EVALUATION OF NITROGENASE GENES AS MARKERS IN SOIL MICROBIAL COMMUNITY STUDIES

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EVALUATION OF NITROGENASE GENES AS MARKERS IN SOIL MICROBIAL COMMUNITY STUDIES

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ABSTRACT

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Molecular tools were developed to overcome some of the constraints to analyzing nitrogen-fixing bacteria in the environment. Two distinct plant-microbe systems were used to develop these tools, and to evaluate their utility and flexibility. The first system focused on nitrogen-fixing bacteria in the rhizosphere of salt-meadow grass (*Spartina patens*), a key plant species in high salt marshes. Members of the ε-subdivision of Proteobacteria were shown to be prominent nitrogen-fixers in this system, with specific populations changing seasonally and in response to the presence of arbuscular mycorrhizal fungi. The second system involved nitrogen-fixing bacteria of the genus

Frankia that form root-nodules in symbiosis with certain woody plants. The diversity of nodule-forming frankiae was assessed in soils from 5 continents, and in natural populations in root nodules of alder trees growing on 3 different mountain tops in central and southern Arizona. Specific difficulties in the development of molecular tools in these two systems were discussed. These studies provided significant databases for specific target sequences, i.e., the *nif*H gene, for both uncultured nitrogen-fixing members of the ε-subdivision of Proteobacteria and cultured and uncultured frankiae, and baseline data that demonstrated the usefulness of the molecular tools for studies on the ecology of both nitrogen-fixing members of the ε-subdivision of Proteobacteria and *Frankia* populations.

CHAPTER 1

Introduction

Introduction

Biological nitrogen fixation is a critical process providing reduced nitrogen resources for the ecosystem. Nitrogen-fixing bacteria convert atmospheric nitrogen gas (N_2) into biologically available ammonium (NH_4+) using the enzyme nitrogenase. Nitrogen fixing bacteria are the primary source of non-anthropogenic nitrogen for the biosphere and therefore determine productivity and plant growth performance in a variety of habitats worldwide. The diversity of nitrogen fixing bacteria spans most bacterial genera and some Archaea due to the ancient divergence and multiple cases of lateral transfer of the genes encoding the nitrogenase enzyme (12, 27, 54). Consequently, microbiological and molecular tools to study the roles and significance of these diverse nitrogen-fixing bacteria in the environment must be correspondingly diverse. Inconsistencies in detection and the analysis of diversity of these bacteria in the past suggest that methodological problems may be the cause.

Microbiological and molecular tools will both introduce selective sampling biases into any ecological studies on nitrogen fixing bacteria in the environment. Microbiological, culture-dependent analyses fail to detect those organisms that cannot be cultured, and select for those for which defined culture media and conditions are available and applied. Molecular, culture-independent tools are similarly selective since

sequence information for the genes used as the “biomarker” is generally limited, and thus specific target sequences allowing detection of undescribed organisms may not be present in those taxa. However, molecular tools do have some advantages over traditional microbiological tools. They circumvent biases of cultivation and can be used directly on environmental samples without retrieving viable organisms first, which is a necessity for reliable ecological studies. However, the usefulness of molecular tools in ecological studies is affected by the biomarker targeted and methodological constraints on the detection of that biomarker.

Biomarkers used for the detection of target organisms are generally macromolecules. Differences in the basic composition of these macromolecules, e.g., sequence differences in phylogenetically relevant macromolecules such as rRNA genes, can then be used in comparative analyses to describe relationships among different organisms with many differences suggesting a more distant relationship than fewer differences. For rRNA genes, these comparative analyses allowed researchers to reliably assign bacteria to different ranks, i.e., the Domain level as the least specific level down to the genus level. More specific classification, i.e., down to the species and subspecies level, however, can usually not be achieved without isolation and physiological testing. Detection of organisms in the environment is generally based on the detection of signature sequences by complementary synthetic oligonucleotides (probes or primers) on phylogenetically relevant macromolecules such as rRNA or their genes that are characteristic for specific phylogenetic levels. Since these signature sequences are often not uniform, i.e., identical in all target organisms on one specific taxonomic or systematic level, degenerate probes or primers are needed and applied. This requirement limits “universal” applications of a

selected (degenerate) probe or primer since sequence information on the vast majority of organisms in the environment is not available and thus probe specificity and coverage cannot be empirically assessed. PCR-based diversity analyses of unknown and uncultured bacterial communities are therefore usually limited by the inherent selectivity resulting from genomic mismatches with those primers.

A second problem of biomarkers such as rRNA is that they only provide phylogenetic information and thus focus on the analyses of community structure or diversity, neglecting any functional aspects of this community. Biomarkers that provide both phylogenetic as well as functional information are represented by genes characterizing functional groups such as sulfate-reducing (*dsr*) or nitrogen-fixing (*nif*H) bacteria. Differentiation between genomic targets (DNA) and transcribed gene targets (mRNA) of these biomarkers opens up the possibility to assess and distinguish between overall diversity of, for example, nitrogen fixing bacteria and those potentially active in the environment. Unfortunately, databases for these biomarkers are even more limited than those for rRNA genes, and thus the problems noted above with probe specificity and coverage even more pronounced. In addition to these target specific problems of detection, the application of many molecular detection and analysis tools is impacted by rather limited sensitivities. Denaturing gradient gel electrophoresis (DGGE), for example, is a community profiling tool in which mixed template PCR product is electrophoresed through a denaturing gradient and resolved into discrete bands. However, DGGE using “universal” primers requires an abundance of at least 9% within the group of target organisms to be detected (47). Thus, organisms making up less than 9% of the target

organism population will not be detected, and a diversity of target organisms larger than 15 will not be assessed accurately (47).

The goal of this research is therefore to develop probe-target systems that allow us to assess functional groups of organisms, both as overall and as active components of the microbial community, on a level of detection that provides accurate information on the structure and function of these organisms in the environment. The focus will be on nitrogen-fixing bacteria using the gene encoding the ATP hydrolysis portion of the nitrogenase enzyme, *nif*H, as target for molecular analyses (24). The probe-target systems to be developed will target nitrogen-fixing bacteria on a phylogenetic level that should provide sequence diversity in a range that is limited and therefore accessible by molecular tools.

This thesis work describes the development of molecular tools to overcome some of these constraints to describing the diversity of nitrogen-fixing bacteria in the environment. Two distinct plant-microbe systems were used to demonstrate the utility and flexibility of these tools. The first system focuses on nitrogen-fixing bacteria in the rhizosphere of salt-meadow grass (*Spartina patens*), a key plant species in high salt marshes particularly in the Northeastern USA. *Spartina patens* relies on its association with nitrogen-fixing bacteria for as much as 50% of its required nitrogen input. The second system involves the nitrogen-fixing, root-nodule-forming bacteria *Frankia* sp., that lives in symbiosis with specific woody plants including alders and sea buckthorn, but also saprophytically in soil, independent of the presence of its host plant. Both systems differ significantly with respect to potential diversity of target organisms (i.e., of

nitrogen-fixing bacteria), and therefore represent systems with contrasting challenges for the development of specific molecular tools.

Nitrogen-fixing bacteria in the rhizosphere of *Spartina patens*

Salt marshes are highly productive systems that have received considerable attention from ecologists as a consequence of their importance to the productivity of estuarine waters (13, 49). Marsh productivity is often based on the growth of grass species (15) such as *S. patens* that rely upon the activity of nitrogen-fixing bacteria to satisfy their annual nitrogen requirements. The activity of free-living nitrogen-fixing bacteria in marsh sediments may account for as much as 50% of the plant's nitrogen requirements (17, 48, 52). The magnitude of nitrogen fixation in marshes depends on soil physiochemical properties and seasonal patterns of plant growth that affect carbon exudation and release into the sediment (7, 40, 41, 50). Populations of phylogenetically distinct taxa in these sediments, such as the α-, β-, γ-, and δ-subdivisions of Proteobacteria and sulfate-reducing bacteria, are dynamic as detected through *in situ* hybridization and can be influenced by seasonal changes in plant growth and abiotic conditions (10, 23). Many nitrogen-fixing bacteria are classified within the α-, β-, γ-, and δ-subdivisions of Proteobacteria (54). Community profiles of nitrogen-fixing bacteria have predominantly been generated using PCR-based profiling tools such as restriction fragment length polymorphisms (RFLP) patterns (9, 10, 43, 51) and denaturing gradient gel electrophoresis (DGGE) (8, 30, 39, 40) to follow community changes though time and treatments. However, these tools that generally targeted *nif*H, the structural gene for the key enzyme nitrogenase reductase (24), have typically failed to detect changes in the

community composition of nitrogen-fixing bacteria in marsh sediments (10, 39, 40, 43). Despite short-term perturbations (2 weeks and 8 weeks following plant trimming or shading), communities of nitrogen-fixing bacteria from the *S. alterniflora* rhizosphere were found to be stable in South Carolina marshes (39), and seasonal changes were not observed for either the potential (those that have the gene, DNA biomarkers) or active (those that have transcribed the gene, mRNA biomarkers) community of nitrogen-fixing bacteria in a New York marsh (10). These results are in conflict with the constituent analysis of phylogenetic groups (10) and appear counterintuitive given the physiological diversity of nitrogen-fixing bacteria present in salt marshes (1, 2, 6). Critical aspects of the diversity of nitrogen-fixing bacteria may not have been described using these molecular tools, which convexes the described stability of this community.

These conflicting results, however, might be a consequence of methodological issues. In addition to sampling constraints (19), PCR-based typing procedures can be affected by the limited sensitivity of analyses that typically attempt to retrieve information on all nitrogen-fixing bacteria; by selective amplification (i.e., by primer bias and differing PCR amplification efficiencies); by the loss of relative abundance information; and by the ambiguity of complex restriction fragment length patterns, since widely varying organisms can produce similar patterns (11, 28, 42). In use, PCR-based techniques often reveal low diversity and high stability of nitrogen-fixing bacteria and cast doubt on the detection sensitivities and applicability of such techniques for the accurate description of these communities (10, 42).

Nitrogen-fixing bacteria of the genus *Frankia*

Bacteria within the genus *Frankia* are nitrogen-fixing actinomycetes that form root nodules in symbiosis with more than 200 species of non-leguminous woody plants in 25 genera of angiosperms (5). Dixon and Wheeler 1986 estimated that the contribution of actinorhizal plants to terrestrial global nitrogen fixation could be as high as 25% (18). Actinorhizal plants are able to grow in regions with restricted nitrogen availability and are successful pioneer plants in disturbed systems and improve overall soil fertility (16, 34). Understanding the interactions between plant host and soil frankiae is key to increasing the efficiency of nitrogen fixation in these systems. *Frankia* have been reported as isolated directly from soil only once (3), in all other cases they have been cultured from plant nodules (20). Descriptions of the genetic diversity of *Frankia* are currently limited by difficulties in culturing strains and the inability to isolate strains from specific host plant nodules (5, 53). DNA-DNA hybridization assays are the accepted way to delineate species in bacterial systematics and require pure cultures of the strain tested and its closest relatives (46). Given the difficulties culturing a wide range of *Frankia*, taxonomic classifications within the genus have not been delineated and different *Frankia* distinguished using informal strain designations (20).

Analyses of the 16S rRNA gene is most commonly used in bacterial systematics to describe and compare various bacteria in a wide range of applications and environments (29, 44). However, the 16S rRNA gene does not have enough nucleotide variation to distinguish different strains within established host-infection groups of *Frankia* (21, 38). Additional attempts at describing *Frankia* diversity have involved comparing cultured and uncultured frankiae from root nodules using fingerprinting techniques such as

random amplified polymorphic DNA (RAPD) (26), rep-PCR (33) and PCR-RFLP (31, 32, 45) analyses. However, these techniques suffer from the lack of comparability and the limited numbers of strains tested, which prevents broad systematic classifications within the genus *Frankia*.

Inferences are made about the presence and diversity of *Frankia* in the soil using plant capture assays to quantify the nodulation units (NU) in a soil (5). However, capture plants demonstrate clear preferences for some *Frankia* strains and inability to nodulate with others and thus may not be able to accurately describe and quantitate diversity of *Frankia* strains in soil (5, 25). The diversity of *Frankia* strains in root nodules may be affected not only by host plant preferences but also by edaphic characteristics, season, and climate (4, 14, 35, 37).

The diversity of *Frankia* represented in nodules of capture plants is only a small fraction of the total *Frankia* population in soil. Zepp et al. 1997, detected one population of *Frankia* in nodules of the host plant at a specific site by *in situ* hybridization, but different populations were detected in soil by PCR targeting specific *Frankia* groups (55). Molecular detection of *Frankia* directly from the soil has been limited due to small databases of potentially discriminative target sequences, and thus focused on analyzing *Frankia* at the genus level or group-specific level (22, 33, 35, 36). The overall diversity of *Frankia* strains present in soil remains undescribed and poorly resolved.

Outline of the thesis

This thesis research has focused on developing molecular tools to address some of the constraints to assessing the diversity of nitrogen-fixing bacteria in the environment.

These goals included the generation of databases containing partial sequences of the *nif*H gene. Retrieval of these databases was different for the two systems under study. While analyses of *nif*H gene diversity in the rhizosphere of *S. patens* required the generation and analysis of gene clone libraries from unknown bacteria as baseline data for more specific analyses, studies on the diversity of *nif*H genes in frankiae took advantage of the availability of pure cultures and natural enrichments in root nodules. Sequence alignments were created in both cases for subsequent detailed phylogenetic analyses and subsequent community analyses.

The objectives of this thesis were as follows:

1. To develop molecular tools to describe the diversity of nitrogen fixing bacteria in the rhizosphere of *S. patens* for groups shown to be active in this system. An initial clonal library was created from reverse transcribed *nif*H gene mRNA, presumably reflecting the active community of nitrogen fixing bacteria. Based on sequences recovered from this library more specific amplification protocols were designed to compare the diversity of a group of nitrogen fixing bacteria hitherto undescribed in this or any system from the ε-subclass of Proteobacteria in 2 different salt marshes with contrasting matrix and history using DGGE, cloning and sequencing (Chapter 2).
2. To assess the usefulness of these tools in an ecologically relevant context by describing the diversity of nitrogen fixing bacteria seasonally in a greenhouse experiment where plant cores were treated with a fungicide to eliminate arbuscular mycorhizal fungi (AMF). Changes in the community structure of the overall nitrogen fixing bacterial community and of a specific group of nitrogen fixing bacteria of the

ε-subclass of Proteobacteria were correlated with AMF presence or absence and plant growth performance characteristics (Chapter 3).

3. To develop molecular tools to describe the overall diversity of frankiae in diverse soils capable of forming root nodules and compare that with the diversity revealed in our culture collection. A plant bioassay using a promiscuous host plant, *Morella pensylvanica*, using soils from 5 continents described the diversity of uncultured root nodule frankiae using a fragment of the *nif*H gene (Chapter 4).
4. To use these tools to describe the diversity of uncultured frankiae in an ecologically relevant context in root nodules of a natural population of alder. Root nodule frankiae from *Alnus oblongifolia* growing on 3 different mountains in central and southern Arizona were sampled to describe the diversity and biogeography of frankiae from this host plant (Chapter 5).

Tools used to assess the diversity of nitrogen-fixing bacteria may have underestimated the diversity of these critical organisms in the environment. This thesis research addressed some of the known limitations in the use of molecular microbial ecology tools. Ultimately we have contributed to advancing the study of nitrogen-fixing bacteria in the environment.

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CHAPTER 2

Analysis of nitrogen-fixing members of the ε-subclass of Proteobacteria in salt marsh sediments [1]

Abstract

Based on phylogenetic analysis of clones retrieved from two *nif*H gene clone libraries that were created using cDNA from suboxic sediment samples densely vegetated with the high salt marsh plant *Spartina patens*, a primer set was designed to target nitrogen-fixing bacteria with sequence similarities to members of the ε-subclass of Proteobacteria. Nested PCR, DGGE and subsequent sequence analysis of re-amplified fragments confirmed the specificity of the primer set by retrieving only sequences of *nif*H for putative members of the ε-subclass of Proteobacteria all of which were characterized by a highly divergent, 27- or 36-bp insertion in both DNA and cDNA.

Analysis of nitrogen-fixing members of the ε-subclass of Proteobacteria in salt marsh sediments

Salt marshes are highly productive systems that have received considerable attention from ecologists as a consequence of their importance to the productivity of estuarine waters (9, 40). Marsh productivity is often based on the growth of C_4 grass species (10)

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such as saltmeadow cordgrass (*Spartina patens* (Ait.) Muhl.), that rely upon the activity of free living nitrogen-fixing bacteria in sediments to satisfy their nitrogen requirements. Nitrogen fixation by these bacteria may satisfy as much as 50% of the plant's nitrogen requirements (11, 39). The magnitude of nitrogen fixation in salt marshes depends on edaphic conditions, soil physiochemical properties, and patterns of plant growth that affect carbon exudation and release into the sediment (3, 41). Seasonal variation in plant root exudation of carbon resources or ions (13, 41), or changes in soil physiochemical conditions that accompany tidal flooding (7) have been shown to affect microbial community structure in sediments as shown for the α-, β-, γ-, and δ-subclasses of Proteobacteria (7). Although these phylogenetic groups contain many previously identified nitrogen-fixing bacteria (44), changes in community structure of nitrogen-fixing bacteria, however, could not be observed in marsh sediments so far (7, 30, 31, 33).

Nitrogen-fixing bacteria have predominantly been analyzed using PCR-based profiling tools such as restriction fragment length polymorphism (RFLP) analysis (6, 7, 33, 42), or denaturing gradient gel electrophoresis (DGGE) (5, 21, 29-31) that typically target *nif*H, the structural gene for nitrogenase reductase (15). PCR typing protocols, however, can be affected by the limited sensitivity of analyses that generally attempt to retrieve information on all nitrogen-fixing bacteria; by selective amplification (i.e., by primer bias); by the loss of relative abundance information; and by the ambiguity of complex restriction fragment length patterns, since widely varying organisms can produce similar patterns (8, 18, 33). The failure to detect changes in community structure of nitrogen-fixing bacteria in salt marshes might therefore be a consequence of methodological issues.

The aim of this study was to develop molecular tools that target members of this functional group on a more specific level. For this purpose, *nif*H gene clone libraries were created using universal primers and reverse-transcribed mRNA (cDNA) from suboxic sediment samples densely vegetated with *S. patens*. Rhizosphere samples were retrieved in July 2006 at a depth of 1.5 – 3.5 cm below surface from two salt marshes with contrasting matrix and history, that is, Piermont Marsh, a natural salt marsh with high organic matter content, and Harrier Meadow, a restored wetland with low organic matter content (26, 36). Both marshes were brackish with salinities between 5 and 15 ppt, and characterized by standing water about 5 cm below the surface which resulted in negative redox potentials at the sampling depth (7).

Table 2.1: Primer combinations targeting *nif*H gene sequences

Primers	Sequence (5' → 3')	Position[a]	Degeneracies	Size (bp)	Reference
NifHforA/	GCI WTI TAY GGN AAR GGN GG	19-38	128	464	(42)
NifHrev	GCR TAI ABN GCC ATC ATY TC	463-482	48		
NifHforB/	GGI TGT GAY CCN AAV GCN GA	112-132	96	371	(42)
NifHrev	GCR TAI ABN GCC ATC ATY TC	463-482	48		
PicenoF/	TAC GGI AAR GGB GGI ATY GG	25-44	12	428	(31)
PicenoR	SAC GAT GTA GAT YTC CTG	436-453	4		
PolF/	TGC GAY CCS AAR GCB GAC TC	115-135	24	362	(32)
PolR	ATS GCC ATC ATY TCR CCG GA	457-477	8		
ENFBf[b]/	GAT GTA TGT AAA CCT GGT GC	223-242	0	252	This study
ENFBr	CTT GTG CTT TTC CTT CAC GG	419-439	0		

[a]Sequence position with reference to the *A. vinelandii nif*H coding sequence (Genbank accession number M20568).

[b]Forward primer contained GC-clamp ($^{5'}$CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCC CGG GGG GCC) at the 5'-end when used for DGGE analyses. Primers without GC-clamps were used for re-amplification of DGGE fragments for sequence analyses. Fragment sizes represent those of fragments generated with primers without GC-clamp.

Nucleic acids were extracted from 0.5-g-sediment samples (n=2 for each site). Cells were lysed by bead beating (14), and nucleic acids purified by sequential phenol,

phenol/chloroform and chloroform extraction (35), subsequent precipitation with 2 volumes of 2.5 M NaCl/20% PEG 8000 (42), which was followed by an additional phenol/chloroform and chloroform extraction, and a final isopropanol precipitation. Nucleic acids were washed twice in 70% ethanol, dried and re-suspended in 40 µl distilled water. Duplicate samples were mixed and split into two portions, i.e., a DNA and RNA sample, respectively. RNA samples were further treated with 4 µl DNase I (Promega, Madison, WI, 1U μl^{-1}) at 37°C for 1 h, following the manufacturer's instructions. Ten percent (4 µl) of the RNA solution was subsequently used to transcribe cDNA using 2 µM of the reverse primer Nifrev (Table 1) (42) and the Reverse-iTMAX RT blend (ABgene, Rochester, NY) according to the manufacturer's instructions. Controls to test for DNA contamination of RNA preparations included the use of the forward primer NifHforA (42) in reverse transcription reactions and attempted 16S rRNA gene amplification from all samples (20).

A *nif*H gene clone library was created using 10% (2 µl) of the cDNA preparations from both salt marshes as template for nested PCR using primer set NifHforA/Nifrev for initial amplification followed by amplification with primer set NifHforB/Nifrev (Table 2.1) (42). PCR product was ligated into pGEM®-TEasy (Promega) and transformed into *E. coli* TOP-10 (Stratagene, Cedar Creek, TX). Fifteen clones from each library were analyzed for *nif*H fragments and sequenced using the CEQ 8800 Quickstart Kit with the addition of 5% DMSO to the reaction mix on a CEQ 8800 sequencer (BeckmanCoulter, Fullerton, CA). This brief census revealed four potential chimeras (28). The remaining 26 sequences were deposited at Genbank under accession numbers EF208162-EF208186 and EF208189. These sequences were aligned with related sequences from Genbank and

EMBL searches (2, 27) using Sequencher 4.2.2 (Gene Codes Corporation, Ann Arbor, MI), CLUSTAL X and MacClade 4.05 (24, 38). Phylogenetic analyses included maximum parsimony (MP), neighbor joining (NJ) and maximum likelihood (ML) methods using nucleic acid or amino acid sequences using PAUP*4.0b10 (37). Confidence in tree topologies was gauged using bootstrap re-sampling methods (BS) in PAUP and only included those values over 70% (12). Additionally, Bayesian methods were used in MRBAYES v 3.0 (16) and a 95% majority rule consensus tree generated in PAUP.

Tree topologies were very similar for each of the phylogenetic methods employed, both for nucleic acid and amino acid sequences, and were not affected by the in- or exclusion of insertions found in several clones (data not shown). A representative MP tree shows that sequences were recovered representing bacteria related to the γ-, δ-, and ε-subclass of Proteobacteria with high bootstrap or probability values supporting the differentiation between these groups (Fig. 2.1). While *nif*H gene sequences representing nitrogen-fixing members of the γ- and δ-subclass of Proteobacteria have commonly been retrieved from other environments such as low salt marshes (4, 21, 22), retrieval of *nif*H genes from environmental samples has not been described for the ε-subclass of Proteobacteria.

In our study, about one third of all clones analyzed clustered well within the ε-subclass of Proteobacteria, with 8 clones retrieved from sediments of Harrier Meadow and 3 from Piermont Marsh (Fig. 2.1). These *nif*H gene sequences, similar to those of all cultured and other uncultured relatives, were characterized by a 36-bp-insertion, except for clone H34, which had a 27-bp-insertion. Sequences of the insertion region were much

more variable than the overall sequence of the *nif*H gene, e.g., with 33, 42 and 56% divergence in the insertion between clone H1 and clones H23, H11, and H34, respectively, while the overall sequences showed 12, 6, and 13% divergence only. Since only four *nif*H gene sequences of cultured bacteria belonging to the ε-subclass of Proteobacteria could be retrieved from the databases, a more specific assignment of clone sequences to clusters within the ε-subclass of Proteobacteria was limited, and not supported by strong bootstrap or probability values (Fig. 2.1).

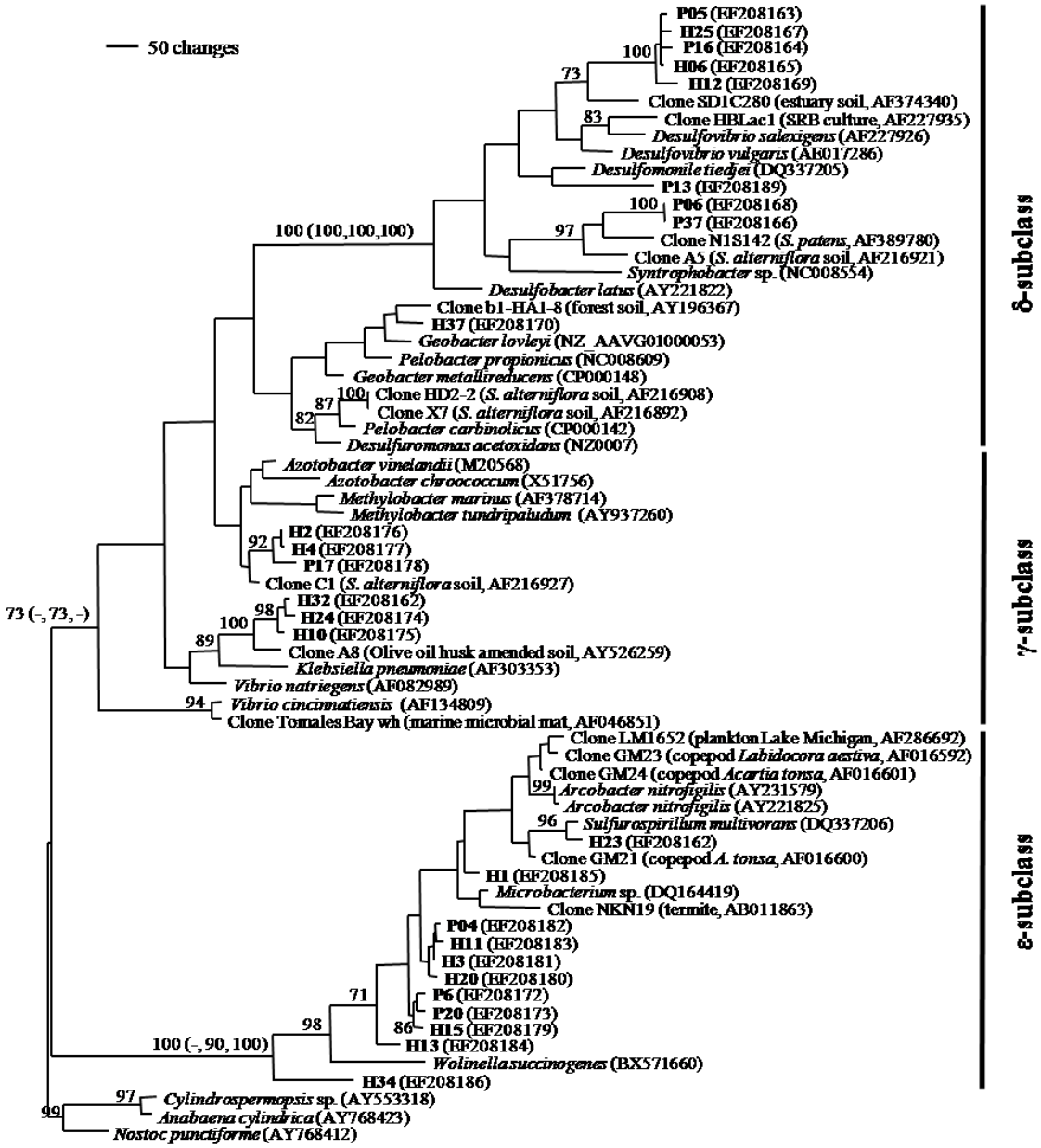

Figure 2.1: Phylogenetic analyses of *nif*H clonal library. Parsimony-based tree showing the phylogenetic position of *nif*H gene clones from two libraries generated from cDNA from rhizosphere samples of *Spartina patens* from Piermont Marsh (P) and Harrier Meadow (H), within the γ-, δ-, and ε-subclasses of Proteobacteria, respectively. The phylogram was created using PAUP*4.0b10 with 10,000 random addition replicates (37). Numbers reflect bootstrap support (BS) measures with 10,000 replicates and only include those measures over 70%. Numbers in parentheses at key nodes represent BS measures and posterior probabilities from neighbor joining, maximum likelihood and Bayesian analyses, respectively. For brevity, these values were omitted from all but the key phylogenetic nodes on the tree. The outgroup was *Cylidrospermopis* sp. (AY553318), *Anabaena cylindrica* (AY768423) and *Nostoc punctiforme* (AY768412).

