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Molecular Characterization of Marine Cyanobacteria from the Indian Subcontinent Deduced from Sequence Analysis of the Phycocyanin Operon (*cpcB-IGS-cpcA*) and 16S-23S ITS Region

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Molecular characterization of ten marine cyanobacterial isolates belonging to the order *Oscillatoriales* was carried out using the phycocyanin locus (*cpcBA*-IGS) and the 16S-23S internally transcribed spacer region. DNA sequences from the phycocyanin operon discriminated ten genotypes, which corresponded to seven morphotypes identified by traditional microscopic analysis. The *cpcB* coding region revealed 17% nucleotide variation, while *cpcA* exhibited 29% variation across the studied species. Phylogenetic analyses support the conclusion that the *Phormidium* and *Leptolyngbya* genera are not monophyletic. The nucleotide variations were heterogeneously distributed with no or minimal informative nucleotides. Our results suggest that the discriminatory power of the phycocyanin region varies across the cyanobacterial species and strains. The DNA sequence analysis of the 16S-23S internally transcribed spacer region also supports the polyphyletic nature of the studied oscillatorian cyanobacteria. This study demonstrated that morphologically very similar strains might differ genotypically. Thus, molecular approaches comprising different gene regions in combination with morphological criteria may provide better taxonomical resolution of the order *Oscillatoriales*.

Keywords: cyanobacteria, genetic diversity, IGS, ITS, *Leptolyngbya*, *Phormidium*, phycocyanin operon, phylogeny, taxonomy

The cyanobacteria are a morphologically distinct group of oxygenic photosynthetic organisms that inhabit terrestrial and aquatic ecosystems. The versatility lies in their ability to grow in extreme environments, their significant role in the biogeochemical cycle, and the production of bioactive compounds. Initially, cyanobacteria were classified as prokaryotic algae due to their phototrophic nature (Anagnostidis and Komárek, 1985). Later, the taxonomic assignments were based on morphological and cytological characteristics (Anagnostidis and Komárek, 1985; Castenholz and Waterbury, 1989; Neilan *et al.*, 1995; Lu *et al.*, 1997). Since cyanobacterial morphology is strongly influenced by environmental stimuli at individual sampling locales, it has been difficult to classify cyanobacteria in appropriate taxonomic groups (Stanier *et al.*, 1971). It has been estimated that as many as 50% of cyanobacterial strains found in culture collections have been misidentified (Komárek and Anagnostidis, 1989) leading to erroneous organism phylogenies.

DNA sequences play an essential role in the reconstruction of evolutionary relationships among organisms and have led to new genetic classifications that may confirm or conflict with traditional taxonomy. Application of molecular techniques to amplify some portions of the genome in order to characterize and deduce phylogenetic relationships of cyanobacteria has increased considerably in the recent years (Neilan *et al.*, 1995; Garcia-Pichel *et al.*, 1996; Orcutt *et al.*, 2002; Taton *et al.*, 2003). At the molecular level, the rRNA genes are the most widely used markers for the identification of bacteria and cyanobacteria due to their conserved function and universal presence. Several researchers have exploited the conserved regions of the 16S rRNA gene for phylogenetic analysis of cyanobacteria (Nübel *et al.*, 1997; Crosbie *et al.*, 2003;

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Salomon *et al.*, 2003). However, the application of 16S rRNA to identify at the species level and below has been contested (Fox *et al.*, 1992). Moreover, the conserved nature of 16S rRNA and the lower evolutionary rate variation compared to the protein-encoding genes makes it less useful for phylogenetic studies of closely related organisms. As a result, researchers have targeted other variable regions such as the 16S-23S internal transcribed spacer region (ITS) and the 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).

