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CHARACTERIZATION AND COMPARISON OF PROKARYOTIC EPIPHYTES ASSOCIATED WITH THREE EAST AFRICAN SEAGRASSES[1]

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Prokaryotic epiphytes on leaves of three seagrass species, *Thalassodendron ciliatum*, *Thalassia hemprichii*, and *Cymodocea rotundata*, from two Kenyan coastal sites, Nyali (a high-nutrient site) and Vipingo (a low-nutrient site), were characterized genetically and morphologically. Denaturing gradient gel electrophoresis (DGGE) and clone libraries of PCR-amplified 16S rRNA gene fragments were used to study prokaryotes associated with these seagrasses. In general, the epiphytic coverage was greater in the high-nutrient site, while the microbial diversity was linked to seagrass species rather than the study sites. Cytophaga–Flavobacteria–Bacteroides (CFB) were associated with *T. ciliatum* and *T. hemprichii* mainly in the nutrient-poor site, while α-, β-, and γ-proteobacteria were associated with all three species at the two study sites. Some bacteria phylotypes were closely related to sequences of microorganisms previously recovered from wastewaters or other contaminated sources, indicating the influx of land-based wastes into these coastal lagoon ecosystems. The abundance of potential nitrogen (N_2)-fixing cyanobacteria on *C. rotundata*, particularly in the low-nutrient site, suggested that this association may have been acquired to meet N demands. Unicellular cyanobacteria were dominant and associated with *C. rotundata* and *T. hemprichii* (with those on *T. hemprichii* being closely related to cyanobacterial symbiotic species), while *T. ciliatum* was almost devoid of cyanobacterial associations at the same site (Nyali), which suggests specificity in the cyanobacteria–seagrass associations. The abundance of prokaryotic epiphytes was considered to be linked to water depth and tidal exposure.

Key index words: **cyanobacteria; diversity; epiphytes; nitrogen fixation; phyllosphere; seagrass**

Abbreviations: **ARB, a data base (from the Latin word *arbor* for "tree"); BLAST, basic local alignment search tool; C:N, carbon:nitrogen; CFB, Cytophaga–Flavobacteria–Bacteroides; DGGE, denaturing gradient gel electrophoresis; NE monsoon, northeast monsoon; NJ, neighbor joining; OTU, operational taxonomic group; SE monsoon, southeast monsoon**

The seagrass community of the East African coastline is composed of 12 species, belonging to 8 genera, and each of these supports on their leaves a diverse phyllosphere microbiology or epiphytic community composed of a variety of macroalgae as well as faunal associations (Isaac 1968, Bandeira 1995, Lindow and Brandl 2003). Attempts to characterize epiphytic associations in seagrass beds have become important due to the recognition that apart from contributing to food webs, they also serve as ecosystem health indicators due to their rapid response to environmental change (Frankovich and Fourqurean 1997). For instance, a survey of several seagrasses, including *Thalassodendron ciliatum*, *Thalassia hemprichii*, and *Cymodocea rotundata*, in intertidal lagoons along Kenyan beaches showed high levels of seagrass growth, productivity, and assemblages of associated macroalgal epiphytes in areas with enhanced seepage of nutrient-rich wastewater (Uku and Björk 2001, 2005), but little is known about prokaryotic epiphytes on seagrasses in eastern Africa.

The use of molecular techniques to characterize prokaryotic phyllosphere or epiphytes in seagrasses is fairly recent, and the usefulness of molecular techniques in the study of seagrass epiphytic associations using 16S rDNA sequences of bacteria was shown by Weidner et al. (1996, 2000) when demonstrating that the leaves of the seagrass *Halophila stipulacea* from Eilat, in Israel, supported a diverse assemblage of bacteria.

Here we aimed at characterizing the prokaryotic epiphytes on the seagrasses *T. ciliatum*, *T. hemprichii,* and *C. rotundata* occurring in two sites (Nyali and Vipingo) with different nutrient status in Kenya, using similar molecular techniques. As previous work in the two sites showed clear differences in macroalgal associations (Uku and Björk 2001, 2005), we hypothesized that there would be similar differences in prokaryotic–seagrass associations caused by differences in nutrient inputs into the

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Kenyan coastal areas. Most studies on epiphytic associations on East African seagrasses have focused on the macroalgal component, and to our knowledge, this is the first time that characterization involving morphology and molecular techniques such as DGGE and cloning assays is being applied to determine and describe in detail the prokaryotic and, specifically, the cyanobacterial associations on East African seagrasses.

MATERIALS AND METHODS

Site description and sample collection. Seagrass samples were collected from Nyali and Vipingo along the Kenyan coast in March 2004. Nyali Beach (4°03′ S, 39°43′ E) is an important tourist center located 2 km from Mombasa Island (Fig. 1). Vipingo (3°45′ S, 39°50′ E) is located 33 km from Mombasa Island and is characterized by few residential houses and one beach hotel.

Early studies from these sites show that the groundwater nitrate inputs reached levels of ~17 μM in Nyali compared to 4 μM recorded for Vipingo for the same period (Uku and Björk 2005). Although most values of ammonia from the water column in Vipingo were ~1 μM, the highest level of ammonia recorded was 5 μM from Vipingo, whereas the highest ammonia levels in Nyali reached ~4 μM (Uku and Björk 2005). Phosphate levels were ~0.6 μM in the two sites (Uku and Björk 2005). The foregoing nutrient data shows predominant nitrate inputs into the nearshore areas in Nyali (Uku and Björk 2005) and ammonia inputs into the lagoon area in Vipingo.

The flora of the Kenyan coast is affected by seasonality caused by the northeast (NE) monsoon that occurs from October to March and the southeast (SE) monsoon from March to October (Moorjani 1977, Uku et al. 1996, Uku and Björk 2001), and samples for this study were collected from the two sites within the NE monsoon. During this period, water temperatures reached 34°C, while light levels varied between 150 and 300 μmol photons $\cdot m^{-2} \cdot s^{-1}$ within the submerged seagrass beds, with levels of 1500 μmol photons $\cdot m^{-2} \cdot s^{-1}$ recorded at the top of the water column.

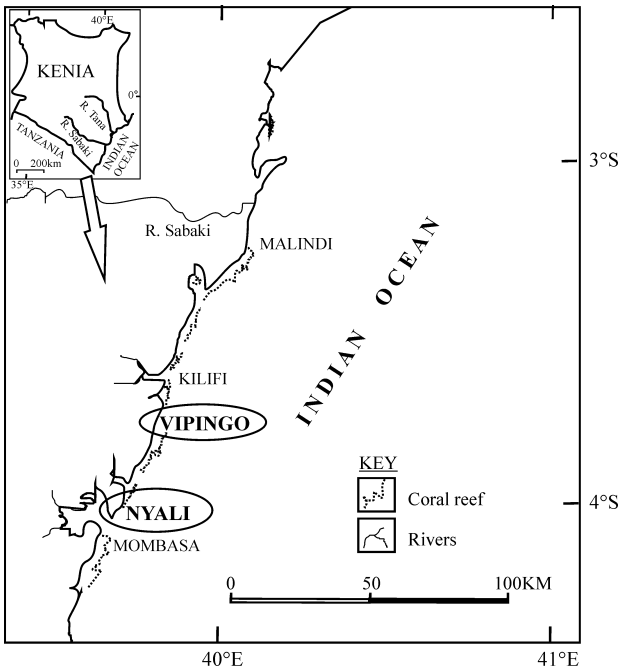

FIG. 1. Map of the Kenyan coast showing the study sites Nyali (high-nutrient site) and Vipingo (low-nutrient site).

