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Annual and Seasonal Variation in the Phyllosphere Bacterial Community Associated with Leaves of the Southern Magnolia (*Magnolia grandiflora*)

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Abstract The phyllosphere contains a diverse bacterial community that can be intimately associated with the host plant; however, few studies have examined how the phyllosphere community changes over time. We sampled replicate leaves from a single magnolia (*Magnolia grandiflora*) tree in the winter of three consecutive years (2007–2009) as well as during four seasons of 1 year (2008) and used molecular techniques to examine seasonal and year-to-year variation in bacterial community structure. Multivariate analysis of denaturing gradient gel electrophoresis profiles of 16S rRNA gene fragments revealed minimal leaf to leaf variation and much greater temporal changes, with the summer (August 2008) leaf community being most distinct from the other seasons. This was confirmed by sequencing and analysis of 16S rRNA gene clone libraries generated for each sample date. All phyllosphere communities were dominated by *Alphaproteobacteria*, with a reduction in the representation of certain *Beijerinckiaceae* during the summer and a concurrent increase in the *Methylobacteriaceae* being the most significant seasonal change. Other important components of the magnolia phyllosphere included members of the *Bacteroidetes*, *Acidobacteria*, and *Actinobacteria*, with the latter two lineages also showing differences in their representation in samples collected at different times. While the leaf-associated bacterial community sampled at the same time of year in three separate years showed some similarities, generally these communities were distinct, suggesting that while there are seasonal patterns, these may not be predictable from year to year. These results suggest that seasonal differences do occur in phyllosphere communities and that broad-leafed evergreen trees such as *M. grandiflora* may present interesting systems to study these changes in the context of changing environmental conditions.

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Introduction

The leaf surface or phyllosphere represents a vast area with abundant bacterial populations [1, 29]. While culture-based studies of phyllosphere bacteria have been an important part of microbiology for years, culture-independent analysis of the phyllosphere is still in its infancy, especially for non-agricultural plants. Over the last few years, molecular surveys have revealed that the leaves of various plants harbor diverse bacterial communities [22, 24, 39], and both molecular and enrichment approaches have shown that these phyllosphere communities vary both spatially [27, 34] and temporally [7, 13, 23]. These studies have shown that phyllosphere communities are more complex than previously understood, but many fundamental questions in their spatial and temporal variability have yet to be addressed [38].

One example of a basic question that has rarely been addressed for the phyllosphere is whether there are seasonal or annual patterns in its community structure. Culture-based studies suggest that there may be consistent seasonal changes in bacterial populations [14, 26, 37]; however, only one study [36] has used molecular approaches to examine seasonal patterns. That study focused on successional changes in the phyllosphere of cottonwood (*Populus deltoides*) leaves over the deciduous growing season from leaf emergence to leaf fall. While temporal variability was high, phyllosphere bacterial communities separated into

early-, mid-, and late-season groups, with mid-season (August) communities being different from those observed early or late in the growing season [36]. Evergreen trees may represent a different environment in that leaves are present year-round so that temporal variation in phyllosphere communities may not be as closely tied to successional changes with leaf development. Evergreen trees also allow for the assessment of leaf-associated bacterial populations outside of a typical growing season.

In this study, we sought to characterize the nature of the bacterial community in the phyllosphere of a broad-leafed evergreen tree using 16S ribosomal ribonucleic acid (rRNA) techniques. More importantly, we attempted to determine if this community shows the same composition at different times of the year (seasonal patterns) and at the same time of the year over three different years (annual patterns).

Materials and Methods

Sample Collection

Leaves were obtained from a small (approximately 5 m tall) magnolia (*Magnolia grandiflora*) tree located in the understory of Bailey Woods, a 20-ha tract of mature woods near Oxford, MS, USA, that is the remains of old-growth forest in the area. The woods are primarily deciduous with magnolia accounting for 1% or less of the total trees present [4]. Because the focus of this study was on temporal changes, to minimize environmental variation on each sample date, four leaves were collected from different branches on the same tree at the same height (2 m) that were within 0.5 m of each other. Leaves were individually placed within sterile sample bags and returned to the laboratory for immediate processing (within 30 min of collection time). Because rainfall has been shown to temporarily influence the composition and activity of phyllosphere communities [22], all collection dates occurred following a period of dry weather for at least 5 days to minimize this source of variation. Collection dates (and mean high/low temperatures over the preceding 5-day period) were February 16, 2007 (8/−3°C), February 28, 2008 (9/−3°C), May 30, 2008 (26/20°C), August 29, 2008 (31/21°C), December 15, 2008 (10/2°C), and March 4, 2009 (5/−5°C). Sampling times were chosen to represent four dates, approximately 3 months apart, during 1 year (seasonal or monthly variation), with one of those times (February) being sampled on three consecutive years (annual variation). February (winter) was chosen for repeated year-to-year sampling because at this time of the year the surrounding deciduous trees are bare so that the magnolia tree itself should be the only source of any phyllosphere populations. The 2009 sampling was scheduled for late February but was delayed until March 4 because of precipitation. For convenience, sample dates are subsequently referred to as Feb07, Feb08, May08, Aug08, Dec08, and Feb09, and replicate leaves on each date designated a–d.

