The ISME Journal (2008) 2, 561–570

www.nature.com/ismej

ORIGINAL ARTICLE

Nitrogen fixation by phyllosphere bacteria associated with higher plants and their colonizing epiphytes of a tropical lowland rainforest of Costa Rica

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Leaf surfaces (phyllospheres) have been shown to provide appropriate conditions for colonization by microorganisms including diazotrophic bacteria that are able to fix atmospheric nitrogen (N_2). In this study, we determined leaf-associated N_2 fixation of a range of rainforest plants in Costa Rica, under different environmental conditions, by tracing biomass N incorporation from $^{15}N_2$. N_2-fixing bacterial communities of the plant species *Carludovica drudei*, *Grias cauliflora* and *Costus laevis* were investigated in more detail by analysis of the *nifH* gene and leaf-associated bacteria were identified by 16S rRNA gene analysis. N_2 fixation rates varied among plant species, their growth sites (different microclimatic conditions) and light exposure. Leaf-associated diazotrophic bacterial communities detected on *C. drudei* and *C. laevis* were mainly composed of cyanobacteria (*Nostoc* spp.), whereas on the leaves of *G. cauliflora* γ-proteobacteria were dominant in addition to cyanobacteria. The complexity of diazotrophic communities on leaves was not correlated with N_2 fixation activity. 16S rRNA gene sequence analysis suggested the presence of complex microbial communities in association with leaves, however, cyanobacteria showed only low abundance. Our findings suggest that cyanobacteria as well as γ-proteobacteria associated with leaf-colonizing epiphytes may provide significant nitrogen input into this rainforest ecosystem.

The ISME Journal (2008) **2,** 561–570; doi:10.1038/ismej.2008.14; published online 14 February 2008
Subject Category: microbial ecology and functional diversity of natural habitats
Keywords: phyllosphere; cyanobacteria; *nifH*; epiphytes; N_2 fixation; tropical rainforest

Introduction

Nitrogen (N) enters ecosystems via atmospheric deposition or biological nitrogen fixation (BNF). The latter pathway is mediated by prokaryotes, so-called diazotrophs. Various bacteria belonging to very different phylogenetic groups share the ability to reduce atmospheric N_2 to ammonium via the enzyme nitrogenase. N_2-fixing bacteria are found in diverse habitats, however, in undisturbed terrestrial systems such as tropical rainforests BNF is considered particularly important for the maintenance of ecosystem N pools (Vitousek *et al.*, 2002). Besides the provision of N_2, plant-associated bacteria are important for supporting growth, health and stress resistance of plants (Lugtenberg *et al.*, 1991).

Biological N_2 fixation in leguminous plants, through a symbiotic relationship with rhizobia, is a highly efficient and well-known system. Recently, Pons *et al.* (2007) found that symbiotic N_2 fixation plays an important role in maintaining high amounts of soil-available N in a tropical rainforest in Guyana. Apart from this symbiotic interaction, nonleguminous plants can harbor endophytic N_2 fixers, as it was shown for agricultural plants (Barraquio *et al.*, 1997; Elbeltagy *et al.*, 2001; Reiter *et al.*, 2003; Knauth *et al.*, 2005).

The leaf surface (phyllosphere) may be colonized by a range of different bacteria and fungi (Lindow and Brandl, 2003). Owing to high humidity and temperature phyllospheres of tropical rainforest plants typically differ from plant species in temperate to boreal ecosystems. They host other, so-called epiphytes consisting to a large extent of algae, bryophytes (mosses, liverworts), lichens, fungi and

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Received 8 September 2007; revised 8 January 2008; accepted 14 January 2008; published online 14 February 2008

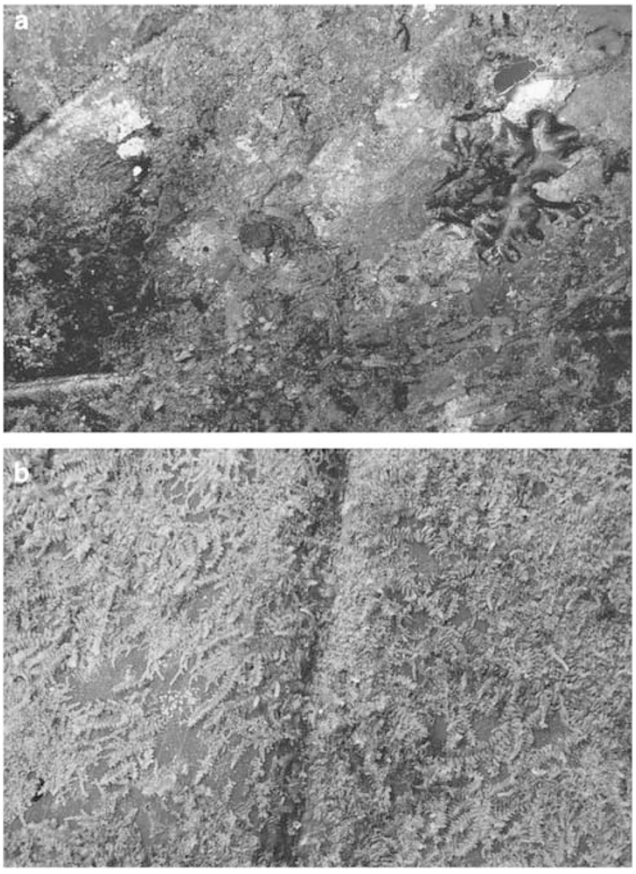

Figure 1 Epiphytic colonization of *P. wendlandii* and *C. laevis* in the Esquinas forest, Costa Rica. (**a**) Senescent leaf (2.5-year old) of *P. wendlandii* densely covered by green algae, fungi, bryophytes and a cyanolichen. (**b**) Mature leaf (1-year old) of *C. laevis* showing intense colonization by bryophytes covering >90% of the leaf surface.

