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Genetic heterogeneity of the marine cyanobacterium *Leptolyngbya valderiana* (Pseudanabaenaceae) evidenced by RAPD molecular markers and 16S rDNA sequence data

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The distribution and diversity patterns of the cosmopolitan marine cyanobacterium Leptolyngbya valderiana (Pseudanabaenaceae, Cyanobacteria) were studied. To asses the level of genetic diversity, morphotypes from different geographical locations (Coast of south India and Andaman) were subjected to randomly amplified polymorphic DNA (RAPD) analysis and partial 16S rRNA gene sequence studies. Morphologically and ecologically, all strains fit the currently circumscribed L. valderiana. However, RAPD primers yielded 100% polymorphism among the studied strains, indicating a considerable degree of intra-specific genomic heterogeneity, with percent similarity between 13 and 82%. Partial 16S rDNA sequence similarity values ranged from 91 to 99%. In conclusion, the genetic variability observed within the marine strains of L. valderiana indicates the presence of cryptic species. Thus, future investigations combining well-characterized ultrastructural morphology with additional molecular markers are essential to characterize and clarify the patterns in the evolution and biogeography.

INTRODUCTION

Cyanoprokaryotes are a morphologically diverse group of oxyphototrophic bacteria containing more than 1000 species inhabiting terrestrial, fresh water and marine ecosystems. The remarkable physiological, biochemical and genetic properties of cyanobacteria enable them to inhabit and often dominate diversified habitats such as hot springs (Ferris *et al.*, 2003; Papke *et al.*, 2003), frozen lakes in Antarctica (Gordon *et al.*, 2000), hyper-saline environments (Dor *et al.*, 1991) and hot deserts (Budel and Wessels, 1991). However, while widely distributed, actual identification of species is often confounded due to phenotypic plasticity, difficulty in choosing diagnostic characters and the presence of cryptic species (Palinska *et al.*, 1995; Casamatta *et al.*, 2003).

Modern molecular approaches have increasingly been employed to elucidate cyanobacterial phylogenies and population level questions (Neilan, 1995; Bartish *et al.*, 2000; Payne *et al.*, 2001; Waltenbury *et al.*, 2005). Randomly amplified polymorphic DNA (RAPD) analysis is one such tool often employed to examine the genetic diversity among cyanobacterial strains (Moschetti *et al.*, 1998; Quiberoni *et al.*, 1998; Bolch *et al.*, 1999; Casamatta *et al.*, 2003). Although RAPD's dominant expression may bias population genetic parameters and some assumptions are required (Lynch and

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Milligan, 1994), the technique is widely used for cluster analysis to study geographic patterns and to evaluate whether correlation exists between morphology and genetic characterization. Actual phylogenetic reconstructions, however, often rely on DNA sequencing, most commonly of the 16S rDNA gene (Casamatta *et al.*, 2005; Gkelis *et al.*, 2005; Comte *et al.*, 2007).

Previous studies on three strains of the marine cyanobacterium *Leptolyngbya valderiana* (Gom) Anagn. et Kom. (formerly *Phormidium valderianum sensu* Gomont) revealed a potentially high degree of dissimilarity (Premanandh *et al.*, 2006a) suggesting the possibility of cryptic species. In order to examine the biogeography and genetic relatedness of cosmopolitan marine cyanobacterium *L. valderiana*, 16 strains fitting the morphological and ecological species circumscription were collected across the coasts of south India and Andaman. These strains were subjected to morphological, RAPD and 16S rDNA gene sequence analysis in order to elucidate the biogeography of this putatively cosmopolitan taxon.

METHOD

Cyanobacterial strains and growth conditions

Sixteen marine isolates collected and deposited in the National Facility for Marine Cyanobacteria (NFMC) from different geographical locations (Fig. 1) were used in this study. The axenic cultures were grown in ASN III media (Rippka *et al.*, 1979) at 25°C, at 200 μmol photons m^{-2}/s illumination. During incubation, the cultures were frequently observed under a microscope to verify the monospecificity of the cultures. Morphological assessments were performed from the cultures in the exponential growth phase using a Leitz DIAPLAN microscope (Leica Microsystems GmbH, Germany). All the 16 strains morphologically fit in the species concept of *L. valderiana* based on the classification strategies proposed by Komarek and Anagnostidis (Komarek and Anagnostidis, 2005).

