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RESEARCH ARTICLE

GENETIC HETEROGENEITY AND PHYLOGENETIC DIVERGENCE AMONG *Nostoc sp.,* BASED ON cpcB-cpcA GENE SEQUENCE

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ABSTRACT

Cyanobacteria belonging to Nostocales family are a highly diverse group in relation to form, function, and habitat. Current cyanobacterial systematics relies on the observation of flexible morphological characters. Accurate and reliable delineation of *Nostoc sp.* has not been possible by traditional methods. Phylogenetic studies of *Nostoc sp.* based on morphological and physiological observations have demonstrated the conflicts in identification and genetic relationships among the Nostocacean strains. Therefore, supplementary genetic information should be incorporated to give a polyphasic classification system for the order Nostocales. In the view of above, the current investigation on the molecular and phylogenetic characterization of three species of the genus *Nostoc* based on cpcB-IGS-cpcA locus of the phycocyanin operon was carried out. The phylogenetic position of these species within order Nostocales had been determined. The results indicated that genus *Nostoc* is heterogeneous. Analysis of the IGS region between cpcB and cpcA showed that *Nostoc* and *Anabaena* are distinct genera, while the taxonomic status of the genera is still unclear. These data indicated that additional analyses and revision of the taxonomic position of *Anabaena, Anabaenopsis* and *Nostoc* species within Nostocales are required.

INTRODUCTION

Cyanobacteria have colonized many ecological niches and have developed diverse strategies to survive in greatly different environments. Early endosymbiotic forms gave rise to the photosynthetic organelles of eukaryotes, thus sharing their ability to perform oxygenic photosynthesis. Extant free living forms exhibit varied physiological properties and range in morphology from simple unicellular to complex filamentous organisms. Under conditions of combined nitrogen limitation, many filamentous strains such as *Nostoc* are able to differentiate heterocysts, specialized cells responsible for the aerobic fixation of molecular nitrogen. This morphological and physiological diversity is mirrored by extensive genetic variability.

Traditionally, the taxonomy of *Nostoc* species has been based on morphological and physiological observations (Vagnoli *et al.,* 1992). The extreme morphological flexibility of *Nostoc* species, which is influenced by the life cycle stage and environmental conditions, makes identification and taxonomy based on morphology alone problematic. Molecular methods have become an indispensable tool for characterization and assessment of evolutionary relations among cyanobacteria in recent decades. The ribosomal RNA genes are well conserved and found in all eubacteria, making them a universal marker for comparison. However, Fox, Wisotzkey and Jurtshuk (1992) concluded that identity in 16S rRNA sequence data was not sufficient grounds for establishing species identity and thus not appropriate for studies at the subgeneric level. As a result, researchers have targeted highly variable intergenic spacer region (IGS) of the phycocyanin (PC) locus (Neilan *et al.,* 1995; Scheldeman *et al.,* 1999; Iteman *et al.,* 2000; Ballot *et al.,* 2004) for studying inter and intraspecific variability in cyanobacteria.

Phycocyanin operon intergenic spacer is the phylogenetic marker commonly employed by phycologists. Distribution of Phycocyanin in aquatic microorganisms makes the study of PC gene heterogeneity ideal for classification of freshwater cyanobacteria. Phycocyanin operon includes the genes responsible for coding of two phycobiliprotein subunits (cpcB and cpcA) and three linker polypeptides. The coding regions show little sequence divergence among closely related species, whereas the spacer regions may exhibit perceptible variability. Therefore, the conserved coding regions of the phycocyanin locus can be used for comparison among cyanobacterial species, while the highly variable spacer region may be used to discriminate strains.

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The current investigation was done to determine the genetic heterogeneity and phylogenetic position of three different *Nostoc sp.* isolated from adjacent water bodies of Madurai city, South India within order Nostocales using the phycocyanin locus cpcB-IGS-cpcA along with those available sequences from other Nostocacean cyanobacteria.

MATERIALS AND METHODS

Cyanobacterial strains and culture conditions

Three species of *Nostoc* genera collected and isolated from water bodies of Madurai city, South India were used in this study. The pure cultures were cultivated in BG11 media at 25^0C for 25 days. A photoperiod of 12:12 (light:dark cycles) with light intensity of 1500-2000 Lux was maintained throughout the growth period. During incubation, the cultures were frequently observed under a microscope to verify the monospecificity of the cultures.

