environmental microbiology reports

Environmental Microbiology Reports (2013) **5**(3), 367–376 doi:10.1111/1758-2229.12031

Epiphytic cyanobacteria of the seagrass *Cymodocea rotundata*: diversity, diel *nifH* expression and nitrogenase activity

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Summary

Seagrasses are photoautotrophic, ecologically important components of many globally widespread coastal ecosystems, in which combined nitrogen may limit their production. We examined the biodiversity and diazotrophic capacity of microbial epiphytes associated with the phyllosphere of the seagrass *Cymodocea rotundata* of the Western Indian Ocean. Light microscopy, 16S rRNA and *nifH* gene analysis revealed the dominance of cyanobacteria in the epiphytic microbial community. Most phylotypes were related to free-living uncultured benthic cyanobacteria, while some to cyanobacterial endosymbionts of marine diatoms. Novel and potentially diazotrophic species, some of known pantropical distribution, were also discovered. Significant diel nitrogenase activities (acetylene reduction assay) were recorded (up to 358 ± 232 nmol C_2H_4 g^{-1} of seagrass FW h^{-1}). The *nifH* gene expression patterns showed that heterocystous phylotypes may be the dominant diazotrophs during the day and non-heterocystous at night. These data show that *C. rotundata* is colonized by diverse diazotrophic cyanobacteria species and suggest that these may be beneficial partners of seagrasses in nitrogen-depleted waters.

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Introduction

Seagrass ecosystems are among the most productive and also among the most threatened coastal ecosystems on Earth. They are estimated to occupy about 0.1–0.2% of the global ocean floor (Duarte, 2002; Björk *et al*., 2008), but with the loss of seagrass meadows accelerating (Orth *et al*., 2006; Waycott *et al*., 2009). In spite of the relatively low coverage on a global scale, the seagrass communities still constitute extremely valuable ecosystems and provide substantial ecosystem services (Torre-Castro and Rönnbäck, 2004). A multitude of human activities in coastal areas are disturbing the diversity and function of seagrasses (Costanza *et al*., 1997; Duarte and Chiscano, 1999; Orth *et al*., 2006; Unsworth *et al*., 2008). To remediate the threat, attempts are made to restore seagrass beds through, for instance, transplantations (Renton *et al*., 2011).

As in all photosynthetically driven ecosystems, nitrogen is a key component for maintaining high seagrass productivity (Vonk *et al*., 2008). Prokaryotic diazotrophs, living on, or in close proximity to seagrasses, have been proposed to sustain their productivity (Goering and Parker, 1972; Capone and Taylor, 1977; 1980). For instance, O'Donohue and colleagues (1991) reported that nitrogen fixers contribute 30–50% of the nitrogen demand of the seagrass *Zostera capricornia* (Moreton Bay). However, the majority of studies have focused on rhizosphere-associated heterotrophic bacteria, and the role of the phyllosphere-associated photoautotrophic diazotrophs has been largely overlooked.

In tropical Western Indian Ocean (WIO) a number of studies have focused on planktonic (Lugomela *et al*., 2001; Lugomela, 2002; Lyimo, 2011) and benthic free-living cyanobacterial communities (Silva and Pienaar, 2000; Bauer *et al*., 2008; Charpy *et al*., 2010), and the majority is based on morphological identification. Previous studies also showed distinct microbial 'biofilms' or 'aggregates', potentially dominated by diazotrophic cyanobacteria, adhering to seagrasses in the WIO (Hamisi *et al*., 2004; Uku *et al*., 2007) and Hamisi and colleagues (2009) provided evidence for epiphytic associated nitrogen fixation to the four seagrass genera, *Halodule*, *Thalassodendron*, *Thalassia* and *Cymodocea*.

Variations in seagrass-associated nitrogen fixation varied with seagrass genus, leaf age, time of the day and season.

We therefore hypothesized that the light-exposed seagrass phyllosphere may be colonized by diverse diazotrophic cyanobacteria. The seagrass *Cymodocea rotundata* was selected as model system as this seagrass is common and widespread in tropical coastal areas worldwide (Short *et al*., 2007; Waycott *et al*., 2009). To identify components of the prokaryotic community, both morphological and molecular approaches were used. More specifically, cyanobacterial specific 16S rRNA and *nifH* genes were examined as well as diel *nifH* gene expression patterns, NifH protein levels and nitrogenase activity associated with the phyllosphere. Sampling was performed in two consecutive years (2007–2008) at the onset of north-east monsoon (October and November), a period previously characterized by having higher cyanobacterial diversity and coverage as well as higher nitrogenase activity, combined with lower levels of dissolved nitrate and phosphate (Hamisi *et al*., 2004; 2009).

Results and discussion

Seagrass–cyanobacteria associations

Cyanobacteria were found associated with the phyllosphere (leaves) of the seagrass *C. rotundata*, but occasionally also on the light-exposed surrounding sediments. The cyanobacteria were recognized as small patches of pigmented microbial aggregates or thin 'biofilms' adhering, more or less firmly, to the *C. rotundata* leaves. The autofluorescence observed under epifluorescence light microscopy (LM) confirmed their presence. Some cyanobacteria appeared more firmly attached to the seagrass leaves, some via basal heterocysts as the genus *Calothrix* (see Fig. 1A), or adhered by other unknown means.

