

DIVERSITY OF *FRANKIA* POPULATIONS IN ROOT NODULES OF
SYMPATRICALLY GROWN *ALNUS* SPECIES

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**DIVERSITY OF *FRANKIA* POPULATIONS IN ROOT NODULES OF
SYMPATRICALLY GROWN *ALNUS* SPECIES**

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ABSTRACT

DIVERSITY OF *FRANKIA* POPULATIONS IN ROOT NODULES OF SYMPATRICALLY GROWN *ALNUS* SPECIES

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The potential role played by the host plant or the soil type in the selection of symbiotic, nitrogen-fixing *Frankia* strains from soil for root nodule formation was assessed by using molecular techniques. The diversity of *Frankia* populations in the root nodules of twelve *Alnus* species grown sympatrically was analyzed using rep-PCR (Repetitive Extragenomic Palindromic polymerase chain reaction). Analysis of 120 root nodules, i.e. 10 root nodules from each plant species revealed three rep-profiles which indicated the presence of only three *Frankia* strains in the root nodules. One profile (further referred to as Type I) was most abundant and was identical within and among the nodules of nine species (*A. incana*, *A. japonica*, *A. glutinosa*, *A. tenuifolia*, *A. rugosa*, *A. rhombifolia*, *A. mandsurica*, *A. maritima* and *A. serrulata*). Type II profiles were found in all nodules of two plant species (*A. hirsuta* and *A. glutinosa pyramidalis*), whereas the

type III profile was unique for *Frankia* populations in nodules of *A. rugosa americana*. No variation was detected in *Frankia* populations among the root nodules of a single plant species, i.e., all nodules from one plant species had identical rep-profiles. Comparative sequence analyses of *nifH* gene fragments from three *Frankia* strains representing each rep-profile (Type I: *A. tenuifolia*; Type II: *A. glutinosa pyramidalis*; Type III: *A. rugosa americana*) revealed differences, with Type I and II clustering with *Frankia* strains of subgroup AII (represented by strain Ag45/Mut15) and Type III with *Frankia* of subgroup AI (represented by strain ArI3). Comparative sequence analysis with 73 clones from a *nifH* gene clone library from frankiae in soil where these 12 alder species were growing, however, revealed that none of these sequences represented frankiae detectable in soil. Sequences from all 73 clones clustered in subgroup AI with similarity values of 93, 97, and 99.6 %, to sequences from populations in nodules of *A. tenuifolia*, *A. glutinosa pyramidalis* and *A. rugosa americana*, respectively. Additional analyses of nodule populations from alders growing on different soils demonstrated the presence of different *Frankia* populations in nodules for each soil, with populations showing identical sequences in nodules from the same soil, but differences between plant species. These results suggest that soil environmental conditions and host plant species have a role in the selection of *Frankia* strains for root nodule formation and this selection is not a function of the abundance of a *Frankia* strain in soil.

INTRODUCTION

Nitrogen is an essential plant nutrient, and in most soils, a limiting factor for plant growth. Although nitrogen comprises nearly 80% of the earth's atmosphere in the form of dinitrogen gas (N_2), plants are unable to use this form of nitrogen for their growth and development. Plants can assimilate nitrogen in the form of ammonium and nitrate. They fulfill their nitrogen requirements from decomposing organic matter, chemical fertilizer or through biological nitrogen fixation by microorganisms. In the process of biological nitrogen fixation, many Bacteria and certain Archaea reduce atmospheric N_2 into ammonium with the help of the enzyme nitrogenase. The total amount of nitrogen fixed by free living and symbiotic microbes is estimated to be 100-175 million metric tons per year (Burns and Hardy 1975).

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 (Dawson 1990). These actinomycetes are gram positive and filamentous in structure. The genus *Frankia* has been broadly divided into three categories depending on the host plant family: an *Alnus* (Betulaceae)/Myricaceae/Casuarinaceae group, an Elaeagnaceae/Rhamnaceae group and a third group including *Frankia* strains nodulating plant species in the families, Coriariaceae, Datisceae and Rosaceae and in the genus *Ceanothus* (Rhamnaceae) (Normand, Orso et al. 1996; Swensen 1996; Clawson and Benson 1999). There are more than 200 species of host

plants that are referred to as actinorhizal plants. They belong to 24 genera, eight families and seven orders of Angiosperms (Schwintzer and Tjepkema 1990; Benson and Silvester 1993) and are distributed all over the world except Antarctica. They can naturally colonize open areas (clear cuts, shrub tundra), forests, riparian habitats, glacial tills, mine spoils and gravel deposits. These plants are successful pioneer plants, frequently establishing themselves after flooding, fires, landslides, glacial activity, as well as after volcanic eruptions (Dawson 1990). They grow on soils with a wide range of properties (Dixon and Wheeler 1983; Dawson 1990). They physically enhance the stability of soils with their well-developed root systems (Knowlton and Dawson 1983) and increase nitrogen mineralization rates in soil thereby increasing nitrogen availability and improving the quality of impoverished soils. Ecologically, actinorhizal plants are therefore useful for reforestation and reclamation of nitrogen-limited soils.

In the plant family Betulaceae, *Alnus* is the only genus that has a symbiotic relationship with *Frankia*. With 34 species belonging to this genus, the genus *Alnus* has a very wide distribution ranging from temperate to alpine ecosystems (up to 2500m). They are drought tolerant and are also capable of regenerating from stumps, e.g., those left after tundra fires (Roy, Khalsa et al. 2007). In temperate regions, *Alnus* species, or alders, are widely used in forestry and agroforestry projects (Gordon and Dawson 1979; Gordon 1983). They serve as nurse trees in mixed plantations with other more valuable tree species, and are also used as an important source of fuelwood and timber (Gordon and Dawson 1979; Dawson 1983; Gordon 1983; Dawson 1986). *Alnus* sp. have been planted in rotation with more valuable crops/trees to maintain soil fertility (Dai, He et al. 2004). Mixed plantations of *Alnus* and pines have been found to improve the yield of the pine

trees (Dai, He et al. 2004) because *Alnus* increases the nitrogen availability in soil. In cold temperate areas indigenous legumes are generally absent, and thus the nitrogen fixed by *Frankia* is very important (Lalonde, Knowles et al. 1976). The symbiosis between actinorhizal plants and *Frankia* can contribute from 60 to 320 kg N ha⁻¹ y⁻¹ through nitrogen fixation (Paschke, Dawson et al. 1989). However, since mainly nitrogen from mineralized organic matter is utilized by the associated tree crops, rates of nitrogen mineralization more accurately reflect the influence of actinorhizal plants on soil fertilization than estimates of total nitrogen input (Dawson 1990). The amount of nitrogen from mineralization of organic matter may range between 48 and 185 kg N ha⁻¹ y⁻¹ for alder stands (Paschke, Dawson et al. 1989).

The establishment of *Frankia* strains in root nodules of alders has been reported to be influenced by environmental factors such as the soil pH (Griffiths and McCormick 1984; Jaman, Fernandez et al. 1992; Crannell, Tanaka et al. 1994), the soil matric potential (Schwintzer 1985; Dawson, Kowalski et al. 1989), and the availability of elements such as nitrogen (Kohls and Baker 1989; Thomas and Berry 1989) or phosphorus (Sanginga, Danso et al. 1989; Yang 1995). In addition to these soil factors, the genotypes of both partners in this symbiosis have been reported to affect the nodulation capacity (Dawson, Kowalski et al. 1989; Navarro, Jaffre et al. 1999). Recently, Van den Heuvel et al. (Heuvel, Benson et al. 2004) suggested that the selection of *Frankia* strains among and within specific host infection groups is primarily the function of the host plant, i.e., the host plants select the particular strains from the soil. The interaction between host plant species and environmental factors thus plays an important role in the selection of *Frankia* strains for root nodule formation (Oakley,

North et al. 2004) which is crucial for an efficient symbiosis between *Frankia* and woody plants of the genus *Alnus*.

Effects of environmental conditions, plant species and isolates of *Frankia* on the establishment of the symbiosis are relatively easy to assess under laboratory conditions and thus a considerable amount of information is available on isolates of *Frankia* and on their interaction with host plant species (Benson and Silvester 1993; Huss-Danell 1997). Quantitative analyses of specific *Frankia* populations originating from soil and their interaction with plants and site conditions, however, are methodologically extremely challenging due to problems encountered with the isolation and identification of *Frankia* strains (Schwintzer and Tjepkema 1990; Benson and Silvester 1993). Consequently, information on the fate and diversity of *Frankia* populations in soil is scarce. Most of the studies on *Frankia* in soil are based on plant bioassays, in which a quantification of the nodulation capacity on a specific host plant (expressed as nodulation unit g⁻¹ soil) is used to describe the infective *Frankia* populations. This approach includes regression and most probable number (MPN) methods in which host plants inoculated with serial dilutions of *Frankia*-containing samples are statistically analyzed on the basis of nodule formation (Huss-Danell and Myrold 1994).