The study sites were characterized by semidiurnal tides, and the tidal range was ~4 m (McClanahan 1988, Obura 2001), which means that there was a wide area of seagrasses exposed during the low-tide periods. The three seagrasses studied here were found growing in intertidal areas close to the beach, were common in the two study areas, and grew in monospecific patches. These seagrasses may also occur in multispecific stands, but in this study, pure stands of each species were targeted.

Determination of the nutrient status of the study sites. Water samples were collected during the period in March 2004 when the seagrass shoots were obtained from the field sites. Five samples were collected during the collection period, which coincided with the low tide. The content of nitrate, ammonium, and phosphate in the water samples was determined using methods described in Parsons et al. (1984), and the results from the two sites were compared using *t*-tests (Fowler et al. 1998).

Determination of epiphytic cover and assessment of tissue C and N. In order to estimate leaf areas under epiphytic cover, the leaves of five air-dried shoots of each seagrass from each site were rehydrated in seawater. The individual leaves in the shoot were separated, and they were then scanned using a computer scanner. Both sides of the leaves were scanned, and the areas covered by the encrusting algae were selected using Photoshop 7.0 (Adobe Systems Inc., San Jose, CA, USA). Area estimates of the selected parts as well as the entire leaf areas were determined using the Carnoy program (Schols and Smets 2001).

For the determination of tissue nutrient content, the leaves of five shoots of each species and from the two sites were cleaned of epiphytes by scraping the leaf surface with a razor blade. After removal of epiphytes, the leaves were dried in an oven at 60°C until they obtained constant weight. The leaves of each shoot were ground using a mortar and pestle. The C and N levels of the leaf tissue (and not the epiphytes) were then determined using an LECO CHN-900 elemental analyzer (LECO Corp., St. Joseph, MI, USA). The differences in epiphytic cover and tissue nutrient levels between the two sites were evaluated using the *t*-test (Fowler et al. 1998).

Nucleic acid extraction from epiphytes on seagrass leaves. The leaves of fresh shoots of each of the seagrass species were preserved in a buffer solution (40 mM EDTA, 50 mM Tris-HCl, 0.75 M sucrose) for nucleic acid extraction. Extractions were made from one shoot of *T. ciliatum*, *T. hemprichii*, and *C. rotundata* from the two sites. Extractions were also made on three additional samples of *C. rotundata* from the two sites to test whether similar patterns would be obtained when the procedure was replicated (data not shown). The epiphytes on the leaves were scraped using a blunt spatula, and the solution was centrifuged (2320*g* for 10 min) to concentrate the epiphytes. In addition, the supernatant was filtered using a 0.2 μm pore-size filter (Durapore, Millipore, Billerica, MA, USA) to capture bacterial cells. Both concentrate and filter were dried with liquid N and then macerated to break down the cells. DNA was extracted from the sample using the GenElute Plant Genomic DNA Mini prep kit (Sigma-Aldrich Sweden AB, Stockholm, Sweden). The integrity of the total DNA was checked by agarose gel (0.8%). Nucleic acid extracts were stored at −70°C until analysis.

16S rDNA–DGGE analysis. Extracted DNA from the three species and the two sites was used as template in a PCR using bacterial primers B341F (with 40 nucleotide GC clamp at the 5′ end) and 907R (Muyzer et al. 1997) as well as cyanobacterial-specific 16S rDNA oligonucleotide primers CYA106F (with 40

nucleotide GC clamp at the 5′ end) and CYA781R (Nübel et al. 1997). Amplified products are of 585 and 675 bp, respectively. The PCR conditions used for the bacterial and cyanobacterial primer sets have been described previously by Muyzer et al. (1997) and Nübel et al. (1997), respectively. The DGGE was used to reveal the associations of bacteria and cyanobacteria occurring on the different seagrass species. The DGGE was carried out with a Dcode system (Bio-Rad Laboratories AB, Sundbyberg, Sweden) as described by Díez et al. (2001). Electrophoresis was run in 0.75 mm thick, 6% polyacrylamide gels (37.5:1 acrylamide:bisacrylamide) submerged in 1× TAE buffer (40 mM Tris, 40 mM acetic acid, and 1 mM EDTA, pH 7.4) at 60°C. Electrophoresis conditions were 75 V for 16 h in a linear gradient of denaturing agents from 40% to 80% for both cyanobacteria and bacteria. After electrophoresis, the gel was stained in 1× TAE buffer containing SYBRGold Nucleic Acid Stain (1:10000 dilution; Molecular Probes/Invitrogen AB, Stockholm, Sweden) to reveal the band patterns and was then scanned using the Typhoon 8600 Variable Mode Imager (GE Healthcare Europe, Uppsala, Sweden). To obtain the sequence of DGGE bands, we proceeded as described by Díez et al. (2001). Sequencing reactions were performed with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Stockholm, Sweden), in an ABI PRISM model 377 (v.3.3) automated sequencer. GenBank accession numbers of each bacterial and cyanobacterial partial 16S rRNA gene DGGE band are DQ072902 to DQ072914 and DQ072894 to DQ072901, respectively. The 18S rDNA eukaryotic partial sequences also recovered from the DGGE were submitted to the GenBank data base with the accession numbers DQ072933 to DQ072935.

16S rDNA clone libraries. Based on the patterns revealed for the bacterial and cyanobacterial associations obtained by DGGE, four of the six samples from the March seagrass collection were cloned to further reveal specific cyanobacterial associations. These samples were *T. ciliatum* from Nyali, *T. hemprichii* from Nyali, and *C. rotundata* from both Nyali and Vipingo. The *T. hemprichii* and *C. rotundata* samples were selected for cloning as they revealed the richest cyanobacterial assemblages, while cloning of *T. ciliatum* was conducted to determine possible cyanobacteria phylotypes that may have been masked due to the large number of chloroplasts in the samples as revealed by the DGGE procedure. The 16S rRNA genes for those samples were partially amplified using the cyanobacterial primers used for DGGE analysis, but without the GC clamp (Nübel et al. 1997). The PCR reaction was performed as specified above. An aliquot of the PCR product was ligated into the prepared vector (pCR 2.1) supplied with a TOPO TA cloning kit (Invitrogen) by following the manufacturer's recommendations. The PCR amplification products containing the right size of insert were digested with 1 U of restriction enzyme MboI and HinfI (Fermentas GmbH, St. Leon-Rot, Germany) for 6 to 12 h at 37°C. Clones that produced the same RFLP pattern were grouped together and were considered members of the same operational taxonomic unit (OTU). Coverage values were calculated for the libraries by using the relative distribution of OTUs and the equation described by Good (1953). Double-stranded plasmid DNAs from selected clones were extracted with a QIAprep Spin miniprep kit (Qiagen AB, Solna, Sweden). Sequencing reactions were performed as above. Two or three clones per each different pattern (OTU) retrieved were sequenced when possible. GenBank accession numbers of cyanobacterial partial 16S rRNA clones are DQ072915 to DQ072940.