DNA extraction and genetic analysis

Total genomic DNA was extracted using the xanthogenate nucleic acid isolation protocol (Tillett and Neilan, 2000). RAPD analysis was performed using Ready-to-Go RAPD analysis beads (GE Healthcare, UK). Each RAPD bead contained 1.5U *Taq* polymerase, 1.5 mM $MgCl_2$, 10 mM Tris–HCl (pH 9.0), 50 mM KCl and 200 μM of each deoxynucleoside phosphate. Genomic DNA from each sample (20 ng) and 25 pmol of respective primer were added to the beads and made up to 25 μL volume with nuclease free water. Four random 10-mer primers (Metabion International AG, Germany) P2 (GTTTCGCTCC), P3 (GTAGACCC GT), P5 (AACGCGCAAC) and P6 (CCCGTCAGCA) were applied in this study. Amplification was performed in a Progene thermal cycler (Techne Cambridge Ltd., UK) programmed as follows: an initial denaturation step at 95°C for 5 min followed by 45 cycles of denaturation (95°C for 1 min), annealing (35°C for 1 min) and extension (72°C for 2 min), with a final extension of 72°C for 5 min. The amplified products were separated by electrophoresis on a 2% agarose gel, stained with ethidium bromide and photographed under UV light.

16S rRNA gene sequencing

For sequencing of 16S rRNA gene region, cyanobacteria-specific primers 1R (AGAGTTTGATCCTGGCTCAG) and 740R (TCTACGCATTTCACCGCTAC) were employed (Seo and Yokota, 2003). Amplification was carried out using Ready-To-Go PCR Beads (Amersham Biosciences, Uppsala, Sweden) containing 10 mM Tris–HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 0.3 μM 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 (Techne Cambridge Ltd, UK) thermal cycler. The cycling profile included an initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation (95°C for 30 s), annealing (65°C for 15 s) and extension (72°C for 60 s) and a final extension of 72°C for 5 min. Five microliter of the amplified products was subjected to 1.5% agarose gel electrophoresis, stained with ethidium bromide and visualized by UV trans-illuminator.

The amplified PCR products were purified using QIAquick PCR purification kit (Qiagen GmbH, Hilden, 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 (Applied Biosystems, Foster, CA, USA) following the protocol recommended by the supplier. The DNA sequences were run on an ABI 310 automated sequencer using the chain-termination method with big-dye terminators (Applied Biosystems, Foster, CA, USA). 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 the SeqScape® software v 2.5 (Applied Biosystems, Foster, CA, USA) and deposited in GenBank (www.ncbi.nlm.nih.gov) (Table I).