protozoa (Ruinen, 1961). Tropical plant leaves can be very densely colonized by these organisms (Figure 1) and it can be assumed that many microbes are associated with epiphytes rather than with the host plant. Bacterial communities on leaves are limited by nutrient availability and it is known that mainly simple sugars, which leach from the interior of plants, are the available C sources (Lindow and Brandl, 2003). The phyllosphere is due to large fluctuations in physical conditions considered as a stressful environment for the associated microflora (Hirano and Upper, 2000). Phyllosphere bacteria may be involved in the N cycle (for example, Jones, 1970; Murty, 1984; Papen *et al.*, 2002) and bacteria-colonizing leaves of tropical rainforest plants have been shown to fix N_2 (Sengupta *et al.*, 1981; Bentley, 1987; Fritz-Sheridan and Portécop, 1987; Carpenter, 1992; Freiberg, 1998).

The diversity of microbial communities and diazotrophs in the phyllosphere has been studied by characterization of isolates (Bentley, 1987; Fritz-Sheridan and Portécop, 1987; Carpenter, 1992; Freiberg, 1998; Yang *et al.*, 2001; Albino *et al.*, 2006) as well as by cultivation-independent methods (Yang *et al.*, 2001; Albino *et al.*, 2006; Soares *et al.*, 2006). The latter approach targets also the unculturable majority of microorganisms and is usually based on the analysis of the 16S rRNA gene or the *nifH* gene encoding nitrogenase reductase.

Although some studies indicated that N_2 may be fixed by phyllosphere bacteria (Bentley, 1987; Fritz-Sheridan and Portécop, 1987; Carpenter, 1992; Freiberg, 1998) and some of the corresponding putative N_2-fixing bacteria have been identified (Bentley, 1987; Fritz-Sheridan and Portécop, 1987; Carpenter, 1992; Freiberg, 1998), a thorough knowledge of the identity and ecology of diazotrophs-colonizing leaves of tropical rainforest plants is still lacking. Additionally, the capacity of phyllosphere communities to fix atmospheric N_2 is not well known. The present study therefore aimed at gaining an insight into the N_2 fixation activity on the leaves of various representative plant species of a tropical lowland forest in Costa Rica and to identify the microorganisms potentially involved in this process. Biological N_2 fixation rates were measured by ^{15}N techniques and plants showing high activity were selected for molecular analysis. Community composition of N_2 fixers and the identity of bacteria-colonizing leaves of these plants were analyzed by cultivation-independent analysis based on the *nifH* and the 16S rRNA genes.

Materials and methods

Field site description and sampling

This study was conducted in the Esquinas forest, Parque Nacional Piedras Blancas, located close to the Golfo Dulce on the Southern Pacific coast of Costa Rica (8°42′46″ N, 83°12′90″ W). The study area is classified as tropical wet forest (Holdridge, 1967) with elevations ranging from 0 to 597 m above sea level. Annual rainfall averages 6000 mm and mean annual temperature is 27.4 °C at the Research Station. Leaf samples were collected in September 2005 when mean monthly rainfall was 700 mm and mean monthly temperature was 27.8 °C. Plants were collected at three forest sites that showed different relative air humidity levels. Sites included a ridge forest (rd) that was assigned to be the driest site of the observation area, a slope forest (s) and a humid ravine forest (rv).

Mixed samples of fully developed but not senescent leaves of each of the following species were collected at the given site(s): *Carludovica drudei* (giant herb, Cyclanthaceae; rv), *Costus laevis* (herb, Costaceae; rd, rv), *Dieffenbachia* sp. (herb, Araceae; rv), *Iriartea deltoidea* (tree-palm, Arecaceae; s), *Grias cauliflora* (canopy tree, Lecythidaceae; rd, rv), *Miconia schlimii* (shrub or treelet, Melastomataceae; rd), *Pentagonia wendlandii* (shrub or treelet, Rubiaceae; rv), *Psychotria elata* (shrub or treelet, subcanopy tree, Rubiaceae; rd), *Psychotria* sp. (shrub or treelet, Rubiaceae; rv), *Tetrathylacium*

macrophyllum (subcanopy tree, Flacourtiaceae; rv), *Vismia macrophylla* (canopy tree, Clusiaceae; rd), *Vismia* sp. (canopy tree, Clusiaceae; rd) and *Zamia fairchildiana* (treelet, Zamiaceae; rd).

^{15}N incorporation experiments

Incorporation of the stable isotope ^{15}N via leaf-associated diazotrophs was assessed by incubation of leaf samples in an artificial $^{15}N_2$:O_2 atmosphere (80:20%, v:v; $^{15}N_2$ at 98 at% ^{15}N, CK Gas Products, Hampshire, UK) in headspace vials (6 ml, butyl rubber septa) for 24 h. Prior to incubation, the leaf samples were cut and leaf areas of these samples, as well as the areas from which epiphytes were scraped off, were measured. Untreated samples were taken to determine the natural abundance of ^{15}N in corresponding leaf and epiphyte samples.

Incubations were conducted with samples of

1. Leaves with epiphytes of all test plants; ambient light conditions.
2. Leaves of *G. cauliflora* (rv), *P. wendlandii* (rv), *C. laevis* (rv) and *C. drudei* (rv) from which epiphytes were scraped off; ambient light conditions.
3. Epiphytes, which were scraped off (see above); ambient light conditions.
4. Epiphyte-covered leaves of *G. cauliflora* (rv) and *C. laevis* (rv); dark conditions.