The use of bioassays for quantitative assessments of *Frankia* populations, however, is hampered by its selectivity, since only nodule-forming populations can be detected. Since a nodule can theoretically be induced by a single spore, a hyphal fragment, or a colony, the correlation of the nodulation unit with cell numbers remains problematic (Myrold, Hilger et al. 1994). Adding to this problem is the uncertainty whether the infectious *Frankia* particle in soil is really an actively growing organism

present in vegetative form (i.e., in long filaments as in pure culture, in short fragments, or in single cell form) or a spore activated by exudates of the capture plant used in the bioassay. In the latter case, nodule formation would reflect properties of the capture plant rather than of the soil to be analyzed. Furthermore, questions on *Frankia* populations belonging to host infection groups other than the test plants (Baker 1987), or on non-nodulating *Frankia* populations of the same host infection group (Hahn, Starrenburg et al. 1988) are neglected. Other drawbacks of the bioassay include the failure to analyze non-competitive *Frankia* populations, including those present in low numbers, the inability to quantify competition for infection on the capture plant between different *Frankia* populations in a sample, and variable compatibilities of host plants to specific *Frankia* populations (Huss-Danell and Myrold 1994; Maunuksela, Hahn et al. 2000). Nodulation capacities can therefore reflect the effect of the plant species on a specific *Frankia* population with respect to nodulation rather than represent a quantitative picture of the overall structure of nodule-forming populations in the soil (Mirza, Welsh et al. in press).

In recent years molecular approaches have increasingly supplemented nodulation-dependent detection methods for studying *Frankia* populations in nature. The most prominent advancement in studies on the ecology of *Frankia* has resulted from the use of the polymerase chain reaction (PCR) which enabled researchers to amplify minute quantities of target sequences on DNA and to rapidly retrieve sequence information from uncultured organisms in the environment. The potential of this technique was initially exploited for the phylogenetic analysis of isolates and uncultured endophytes in nodules but has also been used for the detection and analysis of specific *Frankia* populations in nodules and soil as specific sequence information became available. Generally, genes

with a high phylogenetic significance such as ribosomal RNAs (rRNAs) have been targeted for such analysis. A differentiation of the genus *Frankia* from other nitrogen-fixing organisms has also been achieved by using sequences of *nifH*, the structural gene for nitrogenase reductase (Howard and Rees 1996), the size or the sequences of the intergenic spacer (IGS) between the nitrogenase *nifH* and *nifD* (*nifH*-*D*) genes (Simonet, Grosjean et al. 1991) or the *nifD* and *nifK* (*nifD*-*K*) genes (Jamann, Fernandez et al. 1993), or from other actinomycetes by using the glutamine synthetase II (*glnII*) gene as the target for PCR (Cournoyer and Normand 1994). These new methods revealed much about the genetic diversity and distribution of *Frankia*, and refined and expanded knowledge about endophyte-host specificities. PCR-based approaches have been used to unravel the phylogenetic relationships of isolates, as well as of uncultured endophytes in root nodules of many actinorhizal plants from which no isolates have been obtained. These molecular approaches now open the door to more sophisticated studies of environmental influences on the dynamics of *Frankia* populations in plants and soil, which may lead to advancements in the management of actinorhizal plants and *Frankia* for human benefit.

Because about 70 – 100% of the nitrogen budget of *Alnus* species comes from its symbiotic association with the actinomycete *Frankia*, it is important to develop an optimum symbiotic association which can be achieved only after understanding the role of the host plant and the role of the soil in the selection of the most effective, infective and competitive *Frankia* strain (Dobritsa and Stupar 1989) that results in high plant productivity. Previous studies have shown that the dominance of specific *Frankia* strains in root nodules is influenced by soil and host plant species characteristics (Dawson,

Kowalski et al. 1989; Clawson, Benson et al. 1997; Lumini and Bosco 1999) as well as by the genotype of *Frankia* strains in soil (Hartman, Giraud et al. 1998; Velásquez, Mateos et al. 1999). The extent of the role played by each factor, however, is not fully understood. Studies have also shown that the success of nodulation by a specific *Frankia* strain differs depending on the host plant (Clawson, Benson et al. 1997; Lumini and Bosco 1999; Gtari, Daffonchio et al. 2007) as well as on the soil type (Griffiths and McCormick 1984; Schwintzer 1985; Dawson, Kowalski et al. 1989; Kohls and Baker 1989; Sanginga, Danso et al. 1989; Thomas and Berry 1989; Jaman, Fernandez et al. 1992; Crannell, Tanaka et al. 1994; Yang 1995). Since it has been proven that inoculation with *Frankia* strains has a positive effect on plant growth and productivity (Schwintzer 1985; Prat 1989), it is critical to select the right strain for a particular soil type or host plant in order to establish the most successful interaction. A strain should be selected that is compatible with both the host and the soil, which requires knowledge about basic interactions between these factors.

OBJECTIVE

The objective of this study was to analyze the diversity of *Frankia* populations in root nodules of different alder species grown at the same site in order to retrieve information on the importance of the plant species in the potential selection of the *Frankia* population for root nodule formation. Additional studies included population analyses of frankiae in nodules of selected alder species growing under different environmental conditions in an attempt to either validate the expected plant effect on nodule forming *Frankia* populations or to detect environmental effects. The study also aimed at relating the *Frankia* population in root nodules with those abundant in soil. Three hypotheses were addressed:

- I. Different *Alnus* species that grow sympatrically under the same environmental conditions can select different *Frankia* populations for root nodule formation.
- II. *Frankia* populations of the *Alnus* host infection group that are most abundant in soil, however, dominate the nodules of the *Alnus* plant species.
- III. An *Alnus* species growing in different types of soil select different *Frankia* strains for root nodule formation.

MATERIALS AND METHODS

Study design

In order to test the first hypothesis, root nodules were collected from twelve alder species, i.e., *A. incana*, *A. japonica*, *A. glutinosa*, *A. tenuifolia*, *A. rugosa*, *A. rhombifolia*, *A. mandsurica*, *A. maritima*, *A. serrulata*, *A. hirsuta*, *A. glutinosa pyramidalis* and *A. rugosa americana* growing sympatrically in the Chicago Arboretum, Illinois. These species were growing in soil 107 of the Sawmill series that consists of very deep, generally poorly drained soils formed in alluvium on flood plains within the taxonomic class of fine-silty, mixed, superactive, mesic Cumulic Endoaquolls (<http://www2.ftw.nrcs.usda.gov/osd/dat/S/SAWMILL.html>). Soil type 107 of this series is described as having poor drainage, with a pH of 7.4-7.8 at all sampling sites (Table 1). Ten nodules were selected from each of the alder species. One lobe was obtained from each of the nodules since it has been revealed that only one *Frankia* population is present in a nodule (Zepp, Hahn et al. 1997) and it was assumed to be true for the plants used in this study. The *Frankia* populations inside the lobes were analyzed using a high resolution fingerprinting method (i.e., rep-PCR). This technique allows assessing *Frankia* populations at the strain level.

In order to test the second hypothesis, soil was collected from the site where the aforementioned twelve alder species were growing in the arboretum. Rep-PCR is designed basically for the analysis of pure cultures. Since root nodules are a natural locale of enrichment of one *Frankia* population, rep-PCR could be used to analyze these

population. In contrast, soil harbors a highly heterogeneous community of micro-organisms, which means that rep-PCR cannot be used for the analyses of distinct *Frankia* populations. Consequently, an alternative molecular marker was needed for the analysis of *Frankia* in soil. Although less sensitive with respect to the level of phylogenetic resolution and affected by several methodological biases in any quantitative assessment, *nifH* gene fragments were retrieved from the soil where the plants were growing and a gene clone library was created. Since the profile generated using rep-PCR or any finger printing techniques do not have any phylogenetic information, *nifH* gene fragments from the root nodules of the representative species were sequenced and finally related to a semi-quantitative assessment of the abundance of specific *Frankia* populations in soil.

To test the third hypothesis, some *Alnus* species growing in multiple types of soil in Chicago botanical garden were chosen. In the arboretum *A. glutinosa* was growing in two other soils (soil 534 of the Made soil series, and soil 1107 (wet) of the Sawmill soil series). Similarly, *A. hirsuta* and *A. rugosa americana* were growing in one (soil 194 of the Ozkaukee soil series) and three other soil types (194, 531 of the Markham soil series and 1107), respectively, than soil 107 (Table 1). The *Frankia* population in the root nodules of these plants in these soils were analyzed to see the role played by the host plant in the selection of a particular type of *Frankia* strain.

Table 1. Distribution of host plants in different types of soil. The soils are different in terms of pH level and drainage pattern.