DNA extraction

Cyanobacterial cells were harvested by centrifugation during exponential growth and then suspended in the lysis buffer (50 mM Tris.HCl, 100 mM ethylenediaminetetra-acetic acid [EDTA] Na_2, and 1% sodium dodecyl sulfate; pH = 8.0). Cells were broken by adding Lysozyme and incubated at 70^0C for 15 minutes. The cell lysate was extracted with a mixture of phenol: chloroform (5:1) followed by extraction with chloroform twice. The nucleic acids were precipitated by adding 5M NaCl, ethanol and kept at -20^0C for three hours. Precipitated DNA was purified by adding 70% ethanol and centrifuged at 10,000 rpm at 10^0C for 10 minutes. DNA was stored in 10 mM Tris/HCl and 1 mM EDTA buffer (pH = 8) at -20^0C.

Amplification of PC-IGS region

PC-IGS sequence from cyanobacterial strains were amplified by using the following primer (Neilan *et al.,* 1995).

Forward primer: GGCTGCTTGTTTACGCGACA
Reverse primer: CCAGTACCACCAGCAACTAA

The reaction mixture contained 2.5 mM $MgCl_2$, 20 pmol of each primer, 2 units of thermostable DNA polymerase, 67 mM Tris-HCl, 16 mM $(NH_4)_2SO_4$, 200 mg/ml gelatin, 200 µM deoxyribonucleotide triphosphates and 100 ng of genomic DNA in a 100 µl volume. After preincubation at 94^0 C for 5 minutes, a total of 40 cycles were performed at 94^0C for 20 seconds, 50^0C for 30 seconds and 72^0C for 60 seconds. The temperature cycling was concluded with a final step of 5 minutes at 72^0C. Ten microlitre of the amplified products were subjected to 1.5% agarose gel electrophoresis, stained with ethidium bromide and visualized under UV light in Gel Documentation system (BioRad, USA). PCR products were purified to remove amplification reaction components, including unincorporated primers and nucleotides, and then sequenced using the same set of primers as for amplification. DNA was sequenced with big dye-terminator cycle sequencing kit using ABI DNA sequencer (Applied Biosystems, New Zealand).

Phylogenetic comparison

Nucleotide sequences obtained from DNA sequencing were compared with sequence information available in the National Center for Biotechnology Information (NCBI) data base using BLAST. Based on previously published sequences, the regions of subunit ß (cpcB), IGS and subunit α (cpcA) of the phycocyanin operon were determined. DNA sequences of cpcB and cpcA were translated into amino acid sequences and compared to data available in the NCBI data base using BLASTP. Multiple pair-wise alignment was done using the phylogeny option in CLUSTAL W, which is part of the MEGA 5.05 software. Further, MEGA (Molecular Evolutionary Genetics Analysis) 5.05 was used to phylogenetically analyze the cpcB and cpcA coding regions of the *Nostoc* sequences obtained in this study, along with NCBI Sequences available for other Nostocacean cyanobacteria. Phylogenetic analyses comprised the methods of maximum parsimony, maximum likelihood and neighbor joining algorithms for constructing phylogenetic trees. The sequence alignment was randomly re-sampled 100-fold by setting bootstrap value of hundred to test the reliability of inferred phylogeny. The sequences generated in this study were deposited in DDBJ-EMBL-GenBank database through BankIt under the accession numbers JN646754, JN646755 and JN646756.