After incubation, plant samples were dried at 70 °C for two days, weighed and transported to the University of Vienna. Samples were finely ground in a ball mill (MM2000; Retsch GmbH & Co. KG, Haan, Germany) and aliquots of 1–2 mg dry weight (d.w.) were weighed in tin capsules. N_2 concentrations and ^{15}N abundances (at% ^{15}N) were determined with a continuous-flow isotope ratio mass spectrometer (Delta; Finnigan MAT, Bremen, Germany), linked to an elemental analyzer (EA1110; CE Instruments, Milano, Italy).

Nitrogen fixation rates (ímol N_2 per g d.w. per day) were calculated as follows

$$N_2\text{-Fix} = N_{leaf} \times (\text{at\%}\,^{15}N_{sample} - \text{at\%}\,^{15}N_{control}) / 100 \times 10^3 / M_r / t$$

where N_{leaf} represents the foliar N concentration (mg N per g d.w.), M_r the molecular weight of $^{15}N_2$ and t time of incubation (days). Leaf area-based N_2 fixation rates were calculated by multiplying with the respective specific leaf weight (g d.w. per m^2 leaf area) of the sample. ^{15}N incorporation experiments were performed in triplicates by applying plant material from three different leaves from one plant individual.

Molecular analysis

Treatment of the leaf samples and DNA isolation. At the ravine forest site leaves of *C. drudei* and *G. cauliflora*, which showed high N_2 fixation rates, as well as leaves of *C. laevis* (rv), which exhibited low rates of N_2 fixation were investigated. Leaves, which were analyzed by ^{15}N incorporation experiments, were also used for the analysis of the leaf-associated bacterial microflora.

Epiphyte-laden leaves of the respective plants were put in a plastic bag containing 1 × phosphate-buffered saline (PBS) (0.8% NaCl, 0.02% KH_2PO_4, 0.115% Na_2HPO_4, 0.02% KCL, pH 7.4) and were sonicated for 5 min. Afterward, the leaves were carefully removed and the remaining content of the bag was filtered with a GF/C-grade glass fiber filter with 1.2 μm particle retention size (1.2 μm filters) and additionally with a GF/F-grade glass fiber filter with 0.7 μm particle retention size (0.7 μm filters), to separate larger bryophyte cells with associated cyanobacteria, as well as larger cyanobacterial cells and cell clusters, from those of smaller-sized bacteria.

For bacterial DNA isolation, filters (total filter mass 0.2–0.6 g) as well as epiphyte-covered leaves (0.3 g fresh weight) of the respective samples were put into 2 ml cryotubes or 50 ml centrifuge tubes, respectively, amended with 1 × PBS buffer and 5% phenol in ethanol in a ratio of 1:1 and stored at −20 °C. Samples were amended with approximately 0.7 ml TN150 (10 mM Tris-HCl, pH 8.0, 150 mM NaCl), frozen in liquid N and homogenized in a mixer mill (M2, Retsch GmbH & Co) with two sterile steel beads (5 mm in diameter). DNA was isolated from the homogenized plant material by using the Ultra Clean Soil DNA Kit (MoBioLab, CA, USA) as specified by the manufacturer.

PCR analysis of nifH *and 16S rRNA genes.* PCR amplification of *nifH* genes was performed using a nested PCR protocol described by Yeager *et al.* (2004), except that an undiluted PCR product was used as a template for the second PCR. 16S rRNA gene sequences of phyllosphere bacteria were amplified as described previously (Rasche *et al.*, 2006) using the primers 8f (Weisburg *et al.*, 1991) and 1520r (Edwards *et al.*, 1989).

nifH *terminal-restriction fragment length polymorphism analysis.* Fluorescently labeled *nifH* gene fragments were amplified (as described above) with DNA extracted from epiphyte-covered leaves of *C. drudei*, *C. laevis* and *G. cauliflora* as well as from epiphyte-associated bacteria that were trapped on filters, using a 6-carboxyfluorescein-labeled *nifH*11 primer in the second reaction of the nested PCR amplification. As usually a second band of low intensity appeared, PCR products were loaded on a 1% agarose gel and the bands that corresponded to the correct PCR product size were excised and purified using the QIAquick Gel Extraction Kit (Qiagen, Morgan Irvine, CA, USA). Fluorescently labeled PCR products (200 ng) were digested with *Msp*I (Promega, Madison, WI, USA) at 37 °C for 4 h.

Digests were purified on Sephadex G-50 Medium columns (GE Healthcare Biosciences, Waukesha, WI, USA). Aliquots of 10 µl were mixed with 15 µl HiDi formamide (Applied Biosystems, Foster City, CA, USA) and 0.3 µl of the internal DNA fragment length standard (Rox 500, Applied Biosystems). Prior to electrophoresis, mixtures were denaturated for 10 min at 95 °C and chilled on ice.