Morphological characterization

A total of 19 cyanobacterial morphotypes were encountered (Fig. 1). A variety in the overall morphology, including rich variations in cell and filament sizes and shapes, was apparent. The cyanobacteria identified ranged from filamentous heterocystous (Fig. 1A–E), filamentous non-heterocysts (Fig. 1F–P) to unicellular phenotypes (Fig. 1Q and S). This cyanobacterial diversity largely corroborated previous findings in WIO seagrass ecosystems (Hamisi *et al*., 2004; Uku *et al*., 2007), as well as those of benthic non-seagrass ecosystems in the same waters (Silva and Pienaar, 2000; Lugomela *et al*., 2001; Lugomela and Bergman, 2002; Bauer *et al*., 2008; Charpy *et al*., 2010). This firmly establishes a persistent occurrence and varied biodiversity among benthic cyanobacteria in WIO.

The most frequent morphotypes encountered on *C. rotundata* were species of non-heterocystous filamentous from order Oscillatoriales, with cell widths ranging from 5 to 80 μm (Fig. 1F–J). The most common genera were *Oscillatoria* (filaments without sheath) and *Lyngbya* (filaments with sheaths). Representatives of the Pseudanabaenacea family (cell width < 5 μm) also occurred frequently (Fig. 1K–N). Less frequently observed filamentous genera were *Microcoleus*, with tightly packed trichomes sharing a common sheath, and coiled *Arthrospira* (Fig. 1O and P). Common unicellular morphotypes belonged to the genera *Gloeocapsa*, *Chroococcus* and *Chroococcidiopsis* (Fig. 1Q and S). The cyanobacterial epiphytes were ranked in abundance accordingly: filamentous non-heterocystous > heterocystous > unicellular morphotypes.

Genetic characterization

Partial 16S rRNA and *nifH* gene analysis using cyanobacteria-specific gene oligonucleotide primers described by Nübel and colleagues (1997) and Olson and colleagues (1998), respectively, confirmed the presence of a diverse cyanobacterial community and putative diazotrophic function as shown in the denaturing gradient gel electrophoresis (DGGE) profiles (Figs S1 and S2). The diel stability in the community was further verified in the consistent DGGE patterns throughout the day/night cycle (eight sampling points). The identity of the DGGE bands with identical positions in the gels further strengthened the reproducibility of the method used.

Differences in the cyanobacterial *nifH* DGGE profiles between the 2 years (2007–2008) and even between the 2 months (October and November) were apparent (Fig. S2). This suggests that there were different colonization patterns and/or succession strategies in both space and time, as previously suggested (Hamisi *et al*., 2004). Likewise, open water planktonic cyanobacteria in WIO region are known to be greatly influenced by the seasonal monsoon winds (Lugomela, 2002), which strongly affect ocean currents and local climates (Black *et al*., 2002), as apparently is also the case for the seagrass-associated community.

Furthermore, using the universal bacterial *nifH* gene primers described by Poly and colleagues (2001), out of the 40 *nifH* gene clones obtained approx. 83% originated from cyanobacteria stressing their role as putative diazotrophs in the phyllosphere. Altogether, the bacterial *nifH* gene clone libraries and the cyanobacterial specific 16S rRNA and *nifH* gene DGGE results suggested that cyanobacteria constitute a prominent part of the total epiphytic community.

Phylogenetic reconstructions of sequences based on the cyanobacterial 16S rRNA (Fig. 2) and *nifH* gene