*Nif*H genes for members of the ε-subclass of Proteobacteria have not been detected in environmental samples even though ribosomal RNA sequences or isolates demonstrated their presence in these environments (1, 17, 33, 34, 43). For example, a nitrogen-fixing member of the ε-subclass of Proteobacteria, *Arcobacter nitrofigilis* ATCC 33309, was originally isolated from roots of *S. alterniflora*, a common low salt marsh plant (25). However, nitrogen-fixing members of the ε-subclass of Proteobacteria have not been detected by targeting *nif*H genes in low salt marsh environments so far (4, 21, 22). These studies used different primer sets compared to our study and those of others that detected *nif*H gene fragments in freshwater and marine samples (23, 45) that group with members of the ε-subclass of Proteobacteria in our analyses (Fig. 2.1). These observations suggest that primer bias could be a major cause for the failure of detection.

Due to the limited information available on nitrogen-fixing members of the ε-subclass of Proteobacteria, we chose to focus on this group and develop more specific primers targeting the *nif*H gene of the ε-subclass of Proteobacteria. Forward primer ENFBf and reverse primer ENFBr (Table 2.1) were designed within the NifHforB and Nifrev primer set range from unique regions in the alignment file of the ε-subclass of Proteobacteria, and initially checked for specificity using BLAST and Fasta analyses on Genbank and EMBL databases. While the forward primer showed small mismatches (1 to 3 bases) to corresponding sites on *nif*H gene sequences of some members of the ε-subclass of Proteobacteria such as *Arcobacter* and *Sulfurospirillum* sp., the reverse primer was more divergent, with 3 to 6 mismatches to sequences of *Wolinella* and *Arcobacter* sp., respectively. The specificity of this reverse primer, however, could not be evaluated on sequences for other organisms such as *Microbacterium* and *Sulfurospirillum* sp. since the

sequence information available for these organisms did not cover its binding site. Since these primers were meant to be used in DGGE analyses, the design of degenerate primers was avoided, and instead the specificity of PCR reduced by decreasing the annealing temperature arbitrarily to 4°C below T_m.

PCR reaction mixtures contained 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 µM of each primer with the forward primer carrying the GC-clamp, 5 mg ml^{-1} BSA, 1 x PCR buffer, 2 µl template, and 2 units of *Taq* DNA polymerase (GenScript, Piscataway, NJ). An initial denaturation at 96°C for 10 min was followed by incubation at 80°C for 10 min for *Taq* polymerase addition, and 35 cycles of denaturation at 96°C for 30 sec, annealing at 54°C, and extension at 72°C for 45 sec, and a final extension at 72°C for 30 min. DNA of *Arcobacter nitrofigilis* ATCC 33309 (25) and *Klebsiella oxytoca* (K10, Dept. Microbiology Culture Collection, Wageningen Agricultural University) served as positive and negative controls, respectively.

The amplification conditions allowed us to amplify the *nif*H gene fragment from *A. nitrofigilis* even though the forward and reverse primer showed 1 and 5 mismatches to its target sequence, respectively. However, we failed to amplify *nif*H gene fragments using DNA from environmental samples. This failure was attributed to low template concentrations in these samples, because a nested approach with amplification products generated with either primer set NifHforA/Nifrev, or after nested amplification with primer set NifHforB/Nifrev as templates for the ENFBf/r primer set was successful. Since nested PCR was necessary for the detection, amplification products generated by two additional primer sets, PicenoF/R (31) and PolF/R (32) (Table 2.1) were checked as template for primer set ENFBf/r. Both primer sets produced amplification products with

DNA from *K. oxytoca* and the environmental samples but not with DNA from *A. nitrofigilis*. Nested PCR using the PicenoF/R primer set that was slightly modified from its published version (31) (Table 2.1) did not result in the generation of any amplification product (Fig. 2.2), while that using primer set PolF/R yielded very faint double bands, not suitable for DGGE (Fig. 2.2). These results support the speculation that methodological impacts, i.e., primer bias, might have contributed to the failure to detect *nif*H genes of the ε-subclass of Proteobacteria in previous studies.

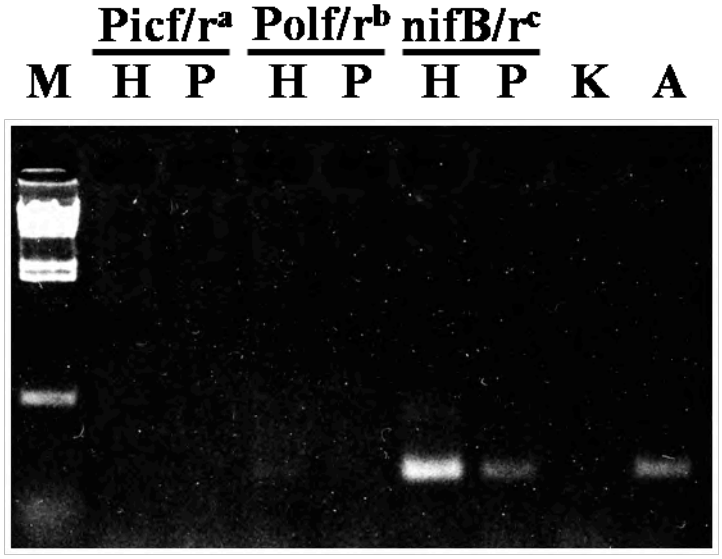

Figure 2.2: PCR products generated using the ENFBf/r primer set in nested PCR. Ethidium bromide stained agarose gel of PCR products generated using the ENFBf/r primer set in a nested PCR with template generated using three different primer sets (PicF/R (31), PolF/R (32), and NifB/r (42)) on DNA from Harrier Meadow (H) and Piermont Marsh (P). Lane "M" contains a λ HindIII size marker, lane "K" a negative control (*Klebsiella oxytoca*) and lane "A" the positive control (*Arcobacter nitrofigilis*).

The specificity of primer set ENFBf/r for members of the ε-subclass of Proteobacteria was tested by DGGE analysis. DNA and cDNA obtained from both sites, i.e., Piermont Marsh and Harrier Meadow, was used as template for primer set NifHforA/Nifrev, and

amplification products directly or after nested PCR with primer set NifHforB/Nifrev used as template for primer set ENFBf/r. Products were analyzed on a DCode Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA). Samples were electrophoresed at 60^{o}C and 180 volts for 10 min, and then at 100 volts for 16 hrs (8% polyacrylamide, 37-47% denaturant). Gels were stained with ethidium bromide (0.5 µg ml^{-1}) in 0.5 x TAE for 30 min, de-stained in water, and visualized on a UV transilluminator.

Amplification products of NifHforA/Nifrev or after nested PCR with NifHforB/Nifrev as templates for ENFBf/r produced very similar profiles in DGGE analyses (Fig. 2.3), indicating that the additional nested PCR can be used to increase template concentrations without inherently generating any spurious by-products or additional artifactual banding patterns. DGGE profiles with either DNA or cDNA used as initial PCR templates were distinctly different. Profiles based on DNA as template were generally more complex than those based on cDNA, but did not necessarily have overlapping profiles (Fig. 2.3). The validity of these results is supported by studies on nitrogen-fixing bacteria on rice where RFLP profiles generated on *nif*H amplicons were very different for DNA and cDNA templates (19), and by results from *nif*H gene clone libraries from lake plankton where most of the cDNA derived sequences did not group closely with those obtained from DNA (43).

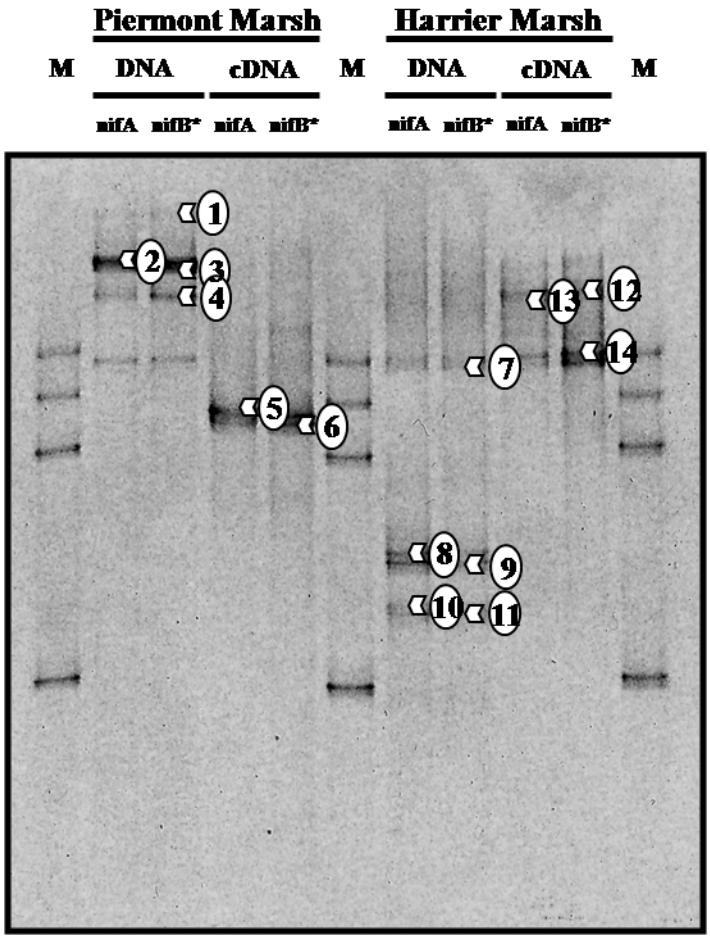

Figure 2.3: DGGE profiles generated using primer set ENFBf/r. DGGE profiles of amplicons generated with primer set ENFBf/r using amplicons generated either with primer set NifHforA/Nifrev (NifA) or after nested PCR with primer set NifHforB/Nifrev on NifHforA/Nifrev amplicons as template (NifB) using DNA or cDNA from rhizosphere samples of *Spartina patens* from Piermont Marsh and Harrier Meadow. The marker (M) is a group of clones from the initial *nif*H gene clone libraries: H15, H11, P04 and H23. Numbers refer to fragments extracted and sequenced.

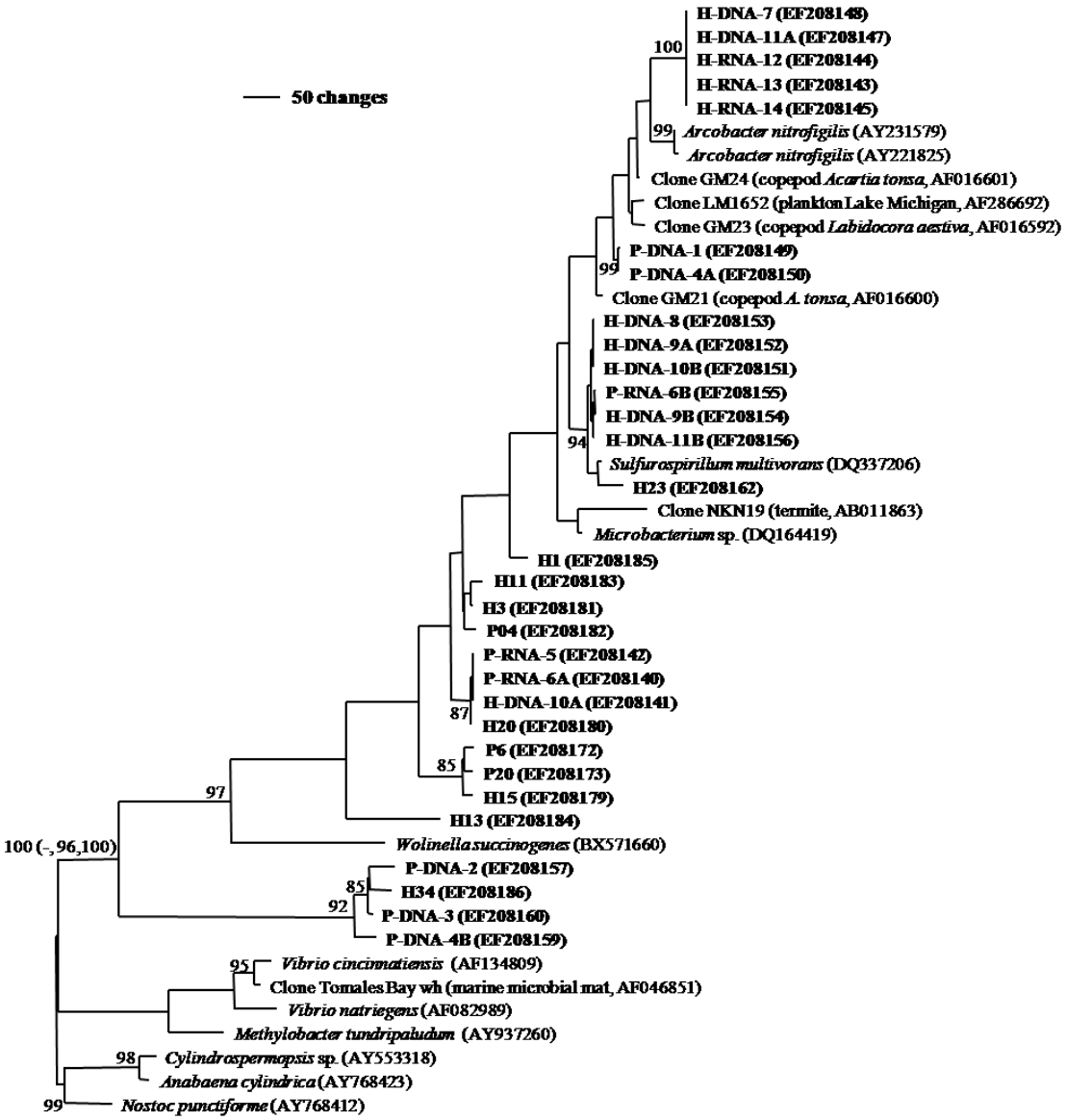

Figure 2.4: Phylogenetic analyses of sequence fragments from the DGGE gel. Parsimony-based tree showing the phylogenetic position of *nif*H gene clones from fragments of DGGE profiles generated using the ENFBf/r primer set (see Fig. 3). Clones are assigned acronyms: "P" and "H" refer to the site, Piermont Marsh and Harrier Meadow, respectively, "DNA" and "RNA" refer to the template for initial amplification (i.e., DNA or cDNA), and a number corresponds to the fragment number in DGGE profiles (Fig. 3). The phylogram was created using PAUP*4.0b10 with 10,000 random addition replicates (37). Numbers reflect bootstrap support measures with 10,000 replicates and only include those measures over 70%. Numbers in parentheses at key nodes represent BS measures and posterior probabilities from neighbor joining, maximum likelihood and Bayesian analyses, respectively. For brevity, these values were omitted from all but the key phylogenetic nodes on the tree. The outgroup was *Cylidrospermopis* sp. (AY553318), *Anabaena cylindrica* (AY768423) and *Nostoc punctiforme* (AY768412).

All DGGE bands were excised, re-amplified and cloned, clones re-analyzed on DGGE and subsequently sequenced. Only those bands with numbers revealed good sequence (Fig. 2.3). Up to 3 clones from each DGGE band were sequenced, analyzed for chimeras, and analyzed by the same phylogenetic methods as described above. Clones generated from the same fragment that had identical sequences were not included in the final phylogenetic analysis. Sequences for DGGE fragments were deposited using accession numbers EF208140-EF208160. All sequences retrieved harbored a 27 or 36 bp insertion in the *nif*H gene and showed high BS support (100%) for their assignment to the ε-subclass of Proteobacteria (Fig. 2.4). More accurate assignments of clones to groups within the ε-subclass of Proteobacteria, however, were hampered by limited database sequences demonstrating that the phylogeny of these clones and isolates needs additional work.

Our study showed that primer set ENFBf/r and the conditions applied in this study were specific enough to retrieve sequence information on defined groups within the ε-subclass of Proteobacteria. This enables us to apply a more refined and targeted approach focusing on this phylogenetic group that may provide a more accurate picture of the dynamics of the nitrogen-fixing microbial community in high salt marsh sediments than obtained in previous studies. Such studies could include the exploitation of the high divergence rate found in the 36-bp (and for a separate group a 27-bp) insertion in the *nif*H gene sequences of members of the ε-subclass of Proteobacteria, because the insertion provides an ideal primer or probe target site for future research aimed at examining these bacteria in the environment more specifically.

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CHAPTER 3

Seasonal analyses of arbuscular mycorrhizae, nitrogen-fixing bacteria and growth performance of the salt marsh grass *Spartina patens* [1]

Abstract

Seasonal variation of arbuscular mycorrhizal fungi (AMF) in roots of the high salt marsh plant *Spartina patens*, the diversity of nitrogen-fixing bacteria in the rhizosphere and plant growth performance was studied at key stages of the growing season coinciding with major plant phenological stages, i.e., vegetative growth, reproduction and senescence. AMF colonization was highest during vegetative growth, with values declining during the growing season to the same level seen at plant dormancy. AMF colonization was reduced at lower depths in the sediments where anoxic conditions were observed and in plants treated with the systemic fungicide Benomyl. Only small changes in diversity of nitrogen-fixing bacteria in general and more specifically of those belonging to the ε-subdivision of Proteobacteria were detected during the season or between treatments by PCR-RFLP of *nif*H gene fragments with DNA as template for amplification; however, greater seasonal changes were displayed when cDNA that was

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used as a proxy for gene expression and thus active bacteria was used as template for amplification. DGGE analyses of *nif*H gene fragments representing nitrogen-fixing bacteria of the ε-subdivision of Proteobacteria using both using DNA and cDNA as template showed highly diverse profiles that changed during the season and in response to treatment. Seasonal changes were observed for a suite of plant growth attributes and differences were observed between treatments, with higher values generally obtained on non-treated plants compared to Benomyl-treated plants. These differences were most pronounced during vegetative growth; however, differences between non-treated and Benomyl-treated plants were reduced seasonally and disappeared by the onset of senescence. This study demonstrates seasonal changes in AMF colonization on *S. patens* and in the community structure of nitrogen-fixing members of the ε-subdivision of Proteobacteria in the plant root zone. Plant growth performance changed seasonally with some effects of Benomyl-treatment.

Introduction

In highly productive salt marsh systems, both positive and negative interspecies interactions that modulate nutrient dynamics are important determinants of plant productivity and successional dynamics (Bertness 1992; Bertness and Callaway 1994; Callaway 1997). The importance of biotic interactions for plant success often increases with the severity of the abiotic environment (Walker and Chapin 1987), and in such cases, mycorrhizal root networks have often been shown to enhance plant growth and acquisition of increasingly limited soil resources (Allen 1991; Hamel 2004; Rozema et al. 1986; Smith and Read 1997). High salt marsh systems are an excellent example of this phenomenon, since they rely on unpredictable tidally-imported nitrogen (N) to reduce N-

limitations, affecting the degree to which the dominant grasses such as *Spartina patens* and *Distichlis spicata* form associations with arbuscular mycorrhizal fungi (AMF) within their roots (Cooke et al. 1993; Hoefnagels et al. 1993; Patriquin and Keddy 1978). In contrast, in low salt marshes with predictable, high tidal N import, dominant species such as *Spartina alterniflora* infrequently form associations with AMF (Hoefnagels et al. 1993; van Duin et al. 1989).

AMF are highly effective at acquiring and supplying limiting nutrients such as phosphorus, but also nitrogen, usually in the form of ammonium, from the soil to the host plant (Ames et al. 1983; Hayman 1986). However, nitrogen fixation might also be an important component of the nitrogen budget of high marsh systems (DeLaune et al. 1989; White and Howes 1994). Free-living or root associated nitrogen fixing bacteria provided with carbon (C) resources from root exudates are likely the primary organisms responsible for nitrogen fixation in this system because salt marsh grasses do not form highly evolved, mutualistic relationships with symbiotic nitrogen fixers (Bagwell and Lovell 2000; Bagwell et al. 1998; McClung et al. 1983; Patriquin and Keddy 1978; Whiting et al. 1986). Because mycorrhizae obtain their C directly within the plant itself rather than by scavenging C that has been released from the root (Graham et al. 1981; Paul and Clark 1996; Smith and Read 1997), AMF may be a more effective competitor for C than rhizosphere microorganisms. Reductions in root C exudation or changes in exudates composition induced by AMF activity could therefore intensify microbial competition leading to altered community structure and metabolic activity. This assumption is supported by studies that showed that the presence of certain species of *Glomus* reduced the biomass of nitrogen-fixing microorganisms, a result of competition

for root exudates (Hamel et al. 1991; Paul and Clark 1996). Changes in populations of functional groups such as nitrogen-fixing bacteria could ultimately impact plant resource acquisition, plant nutrient status and performance.

Nitrogen-fixing bacteria have predominantly been analyzed using PCR-based profiling tools such as restriction fragment length polymorphism (RFLP) analysis (Burgmann et al. 2004; Burke et al. 2002a; Poly et al. 2001b; Widmer et al. 1999), or denaturing gradient gel electrophoresis (DGGE) (Burgmann et al. 2005; Lovell et al. 2001; Piceno and Lovell 2000a; b; Piceno et al. 1999). These analyses that typically target *nif*H, the structural gene for nitrogenase reductase (Howard and Rees 1996), of all bacteria have generally failed to detect changes in the community composition of nitrogen-fixing bacteria in marsh sediments (Burke et al. 2002a; Piceno and Lovell 2000a; b; Piceno et al. 1999). Despite short-term perturbations of their habitat, communities of nitrogen-fixing bacteria were found to be stable in South Carolina marshes (Piceno and Lovell 2000b), and seasonal changes were not observed for either the potential (i.e., those that have the gene) and active (i.e., those that have transcribed the gene) populations of nitrogen-fixing bacteria in composite rhizosphere and bulk soil samples of sites vegetated with mycorrhizal *Spartina patens* (Burke et al. 2002a). These results are in conflict with analysis of specific phylogenetic groups (Burke et al. 2002a) and appear counterintuitive given the diversity of nitrogen-fixing bacteria present in salt marshes (Bagwell et al. 2001; Bagwell and Lovell 2000; Berholz et al. 2001). These conflicting results, however, might be a consequence of methodological issues caused by sampling constraints (Dunbar et al. 2002) or the limited sensitivity or resolution of the analyses (Casamayor et al. 2002; Kisand and Wikner 2003; Poly et al. 2001a).

More specific sample analysis of rhizosphere and bulk soil, for example, revealed differences in RFLP profiles between these samples in the presence of AMF on roots of *S. patens*, but not when AMF activity was suppressed after application of the systemic fungicide Benomyl (Burke et al. 2002b). Seasonal changes in RFLP profiles were noted in a similar study, which indicated substantially different populations during active plant growth compared to those encountered at senescence and dormancy (Burke et al. 2003). These changes in RFLP patterns of nitrogen fixing bacteria were correlated with AMF colonization changes seasonally but there were no detectable differences between samples with AMF and samples where AMF were suppressed, and thus effects of AMF on nitrogen-fixing populations could not be established (Burke et al. 2003).

The objective of the current study was to follow-up on this previous work and to assess the effect of AMF on the diversity of nitrogen-fixing bacteria on more specific levels of taxonomic resolution, and to relate potential changes to plant growth performance. This study took advantage of 1) the availability of plant growth performance data generated during the above-mentioned seasonal study (Burke et al. 2003); 2) replicate sediment samples from this study frozen at -80ºC that were shown to be useful for DNA- and mRNA-based analyses (Welsh et al. 2007); and 3) new profiling approaches for specific groups of nitrogen-fixing bacteria such as nitrogen-fixing members of the ε-subdivision of Proteobacteria that were shown to be dominant in *nif*H gene clone libraries generated from reverse-transcribed mRNA extracted from two salt marshes with different matrix and history (Welsh et al. 2007). One of these marshes, Piermont Marsh (41° 02’30’’ N, 73° 55” 00” W), located approximately 18 km north of New York City, had provided the cores used in the long-term study (Burke et al. 2003).

Materials and Methods

Experimental setup The basic experimental set up has been described in detail in (Burke et al. 2003). Briefly, soil cores with *S. patens* weighing approximately 1 kg fresh weight were taken from Piermont Marsh on March 2, 2000 and fitted into 1-l plastic pots (n=14). These pots were placed into 2.5-l reservoir pots that contained a 5 ppt artificial seawater solution (Instant Ocean® Mentor, OH, USA) amended with 5 ppm NH_4^+ and PO_4^{3-} so that standing water was present 5 cm below the top of the core, which correlated to observed water levels in the field. Half of the cores (n=7) were treated with Benomyl (50% WP; Bonide Products, Inc. Yorkville, NY, USA; 0.2 g per kg of soil dissolved in 100 ml water and applied to the core surface) after a 1-week acclimation period to suppress AMF colonization (Hetrick et al. 1994). Pots of all treatments were placed at random on a greenhouse bench and moved once a week during the growth period for 6 months (March 2 to September 12, 2000) under natural light and temperature conditions to minimize bench effects (Burke et al. 2003).

Soil cores were collected from each pot 8, 16, and 24 weeks after treatment meant to coincide with major plant phenological stages; namely, vegetative growth, reproduction and senescence previously linked to changes in microbial populations under field conditions (Hines et al. 1999; Hines et al. 1989; Rooney-Varga et al. 1997). In addition, soil was collected from pots immediately after they were placed in the greenhouse while plants were still dormant to determine starting conditions. From each pot, 2 soil cores were retrieved using a soil corer (1.5 cm diameter). Samples were collected from cores between 1.5 - 3.5 cm (further referred to as 2.5 cm depth), 4.0 - 6.0 cm (5.0 cm depth),

and 6.5 - 8.5 cm (7.5 cm depth) below the surface of each core. From each of these samples, one portion was retained for root and sediment dry weight determination, one used for the analysis of mycorrhizae, and one retained for microbial analyses, while two additional samples were frozen at -80ºC.

Directly after core retrieval, pH, temperature and redox potential were measured at an approximate depth of 2.5, 5.0 and 7.5 cm using an Oakton® pH/mV meter (Cole-Parmer Instrument Company, Vernon Hills, ILL, USA). Pore water was collected using DET (diffusive equilibration in thin films) gel probes (Krom et al. 1994; Mortimer et al. 1999; Mortimer et al. 1998) and analyzed for sulfide, sulfate and ammonium as described previously (Burke et al. 2003).