Fluorescently labeled terminal restriction fragments (T-RFs) were detected using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) equipped with POP-6 polymer in a 50 cm capillary array (both Applied Biosystems) under the following conditions: 60 s injection time, 5 kV injection voltage and 4000 s run time. T-RFs between 35 and 500 bp long were included in the analysis. T-RFs were determined by comparison with the internal standard using the GeneScan software package (version 3.7, Applied Biosystems). Community profiles were normalized according to the method of Zaid *et al.* (2006).

nifH *and 16S rRNA gene libraries.* Amplified *nifH* genes were cloned using the StrataClone PCR Cloning Kit and StrataClone Solo PackR Competent Cells (Stratagene, La Jolla, CA, USA). Clones were picked and suspended directly in reaction mixtures, containing 1 × reaction buffer (Invitrogen Inc., CA, USA), 200 µM (each) deoxynucleoside triphosphates, 3 mM $MgCl_2$, 0.15 µM of primers M13f and M13r and 2 U of Taq DNA Polymerase (Invitrogen) in a final reaction volume of 50 µl. The reaction cycle started with an initial denaturation at 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 53 °C for 1 min, 72 °C for 2 min and a 10-min extension step at 72 °C. M13 PCR products were purified with Sephadex columns (as described for *nifH* terminal-restriction fragment length polymorphism, T-RFLP analysis). For cloning of bacterial 16S rRNA genes, amplicons obtained from filters were pooled. The working steps were as described above for *nifH* gene libraries.

DNA sequencing and phylogenetic analysis. *nifH* gene sequencing was performed with primer nifH22 (5′-A(AGT)(AT)GCCATCAT(CT)TC(AG)CC-3′) (Yeager *et al.*, 2004), whereas partial 16S rDNA sequencing was conducted with primer 518r (5′-ATTACC GCGGCTGCTGG-3′) (Muyzer and De Waal, 1993), performing the dideoxy chain termination method (Sanger *et al.*, 1977), using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Partial 16S rRNA gene sequences were submitted to the Ribosomal Database Project-II Check Chimera program (Maidak *et al.*, 1999) to detect chimeric sequences. Sequences were subjected to BLAST analysis (Altschul *et al.*, 1997) with the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov). Theoretical T-RF (theor. T-RF) sizes of cloned *nifH* sequences were determined after sequencing.

Phylogenetic analysis of *nifH* sequences was performed using the ARB software package (Ludwig *et al.*, 2004). *nifH* sequences from this study and close reference sequences obtained from the NCBI database by BLAST analysis (Altschul *et al.*, 1997) were used to create two separate ARB databases, for cyanobacteria and non-cyanobacteria. Sequences were automatically aligned, and then corrected manually. Phylogenetic trees of reference sequences (> 300 bp) were calculated by the neighbor-joining method (500 bootstraps) using Felsenstein (1993) and Jukes and Cantor (1969) correction for non-cyanobacteria and cyanobacteria-related sequences, respectively. Shorter sequences were subsequently added to the trees using the maximum parsimony method, without changing the overall tree topology.

Nucleotide sequence accession numbers
Obtained *nifH* gene sequences were deposited in GenBank under protein-id numbers ABR24280–24416 and accession numbers EF547940–548013 for 16S rRNA gene sequences.

Results

^{15}N incubation experiments
N_2 fixation rates of epiphyte-covered leaves under ambient light were highly dependent on host plant species (P-value < 0.001; F-ratio = 20.69; d.f. = 12), assessed by one-way analysis of variance (ANOVA). Highest N_2 fixation rates were associated with the phyllospheres of *G. cauliflora*, *P. wendlandii* and *C. drudei* growing at the ravine forest (rv) site (Figure 2). Lower N_2 fixation activities were found on the leaves of *T. macrophyllum*, *I. deltoidea* and *C. laevis* (rv). N_2 fixation was mainly restricted to the surface of the leaf (Figure 3) as ^{15}N incorporation by epiphyte-covered leaves of *C. drudei*, *G. cauliflora* and *P. wendlandii* from the ravine site was significantly higher compared to the leaves

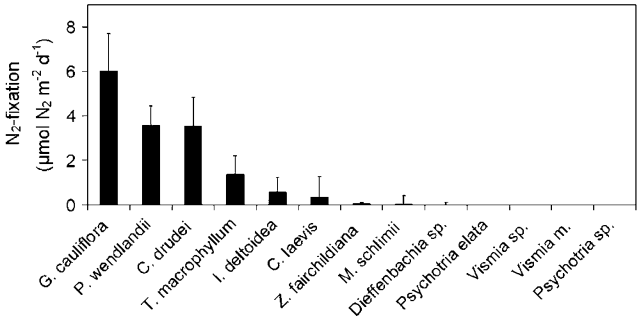

Figure 2 N_2 fixation rates associated with leaves of representative rainforest plants, Piedras Blancas National Park, Costa Rica. Bars represent the means ± 1 s.d. of three replicates. Different letters signify significant differences between means (multiple range test, least significant difference, $P < 0.05$).

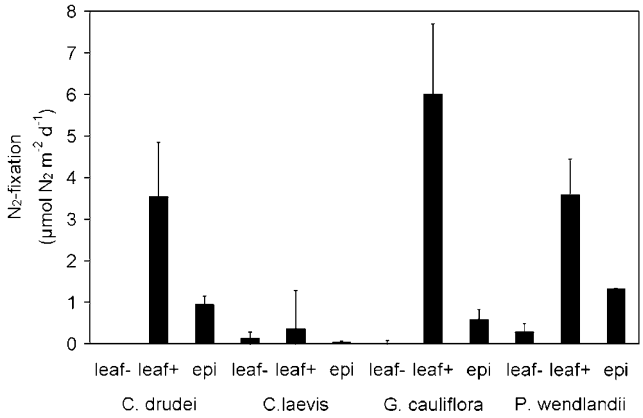

Figure 3 N_2 fixation rates associated with cleaned leaf surfaces (leaf−), epiphyte-covered leaves (leaf+) and epiphyte isolates (epi) of four different plant species grown at the ravine forest site, Piedras Blancas National Park, Costa Rica. Bars represent the mean ± 1 s.d. of three replicates. Multifactor analysis of variance (ANOVA) showed highly significant influences by the factors host plant species (F-ratio = 12.83, *P*-value <0.001, d.f. = 3), leaf fractions (leaf+, epi and leaf−) (F-ratio = 74.72, *P*-value <0.001, d.f. = 2) and the interaction between both factors (F-ratio = 9.70, *P*-value <0.001) on N_2 fixation.

