RESEARCH ARTICLE

Cyanobacterial diversity in the phyllosphere of a mangrove forest

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Abstract

The cyanobacterial community colonizing phyllosphere in a well-preserved Brazilian mangrove ecosystem was assessed using cultivation-independent molecular approaches. Leaves of trees that occupy this environment (*Rhizophora mangle*, *Avicennia schaueriana* and *Laguncularia racemosa*) were collected along a transect beginning at the margin of the bay and extending upland. The results demonstrated that the phyllosphere of *R. mangle* and *L. racemosa* harbor similar assemblages of cyanobacteria at each point along the transect. *A. schaueriana*, found only in the coastal portions of the transect, was colonized by assemblages with lower richness than the other trees. However, the results indicated that spatial location was a stronger driver of cyanobacterial community composition than plant species. Distinct cyanobacterial communities were observed at each location along the coast-to-upland transect. Clone library analysis allowed identification of 19 genera of cyanobacteria and demonstrated the presence of several uncultivated taxa. A predominance of sequences affiliated with the orders *Nostocales* and *Oscillatoriales* was observed, with a remarkable number of sequences similar to genera *Symphyonemopsis*/*Brasilonema* (order *Nostocales*). The results demonstrated that phyllosphere cyanobacteria in this mangrove forest ecosystem are influenced by environmental conditions as the primary driver at the ecosystem scale, with tree species exerting some effect on community structure at the local scale.

Introduction

Mangroves are a coastal transition ecosystem existing between terrestrial and marine environments. These habitats are subject to the influence of tidal regimes and are defined by dense growth of shrubs and trees. Mangroves also sustain highly diverse microbial communities adapted to tolerate high salinity and environmental stressors (Kathiresan & Bingham, 2001; Dias *et al.*, 2010).

Soil and environmental conditions in the mangroves located along the coastline of São Paulo (Brazil) restrict the plant diversity to three dominant tree species: *Avicennia schaueriana*, *Rhizophora mangle*, and *Laguncularia racemosa* (Schaeffer-Novelli *et al.*, 1990a). The phyllosphere is known to harbor complex microbial communities (Lindow & Brandl, 2003; Dulla *et al.*, 2005; Lambais *et al.*, 2006), which are responsive to shifts in plant species and environmental conditions. Even very small differences in microclimate, especially humidity, can influence the community structure of bacteria in the phyllosphere of a tropical rain forest (Freiberg, 1998).

Cyanobacteria are autotrophic, capable of fixation of both nitrogen and carbon (Feller & Sitnik, 1996; Sandau *et al.*, 1996; Juhasz & Naidu, 2000; El-Bestawy *et al.*, 2007), making them theoretically less dependent on the plant exudates for their nutrition. In addition, primary production by cyanobacteria in the phyllosphere may facilitate colonization of the leaf surface by heterotrophic organisms. Previous studies have found cyanobacteria to be the principal nitrogen-fixing epiphytes on tropical

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plants within the Costa Rican rainforest (Freiberg, 1998; Fürnkranz *et al.*, 2008). Understanding the contribution of cyanobacteria to the nitrogen cycle of mangrove ecosystems requires that we understand the ecological factors that govern their abundance and distribution.

Despite their potential importance as keystone species in the microbial ecology of the phyllosphere and their role in the nitrogen cycle in mangroves, there have been no studies to characterize epiphytic cyanobacterial communities colonizing mangrove tree leaves. Therefore, this study was undertaken to assess the diversity of the cyanobacterial community associated with the phyllosphere of *A. schaueriana*, *R. mangle*, and *L. racemosa* in a well-preserved Brazilian mangrove, using two culture-independent molecular approaches: PCR-denaturing gradient gel electrophoresis (PCR-DGGE) to evaluate the structure of cyanobacterial communities associated with the dominant plant species and spatially distinct mangrove habitats; and clone library methodologies, applied to describe the phylogeny of the cyanobacterial groups composing such communities.

Materials and methods

Site description and sampling

Leaf sampling was performed at the mangrove in the Cardoso Island, located in the south of the São Paulo State (Cananéia municipality, Brazil). This natural reserve was created by the Brazilian government on July 3, 1962, enclosing an area of 15 100 ha and contains mangroves and several coastal ecosystems, including the Atlantic Rainforest and restinga (inland forest). The only woody tree species found in this mangrove are as follows: *A. schaueriana* (A), *L. racemosa* (L), and *R. mangle* (R). The climate is humid, with heavy rains occurring between October and March (average temperature of 25 °C), and a less rainy period between April and September (average temperature of 18 °C).