RESULTS AND DISCUSSION

Nostoc and *Anabaena* are traditionally separated based on morphological characteristics and life cycle differences (Rippka, 1988; Tamas, 2000; Turner, 1997; Wilmotte, 1994). 16S rDNA studies have shown that *Nostoc* and *Anabaena* are closely related. However, these studies were unable to clearly differentiate between the two genera (Giovannoni *et al.,* 1988; Turner, 1997; Wilmotte, 1994). Likewise, studies using nifH, ITS1, rpoB and rbcLX gene sequences found in *Nostoc* and *Anabaena* cannot resolve the problematic taxonomy of these genera (Tamas *et al.,* 2000; Turner, 1997; Zehr *et al.,* 1997).The sequence of *Nostoc sp.* generated in this study contained nucleotides coding for cpcB at 3' end, 82 nucleotides representing the intergenic spacer region and nucleotides coding for cpcA at 5' end. Studied regions were identified based on sequence comparison using BLASTN as well as gene prediction analysis using GeneMark software. Determined nucleotide sequences were translated into amino acid sequences and together with others, chosen from the GenBank were used for molecular and phylogenetic analyses. Molecular and genetic characterization was done to determine the variability of the amino acid sequences of β- and α-subunits in different species within the order Nostocales family. The relatively high variability in the two conservative subunits determines the heterogeneity of genus *Nostoc*. To determine the position of the isolated *Nostoc* strains within Nostocacean family, phylogenetic analyses were performed using protein sequences of each coding subunit as well the intergenic spacer region using maximum-likelihood, maximum-parsimony and neighbor-joining algorithms. The phylo-genetic trees constructed by these methods were largely congruent and therefore only the NJ tree is presented.

The topology of Nostocales in the phylogenetic tree based on PC-IGS sequence showed 3 clades (Fig. 1). The first clade is divided into 4 different subclades (1A, 1B, 1C, 1D). *Aphanizomenon ovalisporum* and *Anabaena bergii* formed the first subclade-1A which was supported by the bootstrap value of 100%. Subclade 1B consisted of *Anabaena bergii* strains

Fig. 1. Neighbor joining tree obtained from phylogenetic analysis (MEGA 5.05) of PC-IGS region of *Nostoc sp.* within order Nostocales

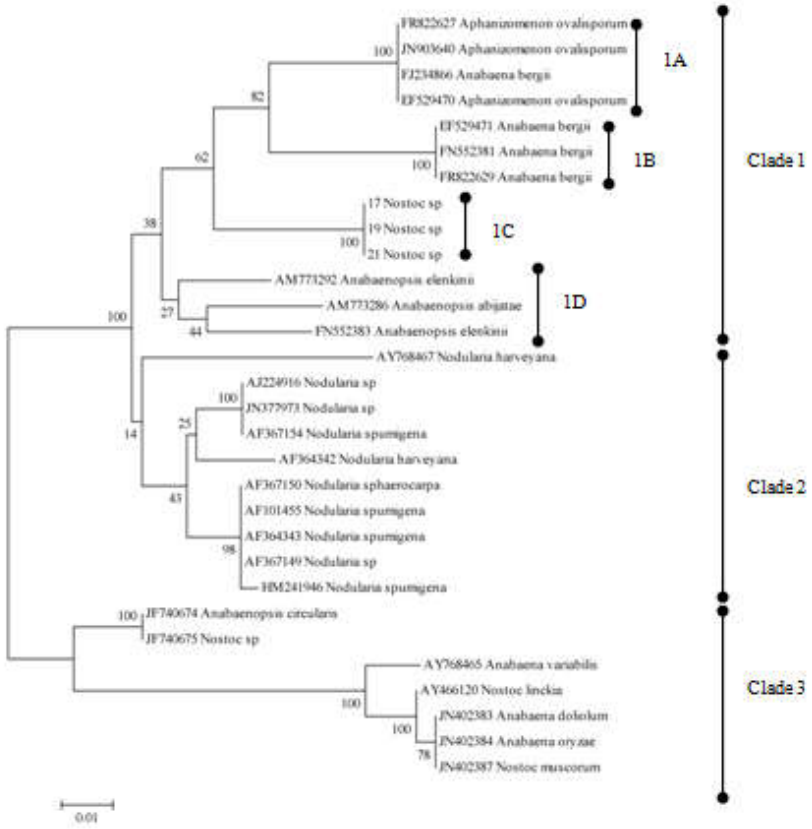


Fig. 2 Neighbor joining tree obtained from phylogenetic analysis (MEGA 5.05) of cpcA region of *Nostoc sp.* within order Nostocales