cleaned from their epiphytes (*P*-value <0.001; F-ratio = 74.72; d.f. = 2). N_2 fixation rates associated with epiphytes were also significantly higher in comparison to cleaned leaves (*P*-value <0.001; F-ratio = 74.72; d.f. = 2). For *G. cauliflora*, N_2 fixation of leaves was clearly influenced by plant growth site as N_2 fixation associated with the leaves collected from the ravine forest was significantly higher than on the leaves of a corresponding plant from the ridge forest (*P*-value <0.001; F-ratio = 32.07; d.f. = 1). A similar trend was observed with *C. laevis*, however, plants grown at both sites showed low fixation rates. Although no significant differences were found between N_2 fixation under light and dark conditions, a tendency of higher fixation rates under light conditions was observed (data not shown).

nifH *T-RFLP analysis*

nifH fingerprints of three fractions, epiphyte-covered leaves as well as from bacteria collected on 1.2 and 0.7 µm filters, were obtained (Figure 4). Both filters showed similar bacterial community profiles. Single profiles contained 12–18 peaks each. T-RFs of 115 and 162 bp were highly abundant in at least one fraction deriving from the different host plants. In addition, clear differences in peak heights of T-RFs were detected. The T-RF of 73 bp showed high abundance in the profile originating from filtered epiphytes from *C. drudei*, whereas a dominant peak at 114 bp was found only in the profile of the epiphyte-laden leaves of *C. laevis*. Notable peaks at 63 and 147 bp were found only in profiles derived from *G. cauliflora*. In many cases T-RFs showed different abundances in different fractions from the same host plant species.

nifH *sequence analysis and identification of diazotrophs*

The predominant diazotrophic colonizers of phyllospheres of *C. drudei* and *C. laevis* were highly related to cyanobacteria, as most of the obtained sequences showed highest homologies to cyanobacterial sequences. Most sequences showed highest homologies to sequences derived from *Nostoc* spp., *Tolypothrix distorta*, *Fischerella* spp. and uncultured, yet unidentified cyanobacteria that live in association with mosses as well as in soils and rice plants (Supplementary Table S1).

On the leaves of *G. cauliflora*, a high number of γ-proteobacteria were found. More than 50% of sequences showed highest similarity to the genus *Klebsiella*, but also a high number of cyanobacteria (*Tolypothrix* sp.) were detected on that host plant (Supplementary Table S1).

Theor. T-RFs were assessed after sequencing and corresponding peaks in T-RFLP profiles were identified (Figure 4; Supplementary Table S1). Theor. T-RFs of cyanobacterial sequences originating from *C. drudei*, *C. laevis* and *G. cauliflora* could be assigned to peaks with relative high signal intensities at 115 bp that confirmed the high abundance of cyanobacteria in diazotrophic phyllosphere communities associated with all three host plants. In profiles of *C. drudei* and *C. laevis*, an additional, cyanobacterial T-RF of 162 bp was identified underlying the predominance of cyanobacteria. A high number of theor. T-RFs from γ-proteobacterial diazotrophs detected in the phyllosphere of *G. cauliflora* were found in the corresponding T-RFLP profiles.

Phylogenetic analysis of obtained nifH *sequences*

Phylogenetic affiliation of diazotrophic bacteria associated with the leaves of tropical rainforest plants was assessed through construction of phylogenetic trees based on *nifH* nucleotide sequences from this study as well as reference sequences. Two trees were constructed, consisting sequences derived from cyanobacteria (Figure 5) and non-cyanobacteria (Figure 6). The majority of cyanobacterial sequences were clustered with sequences from the genera *Nostoc* and *Tolypothrix*. Several sequences were clustered together in distinct lineages, supported by high bootstrap values (Figure 5). All cyanobacterial *nifH* sequences from *G. cauliflora* were clustered together, whereas diazotrophic cyanobacteria associated with other plants showed greater diversity. The majority of non-cyanobacterial sequences formed separate branches within γ-proteobacterial sequences and were only distantly related with *nifH* genes of known cultured bacteria (Figure 6).

16S rRNA gene sequence analysis

Highly dominant leaf-associated bacteria were identified by partial 16S rRNA gene analysis. Sequences were carefully checked for chimeric sequences, which were excluded from further analysis. Only few