In cyanobacteria, the 16S-23S ITS region has variable tRNA genes both in terms of length and nucleotides (Iteman *et al.*, 2000). Therefore, the sequence information of this region can be a valuable tool for use in phylogenetic investigations (Lu, 1999; Li, 2000). However, the existence of multiple rRNA operons in some cyanobacteria has to be considered in those organisms during application in phylogenetic studies (Boyer *et al.*, 2001). Yet another phylogenetic marker commonly employed by phycologists is the phycocyanin operon, which includes the genes responsible for coding of two phycobiliprotein subunits (*cpcB and cpcA*) and three linker polypeptides. The substitution rate of the nucleotides in this region is higher than that of 16S rRNA (Ishida *et al.*, 1997; Nübel *et al.*, 1997; Tillett *et al.*, 2001) and, thus, can be used as a potential genetic marker for phylogenetic studies. 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. Several studies indicate intragenic recombinations and possible exchanges of genetic material between cyanobacterial strains within the phycocyanin operon (Barker *et al.*, 2000a; Manen and Falquet, 2002; Janson and Granéli, 2002; Teneva *et al.*, 2005). These observations called into question the use of the PC-IGS region alone for phylogenetic analyses, especially for phyletically related strains susceptible to homologous recombination (Young, 2001). To avoid the complex evolutionary pattern of the PC-IGS locus within cyanobacteria, Janson and Granéli (2002) suggested inclusion of additional gene loci during phylogenetic analyses.

At the National Facility for Marine Cyanobacteria (NFMC), India, the cyanobacterial cultures collected across different geographical locations within the Indian subcontinent were characterized based on their morphological features. Thus, the present study is an attempt to characterize some oscillatorian strains using the phycocyanin locus *cpcB-IGS-cpcA* and the 16S-23S ITS region, and to compare the morphological and molecular data.

Materials and Methods

Cyanobacterial strains

Axenic cultures from the National Facility for Marine Cyanobacteria (NFMC) were utilized in this study. The strains used and their collection locations are listed in Table 1. The cultures were grown in ASN III media (Rippka *et al.*, 1979) at 25°C for 10 days. A continuous photoperiod with a light intensity of 200 μ mol photons.m^{-2}.s^{-1} was used to grow the cultures. Morphological identification of the cyanobacterial strains was executed by light microscopy.

DNA extraction and PCR amplification

The genomic DNA was extracted using the method described by Wu *et al.* (2000). The primers used in this study cpc_arF; TCG AAG ATC GTT GCT TGA ACG and cpc_arR; TTA GGT CCC TGC ATT TGG GTG were the same as those described by Ballot *et al.* (2004). Amplification of the 16S-23S ITS DNA fragment included the 16SF; TGT GGC TGG ATC ACC TCC TT and 23SR; TCT GTG TGC CTA GGT ATC CAC CGT T primers described by Baurain *et al.*, (2002). The PCR reaction was performed using Ready-To-Go PCR Beads (Amersham Biosciences, Sweden) containing 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 0.30 μ M of each primer, 1.25 units of *Taq* DNA polymerase and 100 ng of template DNA in a 25.0 μl reaction using a Progene thermal cycler (Techne Cambridge Ltd., UK). The cycling profile for the cpcBA-IGS region included an initial denaturation at 95°C for 5 min followed by 35 cycles of 95°C for 25 sec, 60°C for 15 sec, 72°C for 30 sec and a final extension at 72°C for 5 min. For the 16S- 23S ITS region, the thermal cycling was performed with an initial denaturation at 95°C for 5 min followed by 30 cycles of 95°C for 30 sec, 58°C for 15 sec, 72°C for 40 sec and a final extension of 72°C for 5 min. Five microliters of the amplified products were subjected to 1.5% agarose gel electrophoresis, stained with ethidium bromide, and visualized using a UV transilluminator.

DNA sequencing

The amplified PCR products were purified using a QIAquick PCR purification kit (Qiagen GmbH, Germany) as recommended by the manufacturer. The primers employed for PCR amplification were used to determine the sequences on both strands using the DNA cycle sequencing reaction kit according to the protocol recommended by the supplier. The DNA sequences were obtained with an ABI 310 automated

sequencer using the chain-termination method with big-dye terminators (Applied Biosystems, USA). For the sequence data, automated base calls were checked by manual inspection of the electropherograms of both forward and reverse sequences. The base call conflicts were resolved by alignment and comparison of both strands using SeqScape® software v 2.5 (Applied Biosystems, USA).

Nucleotide sequence accession numbersminutes

The nucleotide sequences described in this study have been submitted to the European Molecular Biology Laboratory database (EMBL) under GenBank accession numbers AJ 973256 to AJ 973275 (Table 1).