Sample Processing and DNA Extraction

Upon return to the lab, each individual leaf was aseptically cut into smaller pieces and transferred to a sterile 50-ml centrifuge tube containing 13.5 ml of a high salt DNA extraction buffer [40]. Tubes were frozen until subsequent DNA extraction. DNA was extracted from the leaves following a modified protocol of the high salt/lysozyme/proteinase K method of Zhou et al. [40], as described by Jackson et al. [22] for the analysis of leaf-associated bacterial communities. Briefly, samples were thawed, amended with lysozyme (to 15 $mg l^{-1}$), and shaken for 30 min at 37°C. Proteinase K (100 µl at 10 $mg ml^{-1}$) was added to each sample, which was shaken for a further 30 min (37°C). Samples received 1.5 ml of 20% SDS solution and were incubated at 65°C for 2 h, with gentle inversions every 20 min, and then centrifuged (6,000×*g*, 10 min) and the supernatant collected and transferred to a new tube. Samples were cleaned using chloroform extraction and the DNA precipitated overnight in isopropanol, washed with 70% ethanol, and resuspended in TE buffer.

DNA Amplification

DNA from each of the 24 samples (four replicate leaves taken on each of six sample dates) was used as the template in two sets of PCR amplifications for portions of the bacterial 16S rRNA gene. One set of amplifications amplified an approximately 322-bp region of the 16S rRNA gene for subsequent analysis by denaturing gradient gel electrophoresis (DGGE) using Bac1070f and Univ1392GC primers [15, 20]. The second set of amplifications used Bac8f and Univ1492r primers [20, 22] to amplify a larger portion of the 16S rRNA gene for subsequent cloning and sequencing. Amplification conditions for both sets of reactions were as described previously [20], and each reaction contained approximately 25 ng of template DNA. Essentially we used these amplifications to follow an experimental approach that we have previously used in the analysis of bacterial communities in sediments [18, 21], using DGGE to initially determine similarity in community structure between replicate samples (in this case, leaves sampled on the same date), followed by a more in-depth analysis of selected samples by 16S rRNA gene sequencing.

Denaturing Gradient Gel Electrophoresis and Analysis

Products (approximately 700 ng DNA) of the Bac1070f-Univ1392GC amplifications were analyzed by DGGE using a

C.B.S. Scientific 2001 System (C.B.S. Scientific, Solana Beach, CA, USA). Electrophoresis conditions essentially followed those previously described [18, 19, 21] using 40–70% gradients of a urea–formamide denaturant in 8% acrylamide gels. Gels were electrophoresed at 85 V for 18 h at 60°C. Because of the number and timing of samples, multiple gels were used. Initially, PCR products from leaf samples taken on the same sample date were analyzed in adjacent lanes in order to quickly compare replicate leaves prior to the more in-depth cloning/sequencing analysis. However, for actual analysis of community similarity across all samples, two DGGE gels were ran after the completion of all sampling, each containing 12 random samples (i.e., there should be no bias due to minor differences in run conditions either between or within gels). Following electrophoresis, gels were stained with SYBR Green I and visualized by UV transillumination using a Kodak Gel Logic 200 system running Molecular Imaging Software 4.0 (Eastman Kodak, Rochester, NY, USA). Gel patterns were converted to binary data (presence or absence of particular bands) and similarity (Jaccard similarity) between samples visualized by unweighted pair group method with arithmetic mean (UPGMA) cluster analysis and non-metric multidimensional scaling (MDS) using Gingko (Department of Vegetal Biology, University of Barcelona) and following procedures described previously [18].

Sequence Analysis and Community Comparisons

Based on results from DGGE analysis, one representative phyllosphere sample from each date (Feb07b, Feb08b, May08d, Aug08c, Dec08b, and Feb09a) was selected for cloning and sequencing. For these samples, the PCR products of the Bac8f and Univ1492r primers were used to generate six clone libraries (TA TOPO Cloning, Invitrogen, Carlsbad, CA, USA), and approximately 600 bp of the insert in 96 clones randomly chosen from each library was sequenced. The partial 16S rRNA gene sequences obtained were aligned to the Greengenes database using NAST [9, 10] and subsequently classified. Thirty-five of the 576 sequences were eliminated as erroneous and a further 78 eliminated as they represented chloroplasts from the host plant. Clones from a particular sample that contained the same 16S rRNA gene insert were grouped together, and the frequency of each ribotype used to estimate overall diversity in each library as S_{Chao1} [5] using a web-based interface [25].