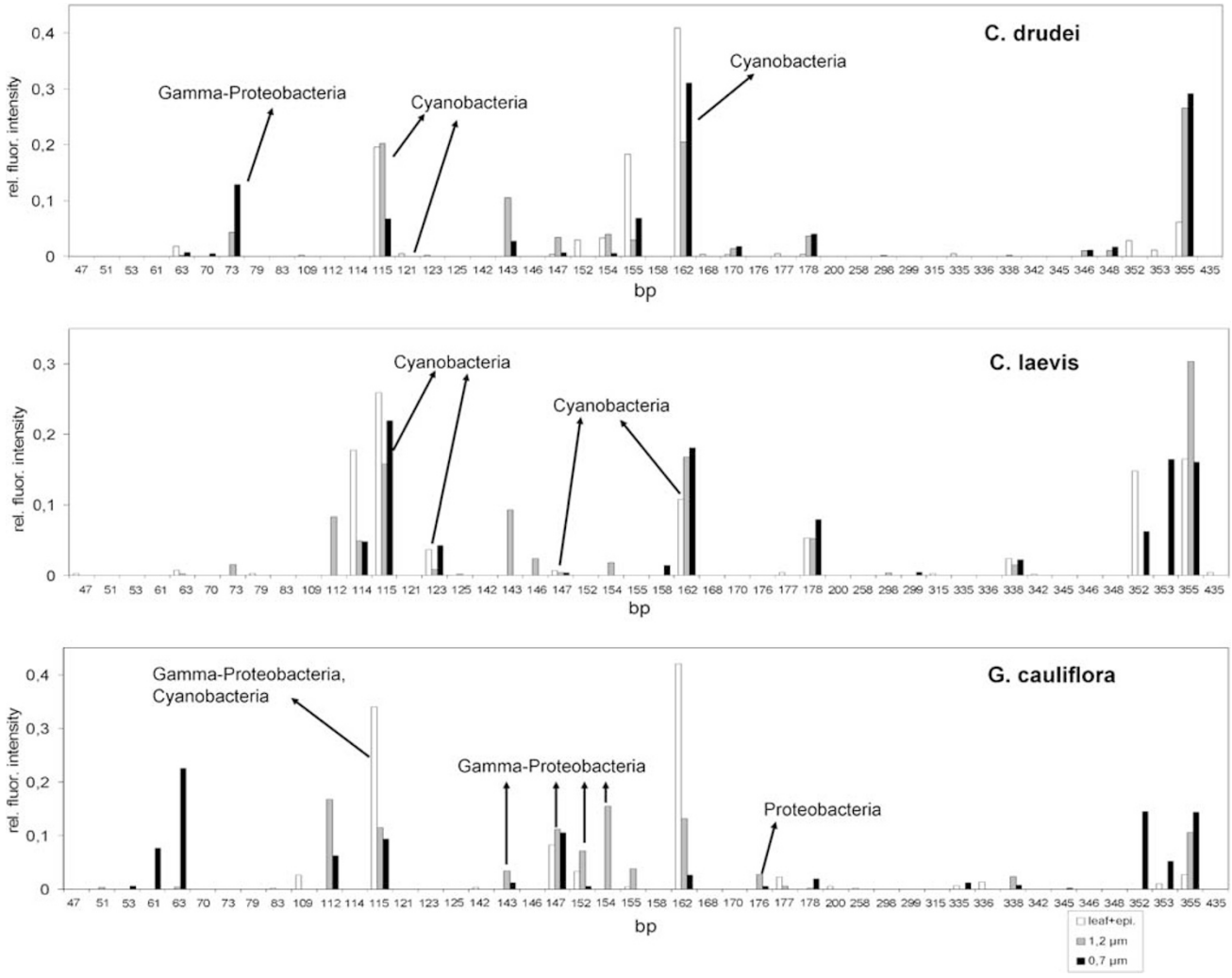

Figure 4 Normalized *nifH* terminal-restriction fragment length polymorphism (T-RFLP) profiles derived from phyllosphere fractions of three host plant species, including a tentative taxonomic affiliation of terminal restriction fragments (T-RFs) on the basis of sequencing of *nifH* genes.

sequences (9%) were derived from plant organelles. Sequences showed 91%–100% homology to NCBI database entries and many of the closest relatives were uncultured bacteria. Most of partial 16S rRNA sequences from bacteria associated with phyllospheres of *C. drudei* and *C. laevis* showed highest similarities to α-proteobacteria, whereas γ-proteobacteria were predominant on the leaf surface of *G. cauliflora* (Supplementary Table S2). Beside representatives of these phylogenetic classes, β-proteobacteria, low G+C Gram positives, high G+C Gram positives, Acidobacteria, Flavobacteria and Verrucomicrobia were identified (Supplementary Table S2). In contrast to *nifH* gene libraries, cyanobacterial sequences were only found in very low abundance.

Discussion

In our study, 13 plant species, corresponding to a wide range of life strategies, of a lowland tropical rainforest in Costa Rica were screened for their leaf-associated N_2 fixation activity. Three plant species, *G. cauliflora*, *P. wendlandii* and *C. drudei*, were found to fix up to 6 µmol N_2 per m^2 per day, whereas other plants showed rather low or no N_2 fixation. These findings are in agreement with a previous study performed in a lowland rainforest at the Caribbean coast of Costa Rica (Bentley, 1987).

In rainforest plants N_2 fixation was reported to occur in the phyllosphere (Ruinen, 1975; Bentley, 1987; Fritz-Sheridan and Portécop, 1987; Carpenter, 1992; Freiberg, 1998), whereas several other plants have been shown to host endophytic diazotrophs (Hurek *et al.*, 2002; Reiter *et al.*, 2003; Knauth *et al.*, 2005). Our results showed that N_2 fixation predominantly takes place on leaves and is mostly mediated by microorganisms associated with epiphytes. Accordingly, bryophytes were typically found on the investigated plants exhibiting high N_2 fixation activities. In our study, no N_2 fixation activity was observed in plants growing on the ridge,