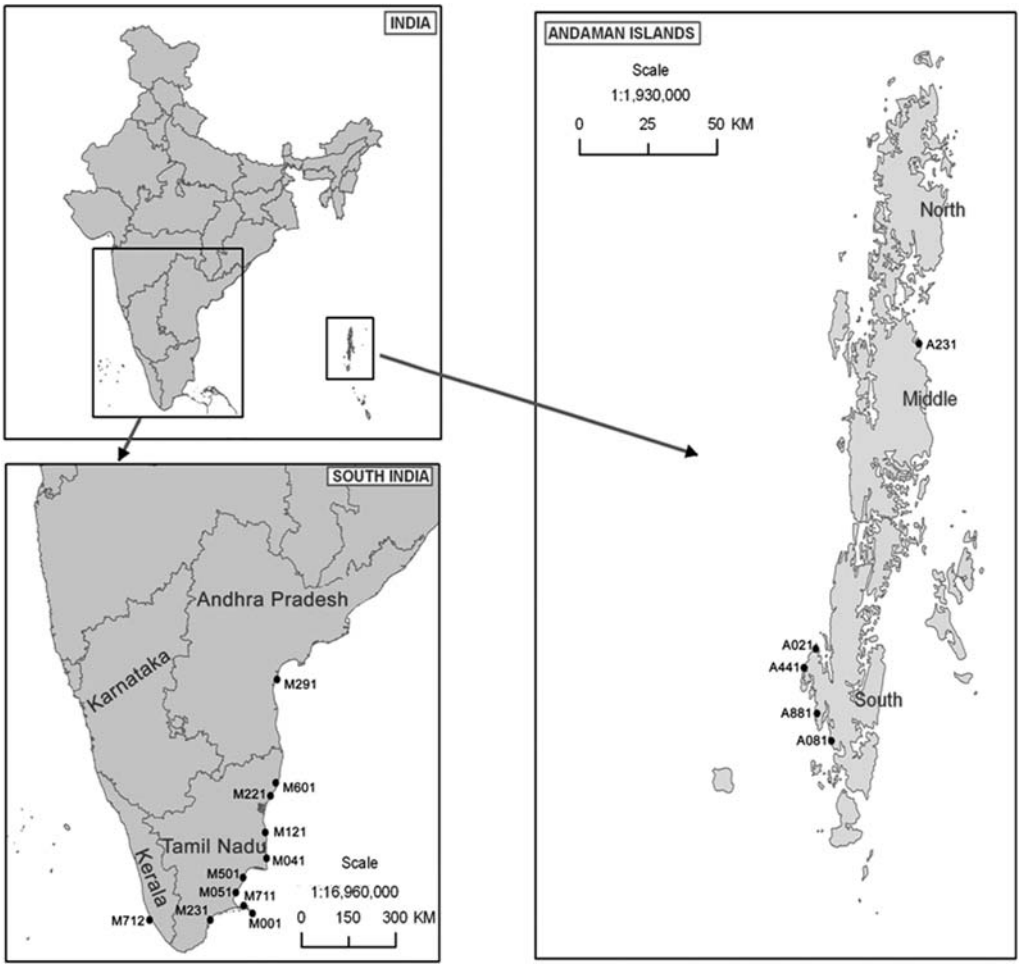

Fig. 1. Map depicting sample locations of 16 *L. valderiana* strains applied in this study.

Data analysis

The RAPD data analysis was performed considering each DNA fragment visualized on a gel as a marker and part of the total RAPD fingerprint generated for each strain. The presence of a band at each position on the gel was scored as 1 and absence as 0 to construct the binomial matrix. The parameters and the percentage of polymorphic loci were calculated using the PAST program (Hammer *et al*., 2001). The genetic similarities between strains were estimated by means of the Dice coefficient (Dice, 1945). The same program was used to construct a dendogram using the unweighted pair-group method with arithmetic averages (UPGMA), which shows the relationships between individuals.

For 16S rRNA gene, the sequences obtained from this study (Table I) were aligned using CLUSTALW. A GenBank BLAST search (http://www.ncbi.nlm.nih.gov) was used to identify the closely related taxa for sequence comparisons. Maximum parsimony trees were generated using a heuristic search constrained by random sequence addition (1000), steepest descent and tree-bisection branch swapping using PAUP v.4.02b (Swofford, 1998). Bootstrap values were obtained from 1000 replicates with one random sequence addition to jumble the data. A maximum likelihood tree employing the general time reversible model with corrected invariable sites (I) and Gamma distribution shape parameters (G) was constructed with 100 rounds (each with its own random addition) of ML analysis.

RESULTS

Morphologically, all the strains illustrated filamentous, flexous trichomes with no constriction at the cross walls, blue-green coloration with a thin sheath, non-capitate, and exhibited a granule on either side of the cross walls (Table I). The taxonomic diversity based on the

Table I: Locations of isolation and morphological measurements of strains used in this study