Analysis of AMF Roots for AM analysis were stained with Trypan Blue in lactic acid using a modification of the procedure outlined by (Kormanick and McGraw 1982) that employed incubation in 5% KOH for 6 hours at room temperature instead of heat for root clearing. At least 30 roots from each sample depth per pot were used for estimations of AM colonization. Roots were mounted on microscope slides and colonization was determined using the slide mount method of (McGonigle et al. 1990). A total of 100 intersections were scored for each sample depth per pot. The root length colonized by mycorrhizal hyphae, arbuscules and vesicles was determined according to the method of (Brundrett et al. 1994) where hyphae are considered mycorrhizal only if visually connected to vesicles or arbuscules.

Analyses of microbial community structure One-g sediment samples from 2 different pots of the same treatment and plant phenological stage were thawed and split into two 0.5-g samples and extracted separately. All samples were obtained from a depth between

1.5 - 3.5 cm representing suboxic conditions (Burke et al. 2003). After cell lysis by bead beating (Hönerlage et al. 1995), centrifugation and re-extraction of the pellet, the respective supernatants of the same sample were mixed and split into 2 equal amounts, one for DNA extraction and one for RNA extraction that were performed as described in Welsh et al. (2007). Briefly, released nucleic acids in the supernatants were purified by sequential phenol, phenol/chloroform and chloroform extraction (Sambrook et al. 1989), and subsequent precipitation with 2 volumes of 2.5 M NaCl/20% PEG 8000 (Widmer et al. 1999), which was followed by an additional phenol/chloroform and chloroform extraction, and a final isopropanol precipitation. Nucleic acids were washed twice in 70% ethanol, dried and re-suspended in 20 µl distilled water and pooled with the concurrent extraction from the same pot and treatment. RNA samples were further treated with 4 µl DNase I (Promega, Madison, WI, 1U μl^{-1}) at 37°C for 1 h, following the manufacturer's instructions.

PCR-based analyses of nitrogen-fixing bacteria were performed using two approaches, i.e., one targeting all nitrogen-fixing bacteria (Widmer et al. 1999), and the second targeting members of the ε-subdivision of Proteobacteria (Welsh et al. 2007). Both approaches were based on nested PCR using amplification products generated with primers NifHforA ($^{5'}$GCI WTI TAY GGN AAR GGN GG) and Nifrev ($^{5'}$GCR TAI ABN GCC ATC ATY TC) (Widmer et al. 1999) as template for the subsequent PCR. Two µl of extracted DNA or 4 µl of cDNA transcribed from mRNA using 10% (4 µl) of the extracted RNA, 2 µM of the reverse primer Nifrev and the Reverse-iTMAX RT blend (ABgene, Rochester, NY) according to the manufacturer's instructions using amplification conditions as described (Widmer et al. 1999). PCR products (464 bp in

size) were detected by electrophoresis on 1% agarose gels, purified using the UltraClean™ 15 DNA Purification Kit (Mo Bio Laboratories, Carlsbad, CA) and dissolved in 10 µl sterile distilled water.

For PCR-RFLP analysis, 1 µl of this solution was used as template in PCR using NifHforB ($^{5'}$GGI TGT GAY CCN AAV GCN GA) and Nifrev as described in (Widmer et al. 1999) to generate 371 bp *nif*H gene fragments meant to represent nitrogen-fixing bacteria in general (Burke et al. 2003). Then the NifHforB/ Nifrev PCR product was cleaned and 1 µl used as template in PCR using ENFBf ($^{5'}$GAT GTA TGT AAA CCT GGT GC) and ENFBr ($^{5'}$CTT GTG CTT TTC CTT CAC GG) as described in Welsh et al. (2007) to generate 252 bp *nif*H gene fragments of members of the ε-subdivision of Proteobacteria. PCR products were purified using the UltraClean™ 15 DNA Purification Kit, and multiple PCR reactions from the same sample pooled such that all DNA or cDNA based samples had similar approximate concentrations as revealed by agarose gel electrophoresis. These samples were then digested either with restriction endonuclease *Hae*III (New England BioLabs, Ipswich, MA) (fragments generated with primer set NifHforB/Nifrev) or with *Dde*I (Promega) (fragments generated with primer set ENFBf/ENFBr) according to the manufacturer's instructions. Digestion products of samples from both pots of the same treatment and plant stage that were prepared separately were mixed to address interpot differences and differential profiling (Nicol et al. 2003) and analyzed on a 10% non-denaturing acrylamide gel after staining with ethidium bromide (0.5 µg ml^{-1} in 0.5 x TAE for 30 min), after visualization on a UV transilluminator.

For DGGE analysis, primer ENFBf was modified with a GC-clamp (Welsh et al. 2007) and used with primer ENFBr to amplify *nif*H gene fragments of members of the ε-subdivision of Proteobacteria. Fragments were analyzed on a DCode Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA). Samples were prepared separately, and again analyzed as mixtures of samples from both pots of the same treatment and plant stage as described above. Samples were electrophoresed at 60^{o}C and 180 volts for 10 min, and then at 100 volts for 16 hrs (8% polyacrylamide, 37-47% denaturant) (Welsh et al. 2007). Gels were stained with ethidium bromide (0.5 μg ml^{-1}) in 0.5 x TAE for 30 min, de-stained in water, and fragments visualized on a UV transilluminator.

GelManager 1.5 (Biosystematica, Ceredigion, UK) was used to identify sites of bands and compare bands between gels. Those bands were scored manually and used to create a presence/absence data matrix for each set of profiles. MacClade 4.05 was used to convert this matrix into a nexus file (Maddison and Maddison 1999). A neighbor joining phylogram was generated in PAUP*4.0b10 for each set of profiles using total character differences to demonstrate the similarity of the different profiles seasonally and by treatment (Swofford 2002).

Analysis of plant growth performance At each sampling interval, ten randomly selected tillers were harvested from each pot and used to determine tiller height, the average number of leaves per tiller, and tiller dry weight. The total number of tillers per pot and mean tiller dry weight were used to estimate total biomass per pot at each sampling interval. Tiller height was estimated from the base of the tiller to the youngest ligule. From the remaining tillers in each pot, two tillers were selected and the second youngest

leaf on each was used to measure leaf areas using a portable leaf area meter (LiCOR 3000, LiCOR Instruments, Lincoln, NE). These leaves were weighed, subsequently dried (90ºC for 48 hours) reweighed to determine specific leaf mass (SLM; leaf mass per unit area in g m^{-2}) and retained for leaf nutrient analysis. At the end of the experiment, all above ground plant material from the pots was harvested and dried for determination of total above-ground biomass.

Total C and N contents of leaves harvested for SLM were determined by the Stable Isotope/Soil Biology Laboratory (University of Georgia, Athens, USA) using a NA1500 CHN analyzer (Carlo Erba Instruments, Milan, Italy). Total leaf P content was determined following wet digestion in H_2SO_4 using an ascorbic acid colorimetric technique (John 1979). Leaf tissue from harvested tillers was dried at 90ºC for 48 hours and reserved for total starch determination following the procedures of Rasmussen and Henry (1990).

Photosynthetic gas exchange was measured at each sampling interval using a portable open-flow infrared gas exchange system (LiCOR 6400, LiCOR Instruments) with a full environmentally-controlled cuvette (Burke et al. 2002b). Net assimilation responses to internal CO_2 concentration (A/C_i curves) were generated to calculate carboxylation efficiency of the mesophyll (the linear portion of the curve; CE) and CO_2 saturated photosynthetic capacity (A_{max}) with least-squares non-linear regression analysis using the exponential model of (Jacob et al. 1995). Control software was used to maintain leaf-to-air vapor pressure deficit at 2.5 kPa, with leaf temperatures adjusted to seasonal mean by altering power to a Peltier-cooling block attached to the cuvette. Cuvette CO_2 concentrations were reduced to 50 ppm, then raised incrementally to saturating levels

(2,000 ppm) by mixing ambient air with CO_2 introduced from an internal CO_2 source. The leaf was allowed to equilibrate for at least 3 min. at each CO_2 concentration before logging data. Spot gas exchange measurements were made to determine instantaneous net assimilation (A_{net}) and stomatal conductance to water vapor (g_s) using a LiCOR 6400 as described above with the cuvette maintained under ambient atmospheric conditions (400 ppm CO_2 , 750 μmol m^{-2} s^{-1} PAR).

Statistical Analyses Two-way ANOVA was used to determine whether differences in growth, tissue C and N content, A_{max}, and CE existed between Benomyl-treated and non-treated plants at three key periods of the growing season (vegetative growth, reproduction, and senescence). Percent tissue N and C content were arcsine transformed and C/N ratios were $\log_{10}$ transformed to meet ANOVA data distribution assumptions (Zar 1998). Post-hoc means comparisons were made using LSD, and general linear contrasts (t-test) were used to test the significance of specific contrasts that underlay any higher-order interaction. The significance level was set at $p=0.05$.

Results

Analysis of pore water and soil samples Values for most physicochemical parameters did not vary significantly during the season or between non-treated and Benomyl-treated cores. Soil temperature was relatively consistent with values from 21 to 26°C during the growing season. No significant differences were found between treatments for sulfide, sulfate or ammonium in pore water though concentrations of these ions changed during the growing season and with depth of the cores. Sulfide concentrations ranged between 0.26 ± 0.08 to 0.64 ± 0.08 mg l^{-1} for non-treated and Benomyl-treated samples, respectively during vegetative growth at the 2.5 cm depth and reached highest levels

during reproduction when concentrations ranged between 2.0 ± 0.7 to 3.4 ± 1.0 mg l^{-1}. Sulfide concentrations generally increased with depth of the cores (up to 4.2 ± 1.0 mg l^{-1} at 7.5 cm). Sulfate concentrations ranged between 929 ± 278 mg l^{-1} and 1779 ± 468 mg l^{-1} over the course of the study, reaching highest levels during reproduction. Lower levels were found with depth (up to 698 ± 102 mg l^{-1} at 7.5 cm). Mean ammonium levels for each treatment ranged from 4.5 ± 0.5 to 5.4 ± 0.5 mg l^{-1} during vegetative growth to 7.4 ± 0.9 to 9.8 ± 2.4 during senescence for non-treated and Benomyl-treated samples, respectively. Ammonium levels were generally lower at 2.5 cm and increased with depth, but these differences were not significant.

Significant differences in physicochemical parameters between treatments were only found for soil pH and redox potential (Fig. 3.1). Values for both parameters displayed contrasting patterns with redox potential generally declining with depth and values for pH increasing. Redox potential in Benomyl-treated cores were always consistently higher than those in non-treated cores at the 2.5 cm depth seasonally ($F=11.26$, $P \leq 0.002$). On the contrary, pH was always lower in Benomyl-treated cores at the 5.0 cm depth ($F=19.7$, $P<0.001$) and at the 7.5 cm depth ($F=22.7$, $P \leq 0.001$). Differences in pH were not significant at the 2.5 cm depth at any sampling time (Fig. 3.1).

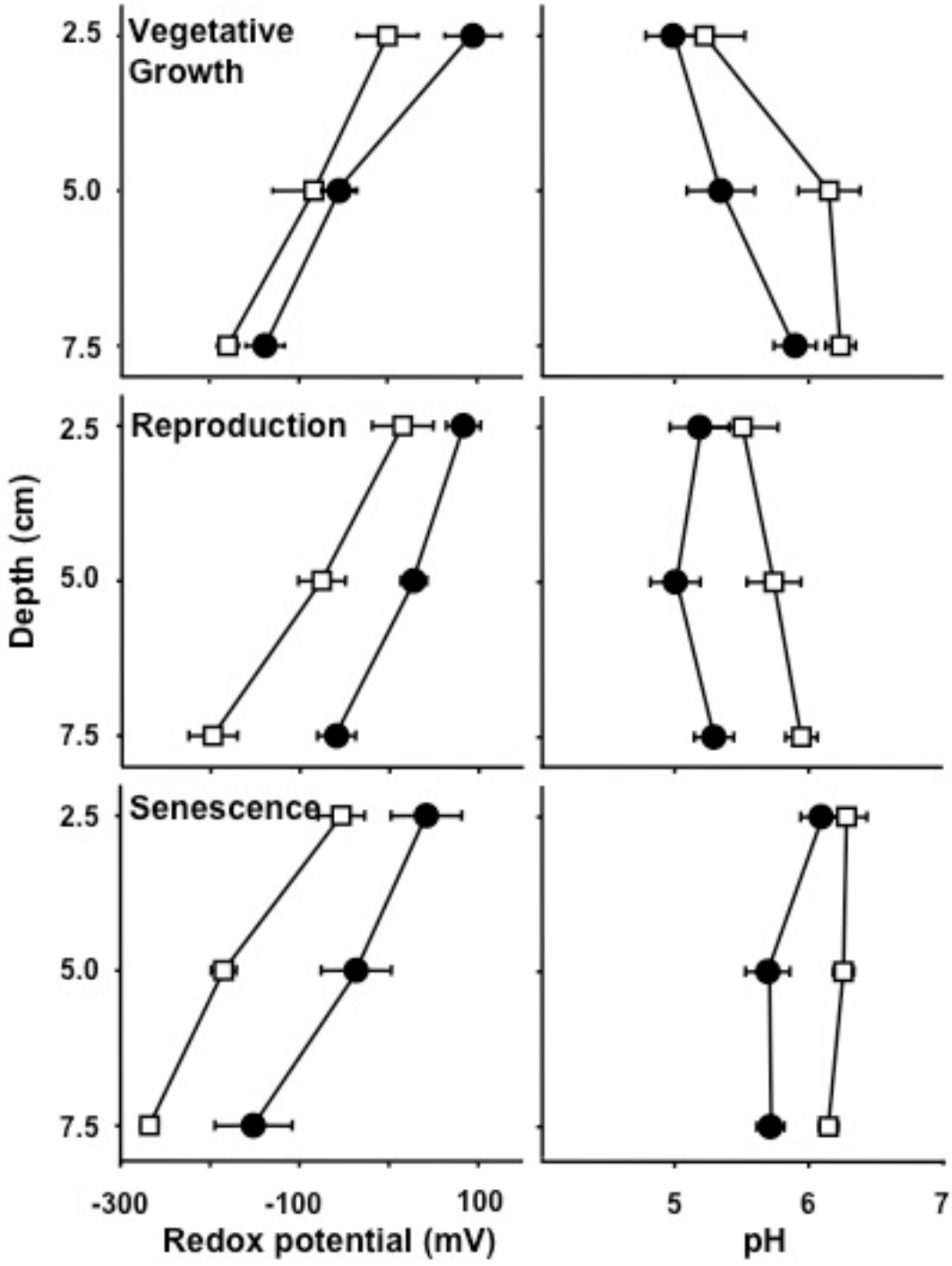

Fig. 3.1: Redox potential and pH soil physiochemical parameters. Depth profiles of redox potential (left panel) and pH (right panel) in Benomyl-treated (●) and non-treated (□) cores at 3 plant phenological stages, i.e., vegetative growth (April), reproduction (May) and senescence (August).

Analysis of AMF Plants in non-treated cores displayed a significant increase in root length colonized by AMF ($F=11.8$, $P<0.001$) compared to plants in Benomyl-treated cores at the 2.5 cm depth during vegetative growth and reproduction (26.6 and 19.9%, respectively). By senescence, this high percentage of colonization was reduced to the original value of about 11.5%, the same level seen when the experiment was set up in the greenhouse (Table 3.1). Benomyl-treatment kept the level of AMF colonization on roots of *S. patens* constant at about 11.5%, with no significant changes with depth or during the season. In contrast to plants in Benomyl-treated cores, root length colonized by AMF on plants in non-treated cores declined significantly with depth ($F=4.28$, $P<0.02$). Although treatment differences were not significant for arbuscular and vesicular colonization, vesicular colonization of plants in non-treated cores was generally higher than that of plants in cores with Benomyl-treatment (Table 3.1).

Table 3.1: AMF on roots of *Spartina patens* grown in cores with and without Benomyl-treatment at key stages of the growing season [X (± SE)] [1]

	Vegetative Growth		Reproduction		Senescence	
	Non-treated	Benomyl-treated	Non-treated	Benomyl-treated	Non-treated	Benomyl-treated
2.5 cm Depth						
Root Length Colonized (%)	26.3(3.7)	11.5 (2.5)	19.9(3.7)	11.3 (2.0)	16.0(2.8)	15.3 (4.0)
Arbuscular Colonization (%)	5.2 (2.4)	2.8 (0.6)	2.7 (0.7)	1.3 (0.7)	2.9 (0.9)	1.0 (0.3)
Vesicular Colonization (%)	5.0 (1.2)	2.5 (0.8)	3.6 (1.1)	1.7 (0.5)	3.7 (0.6)	3.9 (1.2)
5.0 cm Depth						
Root Length Colonized (%)	20.2(7.1)	13.1 (3.1)	15.4(5.5)	9.5 (2.6)	10.6(1.8)	10.2 (2.7)
Arbuscular Colonization (%)	5.8 (2.9)	2.4 (0.7)	3.1 (1.3)	1.6 (0.6)	1.1 (0.6)	0.6 (0.3)
Vesicular Colonization (%)	4.7 (2.0)	3.0 (1.0)	2.2 (1.4)	1.4 (0.6)	2.2 (0.4)	2.5 (0.8)
7.0 cm Depth						
Root Length Colonized (%)	14.1(4.2)	6.4 (2.1)	16.1(3.5)	8.6 (2.0)	7.5 (1.1)	7.9 (1.5)
Arbuscular Colonization (%)	4.4 (1.9)	2.8 (0.9)	0.9 (0.2)	0.6 (0.5)	1.1 (0.5)	1.2 (0.6)
Vesicular Colonization (%)	1.9 (0.8)	1.3 (0.5)	3.3 (0.9)	1.8 (0.5)	1.5 (0.4)	1.9 (0.3)

[1] Mean colonization during dormancy: root length colonized, 11.9 ± 3.6 %; arbuscular colonization, 0.8 ± 0.5 %; vesicular colonization, 2.8 ± 1.3%.

Analysis of plant growth performance Benomyl application resulted in reduced plant growth as evidenced from early (i.e., vegetative growth) and mid-season (i.e., reproduction) above-ground biomass estimations (5.5 ± 0.5 g per pot compared to 9.2 ± 1.5 g and 12.3 ± 1.3 g per pot compared to 17.4 ± 2.6 g, respectively) (Table 3.2, F= 5.0,

P=0.03); however, these differences disappeared by senescence when plants in non-treated cores averaged a total above-ground biomass of 12.6 ± 1.3 g per pot (approximately 100 cm^{-2}) and plants from Benomyl-treated cores 12.3 ± 2.0 g per pot (Table 3.2). Below-ground biomass did not vary with time or treatment in our study (Table 3.2). Similar to above-ground biomass, Benomyl-treatment resulted in fewer tillers on plants during vegetative growth and reproduction compared to plants from non-treated cores but these differences were not significant (F=3.7, P=0.06) (Table 3.2). In all treatments, tiller height increased significantly over time (F=5.2, P=0.01). In the vegetative growth period, tillers from plants in non-treated cores were significantly taller (t-Test; F=2.2, P=0.05) than tillers from plants from Benomyl-treated cores, but this difference disappeared with time (Table 3.2).

Plants in non-treated cores produced a significantly smaller number of leaves (F=5.9, P=0.02) of significantly higher specific leaf mass (SLM; F=7.7, $P \leq 0.01$) than plants from Benomyl-treated cores (Table 3.2). SLM increased significantly (F=38.5, $P \leq 0.001$) for plants in both non-treated and Benomyl-treated cores over the course of the study, however, by the end of the growing season SLM was not different between treatments. This seasonal increase in SLM was matched by a seasonal decrease in leaf area (F=19.9, $P<0.001$). However, there were no differences between treatments.

In both non-treated and Benomyl-treated cores, leaf N content declined significantly with time (Table 3.2). Benomyl application did not affect leaf N content on a dry weight basis (Table 3.2), however, after correcting for SLM, total leaf N concentration on an area basis was greater on plants from non-treated cores. These differences, however, were not statistically significant (F=2.9, P=0.09). Benomyl application did not affect leaf

phosphorus (P) on a dry weight basis, but total leaf P concentration on an area basis was greater, though statistically not different in plants from non-treated cores compared to those on Benomyl-treated cores (F=3.7, P=0.06). Total carbon content did not vary with time or treatment (Table 3.2), while plant starch content rose seasonally with no significant differences between treatments (Table 3.2).

Table 3.2: Growth characteristics of *Spartina patens* grown in cores with and without Benomyl treatment at key stages of the growing season [X (± SE)]

	Vegetative Growth		Reproduction		Senescence	
	Non-treated	Benomyl-treated	Non-treated	Benomyl-treated	Non-treated	Benomyl-treated
Above-ground Biomass (g [100 cm]$^{-2}$)	9.2 (1.5)	5.5 (0.5)	17.4 (2.6)	12.3 (1.3)	12.6 (1.3)	12.3 (2.0)
Below-ground Biomass (mg [g soil]$^{-1}$)	351 (55)	274 (62)	404 (42)	402 (65)	346 (36)	318 (58)
Tiller (# [100 cm]$^{-2}$)	158 (20)	114 (9)	175 (19)	146 (15)	149 (16)	144 (20)
Specific Leaf Mass (g m^{-2})	75.2 (4.7)	63.4 (2.9)	106.0 (6.5)	81.2 (7.0)	120.7 (6.2)	118.3 (15.7)
Tiller Height (cm)	32.1 (1.7)	26.8 (2.4)	35.6 (2.6)	34.9 (3.0)	35.5 (2.1)	36.4 (2.4)
Leaves (# per Culm)	3.3 (0.07)	3.4 (0.1)	3.0 (0.2)	3.6 (0.2)	3.2 (0.3)	3.6 (0.2)
Starch Content (mg g^{-1})	3.6 (0.8)	2.5 (0.5)	2.7 (0.4)	2.1 (0.2)	2.8 (0.3)	3.2 (0.3)
C Concentration (g [100 g]$^{-1}$)	40.0 (1.3)	39.9 (1.1)	38.6 (1.6)	39.6 (2.3)	39.7 (0.2)	41.5 (0.6)
N Concentration (g [100 g]$^{-1}$)	2.3 (0.1)	2.7 (0.1)	1.7 (0.1)	1.9 (0.2)	1.2 (0.1)	1.2 (0.1)
P Concentration (g [100 g]$^{-1}$)	0.13 (0.01)	0.15 (0.01)	0.09 (0.02)	0.09 (0.01)	0.04 (0.01)	0.02 (0.01)
Leaf Canopy N (mmol m^{-2})	121.7 (10.3)	117.6 (8.3)	136.7 (9.9)	104.4 (10.5)	107.4 (8.2)	99.6 (6.6)
Leaf Canopy P (mmol m^{-2})	3.0 (0.2)	3.0 (0.2)	3.2 (0.7)	2.1 (0.2)	1.5 (0.3)	0.7 (0.1)

Pooled across the sampling periods, plants from non-treated cores were not significantly different in A_{max} (32.3 μmol m^{-2} s^{-1}) than plants from Benomyl-treated cores (31.7 μmol m^{-2} s^{-1}). A_{max} increased significantly with time, being lowest during vegetative growth and higher during reproduction and senescence (Table 3.3). However, during reproduction A_{max} was significantly higher (post-hoc t-statistic = 2.34; $P \leq 0.05$) in plants from non-treated cores (38.0 ± 2.4 μmol m^{-2} s^{-1}) compared to those in Benomyl-treated cores (27.4 ± 1.6 μmol m^{-2} s^{-1}), and there was a significant increase in A_{max} from reproductive to senescent sampling periods in plants in Benomyl-treated cores (post-hoc t-statistic = 9.33; $P = 0.0034$) that drove a significant treatment by time interaction (F = 9.81; $P \leq 0.05$). Carboxylation efficiency significantly decreased during the study period (F = 58.9; $P \leq 0.001$; Table 3.3), with no treatment-specific differences over time (Table 3.3). Instantaneous gas exchange measurements of photosynthesis (A_{net}) under ambient conditions were not different between treatments at the time of measurement (Table 3.3). A_{net} declined significantly over time for plants from Benomyl-treated cores (F=8.44, $P \leq 0.01$) but not for plants from non-treated cores (Table 3.3). Stomatal conductances (g_s) did not differ between treatments, but increased significantly over the growing season (F = 25.8; $P \leq 0.001$) (Table 3.3).

Table 3.3: Photosynthetic performance of *Spartina patens* grown in cores with and without Benomyl treatment at key stages of the growing season [X (± SE)]

	Vegetative Growth		Reproduction		Senescence	
	Non-treated	Benomyl-treated	Non-treated	Benomyl-treated	Non-treated	Benomyl-treated
Saturated Photosynthetic Rate (A_{max}, µmol CO_2 m^{-2} s^{-1})	24.9(1.8)	24.9 (1.3)	38.0 (2.4)	27.4 (1.6)	34.2 (6.0)	41.2 (3.8)
Carboxylation Efficiency (CE, µmol CO_2 m^{-2} s^{-1})	500 (30)	609 (50)	280 (60)	240 (2)	140 (60)	160 (30)
Instantaneous Photosynthetic Rate (A_{net}, µmol CO_2 m^{-2} s^{-1})	10.7(1.0)	13.7 (1.4)	9.9 (0.6)	8.5 (0.8)	12.0(3.5)	8.4 (0.5)
Stomatal Conductance (g_s, mmol m^{-2} s^{-1})	58.8(6.9)	81.3 (8.7)	89.1(11.1)	87.1 (7.9)	187.2(37.7)	190.8(13.6)

Analyses of microbial community structure RFLP profiles of *nif*H gene fragments that had been generated by nested PCR with DNA as template and cleaved with endonuclease *Hae*III on average were 86% similar and those profiles generated using cDNA as template were 79% similar (Fig 3.2). However, profiles generated with DNA and cDNA on average were only 42% similar to each other suggesting that the present community reflected in profiles generated using DNA as template was very different from the active community reflected in profiles generated using expressed genes by reverse transcription of mRNA (cDNA) as template (Fig 3.2). On average profiles generated with DNA had 5.5 bands, while those generated with cDNA had only 3 bands and did not necessarily represent subsets of those generated with DNA indicating that the present community was different from the active community (Fig 3.2). Benomyl-treatment had the greatest effect on bacterial community structure at the vegetative point when DNA and cDNA

generated profiles between non-treated and Benomyl-treated samples were only 70% and 80% similar, respectively; at all other time points the profiles were more similar (Fig 3.2).