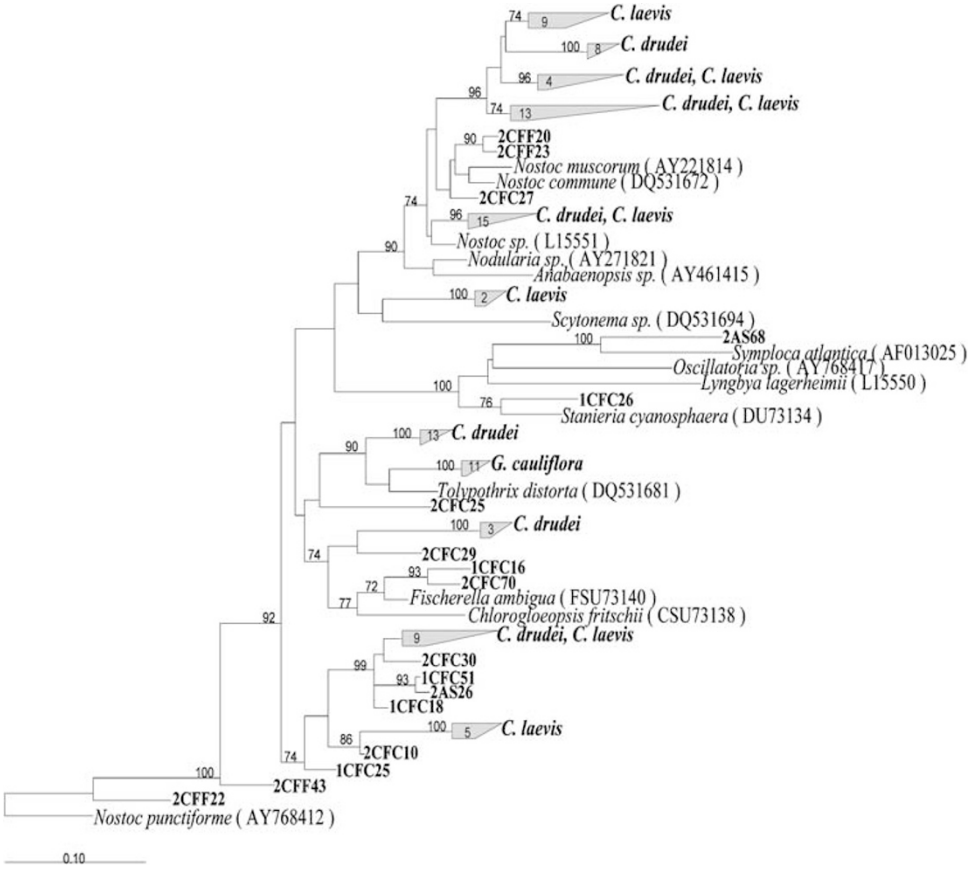

Figure 5 Phylogenetic tree of *nifH* sequences (>205 bp), including cyanobacterial sequences obtained in this study and most closely related *nifH* genes of cultured cyanobacteria. Bootstrap values greater than 70% are shown. *nifH* sequences obtained in this study are shown in bold: grouped sequences are labeled with the names of the plant species on whose leaves they were detected, whereas single sequences are labeled with names of corresponding clones presented in Supplementary Table S1.

whereas high (*G. cauliflora*) or low N_2 fixation rates (*C. laevis*) were found in plants growing in the ravine. This might be explained by the different abundances of bryophytes, which were mainly found on the leaves of plants growing at sites characterized by high humidity such as the ravine forest. Due to the high water storage capacity of bryophytes (Hölscher *et al.*, 2004), leaf surfaces remain wetted for a longer period after rain events. Moreover, desiccated bryophytes leach considerable amounts of organic compounds such as sugars after rewetting (Coxson *et al.*, 1992). Therefore, bacteria-colonizing leaf surfaces probably find appropriate conditions for survival and metabolic activity in association with bryophytes. A correlation between bryophyte biomass and N_2 fixation was also reported by Bentley (1987), and the formation of a dense epiphyte layer may therefore be a prerequisite for high N_2 fixation rates on the leaves of tropical rainforest plants.

Nitrogenase activity on the leaves of trees growing in rainforests of Costa Rica was attributed to the presence of filamentous as well as coccoid cyanobacteria (Carpenter, 1992). In our study, N_2-fixing activity did not differ significantly between leaves incubated under light and dark conditions, however, a trend toward higher N_2 fixation under light conditions was observed. This indicated that cyanobacteria consist an important fraction of the diazotrophic community, as they incorporate N_2 during photosynthesis (Wolk, 1973). Nevertheless, it could be that also γ-proteobacteria associated with *G. cauliflora* (see below) respond to light conditions as recently higher transcript levels of the *nifH* gene of *Herbaspirillum* were observed under light conditions (Mu *et al.*, 2005).

Diazotrophic microbial communities associated with plant species characterized by high (*G. cauliflora* and *C. drudei*) and by low (*C. laevis*) N_2-fixation activity were investigated by molecular means. In general, most diazotrophic bacteria, which were detected in whole-leaf samples, were also found in the epiphyte fractions. However, several diazotrophs were only detected in epiphyte fractions, which might be due to the enrichment of cells on filters leading to the better detection of bacteria with low abundance. Depending on the plant species cyanobacterial and heterotrophic bacterial *nifH* gene sequences were identified, but cyanobacteria consisted the major component of diazotrophic communities. All three plant species analyzed hosted similar cyanobacterial populations,