Strain	Isolation location	Accession number	Morphology
M041	Point Calimare (Bay of Bengal)	FN296229	Filamentous, trichomes flexous, not constricted at the cross-walls, not curved, ends not attenuated, calyptra absent, sheath thin, trichomes blue-green, 2.0 μm wide, 5.3 μm long, cross walls with one granule on either side, non-capitate
M501	Palk Bay (Bay of Bengal)	FN296223	Filamentous, trichomes flexous, not constricted at the cross-walls, not curved, ends not attenuated, calyptra absent, sheath thin, trichomes blue-green, 2.2 μm wide, 5.4 μm long, cross walls with one granule on either side, non-capitate
A441	South Andaman (Bay of Bengal)	FN296230	Filamentous, trichomes flexous, not constricted at the cross-walls, not curved, ends not attenuated, calyptra absent, sheath thin, trichomes blue-green, 2.3 μm wide, 5.4 μm long, cross walls with one granule on either side, non-capitate
M121	Tamil Nadu (Bay of Bengal)	FN296228	Filamentous, trichomes flexous, not constricted at the cross-walls, not curved, ends not attenuated, calyptra absent, sheath thin, trichomes blue-green, 2.0 μm wide, 5.3 μm long, cross walls with one granule on either side, non-capitate
M601	Pondicherry (Bay of Bengal)	FN296226	Filamentous, trichomes flexous, not constricted at the cross-walls, not curved, ends not attenuated, calyptra absent, sheath thin, trichomes blue-green, 2.0 μm wide, 5.3 μm long, cross walls with one granule on either side, non-capitate
A081	South Andaman (Bay of Bengal)	FN296236	Filamentous, trichomes flexous, not constricted at the cross-walls, not curved, ends not attenuated, calyptra absent, sheath thin, trichomes blue-green, 2.0 μm wide, 5.0 μm long, cross walls with one granule on either side, non-capitate
A881	South Andaman (Bay of Bengal)	FN296237	Filamentous, trichomes flexous, not constricted at the cross-walls, not curved, ends not attenuated, calyptra absent, sheath thin, trichomes blue-green, 2.1 μm wide, 5.0 μm long, cross walls with one granule on either side, non-capitate
A021	South Andaman (Bay of Bengal)	FN296238	Filamentous, trichomes flexous, not constricted at the cross-walls, not curved, ends not attenuated, calyptra absent, sheath thin, trichomes blue-green, 2.5 μm wide, 5.5 μm long, cross walls with one granule on either side, non-capitate
A231	South Andaman (Bay of Bengal)	FN296235	Filamentous, trichomes flexous, not constricted at the cross-walls, not curved, ends not attenuated, calyptra absent, sheath thin, trichomes blue-green, 2.0 μm wide, 5.3 μm long, cross walls with one granule on either side, non-capitate
M051	Palk Bay region (Bay of Bengal)	FN296227	Filamentous, trichomes flexous, not constricted at the cross-walls, not curved, ends not attenuated, calyptra absent, sheath thin, trichomes blue-green, 2.5 μm wide, 5.0 μm long, cross walls with one granule on either side, non-capitate
M231	Tamil Nadu (Gulf of Mannar)	FN296232	Filamentous, trichomes flexous, not constricted at the cross-walls, not curved, ends not attenuated, calyptra absent, sheath thin, trichomes blue-green, 2.0 μm wide, 5.1 μm long, cross walls with one granule on either side, non-capitate
M001	Tamil Nadu (Gulf of Mannar)	FN296231	Filamentous, trichomes flexous, not constricted at the cross-walls, not curved, ends not attenuated, calyptra absent, sheath thin, trichomes blue-green, 2.5 μm wide, 5.0 μm long, cross walls with one granule on either side, non-capitate
M221	Tamil Nadu (Bay of Bengal)	FN296234	Filamentous, trichomes flexous, not constricted at the cross-walls, not curved, ends not attenuated, calyptra absent, sheath thin, trichomes blue-green, 2.3 μm wide, 5.1 μm long, cross walls with one granule on either side, non-capitate
M712	Kerala (Arabian Sea)	FN296233	Filamentous, trichomes flexous, not constricted at the cross-walls, not curved, ends not attenuated, calyptra absent, sheath thin, trichomes blue-green, 2.0 μm wide, 5.3 μm long, cross walls with one granule on either side, non-capitate
M711	Palk Bay region (Bay of Bengal)	FN296225	Filamentous, trichomes flexous, not constricted at the cross-walls, not curved, ends not attenuated, calyptra absent, sheath thin, trichomes blue-green, 2.0 μm wide, 5.2 μm long, cross walls with one granule on either side, non-capitate
M291	Bay of Bengal (Bay of Bengal)	FN296224	Filamentous, trichomes flexous, not constricted at the cross-walls, not curved, ends not attenuated, calyptra absent, sheath thin, trichomes blue-green, 2.0 μm wide, 5.5 μm long, cross walls with one granule on either side, non-capitate