Soil type No.	Species	pH	Drainage	Plant age (years)
107	Sawmill soil: Fine-silty, mixed soil. Slope ranges from 0 to 3 percent. Mean annual precipitation is about 40 inches, and mean annual temperature is about 52 degrees F.			
	<i>Alnus incana</i>	7.4-7.8	Poor	26
	<i>A. japonica</i>	7.4-7.8	Poor	47
	<i>A. glutinosa</i>	7.4-7.8	Poor	20
	<i>A. tenuifolia</i>	7.4-7.8	Poor	19
	<i>A. rugosa</i>	7.4-7.8	Poor	22
	<i>A. rhombifolia</i>	7.4-7.8	Poor	17
	<i>A. mandsurica</i>	7.4-7.8	Poor	5
	<i>A. maritima</i>	7.4-7.8	Poor	9
	<i>A. serrulata</i>	7.4-7.8	Poor	26
	<i>A. hirsuta</i>	7.4-7.8	Poor	21
	<i>A. glutinosa pyramidalis</i>	7.4-7.8	Poor	18
	<i>A. rugosa americana</i>	7.4-7.8	Poor	24
194	Ozkauckee soil: Fine, illitic. Slope ranges from 0 to 35 percent. Mean annual precipitation is about 30 inches. Mean annual air temperature is about 49 degrees F.			
	<i>A. hirsuta</i>	6.6-7.3	Well	27
	<i>A. rugosa americana</i>	6.6-7.3	Moderate	24
531	Markham soil: Fine, illitic. Slope ranges from 0 to 20 percent. Mean annual precipitation is about 35 inches, and mean annual air temperature is about 50 degrees F.			
	<i>A. rugosa americana</i>	6.6-7.3	Well	24
534	Made (Clayey): Very fine particles, fine grained minerals			
	<i>A. glutinosa</i>	6.6-7.3	Variable	20
1107	Saw mill wet: Fine-silty, wet, mixed soil. Slope ranges from 0 to 3 percent. Mean annual precipitation is about 40 inches, and mean annual temperature is about 52 degrees F.			
	<i>A. glutinosa</i>	7.4-7.8	Poor	NA
	<i>A. rugosa americana</i>	6.6-7.8	Poor	24

Sample preparation

For each plant species, 10 lobes belonging to 10 different nodules were selected. A single lobe was used for DNA extraction. Lobes were washed with sterile water, and the epidermis was removed. Remaining tissue was homogenized with a mortar and pestle in one ml of sterile water, and the homogenates were transferred to an Eppendorf tube and centrifuged at 14,000 rpm for one minute. The pellets were washed once with 0.1% sodium pyrophosphate in water (wt/vol), followed by two washes with sterile distilled water. Subsequently, the nodule pellets as well as the pellets of pure cultures (approx. 50 mg) used for comparison, were re-suspended in 95 μ l of distilled water, mixed with 5 μ l of proteinase K solution (Promega, Madison, WI, 30 U mg⁻¹, 10 mg ml⁻¹ in water) and incubated at 37°C for 20 minutes. After that, 0.5 μ l of a 10% SDS solution was added and the mixture was incubated at 37°C for another 3 hours which was followed by a final incubation at 80°C for 20 minutes. Nucleic acids were subsequently purified from the lysates using the GenScript QuickClean DNA Gel Extraction Kit (GenScript, Piscataway, NJ), and resuspended into a final volume of 30 μ l. From purified DNA, 2 μ l were used as template in subsequent PCR-based analyses.

Rep-PCR Two μ l of purified DNA was used as template for PCR amplification with primer BoxA1R (5' CTA CGG CAA GGC GAC GCT GAC G 3') (Versalovic, de Bruijn et al. 1998) targeting the BOX element (Martin, Humbert et al. 1992). PCR was performed in a total volume of 25 μ l containing 4 μ l of 5 x Gitschier buffer (83 mM (NH₄)₂SO₄, 33.5 mM MgCl₂, 335 mM Tris/HCl, pH 8.8, 33.5 μ M EDTA, 150 mM β -mercaptoethanol), 1.25 μ l dNTPs (100 mM each, mixed 1:1:1:1), 2.5 μ l di-methyl-sulfoxide (DMSO), 0.2 μ l bovine serum albumin (BSA, 20 mg ml⁻¹), 1.3 μ l of primer

(300 ng μl^{-1}), 2 μl of purified DNA and 0.4 μl *Taq* polymerase (5 U μl^{-1}) (Dombek, Johnson et al. 2000). *Taq* polymerase was added after an initial incubation at 96°C for 10 minutes. Then denaturation at 95°C for 2 minutes was followed by thirty rounds of temperature cycling in a PTC-200 Thermocycler with denaturation at 94°C for 3 seconds and subsequent 92°C for 30 seconds, primer annealing at 50°C for 1 minute, and elongation at 65°C for 8 minutes. This was followed by a final elongation at 65°C for 8 minutes (Rademaker and de Bruijn 1997; Dombek, Johnson et al. 2000). Profiles were analyzed by gel electrophoresis on 2% agarose gels in TAE buffer after staining with ethidium bromide (0.5 $\mu\text{g ml}^{-1}$) (Sambrook, Fritsch et al. 1989).

***NifH* gene** fragments (606 bp) from the *Frankia* strains in root nodules were amplified using primers *nifHf1* (5' GGC AAG TCC ACC ACC CAG C^{3'}) and *nifHr* (5' CTC GAT GAC CGT CAT CCG GC^{3'}) (Normand, Simonet et al. 1988) in a reaction volume of 50 μl , containing 1 μl of a 10 mM dNTP mix, 0.5 μl each primer (0.4 μM), 8.2 μl BSA (30 $\mu\text{g ml}^{-1}$), 5 μl of 10 x PCR buffer with 15 mM MgCl_2 , 2 μl DNA from root nodule or from pure culture, and 0.2 μl *Taq* DNA polymerase (5 U μl^{-1} ; Gene Script, Piscataway, NJ). *Taq* polymerase was 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 second) and final 7 minute incubation at 72°C. Sub-samples of the reactions (10 μl) were checked for amplification products by gel electrophoresis (2% agarose in TAE buffer, wt/vol) after staining with ethidium bromide (0.5 $\mu\text{g ml}^{-1}$) (Sambrook, Fritsch et al. 1989). The size was confirmed using λ DNA cleaved with *HindIII* as size marker. Amplified *nifH* gene fragments 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 (Kukanskis, Siddiquee et al. 1999). Sequences were analyzed on a CEQ 8800 capillary action sequencer (Beckman Coulter).

DNA extraction from soil

Soil samples were collected from soil 107 at the Chicago Botanical garden. For DNA extraction, cells in 0.5-g soil samples were disrupted by agitation in a Mini-Bead-Beater-8 (BioSpec Products, Inc, Bartlesville, OK) for 2 minutes at maximum setting. Beads, soil and cell debris were separated from extraction buffer by centrifugation at 14,000 rpm for 1 minute. The nucleic acids released into the supernatant were purified by sequential phenol, phenol/chloroform and chloroform extraction (Sambrook, Fritsch et al. 1989). One ml of isopropanol and 100 µl of 3M Na- acetate were added to the nucleic acids and incubated at -80°C for 15 minutes. The nucleic acids were precipitated by centrifugation at 14,000 rpm for 10 minutes, washed with 70% ethanol, air-dried and finally re-suspended in 30 µl of sterile distilled water.

The *nifH* gene amplification from soil was carried as described above by using the primers *nifHf1* (5' GGC AAG TCC ACC ACC CAG C3') and *nifHr* (5' CTC GAT

GAC CGT CAT CCG GC^{3'}) (Normand, Simonet et al. 1988). The product of the first PCR reaction was cleaned using the Ultra Clean 15 DNA Purification Kit (MoBio, Carlsbad, CA) and subjected to a nested PCR reaction. In the nested PCR, all the PCR conditions were the same except that primer nifr269 (5' CCG GCC TCC TCC AGG TA^{3'}) was used instead of nifHr. In this reaction, 1 µl of PCR product of the first PCR reaction was diluted in 100 µl of sterile water and 1 µl of the dilution was used as template. The amplified PCR products of the *nifH* gene fragment (269 bp in size) were detected by electrophoresis on 2% agarose gels by comparing with λ DNA cleaved with HindIII as size marker. The band with the right size (269 bp) was cut out from the gel. Then it was purified using the Ultra Clean 15 DNA Purification Kit (MoBio), ligated into pGEM[®]-Teasy (Promega) according to the manufacturer's instructions, and transformed into *E. coli* TOP-10 (Stratagene, Cedar Creek, TX). Clones were analyzed at random for partial *nifH* gene fragments through PCR using the primers seqf (5' TCA CAC AGG AAA CAG CTA TGA C^{3'}) and seqr (5' CGC CAG GCT TTT CCC AGT CAC GAC^{3'}). The reaction volume of PCR was 50 µl, containing 1 µl of a 10 mM dNTP mix, 0.5 µl each primer (0.4 µM), 8.2 µl BSA (30 µg ml⁻¹), 5 µl of 10 x PCR buffer with 15 mM MgCl₂, 2 µl from the *E. coli* culture, and 0.2 µl *Taq* DNA polymerase (5 U µl⁻¹; Gene Script). *Taq* polymerase was 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 (2% agarose in TAE buffer, wt/vol) after staining with ethidium bromide (0.5 µg ml⁻¹) (Sambrook, Fritsch et al. 1989). The size was confirmed

using λ DNA cleaved with HindIII as size marker. Amplified *nifH* gene fragments were purified using the Ultra Clean 15 DNA Purification Kit (MoBio), and sequenced using the CEQ 8800 Quickstart Kit according to the manufacturer's instructions (Beckman Coulter) 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 (Kukanskis, Siddiquee et al. 1999). Sequences were analyzed on a CEQ 8800 capillary action sequencer (Beckman Coulter).

