig. 4. N

Habitat: From culture of paddy field soil.

Thallus seen as a suspension in liquid culture, dark green, trichome single, straight or slightly curved; vegetative cells cylindrical, 3.5- 4.5 µm broad and 3-4.5 µm long, end cells conical with rounded ends. Heterocyst cylindrical, intercalary, 4.5-5 µm broad and 7-8.5 µm long; akinetes on one side of the heterocyst; at first single, barrel shaped later elongate 10-14 µm long and 8-10 µm broad.

Specimens Examined: MBGH 14807 (Bidunampallam, Palakkad)

4.4 GROWTH AND AXENIC CULTURE

The samples collected from different places were brought to the laboratory. For the planktonic masses collected, their preliminary identifications were carried out at laboratory. Serial dilution procedure was carried out for the soil sample collected. BG_0-11 medium suspended in agar plates (4%) is used for the primary isolations of the collected specimens. The growth of the inoculated samples was observed within 3 days of inoculation, the serial diluted soil samples took a bit longer time for their growth. The cultures were heterogenous at first; colony homogenisation was achieved by continuous sub culturing. All the samples that showed growth were morphologically identified and the characters were noted. The desired colonies were identified and individual colonies or filaments were re-inoculated on fresh agar plates with medium for

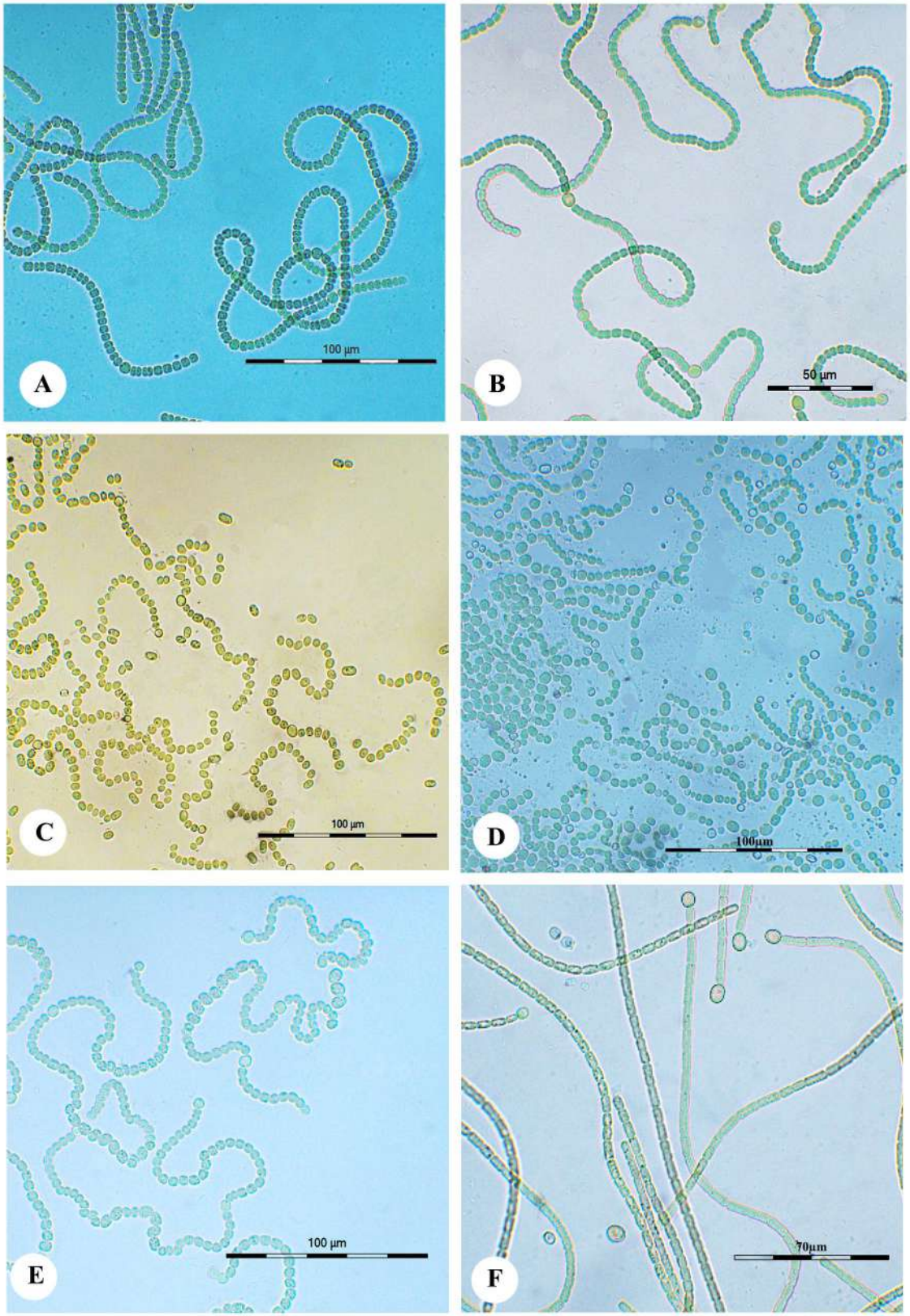


Fig. 3. Morphology of hormogonia of *Nostoc* spp. A. *Nostoc muscorum,* B. *Nostoc paludosum,* C. *Nostoc entophytum,* D. *Nostoc humifusum,* E. *Nostoc linckia,* F. *Nostoc ellipsosporum.*

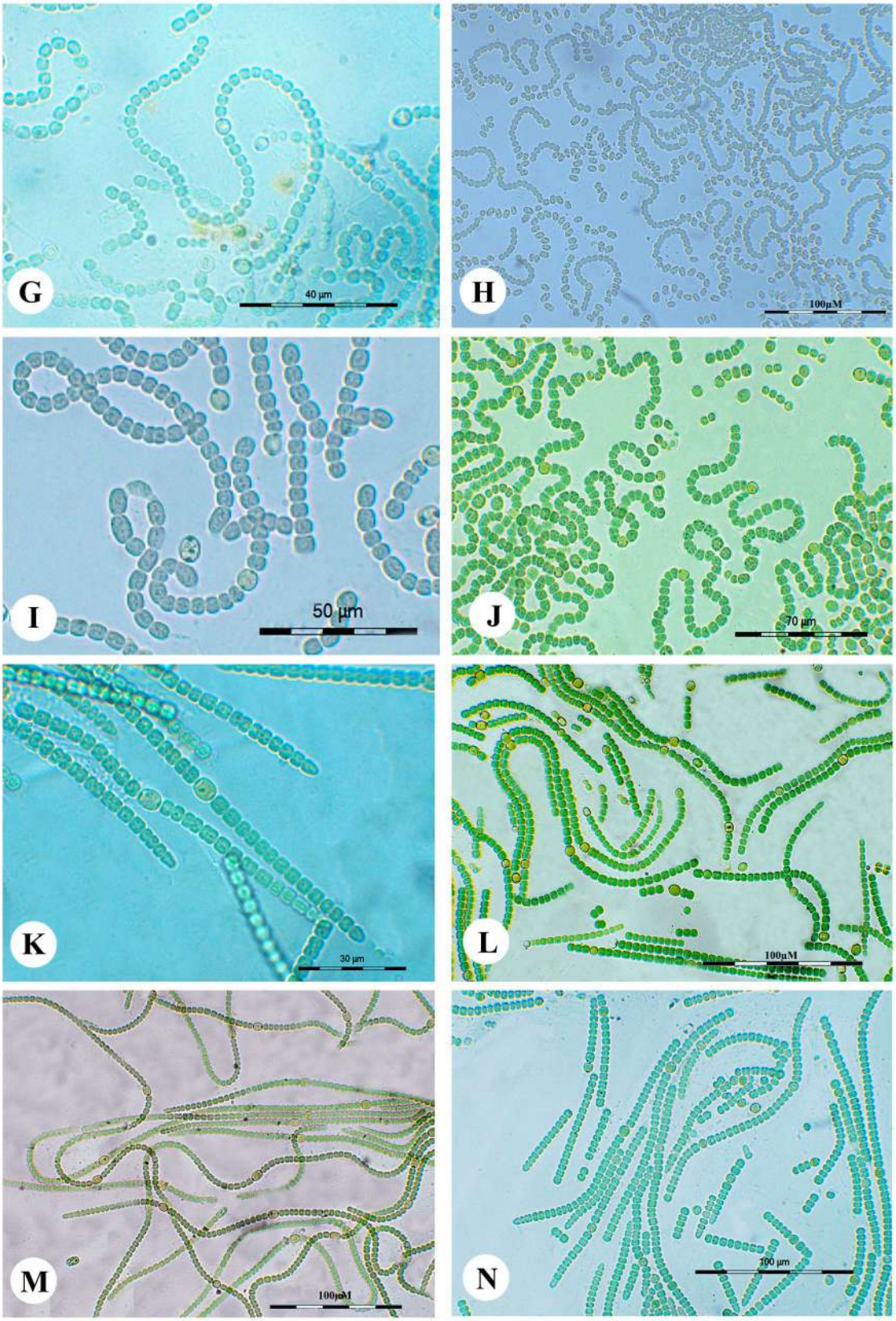


Fig. 4. Morphology of hormogonia of *Nostoc* spp. and *Anabaena* spp. G. *Nostoc* sp., H. *Nostoc punctiforme,* I. *Nostoc spongiaeforme,* J. *Nostoc* sp., K. *Anabaena aphanizomenoides,* L. *Anabaena sphaerica,* M. *Anabaena torulosa,* N. *Anabaena orientalis.*

isolating single species. *Nostoc* and *Anabaena* showed different growth pattern in the agar plates. In case of *Anabaena* the growth was noticed as the filaments growing to the periphery (Fig. 5.). For *Nostoc* growth was seen as colonies or round mucilaginous mass (Fig. 7.). In agar plates, they are seen as small mucilaginous patches or balls; some of them spread on the agar as a mucilaginous layer or unevenly distributed. Some of them even form thick mucilaginous mass without spreading giving a jelly like appearance.

The actively growing cells were taken from the periphery using an inoculation loop and inoculated in the liquid medium. Their growth was monitored and it was noticed that after inoculation, within three days the growth started and they were in the logarithmic phase up to 14-21 days. After which the growth ceases and there occurs the vitrification or yellowing of the samples due to the depletion of nutrients. In majority of the samples this results in the formation of akinetes or the spores in order to tackle the adverse conditions. Prolonged culturing in the medium also leads to the growth of other non heterocystous blue-green algae by utilising the nutrients and the nitrogen fixed by the heterocystous Blue-green algae. Periodic sub culturing is done in order to maintain axenic cultures.

4.4.1 Culture Characteristics

Anabaena and *Nostoc* show different cultural characteristics; *Anabaena* strains grew as planktonic mass that are often attached to the walls of the culture flasks, some of them are seen to grow as a suspension in liquid medium (Fig. 6.). The colour of the colonies ranges from pale green, blue-green, yellowish green to dark green. *Anabaena torulosa* grew as pale green thin hormogonia that are settled at the bottom of the flask, where as the *Anabaena orientalis* were seen as a bluish green evenly distributed suspension that are very difficult to settle down even after high centrifugation. *Anabaena sphaerica* was seen as dark green mass floating on the surface of the medium.

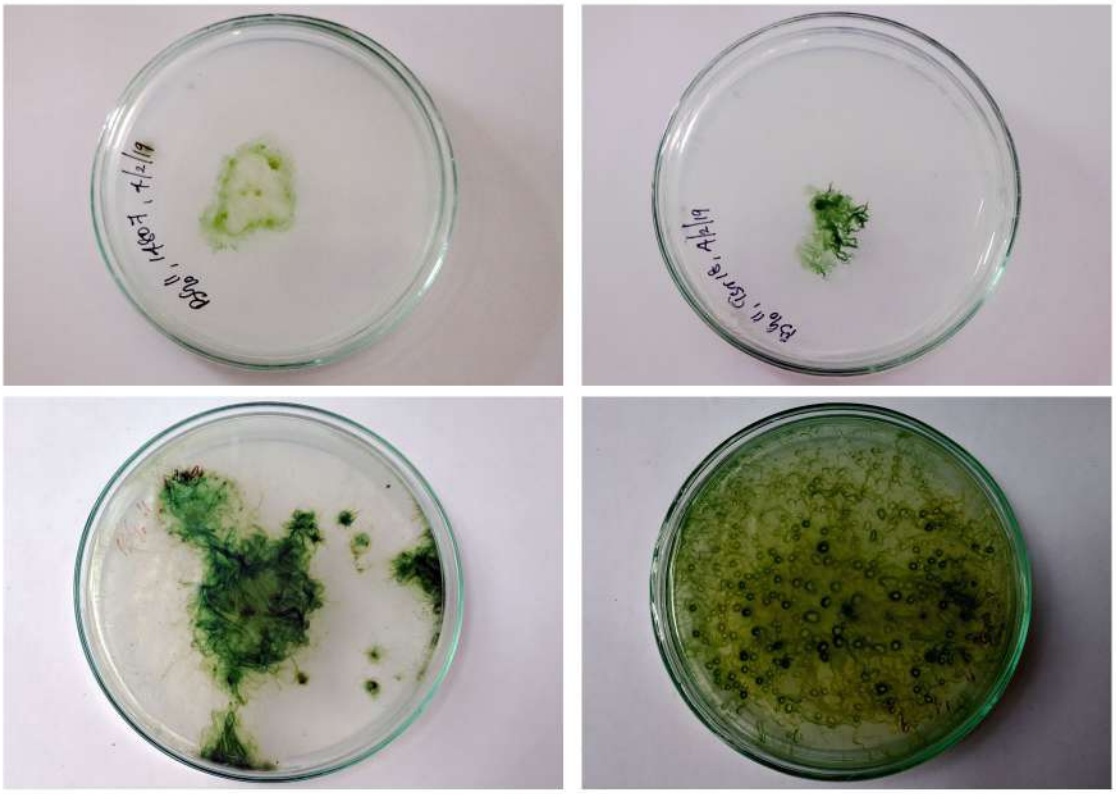


Fig. 5. Growth of *Anabaena* strains in agar plates showing filamentous nature

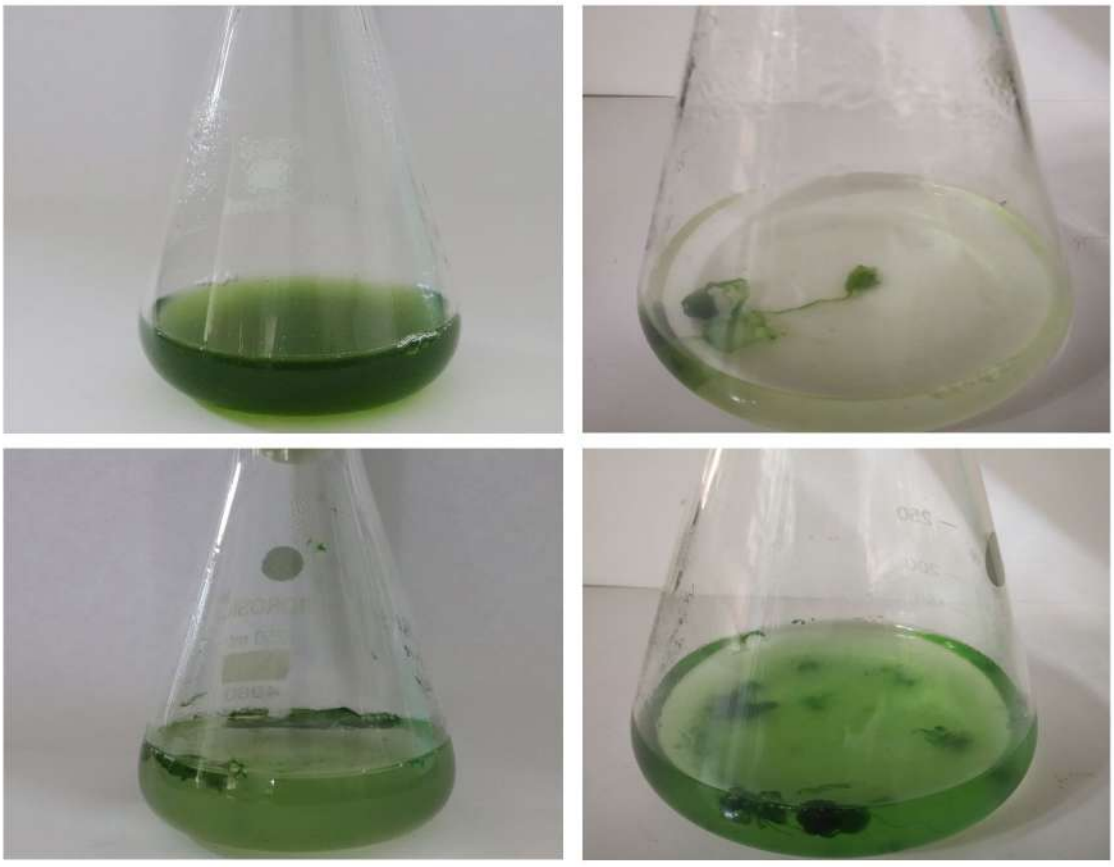


Fig. 6. Liquid cultures of *Anabaena* showing different growth patterns

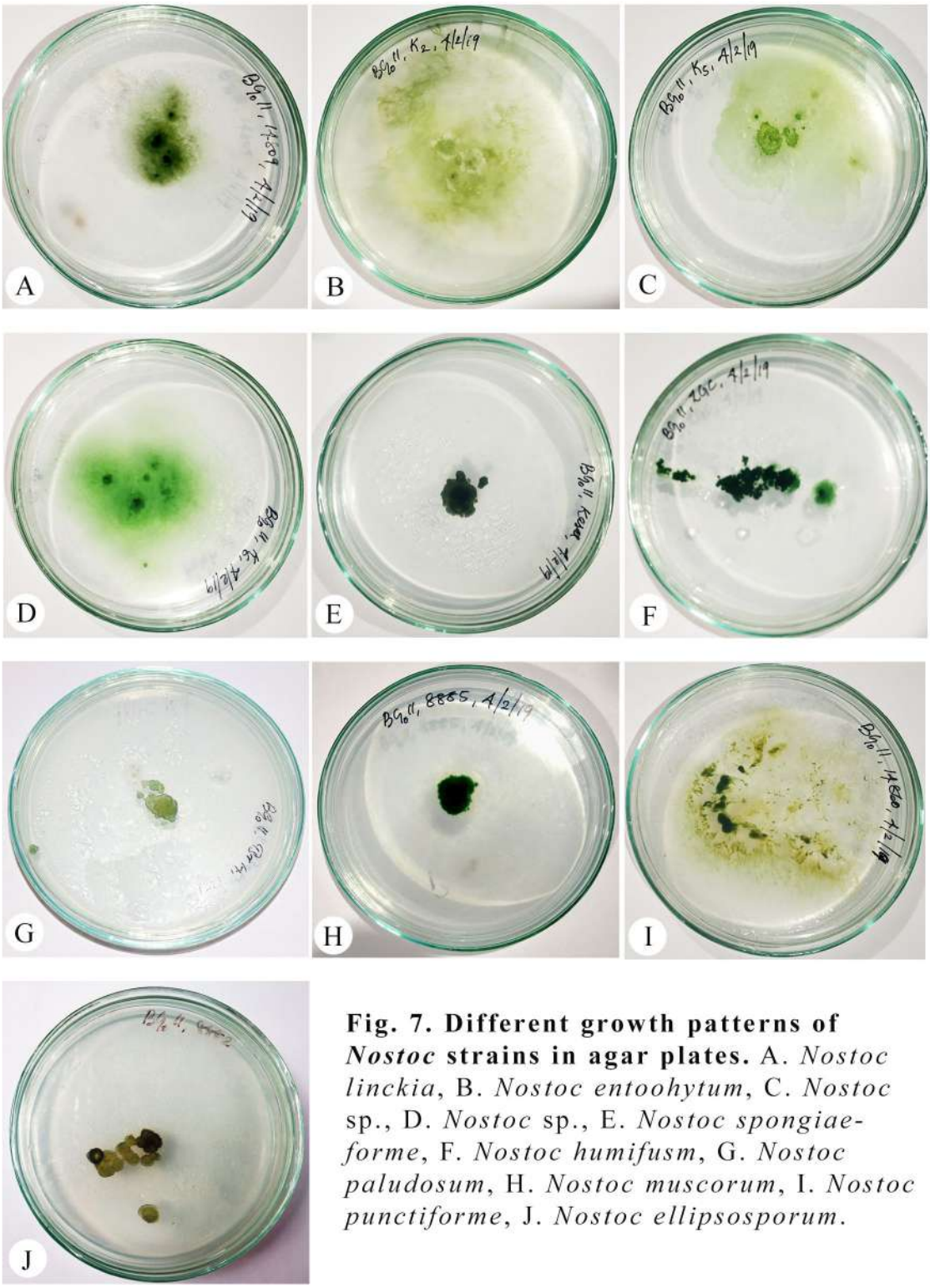


Fig. 7. Different growth patterns of *Nostoc* strains in agar plates. A. *Nostoc linckia*, B. *Nostoc entoohytum*, C. *Nostoc* sp., D. *Nostoc* sp., E. *Nostoc spongiaeforme*, F. *Nostoc humifusm*, G. *Nostoc paludosum*, H. *Nostoc muscorum*, I. *Nostoc punctiforme*, J. *Nostoc ellipsosporum*.

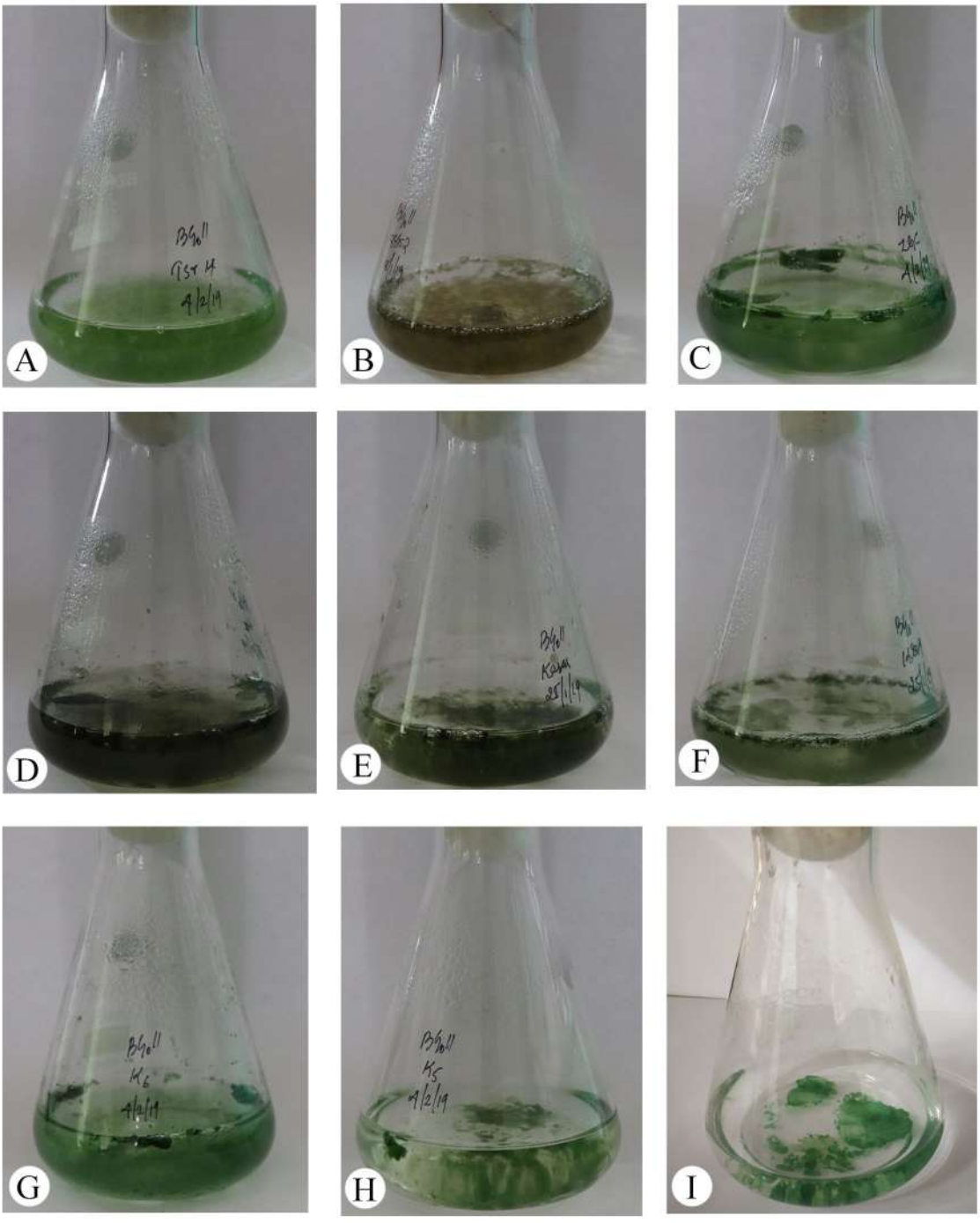


Fig. 8. Liquid cultures of *Nostoc* showing different growth pattern. A. *Nostoc paludosum*, B. *Nostoc ellipsosporum,* C. *Nostoc humifusum,* D. *Nostoc entophytum*, E. *Nostoc spongiaeforme*, F. *Nostoc linckia*, G. *Nostoc* sp., H. *Nostoc* sp., I. *Nostoc muscorum*.

Growth pattern in the agar plates also showed diversity. But majority exhibited thread-like growth with spreading nature, resembling a layer of network on the agar. Some of them were observed as mucilaginous patches that are randomly distributed over the agar surface.

Nostoc spp. showed an entirely different growth pattern from that of *Anabaena*; they grow as small gelatinous balls that later on become expanded to form tuberculate or lobed and vary in colour (Fig. 8.). The colour ranges from pale blue-green, olive green to brownish. They are also seen attached on the walls of the flask. *Nostoc muscorum* grow as blue-green, gelatinous tuberculate or lobed mass that was very slimy and found free floating in the liquid medium, whereas, *N. linckia* was found to be olive green to brownish colour, small gelatinous balls that were free floating and some of them settles down and get attached to the bottom of the flask. *N. ellipsosporum* also forms gelatinous balls in liquid cultures. Some of them have thin leathery mat like appearance e.g., *N. entophytum*. Periodic shaking of the cultures prevents the colonies from attaching to the walls of the flask.

Unlike in liquid cultures, the blue-green algae cultured on the agar plates are found to be a good means for short term storage up to 2-3 months in the culture conditions provided and the storage time could be increased by decreasing the temperature.

4.5 DNA ISOLATION

14-21 days old cultures are used for DNA isolation to ensure the quality and purity of the isolated DNA. The DNA isolation using the modified method proposed in this study was found to be efficient, and was evident from the results. The difficulty in breaking the tough cell walls could be tackled with mechanical lysis using sonicator along with chemical cell lysis using β-mercaptoethanol. All the isolates showed $A_{260/280}$ ratio ranging from 1.5-1.8 (Table 6. &7.). The gel images also showed good quality DNA (Fig. 9.).

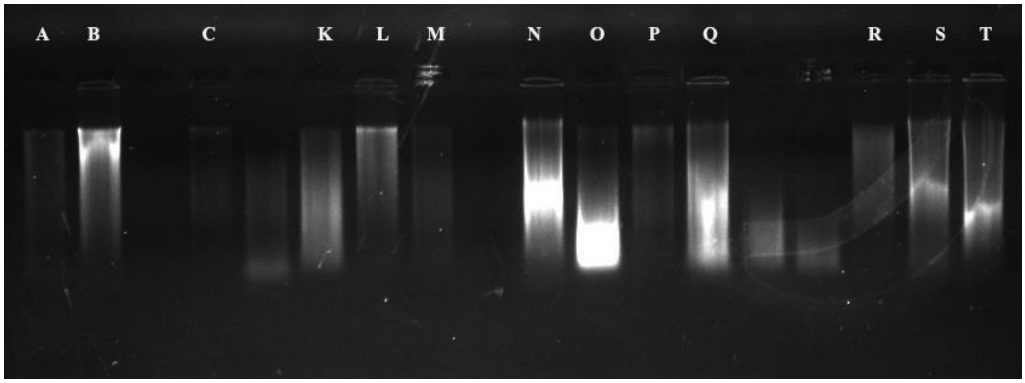


Fig. 9. Agarose (8%) gel electrophoresis image of template DNA isolated from native *Nostoc* and *Anabaena* strains

Table 6. Quantification of isolated DNA of the *Nostoc* samples.