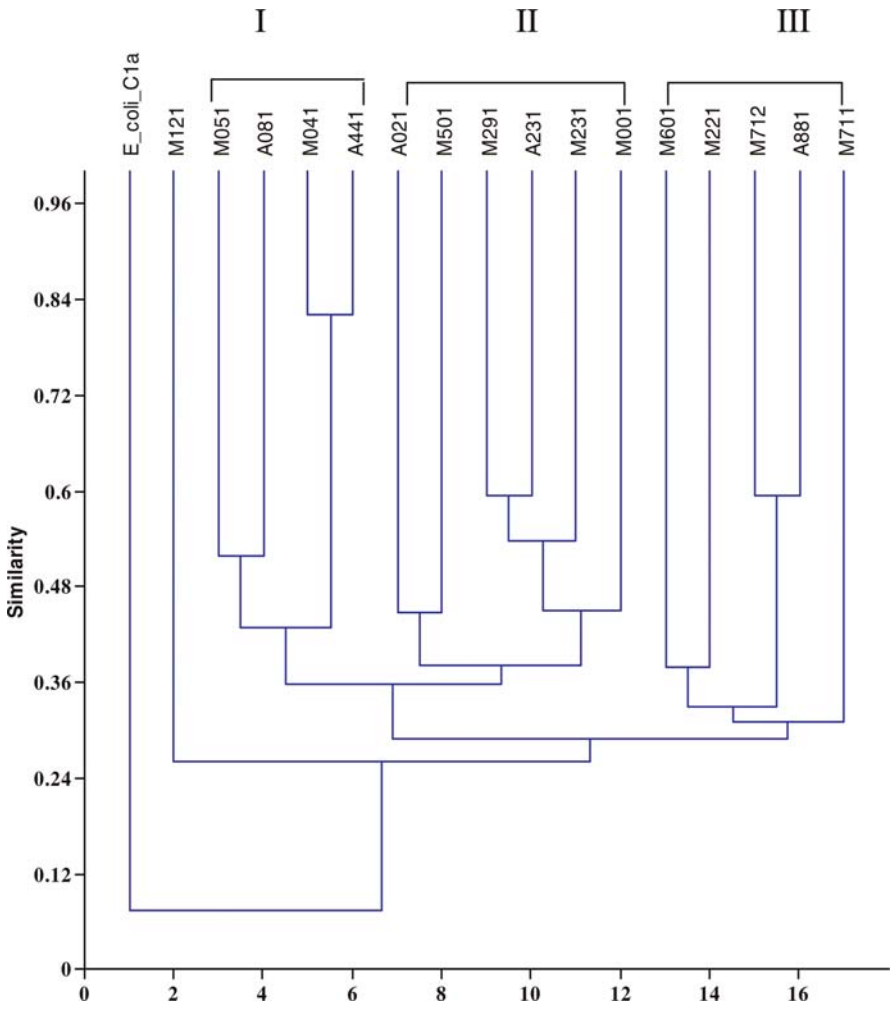

Fig. 2. Dendogram representing the relationships between *L. valderiana* strains based on UPGMA cluster analysis of the RAPD profiles derived using four primers and Dice similarity coefficient.

morpho-species concept was very low and found only in the trichome size.

RAPD analysis of the strains generated 123 bands of 100% polymorphism. Each primer produced from 26 to 38 bands (mean = 31, SD = $\pm$ 5.6). The size of the amplified products ranged from 100 to 1700 bp, while negative controls in which sample DNA was replaced by distilled water did not show any amplification. One of the major drawbacks of RAPD analysis is the reproducing ability of the technique (Penner *et al.*, 1993; Skroch and Nienhuis, 1995). Hence, the reproducibility of the results was assessed by repeating the experiments three times. Other than variations in the intensity of bands, no major variation was observed in the banding profile.

The similarity matrix obtained from the combined RAPD patterns was not associated with the geographical proximity. For example, strains isolated from Andaman were distributed in all the three clades along with the mainland isolates (Fig. 2). The highest percentage of similarity was observed between isolates A441 and M041 (82%), collected from a distance of approximately 1500 km from each other. Conversely, strains M051 and M501, collected within 60 km of each other, revealed only 37% similarity (Table II). Likewise, M712 isolated from a distance of around 1700 km exhibited a similarity of 59% with A881 from the South Andaman region. The lowest percentage of similarity (13.8%) was observed between mainland isolate M711 and A021 (Table II).

A phylogenetic tree was constructed using partial 16S rDNA gene sequence data (520 bp) (Fig. 3). The maximum parsimony tree exhibited discernable biogeographic trends with some exceptions. The samples collected from salt pans within a close proximity (M501 and M051) exhibited 99% sequence similarity and

Table II: RAPD and 16S rRNA gene similarity matrix of strains applied in this study