Alignments and phylogenetic analyses

DNA sequences obtained in this study were aligned and compared with sequence data for other cyanobac-

Table 1. List of cyanobacterial strains applied in this study, their collection locations, and GenBank accession numbers

Species	Strain	Location	GenBank accession numbers cpcBA-IGS	GenBank accession numbers 16S-23S ITS
Leptolyngbya valderiana	BDU 20041	Point Calimare (Bay of Bengal)	AJ 973263	AJ 973273
Leptolyngbya valderiana	BDU 30501	Palk Bay (Bay of Bengal)	AJ 973264	AJ 973274
Leptolyngbya valderiana	BDU 140441	South Andaman Island (Bay of Bengal)	AJ 973265	AJ 973275
Oscillatoria salina	BDU 30411	Palk Strait region (Bay of Bengal)	AJ 973260	AJ 973270
Phormidium boryanum	BDU 92181	Kerala (Arabian Sea)	AJ 973257	AJ 973267
Phormidium boryanum	BDU 141071	Port Blair (Bay of Bengal)	AJ 973256	AJ 973266
Phormidium chlorinum	BDU 140691	South Andaman Island (Bay of Bengal)	AJ 973258	AJ 973268
Phormidium formosum	BDU 91041	Kerala (Arabian Sea)	AJ 973259	AJ 973269
Phormidium subuliforme	BDU 100712	Cuddalore (Bay of Bengal)	AJ 973261	AJ 973271
Phormidium willei	BDU 130511	Vishakapattinam (Bay of Bengal)	AJ 973262	AJ 973272

Table 2. Description of obtained cyanobacterial morphotypes.

Morphotype	Description	Taxonomic assignment
A	Filamentous, trichomes flexous, not constricted at the cross-walls, not curved, ends not attenuated, calyptra absent, sheath thin, trichomes blue-green, 2.0-2.3 μm wide, 5-5.3 μm long, cross walls with 1 granule on either side, non capitate.	*Leptolyngbya valderiana*
B	Filamentous, trichomes coiled, constricted at the cross-walls, 6.5 μm wide, greyish-green, slightly or highly granulated at the cross-walls, cells 5.2 μm wide calyptra absent, non capitate.	*Phormidium boryanum*
C	Filamentous, trichomes straight, slightly constricted at the cross-walls, 4.8 μm wide, bright blue green, attenuated at the ends, slightly granulated, motile with oscillation, calyptra absent, non capitate, end cells nearly obtuse.	*Phormidium formosum*
D	Filamentous, thallus very thin yellowish-green trichomes straight, unconstricted, 3.9 μm wide, without gas vesicles, cross-walls not granulated, calyptra absent.	*Phormidium chlorinum*
E	Filamentous, thallus dull green, trichomes yellow-green, very long flexous, not constricted at the cross-walls, 5.2 μm wide, ends attenuated, cells nearly quadrate, non capitate, calyptra absent.	*Phormidium subuliforme*
F	Filamentous, trichomes grayish blue, bent at the ends, 3.2 μm wide, not constricted at the cross-walls, ends not attenuated, non capitate, not granulated at the cross- walls, cell rounded without a thickened membrane, cells twice as long as broad.	*Phormidium willei*
G	Filamentous, trichomes straight, elongate, rapidly moving not constricted at the cross-walls, 3.4 μm wide blue-green, not capitate, calyptra absent, not granulated, cell content uniformly granular, briefly tapering ending accuminately in a Sharp point, hooked.	*Oscillatoria salina*

teria available in the NCBI database using the CLUSTAL alignment algorithm contained in MegAlign of DNAStar software (DNASTAR Inc., USA). The aligned sequences were studied using the PHYLIP package (Version 3.6) of phylogenetic programs (Felsenstein, 2004). Three methods of phylogenetic reconstruction, maximum parsimony, maximum likelihood (ML), and neighbor-joining (NJ) were applied to all datasets in order to support the validity of the presented phylogenies. Bootstrap values from 100 resamplings were calculated for each set of data. Phylogenetic trees were constructed using the phylogenetic inference protocols SEQBOOT, DNADIST, NEIGHBOR, DNAPARS, DNAML, CONSENSE, and DRAWGRAM of the PHYLIP package. All trees were rooted using the outgroup method. *Cyanidium caldarium* (S77125) was used as an outgroup taxon for the cpcB-cpcA locus and *Cyanophora paradoxa* (M19493) was used as an outgroup for the 16S-23S ITS region.