Leaves were collected in September 2007 from medium size (approximately 4–5 m) individuals of each tree species. Leaves were collected from positions on the tree, which were subjected to variation in sun/shadow conditions throughout the day. The average temperature of the region was 21 °C. Trees of *R. mangle* generally range in height between 5 and 8 m, with leaves about 10–20 cm long and 2.5–7.5 cm broad. *Laguncularia racemosa* has an average size of 3–5 m, with leaves that reach up to 7 cm in length. *Avicennia schaueriana* is characterized by its opposite leaves that are narrow and elliptical in shape (5 cm in length), and the leaves contain glands for salt excretion in the phylloplane (Hogarth, 2007). The sampling sites were located along a 340-m long transect according to the mangrove flood gradient as follows: NS – near the shore (25°05′1.87″S, 47°57′41.1″W), MM – middle of the mangrove (25°05′6.88″S, 47°57′41.42″W) and RT – restinga (25°05′12.21″S, 47°57′41.21″W). Along the transect, differences were observed in the height and density of the trees, with taller trees and greater plant density in the area NS, while smaller trees were observed at the inland forest area RT (Schaeffer-Novelli *et al.*, 1990a, b). *Avicennia schaueriana* individuals were observed only near the shore (NS). At every site, a composite sample of three leaves was collected from three trees of each species, comprising a total of 21 trees sampled (three samples from *A. schaueriana*, nine samples from *L. racemosa* and nine samples from *R. mangle*). The leaves were packaged in sterilized plastic bags and stored at 4 °C until DNA was extracted from epiphytic cyanobacteria. The period from sample collection to DNA extraction was 48 h.

DNA extraction and 16S rRNA gene PCR amplification

Three leaves collected from each tree were placed in a 250-mL Erlenmeyer flask containing 50 mL of ultrapure sterile water and incubated for 1 h at room temperature (~25 °C) under agitation at 150 r.p.m. to promote detachment of cyanobacterial cells from leaf surfaces. For each sample, the liquid containing the cell suspension was transferred to a 50-mL sterile disposable tube and centrifuged at 7000 ***g*** for 20 min. The supernatant was carefully removed, and the total genomic DNA was extracted from the pellet using a phenol/chloroform method. Briefly, the pellet was suspended in 500 μL of TE (Tris–HCl pH 7.8 10 mM, EDTA 1 mM), and amended with 10 μL 10% sodium dodecyl sulfate and 0.1 g glass beads (0.1 mm) (Sigma, Washington, DC). Cell lysis was aided by mechanical disruption using a bead beater (BioSpec, Bartlesville, OK) for 1 min (5000 r.p.m.). Following lysis, total DNA extraction and purification were conducted as described by Ausubel *et al.* (1995).

PCR amplification of the cyanobacterial 16S rRNA gene was performed in a Gene Amp PCR System 2400 (Applied Biosystems, Foster City, CA) using previously published primers 27F1 and 1494Rc and amplification conditions (Neilan *et al.*, 1997). Amplicons were analyzed by electrophoresis on 1% agarose gels, quantified densitometrically (Qubit^{tm} fluorometer; Invitrogen, Carlsbad, CA), and stored at −20 °C.

Nested PCR and DGGE

The structure of the total cyanobacterial community associated with the leaf surfaces of mangrove trees was

assessed by PCR-denaturing gradient gel electrophoresis (PCR-DGGE). A nested PCR approach was applied for the generation and analysis of cyanobacteria-specific 16S rRNA gene fragments. The amplicons obtained with the primers 27F1 and 1494Rc were used as templates in a subsequent PCR for DGGE analysis with the primers CYA359F (with a 40-base of GC clamp) and CYA781Ra and CYA781Rb in equimolar concentrations as described by Nübel *et al.* (1997). For DGGE analysis, 300 ng of each PCR product was used.

DGGE was performed using an Ingeny phor U2 (Ingeny, International BV, Goes, the Netherlands). Polyacrylamide gels (6%; 37.5 : 1 acrylamide-bis acrylamide) were prepared and electrophoresed with 0.5× TAE buffer [0.04 M Tris base, 0.02 M sodium acetate, and 10 mM EDTA (pH 7.4)]. The DGGE gel contained a 20–60% gradient of urea and formamide as denaturing agents [100% denaturant consisted of 40% (v/v) formamide and 7 M urea]. DGGE was conducted at a constant voltage of 100 V at 60 °C for 15 h. The gel was stained with silver nitrate (Blum *et al.*, 1987) and photographed on a white light transilluminator (VariQuest 100; FOTODYNE Incorporated, Hartland, WI). The DGGE band profile was analyzed using an image-analysis system (Bionumerics, Applied Maths NV). Bands were identified and a binary matrix recording the presence or absence of each band in each sample was exported for further analyses.