Phylogenetic analysis of 16S rDNA sequences from DGGE and clone libraries. The DGGE bacterial sequences were aligned in Bioedit using ClustalW (Tom Hall, Ibis Therapeutics, Carlsbad, CA, USA) and manually corrected. All sequences were subjected to BLAST (basic local alignment search tool; Altschul et al. 1997) searches, and the closest relatives from GenBank were included for phylogenetic analysis—only sequences from published studies or culture collections were included. The bacterial 16S rDNA DGGE sequences and the reference taxa were used for phylogenetic reconstructions from distance approximations by the neighbor-joining (NJ) method and Kimura two-parameter (Kimura 1980) in PAUP (version 4.0b10; Sinauer Associates Inc., Sunderland, MA, USA). Branch support was measured by bootstrap analysis using an NJ search strategy with 1000 bootstrap replicates. The 16S rDNA sequences of *Sulfolobus acidocaldarius* and one *Halobacteriaceae* were used as outgroups.

The DGGE cyanobacterial bands and clone partial sequences were added to an alignment of about 50,000 homologous bacterial 16S rRNA gene primary structures as implemented in the ARB software package and corresponding to the released data base available at http://www.arb-home.de (Ludwig et al. 2004). In addition, the same sequences were compared with the available sequences in public data bases by using BLAST in order to retrieve those not included in the ARB data base. All new added sequences were automatically aligned with the alignment tool implemented in ARB (Ludwig et al. 1998) and checked and corrected manually to improve the alignment. The backbone tree shown in Figure 4 was initially reconstructed by using only complete or almost complete gene sequences. The tree was reconstructed by the use of the NJ algorithm with the Jukes–Cantor correction (Jukes and Cantor 1969). Then, environmental 16S rDNA partial sequences of DGGE bands and clone library (~600 bp) were added to the existing tree by the use of the Parsimony Tool as implemented in the ARB package (Ludwig et al. 1998), without allowing changes in the overall topology. Branch support was measured by bootstrap analysis using a parsimony search strategy with 1000 bootstrap replicates.

Light and fluorescence microscopy. Several shoots were air-dried and used for microscopic identification of attached epiphytic algae as well as for the determination of leaf areas covered by epiphytes. The species revealed by the molecular analysis were further located on mature seagrass leaves, using both bright-field and fluorescence microscopy. For microscopic purposes, the oldest (mature) leaf was selected, as this was the leaf with the densest epiphytic associations. Glutaraldehyde-fixed samples and dry samples were used for light and epifluorescence observations under high magnification (×1000). Lower magnification (×200 or ×400) was also used to examine the distribution of the epiphytic cyanobacterial cells on the leaves. Green-light excitation was used to obtain phycobiliprotein fluorescence that was intense red and orange (from emission by phycocyanin and phycoerythrin, respectively), which was primarily due to cyanobacteria. These observations were performed with an Olympus BX 60 microscope (Olympus, Tokyo, Japan) equipped with interference contrast. The images were recorded with an Olympus DP–50 digital camera. The morphological identification was according to Desikachary (1959), Komárek and Anagnostidis (1989, 1999, 2005), and Silva and Pienaar (2000).

RESULTS

Nutrient status of the study sites. Nutrient samples collected from Nyali and Vipingo during this study period revealed that nitrate levels in Nyali peaked at 3 μM, and phosphate levels of 0.3 μM and extremely low ammonium levels were recorded. Samples from Vipingo showed insignificant levels of nitrate, approximately 1 μM of ammonium, and 2 μM of phosphates. The statistical comparison of the

TABLE 1. Gross morphology of the different seagrasses examined at the two study sites and their carbon and nitrogen contents ($n = 5$ for each species).

Characteristic	*Thalassodendron ciliatum*		*Thalassia hemprichii*		*Cymodocea rotundata*	
	Nyali	Vipingo	Nyali	Vipingo	Nyali	Vipingo
Number of leaves per shoot	8.0 ± 0	6.2 ± 0.4	4.0 ± 0.6	3.6 ± 0.5	2.8 ± 0.4	3.0 ± 0
Leaf area ($cm^2 \cdot shoot^{-1}$)	130.9 ± 21.1[a]	38.3 ± 7.9	35.6 ± 3.5[a]	60.5 ± 11.9	17.6 ± 2.9[a]	11.7 ± 1.4
Epiphytic coverage ($cm^2 \cdot shoot^{-1}$)	21.1 ± 7.4[a]	1.1 ± 0.4	1.9 ± 1.4	0.5 ± 0.8	2.5 ± 1.1[a]	0
% Epiphytic coverage on leaves	20	3	5	1	14	0
% Carbon	44.2 ± 2.9	45.6 ± 1.2	42.0 ± 0.03	41.7 ± 1.3	44.7 ± 0.9[a]	46.7 ± 0.7
% Nitrogen	2.2 ± 0.4	2.3 ± 0.4	2.9 ± 0.1	2.5 ± 0.3	3.4 ± 0.2[a]	2.6 ± 0.4
C:N ratio	21:1	20:1	15:1	17:1	13:1[a]	19:1

[a]Denotes a significant difference between the two sites ($P < 0.05$).

nutrient levels showed that all nutrients were significantly different when the two sites were compared ($t = 3.6$, 2.44, and 2.49 for phosphates, nitrates, and ammonium, respectively; $P < 0.05$).

Features of the host seagrass species. Since epiphytes require an attachment substrate, such as the leaves of seagrasses, the influence of leaf size and number was investigated in the three seagrass genera from the two sampling sites (Nyali and Vipingo). As seen in Table 1, the highest number of leaves was observed for *T. ciliatum* growing in the nutrient-rich Nyali site. The leaves of this genus were also larger than those of the other species (Table 1). *Cymodocea rotundata* had the fewest leaves and the smallest leaf area (Table 1). The leaf areas of both *T. ciliatum* and *C. rotundata* occurring in Nyali were significantly different in size from similar species in Vipingo ($t = 4.16$, 9.18 for *T. ciliatum* and *C. rotundata*, respectively; $P < 0.05$), whereas *T. hemprichii* was larger in Vipingo ($t = 4.48$; $P < 0.05$). The dominant epiphytes on these seagrasses were encrusting coralline red algae, and the total epiphytic coverage on shoots of the different seagrasses varied, with the highest cover detected on *T. ciliatum* (Table 1). The epiphytic cover on the leaves of *T. ciliatum* and *C. rotundata* differed significantly between the two sites ($t = 5.23$, 6.01 for *T. ciliatum* and *C. rotundata*, respectively; $P < 0.05$), with a notable absence of encrusting red coralline algae on the leaves of *C. rotundata* found in Vipingo. However, the abundance of these epiphytes on the leaves of *T. hemprichii* was similar at the two sites.

Analysis of nutrient contents of the seagrass leaf tissues revealed significant differences for the seagrass *C. rotundata* based on site. Tissue C content for *C. rotundata* was highest in the low-nutrient site, Vipingo ($t = 3.38$; $P < 0.05$), while the N content was highest in the nutrient-rich Nyali ($t = 4.07$; $P < 0.05$). The C:N ratio was also significantly different when the contents of this species were compared between the two sites, with higher levels occurring in Vipingo ($t = 3.56$; $P < 0.05$) (Table 1). The other two species did not exhibit site-specific differences in the tissue nutrient content.