Results and Discussion

The morphological characteristics of the ten cyanobacterial strains applied in this study (Table 2) and their photographs are shown in Fig. 1. The taxonomic assignments were based on the descriptions of Komárek and Anagnostidis (2005). A total of seven morphotypes were observed (Table 2). Morphologically, no variations were found between the strains of *L. valderiana* (BDU 20041, BDU 30501, and BDU 140441) and *P.*

Fig. 1. Phase-contrast photomicrographs of cyanobacterial morphotypes. (a) *Leptolyngbya valderiana* BDU 140441, (b) *L. valderiana* BDU 20041, (c) *L. valderiana* BDU 30501, (d) *Oscillatoria salina* BDU 30411 (e) *Phormidium boryanum* BDU 92181, (f) *P. boryanum* BDU 141071, (g) *P. chlorinum* BDU 140691, (h) *P. formosum* BDU 91041, (i) *P. subuliforme* BDU 100712, (j) *P. willei* BDU 130511.

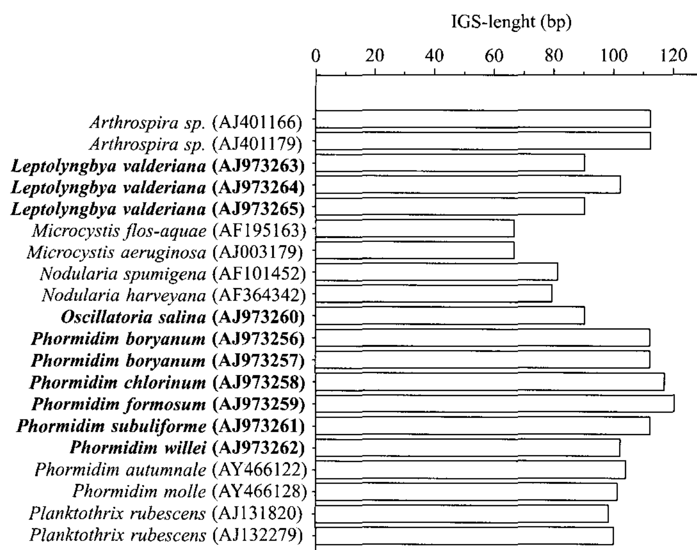

Fig. 2. Length of the IGS region in bases (bp) between the alpha and beta subunit of the phycocyanin gene from all studied species and other members of *Cyanobacteria*. The strains from the present study are given in bold.

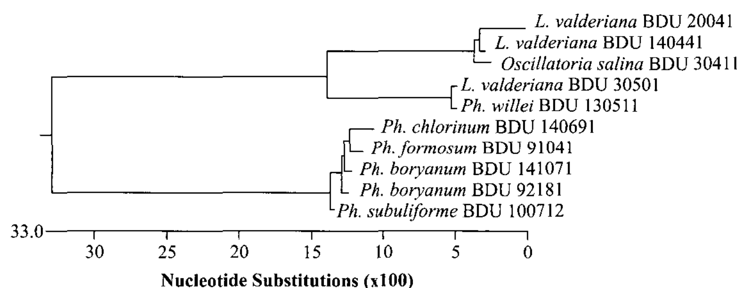

Fig. 3. Phylogenetic affiliations of the studied cyanobacterial strains based on *cpcB-IGS-cpcA* sequences. The phylogenetic tree was reconstructed using the neighbor-joining algorithm as implemented by the Clustal V program. Branches of different length are proportional to the divergence.

boryanum (BDU 92181 and BDU 141071). However, the DNA sequences of the *cpcBA* and the spacer region revealed a higher number of genotypes (compared to the morphotypes) exhibiting variation in the sequences both at the inter- and intra- species levels. The length of the IGS region varied between 90-120 bases (Fig. 2). The spacer region sequences of *L. valderiana* BDU 140441, BDU 20041 and *P. boryanum* BDU 92181, BDU 140171 showed 100% nucleotide similarities within the strains of each species. However, *L. valderiana* BDU 30501 exhibited variation in length (102 bases) and nucleotide sequences forming an identical base pair profile with *P. willei* BDU 130511, while *O. salina* BDU 30411 was clustered with the two *Leptolyngbya* strains. The length of the spacer region of *P. formosum* BDU 91041 was 120 bases, which differed from other species of *Phormidium* studied. The spacer sequences of *P. subuliforme* BDU 100712 showed an identical pattern to that of *P. boryanum* strains. Barker *et al.* (2000b) reported a higher degree of variability in the spacer region of *Aphanizomenon* strains used in their study, leading to the exclusion of this region in the phylogenetic tree. In contrast, our results on the spacer region showed the possibility of discriminating the cyanobacterial strains at the species level, suggesting a varying degree of delineating power of the spacer region within the *Cyanobacteria*.