Aligned bacterial sequences were imported into ARB [32] and added into an existing phylogenetic tree of 8,600 16S rRNA gene sequences using the "Quick add by parsimony" function, a procedure that should minimize distortions that can arise from the analysis of partial, divergent sequences [18, 22]. Sequences in the existing tree were removed and the tree showing just the relationships between sequences obtained in this study was exported from ARB into UniFrac [30, 31], a web application that tests for difference among clone libraries based on 16S rRNA phylogenetic relationships. Overall differences between phyllosphere communities on different dates were examined using the UniFrac metric [30] as well as lineage-specific analyses.

Nucleotide Sequence Accession Numbers

The partial 16S rRNA gene sequences from this study have been deposited in GenBank and have accession numbers GU117110–GU117572.

Results

DGGE Profiles of Phyllosphere Communities

Fragments (bases 1,070–1,392) of bacterial 16S rRNA genes could be amplified from DNA extracted from each leaf sample and were analyzed on DGGE gels. Individual leaves typically showed 20–30 DGGE bands (Table 1). Analysis of DGGE profiles by both UPGMA clustering and ordination by MDS revealed that bacterial community profiles were separated by sample date, and while there was some leaf to leaf variability, this was much less than variability across different seasons or years (Fig. 1). The Aug08 phyllosphere communities were the most distinct from the others, whereas the most similar communities appeared to be those sampled in consecutive seasons in Feb08 and May08, sampling dates which could not be separated by MDS (Fig. 1b). February samples taken in different years grouped in the same general area (high

Table 1 Properties of clone libraries generated from bacterial 16S rDNA amplified from phyllosphere samples of *M. grandiflora* sampled in different months and years

Sample	Clones	Phylotypes	Coverage	Species diversity	
				S_{Chao1}	S_{DGGE}
Feb07	91	29	0.85	41	29±1.9
Feb08	64	28	0.71	67	26±6.7
May08	87	25	0.79	36	22±5.3
Aug08	88	31	0.82	48	17±1.7
Dec08	50	22	0.76	44	22±5.8
Feb09	84	31	0.80	66	20±2.2

Clones indicates the number of sequenced clones from that library that contained valid bacterial 16S rRNA gene fragments, while Phylotypes is the number of distinct phylotypes detected. Species diversity is presented as a nonparametric index determined from clone library data (S_{Chao1}), compared to mean band richness from DGGE gels (S_{DGGE}; ± standard deviation, n=4 leaves per sample date for DGGE). Coverage is the estimated proportion of predicted phylotypes actually sequenced

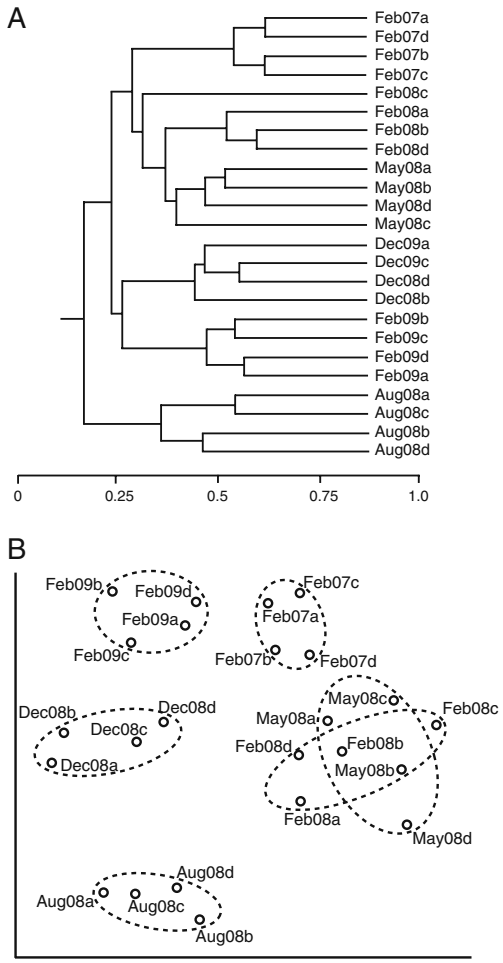

Figure 1 Multivariate plots of 16S rRNA gene DGGE profiles obtained from bacterial phyllosphere communities on individual *M. grandiflora* leaves collected on different dates from 2007–2009. *Lowercase letters a–d* indicate different individual leaves analyzed on that date (four per sample date). Analyses are UPGMA cluster analysis (**a**) and non-metric two-dimensional MDS (**b**; stress 0.05), both determined from Jaccard similarities of DGGE binary data. *Circles* in the MDS plot group individual leaves sampled on the same date

values for axes 1 and 2) when ordinated by MDS but clearly did not harbor very similar bacterial communities according to DGGE profiles (Fig. 1b).