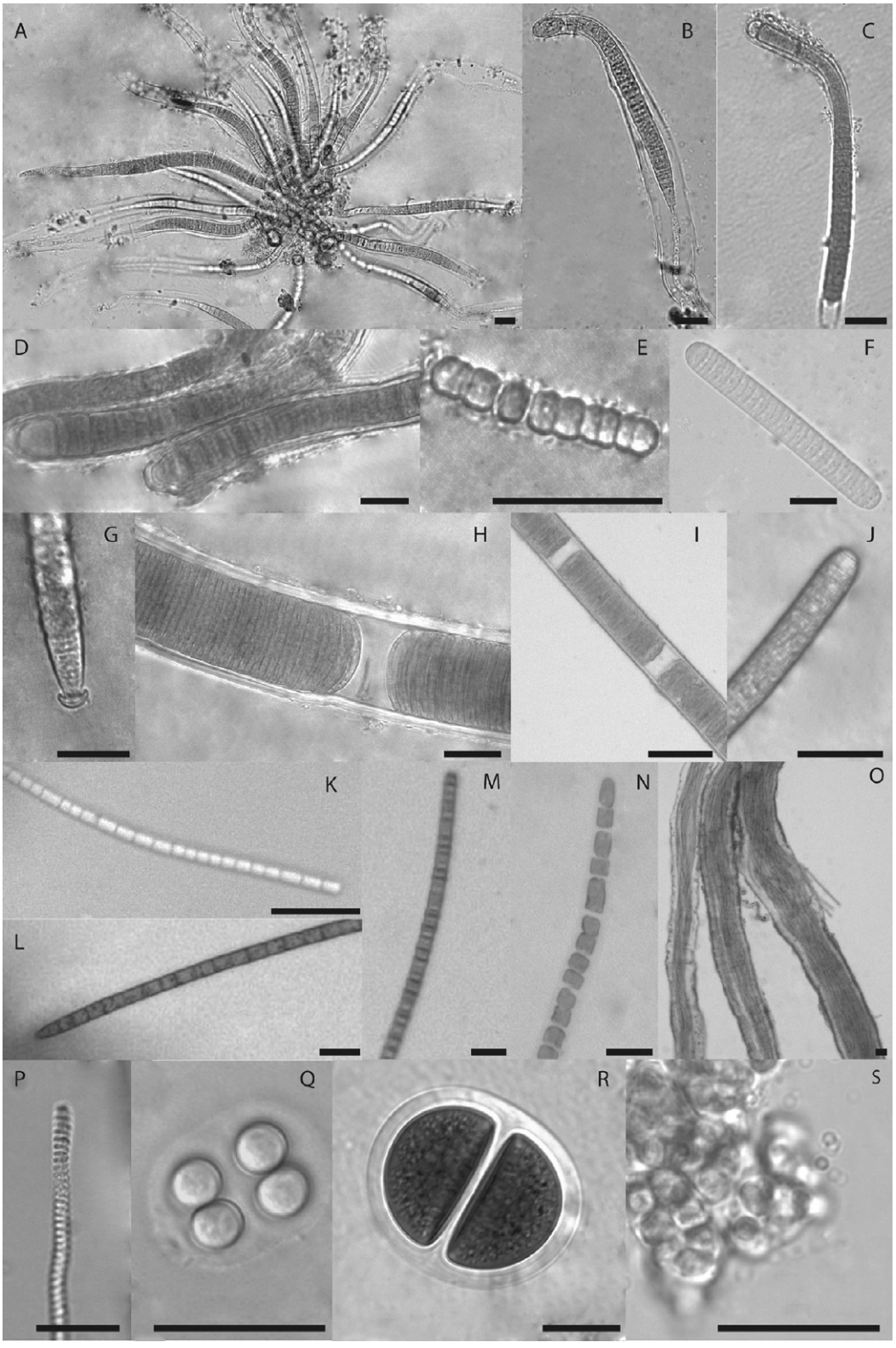

Fig. 1. Representative micrographs of epiphytic cyanobacteria on the phyllosphere of the seagrass *Cymodocea rotundata*: (A–E) heterocystous cyanobacteria (A) *Calothrix* with terminal cells (heterocysts), (B) a close-up of the filament in (A), (C, D) other *Calothrix* morphotypes, (E) *Nodularia*; (F–P) filamentous non-heterocystous morphotypes related to *Oscillatoria* (F, G, J), to *Lyngbya* (H, I) and (K–N) to the Pseudanabaenacea family. (Q–S) Unicellular cyanobacteria (*Gloeocapsa*, *Chroococcus* and *Chroococcidiopsis*), (O, P) *Microcoleus* and *Arthrospira* respectively. Bar = 20 μm.