Multiple pair-wise comparisons of the *cpcB*-IGS-*cpcA* sequences demonstrated a good relationship between the studied oscillatorian species. The evolutionary distances and relative clustering are shown in Fig. 3. The length and the nucleotide sequences of the *cpcB*-IGS-*cpcA* region in the present study correlated very well to the phylogenetic tree constructed by other researchers (Robertson *et al.*, 2001; Crosbie *et al.*, 2003).

The *cpcB* coding region revealed 17% nucleotide

variation while the spacer region and the *cpcA* exhibited 38% and 29% variation across the studied species. Teneva *et al.* (2005) observed a higher percentage of variation in the *cpcB*-IGS-*cpcA* region of *Phormidium sp.* with 35% *cpcB*, 72% IGS and 44% *cpcA* variability. The results imply that the discriminatory power of the phycocyanin region varies across the cyanobacterial species or strains, a finding supported by Bolch *et al.* (1996) and Dyble *et al.* (2002) during their studies on different cyanobacterial strains. Nevertheless, the highest percentage of variation was observed in the spacer region, followed by *cpcA* and *cpcB* as observed by Teneva *et al.* (2005) while a very low percentage of variable informative nucleotide positions in the *cpcB* (1%) and *cpcA* (1.6%) coding regions were detected across the studied *Phormidium* strains. Moreover, the spacer region showed no variation in informative nucleotides, suggesting the possibility of infrequent or no genetic exchanges between the studied strains. Previous analyses of freshwater *Phormidium* species showed a higher percentage of variable informative nucleotide positions in the *cpcB*-IGS-*cpcA* region (Teneva *et al.*, 2005), which may be due to the differences in environmental conditions, as the strains studied here were collected from sandy shore areas.

Because of the low sequence divergence of the *cpcB*-IGS-*cpcA* region in the studied strains, the use of distance methods for tree building might not be optimal. Therefore, maximum parsimony and maximum likelihood analyses were used.

The maximum parsimony tree (Fig. 4) was constructed from the coding *cpcB*-*cpcA* sequences obtained in the current study and sequences of other *Oscillatoriales* available in the GenBank for the phycocyanin operon. The coherent phylogenetic lineage of Indian *Phormidium*