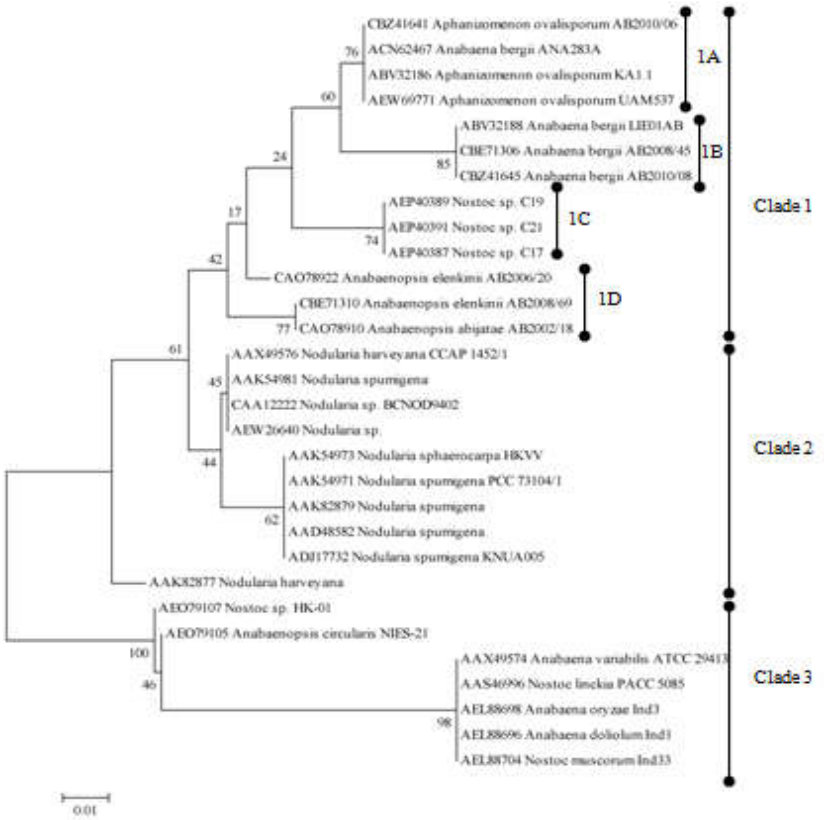
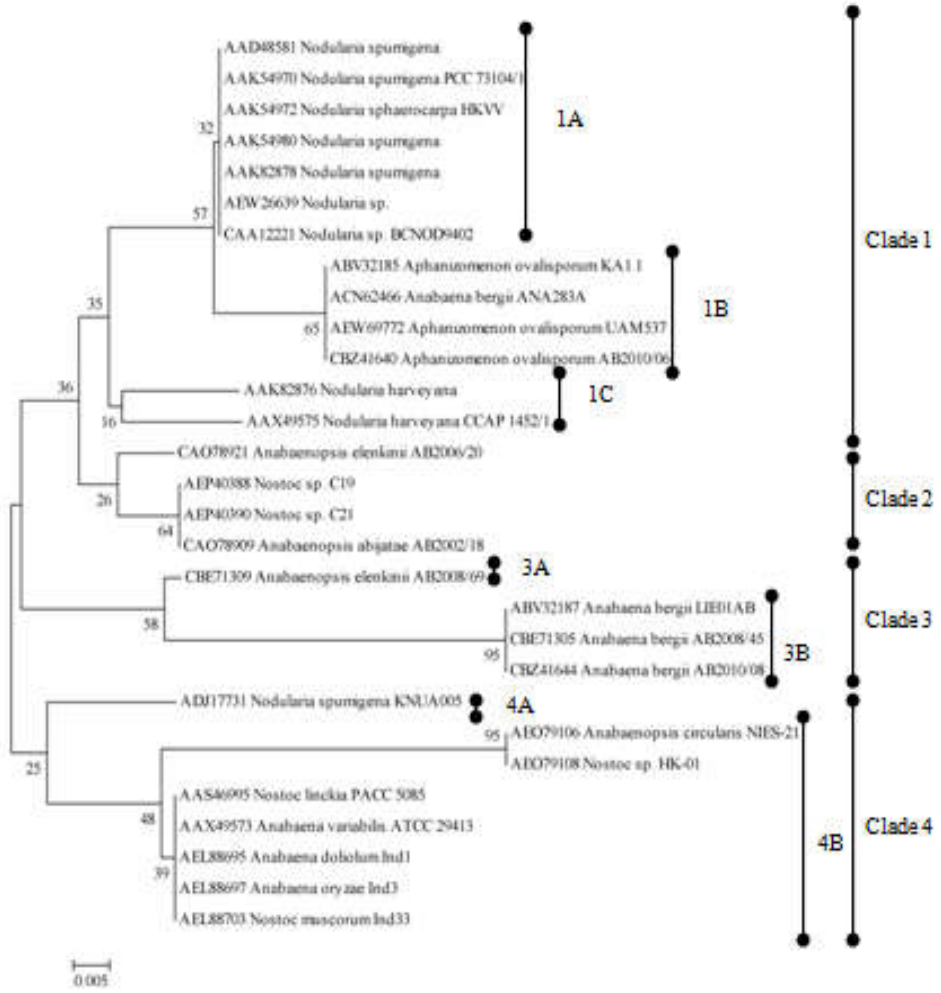