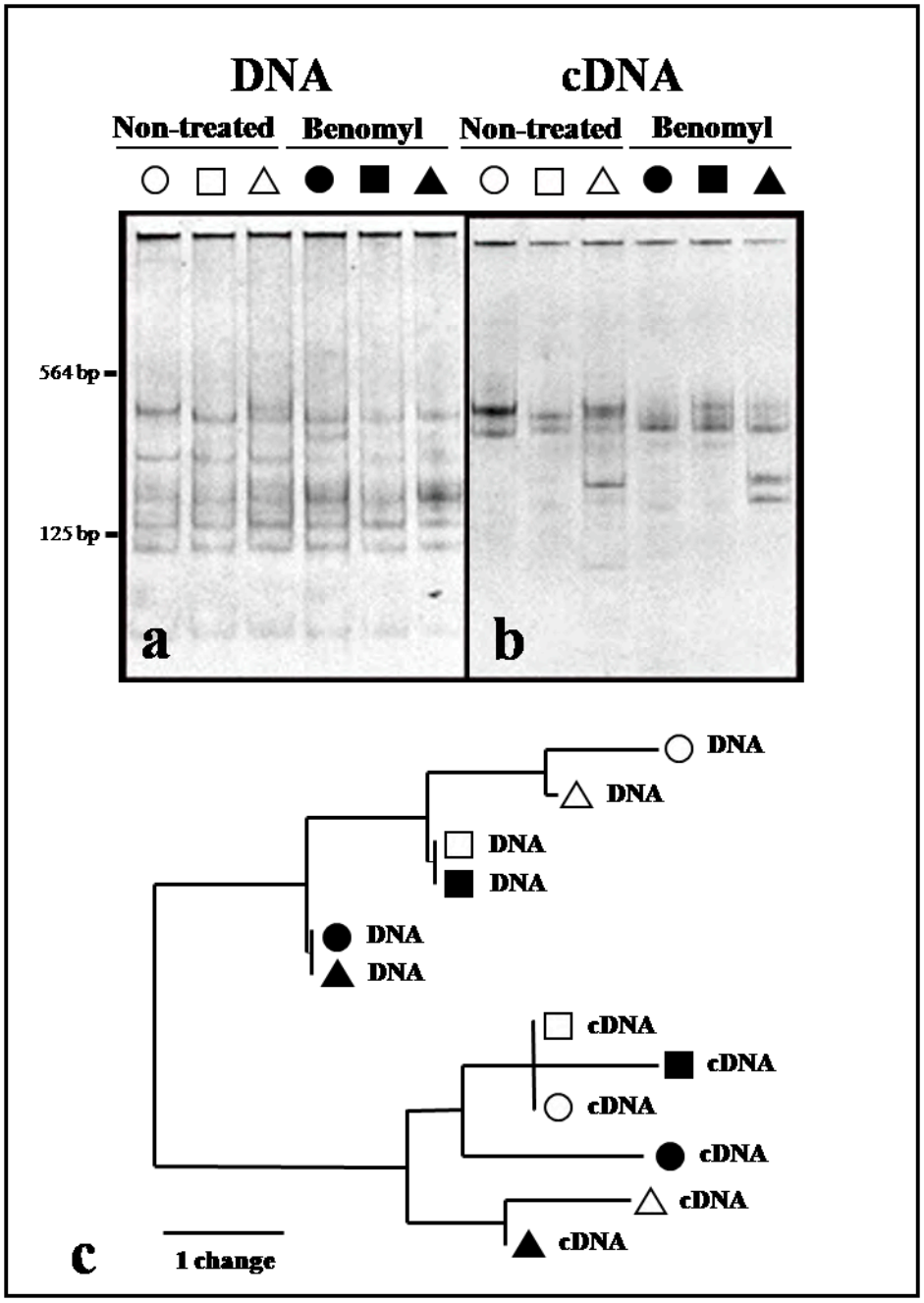

Fig. 3.2: RFLP profiles of all nitrogen-fixing bacteria. RFLP acrylamide gel of *Hae*III restricted *nif*H PCR product generated using the NifHforB and Nifrev primer set (Widmer et al. 1999) in a nested PCR from DNA (a), and cDNA (b) extracted from soil in Benomyl-treated (●) and non-treated (○) cores with *S. patens* at a depth of 2.5 cm corresponding to 3 plant phenological stages, i.e., ○ vegetative growth (April), □ reproduction (May) and △ senescence (August). Each well has digested PCR product from 2 replicate plant cores. The size marker (M) is lambda DNA cleaved with *Hind*III. (c) Corresponding neighbor joining phylogram from comparative analysis of RFLP fragment presence or absence generated using PAUP*4.0b10 (Swofford 2002).

More specific analyses of a subset of these bacteria, i.e. of nitrogen-fixing members of the ε-subdivision of Proteobacteria, by PCR-RFLP of fragments generated with *Dde*I showed that profiles generated with DNA were 91% similar, while those generated with cDNA were only 60% similar suggesting that the active community varied much more seasonally and in response to treatment than the present community did (Fig 3.3). On average the DNA generated profiles were 68% similar to the cDNA generated profiles (Fig 3.3). In contrast to the RFLP analysis of all nitrogen fixing bacteria, the communities of both present and active non-treated and Benomyl-treated samples were more different at the senescence time point than at the vegetative time point. The DNA generated profiles of non-treated and Benomyl treated samples were identical at the vegetative time point but at senescence they were only 80% similar. The cDNA generated profiles of non-treated and Benomyl treated samples were 73% similar at the vegetative time point and only 60% similar at senescence. This indicates changes in the community structure of nitrogen-fixing members of the ε-subdivision of Proteobacteria over the season in response to treatment.

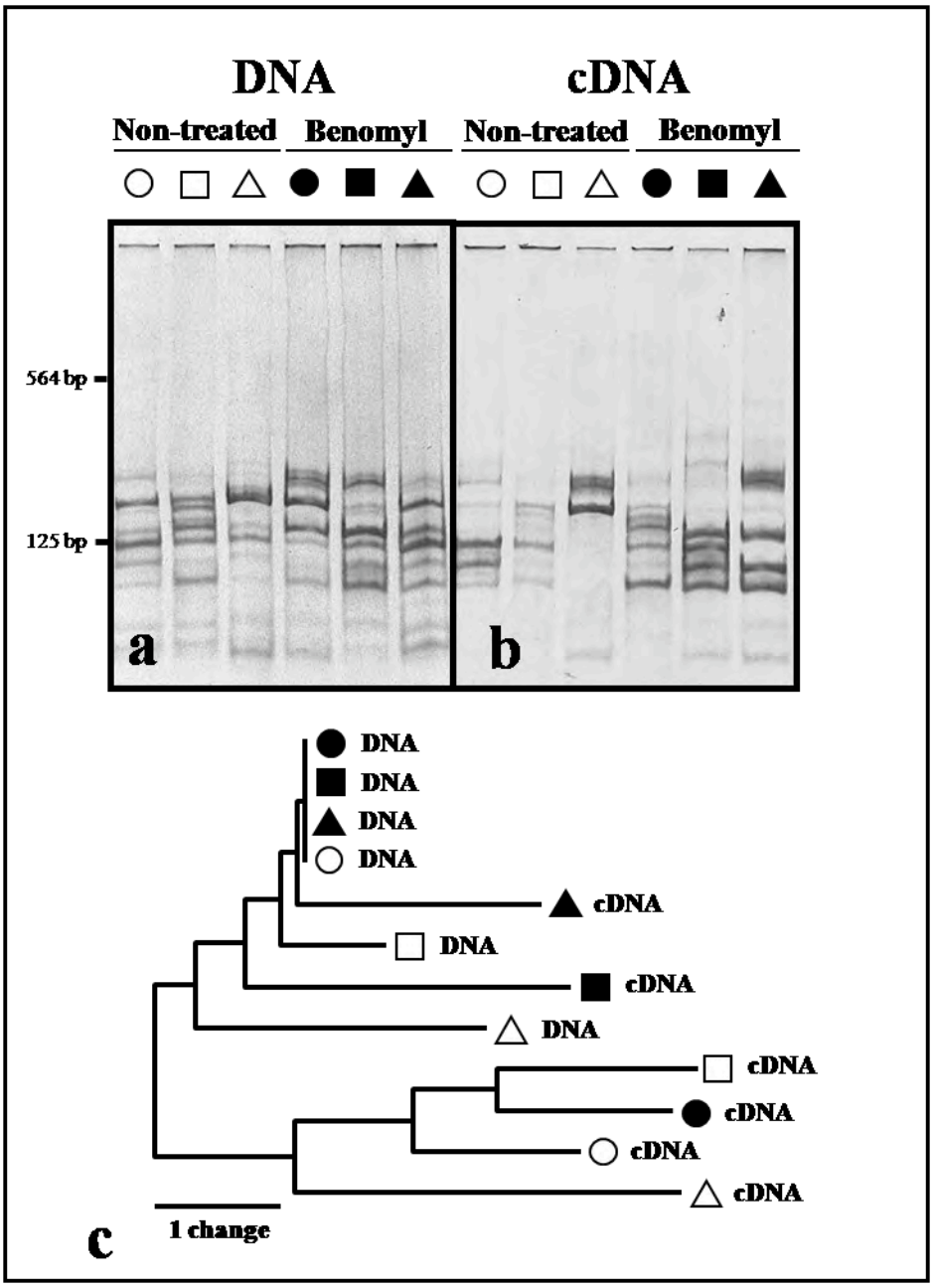

Fig. 3.3: RFLP profiles of nitrogen-fixing bacteria of the ε-subclass of Proteobacteria. RFLP acrylamide gel of *Dde*I restricted *nif*H PCR product for the ε-subclass of Proteobacteria generated using the ENFBf and ENFBr primer set (Welsh et al. 2007) in a nested PCR from DNA (a), and cDNA (b), extracted from soil in Benomyl-treated (●) and non-treated (○) cores with *S. patens* at a depth of 2.5 cm corresponding to 3 plant phenological stages, i.e., ○ vegetative growth (April), □ reproduction (May) and △ senescence (August). Each well has digested PCR product from 2 replicate plant cores. The size marker (M) is lambda DNA cleaved with *Hind*III. (c) Corresponding neighbor joining phylogram from comparative analysis of RFLP fragment presence or absence generated using PAUP*4.0b10 (Swofford 2002).

In contrast to PCR-RFLP analyses, more specific analyses by DGGE to generate profiles of individual of nitrogen-fixing members of the ε-subdivision of Proteobacteria showed that on average the DNA generated DGGE profiles were only 64% similar but the cDNA generated profiles were 78% similar. This suggests that detection of present individuals varied much more seasonally and in response to treatment than the detection of active individuals (Fig 3.4). Additionally, the average number of bands in DNA generated profiles was 8.2 but the average in cDNA generated profiles was 4 bands and did not necessarily represent subsets of those generated with DNA suggesting that the active community was less diverse and different from the present community (Fig 3.4). The DGGE profiles of individual nitrogen-fixing members of the ε-subdivision of Proteobacteria reflected a similar trend in increased differences between treatments over the season as was shown in the RFLP community profiles. DNA generated profiles of non-treated and Benomyl treated samples were 58% similar at the vegetative time point and 54% similar at the senescent time point. The cDNA generated profiles of non-treated and Benomyl treated samples were 87% similar at the vegetative time point and only 75% similar at the senescent time point suggesting there were changes in community structure over the season in response to treatment.

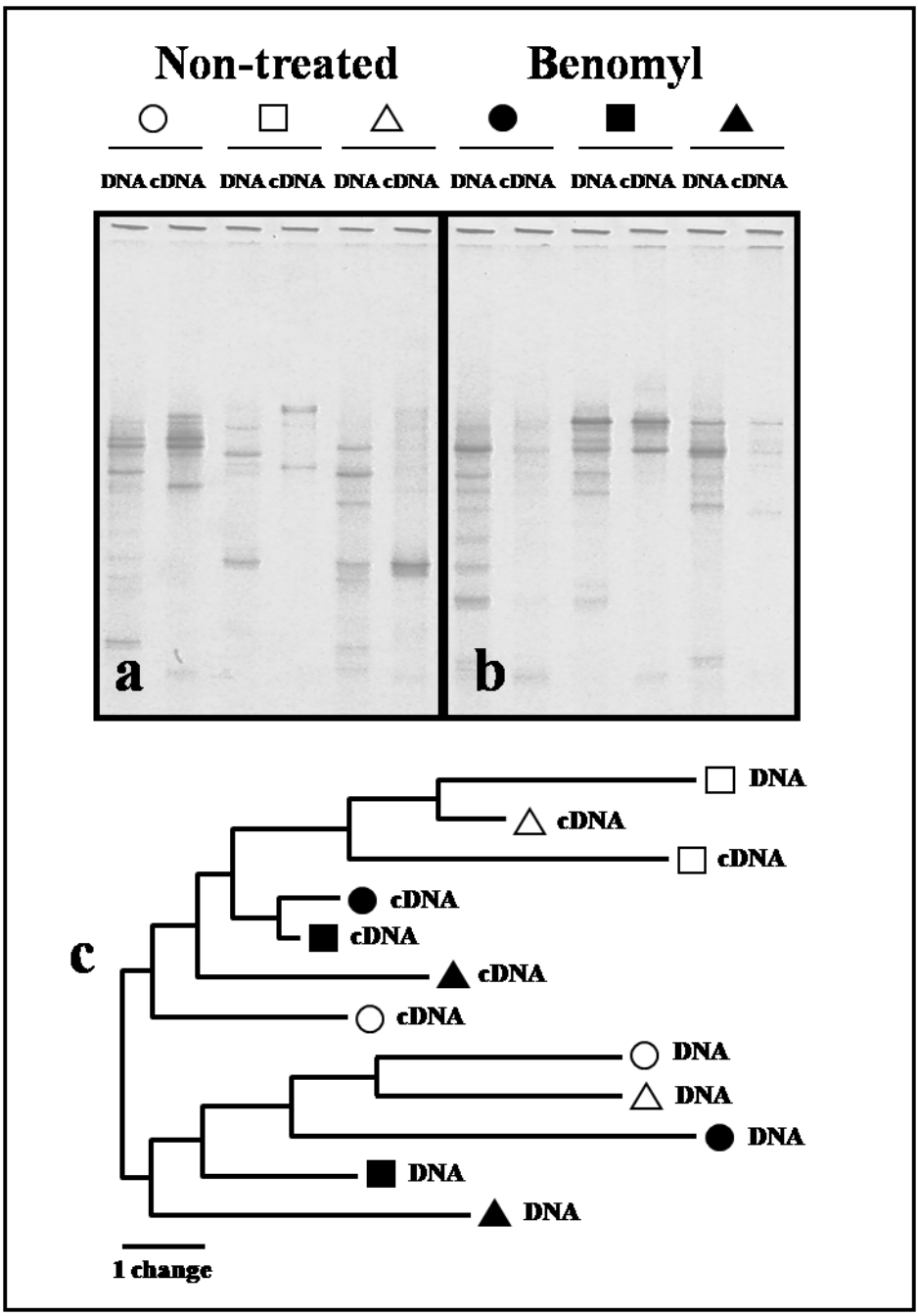

Fig. 3.4: DGGE profiles of nitrogen-fixing bacteria of the ε-subclass of Proteobacteria. DGGE profiles of *nif*H PCR product for the ε-subclass of Proteobacteria generated with the ENFBf and ENFBr primer set in a nested PCR that had been generated with either DNA (a) or cDNA (b) as template extracted from soil in Benomyl-treated (●) and non-treated (○) cores with *S. patens* at a depth of 2.5 cm corresponding to 3 plant phenological stages, i.e., ○ vegetative growth (April), □ reproduction (May) and △ senescence (August). Each well has PCR product from 2 replicate plant cores. (c) Corresponding neighbor joining phylogram from comparative analysis of DGGE fragment presence or absence generated using PAUP*4.0b10 (Swofford 2002).

Discussion

Colonization of *S. patens* roots by AMF showed a strong seasonal dynamic with highest values found during vegetative growth early in the growing season. These results are in agreement with many others that demonstrated seasonal changes in root colonization of plants in different environments (Allen 1996; DeMars and Boerner 1995; Garcia and Mendoza 2007; Lugo et al. 2003), including different wetlands where highest values of AMF colonization on different plant species were generally demonstrated for the major period of growth (Bohrer et al. 2004). Colonization decreased with depth, most likely due to reduced oxygen availability in water-saturated sediments as suggested by more negative redox potentials (Miller 2000). Flooding is meant to be a major factor reducing oxygen availability and thus AMF colonization (Miller 2000), however, fluctuations in the water table or tidal inundation providing periodic aeration might actually increase AMF colonization (van Hoewyk et al. 2001).

Studies investigating the effects of AMF on plant growth performance, competition and resilience to abiotic stress have frequently relied on the use of the systemic fungicide Benomyl to create controls with suppressed mycorrhizal activity (e.g., Callaway et al. 2004; Grogan and Chapin 2000; Hart and Brookes 1996; Hartnett and Wilson 1999; Helgason et al. 2007; Schmidt et al. 2000; Smith et al. 2000; Smith et al. 1999). The application of Benomyl in our study resulted in a significant suppression of AMF on roots of *S. patens* with values that did not change in time or depth, and represented those obtained on dormant plants, similar to our previous studies (Burke et al. 2002b; 2003). The result that Benomyl-treatment did not eliminate AMF is in agreement with those of other studies (Fitter and Nichols 1988; Wilson et al. 2001). Since the level of AMF colonization on *S. patens* roots was kept constant at the same level throughout the

growing season in our study, while plants in non-treated cores displayed a significant increase in colonization during vegetative growth and reproduction with a subsequent decrease to the original level at senescence, a functional suppression of AMF is a likely consequence of Benomyl-treatment in this study.

Similar to our previous studies (Burke et al. 2002b; 2003), the experimental set-up in this study resulted in consistent environmental conditions within and between cores, with values for most physicochemical parameters being not significantly different at comparable depths. Significant effects of Benomyl application were only obtained for pH with lower values and for the redox potential that were consistently more positive than in non-treated cores. For redox potential, these differences were consistent with results of our previous studies (Burke et al. 2002b; 2003); for pH, however, differences were not detected between treatments over the entire depth of the core in our short-term study (i.e., 6 weeks after Benomyl-treatment) (Burke et al. 2002b), or not displayed between treatments at the depth of 2.5 cm even though seasonal changes were observed (Burke et al. 2003). Since redox potential and pH in wetland soils are tightly connected, with increased flooding of acidic, organic soils resulting in increases of pH and concomitant declines in redox potential (Stumm and Morgan 1996), we had speculated in our previous study that focused on the depth of 2.5 cm that the higher pH and lower redox potential values in non-treated cores as compared to Benomyl-treated cores might reflect higher soil oxygenation in Benomyl-treated cores presumably as a consequence of declines in respiration of aerobic AMF.

Growth measurements indicate plant growth was improved in non-treated cores, at least early in the growing season. Mycorrhizal fungi create strong carbon sinks within the

plant-fungal system (Smith and Read 1997) and variation in sink carbon demand can induce change in photosynthetic activity (Stadden et al. 1999; Wright et al. 1998), a situation that could partly explain the variation in the regulation of photosynthetic demand we observed (Table 3.3). Yet, plant growth often does not increase from such stimulation and it is expected that the additional fixed carbon is respired by the fungal symbiont (Stadden et al. 1999; Wright et al. 1998). Final harvest biomass found no overall growth increase in plant mass from non-treated cores, but growth was delayed in Benomyl-treated cores as evidenced from early season growth measurements (Table 3.2). In addition, A_{max} was lower at reproduction, then higher at the senescence stage in plants from Benomyl-treated cores, further suggesting that seasonal physiological development was concurrently delayed with growth, and senescence, very likely as a consequence of AMF suppression.

Leaf N content is generally a good predictor of photosynthetic capacity in C_4 plants (Sage and Pearcy 1987). Seasonal leaf N content declines likely reflected dilution as biomass increases with time. Declines of the magnitude in our study have been previously recorded for *S. alterniflora* (Dai and Wiegert 1997); yet the declines in leaf N content in our study were never so great as to approach the estimated critical N leaf concentration for *Spartina* of 0.7% (Smart and Barko 1980). Correlation between leaf N content and A_{max} were not tight, while changes in mesophyll CE were. A_{max} is usually associated with an increase in the total concentration of carboxylation sites (Huxman et al. 1998; Jacob et al. 1995), while CE is a reflection of the kinematic constraints imposed by the carboxylation enzyme (Von Cammerere and Farquhar 1981). Thus, the seasonal increases in A_{max} and attendant decreases in CE likely reflect an increase in the total

concentration of PEP-carboxylase and Rubisco earlier in the growing period, and an associated down-regulation of enzyme activity during the latter. However, without additional biochemical data, this assertion remains uncertain.

Variation in photosynthetic activity in *S. patens* were attended by a suite of changes in plant growth and leaf attributes which indicates that potential reductions in photosynthetic capacity may have been compensated for by an increase in leaf number in Benomyl-treated cores. These growth limitations are certainly due, in part, to lower total leaf canopy N contents in plants from Benomyl-treated cores. This suggests that Benomyl-treatment and attendant suppression of AMF affected both N-uptake and growth, partly through reduced photosynthesis. The most significant effect AMF may have had therefore was to "jumpstart" plant growth, hastening the periods of active growth as well as senescence.

*nif*H gene diversity (i.e., DNA) was used as a proxy for the diversity of all present nitrogen fixing bacteria and gene transcription and thus mRNA detection as a measure for potentially active bacteria (i.e., cDNA). Comparative analyses suggested that these communities were quite different (Fig 3.2). Community profiles generated using cDNA as template were not merely a less diverse version of the profiles using DNA as template as suggested in comparative studies by others using either ribosomal RNA genes or cDNA of the rRNA as template for amplification (Duineveld et al. 2001; Griffiths et al. 2000; Teske et al. 1996). Our results are corroborated by other studies on the diversity of nitrogen fixing bacteria that used DNA and cDNA as templates, which did not necessarily result in overlapping profiles (Knauth et al. 2005; Mårtensson et al. 2009; Wartiainen et al. 2008). Large differences were also retrieved for *nif*H gene clone

libraries from lake plankton where most of the cDNA derived sequences did not group closely with those obtained from DNA (Zani et al. 2000). In our previous study, profiles for nitrogen-fixing members of the ε-subdivision of Proteobacteria generated with either DNA or cDNA from the same sample also did not necessarily result in similar banding patterns, even though some fragments might be shared or at some sampling times profiles might even match (Welsh et al. 2007). These results suggest that only a small number of those organisms that have the metabolic potential to fix atmospheric nitrogen are in fact expressing those genes and illustrate the importance of targeting potentially active organisms to describe those organisms most likely contributing to plant growth and ecosystem functioning.

Only small changes in the present community structure of nitrogen-fixing bacteria in general and more specifically of those belonging to the ε-subdivision of Proteobacteria were observed when RFLP profiles were used as a proxy for the overall diversity of populations present, i.e. the DNA generated community profiles were 86% and 91% similar, respectively. This result is consistent with those other studies that have generally failed to detect changes in the community composition of nitrogen-fixing bacteria in marsh sediments (Burke et al. 2002a; Piceno and Lovell 2000a; b; Piceno et al. 1999), and not surprising considering the ability of many microorganisms to adapt to adverse environmental conditions by the formation of dormant stages such as spores or dwarf cells that are inactive but remain detectable by molecular methods.

Both DNA and cDNA generated profiles detecting the present community versus the active community of all nitrogen fixing bacteria indicated greater differences in bacterial community structure between non-treated and Benomyl-treated samples at the vegetative

time point than at any other point in the season when the treatment effect of Benomyl on AMF colonization was highest. Although Benomyl is meant to have little effect on populations of many non-target microorganisms, such as algae (Johnstone et al. 2002) or bacterial populations (Allison et al. 2007; Hart and Brookes 1996; Schmidt et al. 2000; Smith et al. 2000), the addition of Benomyl to our cores created an additional variable and thus the differences obtained in diversity between treatments cannot directly be related to potential effects of AMF.

Previous studies in our laboratory on the interaction between the salt marsh plant *Spartina patens*, AMF and associated microorganisms (Burke et al. 2002b; 2003) had shown that AMF had no effect on the abundance of specific phylogenetic groups of bacteria, except for an increase in numbers of members of the γ-subdivision of Proteobacteria. The positive association between this bacterial group and root length colonized by AMF suggested that AMF could affect populations of specific soil bacterial populations in salt marsh sediment (Burke et al. 2003). In this study, the more specific analyses of nitrogen-fixing members of the ε-subdivision of Proteobacteria by both PCR-RFLP and DGGE showed a treatment effect later in the season with greater differences between non-treated and Benomyl treated samples at senescence and not during vegetative growth as indicated for the entire community of nitrogen-fixing bacteria which supports the assumption of seasonal effects on specific groups of microorganisms. Additionally, the DNA generated PCR-RFLP profiles of nitrogen-fixing members of the ε-subdivision of Proteobacteria were 68% similar to the cDNA generated profiles suggesting that this was a much higher resolution analysis in which the active community was better detected within the present community than the PCR-RFLP profiles of all

bacteria were able to reflect where the 2 communities were only 42% similar. These results indicate that broad scale analyses of the entire nitrogen-fixing community may mask seasonal variation of specific bacterial groups that compose the broader community, but also suggests the degree of seasonal variation might or might not be detected depending on the sensitivity of the analysis methods. More refined and targeted approaches focusing on specific phylogenetic groups may provide a more accurate picture of the dynamics of the nitrogen-fixing microbial community than obtained in previous studies. However, this would require replicate analyses in sufficient numbers to reconcile the variation in the detection sensitivities of different molecular tools.

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CHAPTER 4

Diversity of frankiae in root nodules of *Morella pensylvanica* grown in soils from five continents[1]

Abstract

Bioassays with *Morella pensylvanica* as capture plant and comparative sequence analyses of *nif*H gene fragments of *Frankia* populations in nodules formed were used to investigate the diversity of *Frankia* in soils over a broad geographic range, i.e., from sites in five continents (Africa, Europe, Asia, North America and South America). Phylogenetic analyses of 522-bp *nif*H gene fragments of 100 uncultured frankiae from root nodules of *M. pensylvanica* and of 58 *Frankia* strains resulted in a clear differentiation between frankiae of the *Elaeagnus* and the *Alnus* host infection groups, with sequences from each group found in all soils and the assignment of all sequences to four and five clusters within these groups, respectively. All clusters were formed or dominated by frankiae obtained from one or two soils with single sequences occasionally present from frankiae of other soils. Variation within a cluster was generally low for sequences representing frankiae in nodules induced by the same soil, but large between

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sequences of frankiae originating from different soils. Three clusters, one within the *Elaeagnus* and two within the *Alnus* host infection groups, were represented entirely by uncultured frankiae with no sequences from cultured relatives available. These results demonstrate large differences in nodule-forming frankiae in five soils from a broad geographic range, but low diversity of nodule-forming *Frankia* populations within any of these soils.

Introduction

Actinorhizal plants are characterized by their ability to form root nodules in symbiosis with the nitrogen-fixing actinomycete *Frankia*, which enables them to grow on sites with restricted nitrogen availability (2). They therefore resemble typical pioneer plants that frequently establish themselves after flooding, fires, landslides, glacial activity, as well as volcanic eruptions (13). Actinorhizal plants are nearly ubiquitously distributed world-wide, and their occurrence in diverse habitats like deserts and swamps, forests and beaches, at high or low elevations and in many other places between these extremes has been well described (see (6) for review). Information on the geographic distribution and diversity of frankiae, the symbiotic partner of actinorhizal plants, is much more limited, and usually restricted to frankiae in root nodules on specific host plants at a particular site rather than on populations in soil analyzed over a large geographic range (5, 31, 32).

Root nodules represent a natural locale of enrichment of one *Frankia* strain that can easily be characterized by a large variety of methods such as comparative sequence analyses of 16S rRNA genes (10, 46, 56), or genes like *gln*II (11, 22) or *nif*H (21, 38), but also by highly distinctive tools such as Rep-PCR (37, 47, 48), or PCR-RFLPs (36, 40, 50)

that generally require the availability of pure cultures. The usefulness of these tools is largely reduced in soils that represent highly heterogeneous environments with a tremendous diversity of organisms (16, 20, 67, 68) and with frankiae present in low numbers (approximately 10^4-10^5 cells g^{-1} soil) (23, 49) as part of a large microbial community (more than 10^9 cells g^{-1} soil) (8, 71). PCR-based methods, distinctive target genes (e.g., 16S rRNA gene), and specific primers are available for the detection of frankiae on the genus level (24); however, more specific analyses within the genus are hampered by the low abundance of frankiae in soil and the limited resolution of phylogenetically relevant target genes such as rRNAs for assessing diversity within the genus.