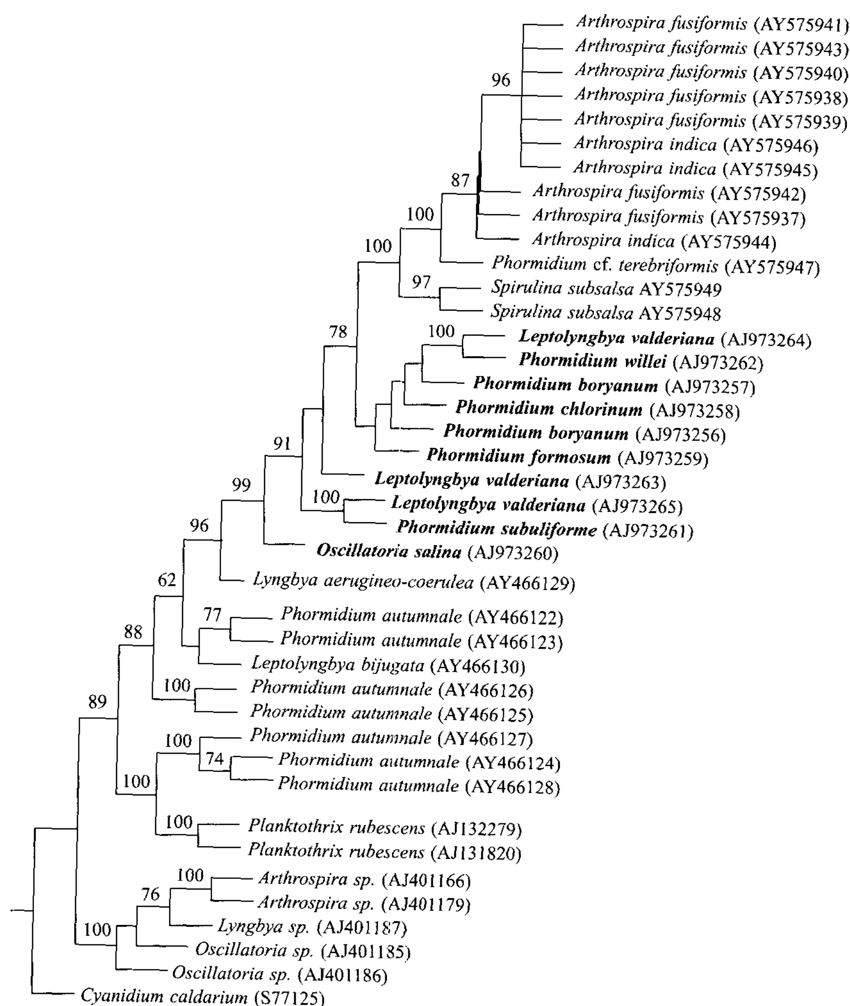

Fig. 4. Phylogenetic estimate of oscillatorian relationships based on maximum parsimony analysis of the *cpcB*-*cpcA* coding regions. Bootstrap values (≥50%) are indicated on the branches. The strains from the present study are given in bold. GenBank accession numbers of cpcBA-IGS sequences are indicated after the taxon names in the tree.

strains with the members of *Leptolyngbya* suggests the polyphyletic nature of this group, and necessitates further molecular studies for better understanding of the systematics. Similar tree topology of clustering was obtained using the method. of maximum likelihood (data not shown).

The present observation on the nature of *Phormidium* is in agreement with the studies of Litivatis (2002) and Teneva *et al.* (2005), who also reported the polyphyletic nature of *Phormidium* using 16S sequences and *cpcB*-IGS-*cpcA* sequences, respectively.

In the case of *Leptolyngbya valderiana,* strain BDU 30501 forms a separate cluster with *P. willei* BDU 130511, supported by 100% bootstrap values, while *Leptolyngbya valderiana* BDU 140441 was clustered with *P. subuliforme* BDU 100712. This evidence suggests that *Leptolyngbya* is not monophyletic. Using 16S rRNA for the taxonomic resolution of *Leptolyngbya,* Payne *et al.* (2001) reported similar results. It is also apparent from the molecular data of three *Leptolyngbya* strains that different genotypes are very similar in terms of morphology. Since morphology may not be controlled by genetics (Saker *et al.*, 1999), a combination of both morphological and molecular data may help to alleviate the discrepancies in taxonomic assignments.

The sequence divergence observed in *L. valderiana* BDU 30501 may result from the ecological conditions prevailing in the collection location. For instance, this strain has been shown to grow in hypersaline conditions of more than 100 ppt (Prabaharan, 1988), which probably resulted in the genetic diversity. Margheri *et al.* (2003) were able to clearly distinguish *Spirulina* and *Geitlerinema* strains from different alkaline, saline, and freshwater habitats and found that hypersaline and alkaline strains are genetically distinct from other marine and freshwater habitats. *L. valderiana* strain BDU 30501 shows 98% sequence similarity with *P. willei* BDU 130511, suggesting possible gene transfer between the taxa (Barker *et al.*, 1999). However, further studies with additional strains and other specific genetic loci could possibly explain the relationships