Diversity of Clone Libraries

Larger fragments (bases 8–1,492) of bacterial 16S rRNA genes were amplified for cloning. Samples (Feb07b, Feb08b, May08d, Aug08c, Dec08b, and Feb09a) were selected based on results of the DGGE analysis and were assumed to represent a typical leaf collected on that sample date. Ninety-six clones from each of the six clone libraries were sequenced, but after the elimination of sequences corresponding to erroneous reads or plant chloroplast DNA, a total of 463 bacterial 16S rRNA gene sequences were characterized, representing from 50–91 clones from each sample (Table 1). These sequences represented 22–31 distinct phylotypes in each library (Greengenes classification, verified visually in ARB). Estimated species diversity (S_{Chao1}) from clone library composition ranged from 36 to 67, most samples being from 36 to 48 with higher values (66–67) calculated for Feb08 and Feb09 (Table 1). These values were higher than diversity estimates obtained by simply counting bands on DGGE gels, and these two methods of estimating species diversity were not correlated ($p>0.05$, $r=0.15$). Rather, S_{Chao1} was related to the number of phylotypes detected in the sequencing sample chosen from a clone library ($p<0.05$, $r=0.80$). Coverage values for the clone library sequencing effort suggested that 71–85% of the phylotypes present were detected (Table 1).

Phylogenetic Analysis

Representatives of the *Proteobacteria* were the most numerous phylotypes in clone libraries derived from leaves on each sample date, accounting for 53–80% of the 16S rRNA gene sequences identified (Table 2). Sequences affiliated with the *Bacteroidetes* also consistently accounted for a substantial portion (11–38%) of the clones sequenced from each leaf. *Acidobacteria* and *Actinobacteria* usually accounted for lower proportions of clone libraries, but certain samples showed increased representation of these groups (e.g., Feb07 had 21% *Acidobacteria*, Feb08 had 17% *Actinobacteria*; Table 2). Within the *Proteobacteria*, *Alphaproteobacteria* were by far the most abundant subgroup, and this lineage alone accounted for 44–67% of the sequences obtained from each library (Table 2).

UniFrac analysis suggested that sample type (i.e., date) had a significant overall influence on the phylogenetic composition of clone libraries (UniFrac metric corrected $p<0.01$). However, when each sample was compared individually to the other samples as a group, only the Aug08 sample was significantly different from the others (environmental distance $p=0.03$ for Aug08; $p>0.05$ for all other samples). Lineage-specific analyses were used to compare samples at different levels of taxonomic resolution. At a broad taxonomic level, sequences affiliated with both the *Acidobacteria* and the *Actinobacteria* ($p<0.05$ for each) were not evenly distributed across sample dates (Table 2). The *Acidobacteria* were much more prevalent in the Feb07 sample compared to the others, accounting for 21% of the phylotypes detected in the clone library compared to 1–7% on other dates (Table 2; Fig. 2). 16S rRNA gene sequences obtained in this study that were affiliated with the *Acidobacteria* all fell within *Acidobacteria* subgroup 1, and while this group were detected on all sample dates, their increased representation in the Feb07 was primarily because of the dominance of a particular sequence (Feb07_04B) that

Table 2 Distribution of 16S rRNA gene sequences belonging to different lineages of *Bacteria* in clone libraries produced from DNA recovered from the phyllosphere of *M. grandiflora* collected on six dates over February 2007–February 2009

Phylogenetic group	Number of 16S rRNA clones recovered from leaf					
	Feb07	Feb08	May08	Aug08	Dec08	Feb09
Alphaproteobacteria	49	38	38	59	31	51
Betaproteobacteria	1	0	5	4	2	0
Gammaproteobacteria	0	0	2	0	0	0
Deltaproteobacteria	1	0	1	7	0	0
Acidobacteria[a]	19	4	3	1	1	6
Actinobacteria[a]	1	11	3	0	5	2
Bacteroidetes	17	7	33	13	8	23
Cyanobacteria	1	0	0	0	0	0
Deinococcus-Thermus	0	0	0	0	0	1
Firmicutes	2	2	2	1	0	1
Nitrospira	0	0	0	1	0	0
Planctomycetes	0	0	0	2	1	0
Verrucomicrobia	0	1	0	0	2	0
Total sequences	91	63	87	88	50	84

[a] Lineages showing significant differences between clone libraries

accounted for 17% of the clone library on that date (Fig. 2). A similar sequence was presented in the Feb08 sample (Feb08_1G, accounting for 6% of the clones analyzed from that sample) but this phylotype was not detected on other dates. Other *Acidobacteria* sequences included a cluster of similar sequences found on different dates that were related to *Terriglobus roseus* and a phylotype limited to the Feb09 sample that showed some similarity to *Edaphobacter* (Fig. 2).