Sl. No.	Strain Code	Organism	A_{260}	A_{280}	$A_{260/280}$
1.	MBG-AL111	*Nostoc muscorum*	0.011	0.007	1.57
2.	MBG-AL114	*Nostoc humifusum*	0.007	0.004	1.75
3.	MBG-AL115	*Nostoc paludosum*	0.006	0.004	1.5
4.	MBG-AL213	*Nostoc entophytum*	0.008	0.05	1.6
5.	MBG-AL214	*Nostoc* sp.	0.011	0.007	1.57
6.	MBG-AL215	*Nostoc muscorum*	0.022	0.012	1.8
7.	MBG-AL217	*Nostoc muscorum*	0.008	0.005	1.6
8.	MBG-AL311	*Nostoc ellipsosporum*	0.022	0.012	1.8
9.	MBG-AL312	*Nostoc spongiaeforme*	0.009	0.006	1.5
10.	MBG-AL313	*Nostoc* sp.	0.134	0.080	1.67
11.	MGB-AL315	*Nostoc punctiforme*	0.584	0.328	1.78
12.	MGB-AL317	*Nostoc linckia*	0.011	0.007	1.57

Table 7. Quantification of isolated DNA of the *Anabaena* samples.

Sl. No.	Strain code	Organism	A_{260}	A_{280}	$A_{260/280}$
1.	MBG-AL113	*Anabaena aphanizomenoides*	0.018	0.010	1.8
2.	MBG-AL116	*Anabaena sphaerica*	0.019	0.013	1.46
3.	MBG-AL212	*Anabaena torulosa*	0.025	0.016	1.56
4.	MBG-AL314	*A.aphanizomenoides*	0.600	0.341	1.75
5.	MBG-AL316	*Anabaena orientalis*	0.620	0.354	1.75

4.6 PCR AMPLIFICATION

The 16S rDNA gene from the isolated genomic DNA was amplified using the primer pair8-27F and 1495R. The size of the amplicon ranged between 1000-1500 kb. The PCR amplification profiles of the isolates are shown in Fig. 10. The amplicons were sequenced.

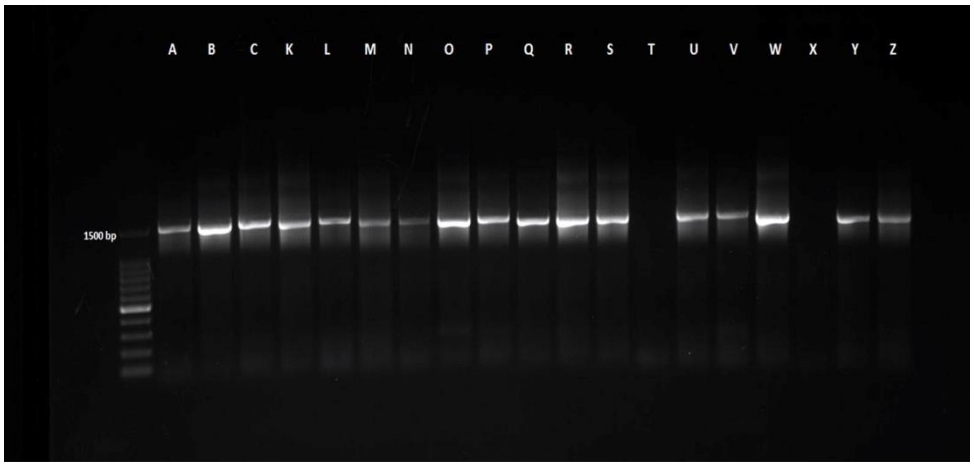


Fig. 10. PCR amplification using 16SrDNA (1.5kb) of extracted genomic DNA electrophoresed in 1% (W/V) agarose gel.

The sequence data of the 16 strains derived from the study were successfully deposited in the GenBank (Appendix I). The 16 strains belonged to 13 taxa and the sequence data of these 13 taxa generated from this study is the first to be deposited in the GenBank from Kerala. The list of GenBank accession numbers of these sequences are mentioned in Table 8. The details of the sequences retrieved from the GenBank for constructing phylogenetic tree is given in Table 9.

Table 8. Details of the 16S rDNA sequences of 17 Isolates considered for molecular systematic analysis.

Sl. No.	Specimen No.	Strain code	Species identity	Gene Bank Accession No
1.	MBGH 8894	MBG-AL111	*Desmonostoc muscorum* Synonym*: Nostocmuscorum*	MG595786
2.	MBGH 14843	MBG-AL113	*Sphaerospermopsis aphanizomenoides* Synonym:*Anabaena aphanizomenoides*	MG595787

3.	MBGH 14839	MBG-AL114	*Nostoc humifusum*	MG595788
4.	MBGH 14841	MBG-AL115	*Nostoc paludosum*	MG595789
5.	MBGH 14837	MBG-AL116	*Anabaena sphaerica*	MG595790
6.	MBGH 14846	MBG-AL212	*Anabaena torulosa*	MG595792
7.	MBGH 14847	MBG-AL213	*Nostoc entophytum*	MG595793
8.	MBGH 14849	MBG-AL214	*Nostoc* sp.	MG595791
9.	MBGH 8885	MBG-AL215	*Desmonostoc muscorum* Synonym*: Nostoc muscorum*	MG595794
10.	MBGH 14850	MBG-AL217	*Desmonostoc muscorum* Synonym*: Nostoc muscorum*	MG595795
11.	MBGH 8882	MBG-AL311	*Nostoc ellipsosporum*	MN100312
12.	MBGH 14852	MBG-AL312	*Nostoc spongiaeforme*	MN149541
13.	MBGH 14848	MBG-AL313	*Nostoc* sp.	Yet to submit
14.	MBGH 14829	MBG-AL314	*Sphaerospermopsis aphanizomenoides* Synonym:*Anabaena aphanizomenoides*	MN149543
15.	MBGH 14860	MBG-AL315	*Nostoc punctiforme*	MN149540
16.	MBGH 14807	MBG-AL316	*Anabaena orientalis*	MN097906
17.	MBGH 14809	MBG-AL317	*Nostoc linckia*	MN149539

Table 9. Details of the 16S rDNA sequences retrived from the GenBank.

Sl. No.	**Taxon designation**	**Strain code**	**Origin**	**GenBank Accession No.**
1.	*N. muscorum*	CENA 61	Amazon Flood plain, Brazil	AY218828
2.	*N. entophytum*	ISC 32	Iran	JN605002
3.	*N. paludosum*	BA033	Water, Minas Gerais state, Brazil	KX423684
4.	*Nostoc* sp.	NQAIF 313	Seasonal creek, Queensland, Australia	KJ636965
5.	*Nostoc* sp.	CENA 105	Waste stabilization pond, Brazil	EF088340
6.	*N. entophytum*	IAM M-267	Japan	AB093490
7.	*N. ellipsosporum*	Lukesova 51_1991	Nezamyslica, field soil, Czech Republic	MG596757
8.	*N. ellipsosporum*	V	Field, Czech Republic	AJ630450

9.	*Nostoc* sp.	PCC 7423	USA	DQ185242
10.	*N. linckia*	NIES-25	Japan	AP018222
11.	*N. linckia*	IAM M-251	Japan	AB074503
12.	*N. spongiaeforme*	Ind 42	Waterbody, Varanasi, U.P, India	HM573463
13.	*N. spongiaeforme*	AUS-JR/DB/NT-007	India	KC556806
14.	*N. humifusum*	U_NEHU	Host *Usnea fragilis*, Shillong, Meghalaya, India	KT232007
15.	*N. humifusum*	AUS-JR/DB/NT-019	Jhumland soil, India	KF322159
16.	*N. entophytum*	MACC-612	Hungary	MH702241
17.	*N. muscorum*	Lukesova 1/87	Czech Republic	AM711523
18.	*N. paludosum*	LCR16	India	EU446016
19.	*N. punctiforme*	roza 5	Iran	LC361246
20.	*N. punctiforme*	NR_114430	Canada	PCC73102
21.	*Sphaerospermopsis aphanizomenoides*	09-03	Reservoir plankton	JQ237771
22.	*A. aphanizomenoides*	1LT27S09	Lake Trasimeno, Italy	FM177473
23.	*Sphaerospermopsis aphanizomenoides*	PMC641.10	Waterbody, France	HQ157698
24.	*A. sphaerica*	PMC 306.07	France	KX580773
25.	*A. sphaerica*	UTEX B1616	Africa	GQ859616
26.	*A. torulosa*	BF1	Rice rhizosphere, India: Aduthurai, Tamil Nadu	GU396091
27.	*A. orientalis*	BTA450	Manipur, India	MH327994
28.	*A. orientalis*	AUS-JR/MT/NT-099	Rice field soil, India	KX670264
29.	*A. torulosa*	A525	India	EF375610

4.7 PHYLOGENETIC ANALYSIS USING 16S rDNA SEQUENCE

The 16S rDNA sequence data for each of 17 strains collected are given in the Appendix I.

ClustalW used for multiple sequence alignment calculated the best match for the selected sequences, and aligned the sequences accordingly. The aligned

sequences of native strains studied showing similarities are given in Appendix II (Fig. 23.). The relationships of the 17 strains were inferred from Neighbour Joining (NJ) and Maximum Likelihood (ML) analysis. The clustering pattern of different taxa showed certain differences in the two trees derived.

Phylogenetic tree of two genera, *Nostoc* and *Anabaena* were considered individually and also in combination to deduce the phylogenetic relation. The native strains studied were also compared with the sequences available in the GenBank and phylogenetic tree reconstructed.

4.7.1 Phylogenetic Analysis of *Nostoc* spp.

The 16S rDNA sequences of the 12 *Nostoc* strains isolated were determined and phylogenetic tree drawn with Neighbour Joining and Maximum Likelihood method.

All the clades were well supported by Bootstrap values. The NJ phylogenetic tree divided into 4 clades (Fig. 11.). The first clade with 6 of 12 *Nostoc* strains; *N. entophytum*, *N. linckia*, *N. spongiaeforme*, *N. ellipsosporum*, *N. humifusum* and *N. paludosum*. This clade further divides and form 2 sister clades of which the first clade with 4 species; where the *N. entophytum* and *N. linckia* form a tight cluster with Bootstrap value of 55, this cluster further branches to two sister clades first one of *N. spongiaeforme* and the next one formed of *N. ellipsosporum*. All these strains have morphological similarity with respect to the colony characters like colour of the trichome and it is reflected in the phylogenetic tree also. The second clade is formed of *N. humifusum* and *N. paludosum* that shows high affinity with bootstrap value of 87.

The second bigger cluster is formed of all the 3 *N. muscorum* strains and a *Nostoc* sp. (MBG-AL214). This clade is formed of two smaller clades one with two *N. muscorum* strains MBG-AL111 and MBG-AL215 with a high bootstrap value (96) and the next clade formed of *N. muscorum* (MBG-AL217) and

Nostoc sp. (MBG-AL214), but here the two strains hardly have any morphological similarity.

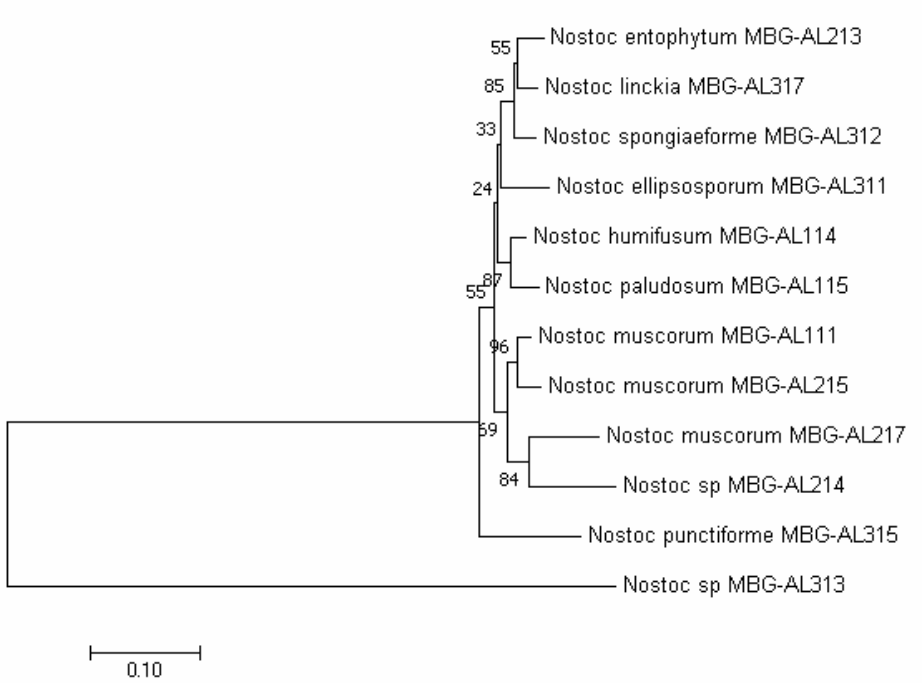


Fig. 11. Neighbour Joining tree based on 16S rDNA sequences (1500 bp) showing clustering of studied *Nostoc* strains. Number near nodes indicates bootstrap values.

The other 2 clades are formed of single strain each, one with *N. punctiforme* and other with *Nostoc* sp. (MBG-AL313) where the *Nostoc* sp. is found to be an out group.

The ML tree showed a different pattern of clade formation (Fig. 12.). In this tree also, there is 4 clades similar to NJ tree. On the upper side of the tree is the larger clade with 7 of the studied strains consisting of all the 3 *N. muscorum* strains, *Nostoc* sp. (MBG-AL214) which was found in the basal part of the NJ tree. Two *N. muscorum* strains; MBG-AL111 and MBG-AL215 do not form a single clade but instead is formed as two sister clades. The *N. humifusum* and *N. paludosum* retains their position and affinity as in the NJ tree but with different BS value. The *N. ellipsosporum* form a sister clade to the larger clade with BS of 49. In the NJ tree the *N. ellipsosporum* clade branch from the nodal portion of *N. entophytum* and *N. linckia* clade and *N. spongiaeforme* clade with

a BS value of 33, but here the branching is from the node of *N. muscorum*, *Nostoc* sp. clade and the *N. humifusum*, *N. paludosum* clade.

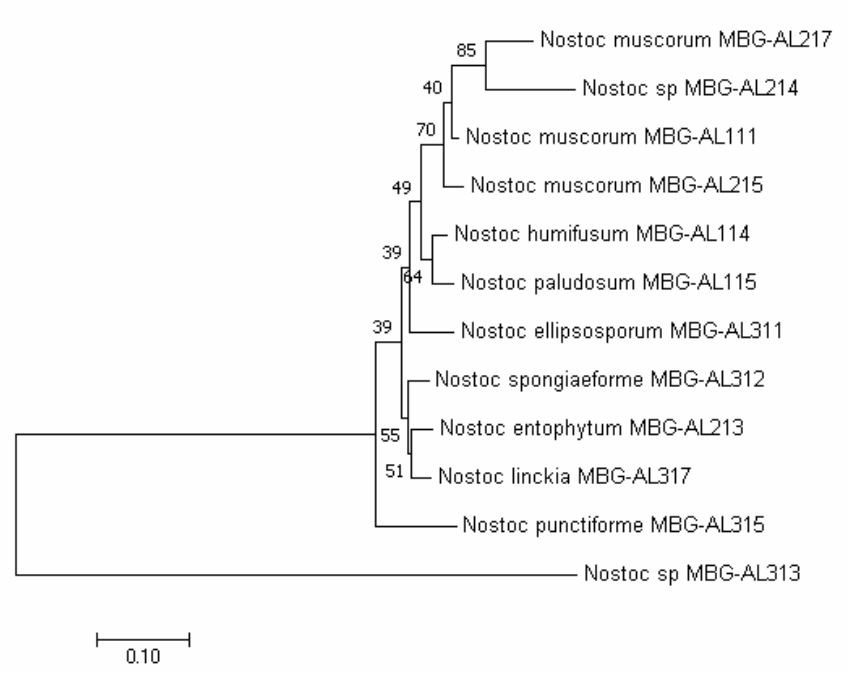


Fig. 12. Most Likely topology resulting from the Maximum Likelihood analysis using 16S rDNA sequences of the studied *Nostoc* strains. Numbers near the nodes indicate Bootstrap values.

When compared with available sequences in the GenBank; the NJ and ML trees derived (Fig. 13. and Fig. 14.). It was noted that majority of the native strains shows affinities with their corresponding strains retrieved from the GenBank except for some cases like; *N. spongiaeforme* that shows affinity and forms sister clade with the *N. linckia* clade, but has no affinity with any of the *N. spongiaeforme* strain retrieved from the GenBank.

Likewise, the *N. punctiforme* does not show any affinity to neither the sequences retrieved from the GenBank nor to any of the native strains, it exists as an independent clade.

The *Nostoc* sp. (MBG-AL313) forms a clade with *N. lickia* strain NIES-25 retrieved from the GenBank with a BS value of 100 but they didnot show any

affinity with the other *N. linckia* strains and also the morphology of MBG-AL313 has hardly have any morphological features of *N. linckia*.

Even though, the *N. ellipsosporum* MBG strain is found within the clade of *N. ellipsosporum* strain retrieved from the GenBank, but they do not form any tight cluster instead is found as a sister clade. All the strains show similar affinities in both NJ and ML trees derived with different bootstrap values.

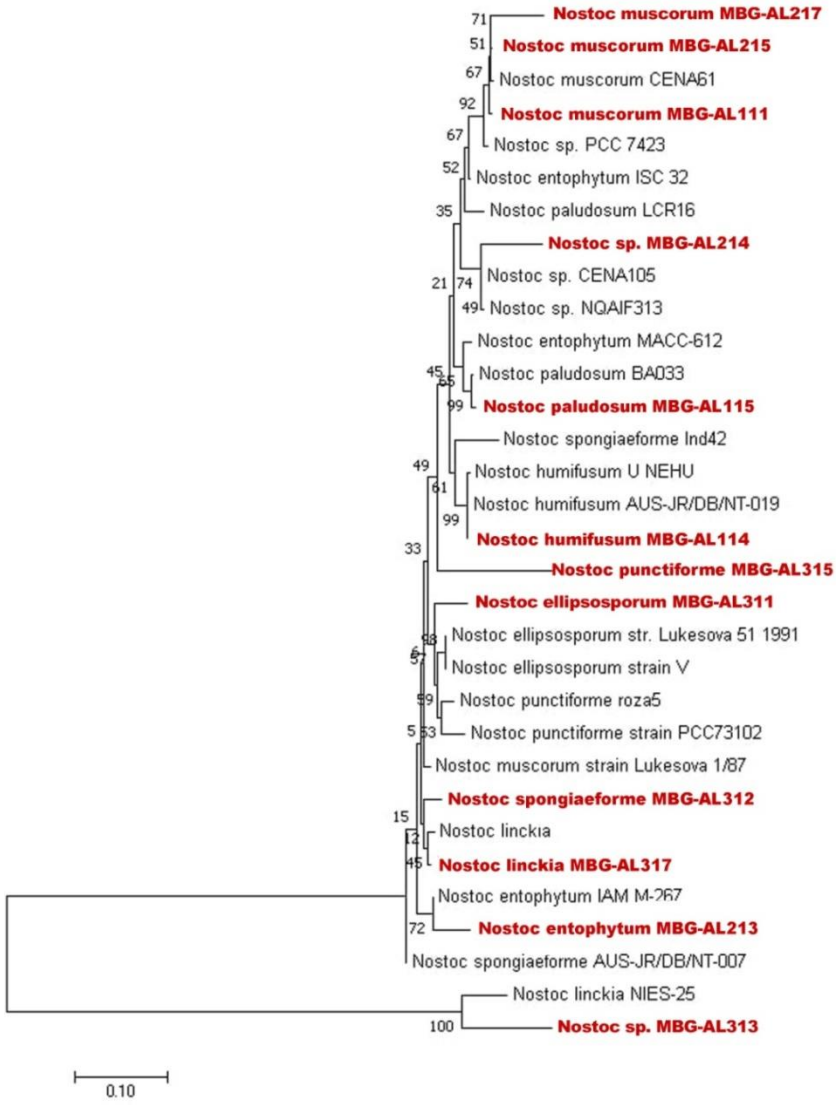


Fig. 13. Neighbour Joining tree of the native *Nostoc* strains 16S rDNA sequences with other sequences available in GenBank. Bootstrap values are indicated in the point at nodes. The names given in colour are the Kerala species studied.

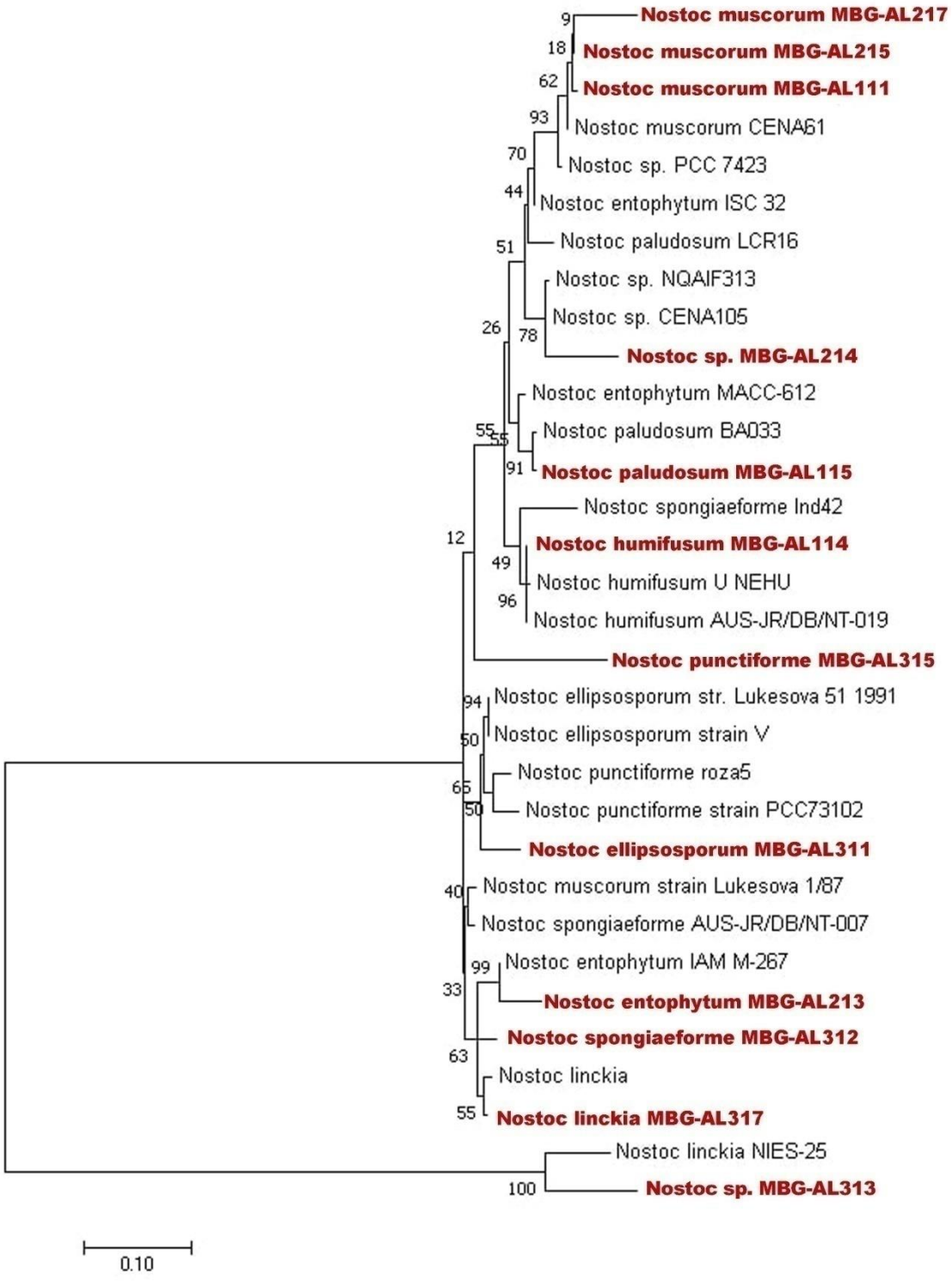


Fig. 14. Most likely topology of the native strains with other sequences available in GenBank resulting from the Maximum Likelihood analysis using 16S rDNA sequences. The names given in colour are the Kerala species studied.

4.7.2 Phylogeny of *Anabaena* spp.

The 16S rDNA sequence for 5 *Anabaena* spp. (*A. aphanizomenoides* MBG-AL113, *A. aphanizomenoides* MBG-AL314, *A. sphaerica* MBG-AL116, *A. torulosa* MBG-AL212 and *A. orientalis* MBG-AL316) were determined. Phylogenetic tree was inferred by Neighbour Joining method and Maximum Likelihood method. The clusters were well supported by Bootstrap analysis and reflect the morphological similarity of the organism to a greater extend.

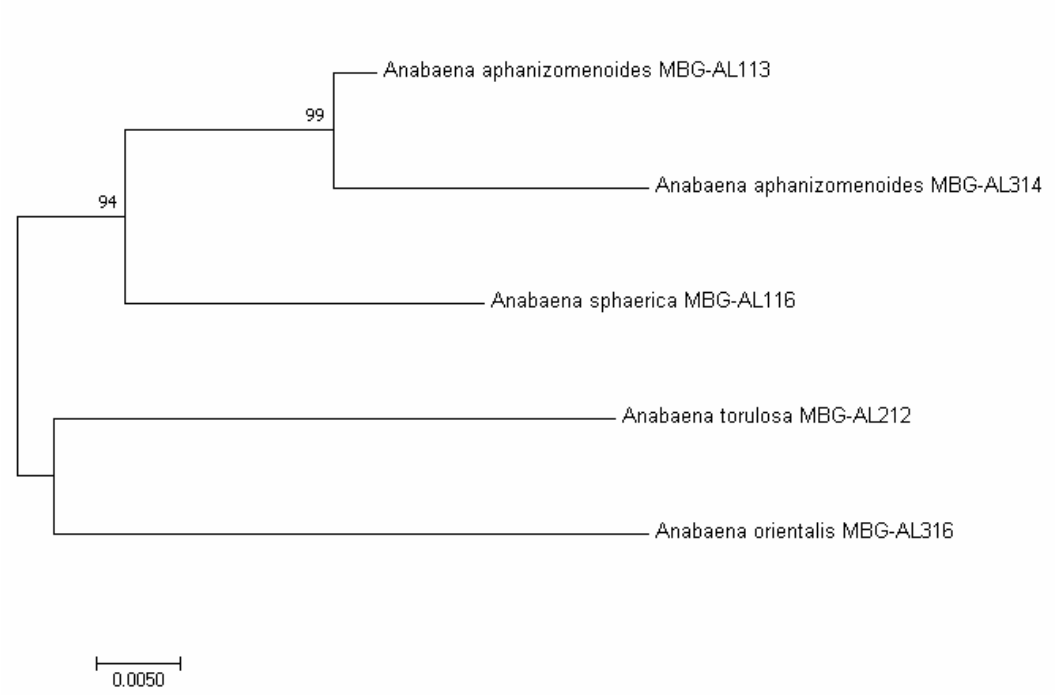


Fig. 15. Neighbour Joining phylogenetic tree of based on 16S rDNA sequences showing clustering of studied *Anabaena* strains. Numbers near nodes indicate bootstrap values.

The *Anabaena* spp. studied here is divided into two clades (Fig. 15.); the first one comprises three of the five strains and the second clade with the remaining two strains. Both the *A. aphanizomenoides* (= *Sphaerospermopsis aphanizomenoides*) strains show BS value 99 similarity genetically also and form a single clade. *A. sphaerica* that has morphological similarity in terms of the position of akinetes with *A. torulosa* shows more affinity to *A. aphanizomenoides* and is seen in the first clade as a sister clade to the *A. aphanizomenoides* clade. In the second clade *A. torulosa* and *A. orientalis* both

the strains forming akinete on both sides of the heterocyst are grouped; both the strains have only little morphological differences which are reflected in the tree also. Both NJ and ML (Fig. 16.) tree show the same clustering pattern with same BS value.

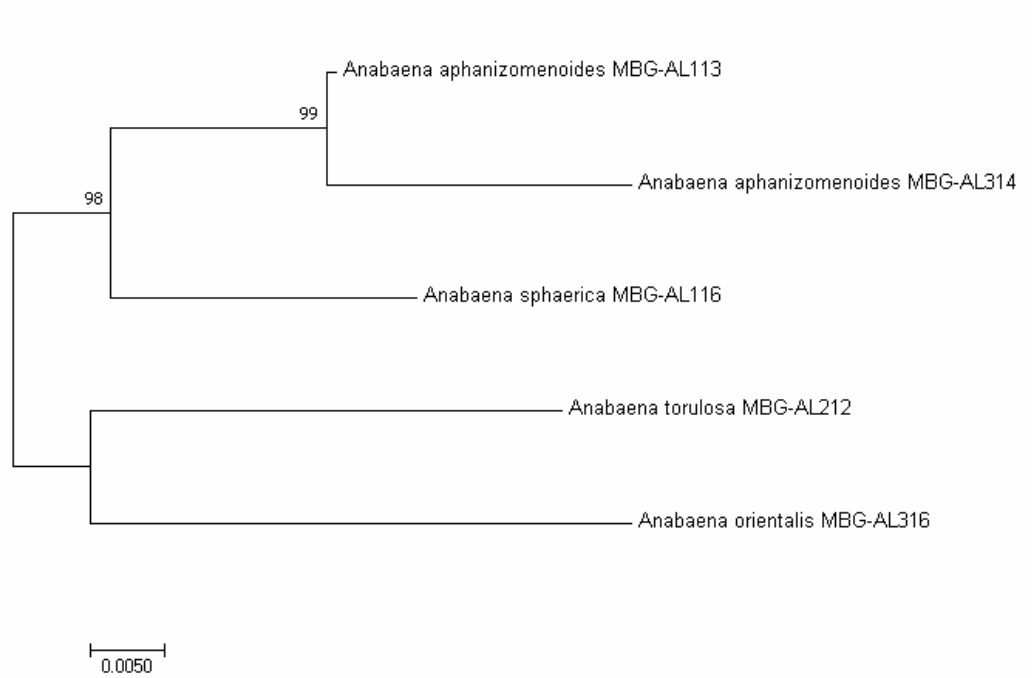


Fig. 16. Phylogenetic tree resulted from the Maximum Likelihood analysis of 16S rDNA gene of native *Anabaena* spp.