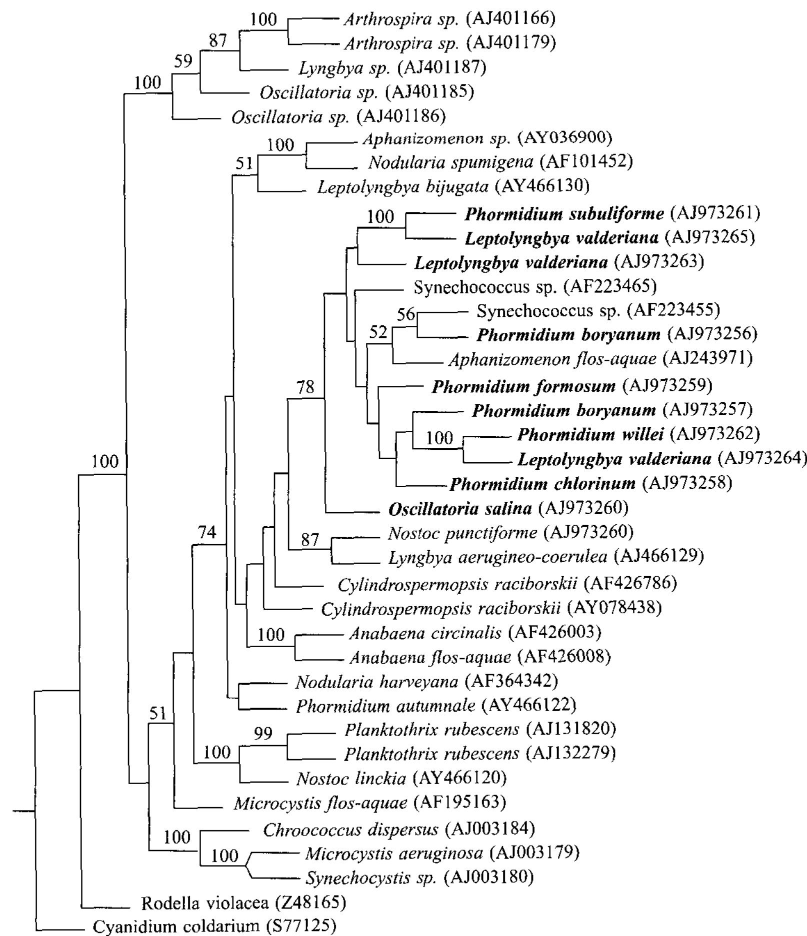

Fig. 5. Phylogenetic estimate of oscillatorian relationships within the *Cyanobacteria* based on maximum parsimony analysis of the *cpcB-cpcA* coding regions. Bootstrap values (≥50%) are indicated on the branches. The strains from the present study are given in bold. GenBank accession numbers of cpcBA-IGS sequences are indicated after the taxon names in the tree.

between these taxa. For such purposes, Janson and Granéli (2002) suggested utilization of the *hetR* gene that encodes a serine protease, and it has been shown to be more specific and lacks sequence anomalies. Additionally, it was found only in filamentous cyanobacteria.

It is obvious from the current study that *O. salina* BDU 30411 formed a separate cluster that was relatively distantly related to the members of the *Phormidium* and *Leptolyngbya* strains. This was supported by a bootstrap value of 99%, advocating the potential use of phycocyanin operon to distinguish closely related cyanobacteria as observed by Bolch *et al.* (1996).

In order to infer the phylogenetic positions of the studied strains within the *Cyanobacteria*, maximum parsimony and maximum likelihood trees were constructed with the addition of non-oscillatorian cyanobacterial *cpcB-cpcA* coding sequences. In the parsimony tree (Fig. 5), all of the oscillatorian strains used in the current study were clustered into one lineage together with two *Synechococcus* strains and *Aphanizomenon flos-aquae*, as supported by 78% of bootstrap replications. The topology of the branches was similar to those obtained for the studied strains within order *Oscillatoriales* (Fig. 4). The dispersed distribution of the oscillatorian specimens in the tree again confirms that order *Oscillatoriales* is polyphyletic and has a complex evolutionary history. This polyphyly of *Oscillatoriales* was also evident in the maximum likelihood tree (data not shown).

Another marker that has been used to infer the phylogeny of cyanobacteria is the 16S-23S ITS region. Boyer *et al.* (2001) reported the existence of multiple rRNA operons in some cyanobacterial species and, therefore, application of this region to infer phylogeny should consider the presence of multiple operons. However, the agarose gel-electrophoresis pic-