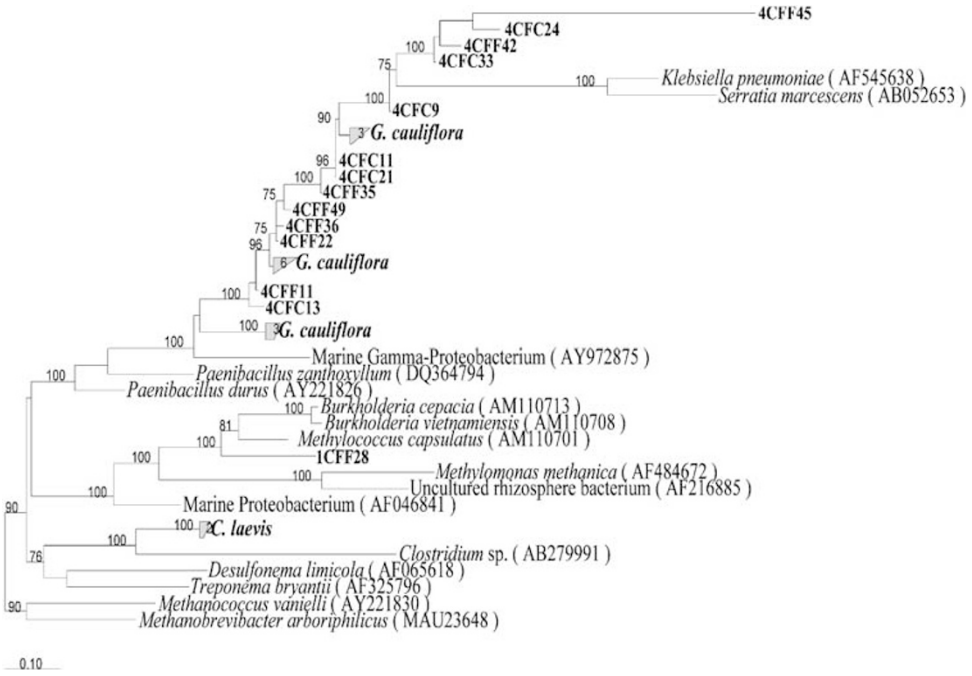

Figure 6 Phylogenetic tree of *nifH* sequences (>205 bp), including non-cyanobacterial sequences obtained in this study and most closely related *nifH* genes of cultured bacteria. Bootstrap values greater than 70% are shown. *nifH* sequences obtained in this study are shown in bold: grouped sequences are labeled with the names of the plant species on whose leaves they were detected, whereas single sequences are labeled with names of corresponding clones shown in Supplementary Table S1.

which indicates that the host plant species has very little influence on the composition of potentially N_2-fixing cyanobacterial communities. *G. cauliflora*, which showed highest N_2 fixation activities, hosted in addition to cyanobacteria a high proportion (about 60%) of bacterial N_2 fixers belonging to the phylum γ-proteobacteria. It is therefore likely that in addition to cyanobacteria other bacteria contribute to N_2 fixation processes in the phyllospheres of tropical rainforest plants.

In tropical regions, N_2-fixing cyanobacteria belonging to the genera *Scytonema*, *Oscillatoria*, *Microcoleus* and *Stigonema* have been reported to colonize the phyllosphere of forest plants (Bentley, 1987; Fritz-Sheridan and Portécop, 1987; Carpenter, 1992; Freiberg, 1998). Analysis of *nifH* sequences in our study indicated a high abundance of the genera *Nostoc*, *Fischerella* and *Tolypothrix*. The filamentous heterocystous genus *Nostoc* is well known to enter symbiosis with bryophytes (Rai *et al.*, 2000). *Fischerella* spp. as well as *Tolypothrix* form filaments and are also able to fix N_2 in heterocysts. *Fischerella* is difficult to distinguish from *Stigonema* by microscopic analysis (see http://silicasecchidisk.conncoll.edu/LucidKeys/Carolina_Key/html/Fischerella_Ecology.html), whereas *Tolypothrix* cells look very similar to *Scytonema* (see http://silicasecchidisk.conncoll.edu/LucidKeys/Carolina_Key/html/Tolypothrix_Main.html). Both *Scytonema* and *Stigonema* have been previously detected as the main cyanobacterial genera in tropical forests in Costa Rica (Carpenter, 1992; Freiberg, 1998) and might have been misidentified. The genus *Fischerella* consists thermophile members and several species have been reported to grow on mosses and tree barks in tropical forests (Asthana *et al.*, 2006a, b).

Despite generally unfavorable environmental conditions in the phyllosphere, a high diversity of bacterial species colonizing the canopy of an Atlantic tree forest was reported (Lambais *et al.*, 2006). We also detected a high number of different bacterial species on leaves, although in our study only dominant community members were analyzed. The majority of detected bacteria belonged to α- or γ-proteobacteria, however, cyanobacteria were detected in only low abundance. This strongly indicates that the potentially N_2-fixing community represents only a subset of the whole microbial community. *G. cauliflora* showed a high abundance of γ-proteobacteria, which was also the dominant phylum among diazotrophs associated with this plant. In contrast to diazotrophic communities, leaf-associated microbial communities analyzed by 16S rRNA gene analysis seem to vary greatly between host plants.

In conclusion, our results showed that N_2 fixation varies among plant species growing in the Esquinas rainforest, and that this variation might be attributed, at least in part, to environmental conditions. N_2 fixation was found to occur mostly on the leaf surfaces (not in the leaf interior) and cyanobacteria associated with epiphytes are likely to represent the key N_2-fixing bacteria in this environment. In addition, bacteria such as diazotrophic γ-proteo-

bacteria may be involved in N_2 fixation processes. Further research based on the expression of *nifH* genes will lead to the identification of actively, N_2-fixing microbial communities. Our results further indicated that cyanobacterial populations are associated and influenced by the epiphyte flora, whereas the whole leaf-associated microbial community is highly diverse and tends to vary with the host plant and environmental conditions.

Acknowledgements

Funding was provided partly by the University of Vienna through its scholarship for short-term research work in foreign countries. We thank the team of the tropical research station La Gamba in Costa Rica for providing living and work space nearby the study site in the tropical rainforest and Dr Eva Schembera for her support during plant species identification and sampling. We also thank Dr Michael Schagerl for providing us cyanobacterial cultures for testing *nifH* gene-specific primers.

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