DGGE profiles were compared using the Sørenson index of similarity (Magurran, 1988; Legendre & Legendre, 1998). A similarity matrix was generated in which the Sørenson's index was determined for all possible pairs of samples. This similarity matrix was used to generate nonmetric multidimensional scaling (NMDS) ordinations to observe patterns of similarity between samples, and the significance of these patterns was tested using the analysis of similarity (ANOSIM) statistics (Clarke & Green, 1988). ANOSIM is a statistical procedure that tests whether the phyllosphere communities in different *a priori* sample groups (e.g. mangrove habitats or mangrove tree species) are significantly different in structure. ANOSIM generates a test statistic, *R*. The magnitude of *R* indicates the degree of separation between groups of samples, with a score of 1 indicating complete dissimilarity and 0 indicating no difference. Permutation of sample labels was used to generate null distributions of *R* values to test the hypothesis that within-group similarities were higher than would be expected if DGGE profiles were grouped at random. Calculation of similarity coefficients and ANOSIM analyses were carried out using PRIMER 6 for Windows (PRIMER-E, Plymouth, UK).

Ubiquitous or abundant bands were excised from the DGGE gel, re-amplified and sequenced for the identification of specific taxa, using a DYEnamic ET Terminator Cycle Sequencing kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK) using the CYA 359F primer (without GC clamp), according to the manufacturer's instructions, and sequences were determined using an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems).

Clone libraries and sequence analysis

Seven clone libraries of cyanobacterial 16S rRNA gene fragments were generated using the same nested PCR approach described for PCR-DGGE analysis, with the exception that the CYA359F primer did not include a GC clamp. PCR amplifications were performed for each sampled tree (21 trees) and the PCR products generated from all trees of the same species at each site were pooled together, resulting in seven amplicon mixtures. These amplicons were purified with GFX PCR DNA and a Gel Band Purification kit (GE Healthcare) and ligated into a pGEM-T Easy Vector (Promega, Madison, WI) at 4 °C overnight, according to the manufacturer's instructions. Ligations were transformed into electrocompetent *Escherichia coli* DH5α cells [One shot TOP 10 Electrocomp *E.coli* (Invitrogen)].

Plasmid DNA was extracted through alkaline lysis (Birnboim & Doly, 1979). The sequencing reaction was: 200 ng of plasmid, 10 pmol of T7 primer, 1 μL of DYEmanic ET Terminator kit (GE Healthcare), 2 μL of buffer (200 mM Tris–HCl pH 9.0 and 5 mM $MgCl_2 \cdot 6H_2O$). The product was precipitated with cold ethanol, resuspended in HI-DI formamide and sequenced in an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems).

Nucleotide sequences were trimmed for the removal of low-quality bases and vector sequences (quality parameter > 20, that is > 1 error in 100 nucleotides); chimeric sequences were also removed using the Bellerophon program (Huber *et al.*, 2004). Sequences were then aligned using the program MEGA 4 (Tamura *et al.*, 2007), and Kimura two-parameter evolutionary distances were calculated (Felsenstein, 1985). The final matrix was used to cluster the sequences into operational taxonomic units (OTUs) based on an evolutionary distance cut-off of 0.03 (> 97% within-group sequence identity) using the program DOTUR (Schloss & Handelsman, 2005). Sequences obtained were compared with sequences deposited in GenBank by performing a BLAST search (GenBank, nr/nt); organisms with the highest values for percent coverage, score, and identity were selected to represent each clone.

To compare libraries, all sequences were grouped together, and OTUs were defined. The phylogenetic tree was constructed by selecting one representative clone for each OTU and combining those with reference cyanobacterial sequences retrieved from GenBank. The phylogeny was built using the Kimura two-parameter substitution

model distance and Neighbor-Joining method (Saitou & Nei, 1987) in the MEGA 4 program (Tamura *et al.*, 2007). Bootstrap analyses involving the construction of 1000 resampled trees were performed (Felsenstein, 1985).

The estimated minimum number of OTUs in the samples was determined through ACE (Chao & Lee, 1992) and Chao1 (Chao, 1984) nonparametric estimators, Shannon's index (maximum likelihood estimator) and rarefaction curves for each clone library were generated using the DOTUR program. SPADE (Chao & Shen, http://chao.stat.nthu.edu.tw/) was used for the determination of estimated sample coverage (ESC). The reciprocal coverage of libraries was analyzed using the program ∫-LIBSHUFF 1.22 (Singleton *et al.*, 2001). A complete alignment, containing the sequences from all seven libraries, was made using CLUSTALX (Thompson *et al.*, 1997) under the default parameters. This alignment was used to calculate Jukes–Cantor evolutionary distances using DNADIST in the PHYLIP 3.63 Package (Felsenstein, 1985). It is assumed that two libraries are considered significantly different when $P < 0.05$. This method uses the Cramer–von Mises statistic to test whether two communities have the same structure (Singleton *et al.*, 2001; Schloss *et al.*, 2004). The significance is tested by permutation of sequences between two libraries (A and B). This test is asymmetrical, making it possible for library A vs. library B to be significant, while library B vs. library A is not; this would indicate that the species mix from which library B was constructed is likely to be a subset of the species mix from which library A was constructed (Yannarell *et al.*, 2006).

Nucleotide sequences accession numbers

The 16S rRNA gene sequences obtained from the clone library and PCR-DGGE were deposited in GenBank under accession numbers JF788626 – JF789246.