Actinobacteria were more common in the Feb08 sample (17% of the detected phylotypes in Feb08 compared to 1–10% in other samples; Table 2; Fig. 3). The *Actinobacteria* sequences obtained in this study were generally similar to those of cultured organisms and included representatives of four families (Fig. 3). *Microbacteriaceae* were detected in clone libraries on all dates apart from August (no *Actinobacteria* were detected in that sample), with a cluster of very similar sequences related to *Frigoribacterium* being present in the Feb08, May08, Dec08, and Feb 09 samples. Representatives of the *Micrococcaceae*, *Propionibacteriaceae*, and *Pseudonocardiaceae* were limited to fewer sample dates (Fig. 3). The February 2008 clone library contained the greatest percentage of *Actinobacteria* (17%) because of the dominance of a specific 16S rRNA gene sequence (Feb08_04H) related to *Propionibacterium*, which accounted for almost 13% of the clones sequenced from that sample (Fig. 3).

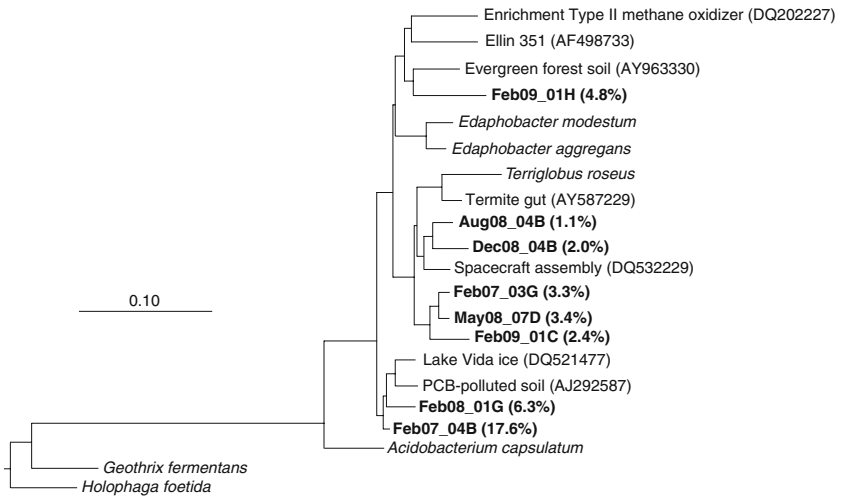

Figure 2 Phylogenetic tree of partial 16S rRNA gene sequences affiliated with the *Acidobacteria* detected in clone libraries generated from the phyllosphere of magnolia leaves sampled from the same tree in February 2007, February 2008, May 2008, August 2008, December 2008, and February 2009. Sequences obtained in this study are shown in *bold*, with percentages indicating the percentage of clones analyzed in that particular library that contained that sequence. Sequences of related environmental clones or cultured organisms are shown for comparison (*number* indicates GenBank accession number where applicable)

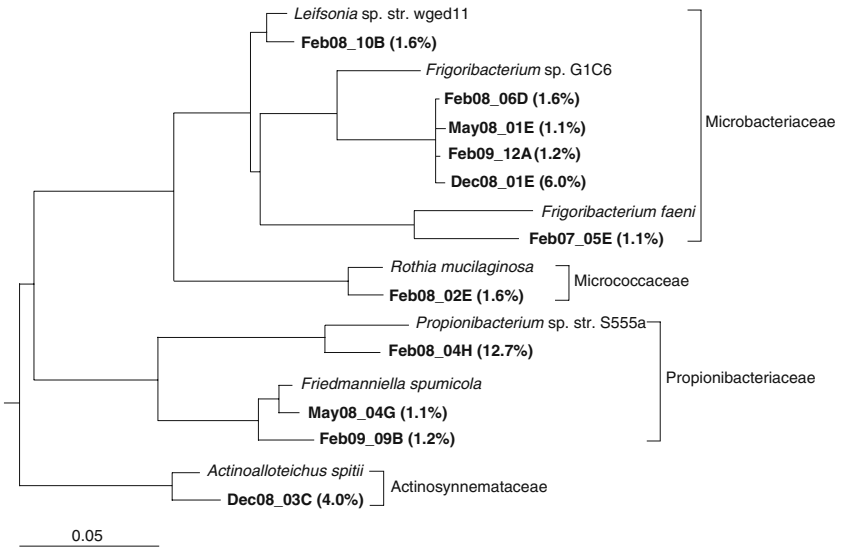

Figure 3 Phylogenetic tree of partial 16S rRNA gene sequences affiliated with the *Actinobacteria* detected in clone libraries generated from the phyllosphere of magnolia leaves sampled from the same tree in February 2007, February 2008, May 2008, August 2008, December 2008, and February 2009. Sequences obtained in this study are shown in *bold*, with percentages indicating the percentage of clones analyzed in that particular library that contained that sequence. Sequences of related cultured organisms are shown for comparison, as are families of *Actinobacteria*