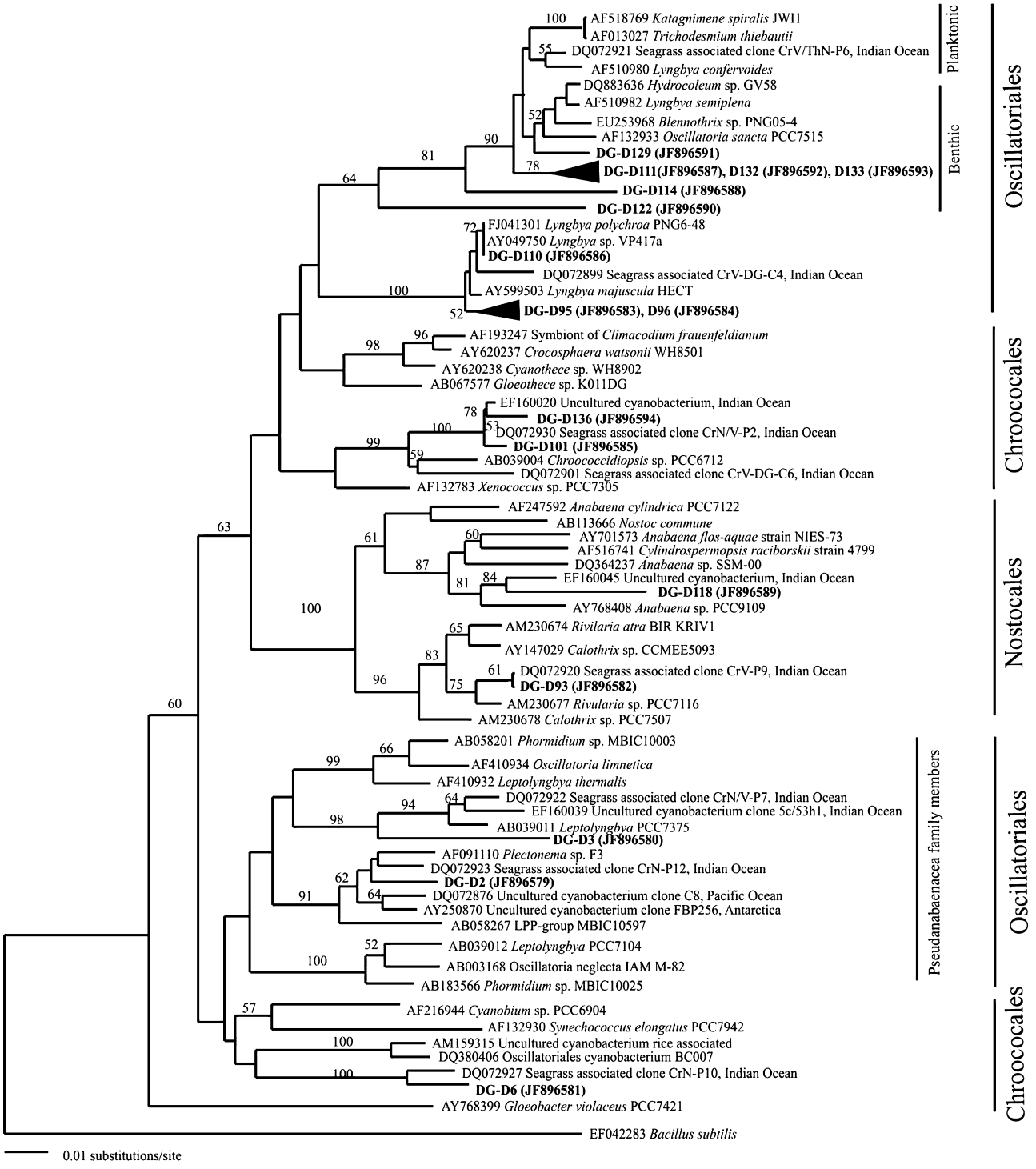

Fig. 2. Phylogenetic reconstruction (nucleotide sequences of 552 bp in length) of epiphytic cyanobacteria associated with the seagrass *Cymodocea rotundata*. The sequences were generated from the 16S rRNA gene – DGGE analysis. The tree was constructed from distance approximations by the NJ method and Kimura two-parameter in PAUP (version 4.0b10) with *Bacillus subtilis* (EF042283) sequence as out-group. Branch support was measured by bootstrap analysis using an NJ search strategy with 10 000 bootstrap replicates. The bold sequences in the tree represent sequences obtained in our study. The numberings are the same as those given in the DGGE profiles in Fig. S1. The prefix 'DG-D' stands for: DG – source of sequence (DGGE) and D – nucleic acid (DNA).