Phylogenetic analyses

Amplified *nifH* gene fragments obtained from uncultured frankiae from the root nodules of all plants analyzed, those from twenty-three pure cultures of *Frankia* and the seventy-three sequences obtained from the soil population through cloning in *E. coli* were trimmed to be 269 bp long and aligned using Sequencher 4.2.2 (Gene Codes Corp., Ann Arbor, MI), CLUSTAL X and MacClade 4.05 (Thompson, Gibson et al. 1997; Maddison and Maddison 1999) and analyzed using maximum parsimony (MP), neighbor joining (NJ), Bayesian and maximum likelihood (ML) methods. For presentation purposes, this dataset was split into three datasets. The first dataset contained sequences representing uncultured frankiae in the root nodules of twelve alder species growing in soil 107 and of pure cultures, the second contained uncultured frankiae in the root nodules of twelve species growing in soil 107, frankiae in soil 107 obtained through cloning and pure cultures, and the third dataset contained uncultured

frankiae in the root nodules of all species growing in different types of soil used in this study, pure cultures and frankiae in soil obtained through cloning (Figures 2, 3, 4, 5 and 6).

MP methods for each dataset began by using MacClade 4.05 to chart the rate of mutation by codon position. Third position changes were downweighted and thus first position changes upweighted to better delineate meaningful changes, i.e., in protein structure, between these sequences. MP analyses of these weighted datasets were completed in PAUP*4.0b10 and included 10,000 heuristic random addition replicates, TBR, and no mul trees (Swofford 2002). 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 (Felsenstein 1985). Only those BS values of at least 70% demonstrate good support measures and thus were retained (Hillis and Bull 1993).

Each *Frankia* dataset was analyzed using NJ methods in PAUP*. Modeltest version 3.7 using the Akaike Information Criterion determined that the GTR+I+G model of sequence evolution fit this dataset best (Posada and Crandell 1998). 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. Additionally, a distance matrix was generated for each dataset in PAUP* to delineate *Frankia* clustering assignments.

Bayesian analyses for the *Frankia* datasets were 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 (Huelsenbeck and Ronquist 2001). 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 (Huelsenbeck, Larget et al. 2002; Swofford 2002).

Aligned sequences were analyzed using maximum likelihood analyses through the RAxML-VI-HPC program (Stamatakis 2006b) 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 (Stamatakis 2006a), invariant sites, empirical base frequencies and 250 bootstrap replicates.

Table 2. List of primers used in the study. BoxA1R targets the BOX element found in all bacterial genomes. *nifHf1* and *nifHr* are the forward and reverse primers targeting the nitrogenase protein coding (*nifH*) gene found in the nitrogen fixing bacteria. *nifr269* targets the partial *nifH* gene. *seqf* and *seqr* are the forward and reverse primers targeting the plasmid fragment.

Primer	Sequence	Target
BoxA1R	5'CTA CGG CAA GGC GAC GCT GAC G 3'	BOX element
<i>nifHf1</i>	5'GGC AAG TCC ACC ACC CAG C 3'	<i>nifH</i> gene
<i>nifHr</i>	5'CTC GAT GAC CGT CAT CCG GC 3'	<i>nifH</i> gene
<i>nifr269</i>	5'CCG GCC TCC TCC AGG TA 3'	<i>nifH</i> gene (partial)
<i>Seqf</i>	5'TCA CAC AGG AAA CAG CTA TGA C 3'	fragment of plasmid
<i>Seqr</i>	5'CGC CAG GCT TTT CCC AGT CAC GAC 3'	fragment of plasmid

RESULTS

I. Different *Alnus* species that grow sympatrically under the same environmental conditions can select different *Frankia* populations for root nodule formation.

The diversity of *Frankia* populations in the root nodules of twelve plant species growing in soil 107 was analyzed with rep-PCR. A total of 120 nodule lobes, i.e., ten lobes from each plant species, growing sympatrically, resulted in three types of rep-profiles (Figure 1). The type I profile was the most abundant one and was detected in the root nodules of nine alder species (*A. incana*, *A. japonica*, *A. glutinosa*, *A. tenuifolia*, *A. rugosa*, *A. rhombifolia*, *A. mandsurica*, *A. maritima* and *A. serrulata*). The type II rep-profile was found in all the nodules from two plant species (*A. hirsuta* and *A. glutinosa pyramidalis*), where as the type III rep-profile was unique and only found in the nodules of *A. rugosa americana*. There was no variation in the rep-profile of *Frankia* found in the different nodules of same species, i.e., all ten nodules taken from a single plant species had an identical rep- profile. The overall results suggest the presence of low diversity and dominance of *Frankia* with the type I rep-profile occupying 75% of the total nodules tested.

Fingerprinting techniques such as rep-PCR provide highly distinctive information that can be used to differentiate organisms on the strain level, but do not have any information on phylogenetic relationships. So, for the phylogenetic grouping and comparison of the *Frankia* populations in root nodules with the pure cultures in the database, *nifH* gene sequences were amplified from the root nodules of three species representing each of the

three rep-PCR profiles. Comparative sequence analyses of *nifH* gene fragments from three *Frankia* strains representing each rep-profile (Type I: *A. tenuifolia*; Type II: *A. glutinosa pyramidalis*; Type III: *A. rugosa americana*) revealed differences, with Type I and II clustering with *Frankia* strains of subgroup A II (represented by strain Ag45/Mut15), and Type III with *Frankia* of subgroup A I (represented by strain ArI3) (Figure 2). The analysis showed a 6% difference in the sequence between frankiae in nodules of *A. rugosa americana* (Arv_{nod}107) and that of *A. tenuifolia* (At_{nod}107), and a 3% difference with that of *A. glutinosa pyramidalis* (Agp_{nod}107).

Each phylogenetic method employed in current analyses, gave a similar grouping of pure cultures and root nodules (data not shown). For presentation purposes only the ML tree was used to demonstrate the relationship between pure cultures and root nodule *Frankia* for each plant analyzed (Figures 2, 3, 4, 5 and 6). High BS and PP values indicate the stability of these groups.

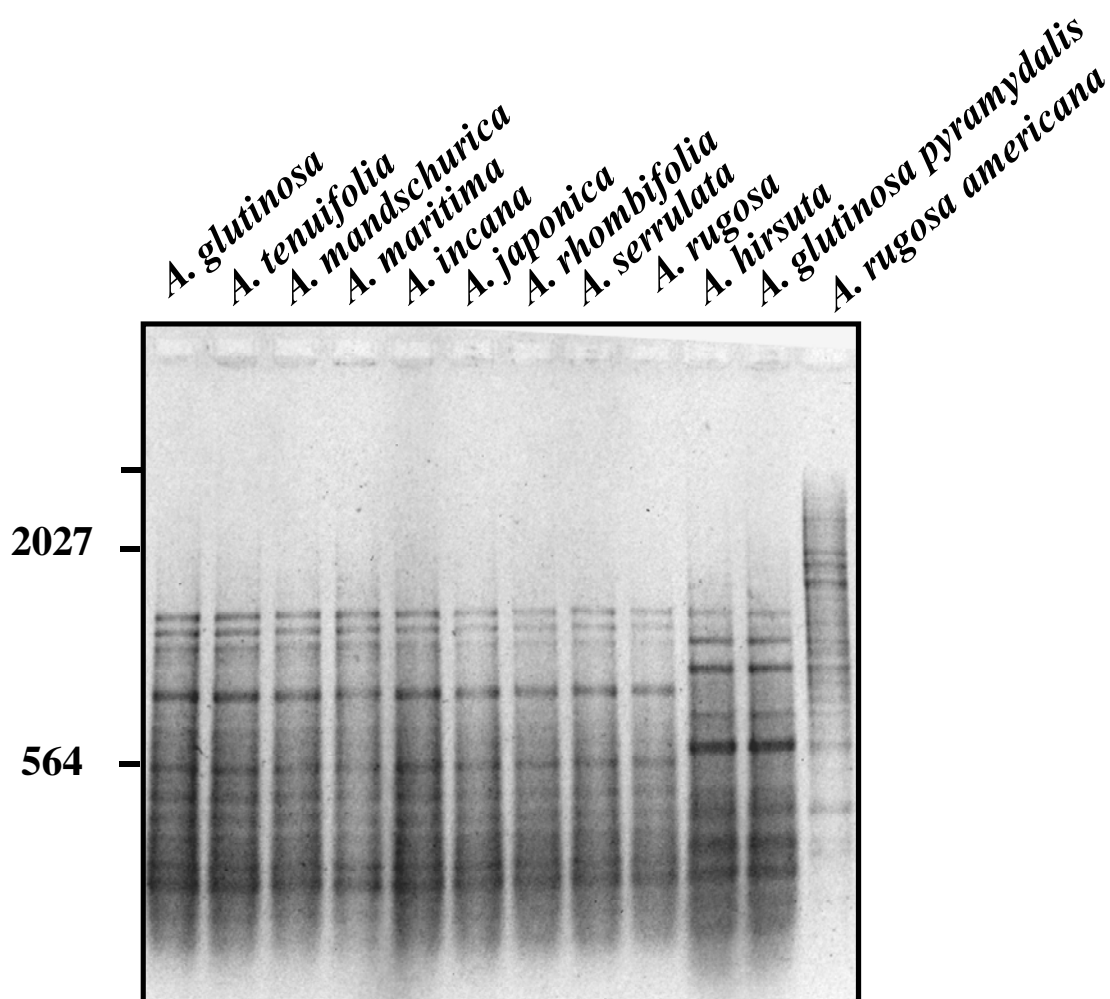


Figure 1. Rep-PCR profiles of *Frankia* in the root nodules of different *Alnus* species. The first nine patterns are identical to each other, third and second from the last are identical and the last pattern is unique. Names on the top are of the alder species from which the corresponding profiles were generated. Fragment sizes on the left represent those of a Lambda HindIII DNA size marker.