Lineage-specific UniFrac analysis also revealed differences in the distributions of specific lineages of *Alphaproteobacteria* at a finer (family-level) taxonomic scale. Based on the representation of 16S rRNA gene sequences in clone libraries, the most prevalent lineages of *Alphaproteobacteria* were typically the *Beijerinckiaceae*, *Methylobacteriaceae*, and *Sphingomonadales*, which together accounted for 43–54% of clones sampled from a particular library (Fig. 4). The *Methylobacteriaceae* showed significant (UniFrac, $p<0.01$) differences between sample dates, with limited presence in clone libraries analyzed on three dates (Feb07, May08, Dec08), compared to accounting for 8–17% of the phylotypes detected on the other dates. A subgroup of the *Beijerinckiaceae* (Fig. 4) also showed significant ($p<0.01$) differences in its distribution in our clone libraries, with only a few phylotypes affiliated with these subgroup being detected in the May08, Aug08, and Feb09 libraries compared to this subgroup accounting for 11–22% of the total number of clones sequenced from the other samples. Sequences affiliated with the third predominant lineage of *Alphaproteobacteria* in these samples, the *Sphingomonadales*, were generally evenly distributed across all sample dates, accounting for 4–11% of total phylotypes detected in clone libraries (Fig. 4).

At a finer taxonomic level, there were clusters of highly similar (>97%) phylotypes that might represent the same species that were found in multiple clone libraries. Only one cluster (consisting of Feb08_04E, May08_05G, Dec08_01G, Feb09_05C, Feb07_03H, and Aug08_04E) was found on all six sample dates and represented a lineage of the *Beijerinckiaceae* (Fig. 4). Generally, this phylotype accounted for just a small percentage of the clones detected in a particular library but was more dominant in the Dec08 library where it accounted for 12% of the clones sequenced. Two clusters were detected on five of the six dates; one affiliated with the *Acidobacteria* (Aug08_04B, Dec08_04B, Feb07_03G, May08_07D, and Feb09_01C; Fig. 2) accounted for 1–3% of the clones sequenced in a particular library; the other cluster (Aug08_01F, May08_03E, Feb07_01B, Feb09_01B, and Dec08_06D), affiliated with the *Beijerinckiaceae* (Fig. 4), was more abundant and accounted for 6–21% of the clone libraries, being particularly dominant in the Aug08 and Feb07 samples. The *Beijerinckiaceae* detected in this study also included two clusters containing closely related sequences detected on four sample dates: one including sequences Feb09_02G, May08_07H, Feb08_12B, and Aug08_05C and the other including sequences Feb07_02F, Feb08_01E, May08_08E, and Dec08_08E (Fig. 4). A third cluster of closely related sequences detected on four dates (Feb08_06D, May08_01E, Feb09_02A, and Dec08_01E) was affiliated with the *Actinobacteria* (Fig. 3). Of the various clusters of sequences found on three sample dates, three clusters included sequences found in each of the 3 years of February sampling but not on other dates. One of these (Feb09_02B, Feb07_03C, and Feb08_02H) grouped with the *Sphingomonadales* (Fig. 4), whereas the other two were *Bacteroidetes* (not shown).

Discussion

Both multivariate visualization of DGGE profile patterns and UniFrac analysis of the composition of 16S rRNA gene clone