	M501	M291	M711	M601	M051	M121	M041	A441	M001	M231	M712	M221	A231	A081	A881	A021
L. valderiana M501	1	0.438	0.277	0.215	0.369	0.317	0.464	0.394	0.269	0.333	0.258	0.323	0.344	0.328	0.242	0.448
L. valderiana M291	0.963	1	0.182	0.4	0.327	0.302	0.508	0.571	0.421	0.5	0.346	0.346	0.593	0.526	0.357	0.526
L. valderiana M711	0.956	0.977	1	0.321	0.214	0.222	0.3	0.246	0.31	0.246	0.302	0.302	0.182	0.31	0.316	0.138
L. valderiana M601	0.961	0.971	0.982	1	0.286	0.296	0.233	0.281	0.276	0.246	0.34	0.377	0.291	0.31	0.351	0.345
L. valderiana M051	0.99	0.965	0.965	0.971	1	0.333	0.367	0.421	0.172	0.281	0.302	0.189	0.291	0.517	0.281	0.172
L. valderiana M121	0.959	0.971	0.978	0.971	0.957	1	0.241	0.255	0.214	0.145	0.275	0.196	0.302	0.286	0.255	0.25
L. valderiana M041	0.957	0.953	0.954	0.961	0.957	0.955	1	0.82	0.29	0.295	0.211	0.246	0.475	0.452	0.361	0.387
L. valderiana A441	0.961	0.965	0.965	0.965	0.959	0.969	0.965	1	0.339	0.31	0.222	0.296	0.464	0.475	0.379	0.407
L. valderiana M001	0.967	0.961	0.969	0.977	0.973	0.975	0.961	0.956	1	0.508	0.291	0.327	0.421	0.267	0.407	0.333
L. valderiana M231	0.972	0.973	0.965	0.971	0.967	0.973	0.963	0.967	0.967	1	0.259	0.444	0.571	0.271	0.31	0.339
L. valderiana M712	0.964	0.944	0.94	0.946	0.959	0.94	0.934	0.944	0.948	0.951	1	0.36	0.192	0.364	0.593	0.255
L. valderiana M221	0.964	0.948	0.948	0.95	0.963	0.944	0.934	0.948	0.948	0.947	0.98	1	0.308	0.255	0.259	0.327
L. valderiana A231	0.953	0.93	0.925	0.929	0.943	0.927	0.921	0.93	0.932	0.936	0.947	0.947	1	0.351	0.357	0.456
L. valderiana A081	0.958	0.936	0.931	0.934	0.949	0.932	0.924	0.934	0.934	0.942	0.953	0.953	0.99	1	0.407	0.3
L. valderiana A881	0.955	0.932	0.927	0.931	0.945	0.929	0.921	0.93	0.93	0.938	0.949	0.949	0.99	0.996	1	0.237
L. valderiana A021	0.951	0.928	0.921	0.927	0.941	0.925	0.919	0.928	0.93	0.934	0.945	0.945	0.994	0.984	0.988	1

Numbers above the diagonal are RAPD similarity values, while numbers below represents 16S values.

grouped with other isolates from the Palk Bay region. Samples from Andaman with an exception of A441 were clustered together with *Phormidium sp.* and are phylogenetically distant from the mainland isolates. Likewise, the isolates from the Gulf of Mannar (M001 and M231) collected from rocky shores grouped with A441 from Andaman and Mainland isolate M041 collected from Andhra Pradesh (Bay of Bengal) which is geographically distant from each other. Similarly, isolates M712 from Kerala (Arabian Sea) clustered with M221 from Tamil Nadu (Bay of Bengal) that is geographically unrelated suggesting their dispersal capabilities. These isolates grouped with type species of *Leplolyngbya* PCC7104 and other Oscillatoriales signifying the non-monophyletic nature of *L. valderiana*. Although, the 16S parsimony tree topology did not match with that generated via RAPD analysis, congruence on genetic relatedness of A441 with M041 and M712 with M221 was observed in both the analyses. A maximum likelihood analysis recovered a tree of similar topology (data not shown). The pair wise similarity values of the sequence data ranged from 91 to 99% (Table II). The low values (91%) indicate putative cryptic species. Indeed, only some strains showed greater than 98.7% sequence similarity, the threshold value for bacterial species boundaries (Stackebrandt and Ebers, 2006).

DISCUSSION

While cyanobacteria are undoubtedly a widely distributed group of organisms, cyanobacterial experts have long debated the existence of cosmopolitan cyanobacteria (Komarek, 1985; Komarek and Anagnostidis, 1999). Many researchers have concluded that many taxa are not cosmopolitan, but have more narrowly defined geographic and ecological ranges than previously thought, and argue that endemism is probably common in cyanobacteria (Komarek, 1999). For example, Cronberg and Komárek (Cronberg and Komarek, 2001, 2004) found that while some planktonic strains of cyanobacteria from lentic African systems appear to be cosmopolitan, the majority of prevalent taxa do not easily fit European species descriptions, and thus infer a high level of regional endemism among both the Nostocales and Oscillatoriales. Branco and Neechi (Branco and Neechi, 1996a, 1996b, 2001) have recently documented many of the common lotic cyanobacterial taxa from Brazil, and point out that many of the taxa are likely new to science. Some lineages apparently have numerous cryptic taxa (Boyer *et al.*, 2002;