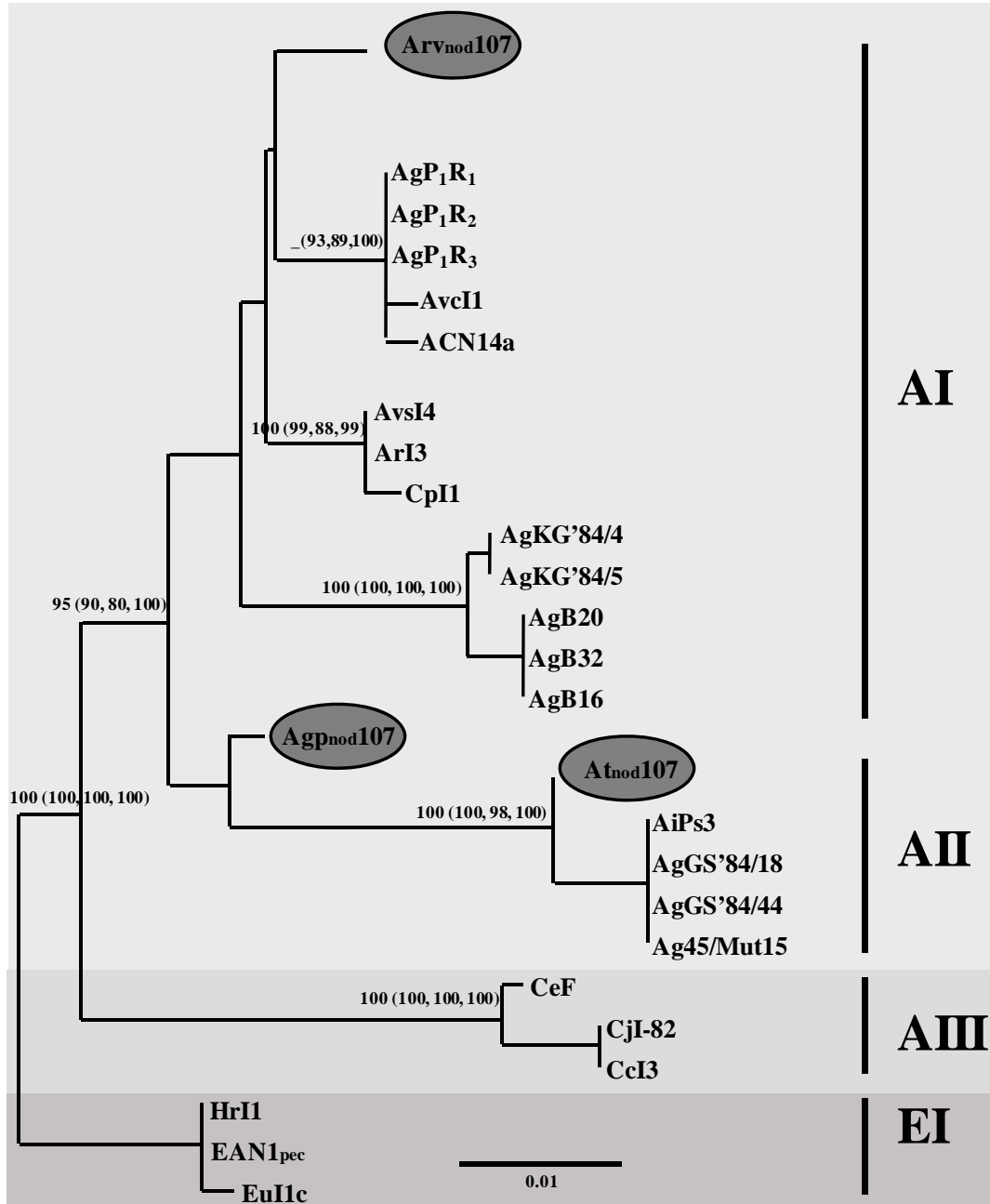


Figure 2. Maximum likelihood-based tree showing the phylogenetic position of uncultured *Frankia* populations in root nodules of *Alnus* species from soil 107 based on comparative sequence analysis of *nifH* gene fragments. The light shaded areas depict sequence assignments to the *Alnus* host infection group with cluster acronyms AI to AIII, AI and AII infecting the *Alnus* species and AIII infecting the *Casuarina* while the darker shaded areas represent sequences assigned to the *Elaeagnus* host infection group with cluster acronym EI. Numbers reflect bootstrap support measures (BS) and numbers in parentheses represent BS measures and posterior probabilities (PP) from maximum parsimony, Bayesian and neighbor joining analyses, respectively. The out group was the sequences from the *Elaeagnus* host infection group. The grey oval areas signify the sequences from the root nodules from the representative *Alnus* species in soil 107.

II. *Frankia* populations of the *Alnus* host infection group that are most abundant in soil, however, dominates in the nodules of the *Alnus* plant species.

To assess the diversity of *Frankia* populations in soil, partial *nifH* gene sequences of 269 bp were amplified through a nested PCR reaction. The PCR product was cloned in *E. coli* and sequenced. The sequence analyses of seventy-three randomly selected clones and three nodule sequences from plant species representative of each rep-profiles showed that all sequences retrieved represented frankiae of the *Alnus* host infection group, with the sequences from soil and the one representing the type III of the rep-profile clustering in subgroup AI and the rest in AII (Figure 3). The three nodular sequences were different from each other, while all clonal sequences were almost identical (Figure 3). Among the three different types of *Frankia* population detected in root nodules, the population in *A. rugosa americana* (Arv_{nod}107) had the closest relationship with the soil population with the minimum difference between sequences of only 0.3%. This root nodule population had the unique rep-profile of type III and was detected in only 8% of the total root nodule population analyzed in this study. The most dominant *Frankia* population in the root nodule (75% of nodules) was not detected in soil by nested PCR. In contrast to this, the least dominant *Frankia* strain in the root nodules was found to be closely related to the most abundant frankiae in the soil. These results suggest that the host plants have strong influence in the selection of a particular *Frankia* strain for root nodule formation regardless of its abundance in soil.