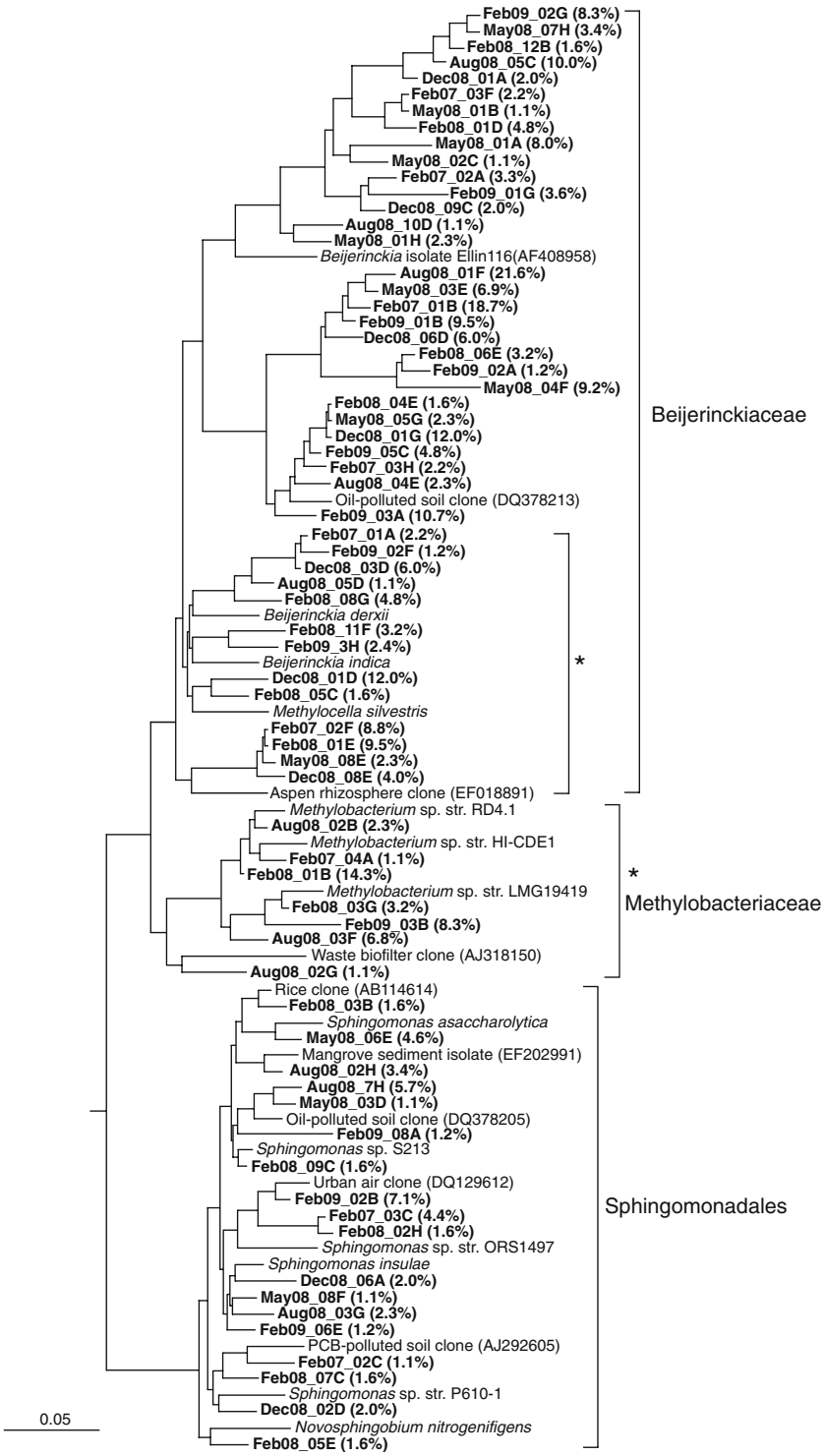

Figure 4 Phylogenetic tree of partial 16S rRNA gene sequences affiliated with the three lineages of *Alphaproteobacteria* (*Beijerinckiaceae*, *Methylobacteriaceae*, *Sphingomonadales*) that were most prevalent in clone libraries generated from the phyllosphere of magnolia leaves sampled from the same tree in February 2007, February 2008, May 2008, August 2008, December 2008, and February 2009. Sequences obtained in this study are shown in *bold*, with percentages indicating the percentage of clones analyzed in that particular library that contained that sequence. Lineages that differed in their distribution across sample dates based on UniFrac analyses are indicated by *asterisk*. Sequences of related environmental clones or cultured organisms are shown for comparison (*number* indicates GenBank accession number where applicable)

libraries showed that the phyllosphere community present on *M. grandiflora* leaves sampled in August was different from the other sample times. This suggests that the August phyllosphere community shows different evolutionary adaptation to that environment compared to the communities present in other seasons. Differences in summer phyllosphere communities compared to other months were also reported for a deciduous tree sampled over a shorter growth season, where mid-season (August) phyllosphere communities were different from those in early or late season [36]. While the August magnolia leaf community was different in terms of its composition, its overall species diversity, as estimated from clone library composition (S_{Chao1} of 48), was right around the average diversity seen across all sample dates.

However, DGGE profiles of the Aug08 samples showed the least number of bands. This discrepancy between diversity estimates (band richness) from DGGE and diversity estimates (S_{Chao1}) derived from clone library composition occurred across all sample dates. Consistently higher S_{Chao1} values for all sample dates show that DGGE fingerprints underestimate overall diversity, likely for reasons that have been previously described [2, 17]. However, the lack of a correlation between S_{Chao1} and S_{DGGE} suggests that these underestimates are not consistent from sample to sample.

The specific differences in community composition that resulted in the separation of the August phyllosphere community from that in other months are difficult to determine. *Alphaproteobacteria* were the most common lineage of bacteria detected in all clone libraries, and members of this lineage such as the methylobacteria have previously been found to be major components of the phyllosphere of various plants [1, 8, 22]. The *Methylobacteriaceae* were more prevalent in the Aug08 clone library than was typical, although the Feb08 and Feb09 libraries also showed greater representation of this group. In contrast, the representation of a subgroup of the *Beijerinckiaceae* (*Alphaproteobacteria*) related to *Methylocella silvestris* and *Beijerinckia indica* was reduced in August compared to other dates in general, although this group was also reduced in the May08 and Feb09 samples. *M. silvestris* is a methanotrophic bacterium that has been previously isolated from acidic soil and only shows growth to 30°C [12]. Other *Beijerinckiaceae* also show limited growth at higher temperatures whereas some members of the *Methylobacteriaceae* grow at higher temperatures [3]. Daily high temperatures averaged 31°C prior to the August sampling, so that the reduction in the proportion of *Beijerinckiaceae* and an increase in the representation of *Methylobacteriaceae* in the August phyllosphere may represent a shift in the dominant methylotrophic populations that is related to high average temperatures in the summer months.