Results

PCR-DGGE analysis

The composition and diversity of the cyanobacterial community in the phyllosphere of mangroves in Cardoso Island were initially evaluated by PCR-DGGE analyses, which revealed greater differences in cyanobacteria assemblages among mangrove habitats (near the shore – NS, middle mangrove – MM, restinga – RT) than among phyllosphere samples collected from each dominant tree species. Comparing samples collected from all the three tree species near the shore (NS) (Fig. 1, samples 1–9), the DGGE patterns from *A. schaueriana* revealed an intense band (Fig. 1, bands 3 and 4) representing a unique cyanobacterial epiphyte population found in the phyllosphere of this tree.

Thirty-one bands were excised and sequenced (Supporting information, Table S1) to characterize the cyanobacteria genotypes represented in the DGGE profiles from

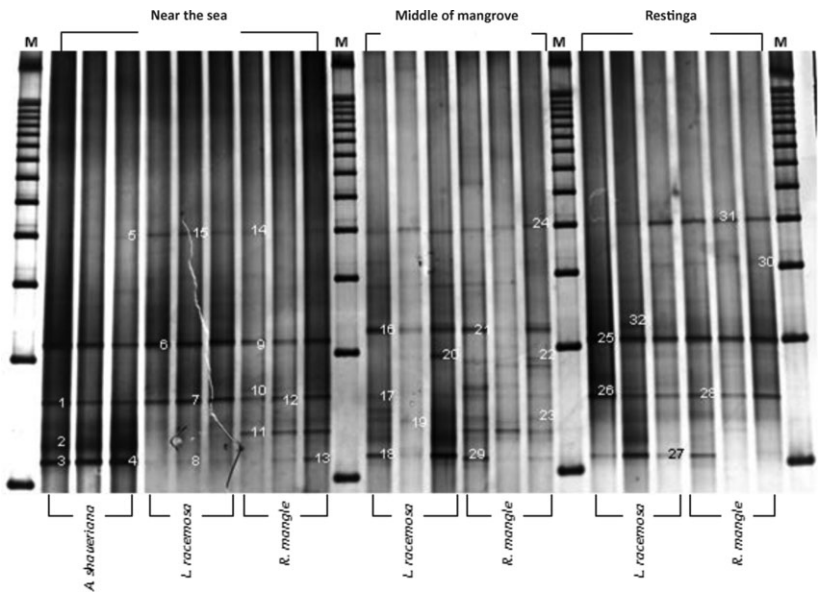


Fig. 1. Denaturing gradient gel electrophoresis (DGGE) profiles of phyllosphere cyanobacteria from the Cardoso Island mangrove forest. M – molecular marker. Numbered bands were excised and sequenced.

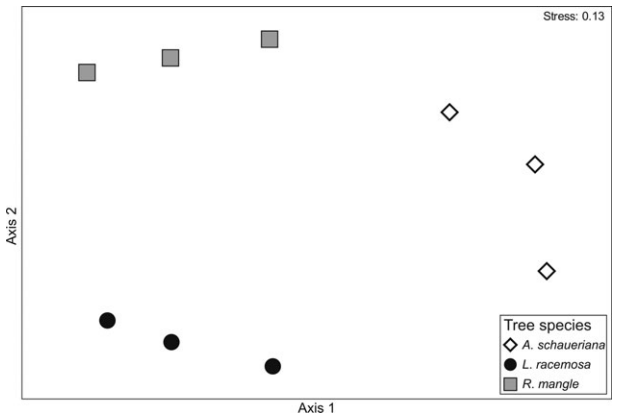


Fig. 2. NMDS analysis comparing the cyanobacterial community structure in the phyllosphere of the three tree species located in the near-shore (NS) habitat of the Cardoso Island mangrove. Each point represents the cyanobacterial assemblage observed in a phyllosphere sample using DGGE. Sørenson's dissimilarity among DGGE profiles is represented as distance in this ordination.

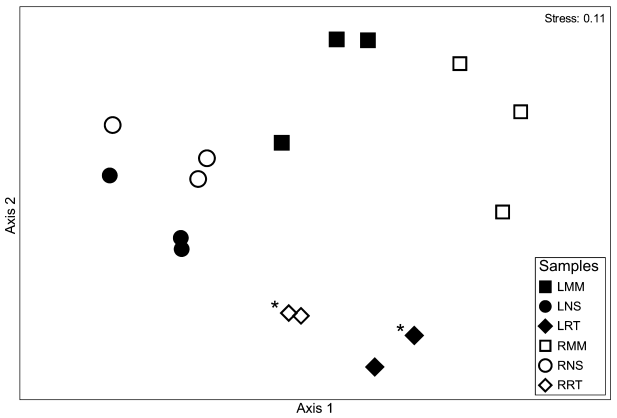


Fig. 3. NMDS analysis comparing the cyanobacterial community structure in the phyllosphere of tree species located in each of the focal habitats within the Cardoso Island mangrove forest: NS – near the shore, MM – middle of the mangrove, RT–restinga. Each point represents the DGGE profiles produced by cyanobacterial assemblages in the two tree species observed in each habitat: *Laguncularia racemosa* (L) and *Rhizophora mangle* (R). *Two overlapping sample points.

the phyllosphere samples. The analyses of those sequences revealed many sequences from sites near the shore (NS) and restinga (RT) similar to *Symphyonemopsis* sp. VAPOR 1 and *Brasilonema* spp. sequences. Additionally, sequences matching uncultured cyanobacteria were found in all of the sites sampled, especially in samples from the middle of the mangrove (MM).