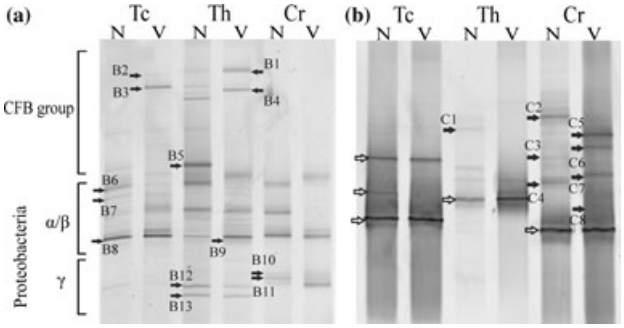

FIG. 2. Denaturing gradient gel electrophoresis images using (a) bacterial primers and (b) cyanobacterial primers on the three seagrass species collected in March 2004. (a) Bacterial bands are denoted by black arrows and the closest match with GenBank (Table S1 in the supplementary material). (b) Chloroplast bands are denoted by open arrows. Cyanobacterial bands are denoted by black arrows. Bacterial and cyanobacterial sequence matches are shown in the phylogenetic tree in Figures 3 and 4, respectively. Tc, *Thalassodendron ciliatum*; Th, *Thalassia hemprichii*; Cr, *Cymodocea rotundata*; N, Nyali (the nutrient-rich site); V, Vipingo (the low-nutrient site); CFB, Cytophaga–Flavobacteria–Bacteroides.

Identification of prokaryotic epiphytes using 16S rDNA–DGGE. The bacterial DGGE patterns obtained demonstrated distinct differences between the three seagrasses as well as between the two sites within the same seagrass genus (Fig. 2). For instance, the bacterial DGGE showed that the Cytophaga–Flavobacteria–Bacteroides (CFB) associations were abundant on *T. ciliatum* and *T. hemprichii* mainly in the low-nutrient site, Vipingo (Figs. 2a and 3). The CFB group matched primarily uncultured bacteria from sewage, garbage compost, and animal farm wastes (bands B1–B4) and from other epiphytic bacteria associated with diatoms, such as band B5 in the Nyali site (where a dominance, of diatoms was observed; Fig. 3). *Cymodocea rotundata* lacked the CFB group at both sites. α- and β-proteobacteria were observed on all seagrasses at both sites. Bands B6 and B7, mainly present in Nyali, were closely related to *Rhizobium* sp., while band B9 was closely related to the Phyllobacteriaceae bacterium from household waste (Fig. 3). Band B8 was the only band closely related to a β-proteobacteria. This band was present in all samples in both sites and

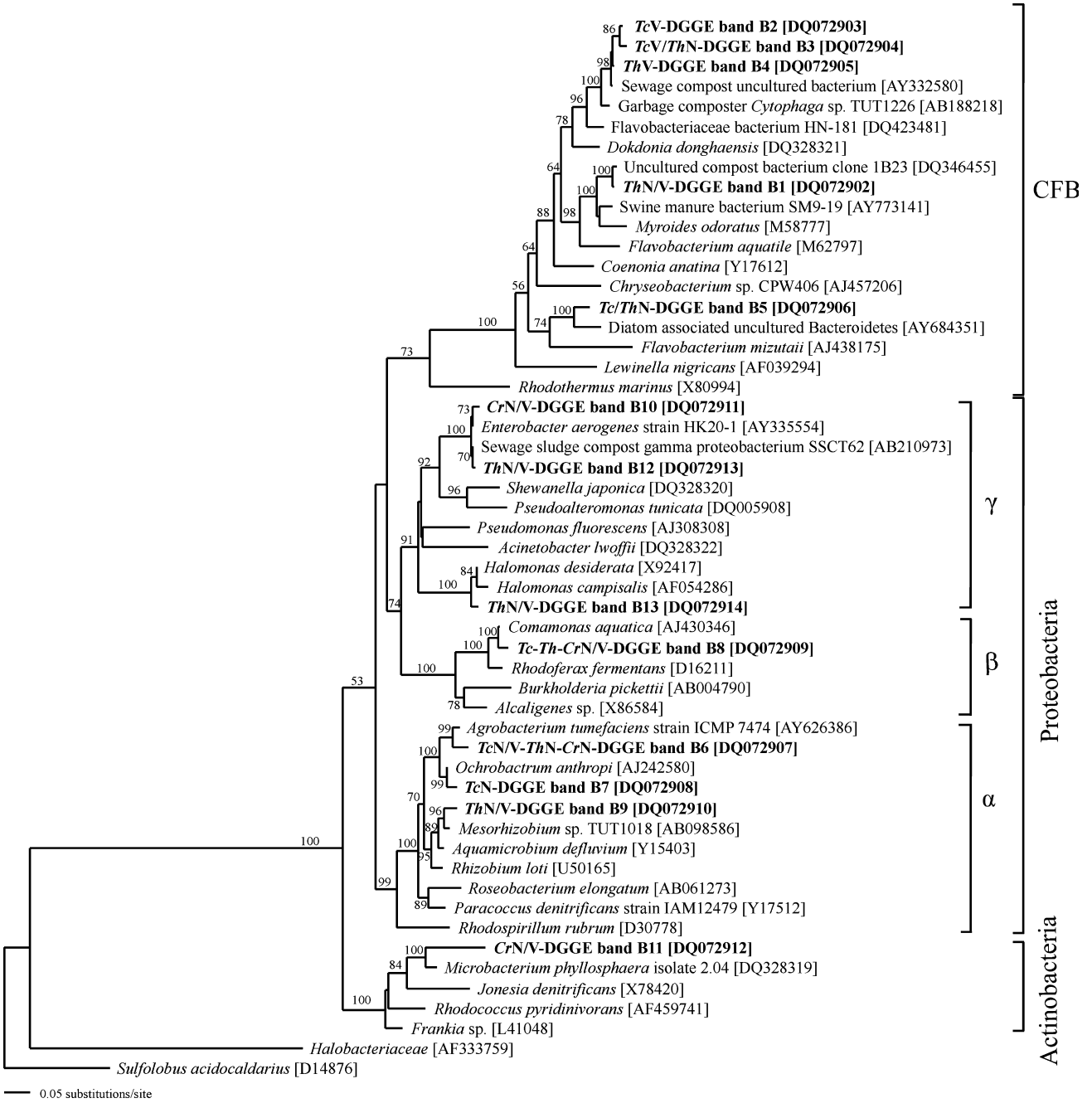

FIG. 3. Phylogenetic affiliation of the bacterial denaturing gradient gel electrophoresis (DGGE) bands obtained from epiphytes on the three seagrasses investigated. The tree was constructed using the neighbor-joining distance method and Kimura two-parameter model. Sequences from the present study are given in bold, and each DGGE band sequence is designated by code as in Figure 2a and by the abbreviation of the seagrass species and the site of collection. Bootstrap support values >50 are shown. Tc, *Thalassodendron ciliatum*; Th, *Thalassia hemprichii*; Cr, *Cymodocea rotundata*; N, Nyali (the nutrient-rich site); V, Vipingo (the low-nutrient site); CFB, Cytophaga–Flavobacteria–Bacteroides.

had a high intensity (relative abundance). γ-Proteobacteria (bands from B10, B12, and B13) were found mainly on *T. hemprichii* and *C. rotundata* at both sites, and most were related to other bacteria also recovered from contaminated sources (Fig. 3). None of the DGGE bands obtained using bacterial primers were affiliated to cyanobacteria.