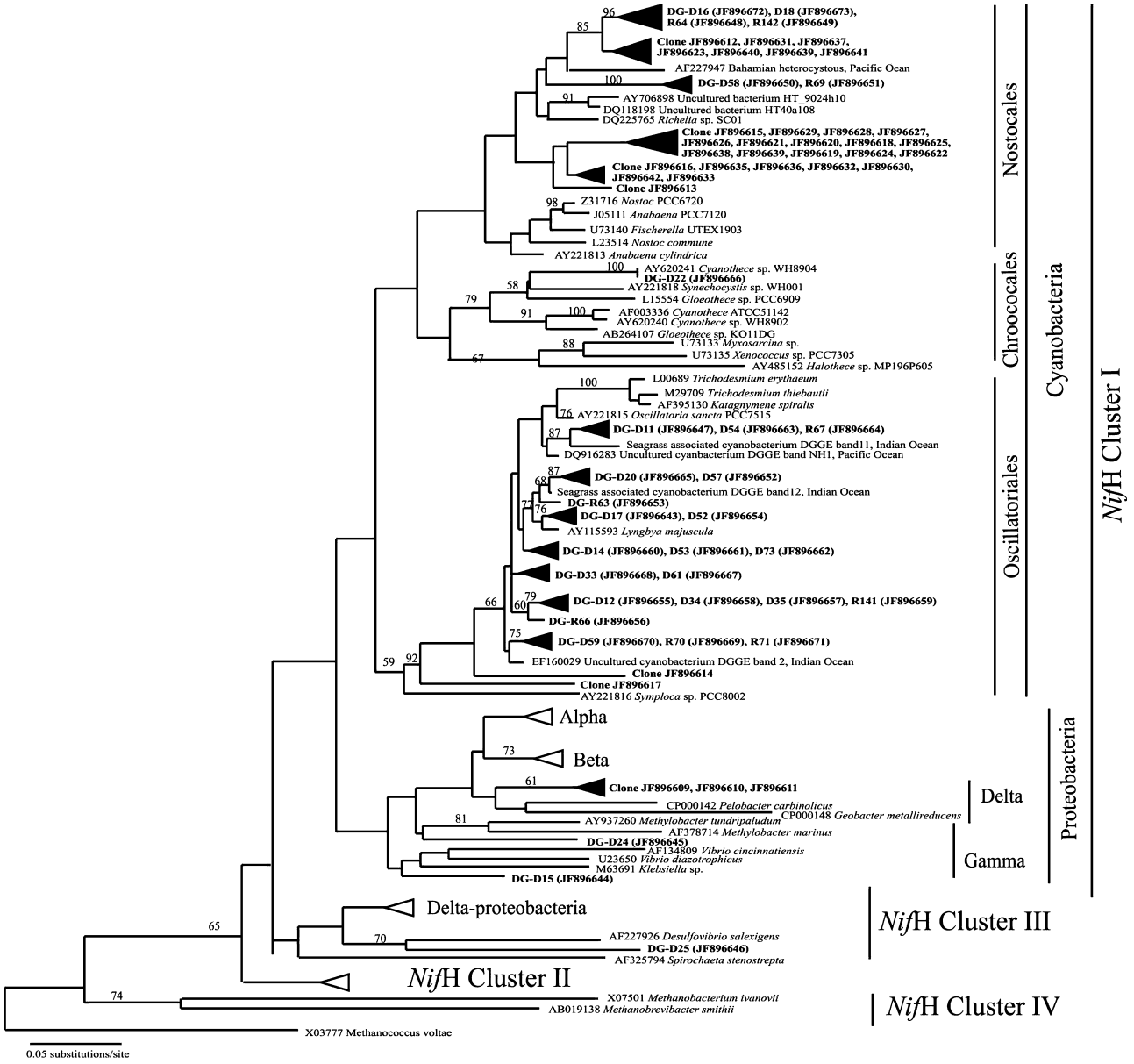

Fig. 3. Phylogenetic reconstruction (nucleotide sequences of 254 bp in length) of epiphytic diazotrophs associated with the seagrass *Cymodocea rotundata*. The sequences were generated from the *nifH* gene – DGGE and clone libraries analysis. The tree was constructed as for the 16S rRNA gene sequences in Fig. 2, but with *Methanococcus voltae* (X03777) sequence as out-group. The bold sequences in the tree represent sequences from this study. The numbers used are the same as in the DGGE profile in Fig. S2. The prefix 'DG-D/R' stands for: DG – source of sequence (DGGE); D – DNA; R – RNA and clone/s denotes sequences generated from November 2008 clone libraries (DNA and RNA).

sequences (Fig. 3), respectively, revealed the presence of uncharacterized cyanobacterial clades. These sequences of cyanobacteria are known to associate with seagrasses (Uku *et al*., 2007) or to benthic communities in tropical marine ecosystems (Steppe *et al*., 2001; Díez *et al*., 2007; Bauer *et al*., 2008; Foster *et al*., 2009). For instance, most of the cyanobacterial non-heterocystous 16S rRNA gene sequences obtained [D111 (JF896587), D114 (JF896588), D122 (JF896590), D132 (JF896592) and D133 (JF896593)] formed a unique clade, distantly related to the benthic Oscillatoriales genera *Blennothrix* and *Hydrocoleum* retrieved in tropical benthic marine environments from New Caledonia (Abed *et al*., 2006). Sequences of 16S rRNA-DGGE bands D95 (JF896583), D96 (JF896584) and D110 (JF896586) appeared in a clade closely affiliated to the genus *Lyngbya*. Sequences from DGGE bands D2 (JF896579) and D3 (JF896580) clustered with members of the uncultured LPP (*Lyngbya,*

Phormidium and *Plectonema*) group (*Leptolyngbya*, *Phormidium* and *Plectonema*). Phylotype D6 (JF896581) belongs to a clade composed of sequences related to unicellular cyanobacteria associated to *C. rotundata* growing along the Kenyan coast (Uku *et al*., 2007) (Fig. 2). This may suggest the existence of specific cyanobacteria–seagrass associations in the WIO. Finally, some sequences [D101 (JF896585) and D136 (JF896594)] were related to phylotypes of the unicellular genus *Chroococcidiopsis* (Fig. 2), which may represent an important part of the community, as observed in our DGGE profiles (bands D101 and D136; Fig. S1), and also verified by the microscopy analyses of samples from year 2007.

Heterocystous cyanobacteria were also well represented in the phylogenetic reconstruction of the 16S rRNA gene (Fig. 2). DGGE band D118 (JF896589) showed high similarity to an uncultured member of the Nostocales from a microbial mat in the WIO (Bauer *et al*., 2008), and to *Anabaena* sp. isolated from a brackish water marsh (Stevenson and Waterbury, 2006). The phylotype D93 (JF896582) was affiliated to a new clade of heterocystous cyanobacteria of the family Rivulariaceae, exclusively composed of seagrass-associated sequences (Uku *et al*., 2007). However, a distant relation was also shown to the heterocystous genera *Calothrix* and *Rivularia* recovered from temperate brackish waters (Baltic Sea; Sihvonen *et al*., 2007), cold freshwaters (Antarctica; Taton *et al*., 2006) and hot springs (Yellowstone National Park; Dillon and Castenholz, 2003). The only unicellular cyanobacterium in the 16S rRNA-based phylogenies belonged to the genus *Chroococcidiopsis* [D101 (JF896585) and D136 (JF896594)].