Fig. 3 Neighbor joining tree obtained from phylogenetic analysis (MEGA 5.05) of cpcB region of *Nostoc sp.* within order Nostocales



with the bootstrap value of 100%. *Nostoc sp.* isolated in this study formed the third subclade 1C. This topology was supported by maximal boot strap value of 100%. Subclade 1D included *Anabaenopsis abijatea* and *Anabaenopsis elenkinii* and the low boot strap value indicated the polyphyletic and heterogenetic nature of this genera. Second clade is completely occupied by *Nodularia sp.* with high boot strap values. This topology confirmed the monophyly of *Nodularia sp.* In contrast, the members of *Nostoc, Anabaena* and *Anabaenopsis* are heterogenous and intermixed within the clade three. The phylogenetic tree based on cpcA was largely congruent with the tree based on PC-IGS region and supported the above mentioned topology with high bootstrap values (Fig. 2). In the phylogenetic tree based on cpcB, the members of order Nostocales are divided into 4 different clades (Fig. 3). The first clade contained 3 subclades (1A, 1B, 1C). *Nodularia sp*. occupied the first subclade 1A which again confirmed the monophyly of *Nodularia sp.* Subclade 1B included *Aphanizomenon ovalisporum* and *Anabaena bergii*. This phylogenetic position repeat the topology observed in the phylogenetic tree based on cpcA and PC-IGS regions. In contrast two strains of *Nodularia harveyana* comprised the third subclade 1C. The *Nostoc sp.* isolated in this study occupied the second clade and formed sister subclade with *Anabaenopsis elenkinii* and *Anabaenopsis abijatae*. Third clade is further divided into 2 subclades of which, *Anabaenopsis elenkinii* alone formed the subclade 3A. *Anabaena bergii* strains constituted another subclade 3B. Clade 4 included 2 subclades 4A and 4B. *Nodularia spumigena* formed subclade 4A but low bootstrap value indicated the lack of support to this topology. Subclade 4B was found to be intermixed with *Nostoc* and *Anabaena sp.* This heterogeneity was noticed in trees constructed using PC-IGS and cpcA sequences. In all the predicted phylogenetic trees *Nostoc sp*. isolated from Madurai water bodies formed sister clades with *Anabaena bergii, Anabaenopsis elenkinii* and *Anabaenopsis abijatae*.

The few conflicting nodes between gene trees received only low bootstrap support. The members of genera *Nodularia* formed monophyletic clusters in all phylogenetic reconstructions. In contrast, the members of *Anabaena, Anabaenopsis* and *Nostoc* are heterogenous and intermixed within the order Nostocales. Similar topology was obtained in other phylogenetic analyses based on 16S rDNA, ITS1, rpoB and rbcLX genes (Gugger *et al.,* 2002; Rajaniemi *et al.,* 2005; Svenning *et al.,* 2005). In summary, the present study indicates that genus *Nostoc* is heterogeneous. Analysis of the IGS region between cpcB and cpcA showed that *Nostoc* and *Anabaena* are distinct genera, while the taxonomic status of the genera is still unclear. Further investigations are necessary to illuminate the similarities and differences among *Nostoc*,

Anabaena and *Anabaenopsis sp*. in order to better understand the relationship between their phenotypes and genotypes.

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