We did not detect *Actinobacteria* in the August sample, but this lineage was poorly represented in most clone libraries, and while there was a significant difference in its distribution across dates, this was clearly being driven by its increased representation in Feb08. Similarly, while the representation of the *Acidobacteria* was significantly different across all sample dates, this was obviously driven by this lineages greatly increased presence in the Feb07 clone library rather than its low representation in August. The August sample did show a greater (but non-significant) representation of *Deltaproteobacteria*, which were generally rare in other samples. While not shown in the phylogenetic trees, these sequences were affiliated with the *Myxococcales* and *Bdellovibrionales*, the latter only being detected in the Aug08 sample. Overall, while the August phyllosphere community was clearly different from that on other dates, there was no single change in a major bacterial lineage that accounted for this difference; rather, this community differed because of changes in various bacterial lineages that as a whole resulted in a significant difference in community composition. Because of this, attributing the changes in August to a specific physiological trait is impossible, but, as suggested earlier, it is likely that differences in environmental factors such as temperature and solar radiation that are thought to affect phyllosphere organisms [24, 38] resulted in a different phyllosphere community during the summer.

While there was some leaf to leaf variation, this was much less than seasonal variation. Other studies have also reported minimal leaf to leaf variation in phyllosphere bacterial communities when analyzed by DGGE [39]. To some extent, this may be a result of our sampling methodology as all leaves were sampled from the same general location within the tree and were likely exposed to similar environmental conditions. It does, however, suggest that the composition of phyllosphere communities is regulated by broader environmental conditions, so that leaves sampled from the same tree at the same time have similar bacterial communities. Whether shorter (daily, weekly) temporal changes in community composition occur was not assessed in this study as we focused on seasonal and year-to-year changes. Appreciable changes in the composition of tree phyllosphere from week to week were reported by Redford and Fierer [36], but even with that finer-scale variability, there were over-riding changes over longer time periods, which together with our study suggests that seasonal patterns in the tree phyllosphere do occur. However, these seasonal patterns may not be consistent from year to year. We sampled the phyllosphere bacterial communities in late winter (February) over three different years and during similar environmental conditions. These bacterial communities were not particularly similar in overall community structure and also showed appreciable differences in the representation of certain groups: The *Actinobacteria* were more prevalent in Feb08 but not other years, and while the *Acidobacteria* were more represented in the February samples than at any other time of year, they were much more dominant in 2007 rather than 2008 or 2009. Comparing other seasons across different years was beyond the scope of this study, but the February data suggest that while seasonal variation in the phyllosphere community does occur, this may not necessarily be predictable from year to year.

The presence of clusters of similar, yet slightly different 16S rRNA gene sequences has been noted in many environmental samples and may represent an example of within-species genetic variation and adaptation of different populations, or ecotypes, of a species to slightly different environments [6, 35]. In this study, clusters of closely related sequences were particularly noticeable when comparing different sample dates and may represent species that are frequent components

of the phyllosphere community, with different ecotypes found on different dates. Assuming that these clusters represent a single species, one species of the *Beijerinckiaceae* was detected at low proportions on all sample dates, but a second species was fairly dominant on five of six dates (only being missed in Feb08). Neither species has cultured close relatives, and the closest sequences matches in databases are to 16S rRNA gene sequences derived from soil clones (the closest being to oil-polluted soil sampled in Romania; DQ378213). A species in the *Acidobacteria* represented by a cluster of five similar sequences was also present in all seasons other than Feb08 and was related to *T. roseus* and a sequence previously detected in clean rooms used for spacecraft assembly (DQ532229; [33]). According to our clone library results, these three bacterial species represent populations that are present in the magnolia phyllosphere year-round. All three of the clusters that we detected that contained four closely related sequences (one within the *Actinobacteria–Microbacteriaceae*, two within the *Beijerinckiaceae*) included phylotypes detected in all months other than August, further suggesting that the phyllosphere population in August is most different from that in other seasons.

Increasing evidence suggests that dominant phyllosphere bacteria such as the *Methanobacterium* are not just transients on the leaf surface but are intimately associated with the host plant [16, 28] and likely respond to both changes in the environment and in tree physiology. Some of these bacteria have been shown to overwinter as endophytes within the plant and migrate to the phyllosphere during the growing season [11], but whether this is a result of changes in the plant or in the environment is unknown. The annual growth and development of the leaves themselves is less likely to be a controlling factor for leaf-associated microorganisms on evergreens than it is for deciduous trees. Thus, broad-leaf evergreen trees such as *M. grandiflora* that combine a typical leaf surface with year-round leaf presence may prove to be effective systems to study the influence of environmental change and seasonal variation on phyllosphere communities. This initial study suggests that while there are year-round bacterial inhabitants of the magnolia phyllosphere, seasonal and year-to-year changes in bacterial community structure do occur.

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