Cyanobacterial-specific DGGE analyses revealed unique patterns of cyanobacterial epiphytes for each seagrass genus (Fig. 2b). The highest diversity was detected on *C. rotundata*, which also showed distinct differences in species composition at the two study sites. In addition, similar DGGE patterns were observed in replicate samples from *C. rotundata*

obtained from both sites (data not shown). Band C1 extracted from the *T. hemprichii* was related to a symbiotic cyanobacterium (see Chroococcales unicellular group II in Fig. 4). C2 was the most representative band on *C. rotundata* in Nyali and was closely related to the unicellular *Chroococcidiopsis* PCC6712 (Chroococcales group II in Fig. 4). Band C6, which was identified on *C. rotundata* from Vipingo, was also closely related to unicellular species related to the Chroococcales group II (Fig. 4). Band C5, associated with *C. rotundata* in Vipingo, was most closely related to filamentous, heterocystous *Calothrix* spp. Bands C3 and C4 (from Nyali) as well as C7 and C8 (from Vipingo) from *C. rotundata* were most closely related to the Oscillatoriales group of filamentous, nonheterocystous cyanobacteria, such as *Planktothrix* sp., *Lyngbya* spp., *Oscillatoria* spp., and *Trichodesmium* spp., respectively. As shown by clone library analyses (see section below), most of these sequences represented the most abundant populations and grouped together in the phylogenetic tree (Fig. 4). Not unexpectedly, chloroplasts from the three seagrasses were also amplified by PCR–DGGE using the cyanobacterial primers (Fig. 2b), and amplification of the samples using eukaryotic 18S rDNA confirmed the relationship to the seagrass families Cymodoceaceae (containing *C. rotundata* and *T. ciliatum*) and Hydrocharitaceae (containing *T. hemprichii*).

Identification of cyanobacterial epiphytes using 16S rDNA clone libraries. Selected samples for cloning based on the results of the cyanobacterial DGGE analyses were from *T. ciliatum* in Nyali, *T. hemprichii* in Nyali, and *C. rotundata* growing in both Nyali and Vipingo. The cloned samples of *T. ciliatum* from Nyali were dominated by chloroplasts, as also evidenced by DGGE analysis, and no cyanobacterial epiphyte clones were retrieved from this species. A more diverse assemblage of cyanobacteria was observed on *C. rotundata* and *T. hemprichii*. After removing clones related to chloroplasts once RFLP analysis was made, a total of the 51 clones (nine different OTUs in *C. rotundata* from Vipingo), 16 clones (nine different OTUs in *C. rotundata* from Nyali), and 44 clones (nine different OTUs in *T. hemprichii* from Nyali) were analyzed. The coverage values were high, ranging from 94% in the *C. rotundata* clone library from Nyali to 98% in *C. rotundata* from Vipingo and *T. hemprichii* from Nyali, from which few cyanobacterial clones were obtained. Comparison of clones from *C. rotundata* between Nyali and Vipingo using the clone libraries showed that 27% of the clones found in Vipingo originated from heterocystous cyanobacteria (Fig. 5), and of this component, 93% were affiliated with the N_2-fixing genus *Calothrix*, and the remainder with the species *Anabaena* cf. *cylindrica* Lemmerm. In the high-nutrient site Nyali, only 6% of the clones were affiliated with heterocystous species (Fig. 5) and with *Anabaena* cf. *cylindrica*. The filamentous, nonheterocystous types were distinct components of both sites on this specific seagrass.

Unicellular cyanobacteria were quantitatively rather equally represented on *C. rotundata* at the two sites (Fig. 5), and only the abundance of each phylotype was different between the two sites. All phylotypes affiliated with the unicellular cyanobacteria in *C. rotundata* were grouped separately in group I and II in Figure 4. As shown in Figures 4 and 5, all together, these comprised about 55% of the clones in Nyali. In Vipingo, the unicellular component was dominated by phylotypes affiliated with the Xenococcaceae family, such as *Chroococcidiopsis* PCC6712 (Chroococcales group II in Fig. 4), which made up 25% of the unicellular component. *Xenococcus* PCC7305 (Chroococcales group II in Fig. 4) comprised 14% of this group, and other unicellular cyanobacteria constituted the remainder.

Filamentous, nonheterocystous cyanobacteria showed a similar abundance (~25%) on *C. rotundata* in Nyali and in Vipingo (Fig. 5). Both sites shared clone P4 and P7 (representative clones of two different OTU patterns): phylotypes affiliated with Oscillatoriaceae and/or Pseudanabaenaceae family species (Fig. 4). Clones related to *Trichodesmium* phylotypes were observed in the two study sites (see clone P6 in Fig. 4), while those related to an uncultured cyanobacterium were only found in Nyali (see clone P12 in Fig. 4). Twenty-seven percent of the clone library associated with *T. hemprichii* in Nyali was related to an uncultured marine bacterium closely related to *Verrucomicrobium* spp. (Fig. 5), while the signal of these bacteria on *C. rotundata* was insignificant (Fig. 5). Unicellular symbiotic associations of *T. hemprichii* comprised 60% of the clone library and affiliated most closely with *Synechococcus* spp. and *Cyanobacterium stanieri* Rippka et Cohen-Baz., closely related to cyanobacterial symbionts of sponges, such as the cyanobacterial symbiont MY17 isolate from *Mycale* sponge (Fig. 5 and Chroococcales group II in Fig. 4). In addition, some sequences of clones affiliated with other *Synechococcus* spp., belonging to different phylotypes of Synechococcaceae family (group IV and V in Fig. 4), were associated with *T. hemprichii*. Heterocystous cyanobacteria were not detected as epiphytes on *T. hemprichii*, while 20% of the clone sequences (Fig. 5) were affiliated with filamentous, nonheterocystous cyanobacteria of the Pseudanabaenaceae family, such as *Leptolyngbya* sp., and with the Oscillatoriaceae family, such as *Lyngbya* spp. and *Trichodesmium* spp. (Fig. 4) (sharing these patterns with *C. rotundata*).

As shown in Figure 6, the number of prokaryotic species (species richness) associated with the leaves of the different seagrasses was not related to epiphytic cover. The highest number of prokaryotes was observed on *C. rotundata* in both Nyali and Vipingo (the only difference being the prokaryotic species present). The cover of encrusting coralline red