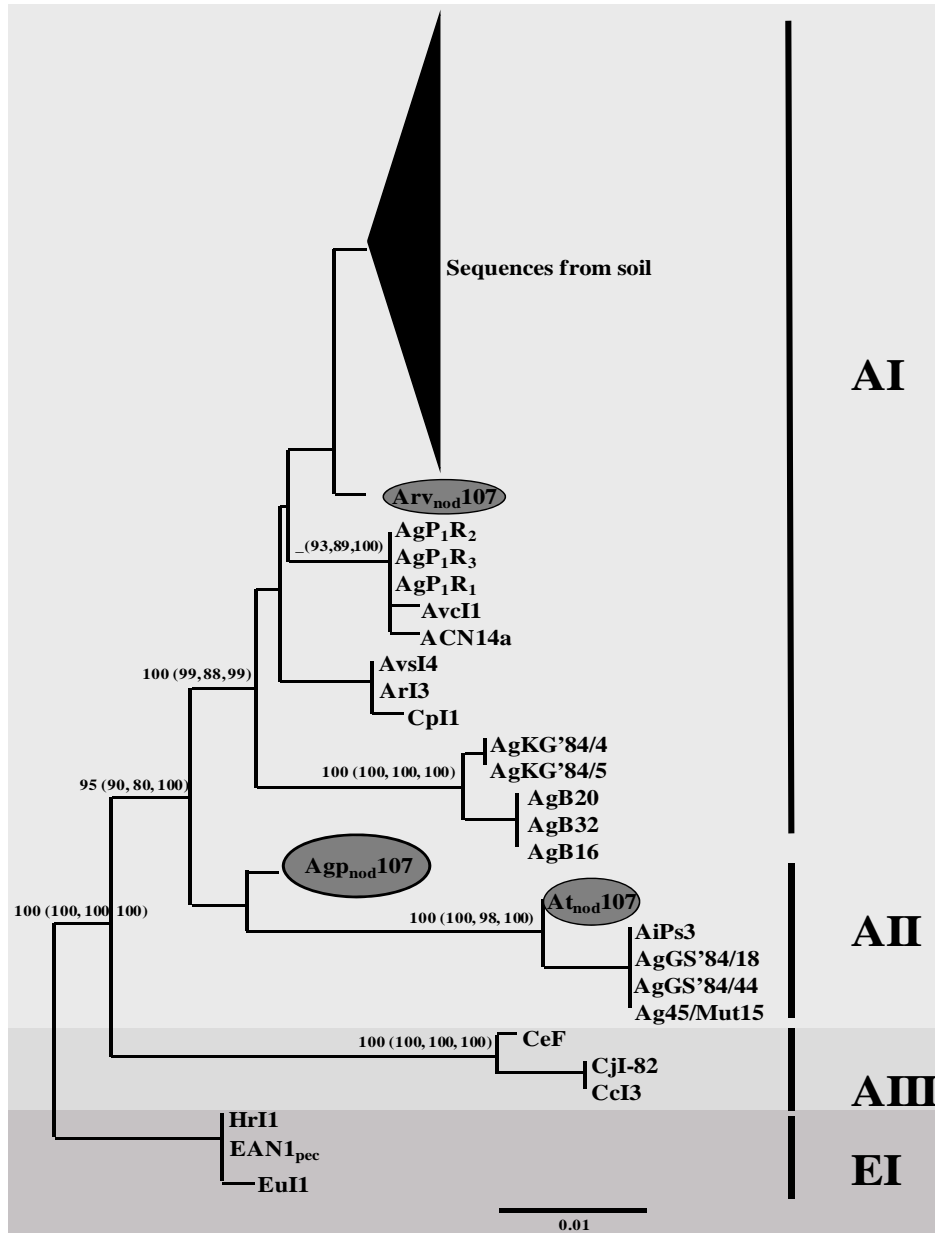


Figure 3. Maximum likelihood-based tree showing the phylogenetic position of uncultured *Frankia* populations in root nodules of *Alnus* species from soil 107 and those in soil 107 based on comparative sequence analysis of *nifH* gene fragments. The light shaded areas depict sequence assignments to the *Alnus* host infection group with cluster acronyms AI to AIII, AI and AII infecting the *Alnus* species and AIII infecting the *Casuarina* while the darker shaded areas represent sequences assigned to the *Elaeagnus* host infection group with cluster acronym EI. Numbers reflect bootstrap support measures (BS) and numbers in parentheses represent BS measures and posterior probabilities (PP) from maximum parsimony, Bayesian and neighbor joining analyses, respectively. The out group was the sequences from the *Elaeagnus* host infection group. The grey oval areas signify the sequences from the root nodules from the representative *Alnus* species in soil 107 and the black triangle represents the seventy-three sequences obtained from the *Frankia* population in soil 107.

III. An *Alnus* species growing in different types of soil select different *Frankia* strains for root nodule formation.

For analyzing the *Frankia* population in the root nodules of the plants growing in other soil types, *A. glutinosa*, *A. hirsuta* and *A. rugosa americana* were chosen as the representative species (depending on the availability of enough root nodules) representing each type of the rep-profile. The analysis of root nodule populations of the three alder species growing on soils with different properties adjacent to the previous site (soil 107) was done through the amplification and sequencing of *nifH* gene. In the arboretum *A. glutinosa* was found in two additional soils. Analysis of the *nifH* gene sequence of *Frankia* At_{nod}107, Ag_{nod}534 and Ag_{nod}1107 in the root nodules of *A. glutinosa* in all of the three soils, 107, 534 and 1107, respectively, showed that the sequences were different from each other with the first and third sequences clustering in subgroup AII and the second in AI within the *Alnus* host infection group (Figure 4). From the 107 soil, the *nifH* sequence of *Frankia* from nodules of *A. tenuifolia* was used in the analysis since it showed the same rep-profile as *A. glutinosa*. *NifH* sequences representing frankiae in nodules of *A. hirsuta* in soil types 107 and 194, Agp_{nod}107 and Ah_{nod}194, were not identical to each other although they clustered in the same subgroup AI (Figure 5). Similarly, the *nifH* gene sequences Arv_{nod}107, Arv_{nod}194, Arv_{nod}531 and Arv_{nod}1107 of frankiae in nodules from *A. rugosa americana* from four different soil types (107, 194, 531 and 1107), clustered in the same subgroup AI within the *Alnus* host infection group. But the sequence obtained from one soil type was not identical to any other sequence from other soil type (Figure 6).

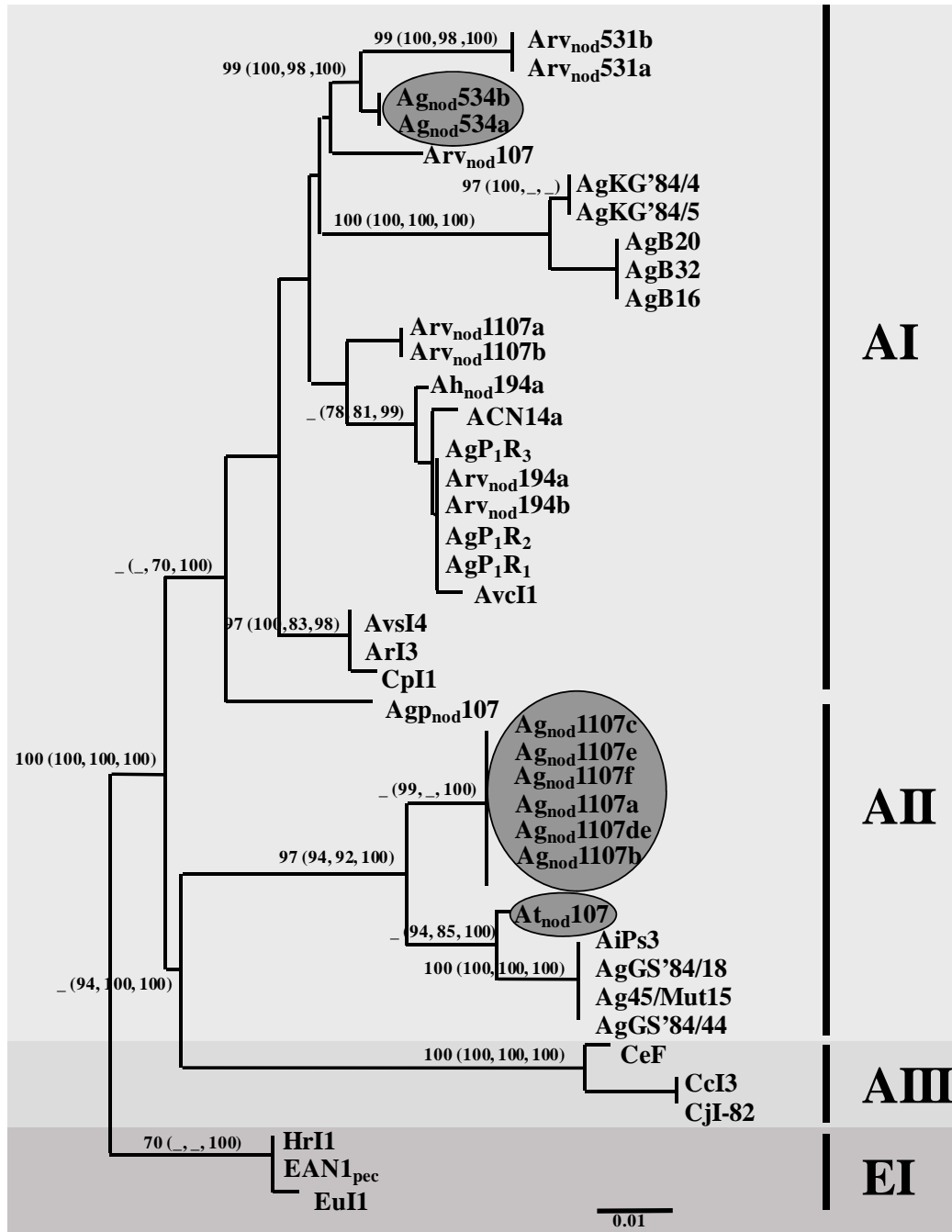


Figure 4. Maximum likelihood-based tree showing the phylogenetic position of uncultured *Frankia* populations in root nodules of *Alnus glutinosa* from different soil types based on comparative sequence analysis of *nifH* gene fragments. The light shaded areas depict sequence assignments to the *Alnus* host infection group with cluster acronyms AI to AIII, AI and AII infecting the *Alnus* species and AIII infecting the *Casuarina* while the darker shaded areas represent sequences assigned to the *Elaeagnus* host infection group with cluster acronym EI. Numbers reflect bootstrap support measures (BS) and numbers in parentheses represent BS measures and posterior probabilities (PP) from maximum parsimony, Bayesian and neighbor joining analyses, respectively. The out group was the sequences from the *Elaeagnus* host infection group. The grey oval areas signify the sequences representing the *Frankia* population from the root nodules of *Alnus glutinosa* species in soils 107, 534 and 1107.