In the NJ tree of the 16S rDNA sequence analysis of the native strains with the strains retrieved from the GenBank (Fig. 17.), the *A. aphanizomenoides* strains MBG-AL113 and MBG-AL314 form a cluster (99) and occupy the top position of the tree but they do not show affinity with any of the sequences retrieved from the GenBank; this clade shows affinity to the *A. orientalis* MBG-AL316 strain (BS 55) which forms an independent clade with *A. orientalis* strain AUS-JR/MT/NT-099. This is in contrast of the NJ tree of the native strains where *A. aphanizomenoides* shows affinity with *A. torulosa*. In this tree *A. torulosa* clade shows affinity with the *A. aphanizomenoides* and *A. orientalis* clade (BS 43). Except for *A. aphanizomenoides* strain, all other native strains show affinity with their corresponding strains retrieved from the GenBank.

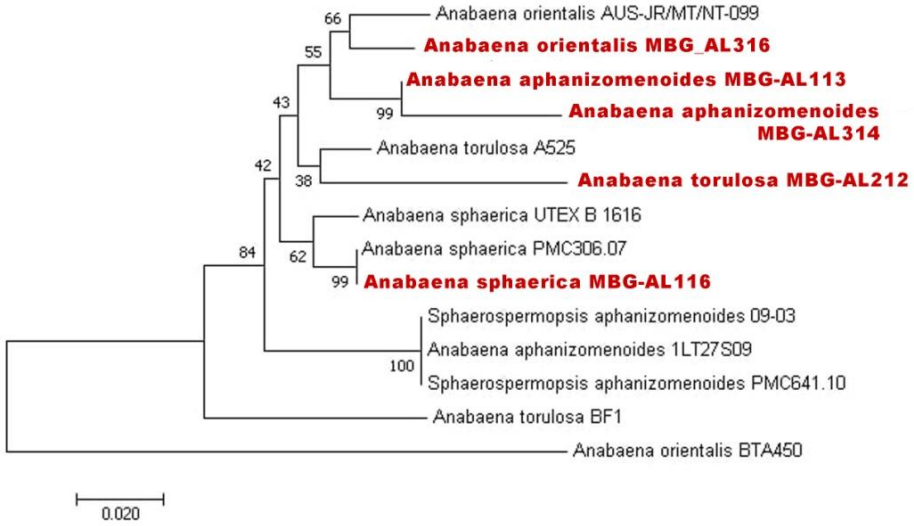


Fig. 17. Neighbour Joining phylogenetic tree of the *Anabaena* spp. 16S rDNA sequences with other sequences available in public databases. Bootstrap values are indicated in the point at nodes. The names given in colour are the Kerala species studied.

In the ML tree (Fig. 18.) the affinities showed by the strains were the same but with different Bootstrap values. The only difference noted was that the first clade comprising of *A. aphanizomenoides* and *A. orientalis* shows more affinity with *A. sphaerica* than with *A. torulosa*.

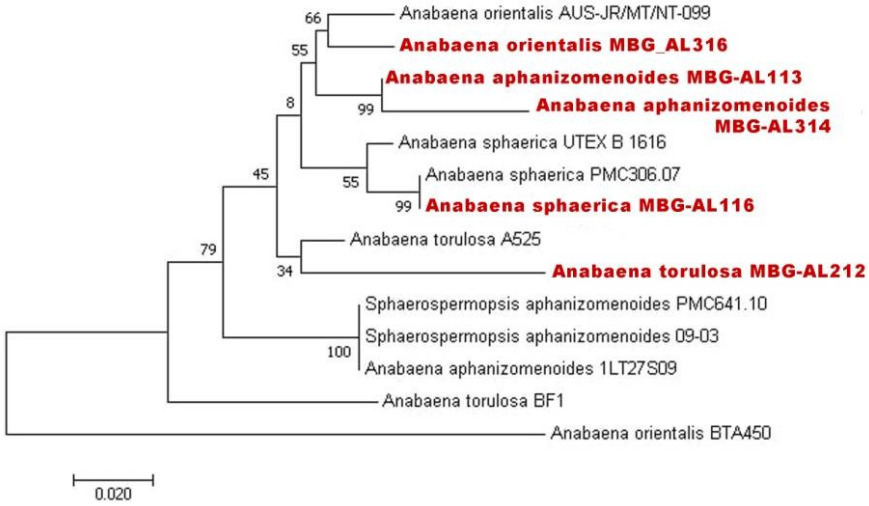


Fig. 18. Maximum Likelihood tree of 16S rDNA sequences of the studied *Anabaena* strains with sequences available in GenBank. Bootstrap values are indicated at nodes. The names given in colour are the Kerala species studied.

4.7.3 Phylogenetic Analysis of *Nostoc* spp. and *Anabaena* spp.

In order to infer the phylogenetic relationship of *Nostoc* and *Anabaena* occurring in Kerala phylogenetic tree of all the 17 strains collected were created by both NJ and ML methods. The phylogenetic tree was well supported by Bootstrap value.

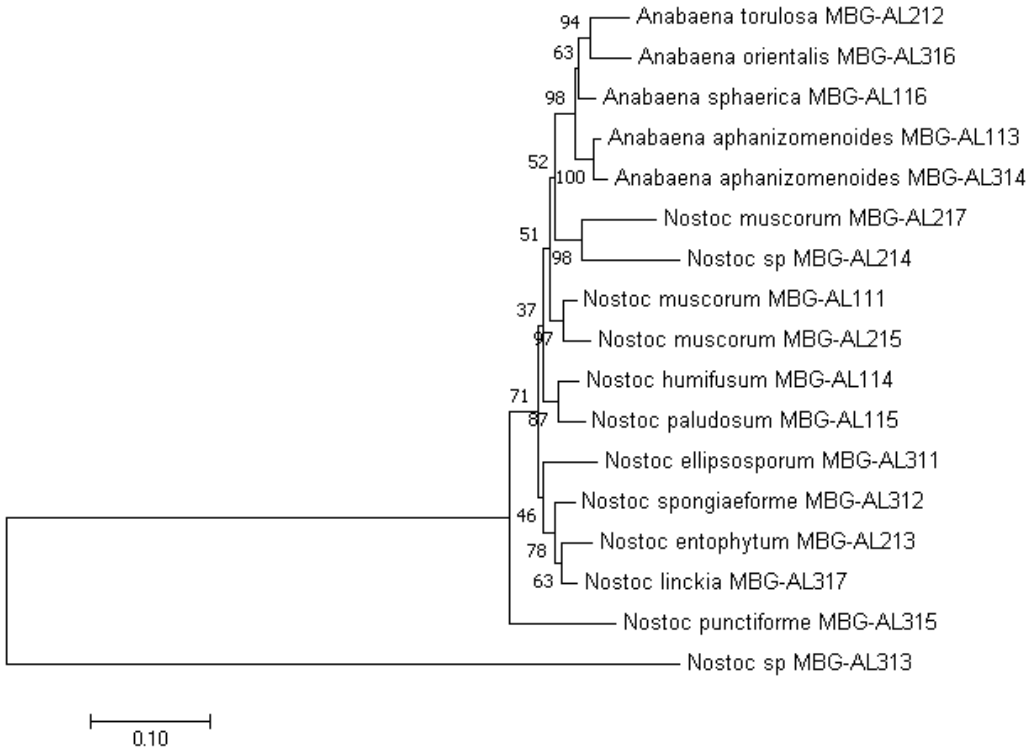


Fig. 19. The optimal tree obtained by the Neighbour Joining analysis using 16S rDNA sequences of 17 strains with a sum of branch length= 1.62389112 was shown. The numbers on the branches represent the support for the branches as bootstrap values.

The NJ tree derived was divided into 6 clusters (Fig. 19.). The first cluster is formed with 7 strains comprising of all the 5 *Anabaena* strains studied and two *Nostoc* strains. This cluster is further divided into two clades; the clade with all the *Anabaena* strains shows affinity with each other. The second clade is formed by *Nostoc* sp. strain MBG-AL214 and *N. muscorum* strain MBG-AL217 (BS 98).

The *Anabaena* clade is further divided into two sub clades; first sub clade with *A. torulosa* and *A. orientalis* (BS 94) and *A. sphaerica* forms a sister clade to

the sub clade. The second sub clade is formed of two strains of *A. aphanizomenoides* (BS 100).

The second cluster is formed by two *N. muscorum* strains that form a clade (97) and this clade have little affinity with the *Nostoc* sp. strain MBG-AL214 - *N. muscorum* strain MBG-AL217. The third cluster with *N. humifusum* and *N. paludosum* forms a clade (BS 87).

The fourth cluster with four *Nostoc* strains that show similar colony characteristics with respect to colour. In this cluster *N. entophytum* and *N. linckia* form tight clade (BS 63); *N. ellipsosporum* and *N. spongiaeforme* forms two sister clades that are on the upper side of the *N. entophytum-N. linckia* clade.

N. punctiforme forms an individual clade without any close affinities with any of the strains. Sixth clade formed by *Nostoc* sp. strain MBG-AL313 that is seen as an out group.

From this tree it is clear that both *Nostoc* and *Anabaena* are paraphyletic group. Even though they are considered as two different genera based on morphological criteria, the phylogenetic tree derived showed a close relatedness and intermixing of *Nostoc* and *Anabaena i.e., N. muscorum* and *Nostoc* sp. strains that is nested within the cluster formed of *Anabaena* and all the studied strains were part of a big cluster except the strain MBG-AL313.

Maximum Likelihood tree resulted in seven clusters where *N. ellipsosporum* forms a cluster without any close affinities and forms the fourth cluster. The fifth cluster is formed of *N. spongiaeforme*, *N. entophytum* and *N. linckia* where *N. entophytum* and *N. linckia* forms a clade with low bootstrap value of 57 (Fig. 20.).

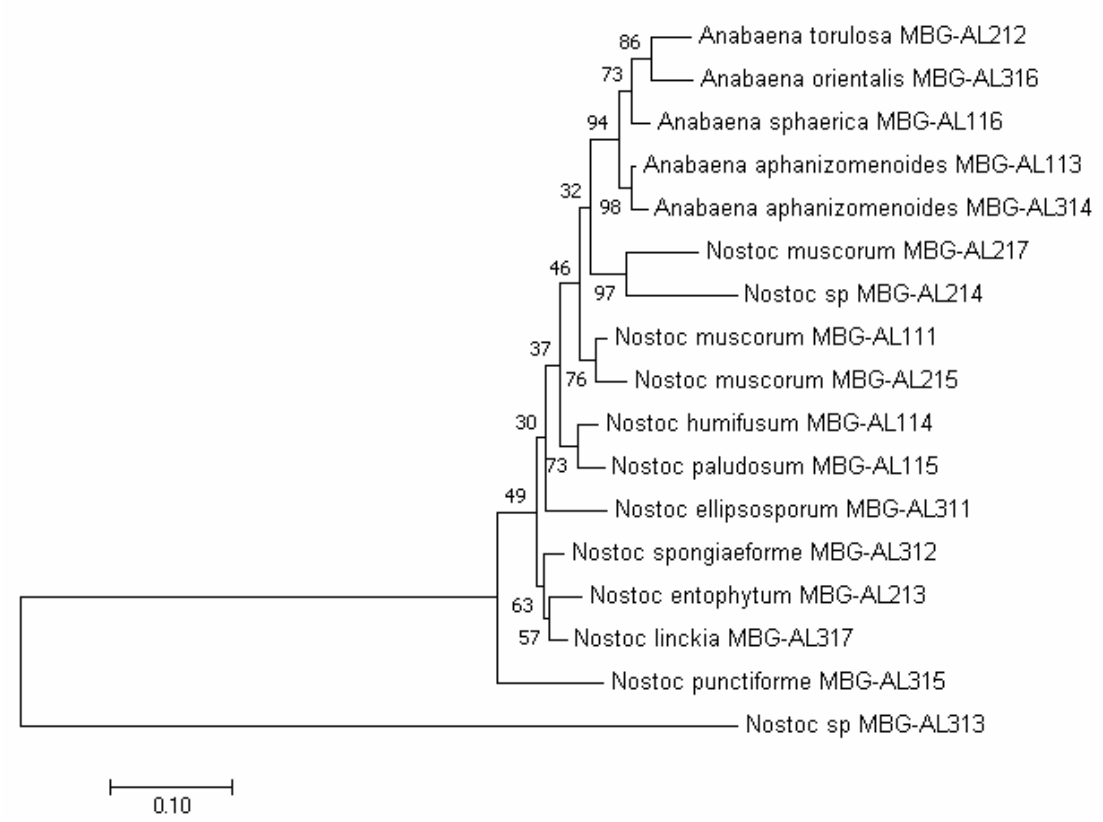


Fig. 20. Most likely topology (with highest log likelihood=-6735.78) resulting from the Maximum Likelihood analysis using 16S rDNA gene sequences of the 17 strains. Bootstrap values are indicated at the nodes.

When compared with the sequences retrieved from the GenBank majority of the native strains shows affinity with their corresponding strains in the GenBank, except for certain cases like; *N. spongiaeforme*, *A. torulosa*, *N. punctoforme* strains studied. The *Nostoc* sp. strain MBG-AL313 shows affinity with *N. linckia* strain NIES from the GenBank, with a high bootstrap value of 100.

A. torulosa strain MBG-AL212 that has affinity with *A. torulosa* strain A525 in the phylogenetic tree of individual *Anabaena* strains (Fig. 17. & Fig. 18.) is not observed in the combined tree instead they were clustered with *Sphaerospermopsis aphanizomenoides* strains retrieved from the GenBank with low bootstrap values in both NJ and ML trees.

Even though the affinities of the strains in both NJ (Fig. 21.) and ML (Fig. 22.) trees are same with difference in the bootstrap values, there are some

exceptions like, *A.torulosa* strain A525 is seen within the *A. aphanizomenoides* MBG strain cluster in ML tree where as they are seen as a sister clade with *A. aphanizomenoides* and *A. orientalis* clade in NJ tree.

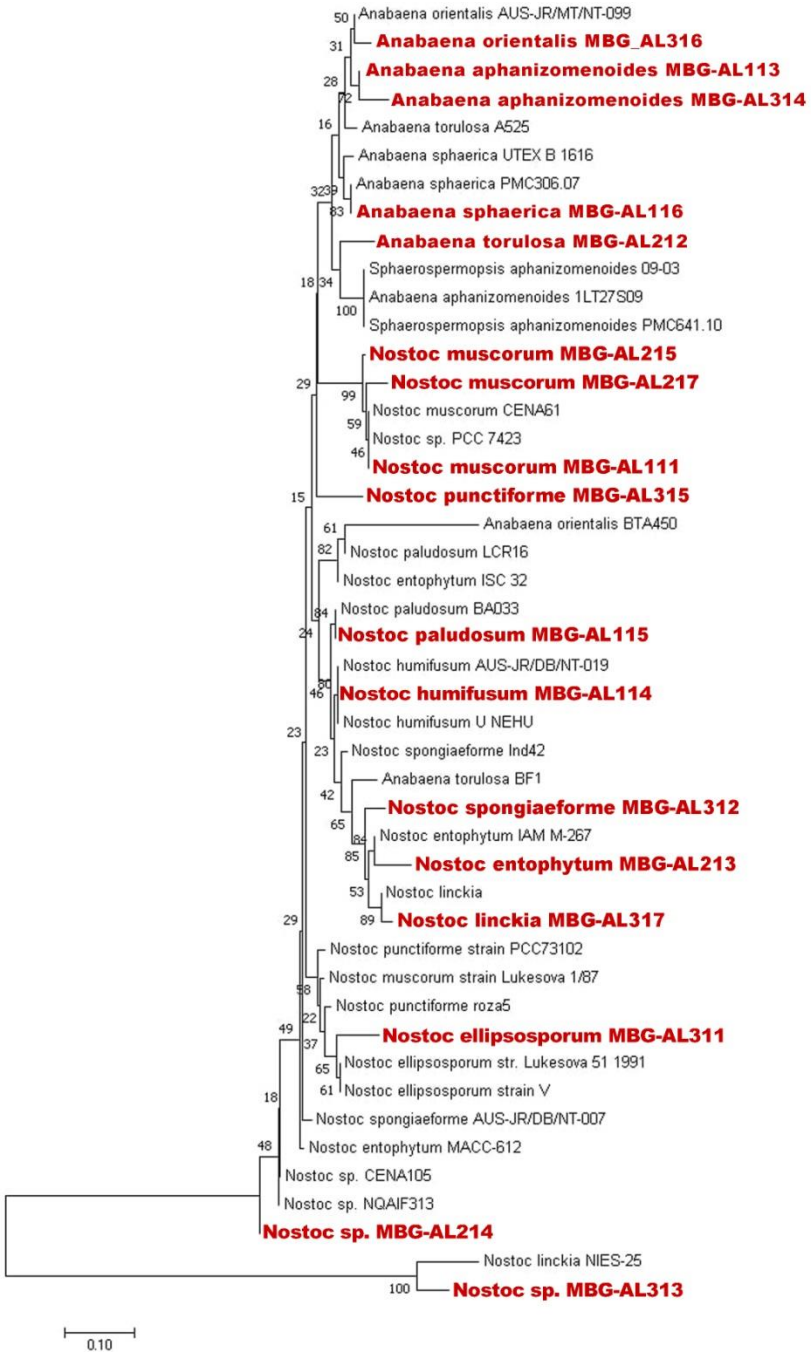


Fig. 21. Neighbour Joining tree based on 16S rDNA showing clustering of all the studied strains with other sequences available in public database. Bootstrap values are indicated in the point at nodes. Strain names are given on right side. The names given in colour are the Kerala species studied.

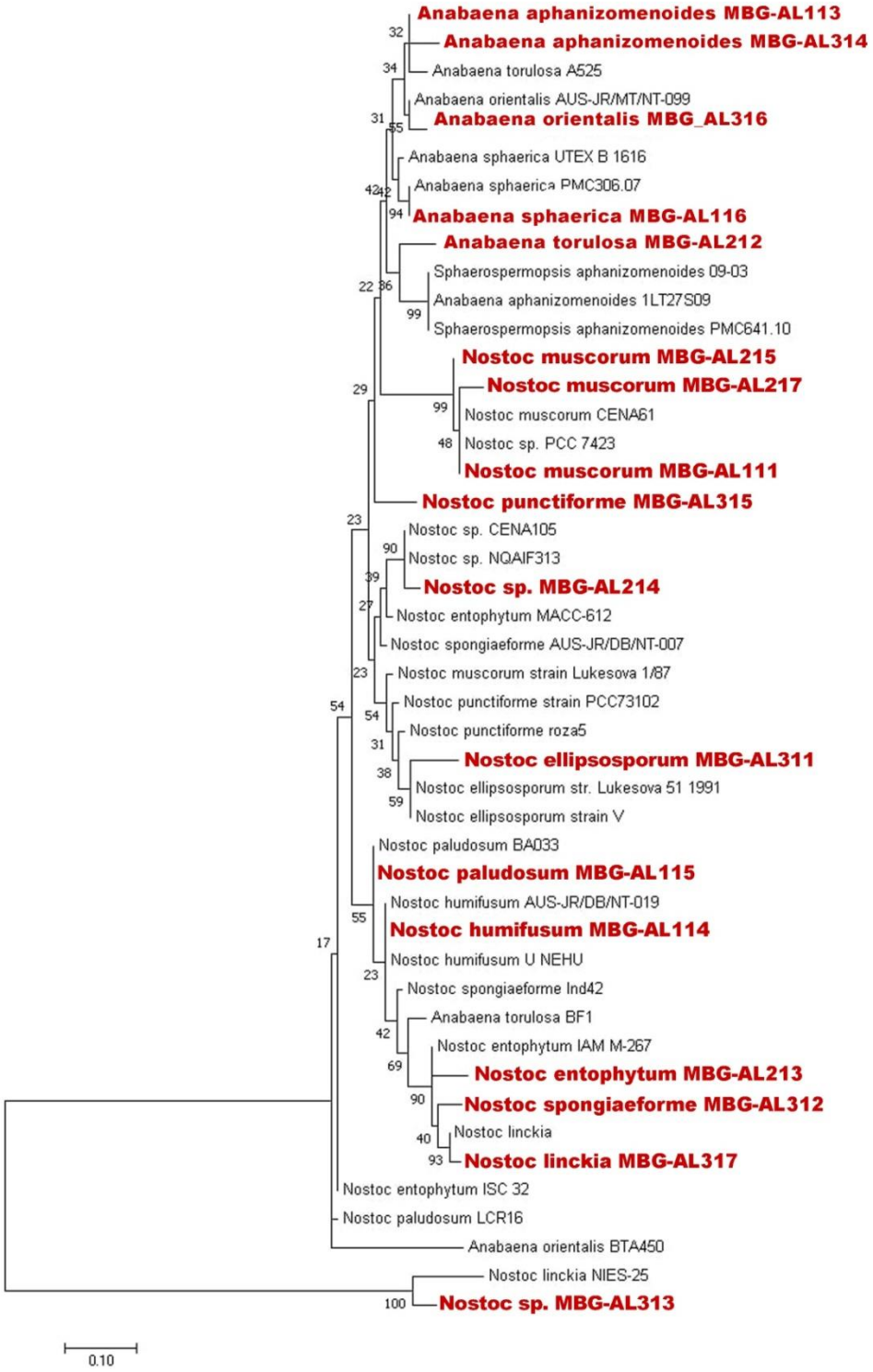


Fig. 22. Tree obtained by Maximum Likelihood analysis of 16 S rDNA sequences of all the 17 strains studied and the sequences available in public database. Strain names are given on the right side and the bootstrap values are indicated in point at nodes. The names given in colour are the Kerala species studied.

N. punctiforme MBG-AL315 is seen as a sister clade to *N. muscorum* in ML tree where as in NJ tree, this was found as an independent clade. In the ML tree the *Nostoc* sp. strain MBG-AL214 is clustered with other *Nostoc* sp. strains retrieved from the GenBank, but in ML tree they exists as an independent clade.

Even some of the retrieved strains do not show any similarity with each other, such as, *A. orientalis* strain BTA450 shows affinity with *N. paludosum* strain LCR16 and *N. entophytum* strain ISC 32. *A. torulosa* strain BF1 is seen towards the basal part of the tree clustered among *Nostoc* strains. *N. muscorum* strain Lukesova 1/87 and *N. entophytum* strain MACC-612 was seen away from their corresponding strains in the trees derived by NJ and ML method.

DISCUSSION

5. DISCUSSION

Molecular Systematics of two genera of the heterocystous blue-green algae *Nostoc* and *Anabaena* (Nostocales) collected from different districts of Kerala were done using partial 16S rDNA sequence analysis. The morphological identification of the materials collected was done using conventional methods such as observing microscopic characters, referring literature, consulting experts, etc.

From the survey and collection of materials the ubiquitous distribution of BGA in all the sites was evident. This result was in accordance with those of Nayak *et al*. (2001) and Rao *et al*. (2008). Tiwari *et al*. (2005) reported cyanobacteria even in the arid regions of Rajasthan. Ubiquitous distribution of BGA in the tropical and sub tropical regions was reported by Vaishampayan *et al*. (2001). The cultivated soil especially the paddy field appears to support the growth of heterocystous BGA since majority of the collected specimens were from the paddy fields.

The water logged conditions of the paddy fields provide a favourable environment for the growth of BGA which in turn provide nitrogen to the plants thus helping in improving the yield and make soil fertile. Algalisation, the process of BGA inoculation helps to provide an environmentally safe agro-ecosystem contributing to economic viability in paddy cultivation reducing cost and energy inputs (Pabby, 2008). The predominance of heterocystous forms in the paddy fields was reported earlier also by Nayak *et al*. (2001; 2004). A contrasting result was obtained by Barman *et al*. (2015) who recorded more non-heterocystous filamentous cyanobacteria from Indian Sundarbans, a mangrove forest. Among the heterocystous BGA, the genus *Nostoc* and *Anabaena* were the common genera, especially in paddy fields. Nikam *et al.* (2013) also reported the abundance of *Nostoc* and *Anabaena* in Maharashtra, while Adhikari and Baruah (2014) reported abundance of *Nostoc* and *Anabaena* in the forest soils of Assam.

In the present study 17 isolates of BGA belonging to the genera *Nostoc* and *Anabaena* were collected and isolated as pure culture from different districts of Kerala, of which 12 of them were *Nostoc* spp. indicating predominance of *Nostoc*. Studies of Venkataraman (1972) also supported the dominance of *Nostoc* in the rice fields of Kerala, Tamil Nadu, West Bengal, Assam and Hariyana. Similar results were reported from different ecological conditions like Lakes of Rajasthan (Borase and Singh, 2013) and Meghalaya (Syiem *et al.*, 2010) whereas; Singh *et al*. (2014) recorded more *Anabaena* spp. from the paddy fields of Chhattisgarh.

In the present study majority of the identifications of the isolates were done from the cultured samples since majority of environmental samples collected are a mixture thus their culturing was essential to get unialgal strains. The identification of the blue-green algae mainly depend on the quantitative characters like the dimensions of vegetative cells and heterocysts, these characters may change in cultures and also akinete induction was not always successful in culture which make it difficult for the identification (Table 10.) as noted by Wright *et al.* (2001).

Table 10. Summary of isolates which showed difficulties in identification.

SI. No.	Isolate	Main ID problem	Identification
1	MBGH 8894 (MBG-AL111)	No akinete formation in cultures	*N. muscorum*
2	MBGH 8885 (MBG-AL215)	No akinete formation in cultures	*N. muscorum*
3	MBGH 14850 (MBG-AL217)	No akinete formation in cultures	*N. muscorum*
4	MBGH 14848 (MBG-AL313)	Cell dimensions differ	*Nostoc* sp.
5	MBGH 8882 (MBG-AL311)	Akinetes rare in cultures	*N. ellipsosporum*
6	MBGH 14849 (MBG-AL214)	Free akinetes	*Nostoc* sp.
7	MBGH 14841 (MBG-AL115)	No akinetes in cultures	*N. paludosum*
8	MBGH 14807 (MBG-AL316)	Akinete formation rare	*A. orientalis*

Once the collection and preliminary identification were made, the next task was to isolate and obtain axenic cultures of the collected *Nostoc* spp. and *Anabaena* spp. for their molecular studies. The isolation of blue-green algae are difficult than other green algae, due to this there are only few studies on this subject.

The ideal culture medium and the optimum culture conditions for the growth of collected specimens were identified in this study. Of the different media combinations tried the BG-11 medium (Rippka *et al.,* 1979) without nitrogen source was found ideal. This medium is the most popular and standard medium used widely. This medium is found very useful nowadays also and among the widely used as reported by many like Lakshmi and Annamalai (2008), Raipuria *et al*. (2016), Guimaraes *et al*. (2017) especially for the heterocystous Blue-green algae. Whereas, Nagle *et al*. (2010) opted for ASN III medium for their studies with non- heterocystous cyanobacteria but in majority of cases ASN III medium is used for the culturing of marine cyanobacteria (Bano and Siddiqui, 2004). Thakare *et al*. (2018) from their studies on the effect of different culture media in the growth of BGA found that Allen & Arnon medium best supported the growth of BGA than BG-11.

According to Bajwa *et al*. (2017) BG-11 medium is best suited for blue-green algal strains for biomass yield, since in the present study biomass yield for the isolation of DNA is required. Thus the choice of the BG-11 medium is found to be most appropriate.

As mentioned earlier, majority of the collected specimens were from the paddy fields that has alkaline pH. The occurrence of cyanobacteria in the alkaline pH was reported earlier itself (Bano and Siddiqui, 2004). The pH of the medium was adjusted to neutral or slightly alkaline (7.2-7.5) which supported the ample growth.

The findings by Parikh and Madanwar (2006) that the carbohydrates synthesised by BGA is used as a carbon source by the contaminants was found

to be true since prolonged culture in the medium resulted in contamination by other non-heterocystous BGA or green algae. In this study axenic cultures are attained by continuous sub culturing while Sachizian and Adrelean (2000) attained axenic cultures of heterocystous cyanobacteria by the addition of antibiotics.

DNA isolation adopted in this study is a modification of the DNA isolation method proposed by Singh *et al*. (2012). In the present study the major difficulty was isolating DNA from Blue-green algae *i.e.,* the effective breakdown of the complex cell wall made up of peptidoglycan and the poor quality of the isolated DNA.