NMDS analysis of the DGGE profiles was performed separately for the near-shore (NS) site because of the presence of *A. schaueriana* at only this site, allowing comparisons of phyllosphere cyanobacterial assemblages among all the dominant tree species (Fig. 2). Comparisons of the effects of discrete mangrove habitats vs. plant species were restricted to the tree species that were found in all the three habitats: *L. racemosa* and *R. mangle* (Fig. 3). Within a mangrove habitat, tree species select distinct cyanobacterial communities (ANOSIM $R = 0.914$, $P = 0.004$). Phyllosphere cyanobacteria associated with *A. schaueriana* were the most distinct from the cyanobacterial assemblages associated with the other tree species (Fig. 2). These results demonstrate that the leaf surface exerts an influence over the cyanobacterial community, with the phyllosphere of each species representing a unique niche that hosts a distinct cyanobacterial assemblage. Nevertheless, comparison of the communities associated with *L. racemosa* and *R. mangle* leaves at distinct sites throughout the mangrove indicated that spatial location had a much stronger impact on the composition of cyanobacterial assemblages than tree species [ANOSIM $R = 0.828$, $P < 0.001$ for comparisons among sites; ANOSIM $R = 0$, $P = 0.85$ (no difference) for comparisons of phyllosphere cyanobacteria communities among the two tree species] (Fig. 3). Within each site, there was some distinction among cyanobacterial assemblages associated with each tree species, but broad patterns in cyanobacterial community composition are more apparent among sites.

Clone libraries

The taxonomic composition of cyanobacterial communities in the phyllosphere of the dominant mangrove vegetation was evaluated on the basis of 590 clones of 16S rRNA gene sequences. Number of clones per site, phylogenetic assignments, and estimates of cyanobacterial diversity and clone library coverage are reported in Table 1. Sequences were assigned to OTUs and subjected to clustering analysis, using a cut-off of 97% sequence similarity. These clusters were used to generate ecological indices of richness and diversity for each community (Chao1, ACE and Shannon) and to estimate sample coverage (ESC) (Table 1). Results showed that *A. schaueriana* had the lowest values for richness and diversity of epiphytic cyanobacteria among all the tree species in this mangrove. At the near-shore site, higher cyanobacteria richness was associated with *R. mangle* than *L. racemosa*, in contrast to the pattern observed in the restinga mangrove habitats (Table 1). These observations are supported by the high estimates of clone library coverage, ranging from 0.871 to 0.953. Such high coverage could also be observed in rarefaction curves (Fig. S1), where most samples reached a plateau. These values also provide validation for library comparisons using ∫-LIBSHUFF.

Table 1. Distribution of cyanobacterial genera sequences, comparison of OTUs and richness and diversity indices derived from 16S rRNA gene clone libraries of phyllosphere cyanobacteria generated from the Cardoso Island mangrove forest

	Near shore (NS)			Middle mangrove (MM)		Restinga (RT)	
Cyanobacteria genera	*A. schaueriana*	*L. racemosa*	*R. mangle*	*L. racemosa*	*R. mangle*	*L. racemosa*	*R. mangle*
Uncultured	23	24	36	77	49	28	22
Anabaena	3					3	
Brasilonema	5						1
Leptolyngbya	12	24	17			19	26
Prochlorococcus	1	2	11	1	2		
Scytonema	1						
Symphyonemopsis	35	32	22	5	6	38	29
Synechococcus	2	1	3	1	1		
Xenococcus		1	2		1		
Fischerella			1				
Planktothrix				3	6		
Cyanobacterium					2		
Microcoleus					1		
Phormidium					2	1	
Rivularia					2		
Oscillatoria					1		
Nostoc				1			1
Hidrocoleum						1	
Acaryochloris							1
Raphidiopsis							1
Total clones	82	84	92	88	73	90	81
OTUs	11	12	23	18	17	17	11
ACE	13.8 ± 2.7	29.8 ± 18.5	52.6 ± 18.7	43.5 ± 19.2	29.0 ± 8.9	47.5 ± 22.0	24.7 ± 13.2
Chao 1	15.0 ± 5.3	36.5 ± 31.1	41.0 ± 14.4	43.0 ± 24.2	27.7 ± 10.3	42.0 ± 24.2	29.0 ± 23.6
Shannon	1.31 ± 0.14	1.55 ± 0.12	2.56 ± 0.10	2.19 ± 0.11	2.04 ± 0.15	1.82 ± 0.14	1.47 ± 0.12
ESC	0.953	0.919	0.871	0.886	0.892	0.888	0.924