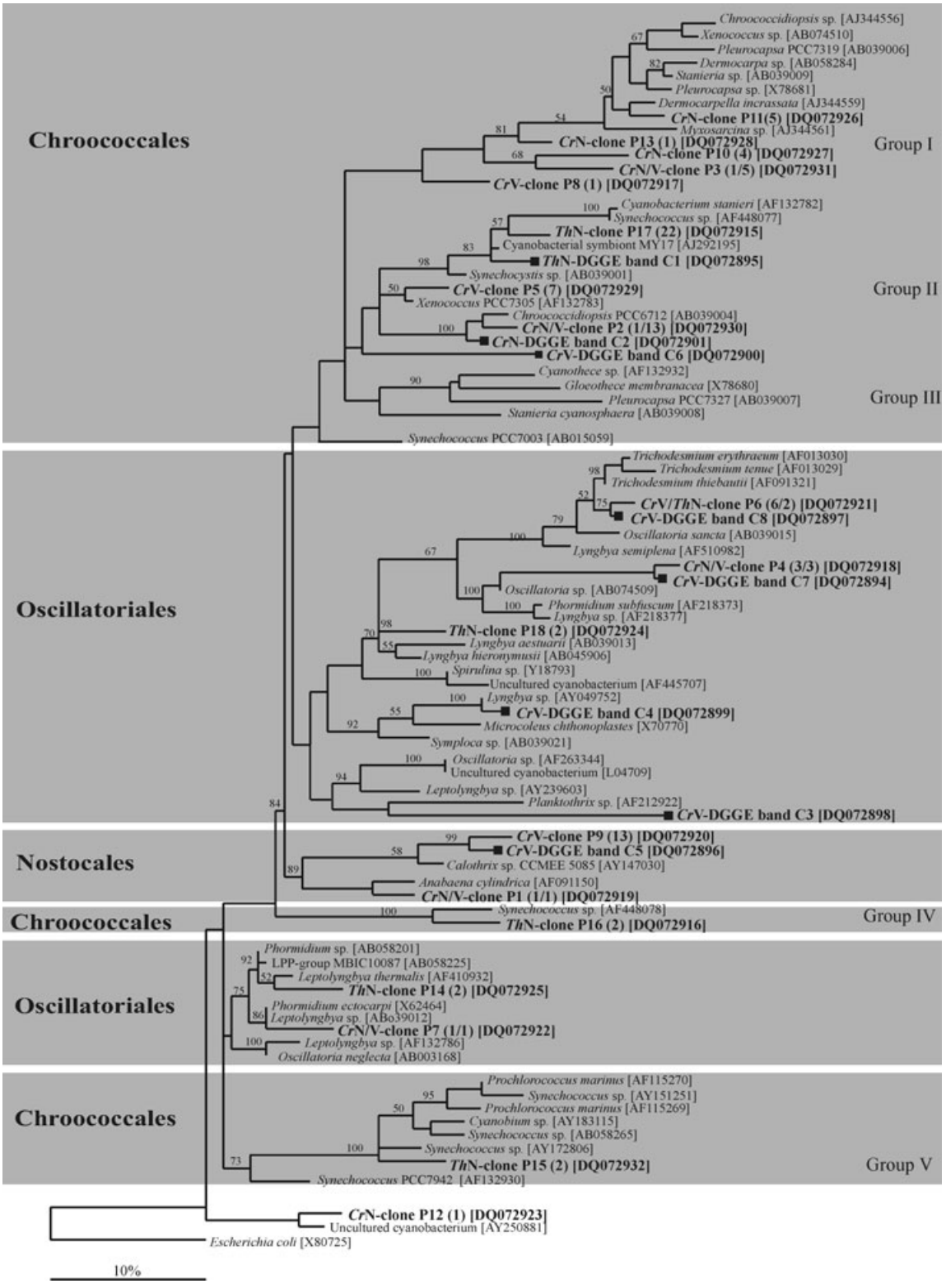

FIG. 4. Phylogenetic affiliation of the 16S rRNA sequences from denaturing gradient gel electrophoresis (DGGE) bands and clones obtained from epiphytes on seagrasses within the cyanobacterial radiation. The DGGE bands and clones [representative of each different operational taxonomic unit (OTU) retrieved after RFLP analysis] are indicated by boldface type and include the seagrass species abbreviation name and the site of collection. Values in parentheses indicate the number of clones in each OTU. A black square symbol was also used to distinguish DGGE bands from clone library sequences. The sequence of *Escherichia coli* (X80725) was used as outgroup. The scale bar represents 10% estimated sequence divergence. Bootstrap support values >50 are shown. Tc, *Thalassodendron ciliatum*; Th, *Thalassia hemprichii*; Cr, *Cymodocea rotundata*; N, Nyali; V, Vipingo.

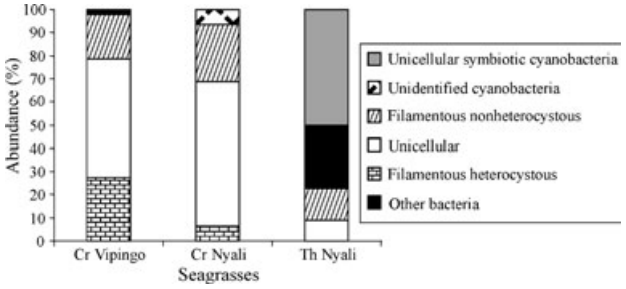

FIG. 5. The distribution of functional microbial groups found using the clone abundance percentage recovered on the seagrasses *Cymodocea rotundata* (Vipingo and Nyali) and *Thalassia hemprichii* in the nutrient-rich site at Nyali. Th, *T. hemprichii*; Cr, *C. rotundata*.

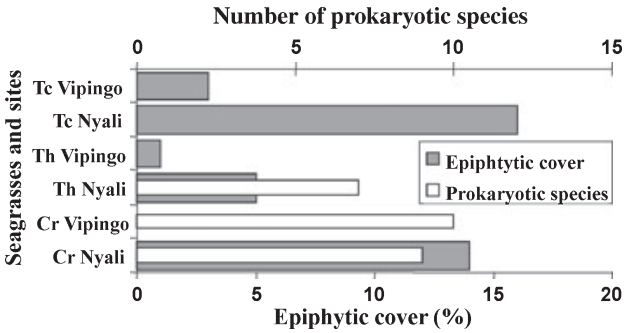

FIG. 6. The percent cover of encrusting coralline red algae on the examined seagrass leaves compared to the number of prokaryotes (species richness) observed. Tc, *Thalassodendron ciliatum*; Th, *Thalassia hemprichii*; Cr, *Cymodocea rotundata*. Nyali is the nutrient-rich site, while Vipingo is the low-nutrient site.

algae on *C. rotundata* was significantly higher in Nyali than in Vipingo. In spite of a comparatively higher cover of encrusting coralline red algae on *T. hemprichii* at the Nyali site, the number of prokaryotes was higher at this site. Although there was a significant difference in the cover of the encrusting coralline red algae on *T. ciliatum* at the two sites, prokaryotic assemblages were lacking at both.

Morphological diversity. Microscopic analysis showed that *C. rotundata* in Vipingo had the highest population of epiphytic cyanobacteria. The rich variety is illustrated in Figure 7 and in Table S1 (see supplementary material), which show representatives from all major morphotypes from unicellular to nonheterocystous and heterocystous types being detected. The cyanobacterial morphotypes identified using LM corresponded to those identified as most abundant in DGGE analyses and cloning (relative percentage of clone abundance and number of species), verifying the usefulness of the different techniques used in this study.

DISCUSSION

The objective of this study was to genetically and morphologically characterize the diversity of epiphytic prokaryotic communities associated with seagrasses along the Kenyan coast and to assess whether these epiphytic assemblages were related to nutrient inputs into the two sites, Nyali and Vipingo. The levels of water-column nutrients confirmed earlier findings, which indicated high inputs of nitrates into the nearshore seagrass beds in Nyali, while inputs into Vipingo were dominated by ammonium. Determination of daily nutrient inputs into the two sites conducted in 2000 and 2002 showed that the nitrate inputs in Nyali were persistent during the low tides, whereas ammonia inputs into the lagoon in Vipingo were not as persistent (J. Uku, unpublished data).

In general, the microbial epiphytic coverage was higher in the more nutrient-rich site Nyali than in Vipingo, while the microbial diversity was related to seagrass species rather than to sites. Associations of α-, β-, and γ-proteobacteria were common, and the CFB group was a distinct component of the epiphytic microbial flora. Our findings further demonstrate that the CFB group was restricted to *T. ciliatum* and *T. hemprichii*. The bacterial assemblages identified here were similar to those of the small seagrass *H. stipulacea* collected from the northern Gulf of Eilat in Israel (Weidner et al. 1996, 2000).