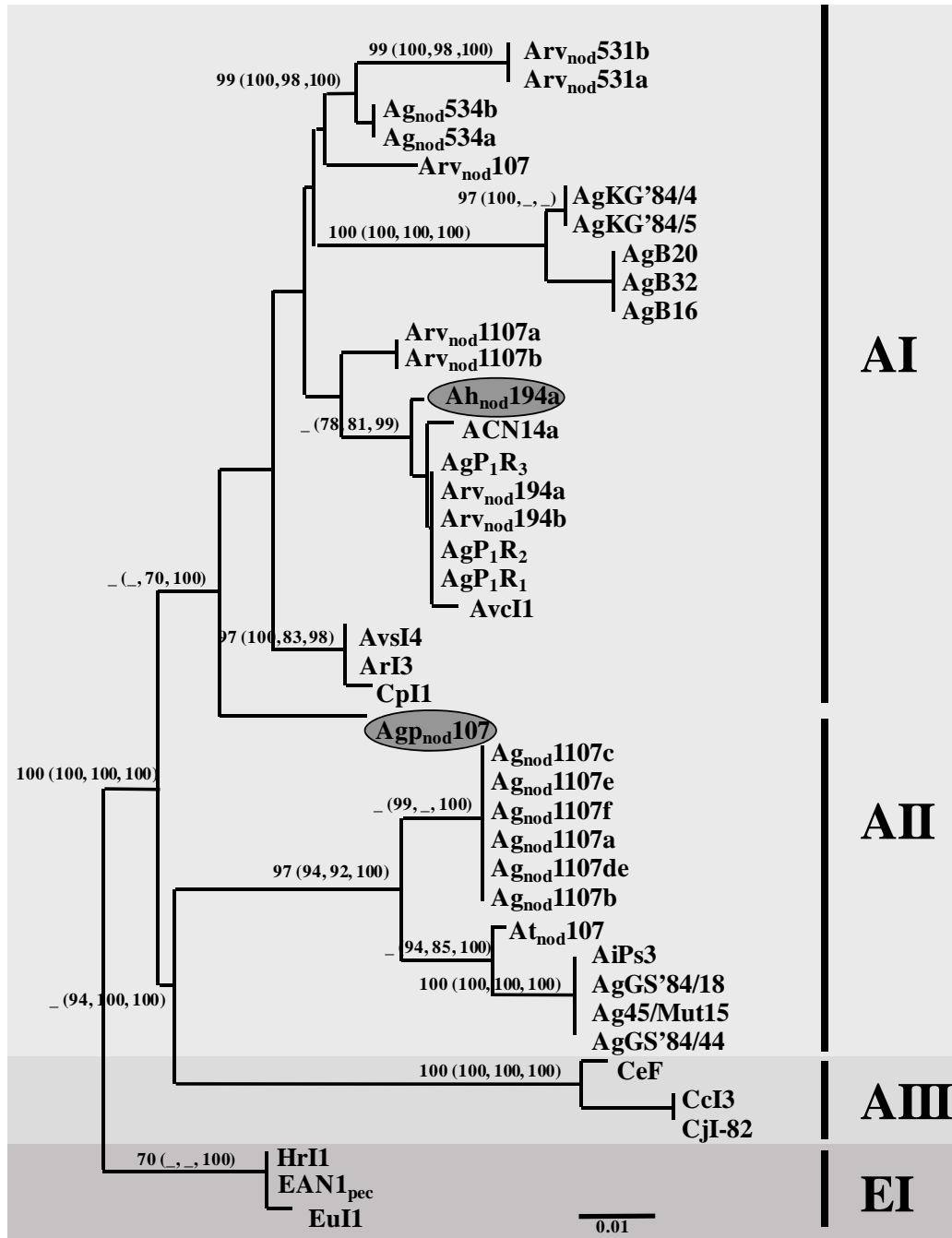


Figure 5. Maximum likelihood-based tree showing the phylogenetic position of uncultured *Frankia* populations in root nodules of *Alnus hirsuta* from different soil types based on comparative sequence analysis of *nifH* gene fragments. The light shaded areas depict sequence assignments to the *Alnus* host infection group with cluster acronyms AI to AIII, AI and AII infecting the *Alnus* species and AIII infecting the *Casuarina* while the darker shaded areas represent sequences assigned to the *Elaeagnus* host infection group with cluster acronym EI. Numbers reflect bootstrap support measures (BS) and numbers in parentheses represent BS measures and posterior probabilities (PP) from maximum parsimony, Bayesian and neighbor joining analyses, respectively. The out group was the sequences from the *Elaeagnus* host infection group. The grey oval areas signify the sequences representing the *Frankia* population from the root nodules of *Alnus hirsuta* species in soils 107 and 194.

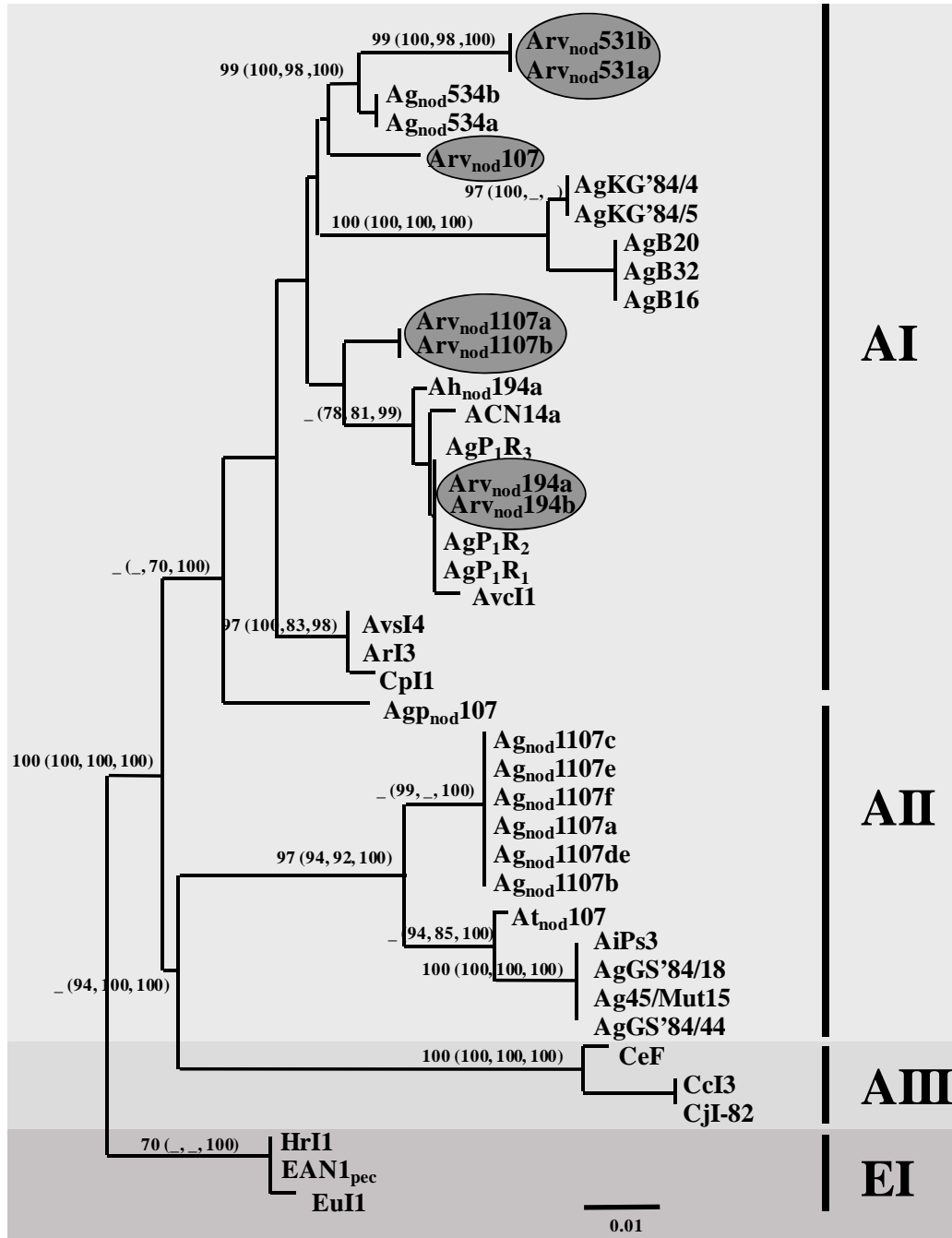


Figure 6. Maximum likelihood-based tree showing the phylogenetic position of uncultured *Frankia* populations in root nodules of *Alnus rugosa americana* from different soil types based on comparative sequence analysis of *nifH* gene fragments. The light shaded areas depict sequence assignments to the *Alnus* host infection group with cluster acronyms AI to AIII, AI and AII infecting the *Alnus* species and AIII infecting the *Casuarina* while the darker shaded areas represent sequences assigned to the *Elaeagnus* host infection group with cluster acronym EI. Numbers reflect bootstrap support measures (BS) and numbers in parentheses represent BS measures and posterior probabilities (PP) from maximum parsimony, Bayesian and neighbor joining analyses, respectively. The out group was the sequences from the *Elaeagnus* host infection group. The grey oval areas signify the sequences representing the *Frankia* population from the root nodules of *Alnus rugosa americana* species in soils 107, 194, 531 and 1107.