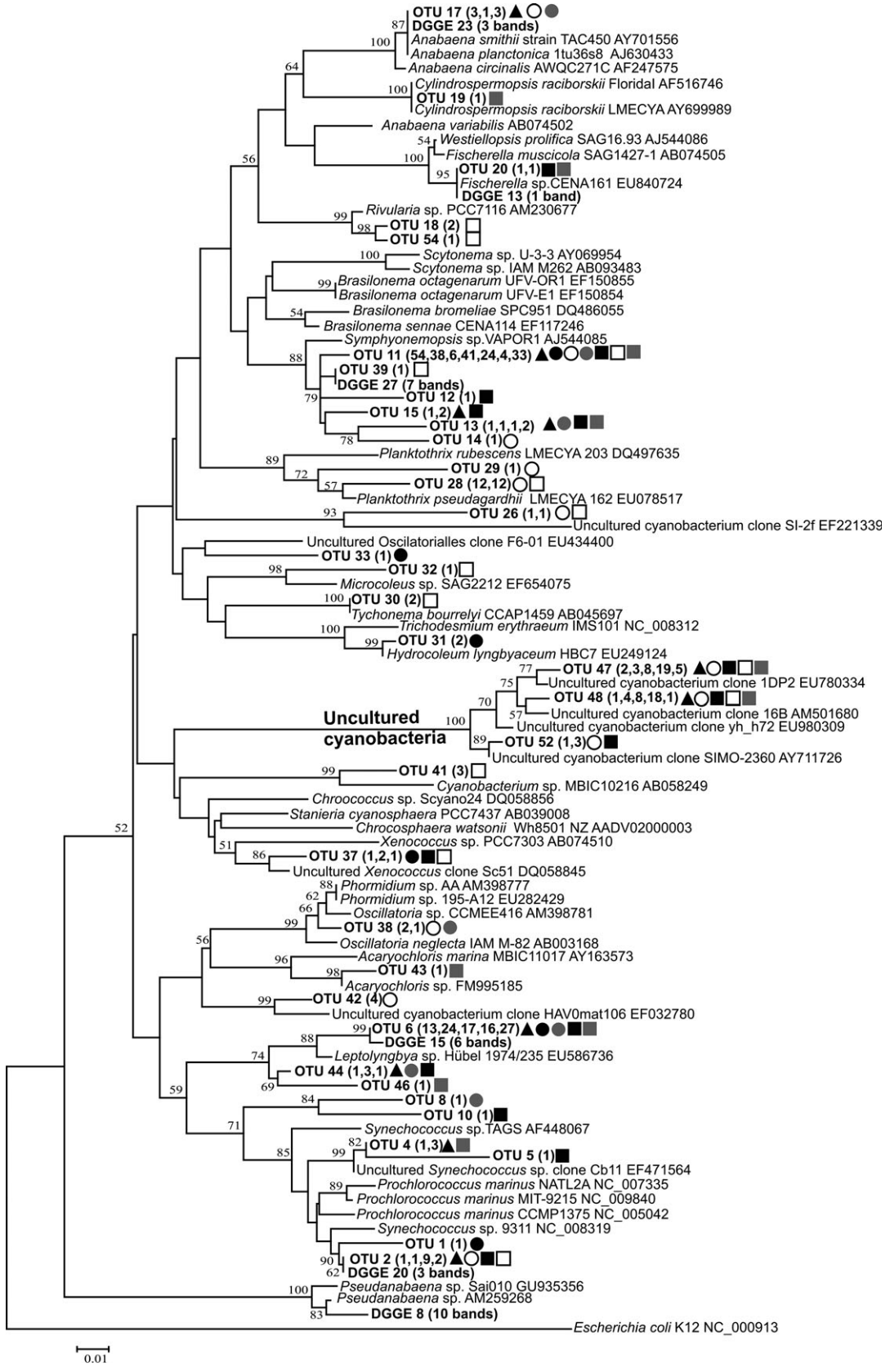
Number of clones of 16S rRNA genes belonging to each cyanobacteria genus detected in the phyllosphere of tree species in different mangrove habitats is indicated.

OTU, operational taxonomic units; ACE and Chao1, nonparametric richness estimators; Shannon, Shannon diversity index; ESC, estimated sample coverage. OTU assignments were based on a 97% similarity threshold.

Comparisons of clone libraries revealed that the phyllosphere cyanobacterial communities found in the middle of the mangrove contained distinct assemblages of populations compared with other habitats ($P < 0.0001$) (Table S2). Interestingly, this site also generated the highest number of sequences affiliated with uncultured cyanobacterial sequences (Table 1). The sequence analysis results contrasted somewhat with the DGGE results, where the NMDS and ANOSIM analyses indicate that the cyanobacterial assemblages in the restinga habitat are least similar to cyanobacteria in other habitats. Additionally, the comparison of clone libraries also showed that sequences generated from the restinga samples were significantly different from sequences obtained from the other habitats (Table S2).