The cell lysis is achieved by various methods in different studies. According to Staskawicz *et al*. (1995) pure enzymatic method resulted in poor quality of extracted DNA but Magana-Arachchi and Wanigatunge (2011) achieved RNA free DNA by adopting pure enzymatic method only. Majority agreed to the opinion that mechanical lysis along with enzymatic disruption aided in the isolation of high quality DNA, Morin *et al*. (2009) and Motham *et al*. (2014), Kumar *et al.* (2018) also has such an opinion and they used enzymes like Proteinase K and Lysozyme for the isolation whereas in this study mechanical lysis along with the use of chemicals were found very effective.

The method proposed in this study, where the cell lysis is achieved with sonication along with the use of chemicals that helped in the breakage of the cell wall to expose the cytoplasm and other nucleic acid materials. Such an observation is made by Magana-Arachchi and Wanigatunge (2011) where chemical treatment along with physical methods were used whereas Rasmussen *et al.* (2007) found that microwave irradiation in presence of detergent was effective than sonication for cell disruption.

There are several methods proposed for the isolation of DNA from cyanobacteria using an array of chemicals that aids in the lysis of cell wall that

includes CTAB, SDS, Xanthogenate-SDS, β- mercaptoethanol, etc (Tillet and Neilan, 2000; Ma *et al*., 2016). In this study also chemicals like SDS, EDTA and β- mercaptoethanol play a significant role in cell disruption where as Chakraborthy *et al.* (2008) used Proteinase K to break the membrane protein. They also found that SDS, EDTA and Proteinase K prevent the activity of DNA degrading nucleases.

In the method adopted by Singh *et al*. (2012) cell lysis was achieved by different steps like crushing with silica gel, homogenising with glass beads and sonication. This long mechanical lysis may cause shearing of high molecular weight DNA. So, in this study the mechanical lysis is restricted to only sonication, which helped in efficient cell lysis and recovery of nucleic acid materials. Cell breakage was attained by the use of detergent coupled with agitation in the presence of glass beads and chloroform (Fawley and Fawley, 2004).

Apart from the cell wall lysis the β- mercaptoethanol used are also responsible for the denaturation of endogenous RNase, thus causing RNA contamination in the isolated DNA (Omega Bio-Tek, 2004). This effect of β- mercaptoethanol could successfully overcome with the use of RNase and phenol treatment. The phenol further eliminated the chances for protein contamination.

High salt concentration in presence of isopropanol was used for the precipitation of DNA by Wu *et al*. (2000) while ethanol treatment with freezing at -20^0 C was found enough in this case. The protocol developed in this study yielded good quality genomic DNA.

One of the major difficulties in the application of these genera in the practical research and the industrial use is their obscure identity. As mentioned earlier one of the main hindrance in giving the name is that they show morphological plasticity. Taxonomy relying mainly on the morphological characters only cannot be trusted fully. A solution for this is the use of a polyphasic approach

which include the information of morphology, physiology and molecular approach. Even though the classical taxonomy treats these as separate genera; there is conflict among different workers, some are with the opinion that *Nostoc* and *Anabaena* could not be considered as separate genera.

The distinction of the genera *Nostoc* and *Anabaena* has always been a topic of debate. These were traditionally separated on the basis of morphological characteristics (Tamas *et al*., 2000), which seems to be insufficient. In the present study an attempt is made to resolve the confusion by phylogenetic method using the native species collected from different districts of Kerala and partial 16S rDNA sequences analysis. For the phylogenetic analysis of the sequences generated in this study Neighbour Joining and Maximum Likelihood methods were adopted and phylogenetic trees are generated. The native strains sequences generated in this study were compared with other strains available in the public database (GenBank) and the phylogenetic trees were reconstructed.

The analysis yielded good clustering that partially reflected the relationship between the taxa. In the phylogenetic tree derived from the native strains it is noted that the genera *Nostoc* and *Anabaena* belonging to family Nostocales form a monophyletic group of heterocystous cyanabacteria as presented by other molecular studies (Rajaniemi *et al.*, 2005; Valerio *et al.*, 2009; Singh *et al*., 2013; 2015). Komarek *et al*. (2014) found that the order Nostocales are a large and monophyletic cluster of filamentous cyanobacteria with specialised cells like heterocysts and akinetes similar observation was made by Tomitani *et al.* (2006) also. Akinete forming heterocystous cyanobacteria formed a distinct clade (Howard-Azzeh *et al*., 2014).

Intermixing of *Nostoc* and *Anabaena* was found in the phylogenetic tree which points to the paraphyletic nature of these two genera, this result was in accordance with Singh *et al*. (2015) who found that there is intermixing found in heterocystous cyanobacteria. Mishra *et al.* (2008) also observed intermixing

of *Nostoc* and *Anabaena* in their study using morphology, SDS-PAGE and 16S rRNA gene where as in this study the two genera were intermixed with overlapping characters when the morphological features were considered.

In the phylogenetic tree derived from the studied native strains both *Nostoc* and *Anabaena* are found in a large cluster, with exception of strain MBG-AL313 which was found as an out group (Fig. 19. & 20.), where all the *Nostoc* strains occupy the basal portion of the tree. This indicated the primitiveness of *Nostoc* with respect to *Anabaena.* Such an observation was made by Svenning *et al*. (2005) also.

In this study *Anabaena* represented by 5 strains belonging to four species (*A. aphanizomenoides, A. torulosa, A. sphaerica and A. orientalis*) which were clustered together along with two *Nostoc* strains as sister clade and forms the first cluster in the phylogenetic tree (Fig. 19. & 20.). Earlier studies have demonstrated that *Nostoc* and *Anabaena* are closely related in 16S rDNA studies but these could not differentiate the two genera (Turner, 1997; Lyra *et al.*, 2001). The present study also indicated the close relatedness of the two genera whereas Galhano *et al.* (2011) clearly characterise *Anabaena, Aphanizomenon* and *Nostoc* based on morphological and 16S rRNA sequence data. The relatedness of these genera was also obtained by partial *nifH* sequences by Turner (1997) and Tamas *et al*. (2000).

Based on their observations Tamas *et al*. (2000) proposed that the two genera should be merged into a single genus whereas the previous studies by Wilmotte and Herdman, 2001 and Rajaniemi *et al*. (2005) based on 16S rRNA gene sequences and *nifD* sequences by Henson *et al*. (2002) well separated the two genera *Nostoc* and *Anabaena*. Teneva *et al*. (2012) is also having the opinion that *Nostoc* and *Anabaena* are distinct genera based on their analysis with IGS region between *cpcB a*nd *cpcA.* According to Minj *et al*. (2017) separation or merger of genus *Nostoc* and *Anabaena* still remained indecisive. The present

study based on the 16S rDNA gene indicated the close relatedness of these two genera suggesting merging of the two but, more studies based on other markers will be needed for the confirmation. Here, in the phylogenetic tree some *Nostoc* strains are found within the *Anabaena*, such a result was obtained by Svenning *et al*. (2005) in their phylogenetic analysis using 16S rDNA.

Apart from the genera *Nostoc* and *Anabaena*, studies by Lehtimaki *et al*. (2000) revealed the close relatedness of *Nodularia* with *Nostoc*, *Anabaena* and *Aphanizomenon* in 16S rRNA gene analysis. The genus *Nostoc* was found to be heterogenic and thus their species level identification was difficult. The heterogenecity of the genus is observed by many workers like Minj *et al*. (2017) and Shariatmadari *et al*. (2017).

Svenning *et al*. (2005) observed *Nostoc* strains both free living and symbiotic formed of large clade whereas in this study *Anabaena* and *Nostoc* strains were found within a large cluster with intermixing.

The two *Nostoc* strains MBG-AL313 and MBG-AL214 whose species identity is not clear, in the phylogenetic tree constructed the MBG-AL214 strain appeared sister to *N. muscorum* MBG-AL217 with a high bootstrap value in both NJ and ML tree, but their morphology does not have any congruence. Similarly the *Nostoc* strain MBG-AL313 form sister clade with *N. linckia* NIES strain retrieved from GenBank. Such confusions were found by many others such as, Rajaniemi *et al*. (2005) who included *Nostoc* strain PCC73102 in a well supported *Nostoc* clade but Lyra *et al*. (2001) obtained a different result in which the strain is clustered with *Anabaena*. Close relatedness of *Nostoc linckia* and *Nostoc punctiforme* were evident from the studies by Teneva *et al*. (2012) these were linked as a sister group to *Planktothrix* and *Lyngbya.*

Based on 16S rRNA sequence analysis Hrouzek *et al*. (2013) described a new genus *Desmonostoc* and placed *N. muscorum* in this genus. In the present study, of the three *N. muscorum* strains described; strain MBG-AL217 form a

clade with *Nostoc* sp. strain MBG-AL214 which is nested in the *Anabaena* cluster. The other two strains form a distinct clade without affinity with any other *Nostoc* strains described. Thus, the observation by Hrouzek *et al*. (2013) is proved correct.

According to Rajaniemi *et al*. (2005) certain *Nostoc* species such as *N. calcicola, N. edaphicum* with high sequence and morphological similarity could be assigned to single species. Likewise, *N. muscorum* and *N. ellipsosporum* were closely related to each in their study but this was not the case in this study where both the species were found in different cluster and also *N. muscorum* strains in this study was found to be related to *Anabaena* strains.

The *N. spongiaeforme* strain described in this study when compared with other strains retrieved from GenBank did not show any relatedness leading to a confusion regarding its status that has to be solved. Even though, morphological data confirms its identity, further studies using different marker will help their clarification.

The genus *Anabaena* which is morphologically differentiated by the position of akinetes, but according to Ezhilarasi and Anand, 2009, the morphological differences within the genus are not reflected at the gene level. Similar observation is made in this study also where *A. sphaerica* which is more similar to *A. torulosa* with respect to the position of akinete, is found within the clade of *A. aphanizomenon*, but in the studies of Ezhilarasi and Anand, 2009 *A. torulosa* and *A. sphaerica* were found in a distinct clade. According to Lyra *et al*. (2001) *Anabaena* producing hepatoxins were phylogenetically different from neurotoxin producing *Anabaena* strains.

In the study two strains of *A. aphanizomenoides* collected from two different populations have different morphology but have high genetic relatedness and forms a single cluster. Such a result was obtained by Shariatmadari *et al*.

(2014) where populations of each species of *Anabaena* were placed close to each other in 16S rRNA gene tree.

The genus *Anabaena* is heterogenous in morphological and ecological point of views (Komarek and Anagnostidis, 1989) their views were proved true in the present study. Certain taxa in genus is separated to other genus based on different morphological characters, among which separation of *Anabaena variabilis* to genus *Trichomus* (Rajaniemi *et al*., 2005), some Wollea species (Kozhevnikov and Kozhevnikova, 2011). Zapomelova *et al*. (2009) proposed a new genus *Sphaerospermopsis* and transferred *A. kisseleviana* and *A. aphanizomenoides* to this genus. Thus *A. aphanizomenoides* is now considered as *Sphaerospermopsis aphanizomenoides*, but when compared with sequences retrieved from GenBank, the native strains studied even though found within the cluster they are not closely related and the *A. torulosa* MBG-AL212 was found as a sister clade to the *Sphaerospermopsis* clade (Fig. 21. & 22.). All these results were in accordance with Minj *et al*. (2017) who proposed that *Anabaena* showed conflicting affiliations in both intra and inter generic perspectives.

According to Shariatmadari *et al*. (2017), the genus *Anabaena* is paraphyletic with *Wollea* and *Trichormus* nesting in them, and *A. oscillarioides* and *A. iyengarii* was found close to *Cylindrospermum.* Paraphyletic nature of the Genus *Anabaena*, *Aphanizomenoides* and *Nostoc* were reported by Costa *et al*. (2001), Fergusson and Saint (2000) and Litvaitis (2002). Here, since only two genera *Nostoc* and *Anabaena* is considered for the study, the paraphyly with other related genera is not clear. Even though *Anabaena variabilis* is separated to genus *Trichomus*, they were found to be similar in both morphological and phylogenetic analysis by Shariatmadari *et al*. (2017).

The inference of the present 16S rDNA sequence analysis indicated the possibilities of merging these two genera. Minj *et al*. (2017) stated that 16S rRNA can be a good identification marker for heterocystous cyanobacteria, but

its use for assessing phylogenetic relationship is limited possibly because of its low resolution in delineating closely related species. Seo and Yokota (2003) found that phylogenies of *gyrB, rpoC1* and *rpoD1* gene support 16S rRNA based classification of cyanobacteria.

According to Shariatmadari *et al*. (2017) the 16S rRNA gene sequence was effective marker in high taxonomic levels such as order and family but in lower taxonomic levels such as genera they are ineffective, this was true in case of genera *Nostoc* and *Anabaena*, but 16S rDNA gene was found to be not effective in discriminating species. This inefficiency of this marker may be due to similarity of genes encoding small sub unit of ribosome. While Kumar *et al.* (2018) and Otzurk *et al.* (2018) is in an opinion that 16S rRNA gene will help in classification and identification of cyanobacterial strains.

The phylogenetic tree derived from the 16S rDNA gene sequences obtained in this study could not place the two genera to separate clades (Fig. 19. & 20.). Whereas, Maximum Likelihood tree obtained from 16S rRNA sequences showed distinction of genus at high accuracy (Karan *et al*., 2017).

The morphological characterisation of these two genera and species were difficult due to their morphological plasticity. The instability of characteristics of cyanobacteria in diverse habitats is reported by (Moisander *et al.*, 2002; Soares *et al.*, 2013; Iranshahi *et al.*, 2014). This instability was observed in cultures also and certain characters such as akinetes were not formed in the culture which further made the identification difficult.

According to Tuji and Niiyama (2010), the validity of species name will be in question when the names of the cultured strains are not corrected or updated. Such confusions regarding the validity of species were observed when phylogenetic tree was reconstructed with available sequences from GenBank such as, *Anabaena torulosa* strain BF1 and *A. orientalis* strain BTA450 which shows affinity with other *Nostoc* strains.

SUMMARY AND CONCLUSION

5. SUMMARY AND CONCLUSION

Blue-green algae are considered to be pioneer microorganisms, economically and ecologically important, with agricultural and biotechnological applications. The genus *Nostoc* and *Anabaena* are among the important genera with diverse applications. The humid tropical climate is ideal for the growth of these Blue-green algae. These genera with little morphological differences have always been a topic of concern. A molecular systematic study of two genera *Nostoc* spp. and *Anabaena* spp. coming under the Order Nostocales was undertaken using 16S rDNA gene primarily to distinguish and characterize the two genera and to deduce the intrageneric and intergeneric phylogenetic relationships among them. The present study is primarily aimed to deduce the relationship between these taxa.

The materials for the present study were collected from different geographical areas of Kerala. Morphological identifications were done using conventional taxonomic methods such as referring classic literatures, consulting expert algologists, etc. From the survey and collection, it was noted that among *Nostoc* and *Anabaena*, the genus *Nostoc* dominated in Kerala with 10 species with frequent distribution and majority of the collected specimens were from paddy fields. A taxonomic key was attempted to discriminate between genera and species based on morphological characteristics. BG-11 medium without nitrogen source and pH range of 7.2-7.5 well supported the growth of the collected specimens. Axenic cultures of the required specimens were done by periodic sub-culturing in the culture medium. DNA isolation was carried out by a modified novel method proposed in this study. 14-21 days old cultures yielded good quality genomic DNA. The 16S rDNA gene was amplified using primer pair 8-27F and 1495R in a PCR thermal cycler. The amplicons obtained were sequenced at RGCB, Thiruvanathapuram and the resulting sequences were assembled to contiguous alignment.

For phylogenetic analysis of the *Nostoc* and *Anabaena* of Kerala using 16S rDNA gene, 17 isolates collected which include four species of *Anabaena* and ten species of *Nostoc* were studied. 16S rDNA sequences of corresponding species from GenBank were used to compare with the native strains. Before going for phylogenetic analysis, the 16S rDNA sequences of all the 17 strains were to be aligned and trimmed.

Sequence alignment was done with ClustalW software. The sequences of the newly sequenced strains were trimmed by using the software BioEdit. The phylogenetic analysis was done with Neighbour Joining method and Maximum Likelihood method in MEGA version 7.

A good clustering pattern was obtained in all the trees and the clusters were well supported with bootstrap values. The main conclusions drawn in the study are:-

1. From the phylogenetic reconstruction using the native strains collected, paraphyly of *Nostoc* spp. and *Anabaena* spp. is revealed.
2. The two genera of heterocystous blue-green algae were found within a single large cluster, with *Nostoc* occupying the basal portion of the tree, indicating the primitiveness of the genus.
3. The tree comprises of six strongly supported clusters, the first cluster with all the studied *Anabaena* strains and two *Nostoc* strains nesting in that cluster.
4. The morphological similarities were not fully reflected in the phylogenetic tree derived from all the studied strains. Except for some *Nostoc* strains having similar colony characters were grouped in a single cluster.
5. The strain MBG-AL313 identified as *Nostoc* sp. which was identified only up to genus level is seen as an out group among the other strains studied. But when compared with other sequences available in the

GenBank, it showed affinity with *N. linckia* strain NIES and formed a cluster with it.

6. Species status of two unidentified strains (of *Nostoc* spp.) viz., MBG-AL214 and MBG-AL313 is under question. Further studies using additional data (physiological, chemotaxonomic or data from other markers like *rbcL, nif* genes, etc) are needed to clarify the doubts.
7. The intermixing of the *Anabaena* strains and *Nostoc* strains shows their close genetic relatedness and points towards merging of these two genera.
8. The two *Sphaerospermopsis aphanizomenoides* strains (Synonym: *A. aphanizomenoides*) when compared with corresponding sequences from GenBank did not show affinity leading to the doubt on the taxonomic status of the taxon.
9. *N. muscorum* was found to be distinct from all other *Nostoc* strains thus supporting their distinction to new genus *Desmonostoc*
10. 16S rDNA gene was found effective in identification upto genus level but is ineffective in distinguishing the species.

Further studies on these genera will be helpful to conclude about their status. The axenic cultures of the strains isolated are well maintained in the laboratory, which will be helpful for further studies. Phylogenetic analysis using 16S rDNA gene from other species that are not included in this study will be informative in clearing doubts regarding the relationships of taxa. In addition, a combined phylogenetic analysis using different approaches like biochemical and molecular data using other markers like *nif, rbcL* etc, will be useful in defining the natural taxa of this region.

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APPENDIX I

Anabaena orientalis strain MBG-AL316 16S ribosomal RNA gene, partial sequence

GenBank: MN097906.1

GenBank Graphics

>MN097906.1 Anabaena orientalis strain MBG-AL316 16S ribosomal RNA gene, partial sequence
GGGGGGGGTCTTTAAAATGCCAAGTCGAACGGACTCTTCGGAAATTAGTGGCGGACGGGTGAGTAACGCG
TGAGAATTTGGCTTTAGGTCGGGGACAACAGTTGGATGCGACTGCTAATACCGGATGTGCCGGAAGGTGA
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TCCGTGCCACCACCCGCGGTAATACGGAGGATGCAAGCGTTATCCGGAATGATTGGGCGTAAAGGGTCCG
CAGGTGGAACTGAAAGTCTGCTGTTAAAGAGTCACGCTCACCGTGATAAAAGCCAGTGGAAACTACAGGA
ACTGGAGTATCGTTCGGGGCAGAAAGTAATTCCTGGTGTTAGCGGTGATAATGCAGTAGATATCAGGAAA
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CAAAAGGAAATAGACAGGGTGACCTCGCCACAAAGCCGGGTGGAAGTTATGGTGGTTTTTAATTTCGATA
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GCTCAGTTCAGATCGAAGGCTGCAACTCGCCTTCGTGAAGGAGGAATCGCTAGTAATTGCAGGTCAGCAT
ACTGCAGTGAATTCGTTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGAAGTTGGTCACTCCCGA
AGTCATTACCCCAACCGCAAGGAGCGGGAGTCCTAACGGTTTGGACCTGTA

Nostoc linckia MBG-AL317 16S ribosomal RNA gene, partial sequence

GenBank: MN149539.1

GenBank Graphics

>MN149539.1 Nostoc linckia MBG-AL317 16S ribosomal RNA gene, partial sequence
TTTAAGGTTGGGGCAGGGAACGGGGTGAGTAACGCGTGAGAATCCTTTTACTTCAGGTCTGGGACAACCA
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TGATTAGCTAGTAGGTGGGGTAAGAGCCTACCTAGGCGACGATCAGTAGCTGGTCTGAGAGGATGACCAG
CCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTCCGCAATGGGCG
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AAGGGAATTCCTGGTGTAGCGGTGAAATGCGTAGAGATCAGGAAGAACACCGGTGGCGAAGGCGCTCTGC
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CTGTAAACGATGGATACTAGGCGTGGCTTGTATCGACCCGAGCCGTGCCGTAGCTAACGCGTTAAGTATC
CCGCCTGGGGAGTACGCACGCAAGTGTGAAACTCAAAGGAATTGACGGGGGGCCCGCACAAGCGGGTGGA
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TCTGAAAGGAAGAGGTGCCTTCAGGAGCGCCGAACCACAGGTGGTGTGTCATTGGGCGTGTCGTCAGCTC
GTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTTTTAGTTGCCAGCATTAAGTTG
GGCACTCTAGAGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCAGCATGCCCCTT
ACGCCTTGGGCTACACACGTACTACAATGCTACGGACAGAGGGCAGCAAGCTAGCGATAGCAAGCAAATC
CCAGAAACCGTAGCTCAGTTCAGATCGAAGGCTGCAACTCGCCTTCGTGAAGGAGGAATCGCTAGTAATT
GCAGGTCAGCATACTGCAGTGAATTCGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGAAGCTG
GCAACGCCCGAAGTCATTACTCCAACCATTCGTGGGGGAGGATGCCCTAACGGGCCCGGTGCCTCGA

Nostoc entophytum MBG_AL213 16S ribosomal RNA gene, partial sequence

GenBank: MG595793.1

GenBank Graphics

>MG595793.1 Nostoc entophytum MBG_AL213 16S ribosomal RNA gene, partial sequence

AGTGGGGGAGGCTATAATCATGCAAGTCGAACGGTGTCTTCGGACATAGTGGCGGACGGGTGAGTAACGC
GTGAGAATCTAGCTTCAGGTCTGGGACAACCACTGGAAACGGTGGCTAATACCGGATGTGCCGGAAGGTG
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GGCTAAATCCCTGCCAGCCAGCCCGCGGTAATACGAGAGGGATTGCAAAGCGTTATCCCGGAATGATTGG
GCGTAAAGCGTCCCGCAGGCGGCTGTGTAAGTCTGCTGTCAAAGAGCAAAAGCTCAAACTTTGTAAAAGG
CAGTGGAAACTACACGGGCTAGAGTGCGTTTCGGGGCAGAGGGAATTTCCTGGTGTAACGGTGAAATGCG
TAAAGATCAAGAAGAAACACCCGGTGGCGAAGGCGCTCTGCTAGGCCGCAACTGACACTGAGGGACGAAA
GCTATGGGGAGCGAATGGGGATTTAAATACCCCCAGTAGTCTCCAGCTGTAAACGATGGATACTAGGCGT
GGCTTGTATCGACCCGAGCCGTGCCGTAGCTAACGCGTTAAGTATCCCGCCTGGGGAGTACGCACGCAAG
TGTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGC
GAAGAACCTTACCAAGGCTTGACATGTCGCGAACTCTTCTGAAAGGAAGAGGTGCCTTCGGGAGCGCGAA
CACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAAC
CCTCGTTTTTAGTTGCCAGCATTAAGTTGGGCACTCTAGAGAGACTGCCGGTGACAAACCGGAGGAAGGT
GGGGATGACGTCAAGTCAGCATGCCCCTTACGCCTTGGGCTACACACGTACTACAATGCTACGGACAGAG
GGCAGCAAGCTAGCGATAGCAAGCTAATCCCATAAACCGTGGCTCAGTTCAGATCGAAGGCTGCAACTCG
CCTTCGTGAAGGAGGAATCGCTAGTAATTGCAGGTCAGCATACTGCAGTGAATTCGTTCCCGGGCCTTGT
ACACACCGCCCGTCACACCATGGAAGCTGGCAACGCCCGAAGTCATTACTCCAACCTTTCGAGGGGGAGG
ATGCCTAACGGGCAGAGGTTCC

Desmonostoc muscorum MBG-AL111 16S ribosomal RNA gene, partial sequence

GenBank: MG595786.1

GenBank Graphics

>MG595786.1 Desmonostoc muscorum MBG-AL111 16S ribosomal RNA gene, partial sequence
GGGTGAGTAACGCGTGAGAATCTGGCTCCAGGTCGGGGACAACAGTTGGAAACGACTGCTAATACCGGAT
GTGCCGAGAGGTGAAAGATTAATTGCCTGGAGATGAGCTCGCGTCTGATTAGCTAGTTGGTGTGGTAAGA
GCGCACCAAGGCGACGATCAGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCC
AGACTCCTACGGGAGGCAGCAGTGGGGAATTTTCCGCAATGGGCGAAAGCCTGACGGAGCAATACCGCGT
GAGGGAGGAAGGCTCTTGGGTTGTAAACCTCTTTTCTCAAGGAATAACTCAATGAAGGTACTTGAGGAAT
AAGCATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATGCAAGCGTTATCCGGAATGATTGG
GCGTAAAGGGTCCGCAGGTGGCAGTGTAAGTCTGCTGTCAAAGAATGAGGCTCAACCTCATCAAGGCAGT
GGAAACTACACAGCTAGAGTACGGTCGGGGTAGAAGGAATTCCTGGTGTAGCGGTGAAATGCGTAGAGAT
CAGGAAGAACACCGGTGGCGAAAGCGTTCTGCTAGGCCTGTACTGACACTGAGGGACGAAAGCTAGGGGA
GCGAATGGGATTAGATACCCCAGTAGTCCTAGCCGTAAACGATGGATACTAGGCGTGGCTTGTATCGACC
CGAGCCGTGCCGGAGCCAACGCGTTAAGTATCCCGCCTGGGGAGTACGCACGCAAGTGTGAAACTCAAAG
GAATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCA
AGACTTGACATGTCGCGAATTTTCTGGAAACAGAAGAGTGCCTTCGGGAGCGCGAACACAGGTGGTGCAT
GGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTTTTAGTT
GCCAGCATTAAGTTGGGCACTCTAGAGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAA
GTCAGCATGCCCCTTACGTCTTGGGCTACACACGTACTACAATGCTCCGGACAGAGGGCAGCAAGCTAGC
GATAGCAAGCAAATCCCGTAAACCGGAGCTCAGTTCAGATCGCAGGCTGCAACTCGCCTGCGTGAAGGAG
GAATCGCTAGTAATTGCAGGTCAGC

Sphaerospermopsis aphanizomenoides MBG-AL113 16S ribosomal RNA gene, partial sequence

GenBank: MG595787.1

GenBank Graphics

>MG595787.1 Sphaerospermopsis aphanizomenoides MBG-AL113 16S ribosomal RNA gene, partial sequence

CGCGTGAGAATTTGGCTTCAGGTCGGGGACAACAGTTGGAAACGACTGCTAATACCGGATGTGCCGGAAG
GTGAAAGATTTATCGCCTGAAGATAAGCTCGCGTCTGATTAGCTAGTTGGTGGTGTAAGGGACTACCAAG
GCGACGATCAGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTAC
GGGAGGCAGCAGTGGGGAATTTTCCGCAATGGGCGAAAGCCTGACGGAGCAATACCGCGTGAGGGAGGAA
GGCTCTTGGGTCGTAAACCTCTTTTCTCAAGGAATAAGAAAGTGAAGGTACTTGAGGAAGAAGCATCGGC
TAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATGCAAGCGTTATCCGGAATGATTGGGCGTAAAGGG
TCCGCAGGTGGAACTGAAAGTCTGCTGTTAAAGAATCACGCTCAACGTGATCAAAGCAGTGGAAACTACA
GAACTGGAGTGCGGTCGGGGCAGAAGGAATTCCTGGTGTAGCGGTGAAATGCGTAGAGATCAGGAAGAAC
ACCGGTGGCGAAAGCGTTCTGCTAGGCCGTAACTGACACTGAGGGACGAAAGCTAGGGGAGCGAATGGGA
TTAGATACCCCAGTAGTCCTAGCCGTAAACGATGGATACTAGGCGTGGCTTGTATCGACCCGAGCCGTGC
CGGAGCTAACGCGTTAAGTATCCCGCCTGGGGAGTACGCACGCAAGTGTGAAACTCAAAGGAATTGACGG
GGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCAAGACTTGACA
TGGCGCGAATCTTCTTGAAAGGGAAGAGTGCCTTCGGGAGCGCGCACACAGGTGGTGCATGGCTGTCGTC
AGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTGTTTAGTTGCCAGCATTA
AGTTGGGCACTCTAGAGAGACTGCCGGTGACAAACCGGAGGAAGGTGAGGATGACGTCAAGTCAGCATGC
CCCTTACGTTTTGGGCTACACACGTACTACAATGCTACGGACAAAGGGCAGCTACACAGCGATGTGAGGC
AAATCTCATAAACCGTAGCTCAGTTCAGATCGAAGGCTGCAACTCGCCTTCGTGAAGGAGGAATCGCTAG
TAATTGCAG