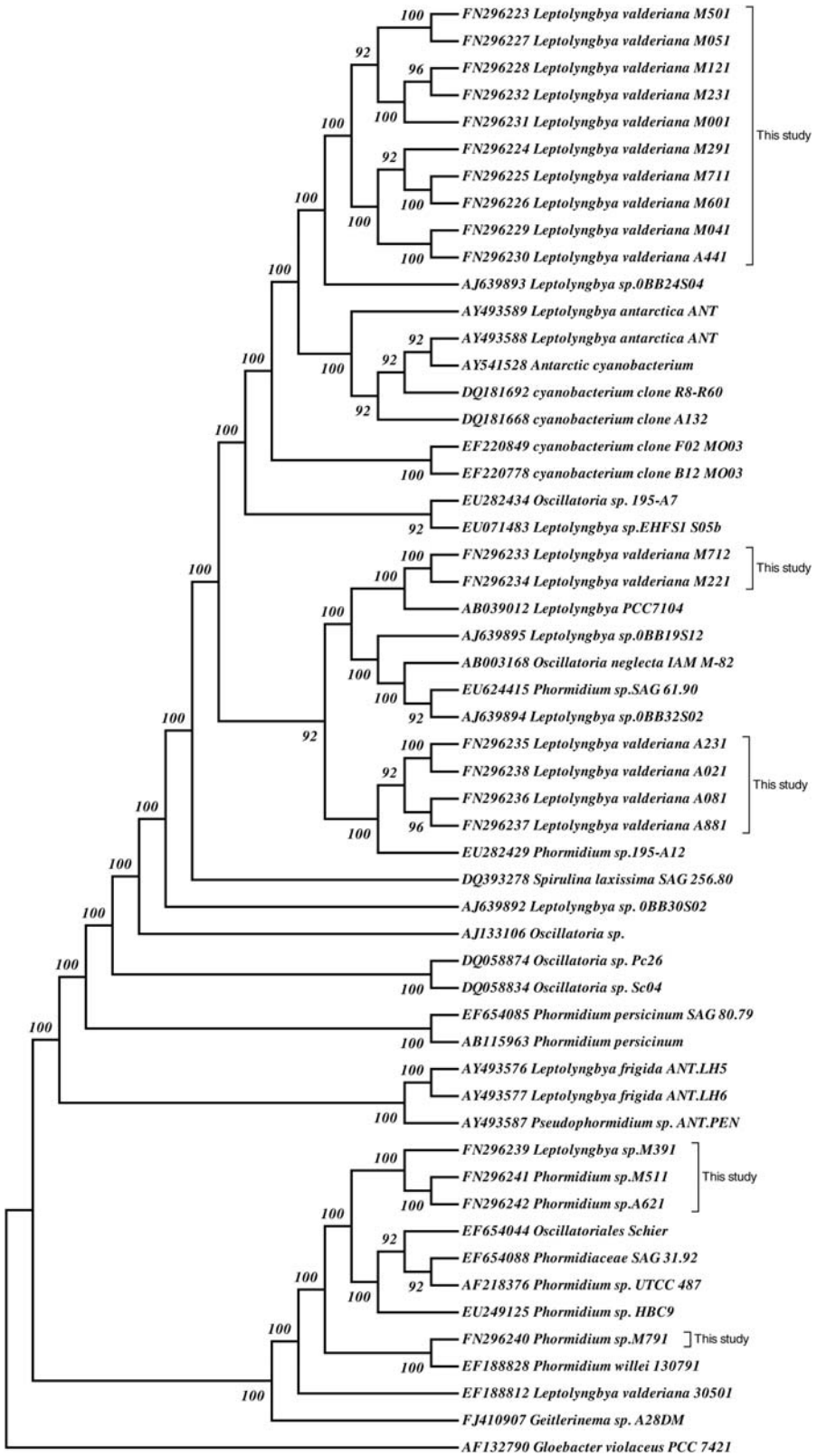

Fig. 3. Phylogenetic estimate on relationships of *L. valderiana* strains based on maximum parsimony analysis of partial 16S rDNA sequences. Values above the lines represent bootstrap values (100 replicates).

Casamatta *et al*., 2003), but it is difficult to reveal such species by morphology alone.

Despite the above findings, many researchers still suggest that cyanobacteria have a low level of endemism (Vincent, 2000). The recent invasion of *Cylindrospermopsis raciborskii* from tropical to temperate regions has been used as evidence that physiologically tolerant cosmopolitan species exist (Briand *et al*., 2004).