nifH gene phylogenetic reconstruction, based on DGGE generated sequences from both DNA (Fig. S2A–D) and RNA (cDNA) (Fig. S2E and F), illustrated a distinct separation between planktonic and benthic phylotypes. This was also the case for the diazotrophic bacterial amplicons generated via cloning (Fig. 3). Most of the non-heterocystous related DGGE phylotypes and clones formed new clades, and some showed similarity to phylotypes previously recovered from WIO benthic habitats (Lundgren *et al*., 2003; Bauer *et al*., 2008) and from other tropical benthic marine ecosystems (Díez *et al*., 2007). A similar scenario was also apparent for the heterocystous cyanobacterial sequences. Only distant relations to uncultured benthic heterocystous cyanobacteria from stromatolites at Bahamas, Atlantic Ocean (Steppe *et al*., 2001) were shown in the *nifH* DGGE generated DNA sequences [D16 (JF896672), D18 (JF896673), D58 (JF896650)] (Fig. 3), and RNA sequences [R64 (JF896648), R69 (JF896651) and R142 (JF896649)] (Fig. 3), as well as most of the *nifH* clones recovered in 2008 (e.g. JF896612, JF896615, JF896633). In addition, relationships were shown to the heterocystous *Richelia* and *Calothrix* living symbiotically within tropical diatoms (Foster and Zehr, 2006) (Fig. 3). As only one sequence was affiliated to the unicellular diazotrophic genus *Cyanothece* in the *nifH* gene analysis [DNA; D22 (JF896666)], and as it likewise was underrepresented in the *nifH* DGGE profiles, its role as a diazotroph in the seagrass community may be minor. The absence of *Chroococcidiopsis* phylotypes in our *nifH* gene analysis implies that it lacks *nif* genes and consequently the nitrogen fixation function, despite being abundant based on 16S rRNA gene and LM analysis (see Fig. 1). This genus has been reported both to fix nitrogen (Friedmann and Kibler, 1980) and to lack this capacity (Billi and Caiola, 1996), the latter supported by our data.

The presence of cyanobacterial sequences related to planktonic phylotypes could be a result of 'coincidental' settlement for planktonic species. Additionally, the discovery of 'new' cyanobacterial clades living epiphytically on seagrasses may suggest the existence of some understudied seagrass-specific phylotypes, and judging from the geographical distribution of the closest BLAST matches found, these phylotypes may have pan-tropical distribution. However, most of the sequences showed low identity percentage (< 95% or even < 90%) to cyanobacterial sequences in the GenBank Database. Exceptions were a few sequences related to cyanobacteria from WIO or other marine tropical regions (Lundgren *et al*., 2003; Díez *et al*., 2007; Uku *et al*., 2007; Bauer *et al*., 2008). These findings stress the presence of novel cyanobacteria, and particularly nitrogen-fixing genera/species, in coastal tropical habitats, also supported by the long branches in the phylogenetic trees (Figs 2 and 3). The latter is also apparent for cyanobacteria associated with tropical coral reefs (Charpy *et al*., 2010) and benthic intertidal lagoons (Bauer *et al*., 2008).

Bacterial diazotrophs were also identified among the *nifH* genes retrieved by DGGE and clone libraries. The recovered bacterial *nifH* sequences [DGGE bands D15 (JF896644), D24 (JF896645)] and clones (JF896609, JF896610 and JF896611) were related to proteobacteria of the nitrogenase type I (Fig. 3). Only one sequence [D25 (JF896646)] was related to anaerobic bacteria of the nitrogenase type III (Fig. 3). Using two different sets of primers, these data verify the presence of other diazotrophic prokaryotes, parallel to the dominance of the diazotrophic cyanobacteria. Indeed, the two photosynthetic organisms (the seagrass and cyanobacteria) may support growth of heterotrophic organisms. However, it can not be excluded that some bacterial *nifH* genes may represent cyanobacteria, as the *nifH* gene of *Microcoleus chthonoplastes* has been shown to originate from a proteobacterium, potentially via horizontal gene transfer (Bolhuis *et al*., 2010). Together, our data clearly verified the common occurrence and dominance of epiphytic