Nostoc humifusum MBG-AL114 16S ribosomal RNA gene, partial sequence

GenBank: MG595788.1

GenBank Graphics

>MG595788.1 Nostoc humifusum MBG-AL114 16S ribosomal RNA gene, partial sequence

GGGTGAGTAACGCGTGAGAATCTAGCTTCAGGTCGGGGATAACTACTGGAAACGGTGGCTAATACCGGAT
GTGCCGAAAGGTGAAAGGCTTGCTGCCTGAAGATGAGCTCGCGTCTGATTAGCTAGTTGGTGTGGTAAGA
GCGCACCAAGGCGTCGATCAGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCC
AGACTCCTACGGGAGGCAGCAGTGGGGAATTTTCCGCAATGGGCGAAAGCCTGACGGAGCAATACCGCGT
GAGGGAGGAAGGCTCTTGGGTTGTAAACCTCTTTTCTCAGGGAATAAGAAAGTGAAGGTACCTGAGGAAT
AAGCATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATGCAAGCGTTATCCGGAATGATTGG
GCGTAAAGCGTCCGCAGGTGGCGATGTAAGTCTGCTGTTAAAGAGCAAAGCTTAACTTTGTAAAAGCAGT
GGAAACTACATAGCTAGAGTACGTTCGGGGCAGAGGGAATTCCTGGTGTAGCGGTGAAATGCGTAGAGAT
CAGGAAGAACACCGGTGGCGAAGGCGCTCTGCTAGGCCGTAACTGACACTGAGGGACGAAAGCTAGGGGA
GCGAATGGGATTAGATACCCCAGTAGTCCTAGCCGTAAACGATGGATACTAGGCGTTGCGAGTATCGACC
CTCGCAGTGCCGGAGCCAACGCGTTAAGTATCCCGCCTGGGGAGTACGCACGCAAGTGTGAAACTCAAAG
GAATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCA
AGACTTGACATGTCGCGAATCCTCTTGAAAGGGAGGAGTGCCTTAGGGAGCGCGAACACAGGTGGTGCAT
GGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTTTTAGTT
GCCAGCATTAAGTTGGGCACTCTAGAGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAA
GTCAGCATGCCCCTTACGTCTTGGGCTACACACGTACTACAATGCTACGAACAGAGGGCAGCAAGCTAGC
GATAGCAAGCAAATCCCGGAAATCGTAGCTCAGTTCAGATCGAAGCTTGCAACTCAGCTTCGTGAAGGAG
GAATCGCTAGTAATTGCAGGTCAGC

Nostoc paludosum MBG-AL115 16S ribosomal RNA gene, partial sequence

GenBank: MG595789.1

GenBank Graphics

>MG595789.1 Nostoc paludosum MBG-AL115 16S ribosomal RNA gene, partial sequence

CGCGTGAGAATCTGGCTTCATGTCGGGGATAACAGTGGGAAACGACTGCTAATACCGGATGTGCCGAAAG
GTGAAAGGCTTGCTGCCTGAAGATGAGCTCGCGTCTGATTACCTAGTTGGTGTGGTAAGAGCGCACCAAG
GCGACGATCAGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTAC
GGGAGGCAGCAGTGGGGAATTTTCCGCAATGGGCGAAAGCCTGACGGAGCAATACCGCGTGAGGGAGGAA
GGCTCTTGGGTTGTAAACCTCTTTTCTCAAGGAATAAGTTCTGAAGGTACTTGAGGAATCACATCGGCTA
ACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATGCAAGCGTTATCCGGAATGATTGGGCGTAAAGCGTC
CGCAGGTGGCCCTGTAAGTCTGCTGTTAAAGAGCAAAGCTTAACTTTGTAAAAGCAGTGGAAACTACAGA
GCTAGAGTGCGTTCGGGGTAGAGGGAATTCCTGGTGTAGCGGTGAAATGCGTAGAGATCAGGAAGAACAC
CGGTGGCGAAGGCGCTCTACTAGGCCGCAACTGACACTGAGGGACGAAAGCTAGGGGAGCGAATGGGATT
AGATACCCCAGTAGTCCTAGCCGTAAACGATGGATACTAGGCGTTGCGAGTATCGACCCTCGCAGTGCCG
GAGCCAACGCGTTAAGTATCCCGCCTGGGGAGTACGCACGCAAGTGTGAAACTCAAAGGAATTGACGGGG
GCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCAAGACTTGACATG
TCGCGAATTCCGGTGAAAGCTGGAAGTGCCTTCGGGAGCGCGAACACAGGTGGTGCATGGCTGTCGTCAG
CTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTTTTAGTTGCCAGCATTAAG
TTGGGCACTCTAGAGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCAGCATGCCC
CTTACGTCTTGGGCTACACACGTACTACAATGCGTCGAACAGAGGGCAGCAAGCCGGCAACGGCAAGCAA
ATCCCGGAAATCGGCGCTCAGTTCAGATCGAAGGCTGCAACTCGCCTTCGTGAAGGAGGAATCGCTAGTA
ATTGC

Anabaena sphaerica MBG-AL116 16S ribosomal RNA gene, partial sequence

GenBank: MG595790.1

GenBank Graphics

>MG595790.1 Anabaena sphaerica MBG-AL116 16S ribosomal RNA gene, partial sequence

TAACGCGTGAGAATTTGGCTTCAGGTCGGGGACAACAGTTGGAAACGACTGCTAATACCGGATATGCCGG
AAGGTGAAAGATTTATCGCCTGAAGATAAGCTCGCGTCTGATTAGCTAGTTGGTGGGGTAAAGGCCTACC
AAGGCGACGATCAGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCC
TACGGGAGGCAGCAGTGGGGAATTTTCCGCAATGGGCGAAAGCCTGACGGAGCAATACCGCGTGAGGGAG
GAAGGCTCTTGGGTCGTAAACCTCTTTTCTCAAGGAAGAAAAAAATGACGGTACTTGAGGAATAAGCATC
GGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATGCAAGCGTTATCCGGAATGATTGGGCGTAAA
GGGTCCGCAGGTGGTGCTGTAAGTCTGCTGTTAAAGAGTCTAGCTCAACTAGATAAAAGCAGTGGAAACT
ACAGGACTAGAGTGCGTTCGGGGCAGAAGGAATTCCTGGTGTAGCGGTGAAATGCGTAGATATCAGGAAG
AACACCGGTGGCGAAAGCGTTCTGCTAGGCCGCAACTGACACTGAGGGACGAAAGCTAGGGGAGCGAATG
GGATTAGATACCCCAGTAGTCCTAGCCGTAAACGATGGATACTAGGCGTGGCTTGTATCGACCCGAGCCG
TGCCGGAGCTAACGCGTTAAGTATCCCGCCTGGGGAGTACGCACGCAAGTGTGAAACTCAAAGGAATTGA
CGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCAAGACTTG
ACATGGCGCGAATTTCGGTGAAAGCTGAGAGTGCCTTCGGGAGCGCGCACACAGGTGGTGCATGGCTGTC
GTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTTTTAGTTGCCAGCA
TTAAGTTGGGCACTCTAGAGAGACTGCCGGTGACAAACCGGAGGAAGGTGAGGATGACGTCAAGTCAGCA
TGCCCCTTACGTCTTGGGCTACACACGTACTACAATGCTACGGACAAAGGGCAGCGAGCTAGCGATAGCA
AGCAAATCTCATAAACCGTAGCTCAGTTCAGATCGAAGGCTGCAACTCGCCTTCGTGAAGGAGGAATCGC
TAGTAATTGCAGGTCAG

Anabaena sp. MBG-AL212 16S ribosomal RNA gene, partial sequence

GenBank: MG595792.1

GenBank Graphics

>MG595792.1 Anabaena sp. MBG-AL212 16S ribosomal RNA gene, partial sequence

GGGCCCCAGCTTACCATGCAGTCGAACGGACTCTTCGGATTAGTGGCGGACGGGTGAGTAACGCGTGAGA
ATTTGGCTTCTGGTCGGGGACAACAGTTGGAAACGACTGCTAATACCGGATGTGCCCAAAGGTGAAAAAT
TTATTGCCTGAAAATAAGCTCTCGTCTGATTATCTATTTGGTGGGGTAAGAGCCTACCAAGGCGACAATC
ACTATCTGGTCTGAGAGGATGATCACCCCCACTGGGACTGAGACACGGCCCACACTCCTACGGGAGGCGG
CAGTGGGGAATTTTCCGCAATGGGCGAAAGCCTGACGGAGCAATACCCCGTGAGGGAGGAAGGCTCTTGG
GTCTTAAACCTCTTTTCTCAAGGAAAAAAAAAGTGACGGTACTTAAGGAATAACCATCGGCTAACTCCGT
GCCACCACCCGCGGTAATACGGAGGATGCAAGCGTTATCCGGAATGATTGGGCGTAAAGGGTCCGCAGGT
GGAACTGAAAGTCTGCTGTTAAAAAGACTGGGTCAACCCAGATAAAAAGCAGTGGTAAACTACAGAACTA
GAGTGCGGTTCGGGGCAAAAAGAAATTCCTGGTGTAGAGGTGAAATGCGTAGAATATCAGGAAAAACACC
GGTGGCGAAAGCGTTCTGCTAGGCCGCAACTGACACTGAGGGACGAAAAGCTAGGGGAGCGAATGGGATT
AGATACCCCAGTAGTCCTAGCCGTAAACGATGGATACTAGGCGTGGCTTGTATCGACCCGAGCCGTGCCG
GAGCTAACGCGTTAAGTATCCCGCCTGGGGAGTACGCACGCAAGTGTGAAACTCAAAGGAATTGACGGGG
GCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCAAGACTTGACATC
CTGCGAATCCTGGTGAAAGGTGGGAGTGCCTTCGGGAGCGCAGAGACAGGTGGTGCATGGCTGTCGTCAG
CTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTTTTAGTTGCCAGCATTAAG
ATGGGCACTCTAGAGAGACTGCCGGTGACAAACCGGAGGAAGGTGAGGATGACGTCAAGTCAGCATGCCC
CTTACGTCTTGGGCTACACACGTACTACAATGCTACGGACAAAGGGCAGCTACACAGCGATGTGATGCAA
ATTTCAGAAACCGTAGCTCAGTTCAGATCGAAGGCTGCAACTCGCCTTCGTGAAGGAGGAATCGCTAGTA
ATTGCAGGTCAGCATACTGCAGTGAATTCGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGAAG
TTGGTCACGCCCGAAGTCATTACCCTCAACCGAAAGGAGCAGGATGCCCTACCGGAAGAATCTTGC

This was later identified as Anabaena torulosa

Nostoc sp. MBG-AL214 16S ribosomal RNA gene, partial sequence

GenBank: MG595791.1

GenBank Graphics

>MG595791.1 Nostoc sp. MBG-AL214 16S ribosomal RNA gene, partial sequence

GGGCCCGGAAAACCATGCAGTCGAGCGGAATCTTTGGGGATTTACTGGCGGACGGGTGAGTACGCGTGAT
AATCTACTTTCACGTCTGGGACAACCATTGAAAACGGTGGCTAATACCGGATGTGCCGGGAGGTGAAAGA
TTAATTGCCTGAAAATGACCTCGCGTCTGATTAGCTAGTTGGTGTGGTAAGATAGCACCGTTGCGACGAT
CATTAGCTGGTCTGAGAGGATGACCAGCCACGCTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGTC
AGGCAGTGGGGAATTTTCCGGCCAACTGGCCGAAAGCCTGAACGGAAGCAATTACCGGCGTGAGGGACGA
AGGCTCTTGGGTCGTAAAACATCCTTTTCTCCAAGGAATTAAGGAATAGGTGAAGGTACTTGACGGAATA
AGCATCGGGCTAACTCCGTGCCAGCAGCCCGCAGTAATACGGAGGATGCAATGCGTTATCCCGGAATGAT
TGGGCGTAAAGGGTCCGCAGGTGGCATTGTGTGTCTGCTATTAAAGAGTTTGGCCTTAACCAGATAAAAG
CAGTGGAAACTACAAAGCTAGAGTGCGTTCGGGGCACAAGGAATTCCTGGTGTAGCGGTGAAATGCGTAG
AGATCAAGAAGAACACCGGTGGCGAAAGCGTTCTGCTAGGCCTGCACTGACACTGAGGGACGAAAGCTAG
GGGAGCGAATGGGATTAGATACCCCAGTAGTCCTAGCCGTAAACGATGGATACTAGGCGTGGCTTGTATC
GACCCCGAGCCCGTGCCGGAGCTAACGCGTTAAATATCCCGCCCTGGGGAGTACGCACGCAAGTGTGAAA
CTCAAAGGAATTGACGGGGGCCCGCACAACCGGTGGAGTATGTGGTTTTAATTAGATGCAAGCGCCAAGA
ACCTTTACCAAGACTAGACATGTCGTGAATTTCTCTGAAAGGAGAAAGTGCCTTAGGGAGCACGAACCCA
GGTGGTGCATGGCGGTTGTCAGTTGGTGTCTTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTC
GTTTTTAGTTGCCAGCATTAAGTTGGGCACTCTAGAGAGACAGCCGGGGACAAACCGGAGGAAGGGGGGG
GGGAAGTCAAGTCAGCATGCGCCCTATGTTGTGGGGTACACACCTATTACAATGGTGCGGACAAAGGGCA
GCTACACAGGGATGTGATGCAAATCCAAGAAAAAGTAGCTCAGTTTAGATAGCAGGGTGCAAATCGCGTG
CGTGAAGGAGGAATCGGTAGTAAATGCAGGTCAGCATATTGCAGGGAATTCGTTCCCGGGCGTTGTACAC
ACCCCCCCTCTCACCACGGAAGAGGGCAACGCCCGAAGTCATTACTCCAACCATTAGGGGGGAGGAGCGC
AACGCTAGTCTTC

Desmonostoc muscorum MBG-AL215 16S ribosomal RNA gene, partial sequence

GenBank: MG595794.1

GenBank Graphics

>MG595794.1 Desmonostoc muscorum MBG-AL215 16S ribosomal RNA gene, partial sequence
CAAACTCCCGTTAAAAAATTCATGCCAAGTCTAACGGAATCTTCGGATTTAGTGGCGGACGGGTGAGTAA
CGCGTGACAATCTGGCTCTAGGTCGGGGACAACAGTTGGAAACGACTGCTAATACCGGATGTGCCGAAAG
GTAAAAGGCTTGCTGCCTAGAGATGAGCTCGCGTCTGATTAGCTAGTTGGTGTGGTAAGAGCGCACCAAG
GCGACGATCAGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTAC
GGGAGGCAGCAGTGGGGAATTTTCCGCAATGGGCGAAAGCCTGACGGAGCAATACCGCGTGAGGGAGGAA
GGCTCTTGGGTTGTAAACCTCTTTTCTCAAGGAATAAGTTCTGAAGGTACTTGAGGAATAAGCATCGGCT
AACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATGCAAGCGTTATCCGGAATGATTGGGCGTAAAGGGT
CCGCAGGTGGGCAACTGTAAGTCTGGCTGTCAAAAGAATGAGGGCTCAACCTTCATCAAGGGCAAGTGGG
AAACTACAGAGCTAGGAGTACGGTCGTGGGTAGAAGGAATTCCTGGTGTAGCGGGTGAAATGCGTAAGAG
ATCAGGAAGAACACCGGTGGCGAAAAGCGTTCTGCTAAGGCCTGTACTTGACACTGAGGGGACGAAAGCT
AGGGGAGCGAATGGGATTTAGATACCCCAGTAGTCCTAGCCGTAAACGATGGATACTAGGCGTGGCTTGT
ATCGACCCGAGCCGTGCCGGAGCCAACGCGTTAAGTATCCCGCCTGGGGAGTACGCACGCAAGTGTGAAA
CTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGGAGTATGTGGTTTAATTCGATGCAACGCGAAAGA
AACCTTACCAAGACTTGACATGTCGCGAATTCCCGGTGAAAGCTGGGGAGTGCCTTTCGGGAGCGCGAAC
ACAAGGTGGTTGCATGGCTGTCGTCAGCTCGTTGTCGTGAGATGTTGGGGTTAAGTTCCCCGCAAACGGA
GCGCCAACCCCTCGTTTTTTTAAGTTGCCAGCCATTTAAAGTTTGGGGCACCTCCTAAAGAAGACTTGCC
CGGTGAACAAAACCGGAAGAAAGGGTGGGGGGAATGAACCGTCCAAGTTCCAGCCAATTGCCCCTTACGT
CTTGGGCTACACACGTACTACAATGCTACGGACAGAGGGCAGCAAGCCGGCGACGGCAAGCAAATCCCGT
AAACCGGAGCTCAGTTCAGATCGCAGGCTGCAACTCGCCTGCGTGAAGGAGGAATCGCTAGTAATTGCAG
GTCAGCATACTGCAGTGAATTCGTTCCCGGGCCTTGTACACACCGCCCTTCACACCATGGAAGCTGGCAA
CGCCCGAAGTCTTTACTCCGAACCCATGGGGCTAGGATGCCTATTCGCAGGTGGTTGCGA

Desmonostoc muscorum MBG-AL217 16S ribosomal RNA gene, partial sequence

GenBank: MG595795.1

GenBank Graphics

>MG595795.1 Desmonostoc muscorum MBG-AL217 16S ribosomal RNA gene, partial sequence
TGGGGCGGCTATACCATGCAAGTCGAACGGATCTTCCGATTTAGTGGCGGACGGGTGAGTAACGCGTGAG
AATCTGGCTTCACGTCTGGGACATCACTTGGAAACGACTGCTAATACCGGATGTGCCCAGAGGTAAAAAA
TTAATTGCCTGAGTATGAGCTCTCGTCTGATTATCTATTTGGTGTGGTAAAAGCGCACCCCGGCCACGAT
CACTATCTGGTCTGAGAGGATGATCACCCACACTGGGACTGAGACACGGCCCACACTCCTACGGGAGGCG
GCAGTGGGGAATTTTCCGCAATGGGCGAAAGCCTGACGGAAGCAAATAACCGGCCGTGAGGGGAGGGAAA
GGCTCTTGAGTTATTAAAACCTCTTTTCTTCAAGGAATAAATTCAATGAAGGTACCTTGAGGAAATAAAG
CATCGGCTAACTCCGTGCGCAGCAGCCCGCGGTAATACGGGAGGGATGCAAGCGTTATCCGGAAATGATT
GGGGCATAAGAGGGTCCACAGGTGGCACTATAAGTCTGCTGTCAAAGAATGAGGCTCAACCTCATCAACG
CAGTAGAAACTACAAAGCTAGAGTACGGTCGGGGTAGAAAGAATTCCTGGTGTAGCGGTGAAATGCGTAG
AGATCAGGAAGAACACCGGTGGCGAAAGCGTTCTGCTAGGCCTGTACTGACACTGAGGGACGAAAGCTAG
GGGAGCGAATGGGATTAGATACCCCAGTAGTCCTAGCCGTAAACGATGGATACTAGGCGTGGCTTGTATA
GACCCGAGCCGTGCCGGAGCCAACGCGTTAAGTATCCCGCCTGGGGAGTACGCACGCAAGTGTGAAACTC
AAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTACGATGCAACGAGAAGAACCT
TTACCCAAGACTTTGACATGTCGAGAATCTTTCTTGAAAGAGAAGAGTGCCCTTTCGGGAGCGCGAACAC
AGATGGTGCCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTTGGGGTTAAGTTCCCCCAAACGAGCGCC
AACCCTCGTTTTTAGGTTGACATCGCATGGAAAATTGGGCACCTACTTAGACAGACTTGCCGGTGAACAA
ACCGGGAAGGAATTGTGTGGGAAATGAGGTTCAAGTTTCAGTCAGTGGGGCCCCTTACCTCTTGGGCCAC
ACATGTAATACAATGTTACGGACAGAGGGCAGATAAACAGAGATGTCAAGCAAATCCTGTAAACAGTAGC
TCAGTTCAGATCGCAGGGTGCAACTCGCGTGCGAGAAGGGGGAATCGATTGTAATTGCAGGTCAGCATAG
GGCAGAGATTTTGTTCCCGTGCCTTGTACACCCCCCCTCTCACCCCATGGAAGGTGGCAACGCCAGAAGT
CATTACTCCAACCTTGTGGAGGAGGATGCCTAATGTTAGGTTTGC

Nostoc ellipsosporum MBG-AL311 16S ribosomal RNA gene, partial sequence

GenBank: MN100312.1

GenBank Graphics

>MN100312.1 Nostoc ellipsosporum MBG-AL311 16S ribosomal RNA gene, partial sequence
CTTTTTATCTATACAAGGTCGGGGACAACCACTGGAAACGGTGGCTAATACCGGATGTGCCCTTGGGTAA
AAGGCTTGCTGCCTGAAGATGAGCTCGCGTCTGATTAGCTAGTAGGTGTGGTAAGAGCGCACCTAGGCGA
CGATCAGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGA
GGCAGCAGTGGGGAATTTTCCGCAATGGGCGAAAGCCTGACGGAGCAATACCGCGTGAGGGAGGAAGGCT
CTTGGGTTGTAAACCTCTTTTCTCAAGGAAGAAAACAATGACGGTACTTGAGGAATCAGCATCGGCTAAC
TCCGTGCCAGCAGCCGCGTGCTATAAATTTATCGGGAAGGATTGCCAAGCGTTATACGGAATGATTGGGC
GTAAAGCGTTCCGCAAGTGGATGGATGTAAGTCCTGCTGTCAAAAGCGTTCTAGCTCAAACTAAGATAAA
GGGCAGTGGAAACTACATGACTAGAGTGCGTTCGGGGCAGAGGGAATTCCTGGTGTAGCGGTGAAATGCG
TAGATATCAGGAAGAACACCGGGTGGCGAAAGCGCTCTGCTAGGCCCGCAACTGACACTGAGGGACGAAA
GCTAGGGGAGCGAATGGGATTAGATACCCCAGTAGTCCTAGCCGTAAACGATGGATACTAGGCGTGGCTT
TGTATCGACCCGAGCCGTGCCGTACTTAACGCGTTTAAGTATCCCCGCCCTGGGGGAGTACGCACGCAAG
TGTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGC
GAAGAACCTTACCAAGACTTGACATGTCGCGAATTGGAGTGAAAGCTTCAAGTGCCTTAGGGAGCGCGAA
CACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAAC
CCTCGTTTTTAGTTGCCAGCATTAAGTTGGGCACTCTAGAGAGACTGCCGGTGACAAACCGGAGGAAGGT
GGGGATGACGTCAAGTCAGCATGCCCCTTACGTCTTGGGCTACACACGTACTACAATGCTCCGGACAGAG
GGCAGCGAGCATGCGAATGCAAGCAAATCCCGTAAACCGGAGCTCAGTTCAGATCGCAGGCTGCAACTCG
CCTGCGTGAAGGAGGAATCGCTAGTAATTGCAGGTCAGCATACTGCAGTGAATTCGTTCCCGGGCCTTGT
ACACACCGCCCGTCACACCATGGAAGCTGGCAACGCCCGAAGTCATTTCTCCCAAACCCATTCCCAAAGA
GAAGCCGGCA

Nostoc sp.

>Nostoc sp MBG-AL313

GGGCCAACCCCCTAACGTTTGGGCATCCTCCCCCCCTGAAGGTTGCATTAATGACTTCGG

GGCGTTGCCATCTTCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACAAATTCAC

TGCAGTATGCTGACCTGCAATTACTACCGATTCCACCTTCAGGCAGGCAATTTGCAACCT

GCGATCTGAACTGAACTCCGGTTTACGGAATTTGCTTGCTATCGCTAGCTTGCTGCCCTC

TGTCCGGAGCATTGTATTACGTGTGTACCCCAAGACTTAGGGGGCATGCTGACTTGACTT

CATCCCCCCCTTCCTCCGGTTTGTCACCGGCATTCTCTCTAAAGTGCCCAACTTAATGCT

GGCAACTAAAAGCGAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGAAACCA

ACTGACGACCGCCATGCACCACCTGGGTTCCCGCTCCCCAAGGGACATACGTTTCAATTC

GATGTATTATGCCAGAGAACAATATCCGCGGACCATGTTCAAGTCTTGCGTCGTCAAAGA

TTATTCCCGGTTGGCATCCAGATTAATACCCACATAACTCCACCCGCGTTGTGGCGAGGC

CCCCCTCAATTCCTTTGAGATTGCACACTTGAGTGCGTACTACCACAGGCGGGGATACTT

AAATGCCTTGGCTCCGGCACAGGCTCGGGTAGATACAACCCACGCCTAGTATCCATCGTT

TACGGGTAGGACTACTGGGGTATCTAATCCCAGTTCGCTCCCCTAGCTTTCGTCCCTCAG

TGTCAATACAGGCCCAAGCAGAAACGCTTTCGCCACCGGTGGTTCCTTCCCTGATCTATA

CGCATTTCACCCGCTACACCCAGGAAATTCCTTCTACCCCCGACCGTACTTCAAGCTAGA

GCAGTTTTCCAACTGCCGTGTATCGAAGGTTTGAACCCTCCAAGTGCTATTTGACACGAC

AGACATTACACTTGCCCACCATGACAGCAACCGCTTTTTAACCCGACCCCCAAAATCAAT

TTTTCACGGGAGATATAAAACGCTTGCATCCTCTGTATTACCGCGGGTGCTGGCACGGAG

TTAGCCGATGCTTATTCATCAAGTACCTTCATTGAGTTATTCCTTGAGAAAAGAGGTTTA

CAACCCAAGAGCCTTCCTCCCTCACGCGGTATTGCTCCGTCAGGCTTTCGCCCATTGTGG

AAAATTCCCCACAGCTGCCTCCCGTAGGAGTATGGGCCGTGTCTCAGTCCCAGTGTGGCT

GATCATCGTCTCAGACCAGCTAATGATGGTAGCCTTGGTGCGCTTCTACCACACCAACAA

GGTAATCAGACGCGAGCTCATCTCCAGGCAATTAATCTTTCACCTATCGGCACATCCGGT

ACTAGCAGTCGCTTCCAAGTGTATCCCCGACCTGGACCCCGACTGTCACGCGTTACTCAC

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Sphaerospermopsis aphanizomenoides MBG-AL314 16S ribosomal RNA gene, partial sequence

GenBank: MN149543.1

GenBank Graphics

>MN149543.1 Sphaerospermopsis aphanizomenoides MBG-AL314 16S ribosomal RNA gene, partial sequence
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Nostoc punctiforme MBG-AL315 16S ribosomal RNA gene, partial sequence

GenBank: MN149540.1

GenBank Graphics

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Nostoc spongiaeforme MBG-AL312 16S ribosomal RNA gene, partial sequence

GenBank: MN149541.1

GenBank Graphics

>MN149541.1 Nostocspongiaeforme MBG-AL312 16S ribosomal RNA gene, partial sequence