Several of the phylotypes recovered, which belong to different groups of bacteria (actinobacteria, γ-proteobacteria, and CFB group), were closely related to epiphytic bacteria associated with the marine macroalga *Ulva lactuca* L. (Burmølle et al. 2006), the rhizosphere of plants (Chung et al. 2005), and diatoms (Grossart et al. 2005). Interestingly, a large group of bacteria phylotypes was closely related to sequences from members of the CFB group and proteobacteria reported to be present in composting household waste (Hiraishi et al. 2003), in wastewater from livestock farming areas, in microbes present in the guts of ruminant animals (Cho and Kim 2000), and in composts of cow and swine manure (Green et al. 2004, Castillo and Bruns 2005).

Hence, our data suggest that the seagrass epiphytes in both Nyali and Vipingo were subject to the influx of land-based wastewater. This conclusion is also supported by the presence of sequences related to Verrucomicrobia typically present in contaminated water or sewage. It may be noted, however, that as these bacteria were amplified using the cyanobacterial-specific 16S rDNA oligonucleotide primers (CYA106 F and CYA781R; Nübel et al. 1997), a careful interpretation of data obtained using these primers is recommended. Although the low-nutrient Vipingo site was selected for being less impacted, our findings show that it may still be influenced by contaminant sources, such as the farming areas present in the vicinity, as evidenced by higher levels of ammonia in the water column. Additionally, transport from other impacted areas may also be a potential nutrient source. The presence of members of the CFB, Verrucomicrobia, and

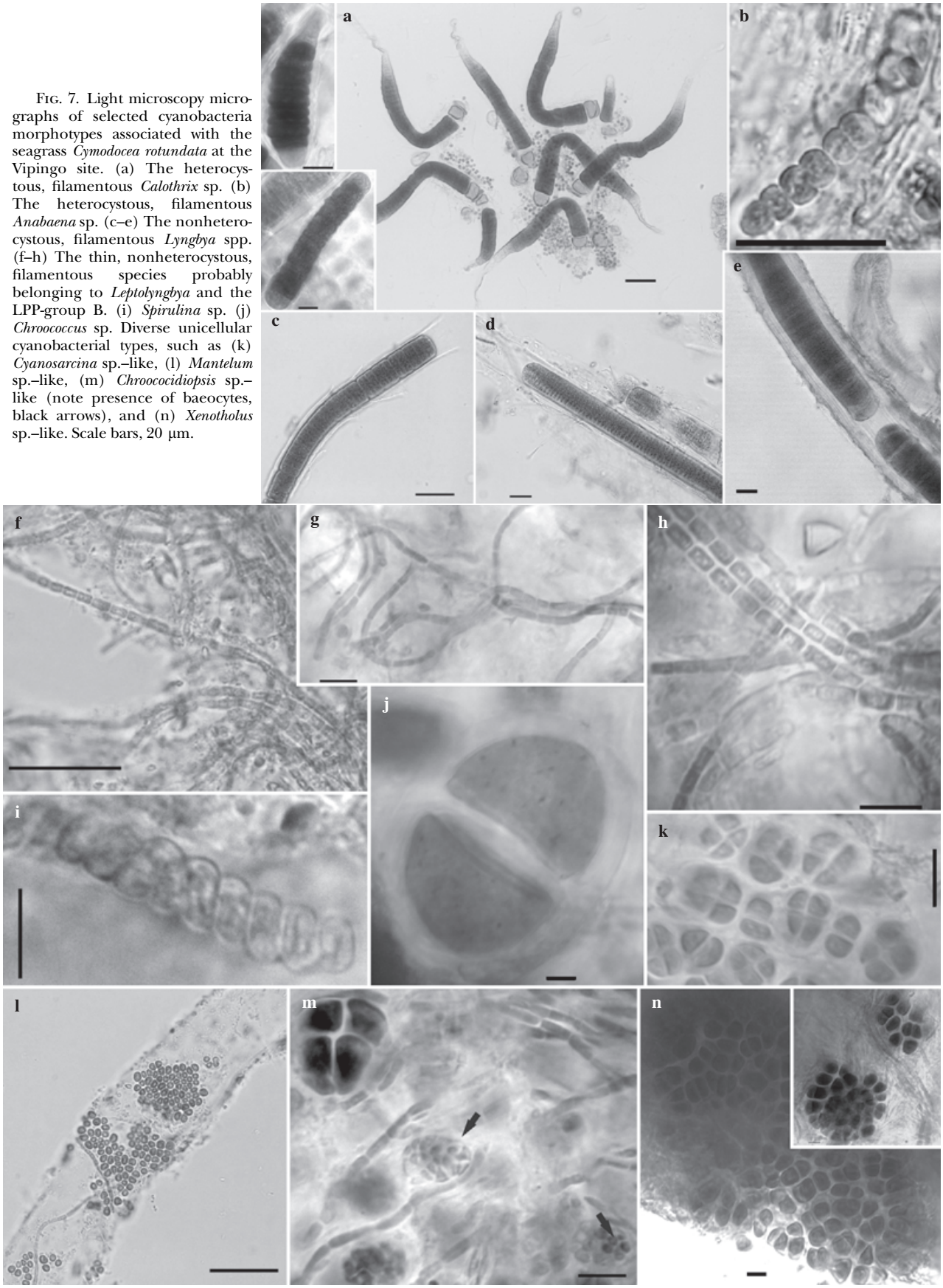

FIG. 7. Light microscopy micrographs of selected cyanobacteria morphotypes associated with the seagrass *Cymodocea rotundata* at the Vipingo site. (a) The heterocystous, filamentous *Calothrix* sp. (b) The heterocystous, filamentous *Anabaena* sp. (c–e) The nonheterocystous, filamentous *Lyngbya* spp. (f–h) The thin, nonheterocystous, filamentous species probably belonging to *Leptolyngbya* and the LPP-group B. (i) *Spirulina* sp. (j) *Chroococcus* sp. Diverse unicellular cyanobacterial types, such as (k) *Cyanosarcina* sp.–like, (l) *Mantelum* sp.–like, (m) *Chroococidiopsis* sp.–like (note presence of baeocytes, black arrows), and (n) *Xenotholus* sp.–like. Scale bars, 20 μm.

other groups of bacteria related to contaminated waters may therefore serve as indicators of anthropogenic inputs and suggest that marine habitats may be impacted, even if activities are not directly at the beachfront (such as in Vipingo).

The nutrient status of the seagrass tissues reflects that the surrounding habitat and plants from low-nutrient environments typically have higher C:N ratios (Duarte 1990). Although, the N-levels of the seagrasses examined here were above the critical N-level of 1.82% per dry weight, suggested as the level below which seagrasses are likely to be nutrient limited (Duarte 1990), there was a clear difference in the tissue N-content of *C. rotundata* at the two sites. This finding indicates some degree of N-limitation in Vipingo, the low-nutrient site, in spite of the higher levels of ammonia in the water column of this site.