In a recent study we used comparative sequence analyses of *nif*H gene fragments to distinguish *Frankia* populations in different root nodules on the same plant species, and to analyze the diversity of *Frankia* populations from the same soil forming nodules on different host plant species (45). The study used plant bioassays, i.e., actinorhizal plants inoculated with dilutions of soil slurries, in order to reduce potential effects of physical and chemical characteristics of the soil on nodule formation. This study demonstrated large host plant effects on the selection of frankiae for root nodule formation as well as a large diversity of nodule-forming *Frankia* populations in one soil that could be distinguished by comparative sequence analyses of *nif*H gene fragments (45).

In this follow-up study, we used the same basic experimental setup including bioassays and comparative sequence analyses of *nif*H gene fragments of *Frankia* populations in nodules formed, to investigate the diversity of *Frankia* in soils over a broad geographic range spanning five continents. The bioassay used *Morella pensylvanica* as the capture

plant because it is known to be highly promiscuous, harboring a wide diversity of *Frankia* in root nodules (4, 14, 28). Additionally, in our previous study *M. pensylvanica* formed nodules with more diverse *Frankia* populations of the *Elaeagnus* host infection group than different *Elaeagnus* plant species, and also with *Frankia* of the second major host infection group, the *Alnus* host infection group (45), In order to adequately assign uncultured frankiae in root nodules to host infection groups and sub-groups, additional analyses focused on retrieving *nif*H gene fragment sequences from a large variety of *Frankia* strains previously isolated from nodules of plants belonging to either the *Alnus* or the *Elaeagnus* host infection group.

Materials and Methods

Soil collection Soils were collected in October 2006 from four plots (10 x 10 m, spaced every 300 m along a randomly-located 900 m transect) at sites located in 5 continents (Africa [Rwanda], Europe [Hungary], Asia [Japan], North America [Alaska], and South America [Peru]) (Table 4.1). From each of the four plots, twenty 10-cm deep soil subsamples were collected, pooled, and homogenized, for a total of four pooled samples (referred to as A, B, C, and D). Between each subplot, soil sampling equipment was sterilized to prevent cross-contamination, and the soils were kept cool and transported to Colorado State University, where the soils were then refrigerated at 4 ^{o}C until used in the experiment.

Table 4.1: Collection sites for soils from around the world

Soil Origin		Latitude, Longitude	Elevation	Description of vegetation
South America	Peru (San Isidro)	S 6°57'25", W 78°22'48"	3,696 m	grazed grassland
Europe	Hungary (Fülöpháza)	N 46°52'24.52", E 19°23'19.64"	106 m	sandy grassland (native *Festuca*)
Africa	Rwanda (Kigali)	S 1°56'45.76", E 30°03'13.40"	1,480 m	grass, forbs, cultivated plants
North America	Alaska (Anchorage)	N 61°10'07.19", W 149°45'39.43"	112 m	upland alder, willow, grasses, forbs
Asia	Japan (Tsuruoka)	N 38°43'8.88", E 139°51'29.00"	15 m	grasses and forbs, near agricultural land

Plant bioassay Plants of *M. pensylvanica* [Mirb.] Kartesz were grown from seeds that were surface sterilized in 30% H_2O_2 solution for 20 minutes and planted in autoclaved sand in propagation flats. Seed propagation flats were housed in the University Greenhouse Facility at Colorado State University with a 16:8 hour light:dark photoperiod. Seedlings were transplanted to individual cone-tainer tubes (3.8 x 21 cm; Stuewe & Sons, Inc., Corvallis, OR) containing sterilized growth media (1:1:1 calcine clay/sand/vermiculite). Plants were fertilized with a complete nutrient solution (34) and grown for about 6 months until a dense root mass filled the tube.

Two weeks prior to inoculation, the nitrogen source was removed from the nutrient solution to encourage nodulation. In December of 2006, each plant was inoculated with 5 ml of a soil slurry (20 cm^3 of soil in 100 ml of sterilized, distilled water) prepared from each of the 20 soils (5 locations x 4 plots). A total of 12 replicate plants were used for

each of the 20 soils (n = 240). Uninoculated sentinel plants were maintained in the greenhouse beside the inoculated plants to monitor contamination from exogenous frankiae. Plants were maintained in the greenhouse with adequate water and N-free nutrient solutions for 6 months. All plants survived, and, upon harvesting, a single nodule was collected from each individual plant (i.e., up to 12 nodules from plants in each soil-subplot treatment combination). Thirty percent (6 of 20) of the sentinel plants harbored nodules. Nodules were preserved in 95 % ethanol and stored at -20 °C until further use.

Sequence analyses For DNA extraction, a single lobe from each nodule collected was washed with sterile water, and the epidermis removed. The remaining tissue was homogenized with a mortar and pestle in 1 ml of sterile water, and the homogenate transferred to an Eppendorf tube and centrifuged at 14,000 x g for 1 minute. The pellets were washed once with 0.1% sodium pyrophosphate in water (wt/vol), followed by two washes with sterile distilled water. Lobe pellets as well as pellets of pure cultures (approx. 50 mg wet weight of a total of 30 strains) were re-suspended in 10 µl of proteinase K (Promega, Madison, WI, 30 U mg^{-1}, 10 mg ml^{-1} in water)/SDS [0.5 %]) solution and incubated at 37 °C for 1 hour. After an additional incubation at 80 ºC for 20 minutes, lysates were stored without purification at -20 ºC until further use.

*Nif*H gene fragments (606 bp) were amplified using *Frankia* specific primers nifHf1 ($^{5'}$GGC AAG TCC ACC ACC CAG C) and nifHr ($^{5'}$CTC GAT GAC CGT CAT CCG GC) in a reaction volume of 50 µl, containing 1 x PCR buffer, 2.0 mM $MgCl_2$, 2.5 % DMSO, 4.95 mg ml^{-1} BSA, 0.2 mM of each dNTP, 0.4 µM each primer, and 2 µl root nodule or pure culture lysate, with 0.5 U *Taq* DNA polymerase (Gene Script, Piscataway, NJ) added after an initial incubation at 96 °C for 10 minutes. The addition of *Taq*

polymerase was followed by 35 rounds of temperature cycling (96 °C for 30 seconds, 60 °C for 30 seconds and 72 °C for 45 seconds), and a final 7 minute incubation at 72 °C. Sub-samples of the reactions (10 µl) were checked for amplification products by gel electrophoresis (1 % agarose in TAE buffer, wt/vol) after staining with ethidium bromide (0.5 µg ml^{-1}) (59).

Amplified *nif*H gene fragments from 20 lobes from each site were purified using the Ultra Clean 15 DNA Purification Kit (MoBio, Carlsbad, CA), and sequenced using the CEQ 8800 Quickstart Kit according to the manufacturer's instructions (Beckman Coulter, Fullerton, CA) with the addition of 5 % DMSO to the reaction mix. The sequencing reaction consisted of an initial incubation at 76 °C for 5 minutes followed by 76 °C for 5 minutes during which primer and master mix were added, a subsequent incubation at 94 °C for 2 minutes, and 35 cycles of temperature cycling (94° C for 30 seconds, 50 °C for 30 seconds and 60 °C for 4 minutes), and a final extension at 60 °C for 10 minutes (39). Sequences were analyzed on a CEQ 8800 sequencer (Beckman Coulter) and deposited at Genbank under accession numbers FJ477419 to FJ477548.

Phylogenetic analyses Amplified *nif*H gene fragments obtained from 100 uncultured frankiae from *M. pensylvanica* root nodules and those of 30 pure cultures of *Frankia* as well as 28 sequences retrieved from public databases were trimmed to be 522 bp long and aligned using Sequencher 4.2.2 (Gene Codes Corp., Ann Arbor, MI), CLUSTAL X, and MacClade 4.05 (41, 66) and analyzed using maximum parsimony (MP), neighbor joining (NJ), Bayesian and maximum likelihood (ML) methods.

MP analysis of this dataset was completed in PAUP*4.0b10 and included 10,000 heuristic random addition replicates, TBR, and no mul trees (64). Confidence in the

topology for this MP tree was gauged using bootstrap re-sampling methods (BS) in PAUP* and included 10,000 replications and a full heuristic search (17). Only those BS values of at least 70% demonstrate good support measures and thus were retained (27).

The dataset was also analyzed using NJ methods in PAUP*. Modeltest version 3.7 determined that the GTR+I+G model of sequence evolution fit this dataset best (55). Specific values for the gamma shape parameter and proportion of invariant sites provided by Modeltest were entered under the distance settings for the NJ GTR model in PAUP*. The BS test in PAUP* included 10,000 replications and a neighbor joining search.

Bayesian analysis for the *Frankia* dataset was completed using MRBAYES version 3.0 and included Metropolis-coupled Markov chain Monte Carlo (MCMCMC) sampling, a GTR+I+G model estimated during the run, 5 million generations, and sampling every 1000 trees (29). A 95 % majority rule consensus tree for the Bayesian output of posterior probabilities (PP) was created in PAUP* with the first 30 trees removed as burn-in (30, 64).

ML analysis was completed using the RAxML-VI-HPC program (63) on the computer cluster of the ‘CyberInfrastructure for Phylogenetic RESearch’ project (CIPRES, www.phylo.org) from the online servers at the San Diego Supercomputing Center. The RAxML program is designed for fast processing of large datasets. Settings included GTR+CAT approximation for rate heterogeneity (62), invariant sites, empirical base frequencies and estimation of the BS replicates necessary for this dataset. TreeView (Win16) was used to display the treefiles (54) and MEGA version 4 was used to collapse branches (65).

Results and Discussion

PCR products of *nif*H gene fragments were obtained from nodules of *M. pensylvanica* with all soils and subplots tested, however, only in about 50 % of the nodules analyzed. The failure to retrieve products from all nodules might be due to a variety of issues. Nodules were extremely small (1 - 2 mm in diameter) which impacted the accurate separation of periderm tissue with potentially contaminating organisms from nodule tissue with frankiae, and thus frankiae could have been removed unintentionally in some preparations. Small nodules have also been shown to be formed by non-nitrogen-fixing, atypical frankiae (25, 69, 70), other actinomycetes (15), or fungi (7), or not be inhabited by any detectable organism at all (51). Thus, nodules that failed to produce amplification products in our study might have been devoid of frankiae potentially detectable by our approach.

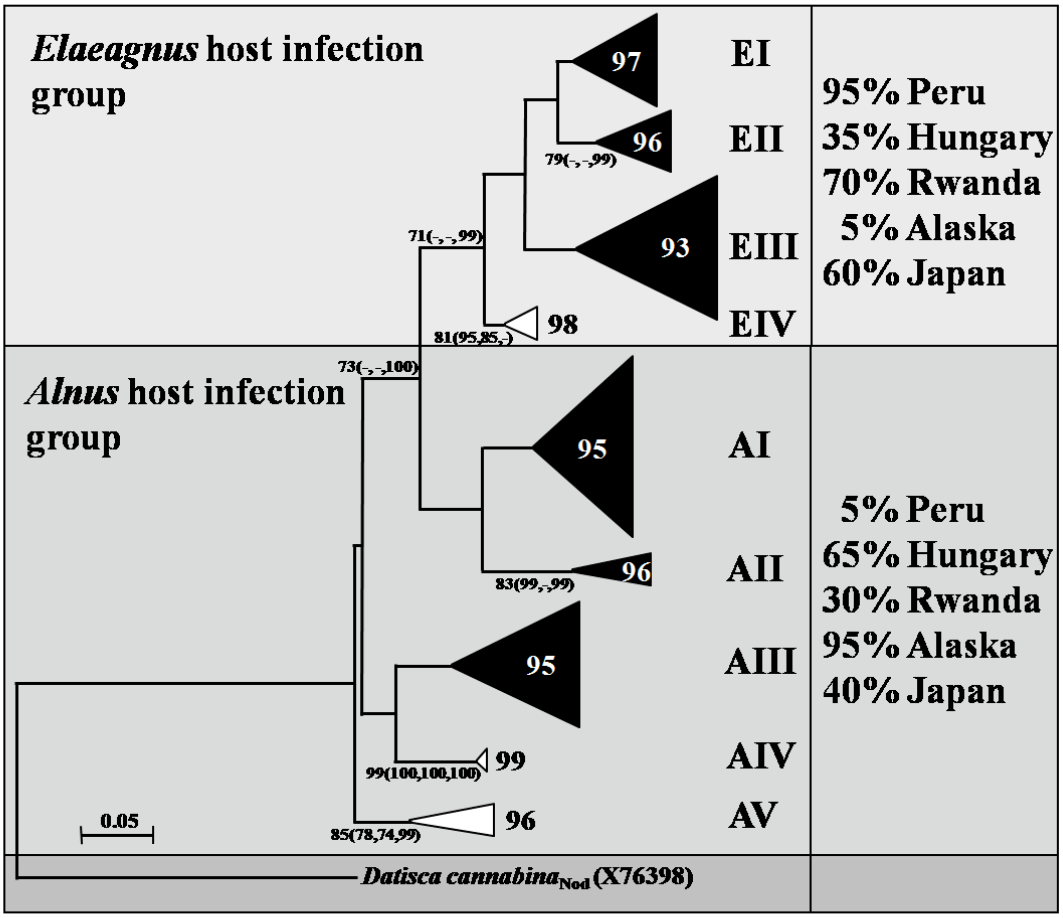

Figure 4.1: Phylogenetic analyses of cultured and uncultured *Frankia*. Maximum likelihood-based tree generated using 522 bp of the *nif*H gene of 100 uncultured *Frankia* populations from root nodules of *Morella pensylvanica* inoculated with soils from 5 continents, and of 58 pure cultures of *Frankia*. Branches were collapsed at key nodes based on topology and bootstrap (BS) support values to give an overall picture of the diversity of *Frankia* recovered. Numbers at nodes reflect BS support measures and numbers in parentheses represent BS measures and posterior probabilities (PP) from neighbor joining, maximum parsimony, and Bayesian analyses, respectively. The outgroup was from an uncultured *Frankia* population from nodules of *Datisca cannabina* (X76398). Cluster designations on the right represent *Frankia* of the *Elaeagnus* host infection group (E I - IV) or the *Alnus* host infection group (A I - V). Black triangles represent clusters where sequences of pure cultures were available, and white triangles those with no sequence information for pure cultures available. Numbers within or beside the triangles represent the percent similarity of sequences in that cluster. On the far right are the percentages of nodules retrieved from each soil site for both host infection groups.

Sequences of amplification products were obtained from nodules of all soil-subplot treatment combinations resulting in a total of 20 sequences from nodules for each of the soils. Phylogenetic analyses on the sequence dataset of *nif*H gene fragments of 100 uncultured frankiae from root nodules of *M. pensylvanica* and of 58 *Frankia* strains produced similar topologies independent of the methodology used (data not shown). Frankiae of the *Elaeagnus* and *Alnus* host infection groups were separated from each other, supported by a BS value of 73% (ML analysis only) and a PP value of 100 % (Bayesian analysis) (Fig. 4.1). These results are consistent with comparative sequence analyses of 16S rRNA genes (53). BS values of 71 % (ML) and PP values of 99 % also supported frankiae of the *Elaeagnus* group as a monophyletic group (Fig. 4.1). Within the *Elaeagnus* host infection group, 4 major clusters of frankiae (E I to E IV) were identified, and 5 clusters of frankiae within the *Alnus* host infection group (A I to A V) (Fig. 4.1).

Sequences within both host infection groups were clustered based on criteria chosen somewhat arbitrarily using tree topology, sequence similarity and BS and PP support values (Fig. 4.1). Therefore, clusters are designated by a range of sequence similarity values from 93 to 99 %, rather than a specific similarity value. Defining a specific value for cluster assignments such as, for example, sequence similarity values above 97 % that are often used for 16S rRNA gene sequence similarities to assign species (58, 61), would definitely increase the number of clusters and thus diversity detected. However, similar to values for 16S rRNA gene sequences (19, 42), defined similarity values for sequences of *nif*H gene fragments do not consistently correspond to accurate definitions of genomic groups. While some *Frankia* strains belonging to one genomic

group (e.g., strains AvcI1, An2.1, MpI1, and ArI3 belonging to the *Alnus* infection group) (1, 3, 18) have *nif*H gene fragment similarity values above 97%, others with even higher similarity values (e.g., 98.8% for strains Ea1.12 and EAN1pec of the *Elaeagnus* host infection group) have been assigned to different genomic groups (18). For our purpose to show major differences between uncultured frankiae populations in soils, the approach to use more loosely defined values for clustering is therefore adequate.

All soils produced nodules belonging to the *Elaeagnus* and the *Alnus* host infection groups on *M. pensylvanica* (Fig. 4.1, 4.2, 4.3). The diversity of frankiae among nodules, however, depended on the soil used as inoculum. Nodule-forming frankiae in the Peruvian soil were dominated by the *Elaeagnus* host infection group, while those of the Alaskan soil were dominated by the *Alnus* host infection group, with 95 % of all the sequences retrieved belonging to one host infection group. Frankiae in nodules formed after inoculation with soils from Hungary, Rwanda, and Japan retrieved *Frankia* from both host infection groups with no clear dominance of host infection group (Fig. 4.1). Variation in populations among subplots (A, B, C, or D) of one soil was minimal because sequences that clustered together were generally obtained from several, even though not necessarily all subplots (Fig. 4.2, 4.3). Potential detection of variation in *Frankia* populations between subplots could have been impacted by asymmetric sample analyses because numbers of sequences retrieved were not identical for the subplots (i.e., 5 sequences for each of the subplots), but varied (i.e., between the extremes of 2 to 10 sequences per subplot).

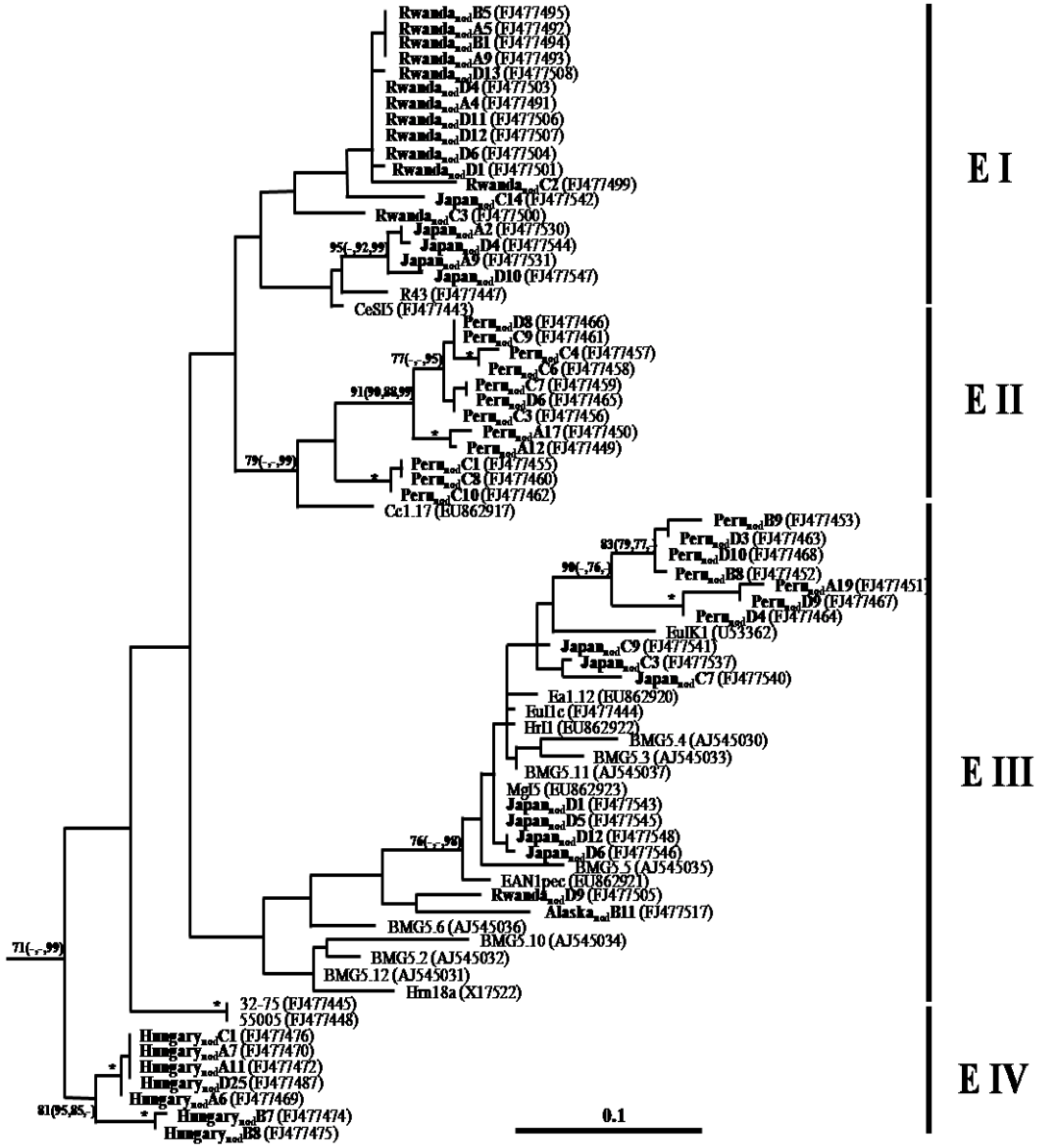

Figure 4.2: Phylogenetic analyses of cultured and uncultured *Frankia* of the *Elaeagnus* host infection group. Maximum likelihood-based tree generated using 522 bp of the *nif*H gene of 100 uncultured *Frankia* populations from root nodules of *Morella pensylvanica* inoculated with soils from 5 continents (in bold print), and of 58 pure cultures of *Frankia* (in plain print) cut to only show those sequences in the *Elaeagnus* host infection group. Sequences from uncultured *Frankia* populations from a given subplot within a continent are designated A, B, C, or D. Numbers at nodes reflect bootstrap support measures (BS) and numbers in parentheses represent BS measures and posterior probabilities (PP) from neighbor joining, maximum parsimony, and Bayesian analyses, respectively. Asterisks at tip nodes reflect high BS or PP values from 3 of the 4 phylogenetic methods utilized in these analyses. The outgroup was from an uncultured *Frankia* population from nodules of *Datisca cannabina* (X76398). Cluster designations (E I - IV) are listed on the right.

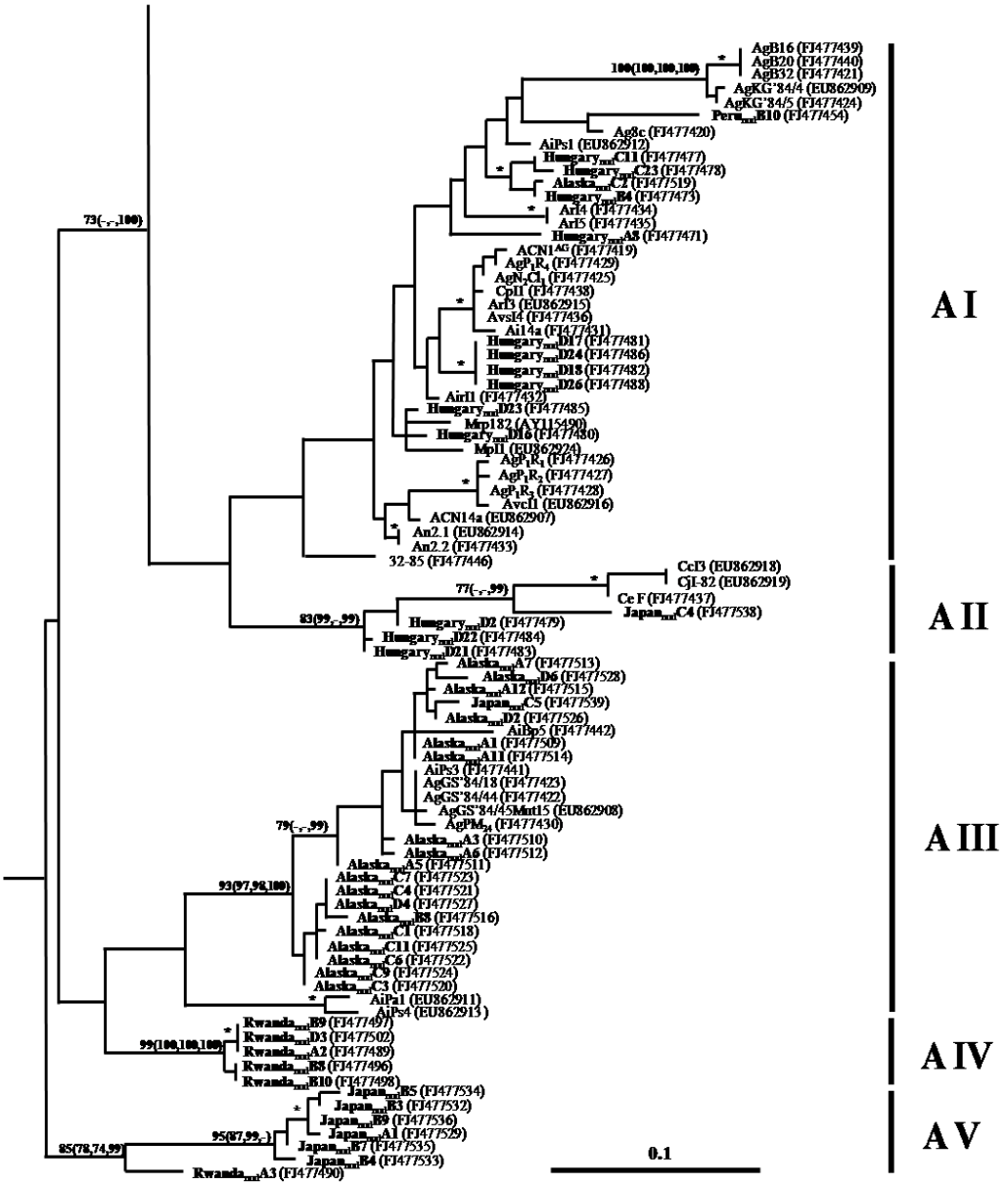

Figure 4.3: Phylogenetic analyses of cultured and uncultured *Frankia* of the *Alnus* host infection group. Maximum likelihood-based tree generated using 522 bp of the *nif*H gene of 100 uncultured *Frankia* populations from root nodules of *Morella pensylvanica* inoculated with soils from 5 continents (in bold print), and of 58 pure cultures of *Frankia* (in plain print) cut to only show those sequences in the *Alnus* host infection group. Sequences from uncultured *Frankia* populations from a given subplot within a continent are designated A, B, C, or D. Numbers at nodes reflect bootstrap support measures (BS) and numbers in parentheses represent BS measures and posterior probabilities (PP) from neighbor joining, maximum parsimony, and Bayesian analyses, respectively. Asterisks at tip nodes reflect high BS or PP values from 3 of the 4 phylogenetic methods utilized in these analyses. The outgroup was from an uncultured *Frankia* population from nodules of *Datisca cannabina* (X76398). Cluster designations (A I - V) are listed on the right.

Frankia populations in nodules induced by the Alaskan soil were dominated by frankiae belonging to subgroup A III (90 %), while frankiae of the remaining soils were represented by 2 or more prominent subgroups (Fig. 4.2, 4.3). The Alaskan soil is the only soil with actinorhizal plants in the sampling area, i.e., an alder stand, while all other soils were obtained from more disturbed sites that were devoid of known actinorhizal plants (Table 4.1). The presence of one type of frankiae belonging to the *Alnus* host infection group in the Alaskan soil is consistent with other studies that found a low diversity of *Frankia* dominated by one or two types in alder stands (12, 35). Similarly, frankiae in nodules of closely spaced *Colletia hystrix* plants were dominated by one ribotype which was the same as strain ChI4 isolated from the same place years before indicating the dominance and stability over time of this *Frankia* strain in the presence of their host plant species (9). Thus, the presence of an actinorhizal plant may enrich the soil with one kind of *Frankia* compatible with this host and may therefore account for the comparatively low diversity of *Frankia* found in the Alaskan soil, i.e. *Frankia* compatible with this alder host.