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APPENDIX II

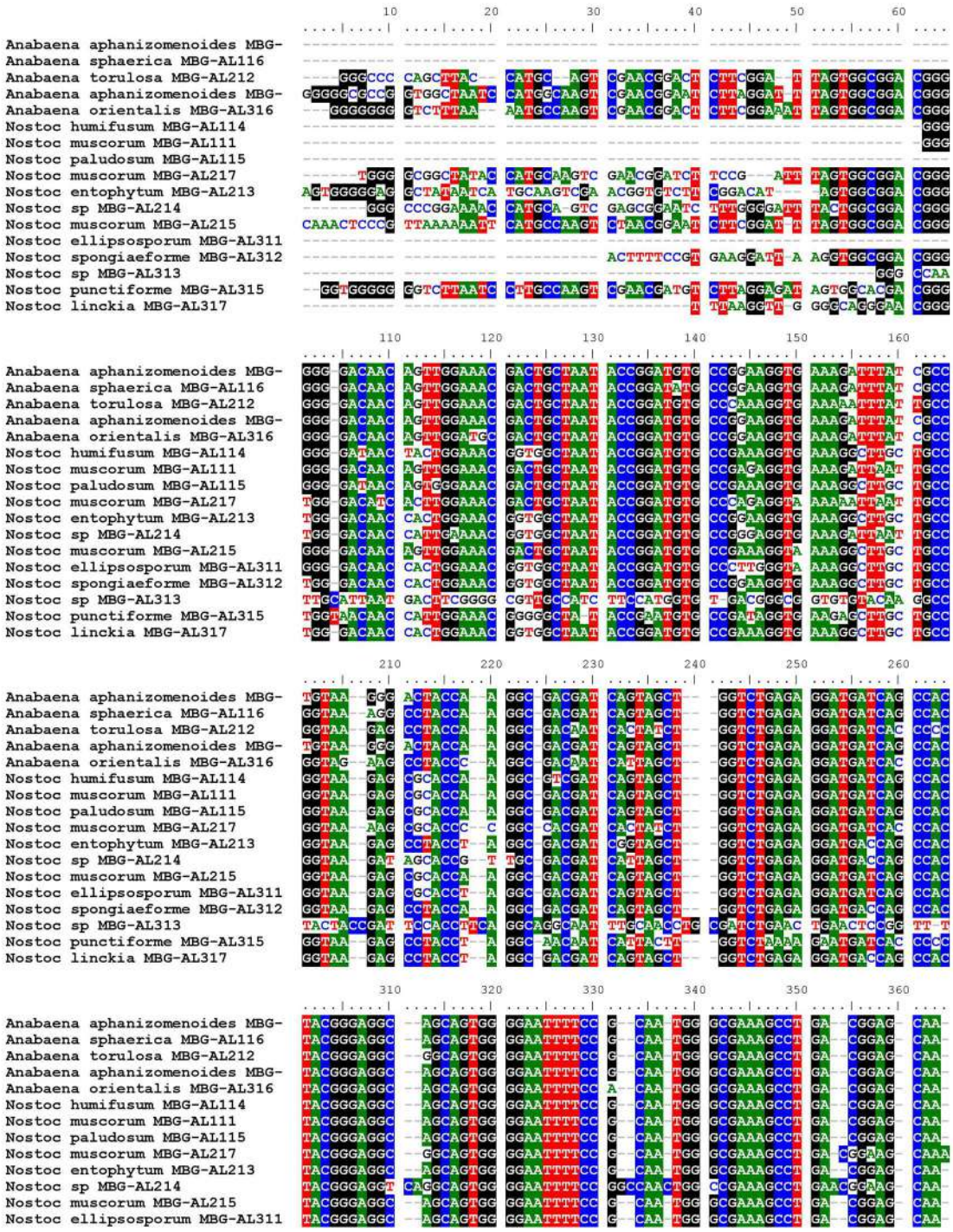

Fig. 22. ClustalW multiple sequence alignment of the native strains

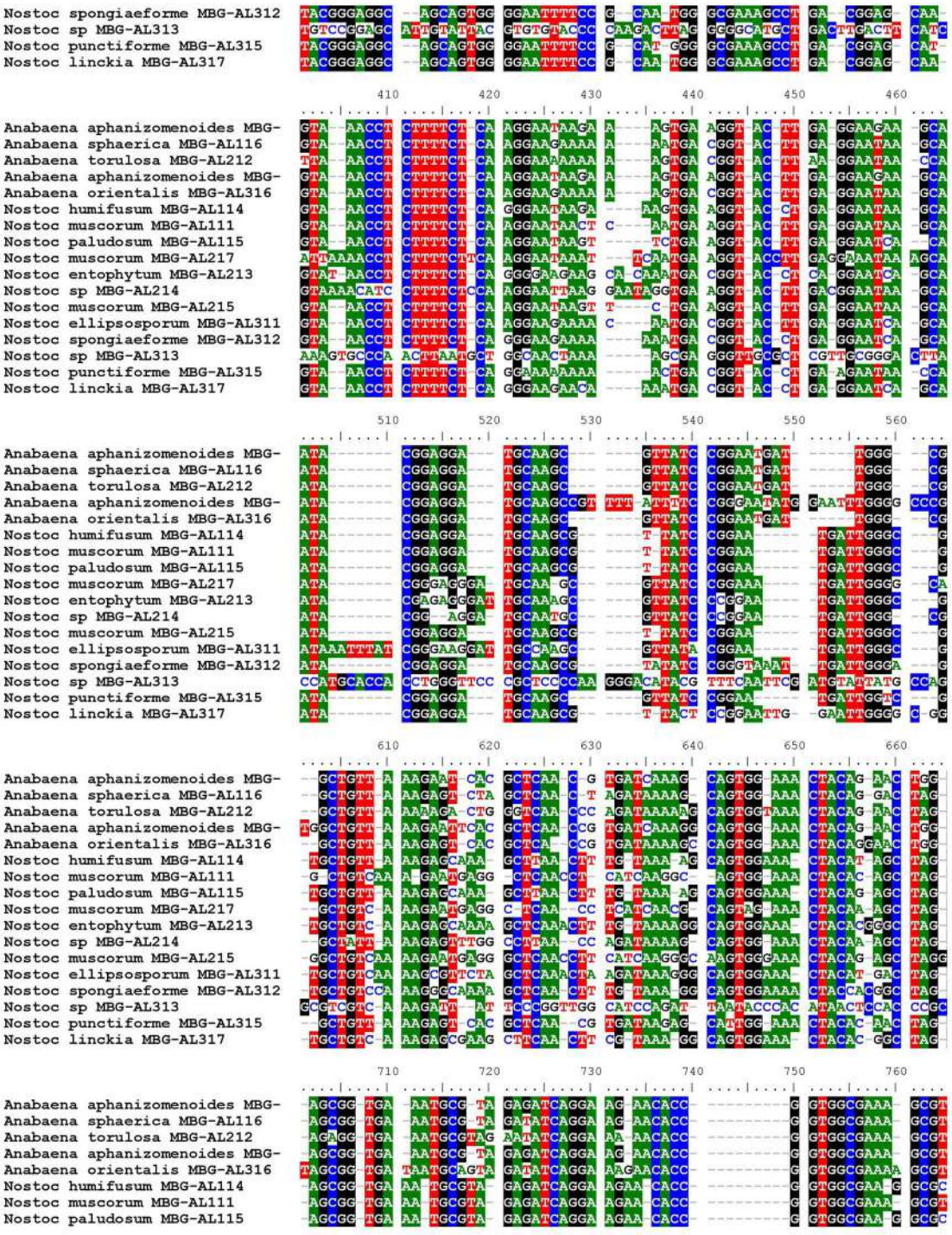

Fig. 22. ClustalW multiple sequence alignment of the native strains

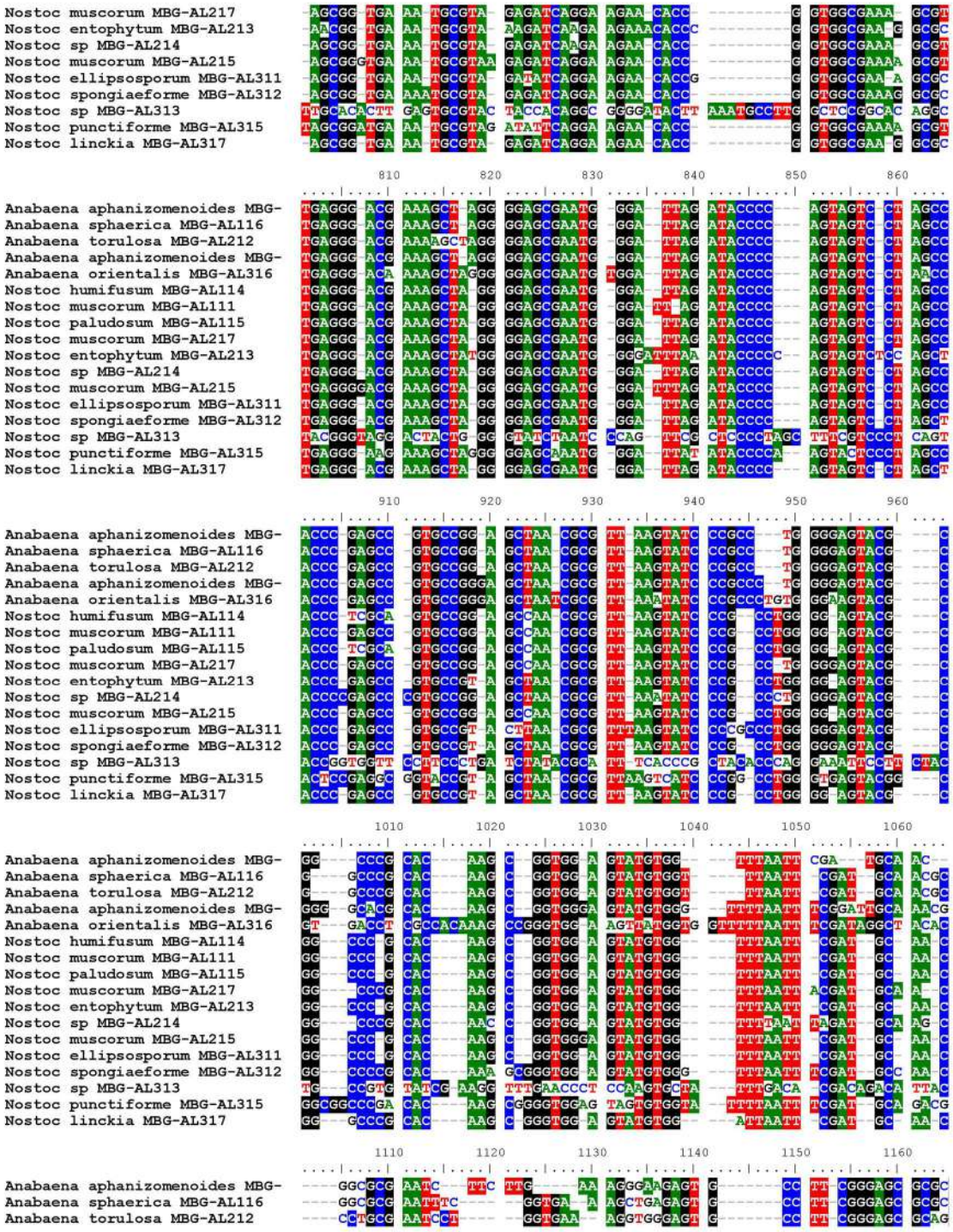

Fig. 23. ClustalW multiple sequence alignment of the native strains

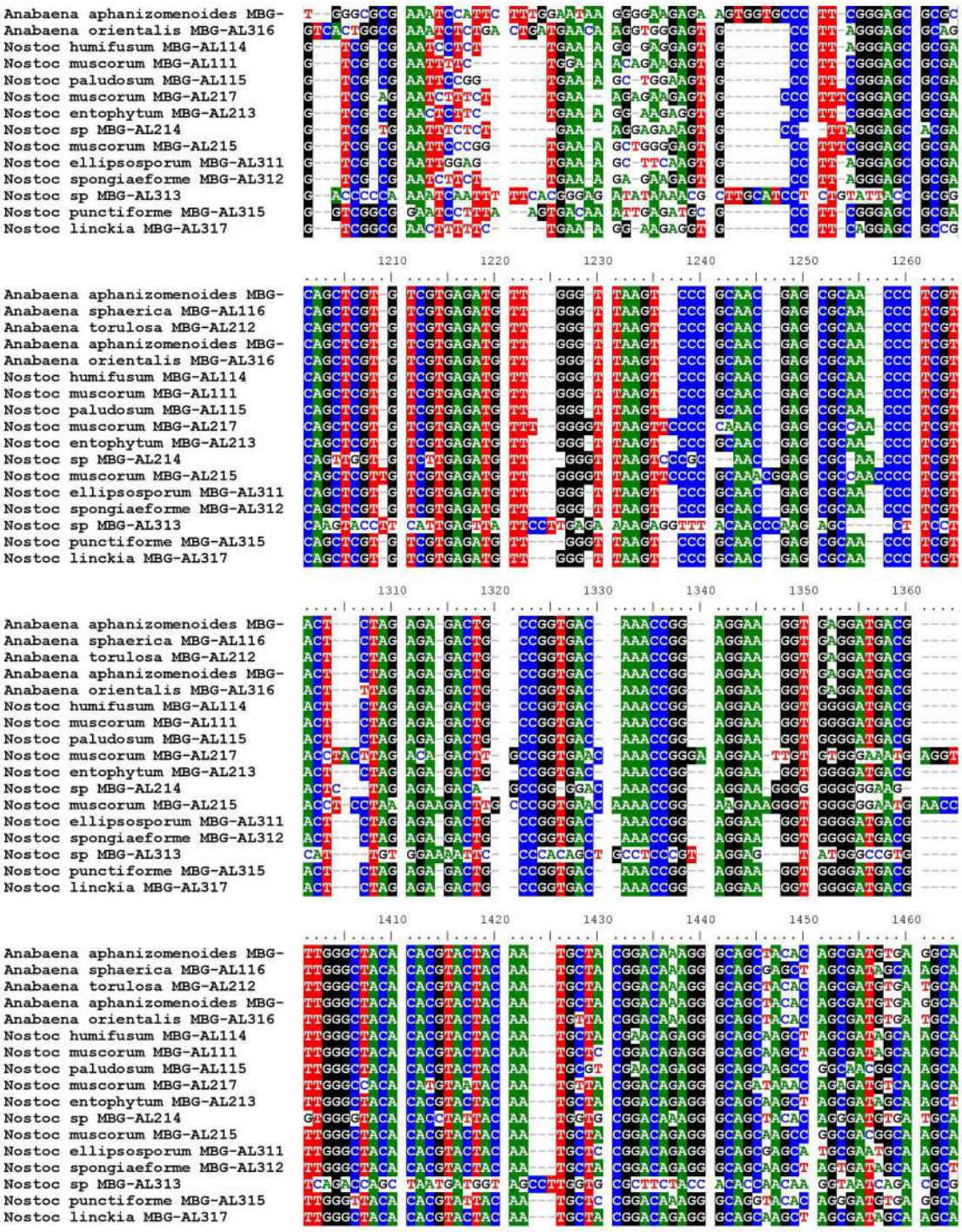

Fig. 22. ClustalW multiple sequence alignment of the native strains

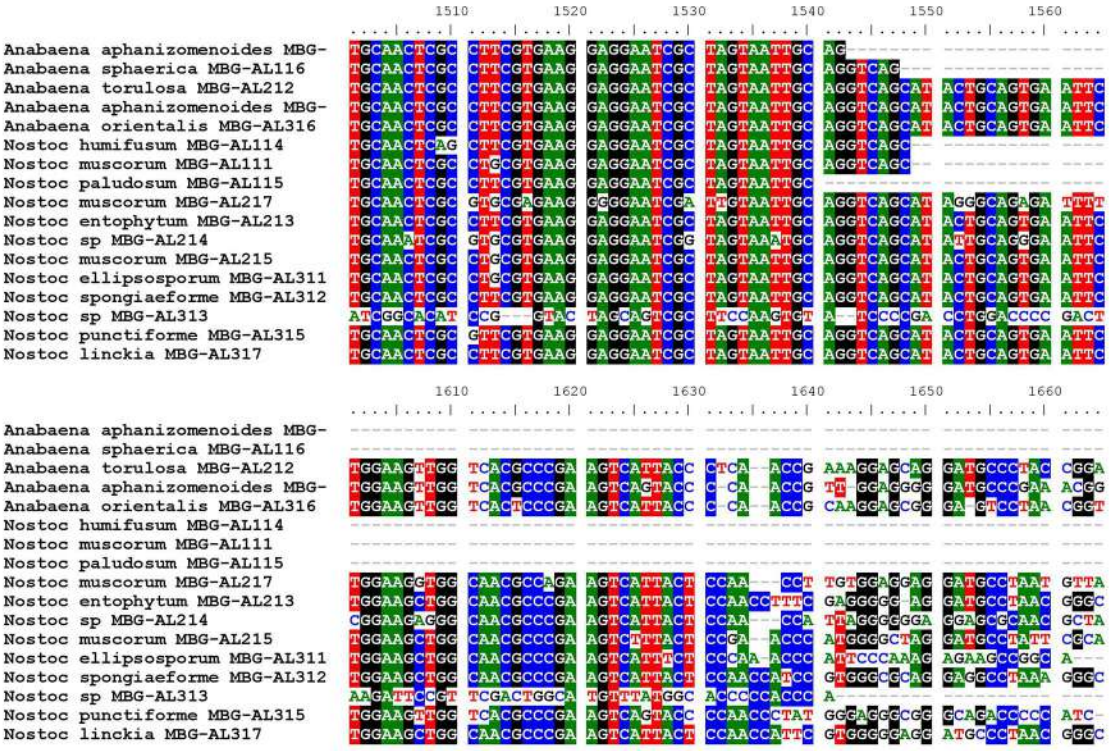

Fig. 22. ClustalW multiple sequence alignment of the native strains

APPENDIX III

List of Papers published

1. Swetha Thilak T., P.V. Madhusoodanan, R. Prakashkuma,r N.S. Pradeep. **An Improved Method for the Isolation of High Quality Genomic DNA from Axenic Cultures of Filamentous Blue-green algae (Cyanobacteria), Nostoc and Anabaena.** *Research & Reviews: A Journal of Life Sciences*. 2019; 9(2):32–38p.

2. Swetha Thilak T., Anusree, P., Madhusoodanan, P.V., Prakashkumar, R. **Isolation and axenic culture of nostocales (*Nostoc*spp. and *Anabaena* spp.) of Kerala. Advances & Challenges in Plant Breeding, Biotechnology & Conservation**, Smitha *et al.(Eds.),* 336-341 MBGIPS 2016

3. Thilak ST, Anusree P, Jayaprakash CMSB, Smitha RB, Divakaran M, Madhusoodanan PV, Prakashkumar R. ***In Vitro* conservation and propagation of the endemic species of "floating hearts" (*Nymphoides krishnakesara* Joseph and Sivar. - Menyanthaceae) as a conservation strategy**. J App Biol Biotech. 2018; 6(04) :9-13. DOI: 10.7324/JABB.2018.60402

4. Swetha Thilak T, Indulekha P. ***In vitro* Responses of *Indigofera tinctoria* Linn. (Neela Amari)**. *Research & Reviews: Journal of Herbal Science*. 2017; 6(1): 5–9p.

List of papers presented

1. **'Diversity of Blue-green algae (Cyanobacteria)- Nostocales of Kerala'** in National Seminar on 'PLANT DIVERSITY CONSERVATION AND BIOPROSPECTING IN WESTERN GHATS' held at Govt. College Kasargod.

2. **'Isolation and Axenic culture of Nostocales (Nostoc spp. and Anabaena spp.) of Kerala'** in National seminar on 'PATENTS, PLANT BREEDING , BIOTECHNOLOGY AND CONSERVATION' held at MBGIPS in association with Gregor Mendel Foundation, University of Calicut, Patent Information Centre (PIC) & KSCSTE.

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An Improved Method for the Isolation of High Quality Genomic DNA from Axenic Cultures of Filamentous Blue-green algae (Cyanobacteria), *Nostoc* and *Anabaena*

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Abstract

Blue-green algae are one of the important organisms having different ecological and economic important properties and are among one of the least explored one. The major difficulty is met with their exact identity; this could be tackled to an extent with the use of molecular techniques. Isolation of high quality DNA is essential for molecular biology applications. In the present study an efficient protocol for the extraction of genomic DNA from cultured samples of Nostoc and Anabaena collected from different districts of Kerala is developed. The DNA was found suitable for PCR amplification. The $A_{260/280}$ of the isolated DNA was found to be 1.5–1.8 indicating their purity.

Keywords*: Isolation, DNA, blue-green algae, PCR amplification, axenic culture*

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INTRODUCTION

Algae are the simplest members of the Plant kingdom and are the first photosynthetic organisms to release elemental oxygen to the environment. They are the primary producers of the ecosystem. Blue green algae are the simplest among the algae, unlike other algae these lack membrane bound cell organelles and possess cell wall material (peptidoglycan) similar to bacteria and hence they are now considered as cyanobacteria. They get the characteristic blue green colour from the pigment phycocyanin. Cyanobacteria have an ancient history dating about 3.5 billion years and diversified extensively to become one of the most successful and ecologically significant organisms on earth [1]. Their ability to perform oxygenic photosynthesis distinguishes them from other prokaryotes and also they are the first organism to evolve elemental oxygen. The most important property of the blue green algae is their ability to photosynthesize like that of vascular plants [2] and they are pioneer primary producers in many environments. The nitrogen fixing capacity of cyanobacteria makes them economically important [3, 4]. These organisms are also important as model organisms to investigate various biological processes like photosynthesis, nitrogen fixation, biofertilizers and other biological processes [5, 6, 7]. The identification and classification of cyanobacteria remain a cumbersome and confusing task leading to uncertain identifications; this could be tackled with the use of molecular methods [8]. Polyphasic approach using different methods such as morphologic, physiologic and molecular methods using genomic DNA are proved to be appropriate in the identification and classification of Cyanobacteria. The success of the use of molecular methods lies on the efficient and reproducible method for the isolation of genomic DNA.

Genomic DNA isolation from filamentous cyanobacteria poses problems due to additional structures such as mucilaginous sheaths, slime, etc. The major hindrance in the DNA isolation of blue green algae is the lysis of the tough cell walls made up of polysaccharides which

requires enzymatic and mechanical disruption of the cell walls. Several methods are proposed for the isolation of DNA which often uses chemicals such as CTAB, PVP, PEG, etc. Some suggested methods without using these chemicals that are found to be causing irritation and other health problems [9].

The aim of the present study is to develop a simple method to extract DNA from live samples (Axenic cultures) of heterocystous filamentous cyanobacteria of Nostocales.

MATERIALS AND METHODS

Isolations of Blue green algal samples were obtained either from direct mass (colony) or soil isolations (Serial dilution method) from different districts of Kerala. The details of the isolates are given in Table 1. All these were grown and maintained in BG-11 medium [10] without the nitrogen source.

The strains were cultured under controlled conditions of light (12 h of light and darkness) conditions provided by cool white fluorescent lamps (3000 lux) and a temperature of 25±2°C. The cultures were periodically observed and sub cultured to maintain an axenic culture. The cells were harvested in the exponential stage (14^{th}- 20^{th} day) and used for the DNA isolation studies.

Isolation of DNA

Different methods were tried for the efficient isolation of genomic DNA from Cyanobacteria. Several difficulties were encountered either in the quantity or quality of DNA. We tried to develop a method for the efficient recovery of DNA by modifying the phenol free method [9]. The improved method is as follows:

Cells in the logarithmic growth phase were centrifuged at 12000 rpm for 5 minutes at 25°C and collected the pellets.

The pellets were washed with 1 ml of a solution containing 50 mM Tris HCl pH 8.0 and 50 mM EDTA.

Table 1: *Details of the Strains used for the Isolation of Genomic DNA and PCR Amplification.*

Sample Code	Locality	Source	Identity
MBG-AL111	Malampuzha canal, Palakkad	Colony	*Nostoc muscorum*
MBG-AL114	Kothamangalam, Ernakulam	Direct mass	*Anabaenopsis circularis*
MBG-AL115	Anthikad, Trissur	Direct mass	*Nostoc paludosum*
MBG-AL116	Thalappara, Malappuram	Direct mass	*Anabaena sphaerica*
MBG-AL113	Anthikad, Trissur	Isolated from soil	*Anabaena aphanizominoides*
MBG-AL212	Kuruvattur, Kozhikode	Direct mass	*Anabaena torulosa*

The pellets were then suspended in 200 µl of the same solution and the cells were lysed with the help of an ultra sound probe sonicator by sonicating for 2 minutes.

After sonication 500 µl of lysis buffer were added (50 mM Tris HCl pH 7.2, 50 m EDTA, 3% SDS and 1% v/v 2-β mercaptoethanol) and transferred to a fresh Eppendorf tube and incubated in 60°C water bath for 30 minutes.

The mixture was then cooled to room temperature and 700 µl Chloroform: isoamyl mixture (24:1) were added, mixed thoroughly to form an emulsion and centrifuged at 12000 rpm for 15 minutes, 25°C.

Upper layer was then transferred to a fresh Eppendorf tube and added equal amount of chloroform and centrifuged at 12000 rpm for 10 minutes at 4°C and the upper layer transferred to a fresh Eppendorf tube and 100% chilled ethanol was added through the sides and the mixture was kept in -20°C for 30 minutes.

The mixture was then centrifuged at 10000 rpm for 15 minutes at 4°C and the pellets were collected. Pellets were further washed with 70% ethanol twice and dried under vacuum.

The pellets were then suspended in 50 µl TE buffer. 6 µl RNase was added and incubated at 37°C for 1.30 hr in a water bath. 500 µl Phenol: Chloroform : Isoamyl mixture (24:25:1) was added to this and mixed gently and centrifuged at 10000 rpm for 10 minutes at 4°C, supernatant taken in a fresh Eppendorf tube. Add 500 µl Chloroform, mixed gently, centrifuged and supernatant collected.

To this supernatant double volume absolute alcohol and 1/1[1]°(50 µl) 3M Sodium Acetate was added and kept overnight at -20°C, collected the pellets by centrifugation at 10000 rpm for 15 minutes at 4°C, washed the pellet in 70% ethanol and re-suspended in TE buffer (50–100 µl).

Conventional DNA isolation method for plants using CTAB was also tried. Fresh samples in the exponential growth phase were centrifuged and ground, for the mechanical lysis of the cell walls grinding with liquid nitrogen and sonication were also tried. Fresh samples were used in case of blue green algae unlike the deep freezed or silica dried samples used in case of plant samples.

DNA Quantification

Genomic DNA was quantified by measuring optical density at 260 and 280 nm in UV Spectrophotometer (Systronics). The ratio of A_{260nm} to A_{280nm} was calculated to check the purity level.

The quantity of the DNA isolated was checked using agarose gel electrophoresis. 1 µl of gel-loading dye mixed in 5 µl of the DNA. The samples were loaded to 0.8% agarose gel prepared in 1X TAE (Tris-Acetate-EDTA) buffer containing 0.5 µg/ml ethidium bromide. Electrophoresis was performed with 1X TAE as electrophoresis buffer at 90 V until bromophenol dye front has migrated to the bottom of the gel. The gels were visualized in a UV transilluminator (in vitro Gen) and the image was captured under UV light using Gel documentation system.

PCR Amplification

PCR amplification of approximately 1.5 kb of 16S ribosomal DNA (rDNA) of the selected isolate was performed using EmeraldAmp® GT PCR master mix (Takara, Japan) in BioRad DNA thermal cycler (USA) with eubacterial primers 8–27F (*Escherichia coli* positions 8 to 27), and 1495R (*E. coli* positions 1495 to 1476) that were modified from primers fD1 and rP2 respectively [11]. The reaction mixture contained 50 ng of DNA, 1xEmeraldAmp® GT PCR master mix and 1 µl of 10 µM of each primer. PCR conditions consisted of an initial denaturation at 98°C for 1 mins; 30 cycles at 98ºC for 10 sec, annealing 58ºC for 30 sec and 72ºC for 1.30 sec; and final 10 mins extension at 72ºC. The amplification products were examined by agarose gel electrophoresis.

RESULTS

Obtaining pure culture is the pre requisite to acquire high quality genomic DNA. This was achieved by periodic sub culturing in liquid medium. Once the inoculation is done, the cells start to develop after 3 days. After which they start to grow fast by consuming the medium and split to form a large number. After 14 -20 days it was noticed that their growth depleted. The day between 14 and 20 is considered to be their logarithmic or exponential phase. After this the nutrient get depleted as such the growth get depleted at this stage there is the formation of akinites or the spores to tackle the adverse conditions. To ensure the quality and purity of DNA, cells in the logarithmic phase is selected for the isolation procedure.

The efficient isolation of genomic DNA by using the modified method was evident from the results. All the isolates used in this study showed $A_{260/280\ nm}$ ratio ranging from 1.5–1.8 (Table 1). The gel images also showed good quality of DNA (Figure 1), and also in the gel image there was only a little fluorescence in the loading walls indicating low polysaccharide, protein or phenol contamination.

Successful PCR amplifications were obtained by the 16S rDNA primers (Figure 2) indicating that the isolated DNA is suitable for PCR amplifications. The amplified products could be successfully sequenced.

Even though the CTAB method was found to be not effective for the isolation of DNA, when the mechanical lysis was replaced with sonication two out of the six samples used for isolation gave better results. The quality and quantity of the isolated DNA was not good enough (Table 2 and Figure 3) that the PCR amplifications were not successful.