BLAST analyses revealed 19 cyanobacterial genera, with representative of five orders according to the classification published by Hoffmann *et al.* (2005). Genera from the orders *Oscillatoriales* (five genera) and *Nostocales* (eight genera) were the dominant taxa observed in the samples analyzed, while sequences of *Chroococcales* (two genera), *Pseudanabaenales* (one genus), and *Synechococcales* (three genera) were observed in lower numbers. Among the *Nostocales*, high numbers of sequences with high similarity (96–100%) to *Symphyonemopsis* sp. VAPOR1/*Brasilonema* were observed, especially from the samples collected from near-shore and restinga habitats (OTUs 11–15, and 39, Fig. 4). This taxon was also observed in the sequences derived from excised DGGE bands (Fig. 1, Table S1). Excised DGGE bands generated sequences with similarity to nine genera represented in clone library.

In the phylogenetic analysis, OTUs representing mangrove phyllosphere cyanobacteria and sequences retrieved from DGGE bands aligned with representative 16S rRNA gene sequences of previously described cyanobacterial taxa (Fig. 4). Additionally, a separate clade was formed with 73 sequences, containing three OTUs which best matched sequences of uncultured cyanobacteria.



Discussion

The structure and spatial variability of the phyllosphere cyanobacterial community associated with mangrove trees were evaluated for the first time, using culture-independent approaches. The present study provides new insights into the ecology of cyanobacteria that inhabit phyllosphere niches in mangrove ecosystems. The comparisons of both clone library and PCR-DGGE results among habitats and among plants revealed that a hierarchy of ecological filters determines cyanobacterial community structure. At the ecosystem scale, position along a transect from the seashore to the upland forest is the strongest determinant of cyanobacterial assemblages. At the local scale, each tree species selects a distinct assemblage from the cyanobacterial metacommunity present within each habitat, indicating that the phyllosphere cyanobacteria communities are structured by deterministic niche-based processes (Dumbrell *et al.*, 2010; Caruso *et al.*, 2011).

The phyllosphere is an environment of great importance because of the abundance of plants in the world, and the activities of microbial communities in this niche influence plant health and ecosystem productivity (Lindow & Brandl, 2003). While several studies have assessed the diversity of bacterial communities in the phyllosphere (Lindow & Brandl, 2003; Lambais *et al.*, 2006), only limited research has been conducted to evaluate cyanobacteria residing in the phyllosphere, with a few studies on seagrasses (Uku *et al.*, 2007) and tropical forest (Fürnkranz *et al.*, 2008). To our knowledge, phyllosphere bacteria in mangroves have not been previously described, but exploratory studies such as this is the first step in better understanding the ecological relationships that sustain these important coastal habitats.

The data obtained in this study indicate that overall, the cyanobacterial community associated with mangrove phyllospheres is similar between *R. mangle* and *L. racemosa* species, although some plant-specific differences in cyanobacterial community structure are apparent within each mangrove habitat. DGGE patterns from *A. schaueriana*, found only in the area closest to the seashore, indicate that this plant is colonized by a unique cyanobacterial epiphyte population not observed in the phyllosphere of the other trees. This observation may be linked to plant metabolism; *A. schaueriana* leaves have salt glands responsible for osmotic control (Shimony *et al.*, 1973; Clough, 1984), a trait that is absent in leaves of the other two genera. Therefore, salt accumulation on the leaf surface may create a distinct niche that is colonized by the unique cyanobacterial population.

Freiberg (1998) reported that the composition of leaf exudates and nutrient accessibility in the phyllosphere influences leaf colonization. However, these resources are patchily distributed, and much of the leaf surface is considered to be a "nutritional desert" (Mercier & Lindow, 2000; Dulla *et al.*, 2005). In this environment, cyanobacteria, capable of both nitrogen and carbon fixation, may have a distinct advantage over heterotrophic bacteria. In addition, colonization by cyanobacteria may expand the phyllosphere niche space available to heterotrophs by providing fixed N and C. Thus, cyanobacteria can be viewed as keystone species in the ecology of the phyllosphere. However, even considering that these organisms are less dependent on plant exudates for their nutrition, a previous study reported clear differences in cyanobacterial colonization among leaves of different seagrasses (Uku *et al.*, 2007).

In the present study, the plant and cyanobacterial community both reflect the influence of environmental gradients within the mangrove ecosystem. Differences in plant structure were observed along the shore to upland transect; taller plants were observed in the near-shore area, and a decrease in plant height was observed proceeding inland. This demonstrates the plants adaptive physiology in response to changes in the environment, which may alter the composition of the cyanobacterial community in the phyllosphere. Spatial patterns in the composition of phyllosphere cyanobacteria may result from direct influence of environmental gradients on these communities, or from the indirect influence of the environment, as plant response to the environment modulates the conditions in the phyllosphere niche.