DISCUSSION

Variation among *Frankia* strains in the root nodules within a host infection group as well as among host plant species grown in different types of soil had been documented, but the exact role played by the host plant species or the soil characteristics in the selection of a particular type of *Frankia* strain for root nodule formation had not been comprehensively investigated. Several factors were proposed to effect the selection of a particular type of *Frankia* strain for root nodule formation like host-symbiont genotype (Dawson, Kowalski et al. 1989; Navarro, Jaffre et al. 1999), soil pH (Griffiths and McCormick 1984; Jaman, Fernandez et al. 1992; Crannell, Tanaka et al. 1994), soil moisture content (Schwintzer 1985; Dawson, Kowalski et al. 1989), and organic matter content (Huguet, Batzli et al. 2001) . On analyzing the *Frankia* population in the root nodules of the twelve different alder species growing in the same type of soil (107) through rep-PCR, three different rep-profiles were detected. Rep-PCR profiles of *Frankia* populations in root nodules of 9 alder species, i.e., *A. incana*, *A. japonica*, *A. glutinosa*, *A. tenuifolia*, *A. rugosa*, *A. rhombifolia*, *A. mandshurica*, *A. maritima* and *A. serrulata* were identical within and between plant species. Similarly, the rep-PCR profiles of *A. hirsuta* and *A. glutinosa pyramidalis* were identical within and between species but different from the profile of aforementioned 9 species. Likewise rep-PCR profiles of *Frankia* populations in root nodules of *A. rugosa americana* were identical but unique compared to profiles of *Frankia* populations in root nodules of other *Alnus* species. This suggest

that there is no diversity in the *Frankia* strains within a host plant. In contrast to this result, a study had reported that different *Frankia* strains co-exist in the same host plant under natural conditions (Dai, He et al. 2004). In this study, when *Frankia* populations in the all root nodules of a plant were compared, a particular *Frankia* strain was dominating. Such dominance was also reported in the past in an *Alnus* stand and the dominating strain was thought to be more competitive for that particular environment (Clawson, Gawronski et al. 1999). It was not understood whether such dominance was linked with soil characters or host plant or population in soil or combination of two or more factors. The presence of three different *Frankia* strains in the root nodules of twelve plants grown sympatrically denotes that the host plants can select a particular type of *Frankia* strain for root nodule formation. Similar findings was observed by Oakley et al. 2004 (Oakley, North et al. 2004) where he concluded that host species was the significant variable which distinguishes phylogenetic groupings of *Frankia* at a local level after looking at the influence of geographic separation, host specificity and environment. In our study, soil 107 had the pH of 7.4-7.8 and poor drainage and all the plants were exposed to the same soil conditions. However, different species were found to harbor different types of *Frankia* population. With this result, it can be inferred that different *Alnus* species growing sympatrically under the same environmental conditions can select different *Frankia* populations for root nodule formation.

Rep-PCR allows distinguishing *Frankia* population at the strain level. It is also cheaper than gene sequencing. However, finger-printing techniques like rep-PCR do not provide any phylogenetic information. In order to detect differences between the strains, rep-PCR was done and then to compare those strains with other sequences from pure

cultures, *nifH* gene fragments were amplified and sequenced. Phylogenetic analyses based on *nifH* sequences are consistent with those of 16S rRNA and *GlnII* sequence analyses (Gtari, Daffonchio et al. 2007). The *nifH* gene sequences of *A tenuifolia*, *A glutinosa pyramidalis* and *A rugosa america* representing the three different rep-profiles clustered in different subgroups within the *Alnus* host infection group in the phylogenetic tree. Here the variation among *Frankia* strains even within a host specific group seems to be the function of host species identity as was reported in Oakley et al. 2004 (Oakley, North et al. 2004). However, with only these results we cannot say whether it's totally a function of host species or is the influence of the dominant population in soil unless the root nodular populations are compared with the *Frankia* population in soil.

Despite being the natural locale enrichment of *Frankia*, root nodules represent only a fraction of physiologically active *Frankia* population that can infect the host plants (Hahn, Nickel et al. 1999). The other significant number is found in soil growing saprophytically. Study of *Frankia* in only root nodules thus underestimates the overall diversity of *Frankia* populations. Relatively few studies have been conducted on the *Frankia* populations in soil. So, it's important to study the population in soil in order to assess the overall diversity of *Frankia* and see if there is any kind of relationship between these two populations. In soil, *Frankia* are present in low number and are found along with a large numbers of other microorganisms. The isolation of *Frankia* strains from soil is therefore very difficult. Studies of *Frankia* in soil in the past are largely based on plant bioassay. This technique captures only the nodule forming strains which are compatible with the test plants chosen for the study (Hahn, Nickel et al. 1999). So the actual diversity is underestimated in plant bioassay. In this study the soil populations were studied using

nifH gene clone library analyses, and through this technique the numerically most prominent *Frankia* strains with sequences compatible with the primers could be assessed irrespective of their compatibility with the host plant. Comparative sequence analysis of *nifH* gene fragments obtained from the *Frankia* populations in soil 107 revealed that the dominant *Frankia* strains in the soil formed a different subgroup than the root nodule population (Figure 3). The strain dominating the root nodule population, i.e., the strain represented by frankiae in all nodules of nine alder species including *A. tenuifolia* belonged to a different subgroup than the clones from the soil. The sequence of *Frankia* in root nodules of *A. rugosa americana* was in the same subgroup as were the sequences of the *Frankia* population in soil with very high sequence similarity. However, this sequence was only found in nodules of one out of twelve alder species found in soil 107 and the sequence of eleven other species represented by frankiae in nodules of *A. tenuifolia* and *A. glutinosa pyramidalis* belonged to different subgroups. Dai et al. (2004) had found dominant patterns of *Frankia* in *A. nepalensis* in their study and had suspected that the same *Frankia* strain might be dominating in soil resulting in higher probabilities to produce nodules on a respective host plant. The results obtained in our study, however, did not show any relationship between the dominant population in soil and those in root nodules. This shows that host plants have a strong role in the selection of a particular *Frankia* strain for the formation of root nodules irrespective of the population present in soil.

Follow-up studies of the root nodule populations of three alder species representative for each distinctive rep-PCR profile (i.e., *A. glutinosa*, *A. hirsuta*, and *A. rugosa*) that were growing on soils with different properties adjacent to the previous site

was done through the amplification and sequencing of *nifH* gene. In the arboretum *A. glutinosa* was found in two additional soils. Analysis of the *nifH* gene sequence of *Frankia* in the root nodules of *A. glutinosa* in all of the three soils (107, 534 and 1107) showed that the sequences were different from each other (Figure 4). *A. hirsuta* in the two soil types (107 and 531) had different sequences from one another (Figure 5). Similarly, the *nifH* gene sequences of *A. rugosa americana* in four different soil types (107, 194, 531 and 1107) were found to be close to each other but not identical. This result indicates a plant-specific selection of *Frankia* strains dependent on environmental conditions.

The soil parameters like pH, moisture level and organic matter content have been found to frequently alter the diversity of *Frankia* populations in root nodules (Huguet, Batzli et al. 2001). In our study, all sequences obtained from nodules of alders growing at different sites, were genetically different which suggests a potential role of soil characteristics such as soil pH and moisture conditions on the selection of the *Frankia* strain by the host plant species. However, no specific grouping was observed in relation to soil hydrology or pH. One possible reason behind it could be the small difference in the pH of the soil where the plants were growing and the small data set for both parameters. But still it is obvious that the presence of a particular type of *Frankia* strain in a root nodule of *Alnus* species involves the interactions between the host species specificity and environmental parameters. Past studies have reported that the genetic differences are stronger than the environmental differences in the determination of host/endophyte symbiosis (Huguet, Mergeay et al. 2004). However, since this study shows the interactive role played by host plant species and soil characteristics, the extent

of the role played by the host plant species and different environmental parameters still remains unanswered.

PCR based methods allowed us to analyze *Frankia* population diversity in root nodules and in soil. The primers that have been used, however, had been designed based on the sequences of few isolates and /or of some non-cultured endophytes in root nodules. Therefore, the specificity of these primers could not be verified quantitatively, and thus some yet uncultured and undescribed populations of frankiae may have been escaped detection (Hahn, Nickel et al. 1999). The primers used in this study, however, have successfully amplified the uncultured *Frankia* populations in the root nodules of all alder species chosen in this study. The primer used in nested PCR was confirmed to be specific for frankiae by comparative sequence analyses of our database and searches in Genbank. Still the dataset obtained may not contain the complete diversity of *Frankia* populations in soils. Therefore, it's very important to update the primer design based on the availability of new sequence in the DNA databases (Normand and Chapelon 1997).

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