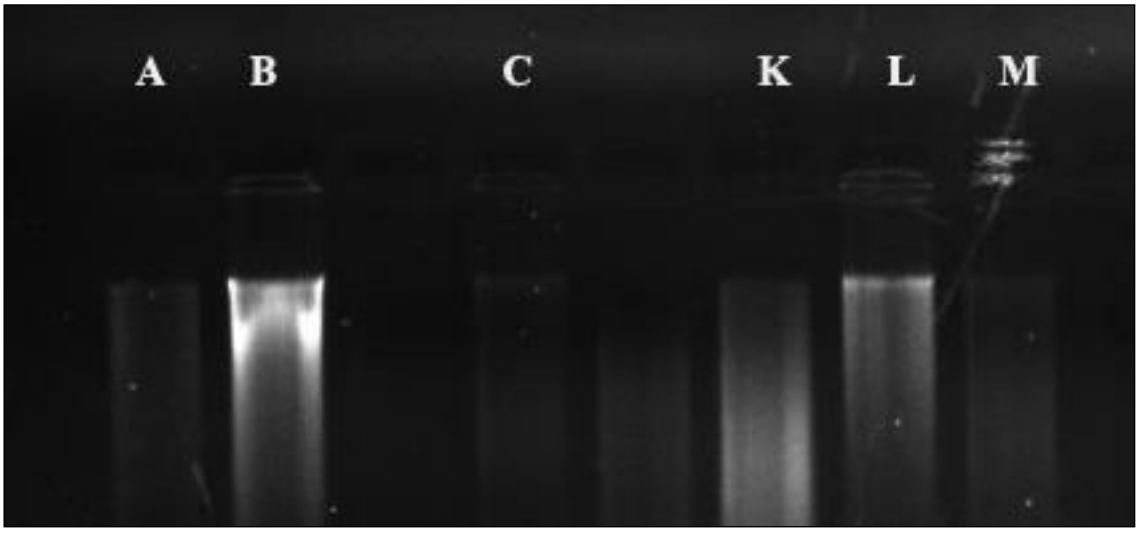

Fig. 1: *Agarose Gel Image of Isolated DNA using the Modified Method, Lanes A—N. muscorum, B—N. humifusum , C—N. paludosum, K—A. sphaerica, L—A. aphanizomenoides and M—A. torulosa.*

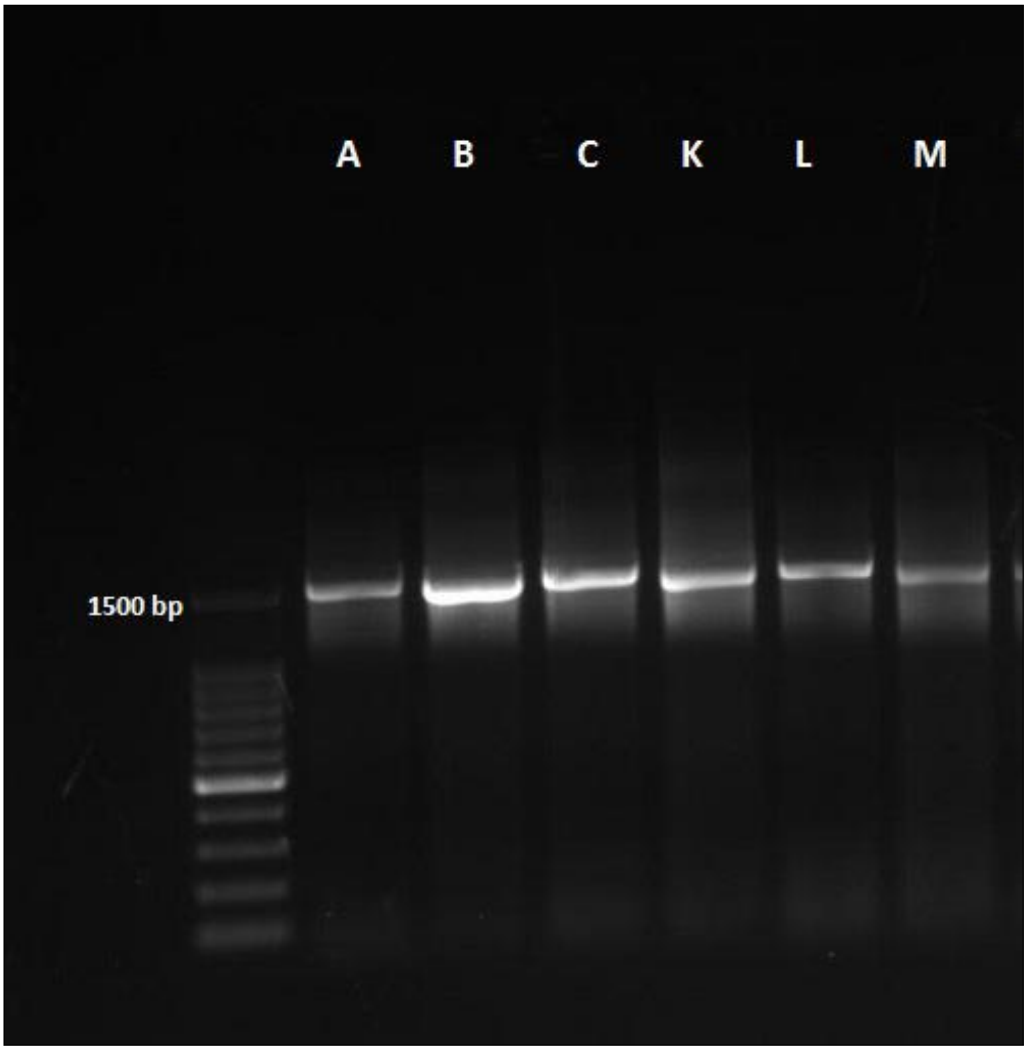

Fig. 2: *Specific Amplification of 16S rDNA (1.5 kb) of Extracted Genomic DNA Electrophoresed in 1% (w/v) Agarose Gel.*

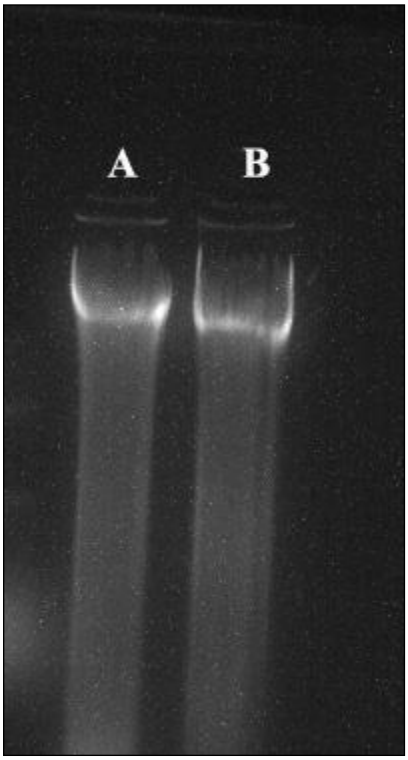

Fig. 3: *Agarose Gel Image of the Isolated DNA by the Conventional CTAB Method. Lanes A—N. muscorum and B—N. linckia.*

Table 1: *Quantitative Determination of Genomic DNA from the Selected Species.*

Organism	**A_{260}**	**A_{280}**	**$A_{260/280}$**
Nostoc muscorum	0.011	0.007	1.57
Nostoc humifusum	0.007	0.004	1.75
Nostoc paludosum	0.011	0.007	1.57
Anabaena sphaerica	0.008	0.005	1.6
Anabaena aphanizomenoides	0.018	0.010	1.8
Anabaena torulosa	0.025	0.016	1.56

Table 2: *Quantitative Determination of DNA Isolated by the Conventional CTAB Method.*

Organism	**A_{260}**	**A_{280}**	**A_{260}/A_{280}**
Nostoc muscorum	0.0083	0. 0060	1.38
Nostoc linckia	0.0059	0.0039	1.5

A comparison between the Phenol free method and the new modified method is given in Table 3.

DISCUSSION

One of the major steps in the isolation of DNA from blue green algae is the effective break down of the cell walls as such to expose the protoplasm and nucleic acid materials. In this new method, two major problems involved in the DNA isolation from cyanobacteria, i.e., poor cell lysis [12] and poor quality of the isolated DNA could be tackled. Blue green algae possess exo-polysaccharide cell wall similar to that of the bacteria. There are different views regarding the methods for the cell lysis, some are in a view that a pure enzymatic method resulted in a poor quality of DNA [13] while some others proved that mere enzymatic method could result in a RNA free DNA [14]. In contrast majority are of the opinion that mechanical lysis along with enzymatic disruption aided in the isolation of good quality DNA [15, 16]. In this paper cell lysis was achieved with sonication along with chemical treatment. This method was devoid of the use of any lysozyme and liquid nitrogen and resulted in a lesser amount of debris.

A variety of methods for the isolation of DNA is being proposed by many workers using an array of chemicals for the mechanical lysis which includes CTAB, SDS, xanthogenate –SDS mercaptoethanol, etc. [17,18] all these chemicals are known to enhance the cell lysis. The new method proposed here is a modification of the method proposed by Singh *et al.,* 2012 where cell lysis was achieved by adopting different methods like crushing with silica gel, homogenizing with glass beads along with sonication simultaneously even though the silica helps in enhancing the cell lysis it may cause shearing of high molecular weight DNA, this step was modified in the present protocol where, the sonication was adopted in the first step itself which helped in the efficient cell lysis and recovery of the nucleic acid materials. The β-mercaptoethanol used for the cell lysis are also responsible for the denaturation of endogenous RNase, thus there is an increased possibility of RNA contamination [19]. This effect of the mercaptoethanol could successfully overcome with the use of RNase treatment and the phenol step eliminated the traces of protein also. Ethanol precipitation for 30 minutes at -20ºC was found to be important for the precipitation of DNA which was in contrast to high salt concentration used for the precipitation of DNA in the presence of isopropanol [20]. The new protocol developed in this study isolated good quality genomic DNA with less degradation; the gel images further revealed the less degradation in the form of shearing.

Table 3: *Comparison of Phenol Free Method and the New Modified Method.*

Sl. No.	Phenol Free Method	Modified Method
1. .	Freshly grown cultures, centrifuge	Freshly grown culture centrifuged
2.	Pelleted cells washed with washing buffer (50 mM Tris HCl pH 8.0, 50 mM EDTA, 50 mM NaCl)	Pellets washed with solution containing (50 mM Tris HCl pH 8.0, 50 mM EDTA)
3.	Crushed with 100 mg Silica gel	Suspended in 200 µl of the same solution
4.	Homogenised using sterile glass beads on a vortex mixture for 5 min	Sonication under pre chilled conditions
5.	Homogenate centrifuged	Suspension was added with 500 µl lysis buffer
6.	Supernatant containing impurities decanted	Incubated in 60ºC water bath for 30 minutes
7.	Pellets washed with washing buffer	Mixture cooled to room temperature
8.	Suspended in 200 µl	Added 700 µl Chloroform: Isoamyl alcohol mixture, mixed to form emulsion
9.	Sonication under prechilled conditions, suspension added with 500 µl lysis buffer	Centrifugation
10.	Incubated at 60 ºC waterbath for 30 min	Upper layer transferred to fresh micro centrifuge tube
11.	Mixture cooled to room temp	Added equal volume chloroform and mix
12.	Added 700 µl Chloroform: Isoamyl alcohol, mixed to form emulsion	Centrifugation
13.	Centrifuge	Collect supernatant
14.	Upper layer transferred to sterile centrifuge	Added 500 µl chilled ethanol
15.	Added equal volume chloroform and mix	Kept in -20ºC for 30 minutes
16.	Centrifugation	Centrifugation
17.	DNA precipitated by adding 500 µl chilled ethanol	Collect pellets
18.	Precipitate centrifuged	Washed with 70 % ethanol
19.	Pellets collected	Dried under vacuum
20.	Washed with 500 µl 70% ethanol	Pellets suspended in 50 µl TE buffer
21.	Suspended in 50 µl TE buffer	Added 3–6 µl RNase and incubate at 37ºC for 1.30 hr
22.		Added 500 µl Phenol:Chloroform: Isoamyl alcohol, mix well
23.		Centrifuged and collected supernatant
24.		Added 500 µl chloroform, mix and centrifuged
25.		Supernatant collected and added double volume absolute alcohol and 50 µl 3M sodium acetate
26.		Kept over night
27.		Collect pellet by centrifugation
28.		Wash pellet in 70% ethanol
29.		Re suspend the pellet in 50 µl TE buffer
Advantage	Contamination of RNA and Protein	Good quality DNA free from RNA and Protein

CONCLUSION

An efficient method for the isolation of genomic DNA from the pure cultures of filamentous blue green algae *Nostoc* and *Anabaena* is proposed which includes a mechanical lysis along with chemical treatment for the cell breakage and RNase treatment with phenol extraction to prevent any RNA and protein contamination.

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Isolation and axenic culture of nostocales (*Nostoc* spp. and *Anabaena* spp.) of Kerala

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ABSTRACT: *Blue green algae (Cyanobacteria) are the ubiquitous pioneer autotrophic organisms among the Plant Kingdom that represent an interesting group as capable of both Photosynthesis and N2 fixation simultaneously. The aim of the present study was to isolate, identify and characterise the heterocystous cyanobacteria (Nostoc spp. and Anabaena spp.) occurring in the rice fields of the Malabar region of Kerala (Kozhikode, Kannur, Malappuram and Thrissur Dist.) The samples were collected from the field as blue green mass or soil samples. From soil the algae were isolated using serial dilution and streak plate method. The medium used in this study was BG11 medium (Rippka et al. 1979) with the omission of NaNO3. Solid medium was found ideal for the long term maintenance. Unialgal cultures were raised. In the liquid media the cultures were examined regularly to check the purity of the culture. The organisms were identified using standard manuals and by consultation with expert algologists. After identification the isolated colonies were subcultured. 6 pure cultures were raised viz; Anabaena torulosa, A. variabilis, A. oscillrioides, Nostoc muscorum, N. linckia, N. spongiaeformae. The distinction of these genera based on morphological feature is difficult due to morphological plasticity in nature as well as in cultures. Majority of the species were distinguished based on the characters of hormogonia and spore morphology but in culture conditions the sporulation was not very common. To solve such problems molecular tools are being used.*

Key words: *Blue-green algae, Axenic, Hormogonia, BG-11*

Introduction

Algae are the simplest members of the Plant Kingdom, and the Blue Green algae are the simplest among them. Cyanobacteria (until recently being known as blue green algae) owing to its bacterial characters such as peptidoglycan cell wall, prokaryotic nature, etc. are ubiquitous organisms, capable of photosynthesis and nitrogen fixation simultaneously. They are more related to eubacteria in their structure and chemical composition. The division Cyanophyta includes a large number of algae which are characterised by a low state of cell organisation. The cell lacks the well defined nucleus in contrast with other algae which are typically eukaryotic (Desikachary 1959).

Cyanobacteria fix atmospheric nitrogen and convert it into ammonia with the help of nitrogenase enzyme produced in specialised cell called heterocyst. The heterocystous cyanobacteria thus play an important role in enhancing the soil fertility. The role of algal inocula in the rice fields as bio-fertilizer was studied by Venketaraman (1979).

Various methods have been adopted to efficiently isolate and purify the freshwater blue-green algae by Ferris and Hirsch (1991). Among different factors that influence the growth of blue green algae the pH of the medium plays a major role. The growth and nitrogen fixing potential of acid tolerant cyanobacteria were investigated by Madhusoodanan and Dominic (1996). The present study aims the isolation, characterisation and axenic culture of *Nostoc* and *Anabaena* species occurring in the rice fields. The major distinguishing feature of the heterocystous cyanobacteria is the position of spores. But in culture conditions these members do not show sporulation in common. So their species distinction is difficult. To solve such problems the use of molecular tools will be helpful. For such molecular studies, axenic culture is a prerequisite. So this study is a preliminary step towards the molecular characterisation.

Materials and methods

Collection of Samples

The samples were collected from different areas of Thrissur, Malappuram, Kannur and Kozhikode either as soil samples or as blue green (mucilaginous) algal mass. The collected materials were isolated for which BG.11 medium (Rippka et al. 1979) with the omission of nitrogen source was used. The isolation techniques include direct isolation, by inoculating the samples on the medium while that of indirect method include the inoculums prepared by serial dilution method from the soil. In both the cases, they were inoculated on agar plates containing 8 gm/l agar. The growth of algae was visualised by naked eyes by their gliding movement and phototactic migration in the agar plate. Once growth noticed in solid medium, they were inoculated to liquid medium. The liquid media were examined periodically for any contamination. The medium were autoclaved at 121 lbs pressure for 20 minutes before autoclaving the pH were adjusted to 7.2 ± 2 with 1N HCl. The cultures were maintained in conditions of 23 ± 2^0 C under a 12/12 hr photoperiod supplemented from white fluorescent lamps. The unialgal cultures are raised by continuous sub culturing. The purity of the cultures was checked under light microscope. The unialgal stocks were maintained in 250 ml Erlenmeyer flasks with 100 ml $BG_0$11 medium (nitrogen free) and also in agar plates for long term maintenance.

Results

12 samples were collected from different paddy fields of Kerala in which 3 species of *Nostoc* and 3 species of *Anabaena* could be identified upto species level. The *Nostoc* spp. are;

***Nostoc linckia* (Roth) Bornet ex Born.*et* Flah.** (Fig. 1 A)

Thallus varying size, globose later irregularly expanding, blue-green to blackish green, filaments densely entangled. Cells short barrel shaped, heterocyst sub spherical; spores sub spherical, 6-7 µm broad.

***Nostoc muscorum* Ag.ex Born.*et* Flah.** (Fig. 1 B)

Thallus membranous, olive green or brown, trichome 3.5- 5 µm broad, cells variable in shape, spores oblong, many in chains 4-5 µm broad.

***Nostoc spongiaeforme* Agardhex Born.*et* Flah.** (Fig. 1 C)

Thallus gelatinous, globose at first trichome 4-5 µm broad, cells elongate-cylindrical or barrel shaped 5-6 µm long. Spores seen as a chain away from heterocyst. Heterocyst terminal or intercalary. These were distinguished based on position of spores and the nature of trichome.

The three Anabaena species isolated and identified were:

***Anabaena oscillarioides* Bory ex Born. *et* Flah.** (Fig. 1 D)

Thallus dark green, cells barrel shaped end cells rounded, spores on both side of the heterocyst at first oval later rounded- cylindrical, 8-9 µm broad.

***Anabaena torulosa*(Carm.) Lagerh. ex Born. *et* Flah.** (Fig. 1 E)

Thallus mucilaginous, thin, pale blue green, trichome 4-5 µm broad, apical cell acutely conical, cells barrel shaped, heterocyst ovoid, spores on either side of the heterocyst sub-cylindrical with rounded ends.

***Anabaena sphaerica* Bornet. *et* Flah.** (Fig. 1 F)

Thallus soft gelatinous, blue green, trichome straight 4-6 µm broad, cells barrel shaped, end cells rounded, heterocyst sub spherical, spores on either side of the heterocyst, spherical to oval in shape.

The generic identity and distinction of *Anabaena* from *Nostoc* has always been cumbrance owing to their plasticity, which varies according to the habitat characteristics such as temperature, salinity, pH, etc. Hence, an attempt is initiated inorder to distinguish these genera based on all possible taxonomic evidences including the cellular features, hormogonial characters, pigment content, etc.Culture characteristics of the Cyanobacterial species in BG_0-11 medium are given in Table 1.

In the present work three species of *Nostoc* and three species of *Anabaena* isolated from paddy fields of Kerala and 12 more species are yet to be identified owing to obscure characters.

Table1: Culture characteristics of the Cyanobacterial species in BG_0-11 (nitrogen free) culture medium- both solid and liquid

Sl. No.	Cyanobacterial species	Place of Collection	Time taken (days)		Thallus characteristics
			Visible Colony	Complete spreading on agar plate	
1.	*N. linckia* (Roth) ex. Bornet. *et* Flah.	Kozhikode	10-12	30-35	Globose colony, blue green to dark green colour
2.	*N. muscorum* Ag. ex Born. *et* Flah.	Thrissur kole field	6-8	30	Olive green to brownish colonies spreading the entire plate
3.	*N. spongiaeformae* Agardhex. ex Born. *et* Flah.	Payyannur Kannur	7-8	30	Olive green to blue-green colonies
4.	*A. oscillarioides* Bory ex Born. *et* Flah.	Kozhikode	7	28-30	Blue green colonies form as a mat on the agar surface
5.	*A. torulosa* (Carm.) Lagerh. Ex Born. *et* Flah	Malappuram	7	28-30	Blue green colonies on the surface spreading. Distinguished from *A. oscillaroides* by the shape of spores
6.	*A. sphaerica* Bornet. *et* Flah.	Thrissur	5-6	28-30	Mucilaginous blue-green mass formed on the surface of the agar. In the liquid medium the colonies found floating

Discussion

Heterocystous cyanobacteria have been already studied for their diversity in rice fields of Kerala by Umamaheswari (2005) but limited works have been reported. The genus *Nostoc* is being distinguished from *Anabaena* by the coiling or wavy nature of the hormogonia and shape of the heterocysts. *Nostoc* spp. showed more coiling whereas the *Anabaena* hormogonia are rather straight or wavy and with the heterocysts ovoid or elongated which was in accordance with Desikachary (1959). The morphology of strains could not be considered for the distinction of genus because at certain times environmental conditions and culture conditions also affect the morphology.

Morphology of strains may change depending on environmental conditions and the diversity of the strains can be altered by selective culture conditions (Palinska et al. 1996).

Simple thallus organisation of cyanobacteria, except for the presence of unique structures like heterocyst and akinite based on which the classification is done is a limiting factor. The existing classification is inadequate to delimit the species (Roger 1991). In these contexts, the use of molecular markers for the systematic studies becomes important. Molecular studies have undertaken by Rajaniemi et al. (2005) in which they separated *Nostoc* strains from *Anabaena* strains using molecular marker such as 16s rRNA. Prasanna et al. (2006) differentiated *Anabaena* strains by employing molecular markers. For such molecular studies the prerequisite is the unialgal cultures from which DNA could be successfully extracted. Such works are in progress in the laboratory.

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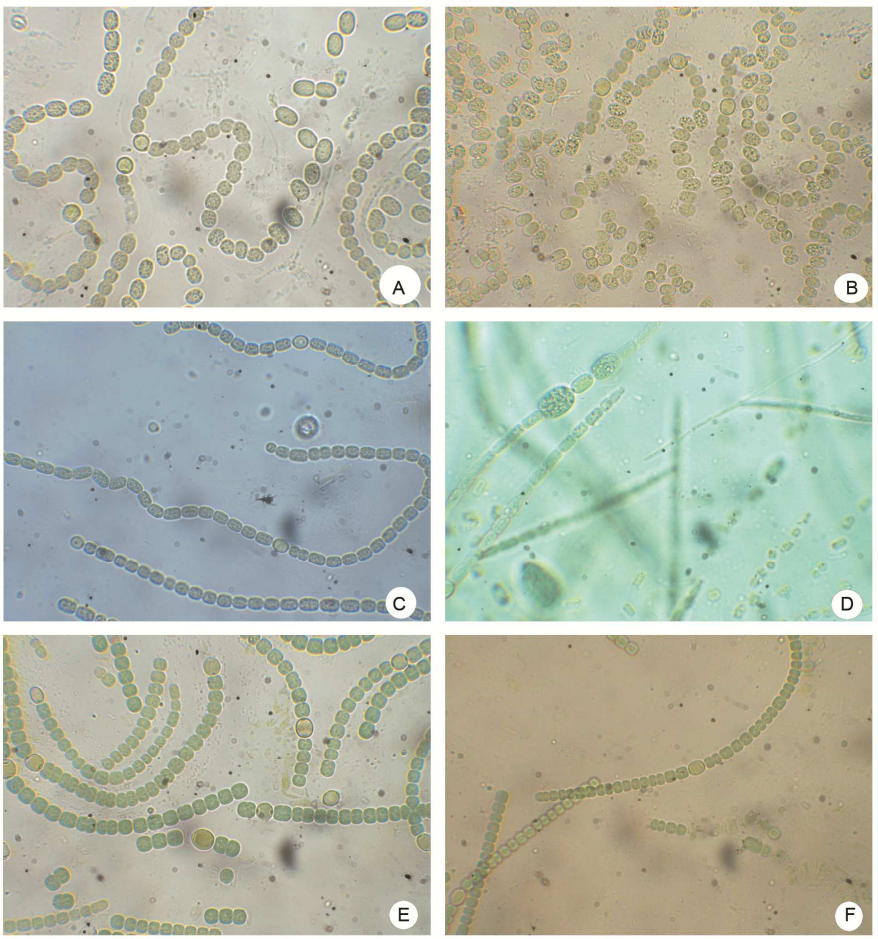
Figure 1: A. *N. linckia,* B. *N. muscorum*, C. *N. spongiformae*, D. *A. oscillaroides*, E. *A. torulosa*, F. *A. sphaerica*



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In vitro conservation and propagation of the endemic species of "floating hearts" (*Nymphoides krishnakesara* Joseph and Sivar. - Menyanthaceae) as a conservation strategy

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ABSTRACT

Nymphoides krishnakesara Joseph and Sivar. (Menyanthaceae), an endangered aquatic angiosperm, endemic to Kerala, was multiplied and propagated through *in vitro* culture. It is a rare species with very restricted distribution occurring in a single location. It is an annual herb, grows in shallow temporary pools on laterite. The nodal explants when cultured in Murashige and Skoog (MS) medium attained bud break in 20 days. Maximum multiple shoot proliferation observed in 1.0 mg/L benzyl amino purine (80 shoots/explant). Shoots developed *in vitro* were rooted in MS medium with both indole 3-butyric acid (IBA) (1.0 mg/L) and naphthalene acetic acid (mg/L). Roots developed in IBA are found to be more favorable based on the histological studies. The rooted plantlets were then transferred to the field after hardening and they flowered after 2 months. Total time taken from explants to flowering is 10 months. This work standardizes an easy protocol for mass production of plantlets, and thus enhances conservation of this endemic and rare aquatic plant. The hardened plants were successfully reintroduced and recorded 100% survival.

1. INTRODUCTION

India is rich in its aquatic flora, of which majority comprises from South India. Among the aquatic plant diversity *Nymphoides* Seg. is an interesting genus of about 20 spp. [1], widely distributed in the tropical and temperate regions of both the Old World and the New World. *Nymphoides krishnakesara,* an endemic emergent herb found in shallow waters of seasonal ponds of lateritic hills, is an interesting dioecious plant [2]. *N. krishnakesara* was originally reported from Madayipara, a midland lateritic hill in Kannur District, Kerala, South India [2]. Habitat of the species is threatened due to environmental modifications and urbanization. Its occurrence in a single location made this plant endemic to Northern Kerala and also included in the IUCN Red list of threatened species version 2011 [3]. The plant being dioecious and unavailability of male and female plants in the same location restricts the natural propagation through seeds. In traditional folklore medicine, the flowers and roots of this plant are used as a febrifuge [4].

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The genus can be easily distinguished from the similar looking water lilies (*Nymphaea* spp.) by its petiole-like uniphyllous sympodial branches bearing a cluster of flowers at the nodal region. Only few works were undertaken on the micropropagation of *Nymphoides* spp. The genus *Nymphoides* is a less exploited one. A protocol for rapid shoot organogenesis from petiole explants of the ornamental aquatic plant *Nymphoides indica* L. [5] and indirect regeneration of *Nymphoides cristatum* floral buds [6,7] are some works on the genus. The application of tissue culture as a tool for the conservation of rare and endangered plants has gained huge trust in the recent decades. The present study was undertaken to formulate a standard protocol for micropropagation of the endangered and endemic species *N. krishnakesara* Joseph and Sivar. and its introduction to the field, thereby helping habitat restoration.

2. MATERIALS AND METHODS

2.1. Bud Break and Proliferation

A healthy growing plant in the Aquagene (Aquatic Plant Conservatory of Malabar Botanical Garden and Institute for Plant Sciences) introduced from the original locality is used as the explant source. Nodal cuttings from fresh sprouts (3rd and 4th leaf) were used as explants [Figure 1a]. The leaves were collected, washed in running water for 25 min and the nodal region was separated and treated with Tween 20 (2–3 drops in 1 L distilled water) for 15 min followed by washing with double distilled

water 3–5 times. Surface sterilization of explants was done with 0.1% (w/v) $HgCl_2$ for 2–3 min and washed thrice with sterile double distilled water before inoculation inside the laminar air flow chamber. The nodal segments were trimmed into two equal vertical halves of about 1 cm long aseptically and used directly as the explant. The surface sterilized explants were inoculated in Murashige and Skoog (MS) basal medium [8] and MS medium with combinations of auxins (indole 3-butyric acid [IBA]*, naphthalene acetic acid [NAA]**) and cytokinins (benzyl aminopurine [BAP]***) were tried for shoot or root induction. Once shoot formation was noticed, they were subcultured in the rooting medium with auxin after the formation of roots and they were transferred to basal MS medium containing low concentration (4.5 mg/L) of agar. After 2–3 weeks, the fully developed plants were transferred to pots with sterile clay and kept in the greenhouse for hardening.

The media used were fortified with 3% sucrose and the pH adjusted to 5.8, before the addition of agar and followed by autoclaving at 121°C for 20 min. The cultures were maintained at 25 ± 1°C with 70% relative humidity and a photoperiod of 12/12 h 35–40 µ/mol m^{-2} s^{-1} irradiance provided by cool white fluorescent tubes.

2.2. Anatomical Studies

The roots developed *in vitro* were investigated anatomically. Hand sections of root formed in medium supplemented with 0.5 mg/L IBA and 0.5 mg/L NAA were taken, stained with 1% safranine and observed under microscope and photographed.

3. RESULTS AND DISCUSSION

3.1. Establishment of Explants and Bud Initiation

The nodal segments inoculated on basal MS medium showed bud break after 20 days. After the bud break, direct regeneration of a single shoot was noticed on the 30th day of inoculation in MS medium without any PGR [Figure 1b]. Once the regeneration is noticed, they were transferred to the multiplication medium. Different concentrations (0.25–1.0 mg/L) [Figures 2 and 3] of cytokinins (BAP) were tried for shoot proliferation. Highest shooting percentage (90%) with maximum shoot proliferation (80 shoots/explant) is attained in medium supplemented with 1.0 mg/L BAP [Figure 1c]. 0.25 and 0.5 mg/L BAP gives 20 and 50 shoots/explant and 60% and 80% of shooting response, respectively. Initially, the concentration of agar used was 6.5 g/L in which the *in vitro* shoot development showed stunted growth with brittle leaves [Figure 1d]. A combination of BAP and IBA both 0.5 mg/L showed rhizogenesis after 10 days along with multiple shoots where the shooting percentage noticed was the same as that of 0.5 mg/L fortified medium.