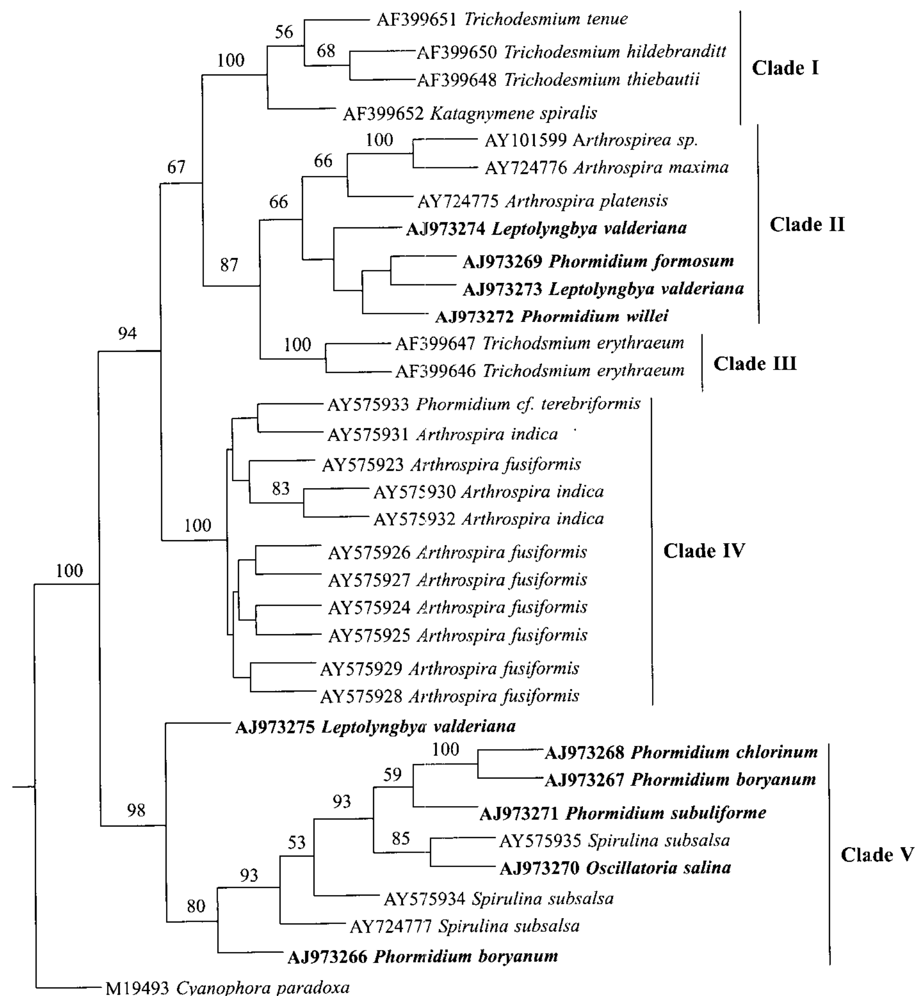

Fig. 6. Phylogenetic estimate of oscillatorian relationships based on maximum parsimony analysis of the 16S-23S *ITS* region. Bootstrap values (≥50%) are indicated on the branches. The strains from the present study are given in bold. GenBank accession numbers of the 16S-23S *ITS* sequences are indicated before the taxon names in the tree.

ture of the amplified PCR products from the current study consistently produced a single band from all of the strains advocating the presence of single operon (data not shown). Results from parsimony analysis of the 16S-23S ITS sequences within *Oscillatoriales* are presented in Fig. 6. The consensus tree includes five well-supported main clades. Two *Leptolyngbya valderiana* strains (BDU 30501 and BDU 20041) were clustered together with *Phormidium formosum* BDU 91041 and *Phormidium willei* BDU 130511, but this branch is not supported by a sufficient bootstrap value. Three *Arthrospira* strains formed a sister group in the same lineage, and was supported by 66% of the bootstrap trees (clade II). The remaining studied *Phormidium* strains are in a cluster that also contains three strains of *Spirulina subsalsa* (clade V) in 80% of the bootstrap trees. *Leptolyngbya valderiana* BDU 140441 is located in a separate branch, and the bootstrap support for this finding was 98%. The same pattern of clustering was obtained from the maximum likelihood analysis. The only variable clustering observed was for *Oscillatoria salina* BDU 30411, which clustered more distantly than the other studied strains (data not shown).

Results from analyses of both molecular datasets (*cpcB-IGS-cpcA* and 16S-23S ITS) strongly support the conclusion that order *Oscillatoriales* is polyphyletic despite the differences in the branching patterns. Hence, the morphological criteria used for taxonomic identification of the oscillatorian cyanobacteria within the "LPP-group" should be reexamined.

The differences between morphotypes and genotypes may also result from the variation induced during culturing (Otsuka, 2000), or differential selection may favor the maintenance of different phycocyanin genotypes in the population.

Despite the low sample numbers, the genetic data support the recent classical taxonomic revisions of Komárek and Anagnostidis (2005). Additional genetic studies are planned using other markers such as 16S rRNA, *hetR* and *rpo* genes to resolve the relationships between *Phormidium, Leptolyngbya,* and other *Oscillatoriales*.

In conclusion, the molecular analysis of *cpcBA*-IGS and the 16S-23S ITS region supports the polyphyletic nature of *Phormidium* and *Leptolyngbya* species. More studies comprising different gene regions in combination with well-characterized morphology from natural and cultivated populations may provide a better understanding of the systematics of order *Oscillatoriales*.

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