While some degree of variation is likely to occur even among clonally related isolates (Hyttia *et al*., 1999; Roberts and Crawford, 2000), the polymorphic profiles generated by RAPD analysis suggest considerable degree of intra species heterogeneity despite morphological similarity. Since morphology might not be strictly controlled by genetics (Saker *et al*., 1999), genotypic variations are virtually indistinguishable by morphological means. The failure of morphological features to distinguish cyanobacterial strains agrees with previous findings on *Phormidium retzii* using RAPD markers (Casamatta *et al*., 2003) and *Leptolyngbya* utilizing 16S rRNA gene sequences (Payne *et al*., 2001). Other authors report that ultrastructural features (e.g. thylakoid arrangement) are useful characters to define taxonomic relationships among closely related taxa within cyanobacterial families (Casamatta *et al*., 2005). However, more recent study on the thylakoid arrangement of *Phormidium* suggests that some taxa cannot be distinguished based on their cellular ultrastructure (Marquardt and Palinska, 2007). Thus, a combination of both morphological and molecular data may facilitate establishing taxonomic positions of this problematic group.

Our previous study on the presence of microcystin synthetase genes supports the present pattern of genomic variations (Premanandh *et al*., 2006b). Out of the 21 strains of *L. valderiana* screened, only four appeared to be positive for the microcystin synthetase gene by PCR analysis suggesting variability among strains. The variations may have resulted in response to environmental stress on the ecotypes. For example, *L. valderiana* BDU 30501 is capable of utilizing ampicillin and phenol in nitrogen stress environments (Prabaharan *et al*., 1994). Likewise, degradation of lignin model dye Poly R-478 and organophosphorous pesticide by *L. valderiana* BDU 140441 and azo dyes (orange G) by *L. valderiana* BDU 20041 (Priya *et al*., 2006) signify the genetic differences leading to the adaptability of strains to various environmental conditions. It is also apparent from the cluster analysis that the patterns of genetic differences were not well associated with eco-geographical variables. For example, the five strains isolated from Andaman do not correspond to distinct geographic areas and distributed throughout the dendogram (Fig. 2). Even physical proximity of the strains A081 and A881 was not a good predictor of genetic resemblance. The possible reason may be attributed to the technique itself since the RAPD method derives information from whole bacterial genome where regions with higher variability are present. Nevertheless, the genetic relatedness of A441 with M041 corroborated well with our previous findings using the phycocyanin operon (Premanandh *et al*., 2006a).

In the case of 16SrRNA gene phylogeny, distinct environmental conditions such as salinity (Palk Bay region) and rocky shores rich in organic matter (Gulf of Mannar) have contributed to the genetic relatedness leading to discernible ecological trends among the isolates as observed by Miller *et al*. (Miller *et al*., 2007). The geographical separation of Andaman isolates from the Mainland isolates into separate sequence clusters (Fig. 3) may be due to the lack of opportunity to exchange genes with other sub populations.

Nevertheless, 16S rRNA gene sequences of many isolates revealed genetic differentiation on the order that is normally encountered between other species. These results indicate that several morpho-species in reality composed of genetically divergent population or cryptic species as observed in other closely related taxa (Casamatta *et al*., 2003). Therefore, we propose to address *L. valderiana* at the generic level until further study proves otherwise.

Besides adaptive mutations leading to ecotypes, variations may also be due to the ongoing taxonomic refinements of this group. The recent establishment of the genus *Leptolyngbya* (Anagnostidis and Komarek, 1988) was the result of reassignment of many species with thin filaments into this genus. Hence, it can be speculated that morphological simplicity of this group may perhaps have resulted in two or more ecological groups. For instance, the uncertainty on the generic identity of some cyanobacteria with narrow trichomes places them either within the genus *Leptolyngbya* or *Pseudophormidium*. However, 16S rDNA gene sequence analysis of these morphotypes exhibits distinction above genus level. Such designations remain provisional awaiting results from further characteristic studies of large numbers of morphotypes and ecotypes (Komarek and Anagnostidis, 2005).

However, more samples from geographically distinct locations and habitats may reveal further information on the taxonomic clarification of this putative group.

In conclusion, the genetic variability observed within the marine strains of *L. valderiana* indicates the presence of cryptic species. Since many morpho-species of this group are potentially the subject for biotechnological application, future investigations combining well-characterized

ultra structural morphology with additional molecular markers are essential to characterize and clarify the patterns in their evolution and biogeography.

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