3.2. Effect of Auxin in the Development of Roots

The *in vitro* regenerated shoots were excised and transferred to rooting medium with different concentrations (0.5 and 1.0 mg/L) [Table 1] of auxins (IBA and NAA). Both IBA and NAA were found to be effective in rhizogenesis. *In vitro* developed shoots when subcultured in a medium with 0.5 mg/L NAA showed rhizogenesis after 5 days of inoculation [Figure 1e] with an average of 14 roots while that of media fortified with 0.5 mg/L of IBA produced roots after 1 week [Figure 1f] with an average of five roots. The average number of roots were high in NAA and the number showed a considerable increase when the concentration of NAA was raised to 1 mg/L. There was no callus formation at the base, and hence, the rhizogenesis is direct. Highest rooting percentage was noticed in medium fortified with 1.0 mg/L NAA with an average of 24 roots.

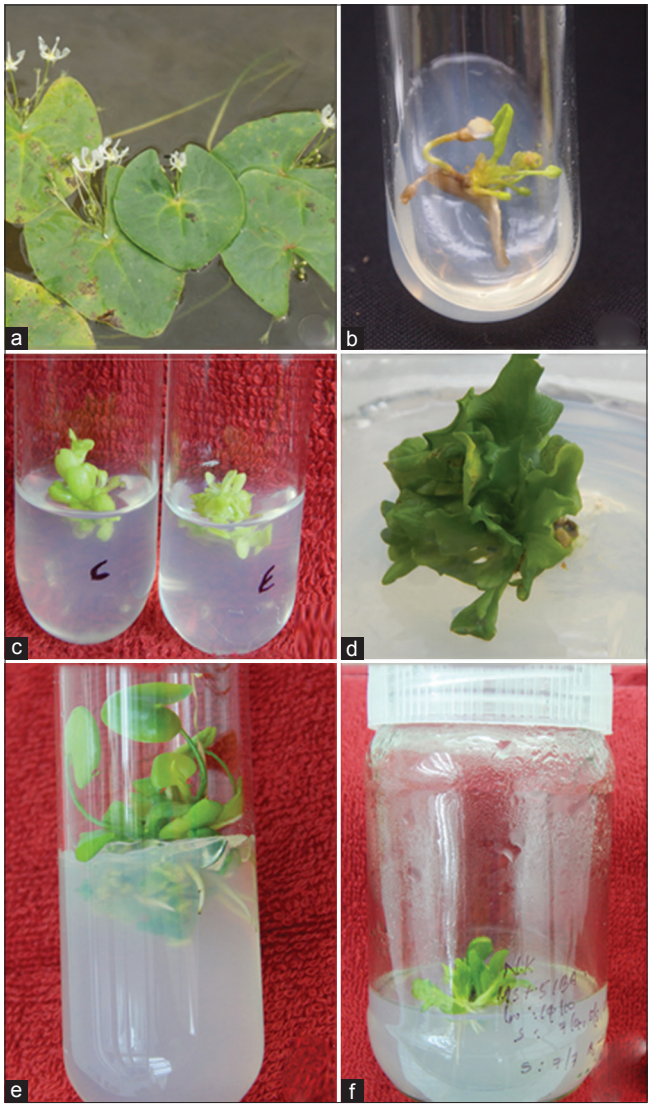

Figure 1: (a) Habit, (b) direct organogenesis, (c) multiple shoot formation in 1mg/L benzyl amino purine, (d) short and brittle leaves in 6.5 mg/L Agar, (e) induction of roots in 0.5 mg/L Naphthalene acetic acid. (f) induction of roots in 0.5 mg/L Indole 3- butyric acid.

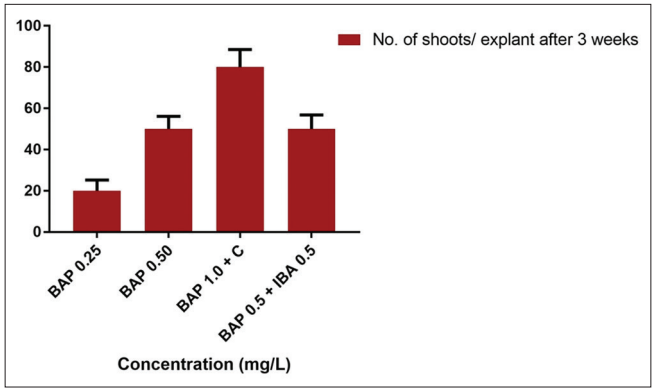

Figure 2: The number of shoots in different concentration of benzyl aminopurine.

Table 1: Effect of different concentration of auxins promoted rooting of the plant.

Hormone	Concentration	Root induction (%)	Rooting in number of days	Number of roots
IBA	0.5	80	7	5
	1.0	80	7	10
NAA	0.5	70	5	14
	1.0	80	7	24

IBA: Indole 3-butyric acid, NAA: Naphthalene acetic acid.

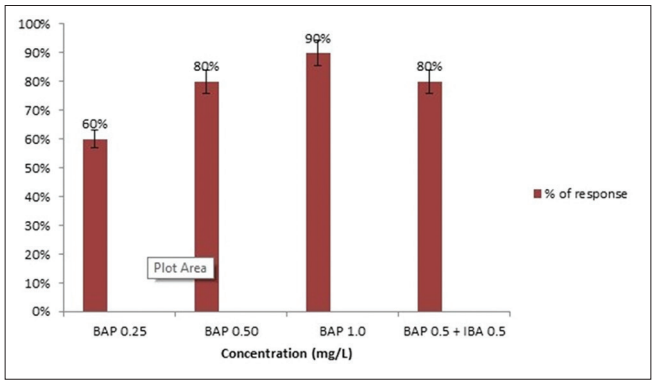

Figure 3: Percentage response of shoot formation in different concentration of benzyl aminopurine.

The roots formed in NAA were soft, spongy, and numerous while the roots formed in IBA were strong with lateral roots. Once the roots were formed, they were transferred to medium with low concentration of agar (4.5 g/L) for the better absorption and root development [Figure 4a and b]. In this concentration of agar, the normal leaf with long petiole, which helps the lamina to float on the water surface, was developed. Among the plants developed *in vitro,* those developed roots in medium containing IBA showed better establishment under field conditions.

3.3. Acclimatization of Plantlets Developed *In Vitro*

For the hardening process, the individual plantlets were separated from the medium and cleaned with sterile water to get rid of the excess agar [Figure 4d]. The plants were first transferred to sterilized tap water and kept in culture conditions [Figure 4c]. After 2 weeks, they were planted in cups with sterile clay with 1 cm deep water and kept in the greenhouse [Figure 4e].

The plants developed *in vitro* flowered within 2 months of introduction to the field [Figure 4f]. Of these, the plants rooted in medium with IBA showed better development in the field conditions, and initially, these were smaller in size than the normal ones.

3.4. Histological Studies

In histology, the root developed in NAA has more number of aerenchyma (air spaces) in the cortical region than that of the roots developed in the medium with IBA [Figure 4h and i]. The C.S. of the control root also showed a lesser number of aerenchyma [Figure 4g] indicating that lesser number of aerenchyma favored better establishment of roots in field conditions.

4. DISCUSSION

The aquatic systems in Kerala currently face a serious threat of extinction due to rapid urbanization and industrialization. Developmental initiatives by filling wetlands have seriously affected the rich aquatic biodiversity of Kerala which include several endemic and endangered species, many of which are reported from transient pools developed on the laterite hills of midland region during the rainy seasons but become dry during the summer season. *N. krishnakesara* is reported from such a unique habitat which survives the summer drought through dormant shoots and regenerates during next monsoon with vigor. Since the plant is a dioecious one, seeds develop only when both the male and female plant exists in the same region. This taxon is highly endemic and reported from only one locality in the Northern Kerala.

Only few works were undertaken on the micropropagation of aquatic plants. Of these, major works were on the micropropagation of medicinal plants such as *Bacopa monnieri* [9,10] and *Acorus calamus* [11,12]. For *in vitro* clonal propagation, the common explants used are the nodal segments. In the present study also, the explant selected was the nodal region, from where direct organogenesis is achieved, similar result was obtained in *N. indica* [5] also, while floral buds of *N. cristatum* produced friable callus from which organogenesis was achieved [6,7]. In this study, even though growth regulator-free MS medium was able to induce bud break and shoot formation, the number of shoots formed from single explant was found to be less in number. BAP was the single cytokinin used for the multiple shoot formation. Several reports point out the capacity of BAP for bud proliferation and multiple shoot formation in many plants such as *B. monnieri* [12] and *Avicennia marina* [13]. Averages of 80 shoots were obtained from 2-week-old cultures in medium with 1.0 mg/L BAP while 60 shoots were obtained from per piece in 50 days of culture in *Passiflora caerulae* L. [14].

Two auxins (IBA and NAA) were tried for the root induction, both showed favorable results. It is reported earlier that the auxins at lower concentration facilitate better root formation [15]. Even though the time taken for rhizogenesis in MS medium with NAA was less and produced more roots, the roots produced in IBA were strong both in morphological and anatomical studies. Similarly, the increase in the rooting percentage and the better rooting in the medium containing IBA were reported in *Alnus glutinosa* [16]. Earlier reports indicate that NAA also induces callus tissue; hence, establishment of plants in the field is hindered by the interfering callus tissue [17,18].

The institute has undertaken several studies on different aspects of the aquatic vegetation of South India with a view to develop a comprehensive conservation protocol for aquatic and wetland plants of the country. The study also involves micropropagation of aquatic plants of rare, endangered, and threatened category through *in vitro* cloning. The present study facilitates an easy protocol for the production of rooted multiple shoots up to 80 from a single explant in basal MS medium supplemented with 1.0 mg/L BAP. It is therefore established that the most reliable way for rapid clonal propagation of *N. krishnakesara* is through direct organogenesis.

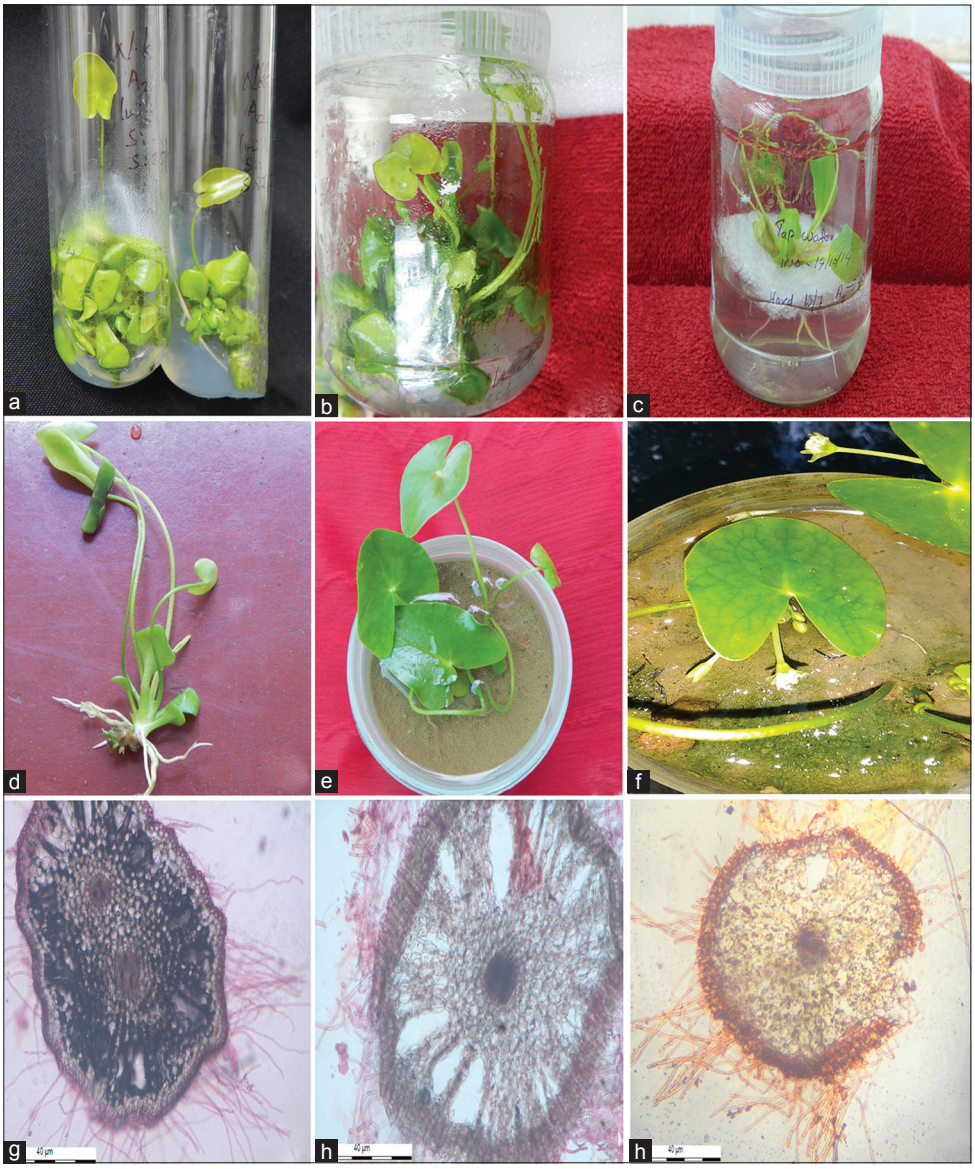

Figure 4: (a and b) Normal leaves in 4.5 mg/L agar, (c) hardening in tap water, (d) plantlet before planting, (e) plant in greenhouse for hardening, (f) flowering in field, (g) C. S. of control root, (h) C. S. of root in Naphthalene acetic acid and (i) C. S. of root in Indole 3- butyric acid showing lesser number of air chambers.

5. CONCLUSION

In the present situation, there is a great need for the conservation of aquatic plants since the rapid urbanization has led to the loss of our valuable aquatic and wetland habitats. In this context, the present study suggesting a simple protocol for micropropagation of rare and endemic aquatic plants owes its importance. This study is the first attempt on the micropropagation and the successful field establishment of the endemic plant *N. krishnakesara* Joseph and Sivar.

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In vitro Responses of *Indigofera tinctoria* Linn. (Neela Amari)

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Abstract

Indigofera tinctoria is an important medicinal plant of South and South-East Asia. These plants showed a low germination rate and scanty rooting of seedlings in conventional propagation. The tender leaves showed callus formation in MS medium with single auxin [Naphthalene Acetic Acid (NAA); 2, 4 Dichloro phenoxy Acetic acid (2, 4-D)]. Lower concentration of auxin showed better result. The explants showed callusing frequency of 95%. Even though both the auxins induced callus, they showed a difference in the days for response. Rhizogenesis along with callus was noticed only in NAA supplemented medium. It was noticed that NAA showed better result in case of initial callusing and also rhizogenesis.

Keywords: *In vitro, rhizogenesis, callus, explants, NAA*

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INTRODUCTION

Indigofera tinctoria Linn. (Neela Amari) of the family Papilionaceae is one of the important medicinal plants distributed in South and South-East Asia, Tropical Asia and also introduced in Tropical America. The root, stem and leaves are bitter, thermogenic, laxative etc. and are useful for promoting growth of hair. The plant yields a valuable dye stuff called indigo. Indigo has a long history as a dye. It was so valuable that it was called Blue gold. Newton named one of the colours of the spectrum after the colour of the dye. The indigo Revolt of 1858 in Bengal was considered by many historians as the precursor to the struggle for independence in India. The dye obtained is marketed as natural colouring dye. The plant is not palatable to cattle; it is grown as a cover or green manure crop in coffee plantations and rice fields in South India [7].

In Ayurvedic practice, *I. tinctoria* is considered as a reputed drug for the promotion of hair growth, and its antitoxic property makes it a good remedy for all poisonous infections and various skin diseases. It forms a major ingredient of Ayurvedic preparations such as Neelibringadi oil, Neelithulasiyadi kashayam etc. It is a source of dye indigo which is used to colour silk and other textiles. The juice of the young branches mixed with honey is recommended as application for the ailments of the mouth of the children [6]. The plant is a legume, so it is rotated into the fields to improve the soil nitrogenous reserves.

The leaves of the plant and indigo have been used in hepatitis, epilepsy and other nervous affections. The leaves rubbed in water and applied to abdomen are efficacious in promoting urine. The root when boiled in milk is used as a purgative, and a decoction of the stem used as gargle is considered efficacious in mercurial salivation. The plant is popularly regarded in parts as preventive of hydrophobia and administrated internally in infections and the juice is applied internally to the part in insect bites [4]. The plant contains rotenoids, deguelin, dehydrodegueli, rotenol, rotenone, tephrosin and sumatrol.

The conventional propagation of this plant is not in pace to meet the need by pharmaceutical industries due to low rate of germination, scanty and delayed rooting of seedlings. The objective of the present study was to develop a protocol for the *in vitro* callusing from the leaf explants.

MATERIALS AND METHODS

The leaf explants of *I. tinctoria* were collected from the plantlets grown in Chevayur, Kozikode East, India. Tender leaflets (especially 3rd–4th from the apex) were collected from the active flushes of *Indigofera.* The leaflets were washed under running tap water and washed with soap solution (Tween 20) for 5 min followed by rinsing in double distilled water for 3–5 times. Then these were surface sterilized using 0.1% mercuric chloride [$HgCl_2$ (w/v)] solution for 5–10 min inside the sterile hood and later thoroughly rinsed with sterile water thrice. Surface sterilized explants were cultured on MS medium supplemented with different concentrations of growth regulators. The growth regulators used for the study were two auxins, 2, 4-Dichloro phenoxy acetic acid (2,4-D), Naphthalene Acetic Acid (NAA) and cytokinine, 6-Benzyl Amino Purine (BAP). The details regarding the types and concentrations of growth regulators used are summarized in Table 1.

Table 1: *Concentrations and Types of Growth Regulators Used for Callusing for Indigofera tinctoria Leaflet.*

Plant	Explant	Basal medium	Auxin	Con. (mg/l)	Cytokinin	Con. (mg/l)
Indigofera tinctoria	Tender leaflet	MS	2,4-D	2	BAP	0.5
				4		
			NAA	2	BAP	0.5
				4		

The pH of the prepared medium was adjusted to 5.7±0.1 with the help of an electronic pH meter (MS Electronics India Pvt. Ltd) using 0.1 N HCl or 0.1 N NaOH. The prepared media was solidified by adding 0.6% agar (Himedia Laboratories, Bombay, India).

Culture Conditions

The cultures were incubated under controlled conditions of temperature, light and humidity in the culture room provided with culture racks. For callusing, the tender leaf segments were maintained in complete darkness at a temperature of 25 ±1 ºC and relative humidity of 70%. The cultures were observed regularly at an interval of 5 days. The percentage of responding explants, nature of response including tissue enlargement, callusing, change in colour and texture of calli was noted. Photographs were taken using a Canon PowerShot 8515 digital camera in the department of botany. The fresh weights were recorded at an interval of 5 days and the growth index (GI) was calculated using the following formula:

$$\text{Growth Index} = \frac{\text{Final fresh weight} - \text{Initial fresh weight}}{\text{Initial fresh weight}} \times 100$$

Histological Study

Internal structure of callus was analysed for histological evidences. Squashes as well as hand sections of callus were prepared and stained with safranin and observed under microscope. Photographs were taken using a Motic Image Plus digital camera, connected to a high resolution microscope and PC at 100x.

RESULTS & DISCUSSION

Callus Induction

The tender leaf explants showed callus formation in MS media supplemented in presence of single auxin (NAA or 2,4-D) with BAP (0.5 mg/l). Two concentrations of auxins were tried and it was found that callusing occurred in a lower concentration (2 mg/l) of auxin only. The explants showed differential responses in two different auxins in terms of callus induction, callus growth and GI. Of the two auxins 2, 4-D and NAA both showed similar responses with respect to callusing frequency (number of explants callused/total number of explant) which was almost 95% (Figure 1). The callus showed a high rate of proliferation. There was only very small rate of contamination and it was manageable. The green colour of the explants was retained after callusing.

Effects of NAA

MS medium in presence of NAA (2 mg/l) showed a callus induction within 5 days after inoculation (DAI). On 15 DAI, there was direct rhizogenesis from the callus. NAA showed higher response with respect to fresh weight increase and GI, than 2, 4-D. On 25 DAI, a fresh weight increase was observed from an initial explant weight of 0.0065 g to 2.632 g. On 35 DAI, fresh weight was increased upto 3.817 g. GI also showed a marked increase from 163.2% in 25 DAI to 281.7% in 35 DAI (Figure 2).

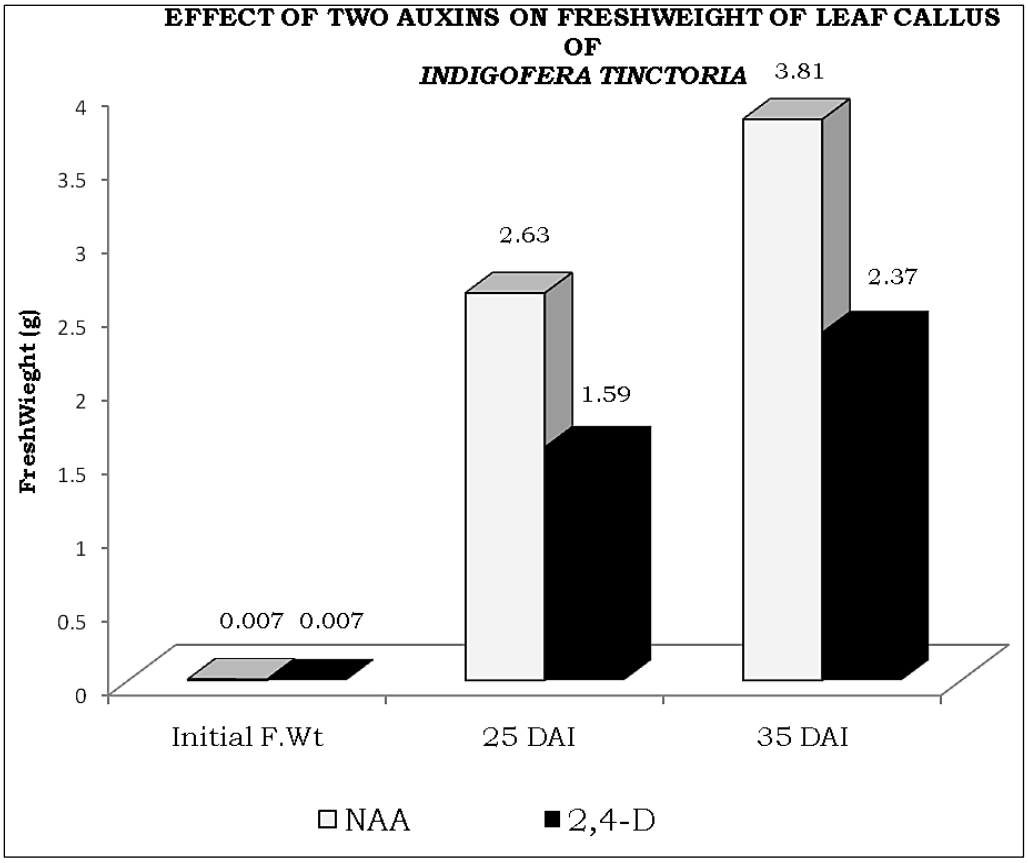

Fig. 1: *Effect of Two Auxins on Fresh Weight of Leaf Callus of Indigofera tinctoria.*

Fig. 2: *Callus on 35 DAI with in vitro Rhizogenesis.*

Fig. 3: *Callus Induction after 7 DAI.*

Effects of 2,4-D

In MS medium supplemented with 2,4-D (2 mg/l), the explants showed tissue enlargement and callus induction within 7 DAI (Figure 3). No rhizogenesis was noticed. 2, 4-D was found to be less efficient as compared to that of NAA in both callus induction and fresh weight increase.

On 25 DAI, the fresh weight increase was observed from an initial explant weight of 0.0065 g to 1.590 g; and on 35 DAI, fresh weight was increased upto 2.376 g.

The GI also showed an increase from 59% in 25 DAI to 137.6% in 35 DAI (Figure 4, Table 2).

Nature of Callus

The callus originated from the midrib region and also from leaf margins. The callus was soft and friable. But the central callus mass was strongly attached to the explant. The colour of the callus was white which later turned into brown. The callus was lobed. In media supplemented with NAA (2 mg/l) there was *in vitro* formation of roots. The roots developed from all over the callus and growth of the roots were noticed into the medium also. The *in vitro* roots were white in colour with profuse root hairs.

Histology of Callus

The histological section of the callus showed irregularly arranged mass of cells. Towards the central region there were compact thick cells. In the periphery there were loose cells. In MS media with 2,4-D medium, the callus was composed of loose cells (Figure 5).

Histology of *in vitro* Root

The cross-section of root showed outer thin walled cells and central thick walled cells. Root hairs were present. In the lateral section of root, the root hairs were found to be unicellular. Vascular elements, most probably, xylem trachieds was observed in the central callus mass, which connected the callus mass to the parent explant. The objective of the study was to initiate callus from leaf explants of *I. tinctoria*, using two different growth hormones (NAA and 2,4-D).

In *Indigofera*, of the two auxins NAA (2 mg/l) was found to produce more favourable results with reference to fresh weight increase. In both the auxins the callus showed a faster growth. NAA was slightly more efficient with regard to initial callusing. Of the two auxins rhizogenesis along with callus proliferation was observed only in NAA, while 2,4-D showed only callus proliferation with rhizogenesis.

Fig. 4: *Callus after 35 DAI.*

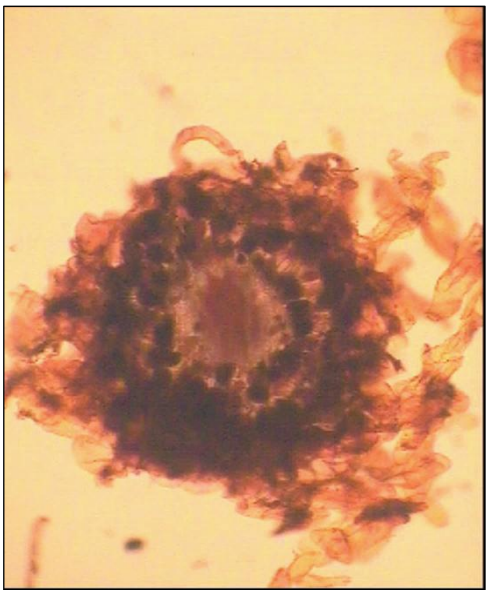

Fig. 5: *Histology of Callus.*

Table 2: *Increase in Fresh Weight and Growth Index in Indigofera tinctoria Leaflet Explant.*

Explant	Concentration of auxin	Initial explant weight	25 DAI	Growth index** (%) 25 DAI	35 DAI	Growth index** (%) 35 DAI
Indigofera tinctoria leaflet	NAA(2 mg/l)	0.0065	*2.632[1]	163.2	*3.817[2]	281.7
Indigofera tinctoria leaflet	2,4-D (2 mg/l)	0.0065	*1.590[3]	59	*2.376[4]	137.6
SD: (1)0.0342 (2)0.0250 (3)0.024 (4)0.0107						

** Mean of 20 Replicates*

** $Growth\ Index = \frac{Final\ fresh\ weight - Initial\ fresh\ weight}{Initial\ fresh\ weight} \times 100$

The results obtained from the present study were in agreement with some of the earlier reports in *Indigofera* species as well as in some other related plants such as study by Howell *et al.* [3] in *Indigofera potaninii* and Guo *et al*. [2], [5] in *Saussurea involucrata* leaf explants. Bharal and Rashid (1979) [1] reported shoot bud differentiation from excised leaves, green cotyledons and hypocotyl of immature seeds of *Indigofera enneaphylla* Linn. on a defined medium containing NAA and BAP.

The morphology of the callus was friable type. With the development of vascular elements the original callus mass was strongly attached to the explant but later when callus proliferated, several loose lobes were observed. In histology, callus tissue showed development of vascular elements which indicated a strong connection between the callus mass and leaf explant.

The morphological nature of the callus was common to both the auxins in the study. Since the central callus mass was compact and connected to the explant with the help of vascular elements, the type of callus can be embryogenic. The rhizogenesis also indicated the embryogenic potential. The peripheral loose callus lobes indicated rapid proliferation and meristematic activity of callus cells. With the help of an appropriate media composition and protocol the callus may be directed towards somatic embryogenesis in future.

CONCLUSION

In the present study NAA proved to be the best auxin in *Indigofera* for callusing. In NAA medium, rhizogenesis was observed along with callus proliferation. Vascular elements inside the callus proved a strong connection with the explant and embryogenic potential. The friability may be associated with the strong meristematic activity. *Indigofera* is medicinally and economically valuable and is an ideal system for adopting the tissue culture propagation. Callus culture method and rhizogenesis can be exploited for secondary metabolite production, which can avoid the destruction of valuable germplasm for their extraction, and thus helps to conserve biodiversity and ecological balance.

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