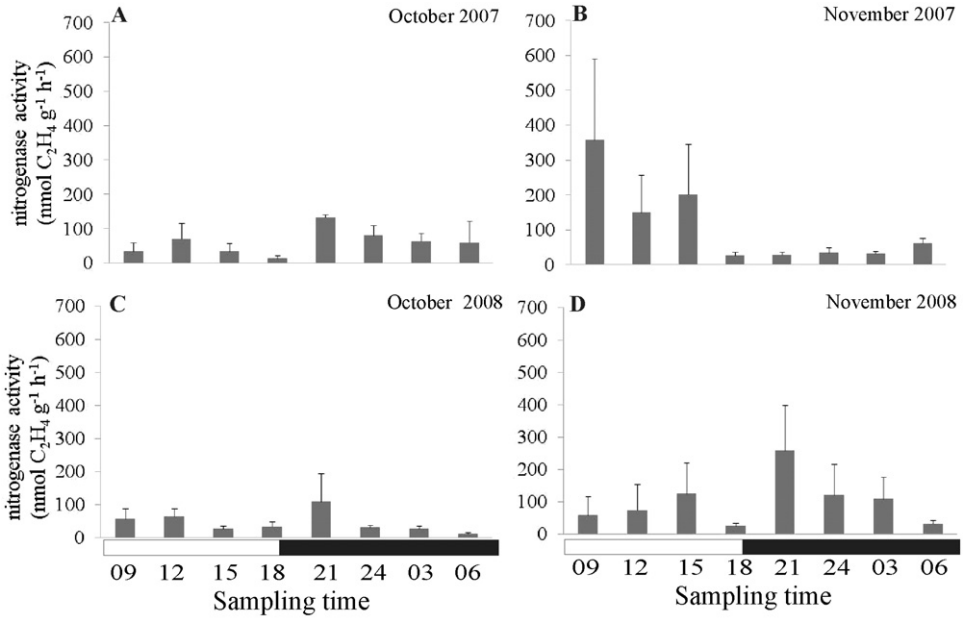

Fig. 4. Diel nitrogenase activity (acetylene reduction assay) by epiphytes associated to the seagrass *Cymodocea rotundata*. The graphs represent nitrogenase activity during the different months (October and November) and years (2007–2008) investigated. The nitrogenase activity is expressed as the ethylene produced per gram wet weight of the seagrass per hour; SD (±) is given as bars.

cyanobacterial morpho- and phylotypes, revealed the presence of uncharacterized taxa and confirmed taxonomic relationships.

Diel nifH gene expression and protein patterns

Next, the expression of the *nifH* gene was examined (October and November 2008). *nifH* gene expression differed between day and night (Fig. S2E). Non-heterocystous phylotypes [R63 (JF896653), R66 (JF896656), R67 (JF896664) and R70 (JF896669)] and a few heterocystous phylotypes [R64 (JF896648), R69 (JF896651)] were the apparent active diazotrophs (Fig. 3). In November, *nifH* gene expression was more even over the diel cycle (Fig. S2F), and originated from a less diverse community composed of few heterocystous [R142 (JF896649)] and non-heterocystous [R141 (JF896659)] phylotypes (Fig. 3). This may be attributed to the differences caused by the monsoon winds, which affect physico-chemical parameters of the ocean even between the consecutive months. Nevertheless, both October and November are on the onset of the north-east monsoon, the season characterized by having lower levels of dissolved nitrate and phosphate that favours nitrogen fixers' colonization (Hamisi *et al*., 2004; 2009).

Moreover, a single band of approx. 38 kDa was apparent in Western blot analyses using an anti-NifH antibody on total cell extracts of the epiphytes. Diel NifH protein levels peaked at 21:00 h in October 2008 samples (Fig. S3), i.e. at about the same time as the highest *nifH* gene expression occurred (Fig. S2E).

Diel nitrogenase activity

The nitrogenase activity in the phyllosphere of *C. rotundata* was examined during 24 h cycles (2007 and 2008) using the acetylene reduction assay. The activity (per gram wet weight seagrass leaf) over the diel cycle ranged from 10.7 ± 4.3 to 358 ± 252 nmol C_2H_4 g^{-1} h^{-1} (Fig. 4A–D). These activities corroborate those found previously for *C. rotundata* epiphytes in the same area [max. 75 nmol N g^{-1} h^{-1} in Hamisi *et al*. (2009); equivalent to 300 nmol C_2H_4 g^{-1} h^{-1}]. However, the levels exceed those reported from other tropical 'non-seagrass' benthic ecosystems (Lugomela and Bergman, 2002; Kyaruzi *et al*., 2003; Lyimo and Lugomela, 2006), which may suggest some synergy effects in the cyanobacteria–seagrass association. The nitrogenase activity did not vary significantly between the years (Mann–Whitney test, $P = 0.56$, $U' = 144$), or the months tested ($P = 0.95$, $U' = 33$ and $P = 0.83$, $U' = 49$, for 2007 and 2008 respectively), nor between day and night ($P = 0.78$, $U' = 35$, both years). The highest nitrogenase activity was recorded in November both years, being 358 ± 232 and 258 ± 139 nmol C_2H_4 g^{-1} of seagrass FW h^{-1} at 09:00 h in 2007 and at 21:00 h in 2008 respectively (Fig. 4B and D). The diel activity patterns differed in November between the 2 years, being higher during the day in 2007, and at night in

2008, while in October, the higher nitrogenase activity was found during night in both years, reaching 132 ± 8.9 and 108 ± 84 nmol C_2H_4 g^{-1} of seagrass FW h^{-1} in 2007 and 2008 respectively. The nitrogenase activity in general coincided with higher *nifH* gene expression intensity (Fig. S2E and F) and NifH protein levels (Fig. S3). These patterns suggest that the nitrogenase activity may originate from both non-heterocystous (*Lyngbya*-type; typical fixers at night) and heterocystous (*Calothrix*-type; typical fixers during the day) (Bergman *et al*., 1997).