Although the diversity of frankiae in nodules induced by soils from Japan, Hungary, Peru, and Rwanda had 2 or more prominent subgroups and was higher than that in nodules induced by the Alaskan soil, overall diversity of nodule-forming populations in each soil was relatively low, which is consistent with results from other studies (33). Only a few populations were present in more than one or two soils. All clusters were formed or dominated by frankiae obtained from one (E II, Peru; E IV, Hungary; A I, Hungary; A II, Hungary; A III, Alaska; A IV, Rwanda; A V, Japan) or from two soils (E I, Rwanda, Japan; E III, Peru, Japan) with single sequences occasionally present from

other soils (E III, A I, A II, AIII and A V) (Fig. 4.2, 4.3). Variation within a cluster was generally low for sequences obtained from nodules induced by the same soil, but larger between sequences of frankiae originating from different soils (Fig. 4.2, E I and E III). These results might reflect adaptations of *Frankia* populations to edaphic conditions where differences between soils result in distinct populations compared to more uniform populations in the same soil (31, 37). This assumption would presuppose that specific populations enrich in the absence of host plants and thus that *Frankia* populations grow saprophytically in these soils. While the potential for saprophytic growth by *Frankia* (44, 57, 60) as well as effects of edaphic conditions on specific populations (31, 43, 51, 52) has been demonstrated in several studies, direct proof for this assumption, i.e., tracking of dynamics of specific *Frankia* populations in soil, is still lacking.

Three of the observed clusters, one within the *Elaeagnus* (E IV) and two within the *Alnus* (A IV and A V) host infection groups, were entirely represented by uncultured frankiae with no sequences from cultured relatives available, which is similar to results of comparable studies (26). While sequences of pure cultures were relatively evenly distributed over the remaining clusters within the *Alnus* host infection group (Fig. 4.3), representation of pure cultures was asymmetric in the remaining clusters of the *Elaeagnus* host infection group. Here, sequences of only one (strain Cc1.17) or two (CeIS5 and R43) strains grouped with 12 and 18 sequences, respectively, of uncultured frankiae to form clusters E II and E I, while 17 (85 %) of the sequences of pure cultures grouped with cluster E III, the most diverse with 93 % sequence similarity within this cluster (Fig. 4.2). This cluster is also most diverse with respect to the origin of uncultured frankiae, with 7 sequences from Peruvian, 7 sequences from Japanese, and one each from

Alaskan and Rwandan soils. While these results, together with the widespread origin of the strains with respect to host plant species and location indicate a certain prominence of members of this cluster in nature, they might be entirely due to the limited dataset of sequences available from cultured strains of the *Elaeagnus* host infection group.

Although our database of *nif*H gene sequences represents more than 50 pure cultures, these sequences do not adequately reflect the diversity of frankiae in nature, and thus sequences from additional pure cultures isolated from different host plant species and from different locations are needed. Our database of *nif*H gene sequences that includes those of cultured and uncultured frankiae, however, can be used as a basis for taxonomic studies within the genus *Frankia*, and potentially clarify the taxonomic position of isolates. Strains R43 (NRRL B-16306), CeS15, 32-75 (NRRL B-16412), and 55005, for example, were all isolated from roots of *Casuarina* species, yet all group well within the *Elaeagnus* host infection group and not with *Casuarina*-infective strains within the *Alnus* host infection group. Confirmation of these results, however, requires additional studies with other genes and new cultures of these strains. The database also allows us to search for primer binding sites that might be useful in developing nested PCR approaches to retrieve specific sequence information on frankiae from heterogeneous environments like soils, and to compare diversity of nodule-forming populations with entire populations in the environment.

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CHAPTER 5

Diversity of *Frankia* in root nodules of geographically isolated Arizona alders in central Arizona (USA)[1]

Abstract

The diversity of uncultured *Frankia* populations in root nodules of *Alnus oblongifolia* geographically isolated on mountain tops of central Arizona was analyzed by comparative sequence analyses of *nif*H gene fragments. Sequences were retrieved from *Frankia* populations in nodules of 4 trees from each of 3 mountain tops (n=162) and their diversity compared using spatial genetic clustering methods and single nucleotide, 1, 3 or 5% sequence divergence thresholds. Sequence diversity was very high on the single nucleotide level, with 45 different sequences retrieved with significant differences between the mountain tops and the southern site partitioning in a separate population from the two other sites. Some of these sequences were identical in nodules from different mountain tops and to those of strains isolated from around the world. A high diversity was also found on the 1% divergence level that resulted in the assignment of 14

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clusters of sequences. Single nucleotide and 1% divergence levels thus demonstrate microdiversity of frankiae in root nodules of *A. oblongifolia* and suggest a partitioning of diversity by site. At the 3 and 5% divergence levels, however, diversity was reduced to 3 or 1 cluster(s), respectively, with no differentiation by mountain top. Only at the 5% threshold level all *Frankia* strains previously assigned to one genomic group cluster together.

Diversity of Frankia in root nodules of geographically isolated Arizona alders in central Arizona (USA)

Frankia sp. are nitrogen-fixing actinomycetes that form root nodules in symbiosis with more than 200 species of non-leguminous woody plants in 24 genera of angiosperms (5, 27, 47). These actinorhizal plants have an almost worldwide distribution and can live in soils with low nitrogen availability and thus exploit habitats not favorable for growth of many other plant species (13). *Alnus oblongifolia* Torr. (Arizona alder) is an actinorhizal plant that can be found in mountainous regions in northern Mexico and the southwestern United States. Within the southwestern United States, isolated populations of Arizona alder are frequently found along streams draining the southern edge of the Colorado Plateau and the scattered mountain ranges found throughout central Arizona. The alder sites are in mountains that are surrounded by deserts, grasslands, brush or woodland types, and forests and as such are home to many endemic species that have developed as a consequence of geographic isolation (52).

Alnus oblongifolia grows in unique moist environments in this desert region, specifically along perennial streams of canyons, primarily at elevations between 1,400

and 2,300 m and have been shown to form effective root nodules in nature (14). Because mountainous sites inhabited by *A. oblongifolia* are geographically isolated, analyses of *Frankia* populations in nodules of *A. oblongifolia* growing on different mountain tops may provide a unique opportunity to get new insights into the diversity and biogeography of these *Frankia* populations.

Specific factors that drive *Frankia* diversification are presently unclear, even though there are preferences among *Frankia* strains for specific host plants, separating strains into host infection groups and subgroups (16, 24, 31). *Frankia* strains infecting *Casuarina* plants have been shown to have co-evolved with their host plant, illustrating the importance of the host plant in shaping the diversity and evolution of these strains (48). However, for most *Frankia* strains, no simple pattern of co-evolution is present (3). While phylogenetic analyses reveal 3 clades for each of the partners in this symbiosis, *Frankia* within one clade may form root nodules with plants in more than one clade (4). This lack of correlating phylogenies is likely due to *Frankia* occupying 2 distinct ecological niches, root nodules and soil, where symbiotic or saprophytic growth conditions may drive diversification of *Frankia* differently (3). Thus, the complex divergence patterns in *Frankia* phylogeny may best be explained in a geographic mosaic theory of co-evolution in which multiple confounding factors like geographic isolation, plant host preferences, and environmental factors converge to shape the evolutionary patterns of *Frankia* (3, 50).

One aspect of the geographic mosaic theory of co-evolution is allopatric speciation, the divergent evolution of geographically isolated populations, which may be a potential driver of *Frankia* diversification (40, 55). Comparative analysis of *Frankia* on

isolated mountainous habitats may be a unique opportunity to test if geographic divergence is indeed driving *Frankia* evolution. The isolation of *Frankia* in root nodules of trees growing on different mountain tops may permit differentiation as the effects of neutral drift, population bottlenecks and adaptation to even slight environmental differences cause the accumulation of mutations which may lead to allopatry (40), as indicated for other bacteria (39, 56). However, *Frankia* are capable of forming spores that allow them to survive transport from one hospitable habitat to another (29). Additionally, *Frankia* strains, particularly those of the *Alnus* host infection group, seem to have a cosmopolitan distribution (4) because strains from the same genomic group have been isolated from all over the world (see 1, 20) and have been found in soils with no extant actinorhizal plants (10, 28, 33, 42).

The aim of this study was to determine if uncultured *Frankia* populations in root nodules of *A. oblongifolia* isolated on mountain tops within different geologic regions of Arizona showed signs of endemism in a functional gene, *nif*H, and whether that unique diversity could be correlated with differences in *Frankia* populations from root nodules among mountain tops. Nodules were collected in June of 2008 from 4 trees at each of 3 mountain top sampling sites, each separated from the nearest by 150 km proceeding from north to south within 1° longitude of each other along a 300 km latitudinal gradient in central and southern Arizona (Fig. 5.1). Site 1 (Oak Creek in the Coconino National Forest, N 35°00.6’ W 111°44.3’) was a sandy alluvial soil located near Oak Creek at an elevation of 1703 m, site 2 (Workman Creek watershed in the Sierra Ancha Experimental Forest of Tonto National Forest, N 33°49.1’ W 110°55.8’) a streamside soil high in organic matter at an elevation of 2073 m, and site 3 (Sabino Canyon in Coronado

National Forest, N 32°26.1’ W 110° 45.5’) a sandy loam soil adjacent to Sabino Creek at an elevation of 2310 m. Each site was in a different geologic province: the Colorado Plateau Province in the north, the Central Highlands Province as a transition zone and the Basin and Range Province within the Madrean Archipelago in the south (9, 34). Nodules were stored in cold 95% ethanol until analyzed.

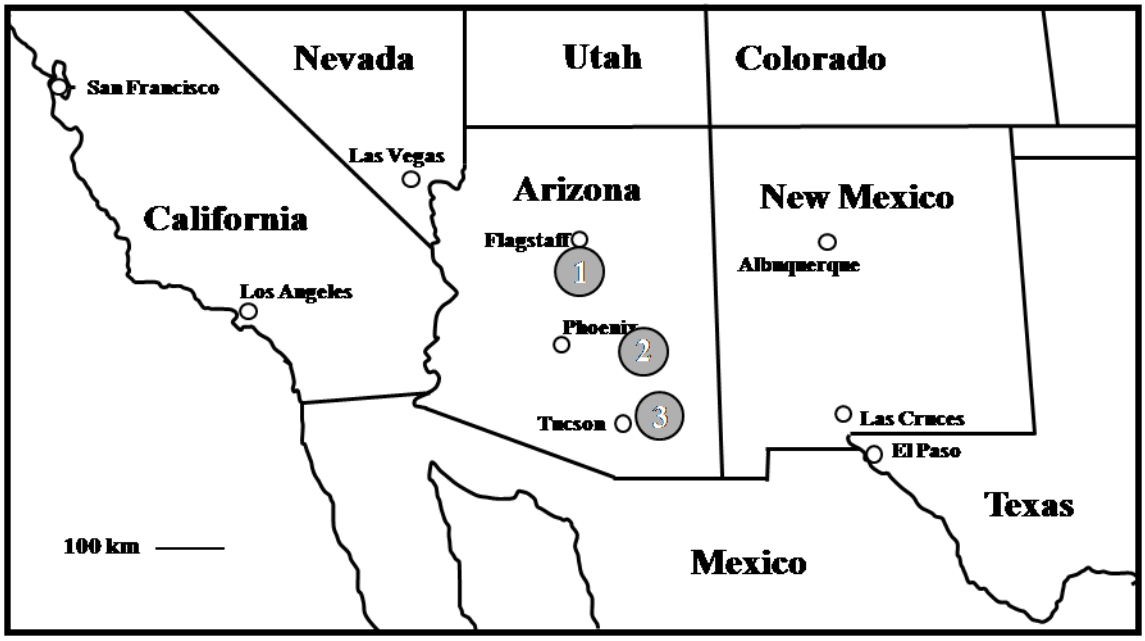

Figure 5.1: Sampling sites in central Arizona. Locations of the mountain tops in central and southern Arizona sampled for uncultured *Frankia* from the root nodules of *Alnus oblongifolia* growing near perennial streams on these mountains. Scale indicates 100 km. Site 1 is Oak Creek in the Coconino National Forest (N 35°00.6' W 111°44.3'), Site 2 is the Workman Creek watershed in the Sierra Ancha Wilderness of Tonto National Park (N 33°49.1' W 110°55.8'), and Site 3 is the Sabino Canyon watershed in the Coronado National Forest (N 32°26.1' W 110° 45.5').

DNA was extracted from individual lobes of different root nodules and a 606 bp fragment of the *nif*H gene was amplified and sequenced using *Frankia* specific amplification protocols as described previously (54). Initially, *nif*H gene sequences were obtained from 24 nodules from 1 tree from each mountain top to determine the level of sampling required to capture the diversity present. A rarefaction curve was generated using DOTUR (45) and a threshold level of divergence set to 3% which was found to group *Frankia* strains into appropriate genomic groups in a previous study (35). Based on rarefaction analyses, 10 nodules were sampled from the remaining 3 trees from each mountain top for a total of 54 nodules from each mountain. Sequences of amplified *nif*H gene fragments of uncultured *Frankia* from 54 *A. oblongifolia* nodules from each of 3 mountain tops (Genbank accession numbers FJ977167-FJ977328) were aligned with those of the 3 pure cultures of *Frankia* (FJ977329-FJ977331) and sequences of 46 other strains or uncultured populations analyzed in previous studies (35, 54) or retrieved from public databases, and analyzed using maximum likelihood, maximum parsimony, neighbor joining and Bayesian analyses as described previously (54).

Phylogenetic analyses of this dataset of 211 sequences produced similar topologies independent of the methodology used (data not shown) and assigned all sequences in nodules of *A. oblongifolia* to frankiae of the *Alnus* host infection group (Fig. 5.S1, supplemental material). The analysis retrieved 45 different sequences in these nodules from *A. oblongifolia*, differing from each other by at least one nucleotide. For presentation purposes to show the relation of frankiae from root nodules of *A. oblongifolia* to available pure cultures, the complete dataset was reduced to 51 representative sequences including 21 sequences from frankiae in root nodules of *A.*

oblongifolia and was re-analyzed using the above parameters (Fig. 5.2). Several of the sequences obtained from root nodules were identical or nearly identical (i.e. single nucleotide differences) to those of strains or uncultured *Frankia* populations from other parts of the world (Fig. 5.2). For example, sequence AO3-14nodF was identical to *nif*H gene fragment sequences from 4 *Frankia* strains isolated from around the world [CpI1 in Massachusetts (8), ArI3 in Oregon (6), AvsI4 in Washington (2), and Ai14a in Finland (53)]. Identical sequence does not mean that these are identical strains, but does suggest that certain genotypes may have a ubiquitous distribution (35).

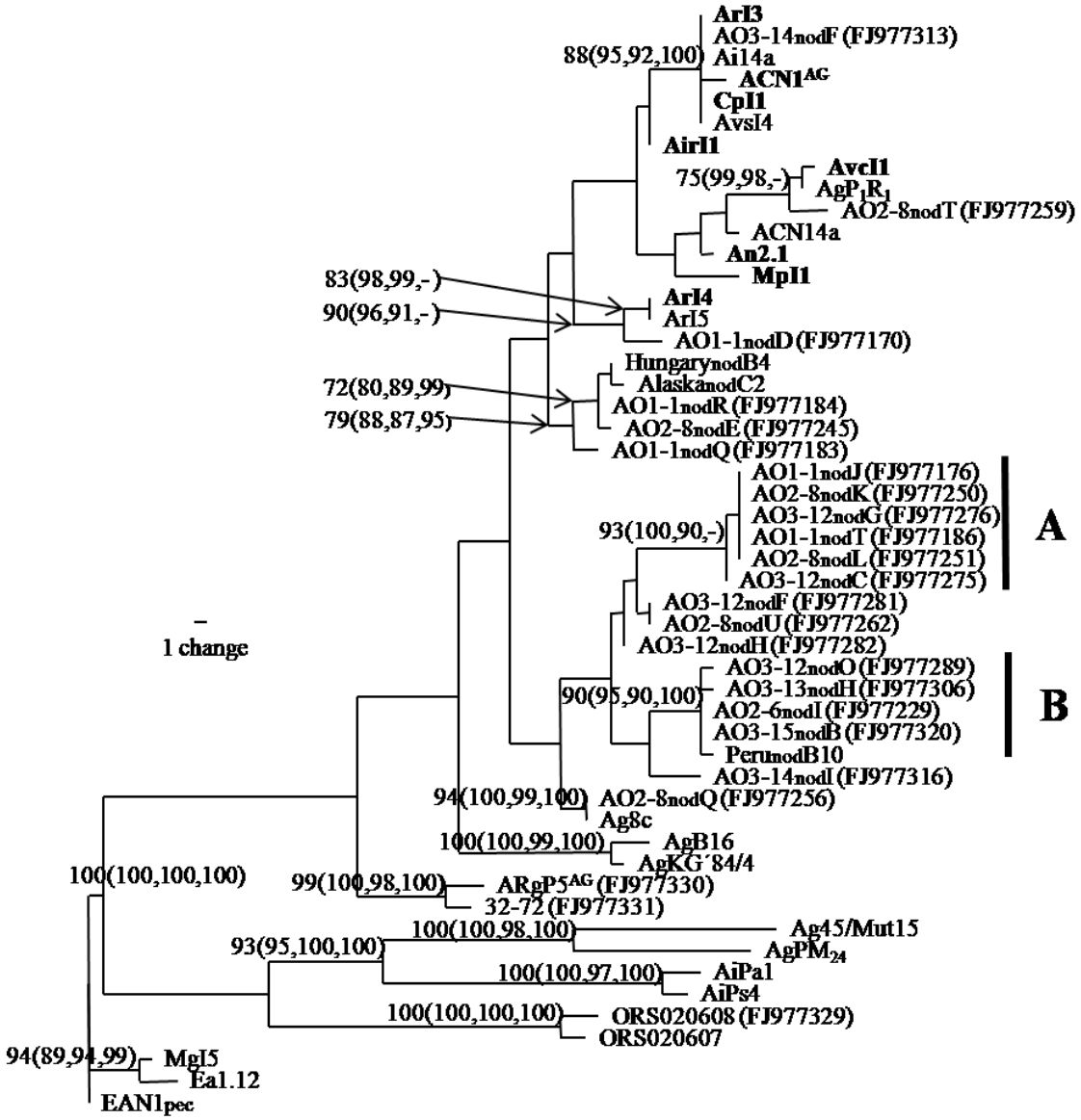

Figure 5.2: Phylogenetic analyses of cultured and uncultured *Frankia* from root nodules of *Alnus oblongifolia*. Maximum parsimony tree of a subset of uncultured *Frankia* from root nodules of *Alnus oblongifolia* and other pure cultures and uncultured *Frankia* created using 522 bp of the *nif*H gene. Numbers reflect bootstrap support (BS) values and numbers in parentheses reflect BS or posterior probabilities from neighbor joining, maximum likelihood and Bayesian analyses, respectively. Representatives from clusters A and B are indicated. Strains in boldface belong to *Frankia* genospecies 1 (see ref. 23 for summary). Strain EAN1pec was used as the outgroup.

Sequences retrieved from *Frankia* in root nodules of *A. oblongifolia* were organized into populations by tree they were isolated from and by mountain top (19) for AMOVA analysis using Arlequin ver 3.01 (18). AMOVA settings included 16,000 permutations and more conservative proportion of differences matrix criteria. AMOVA analysis includes both differences in sequences at the single nucleotide level and differences in abundance of sequences present (44) and indicated significant differences in sequence diversity within trees ($p<0.001$), with most of the variation in diversity (83.6%) found within populations of *Frankia* from each *A. oblongifolia* tree. Differences in sequence diversity among trees on each mountain top, however, were not significant ($p=0.165$). Significant differences in the diversity of *Frankia* were also recovered among mountain tops accounting for 14.6% of the variation in diversity suggesting differences by site in the diversity of *Frankia* recovered at the single nucleotide level of differences.

To explore this geographic component in more detail spatial genetic clustering methods were used in GENELAND version 3.1.4 which utilizes a Bayesian algorithm to make population assignments and weights using geographic coordinates (22). The analyses proceeded in 2 stages similar to Coulton *et al*. 2006, except that K initially fluctuated between 1 and 24 and 1 million generations were run with a burnin of 100,000 (11). Spatial and non-spatial settings were used and uncertainty in coordinates was tested at 3 m and 10 m, however, all analyses yielded two populations in the dataset (K=2) and assigned individuals in the same way. Sequences of 108 root nodule *Frankia* were unambiguously assigned to one population corresponding to those from sites 1 and 2 and 54 sequences of root nodule *Frankia* were unambiguously assigned to the second population corresponding to site 3. Site 3 is most isolated from the other 2 sites by being

surrounded by desert on 4 sides. Sites 1 and 2, however have some connectivity by forest along the Mongollon Rim suggesting that they were more recently separated than site 3, and thus might be more similar for that reason (9).

In an attempt to obtain a more ecologically relevant discrimination of *Frankia*, i.e. on a level representing species or subspecies (35, 54), three additional threshold levels, i.e., 1, 3 and 5% divergence levels, were subsequently used to compare diversity of nodule populations among mountain tops. While the 1% level, corresponding to ~5 nucleotide differences, was arbitrarily chosen, the 3 and 5% levels represented thresholds previously used to assign *Frankia* strains of the same genomic group (35) and uncultured root nodule frankiae of the *Alnus* host infection group (54) into the same cluster based on comparative sequence analyses of *nif*H gene fragments, respectively. To formulate the assignment of clusters at these three levels of differentiation, the complete dataset was reduced by removing all sequences but those representing frankiae in nodules of *A. oblongifolia*. This dataset of 162 *nif*H gene fragments was executed in PAUP* 4.05b where an uncorrected distance matrix was created and analyzed in DOTUR and then in SONS (46). A similar DOTUR/SONS analysis was completed on the entire dataset of 211 sequences to describe the groupings of uncultured nodule populations with pure cultures representing various genomic groups. Microsoft Excel was then used to generate pie charts displaying the clusters of frankiae in root nodules of *A. oblongifolia* (Fig. 5.3).

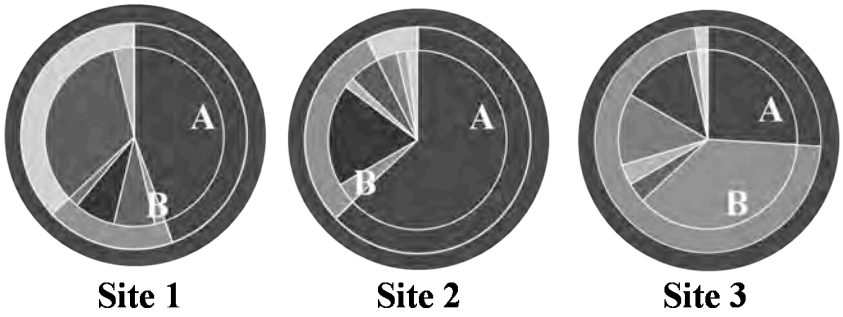

Figure 5.3: Clusters of uncultured *Frankia* from root nodules from *Alnus oblongifolia*. Graphical representation of output from SONS (46) for clusters from uncultured *Frankia* from root nodules from *Alnus oblongifolia* (n=54) isolated on three different mountain tops in central and southern Arizona (sites 1-3). The inner circle represents 14 clusters recovered using a 1% diversity threshold, the middle circle represents 3 clusters recovered using a 3% diversity threshold and the outer circle represents 1 cluster recovered using a 5% diversity threshold. A and B designate the only clusters at the 1% diversity threshold, ~5 nucleotide differences, of uncultured frankiae recovered from all 3 mountain tops.

At the 1% threshold level, analyses using the SONS program demonstrated the presence of 14 clusters of sequences (Fig. 5.3). Half of these clusters were represented by 3 or fewer sequences. Seven sequence clusters were found only in nodules of trees from one mountain top, 5 were present in nodules from trees of 2 mountain tops, and 2 were detected on all 3 mountain tops (identified as A and B in Fig. 5.3). Cluster A was dominant overall (n=73, 44% of all nodules recovered) and was found on all 3 mountain tops at varying frequencies (Fig. 5.3). Cluster B was also found on all 3 mountain tops (n=23, 14% of all nodules recovered) but was dominant on one site (site 3, Sabino Canyon) and barely detected on the other mountain tops supporting the geographic uniqueness of this site (Fig. 5.3). The number of clusters decreased to 3 when a 3% divergence threshold was used. All three clusters were present on all 3 mountain tops, but

in varying frequencies (Fig. 5.3). At this threshold, however, pure cultures belonging to the same genomic group still did not cluster in the same group. Only when the threshold was set to 5% did all the pure cultures from *Frankia* genomic group 1 (see (23) for summary) cluster together. At a threshold of 5%, all *Frankia* populations in nodules of *A. oblongifolia* were placed in one group, suggesting no differentiation by mountain top and a limited overall diversity with one cluster present compared to the potential presence of at least 6 clusters of frankiae within the *Alnus* host infection group described in previous studies (35, 54). Low overall diversity has also been described in other studies of natural *Frankia* diversity in root nodules of various alder species (12, 26, 30, 32).

Differences in nitrogenase activity and nodulation capacities have been reported for *Frankia* strains of the same genomic group on the same alder species in the same soils (15, 16). These differences in effectiveness and infectivity of strains in the same species group have been suggested to be evidence of the effects of plant host shaping symbiotic *Frankia* diversity in different environmental conditions (3). Thus, the variations in diversity and abundance seen at the single nucleotide or 1% diversity level may reflect preferences by *A. oblongifolia* for one strain over another in the different environmental conditions on each mountain top and microscale differences among trees on the same mountain top. Genetic differences among Arizona alder populations are unknown, as is the extent to which seed and pollen dispersal occur among these isolated populations. Nonetheless, there were no morphological differences among trees in populations sampled and they were all the same species. In contrast, *Frankia* diversity in root nodules has been shown to be affected by different edaphic conditions like soil type and pH (37, 49) or environmental effects like elevation (30, 32), which are different on each mountain

top. However these variations in diversity may also reflect random chance and low sample size (17), because rarefaction analysis at the 1% diversity level did not indicate saturation of sampling for any mountain top (data not shown).

Determination of reasons for selective nodulation by specific strains of *Frankia* becomes highly speculative. Some evidence suggests that active *Frankia* in the soil may be preferentially selected by the host for nodulation (36, 38). Previous research in our lab has confirmed the importance of the plant host in selecting *Frankia* strains for symbiosis when the same soil was inoculated into 6 different actinorhizal plant species and resulted in 6 different diversity profiles (35). Additionally, we have shown that the same actinorhizal plant species inoculated with soils from 5 different continents resulted in 5 different diversity profiles demonstrating the effects of soil type and history on root nodule *Frankia* diversity (54).

Frankia micro-diversity in root nodules of *A. oblongifolia* recovered in this study does show a clear geographic pattern but reasons for these patterns are unclear. The limited *Frankia* diversity in *A. oblongifolia* root nodules is likely due to a combination of factors including saprophytic growth capabilities, host plant preferences and edaphic conditions acting at the micro-ecosystem level on *Frankia* populations.