Leaf growth rates of the three seagrasses evaluated in an earlier study showed that *C. rotundata* experienced similar growth rates of approximately 12 mm $\cdot$ shoot$^{-1}\cdot$d^{-1} in the two study sites, whereas the growth rates of the other two species differed significantly when the two sites were compared (Uku and Björk 2005). *Thalassodendron ciliatum* had growth rates of 17 mm$\cdot$shoot$^{-1}\cdot$d^{-1} in Nyali and 12 mm $\cdot$ shoot$^{-1}\cdot$d^{-1} in Vipingo, whereas *T. hemprichii* had growth rates of up to 28 mm$\cdot$shoot$^{-1}\cdot$d^{-1} in Nyali and 17 mm$\cdot$shoot$^{-1}\cdot$d^{-1} in Vipingo (Uku and Björk 2005). The site-specific nutrient differences appeared to influence growth of two of the seagrasses apart from *C. rotundata*.

The occurrence and abundance of heterocystous N_2-fixing *Calothrix* sp.—as well as other common potentially N_2-fixing unicellular cyanobacteria—such as *Chroococcidiopsis* sp. (Bergman et al. 1997), and nonheterocystous, filamentous cyanobacteria—on *C. rotundata* in Vipingo is therefore likely linked to the demand for N to maintain the growth rate of *C. rotundata* at levels comparable to those for the same species in Nyali. The prevalence of heterocystous cyanobacteria on seagrasses in Vipingo was previously reported by Uku and Björk (2001), indicating that this is a persistent association. Hence, N_2-fixing cyanobacteria, such as *Calothrix* sp., as well as other potential N_2 fixers may enable *C. rotundata* to sustain its N needs. Although seagrasses can obtain nutrients from the water column and sediments, these nutrient pools may be inadequate to meet growth requirements (Pereg et al. 1994, Herbert 1999, Welsh 2000), which also indicates the importance of associations with N_2-fixing microbial consortia. The N_2-fixing potential of associated epiphytes on seagrass leaves has been previously documented (Goering and Parker 1972, Capone 1982, Moriarty and O'Donohue 1993, McClathery et al. 1998, Pereg et al. 2002). Herbert (1999) showed that epiphytic cyanobacteria may contribute up to 50% of the N required of certain seagrasses, and that N_2 fixation was higher on leaves of *Thalassia testudinum* associated with *Calothrix* sp. than on leaves lacking this epiphyte (Capone et al. 1979). A comparative study in East Africa on the occurrence of cyanobacteria in seagrass meadows (Hamisi et al. 2004) in areas of varying nutrient inputs also showed that the coverage of N_2-fixing cyanobacteria was higher in the nutrient-poor site, thereby confirming our findings of N_2 fixers in our nutrient-poor site, Vipingo.

The different growth locations as well as the anatomy of the different seagrasses examined may play a role in maintaining the prokaryotic associations. *Cymodocea rotundata* grows higher up in the intertidal zone (Bandeira 2002) and is often exposed to high light levels during low tides. In contrast, *T. hemprichii* and in particular *T. ciliatum* occur in deeper pools and possess more leaves, possibly contributing to self-shading and lower light levels. As N_2-fixing cyanobacteria (both heterocystous and nonheterocystous forms) require high irradiances to sustain the energy-demanding N_2-fixation process (Gallon and Stal 1992), locality in terms of water depth and tidal exposure may also contribute to the richer cyanobacterial association to *C. rotundata*.

In terrestrial plants, variations in physical and nutritional characteristics of the phyllosphere (leaf surface) have been shown to influence epiphytic populations of bacteria (Lindow and Brandl 2003). A study on microbial phyllosphere populations by Yang et al. (2001), based on DGGE analysis of PCR 16S rRNA genes, showed that phyllosphere microbiota of a given species were more related than microbiota occurring on leaves of different plant species, indicating an adaptation to the specific leaf features. Although exudates from seagrasses have been documented in several studies (Zapata and McMillan 1979, Harrison 1982, Wahbeh and Mahasneh 1984) and certainly could have an effect on the microbiotic species composition, there is little evidence to show that seagrasses are able to control epiphytic settlement and growth in such a way (Hemminga and Duarte 2000).

The leaves of *C. rotundata* and *T. hemprichii* have a turnover time of about 20 d, while leaves of *T. ciliatum* turn over in 45 d (Uku and Björk 2005). The fact that there is a high cyanobacterial assemblage on *C. rotundata,* which possesses a smaller leaf area, and a lower one on *T. ciliatum,* which possess a larger leaf area, may indicate that there are other factors besides the longevity of the leaves and size that determine initiation and persistence of the cyanobacterial–seagrass associations. This finding is also supported by the fact that the presence of encrusting coralline red algae on leaf surfaces did not deter cyanobacterial settlement. Both *T. ciliatum* and *C. rotundata* in Nyali had a comparable cover of encrusting coralline red algae, but *C. rotundata* was consistently associated with more epiphytic cyanobacteria.

Interestingly, the dominant cyanobacterial epiphytes associated with *T. hemprichii,* which were clearly different from the ones associated with

C. rotundata (see Fig. 5), were related to the genus *Synechococcus* and other unicellular cyanobacteria forming symbioses with sponges, such as the cyanobacterium symbiont from the Synechococcaceae family in Fig. 4 (Steindler et al. 2005). The presence of the sponges, such as *Mycale* sp., has been documented in Malindi on the north coast of Kenya (Barnes and Bell 2002), and although it is not unusual that sponges associate with seagrasses in the study sites (J. Uku, personal observation), this symbiont may have been extracted from the seagrass. The fact that many sequences recovered were from seagrass chloroplasts provides evidence that seagrass tissue was scraped off during the sample preparation, and some of this tissue may have contained symbiotic cyanobacteria. The intimacy of this cyanobacterium–*T. hemprichii* association is therefore of interest for examination in greater detail.

The DGGE fingerprinting technique was used in this study as a rapid screening method for the presence of cyanobacteria before performing the clone libraries analysis. However, the DGGE technique does not recover populations that are in low abundance. The number of eukaryotic chloroplasts from the seagrass tissue and the macroepiphytic encrusting algae in the samples may have been responsible for hiding the DGGE cyanobacteria signal. A clearer cyanobacterial signal was only possible by the use of clone libraries. Therefore, the use of RFLP analysis resulted in a fast screening to distinguish the chloroplast patterns from the nonchloroplast clones for a better identification of cyanobacteria.

CONCLUSIONS

We conclude from our genetic and morphological data that there is a varied and distinct prokaryotic epiphytic community present on seagrasses, composed of both bacteria and cyanobacteria, and although the microbial community shows similarities between seagrass species, enough distinct differences exist to suggest that there are specific components involved in the establishment of the associations. The fact that the nutrient-poor site was characterized by an abundance of potential N_2-fixing cyanobacteria suggests that these may contribute to the nutrient demands of the seagrass. It is also apparent that prokaryotic epiphytes develop as a response to the surrounding environment, and that some may function as biomarkers for water intrusions from land sources in coastal lagoon ecosystems.

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Supplementary Material

The following supplementary material is available as part of the online article from http://www.blackwell-synergy.com:

Table S1. Morphological characteristics of selected cyanobacteria distinguished under the microscope (the sample number corresponds to the label on the photographic images; all images were taken from the seagrass *Cymodocea rotundata* obtained from Vipingo, the nutrient poor-site). Classification was done according to Desikachary (1959), Komárek and Anagnostidis (1989, 1999, 2005), and Silva and Pienaar (2000). Corresponding classification according to *Bergey's Manual of Systematic Bacteriology* (Castenholz 2001) is given in brackets when the systems differ.

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