The phylotypes of the sequences obtained from both clone libraries and DGGE bands suggest that the presence of both heterocytous and homocytous filamentous cyanobacteria, with affiliation to groups previously described as diazotrophs. This might indicate a possible ecological role of cyanobacteria in the phyllosphere of mangrove trees, where they can possibly contribute to the cycling and availability of nitrogen to the plant. Diazotrophs are particularly important in environments, such as mangroves, where nitrogen is a limiting factor for primary produc-

Fig. 4. Phylogenetic tree based on Neighbor-Joining analysis of the partial 16S rRNA gene sequences of phyllosphere cyanobacteria from the Cardoso Island mangrove forest, in relation to sequences obtained from GenBank. Bootstrap values (expressed as percentages of 1000 resamplings) > 50% are shown at branch points. The scale indicates the substitutional distance based on Kimura's two-parameter model. The sequences that were obtained from this study are shown in bold. Numbers in parentheses represent the number of sequences retrieved from each sample: ▲ *Avicennia schaueriana* near the shore; ● *Laguncularia racemosa* near the shore; ○ *L. racemosa* middle of the mangrove; ● *L. racemosa* restinga; ■ *Rhizophora mangle* near the shore; □ *R. mangle* middle of the mangrove; ■ *R. mangle* restinga.

tion (Karl *et al.*, 2002). Abril *et al.* (2005) reported that the interface between leaf surfaces and the atmosphere is a fundamental pathway for nutrient, especially nitrogen, exchange and is perhaps the main mechanism of nitrogen inputs in tropical environments. The most abundant sequences recovered from the phyllosphere of mangrove trees matched those of the order *Nostocales* (including diazotrophic genera such as *Anabaena*, *Brasilonema*, *Scytonema*, *Symphyonemopsis*, *Fischerella*, *Rivularia*, and *Nostoc*), whose members have specialized cells (heterocytes) for nitrogen fixation (Kumar *et al.*, 2010), possibly indicating their role in this ecosystem.

Sequence analyses of cyanobacteria present throughout the mangrove have generated a large number of sequences (OTUs 11, 12, 13, 14, 15, and 39 and DGGE 27) with similarity to *Symphyonemopsis*, a genus belonging to the nitrogen-fixing order *Nostocales* (Komárek & Hauer, 2010). The only *Symphyonemopsis* sequence available is the VAPOR1 strain that is closely related to *Brasilonema*, a commonly distributed genus, particularly in subaerophytic habitats within the Brazilian Atlantic rainforest (which encompasses the Cardoso Island mangrove ecosystem). *Brasilonema* has been isolated from leaves of bromeliads (*B. bromeliae*), eucalyptus (*B. octagenarum* UFV-E1) and orchids (*B. octagenarum* UFV-OR1), while the *Symphyonemopsis* sp. VAPOR1 was retrieved from a cave in Spain (Hoffmann *et al.*, 2003; Fiore *et al.*, 2007; Aguiar *et al.*, 2008; Sant'Anna *et al.*, 2011). In addition to these ecological differences, no bootstrap support for the phylogenetic separation of the *Symphyonemopsis*/*Brasilonema*/OTUs was found in this study. Therefore, owing to the close relationship of *Symphyonemopsis* and *Brasilonema*, and the short sequence length (400 bp) comparison, it is possible that the OTUs affiliated with *Symphyonemopsis* sp. VAPOR1 most likely belong to the genus *Brasilonema* and represent phyllosphere specialists in tropical ecosystems.

The present study has pioneered exploration of diversity of cyanobacteria in the mangrove phyllosphere using culture-independent approaches. We describe for the first time the genotypes of cyanobacteria that colonize leaves of trees in mangrove ecosystems and identify environmental factors that shape the composition of these communities. The results suggest that environmental gradients, such as tidal regime, salinity, and marine aerosols may control community composition at the ecosystem scale, while plant species may select a subset of the cyanobacterial metacommunity and impose structure on a more local scale. While phylogeny does not always predict function, our phylogenetic analysis has revealed the presence of potential diazotrophs in the phyllosphere of mangrove trees. This study provides the initial steps toward describing the role of these organisms in sustaining ecosystem function of this important coastal habitat.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Rarefaction curves of partial sequences of cyanobacterial 16S rRNA gene clone libraries from the phyllosphere of the Cardoso Island mangrove forest, calculated by DOTUR with a 3% evolutionary distance cutoff.

Table S1. Best match among 16S rRNA gene sequences obtained from the DGGE analysis to those available in the GenBank database.

Table S2. *P*-value from the multiple comparisons among 16S rRNA gene clone libraries of phyllospheric cyanobacteria communities from the Cardoso Island mangrove forest (evolutionary distance 0.03).

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