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CHAPTER 6

Discussion

Discussion

Molecular tools were developed that have allowed us to retrieve sequence information on a variety of nitrogen-fixing bacteria in the environment. The database of sequences of uncultured nitrogen-fixing bacteria of the ε-subdivision of Proteobacteria was expanded and we demonstrated that they are active nitrogen-fixing bacteria in the environment. Additionally, we have expanded the database of *nif*H gene sequences for both cultured frankiae and uncultured frankiae from root nodules, and used comparative sequence analyses to assess the geographic distribution of specific nodule-forming populations on *Alnus oblongifolia*.

A number of difficulties were encountered in the development and application of the molecular tools that were used to retrieve the sequence databases. This is a discussion of 2 of the most prominent problems we had in the development and application of molecular tools to describe nitrogen-fixing bacteria in the environment. The data presented was beyond the scope of previous chapters and yet warranted reporting as these difficulties constrained the application and interpretation of data generated using molecular tools for ecological studies.

One of these difficulties involved the design and the application of appropriate sampling techniques. The bacterial community analyses completed in Chapter 3 consisted of independently prepared and then mixed samples from 2 replicate plant core treatments to account for potential sample variability. The data generated from these 2 different samples examined individually prior to mixing illustrated some of the inherent problems in sampling and sample processing involved in using molecular tools.

Analyses using PCR-RFLP of *nif*H amplicons of all nitrogen-fixing bacteria with replicate treatment samples viewed individually showed that the present community, those detected using DNA as template, and the active community, those detected using reverse transcribed mRNA (cDNA), were similar (Fig 6.1 a and b). Half of the community profiles generated using cDNA were not different from the profiles generated with DNA which were all the same, i.e. they have greater than 70% similarity in the dendrogram analyses (Fig 6.1c). At the vegetative growth time point when the effects of Benomyl-treatment on the suppression of AMF were most pronounced, the community profiles of the two samples treated with Benomyl and of one of the non-treated samples were identical indicating no treatment effect. Overall the profiles generated using cDNA as template had fewer bands than those generated using DNA, and the profiles generated with cDNA were more variable indicating the active community was more different seasonally than the present community.

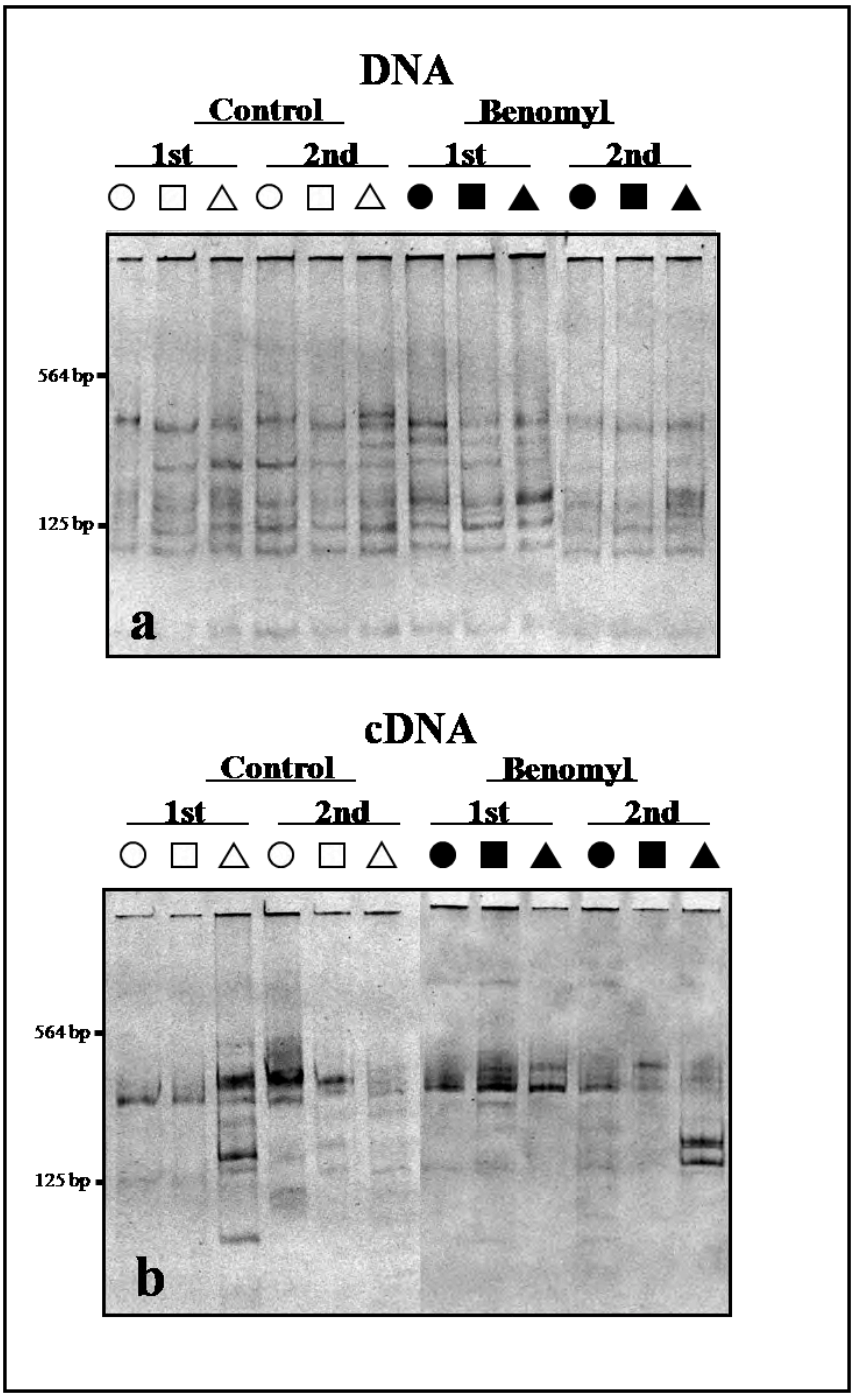

Fig. 6.1: RFLP profiles of all nitrogen-fixing bacteria for replicate treatments. RFLP acrylamide gel of *Hae*III restricted *nif*H PCR product generated using the NifHforB and Nifrev primer set (46) in a nested PCR from DNA (a), and cDNA (b) extracted from soil in Benomyl-treated (●) and non-treated (○) cores with *S. patens* at a depth of 2.5 cm corresponding to 3 plant phenological stages, i.e., ○ vegetative growth (April), □ reproduction (May) and △ senescence (August). Each well has digested PCR product from 1 plant core. 1st and 2nd refers to samples from replicate plant cores analyzed independently. The size marker (M) is lambda DNA cleaved with *Hind*III.

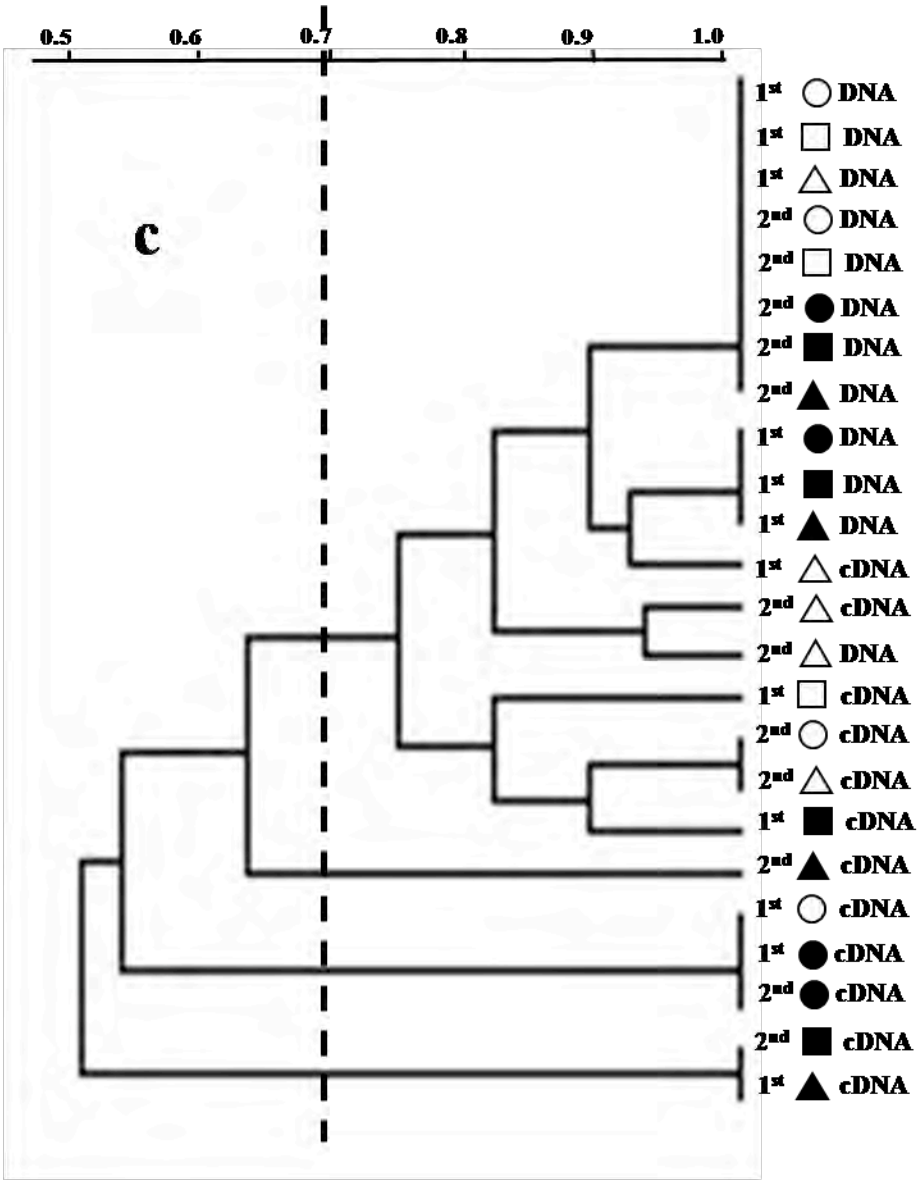

Fig. 6.1 continued: RFLP profiles of all nitrogen-fixing bacteria for replicate treatments. (c) Corresponding UPGMA dendrogram from RFLP fragments generated using GelManager v 1.5 (Biosystematica). The dashed line marks the 0.7 borderline above which patterns were assumed to be the product of the same population.

In contrast to PCR-RFLP analyses of the entire community of nitrogen-fixing bacteria, profiles of nitrogen-fixing members of the ε-subdivision of Proteobacteria analyzed by RFLP and DGGE were highly variable, with different profiles in time and between treatment and template for most samples. RFLP and DGGE analyses conveyed conflicting results about the diversity and treatment effects for this group of nitrogen-fixing bacteria. RFLP analyses suggested that the profiles of nitrogen-fixing members of the ε-subdivision of Proteobacteria generated using DNA as template indicated 4 different communities in total, and the profiles using cDNA also indicated the presence of 4 communities suggesting that the present and active communities had similar differences in community structure; the active community was not more variable than the present community (Fig 6.2). There was a trend in RFLP profiles of an increase in differences in community structure over the season which suggested the active and present communities might increase in diversity over the season, but no clear effect of treatment.

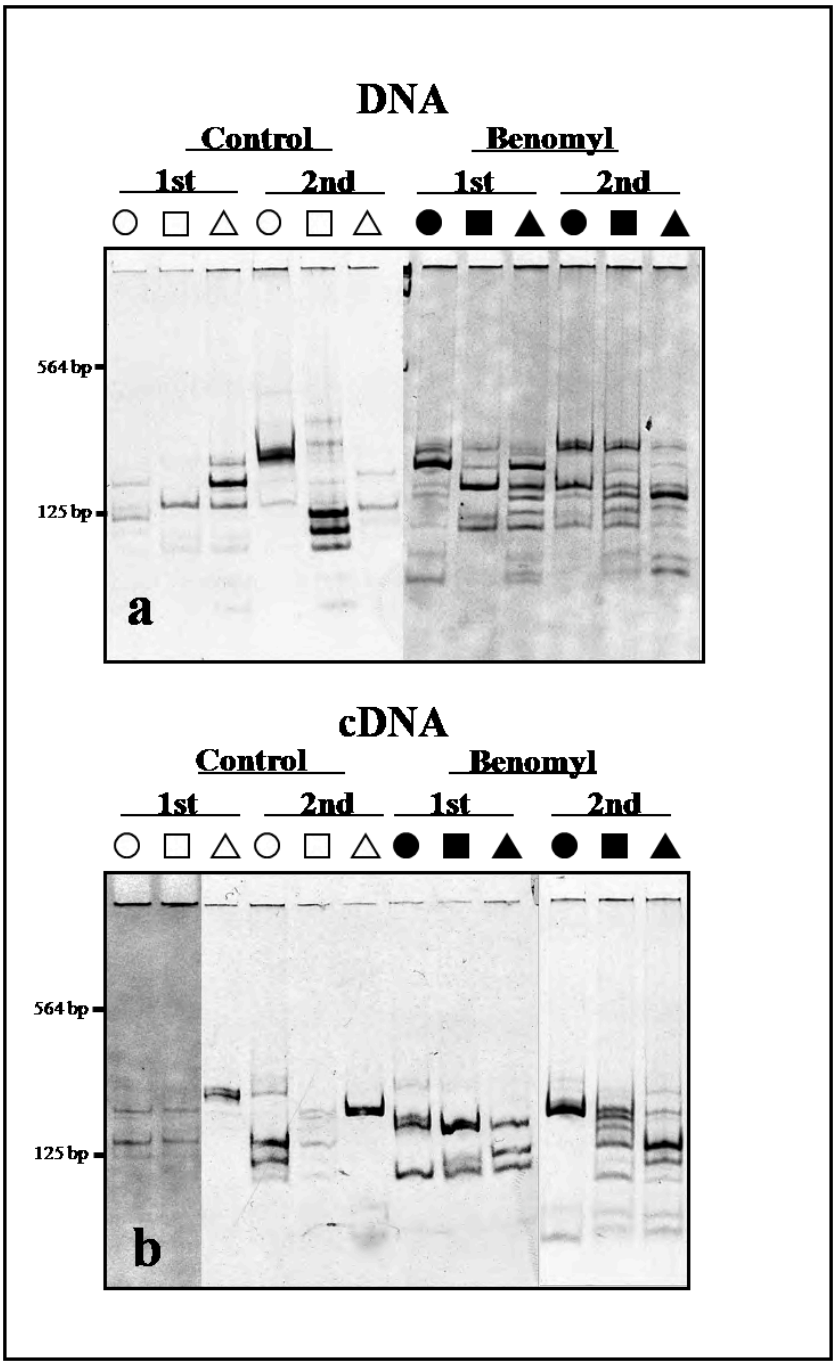

Fig. 6.2: RFLP profiles of nitrogen-fixing bacteria of the ε-subclass of Proteobacteria for replicate treatments. RFLP acrylamide gel of *Dde*I restricted *nif*H PCR product for the ε-subclass of Proteobacteria generated using the ENFBf and ENFBr primer set (43) in a nested PCR from DNA (a), and cDNA (b), extracted from soil in Benomyl-treated (●) and non-treated (○) cores with *S. patens* at a depth of 2.5 cm corresponding to 3 plant phenological stages, i.e., ○ vegetative growth (April), □ reproduction (May) and △ senescence (August). Each well has digested PCR product from 1 plant core. 1st and 2nd refers to samples from replicate plant cores analyzed independently. The size marker (M) is lambda DNA cleaved with *Hind*III.

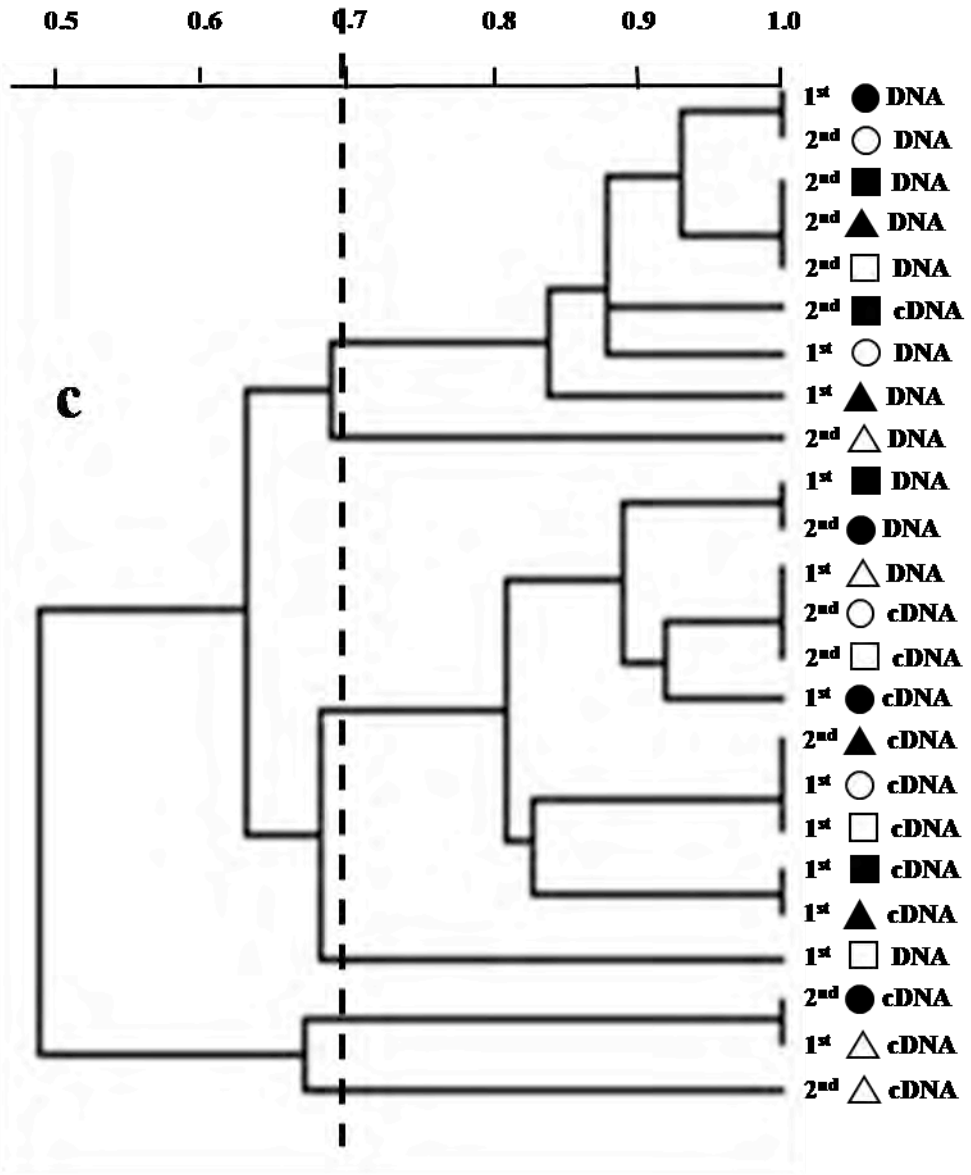

Fig. 6.2 continued: RFLP profiles of nitrogen-fixing bacteria of the ε-subclass of Proteobacteria for replicate treatments. (c) Corresponding UPGMA dendrogram from RFLP fragments generated using GelManager v 1.5 (Biosystematica). The dashed line marks the 0.7 borderline above which patterns were assumed to be the product of the same population.

The DGGE profiles of *nif*H gene fragments representing nitrogen-fixing members of the ε-subdivision of Proteobacteria, however, suggested that the profiles generated from DNA were more variable from one another than the cDNA profiles are; thus, the total community of nitrogen-fixing bacteria present was more variable than the active community. As in the RFLP profiles, no clear treatment effect could be discerned in the patterns of the DGGE profiles (Fig 6.3). Profiles generated from DNA and cDNA from the same time and treatment did not necessarily overlap and the profile based on cDNA was not necessarily a less complex version of the corresponding profile based on DNA (Fig. 6.3 a and b). There was no trend in the DGGE profiles generated by either cDNA or DNA to suggest that there was an increase in differences in community structure over the season. The DGGE profiles were highly variable throughout the season and it was very difficult to discern any patterns in community structure.

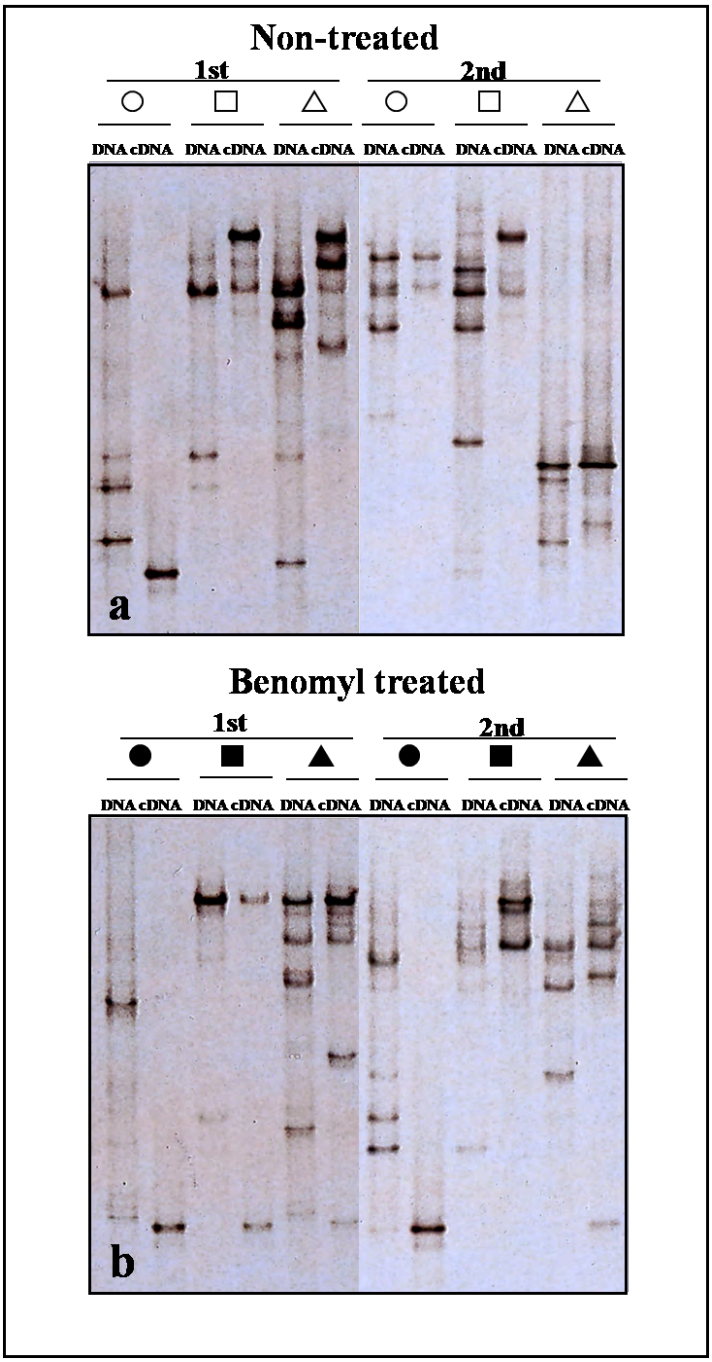

Fig. 6.3: DGGE profiles of nitrogen-fixing bacteria of the ε-subclass of Proteobacteria for replicate treatments. DGGE profiles *nif*H PCR product for the ε-subclass of Proteobacteria generated with the ENFBf and ENFBr primer set in a nested PCR that had been generated with either DNA (a) or cDNA (b) as template extracted from soil in Benomyl-treated (●) and non-treated (○) cores with *S. patens* at a depth of 2.5 cm corresponding to 3 plant phenological stages, i.e., ○ vegetative growth (April), □ reproduction (May) and △ senescence (August). Each well has digested PCR product from 1 plant core. 1st and 2nd refers to samples from replicate plant cores analyzed independently.

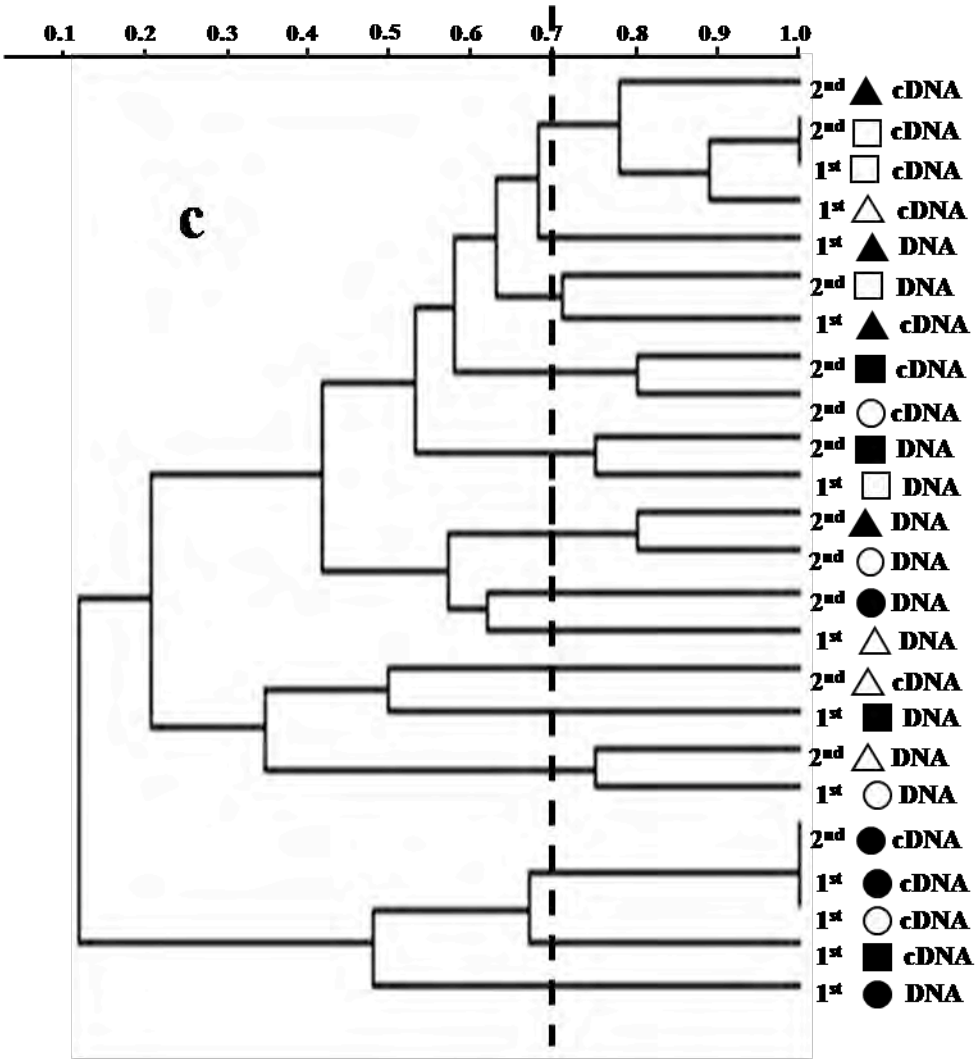

Fig. 6.3 continued: DGGE profiles of nitrogen-fixing bacteria of the ε-subclass of Proteobacteria for replicate treatments. (c) Corresponding UPGMA dendrogram from DGGE profiles generated using GelManager v 1.5 (Biosystematica). The dashed line marks the 0.7 borderline above which patterns were assumed to be the product of the same population.