Conclusions

The data demonstrate that a tropical seagrass, *C. rotundata*, supports a diversity of epiphytic cyanobacteria in the phyllosphere, including several previously uncharacterized cyanobacterial diazotrophs, some of which may be of a pantropical distribution. The variation in biodiversity was also reflected in the intricate diel patterns of *nifH* gene expression, NifH protein levels and the nitrogenase activity. It may also be concluded that the seagrass epiphytical prokaryotes and activity is highly dynamic and the cyanobacteria–seagrass association may therefore not represent a true symbiotic relationship. Still, some specificity between the partners and mutual interactions may exist: the seagrass may benefit from the diazotrophic nature of the epiphytes and the diazotrophic activity of the epiphyte may be stimulated as indicated by the high nitrogenase levels recorded. To fully understand the intimacy and true nature of the coexistence between the partners, better defined aspects of the seagrass colonization, the interactions between the partners, as well as the stability of the system are warranted. Further, it is also of key importance to determine whether the nitrogen fixed by the epiphytes underpins the ecological success of the seagrass community.

Sampling strategies and methods

Sampling and field experiments were conducted along the WIO coast in October and November 2007 and 2008 within an intertidal seagrass meadow in Mjimwema (06°50′S, 39°21′E), located along the coast south of Dar es Salaam, Tanzania. Sampling period and seagrass species (*C. rotundata*) were selected based on the highest nitrogenase activities detected in previous investigations (Hamisi *et al*., 2004; 2009). Samples from the phyllosphere (leaves) of *C. rotundata* were collected in 3 h intervals from 09:00 h in the morning till 06:00 h the next day, i.e. four times in the light period and four times in the dark period, covering 24 h diel cycle.

Nitrogenase activity was measured by acetylene reduction assays as described by Capone (1993). Randomly selected three to five seagrass leaves (approx. 8–10 cm in length) were collected and placed in 17 ml glass incubation bottles (seven replicates). The samples were incubated for 2 h in the tidal pools at the sampling point. After incubation, 5 ml of the head gas phase was withdrawn from each bottle and the ethylene concentration was analysed by gas chromatography (Shimadzu GC-8A, Kyoto, Japan, equipped with a Porapac N column). The rates of ethylene produced were normalized to the seagrass wet weight. Background ethylene levels in the acetylene gas from control bottles with no seagrasses were subtracted. After incubation, leaves were cut into small pieces (approx. 0.5–1.0 cm), which were mixed and stored accordingly for subsequent microscopy, nucleic acid (DNA and RNA) extraction and protein analyses. Each subsample collected at different times along the dial cycle was treated as biological replicates for both morphological and genetic characterizations.

Morphological identification of the epiphytic cyanobacteria was performed according to Desikachary (1959); Komárek and Anagnostidis (1998; 2005) and Silva and Pienaar (2000). Total DNA and RNA (seagrass and epiphytes) were extracted using GenElute Plant Genomic DNA Mini prep kit (Sigma-Aldrich Sweden AB, Sweden) and RNeasy Plant mini kit (Qiagen, German) respectively. DGGE was carried out using a Dcode system (Bio-Rad) as previously described by Díez and colleagues (2007), and the clone libraries were generated using the commercial TOPO TA cloning kit (Invitrogen, USA). The protein concentration in the supernatant was determined using RC DC protein assay (Bio-Rad, USA) and immunoblot analysis was run according to Klint and colleagues (2007).

Acknowledgements

We thank the Swedish Sida/SAREC financed Bilateral Marine Sciences Program between University of Dar es Salaam (Tanzania) and Stockholm University (Sweden), The Swedish Research Council, The Swedish Foundation for International Cooperation in Research and Higher Education and the K. and A. Wallenberg Foundation. Dr L. Ran (Stockholm University) is greatly acknowledged for technical advice and assistance.

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Supporting information

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

Fig. S1. Diel 16S rRNA gene – DGGE profiles of epiphytic cyanobacteria associated with *Cymodocea rotundata*. Samples were collected at eight time points during 24 h cycles. The bands marked with black dots and numbers were excised, sequenced and used for the phylogenetic reconstructions in Fig. 2 (As the same time points were used for the diversity analyses these could be regarded as biological replicates.).

Fig. S2. Diel *nifH* gene (based DNA and RNA) – DGGE profiles of epiphytic cyanobacteria associated with *Cymodocea rotundata*.

A–D. Diel DNA-based gel patterns in 2007 and 2008; and (E–F) diel RNA-based gel patterns in 2008. Samples were collected at eight time points during 24 h cycles. The bands marked with black dots and numbers were excised, sequenced and used for the phylogenetic reconstructions in Fig. 3.

Fig. S3. Diel NifH protein profile of epiphytes associated with the phyllosphere of *Cymodocea rotundata*. Total protein extracts from epiphytes collected in October 2008 were examined using SDS-PAGE and immunoblotting. The 40 kDa size ladder marker is given in Lane 1 (arrow). The molecular weight of the NifH protein was estimated to ~ 38 kDa.

Sequences accession number in word file.