These results from replicate plant cores of the same treatment analyzed individually demonstrated different ecological implications of the seasonal effects of Benomyl application and AMF reduction on the community structure of nitrogen-fixing bacteria overall and specifically of nitrogen-fixing members of the ε-subdivision of Proteobacteria than were interpreted from analyses on mixed samples of the same treatment.

The analyses on the overall nitrogen-fixing community structure using samples mixed from replicate treatments suggested that the active nitrogen-fixing community, i.e. those detected using reverse-transcribed *nif*H mRNA (cDNA), was very different from the present community, i.e. those detected using the *nif*H gene (DNA). In the RFLP analyses of all nitrogen-fixing bacteria with replicate treatments analyzed individually, the active community was not different from the present community (Fig 6.1). Both kinds of analyses, mixed samples and samples analyzed individually, showed that the active community was different seasonally and the present community did not change seasonally as might be expected because DNA can be detected even in dormant and dead cells (21). However, concerning bacterial community structure, the data might or might not indicate that the active community of nitrogen-fixing bacteria was distinctly different from the entire community of nitrogen-fixing bacteria present in the environment.

Specific analyses of nitrogen-fixing members of the ε-subdivision of Proteobacteria in samples mixed from replicate treatments using both RFLP and DGGE suggested that the active population varied greatly over the season and that populations in the non-treated samples were more similar to populations the Benomyl-treated samples at the beginning of the season than at the end of the season implying bacterial succession acting

over the season in response to treatment (2). However, when analyzed individually, the trend of increased differences between non-treated and Benomyl-treated samples was not supported (Fig 6.2 and 6.3). For example, comparing RFLP profiles from non-treated and Benomyl-treated samples using the first replicate showed that the active community was the same for all treatments and time points, except for the profile from the non-treated sample at senescence. However, comparing the RFLP profiles of the second replicate indicated that populations in the non-treated and Benomyl-treated samples were different at each time point and no more different at the vegetative growth time than at the senescence time point.

RFLP and DGGE analyses of replicate treatments that were mixed and then analyzed suggested that seasonal changes in plant performance and presence of AMF coincided with increased differences in the community structure of nitrogen-fixing members of the ε-subdivision of Proteobacteria. If viewing these same samples individually there was no coincidence of increased community differences, AMF and plant performance, and the trends in community structure change of specific nitrogen-fixing members of the ε-subdivision of Proteobacteria were much more difficult to interpret.

These analyses of individual or mixed sample treatments clearly illustrated the effects of sampling and sample processing on interpretation of data on bacterial diversity in the environment recovered using molecular tools. These conflicting interpretations of bacterial diversity change might simply have resulted from how we chose to sample, and not reflect treatment or seasonal effects at all. Edaphic conditions like pH are critical for shaping soil bacterial community structure overall (6, 8, 41) and specifically for the structure of present nitrogen-fixing bacterial communities (19). Microsensor analyses

have shown profound gradients in O_2, NO, or H_2S at the millimeter scale in submerged rhizosphere sediments (34) or marine sediments (40) and these changes in physiochemical parameters have been linked to changes in bacterial community structure. Even in less extreme environments like the rhizosphere of arable soils micro-scale gradients in oxygen have been demonstrated (14). Nicol *et al*. (31), examined the diversity of archaeal community structure using a sampling scheme over 2m intervals along triplicate 8m transects in 10g, 1g, and 0.1g samples both using intact cores and soil core homogenates (31). DGGE profiles of 10g samples from intact cores were more similar with decreased distance and within a grassland treatment, but profiles from the 1g and 0.1g samples of intact cores showed no similarity by location or treatment. When soil samples were homogenized the community profiles for each sampling size were similar, indicating that micro-site heterogeneity within intact soil cores was high (31). In addition, homogenization and sampling did not detect unique diversity only present in specific microenvironments, which may be due to preferential amplification of more abundant sequences in PCR and low detection thresholds of DGGE (1, 28, 31). Thus micro-site heterogeneity perhaps due to micro-scale environmental gradients can affect the diversity of detected soil microbial community structure.

If bacteria do indeed demonstrate a taxa-area relationship, then it is likely that -as the area sampled increases- there will be greater variety in environmental gradients, thus bacterial diversity will increase (16, 42). Biogeographic studies attempting to link spatial distance and environmental heterogeneity have correlated specific bacterial diversity and specific environmental conditions, but most of the variation in overall bacterial community structure (42) and in *Burkholderia* genotypes (33) was unexplained. This

unexplained variation was suggested to be due to unmeasured environmental variability and sampling effects (33). Thus the variations we detected in profiles of nitrogen-fixing bacteria and specific nitrogen-fixing members of the ε-subdivision of Proteobacteria may reflect environmental heterogeneity induced by seasonal or treatment effects but it is also possible that this variation is due to undetected environmental heterogeneity or sampling itself.

Soil micro-site abiotic environmental heterogeneity can drive bacterial diversity and community structure but diversity can also be shaped by biotic microbial interactions. Examination of the distribution of ammonia- and nitrite-oxidizing bacteria along a millimeter soil transect showed that these two groups were not independently or randomly located spatially, presumably because nitrite oxidizers use the metabolites ammonia oxidizers produce (9). These organisms together had a spatial range of 2 to 4 mm which is much smaller than the usual scale for soil sampling in diversity studies of several grams and for measuring soil parameters like pH and redox potential (9). Additionally, specific strain detection within the family Vibrionaceae was correlated with micro-site habitat and bacterial lifestyle (i.e. free living, particle associated, or zooplankton associated) (18). However, some bacterial taxa detected using micro-site sampling techniques do show presumably random distributions. Diversity of organisms of the genus *Nitrobacter* sampled at a submillimeter scale in soil was almost that found for the whole genus (10). Diversity of specific strains of *Bacillus subtilis* was as high using micro-scale detections (i.e. $1cm^3$ soil samples progressively divided into smaller sections and analyzed) as on the macro-scale (i.e. meters to kilometers) (39). Some bacteria may show non-random distributions at the micro-scale in relation to other

organisms or specific environmental conditions while other bacteria may be truly ubiquitous even at the micro-site level of distribution and detection. Thus, bacterial diversity estimates can also be confounded by differences in distribution profiles and lifestyle for different bacterial groups particularly if detection techniques do not target active organisms.

Multiple studies using a variety of molecular tools like gene arrays (51), sequencing of clonal libraries (48), T-RFLP (22), and DGGE (25) have shown that the active nitrogen-fixing bacterial community, i.e. those expressing the *nif*H gene as detected by reverse transcription of *nif*H mRNA, were distinctly different from the community structure suggested by those with the metabolic potential for nitrogen fixation, i.e. those detected based on the *nif*H gene. The active population of nitrogen-fixing bacteria was represented by only a small fraction of a 56-oligonucleotide probe microarray (51). This limited detection may be due to low diversity of active nitrogen-fixing bacteria or lack of probes on the array with homology to these active organisms (51).

Detection sensitivity is difficult to determine for the protocols we have developed targeting nitrogen-fixing members of the ε-subdivision of Proteobacteria where we were unable to quantify the specificity of these amplification protocols due to the lack of available pure cultures (43). Based on the diversity of sequences retrieved by DGGE, it is likely that we were able to target multiple genera within the ε-subdivision of Proteobacteria, and thus we could detect even minor changes in this group (43). Protocols targeting all nitrogen-fixing bacteria in an environment are correspondingly less sensitive because they amplify such a wide diversity of sequences that only changes in dominant populations are able to be detected (19). The comparatively increased sensitivity of our

DGGE analyses further complicates the patterns observed in community structure changes with season and treatment because changes in structure due to unexplained variation like environmental micro-heterogeneity and sampling effects are likely able to be detected and are not lost in the noise of universal, overall community changes.

The sampling scale used in this study was 1.5cm soil cores from 1 kg plant cores fixed in 13x13x16 cm^3 pots sampled from a 1.5 to 3.5 cm depth in one gram portions. Then another plant core of the same treatment was sampled similarly at the same time point. If the diversity of nitrogen-fixing members of the ε-subdivision of Proteobacteria has a non-random spatial distribution on a scale of millimeters (9, 18) and is shaped by micro-site environmental heterogeneity (31) then it is highly unlikely that 2 different plant cores could possibly support the same micro-site variation. In fact we could probably not get the same DGGE profiles for active nitrogen-fixing members of the ε-subdivision of Proteobacteria even if we sampled within the same 1.5 cm soil core.

For our study, the seasonal effect on the overall community structure of all nitrogen-fixing bacteria and of a specific group of nitrogen-fixing bacteria of the ε-subdivision of Proteobacteria in non-treated and Benomyl-treated samples remains unclear. The profiles generated by analyses of individual samples likely represent micro-site variations in environmental parameters that may obscure the overall diversity trends (10, 18, 31). The profiles generated by analyses of mixed samples of the same season and treatment likely do not capture all the micro-heterogeneity due to the season or treatment because only 2 replicate samples were mixed (31). The most accurate representation of the overall diversity present would likely be generated by dozens of micro-scale samples both within the same plant core and between multiple plant cores of the same treatment processed and

analyzed separately then subjected to a meta analysis to look for broad overall patterns of diversity with season and treatment in addition to fine scale sequence based analyses to link individual sequences with very specific micro-sites and conditions.

In the frankiae system, we successfully applied tools to study the diversity of frankiae in root nodules but were unable to directly study the diversity of frankiae in soil. This was not due to sampling constraints or lack of available target sequences from which to design amplification protocols but rather was due to difficulties in the specific application of these molecular tools for frankiae detection. We attempted to establish a DGGE protocol for the detection of frankiae similar to the *Spartina* system in which DGGE profiles generated using reverse transcribed mRNA of the nitrogenase gene *nif*H (cDNA), representing those organisms active in the soil, could be generated beside *nif*H gene profiles (DNA), representing those organisms present in the soil, and generated beside *nif*H gene profiles from uncultured frankiae in root nodules from plants grown in that soil. DGGE profiles from frankiae in soil generated using *nif*H cDNA may have shown that frankiae are part of the free-living soil nitrogen-fixing bacterial community. It might also have shown that distinct populations are active, while others are not (i.e. in a comparative analysis with DNA and cDNA as template). The former might then be those forming root nodules, while the latter might not and this could have been demonstrated by comparative analyses of soil and root nodule populations using DGGE.

Amplification of frankiae from soil has been shown to be difficult. This is most likely due to the small population sizes of frankiae in soils that required nested PCR in previous studies (26, 29). Additional primers for frankiae were designed internal to the 606 bp region previously developed for the amplification of the *nif*H gene for frankiae (45) based

on an alignment of over 80 pure cultures of *Frankia*. For amplification of frankiae directly from soil this 606 bp product was amplified as described (45) and used as template in a second PCR with newly designed frankiae primers FraNif256f ($^{5'}$ACC TCC ATC ACC TAC CTG GAG) and nifHr as described (45) using an annealing temperature of 64°C. The protocol was successful in amplifying *nif*H PCR product from 10 pure cultures from different frankiae genomic groups and in amplifying frankiae directly from soil from Peru and 2 different soils from Colorado.

The nested PCR protocol for detection of frankiae in soil was designed for DGGE analysis. The amplified region for DGGE was designed with one melting domain as determined by using the program Meltmap (24). This resulted in an amplicon with a guanine + cytosine (GC) nucleotide content of about 63% and a T_m of about 80°C which was right at the threshold able to be distinguished by DGGE (47). The reverse primer nifHr (45) had a 60-bp GC-clamp which is longer than the typical 40-bp GC-clamp used for typical DGGE analyses (28) to aid in high GC-content template separation (47). This amplicon was 411-bp long with the GC-clamp and well within the size limits suggested for DGGE (28).

Development of the frankiae *nif*H gene DGGE protocol was executed as described (44). The denaturing gradient, template amount, electrophoresis time, and voltage were all optimized (3, 5, 7, 37). Despite numerous optimizations, all the frankiae *nif*H gene DGGE gels were characterized by 3 large smeary bands low in the gel, presumably caused by single stranded DNA, and comparatively faint, multiple bands even from pure cultures (6% acrylamide, 60-80% denaturant, 7hrs at 180 volts) (i.e., Fig 6.4).

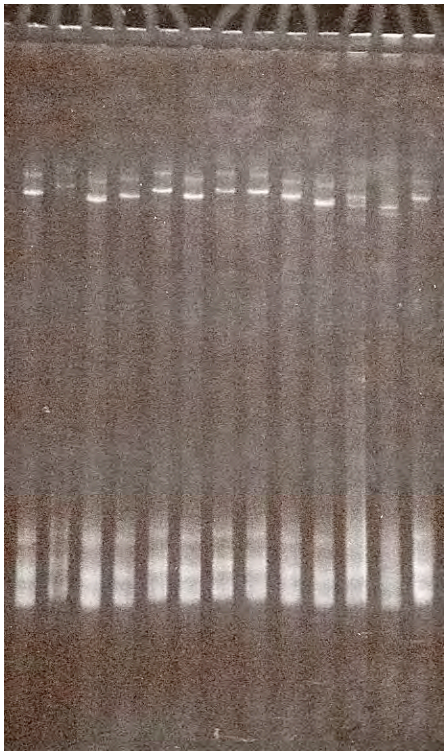

Fig. 6.4: DGGE profiles of cultured and uncultured soil frankiae. DGGE profiles of frankiae amplicons generated with primer set FraNif256f/GC-nifHr after nested PCR with primers nifHf1/nifHr of frankiae strains Ea1.12, CcI3, 32-85, AgP_1R_2, Ag45/Mut15, AiPs1, AgB16, EuI4, ArI3, AiPs4, and Peruvian soil and 2 Colorado soils, respectively.

Ultimately, the DGGE technique could not be applied to the *nif*H gene of frankiae because the multiple bands present in pure cultures and the large smears low in the gel for both pure cultures and for soil samples could not be eliminated. Extraneous bands in DGGE have been attributed to heteroduplex formation (5, 38) which may have been a consequence of the nested PCR we used to generate DGGE PCR product. However, for the pure cultures included in these analyses multiple bands were present using nested or single amplification PCR protocols from DNA as template. These multiple bands high in the gel and smears low in the gel may be due to single stranded DNA generated during PCR (4, 20) or due to some complex interaction of various types of unwanted amplicons

(32). Additionally due to the high GC-content of the *nif*H gene of frankiae, the denaturant concentration needed to cause this double stranded PCR product to denature and become lodged in the gel as a distinguishable band was likely very close to the concentration needed to completely denature the PCR product even with a 60-bp GC-clamp. Therefore, the smears low in the gel may be due to denaturation of the target PCR product. This theory is supported by the bright bands low in the gel for all strains and soils amplified.

Development of DGGE protocols for frankiae using a gene other than the *nif*H gene was considered. Analyses of the 16S rRNA gene is most commonly used in bacterial systematics to describe and compare various bacteria in a wide range of applications and environments (23, 36). However, the 16S rRNA gene does not have enough variation to delineate different species within established host infection groups of frankiae (12, 30). The 23S rRNA gene insertion in Domain III, present in all bacteria with a high G+C DNA content, has been used to differentiate frankiae into host infection groups and subgroups due to its high variability (15). These host infection subgroups have been used to describe natural populations of frankiae in root nodules and soils using PCR with 23S rRNA gene primers specific for different host infection subgroups (26, 50) and hybridization based analyses with probes specific for infection subgroups (15, 26, 49, 50). However, the 23S rRNA gene insertion is not under any selection pressure to produce gene product and may vary unpredictably from one strain or species of *Frankia* to another (35). Thus, the 23S rRNA gene insertion may not provide the necessary resolution to describe broad phylogenetic relationships within frankiae.

Initial investigations on the usefulness of the 23S rRNA gene insertion for DGGE analysis were more favorable than that for the *nif*H gene. Sequence analysis of the 23S

rRNA gene insertion of a group of 10 pure cultures of frankiae indicated that the gene had one melting domain (24), had a T_m of about 76°C and was about 57% GC. This T_m is still quite high but below the threshold that has been used for detection using DGGE (47). Additionally, the 23S rRNA gene insertion amplicon was 191 bp long, well within the size limits suggested for use in DGGE (28).

Before committing to developing a DGGE protocol for frankiae using the 23S rRNA gene insertion, we completed comparative phylogenetic analyses for the same pure cultures of frankiae and uncultured frankiae from root nodules of *Alnus oblongifolia* for both the *nif*H gene and the 23S rRNA gene insertion as described (27). Based on bootstrap and posterior probability values, the 23S rRNA gene insertion analysis had lower resolution as compared to the *nif*H gene analyses (Fig. 6.5). Only bootstrap values over 70% (13) or Bayesian posterior probabilities over 95% (17) represent well supported phylogenetic relationships. The *nif*H gene analysis had 4 well supported internal nodes and 12 well supported tip nodes whereas the 23S rRNA gene insertion analysis had 2 well supported internal nodes and only 4 well supported tip nodes (Fig 6.5). Additionally, the analysis of the 23S rRNA gene insertion did not support the separation of strains ARgP5^{AG} and ORS020607 from strains like ArI3 and MpI1 (Fig. 6.5, in bold) which are in different genomic groups (11), but the *nif*H gene analysis did separate these strains. Thus, the 23S rRNA gene insertion of frankiae did not have the phylogenetic resolution of the *nif*H gene. The 23S rRNA gene insertion analysis could not be used to find a link between diversity and geography of uncultured frankiae in root nodules of *A. oblongifolia* on different sky islands in southern Arizona. Subsequent analyses using both the *nif*H gene and 23S rRNA gene insertion on 34 pure cultures also showed that the 23S rRNA

gene insertion did not have the same power to resolve relationships between closely related frankiae strains that the *nif*H gene had (27).

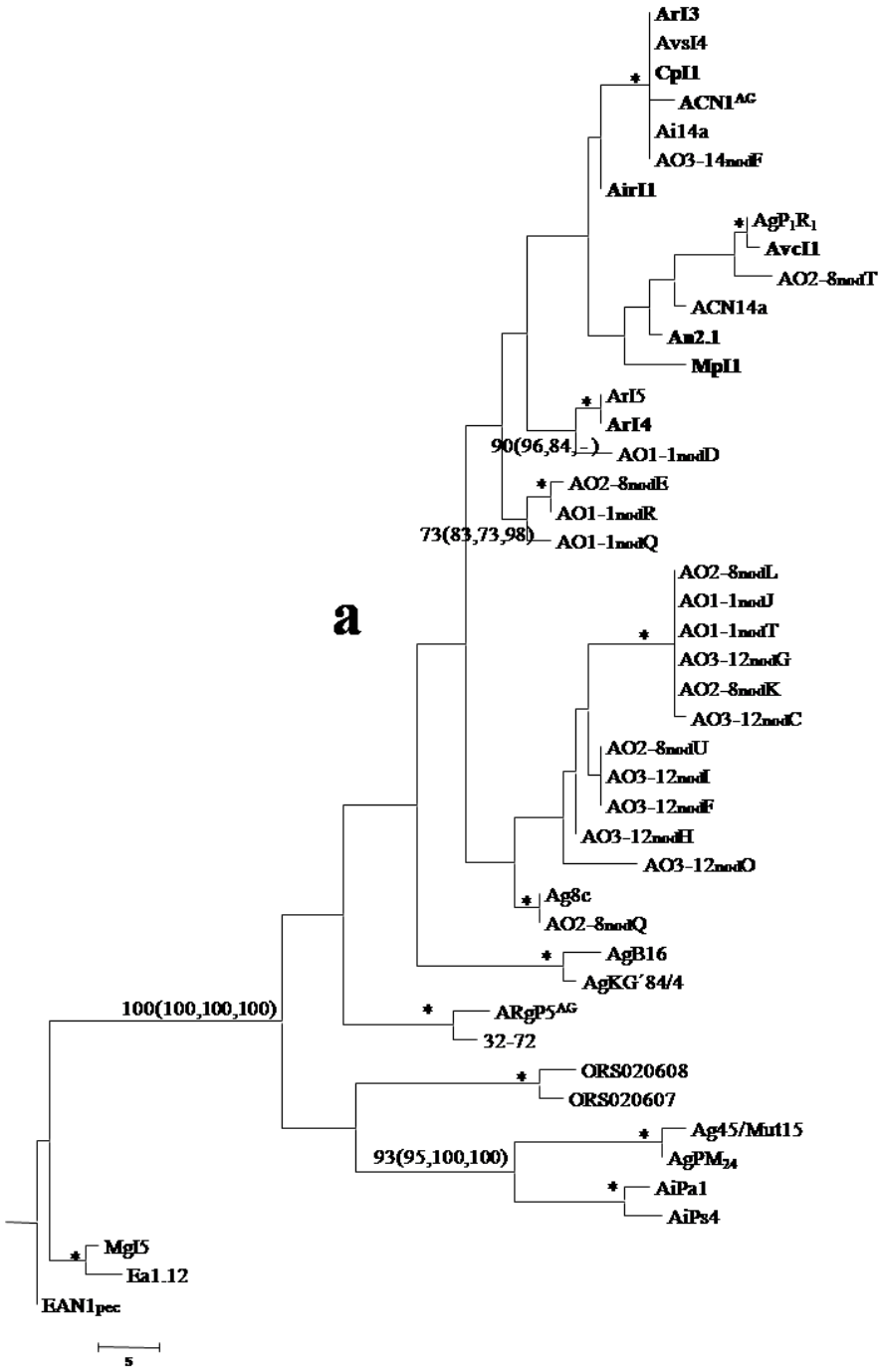

Fig. 6.5: Phylogenetic analyses of selected cultured and uncultured *Frankia* from root nodules of *Alnus oblongifolia*. Maximum parsimony trees of uncultured *Frankia* from root nodules of *Alnus oblongifolia* and other pure cultures created using 522 bp of the *nif*H gene (a) and using 152 bp of the 23S rRNA gene insertion specific for actinomycetes (b). Numbers reflect bootstrap support (BS) values and number in parentheses reflect BS or posterior probabilities from neighbor joining, maximum likelihood and Bayesian analyses, respectively. Strains in boldface belong to *Frankia* genospecies 1 (11). Strain EAN1pec was used as the outgroup.

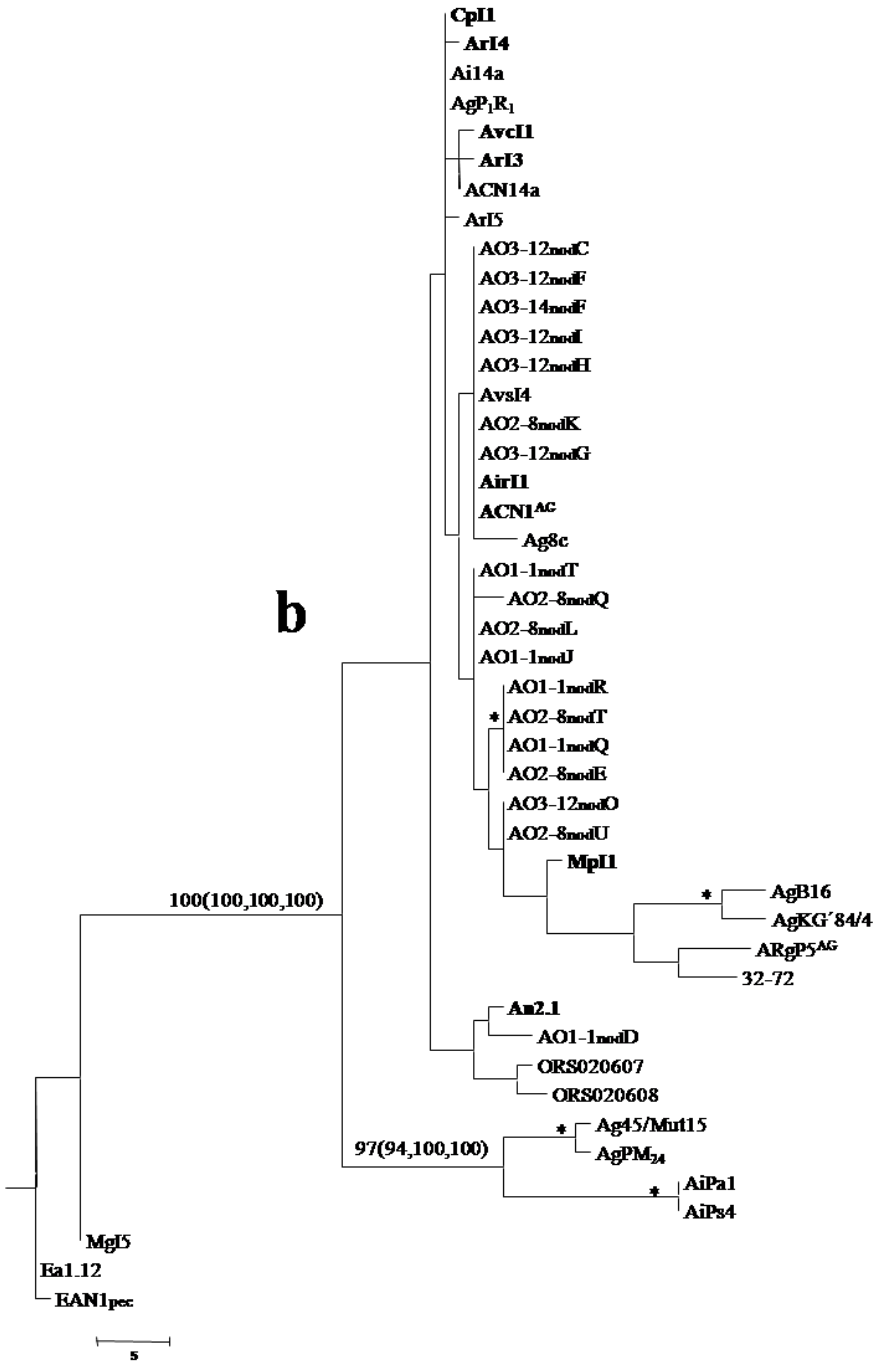

Fig. 6.5 continued: Phylogenetic analyses of selected cultured and uncultured *Frankia* from root nodules of *Alnus oblongifolia*. Maximum parsimony trees of uncultured *Frankia* from root nodules of *Alnus oblongifolia* and other pure cultures created using 522 bp of the *nif*H gene(a) and using 152 bp of the 23S rRNA gene insertion specific for actinomycetes (b). Numbers reflect bootstrap support (BS) values and number in parentheses reflect BS or posterior probabilities from neighbor joining, maximum likelihood and Bayesian analyses, respectively. Strains in boldface belong to *Frankia* genospecies 1 (11). Strain EAN1pec was used as the outgroup.

Sequences of the *nif*H gene might provide the resolution to distinguish closely related *Frankia* strains, but the gene was not conducive to DGGE analyzes likely due to the high GC-content. The 23S rRNA gene insertion did not have the resolution to distinguish closely related frankiae strains even though it had lower GC-content and might be conducive to DGGE analyses. DGGE requires both a gene that is phylogenetically informative and a gene amenable to DGGE analyses. This gene has yet to be identified for frankiae.

The major advantage that molecular tools have over traditional microbiological techniques in ecological studies is that information can be retrieved on uncultured bacteria in the environment circumventing the biases of isolation and laboratory cultivation. However, molecular tools also have biases and limitations. We have demonstrated the inherent problems in sampling and interpreting data on bacterial community structure in the environment with differing sampling schemes and analyses. Additionally, we have shown that some genes for some bacteria are not amenable to some types of molecular tools like DGGE. Despite these challenges to the interpretation and application of molecular tools we have greatly expanded the database of sequences of *nif*H genes for both nitrogen-fixing members of the ε-subdivision of Proteobacteria and cultured and uncultured frankiae and have gathered baseline data that demonstrated the usefulness of these databases and molecular tools for studies on the ecology of both nitrogen-fixing members of the ε-subdivision of Proteobacteria and *Frankia* populations.

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