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Effect of Freezing of Soils on Nodulation Capacities of Total and Specific *Frankia* Populations

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Abstract

Freezing of soils from pine-, spruce-, and birch-stands that had previously been analyzed for nodulation capacities with *Alnus incana* as capture plant, and subsequent storage at –20°C for four years resulted in a reduction of infective frankiae. In bioassays with *A. incana*, nodulation capacities of frozen soils decreased to 2, 7, and 4%, respectively, of the nodulation capacities of the fresh soils. *In situ* hybridization with four *Frankia* group-specific probes identified the *Frankia* populations in about 90% of the root nodules. Based on rep-PCR analysis, unidentified nodule populations were clustered into two groups outside the analyzed *Frankia* groups, but within frankiae of the *Alnus* host infection group. The analysis of specific *Frankia* populations in nodules on *A. incana* showed that the reduction in nodulation capacities of frozen soils was accompanied by shifts in nodule-forming *Frankia* populations. This indicates an effect of freezing and storage not only on the total *Frankia* population, but also on specific populations of *Frankia* in soil. Comparative analysis with *A. glutinosa* as capture plant demonstrated variable compatibilities of *A. incana* and *A. glutinosa* for infection with the total *Frankia* population in soil. Although statistically significant differences in nodulation capacities were not observed for soil from the spruce-stand, soils from

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pine- and birch-stands displayed about ten times higher nodulation capacities with *A. glutinosa* than with *A. incana*. Specific *Frankia* populations in root nodules on both plant species were not significantly different after inoculation with soil from the spruce- and birch-stand. Larger differences, however, were obtained for soil from the pine-stand suggesting variable compatibilities of the host plants for infection also by specific *Frankia* populations.

Keywords: alder, *Frankia*, *in situ* hybridization, nodulation capacity, population, rep-PCR

1. Introduction

Members of the actinomycetous genus *Frankia* are generally characterized as nitrogen-fixing organisms that form root nodules in symbiosis with more than 200 species of non-leguminous woody plants in 25 genera of angiosperms (Schwintzer and Tjepkema, 1990). *Frankia* populations inhabit two distinct ecological niches, the root nodule and the soil. While a considerable amount of information is available on *Frankia* strains isolated from root nodules and on their interaction with their host plants (see Benson and Silvester, 1993; Huss-Danell, 1997 for review), data on *Frankia* populations in soils are scarce (Hahn et al., 1999). Studies of *Frankia* populations in soil have until recently been based mainly on plant bioassays in which a quantification of the nodulation capacity on a specific host plant (expressed as nodulation units g^{-1} soil) is used to describe the infective *Frankia* population. 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). By these methods, nodulation units (nu) between 0 and 4,600 nu g^{-1} soil [dry wt] have been obtained for different soils (Myrold et al., 1994).

Plant bioassays also demonstrated that members of the genus *Frankia* can survive and remain infective in soils that are devoid of host plants (Arveby and Huss-Danell, 1988; Elo et al., 2000; Huss-Danell and Frej, 1986; Maunuksela et al., 1999; Smolander, 1990; Smolander et al., 1988; 1990; Smolander and Sarsa, 1990; Smolander and Sundman, 1987). Nodulation capacities of soils from birch, pine- or spruce-stands, for example, analyzed with *Alnus incana* as capture plant resulted in very high nodulation capacities of 3160±7, 2267±13, and 2747±6 nu g^{-1} soil [dry wt], respectively (Maunuksela et al., 1999). These results, however, were not consistent since earlier studies found the nodulation capacity of the soil from the birch-stand to be much higher than that of the soils from the pine- or spruce-stands (Smolander, 1990; Smolander et al., 1990; Smolander and Sarsa, 1990). Previous studies have demonstrated that results of bioassays depended on the cultivation conditions as well as on the capture

plant used, with nodule numbers consistently being greatest on *A. rubra*, less on *A. incana*, and least on *A. glutinosa* (Huss-Danell and Myrold, 1994). Other drawbacks of bioassays include the failure to analyze specific *Frankia* populations, the inability to quantify competition for infection between *Frankia* populations in a sample, and variable compatibilities of host plants to *Frankia* populations (Hahn et al., 1988; Huss-Danell and Myrold, 1994). Nodulation capacities of soils inoculated with frankiae have been shown to be impacted by storage conditions such as temperature regime and moisture contents, although the impact depended on the *Frankia* strain (Sayed et al., 1997). While nodulation capacity of one strain, for example, decreased in time under a large variety of temperature and moisture conditions, nodulation capacities of others were only decreasing by a small subset of conditions (Sayed et al., 1997).

In this study we tried to assess the effect of freezing and subsequent low temperature-storage of soils on the nodulation capacities of total and specific *Frankia* populations. The study was based on bioassays using *Alnus incana* (L.) Moench. and *Alnus glutinosa* (L.) Gaertn. as capture plants for the analysis of nodulation capacities of soils from birch- (*Betula pendula* Roth), spruce- (*Picea abies* (L.) Karsten) and pine- (*Pinus sylvestris* L.) stands. Specific populations in root nodules of the capture plants were analyzed by *in situ* hybridization and non-identified populations further characterized by rep-PCR.

2. Materials and Methods

Analysis of nodulation capacity

The soils used in this study were initially analyzed for nodulation capacities using *A. incana* as capture plants directly after sampling in summer 1994 (Maunuksela et al., 1999). Remaining soil samples were frozen and kept at –20°C for four years. In summer 1998 they were thawed and again analyzed for nodulation capacities using *A. incana* and *A. glutinosa* as capture plants. *A. incana* and *A. glutinosa* seedlings were sterilized, germinated and grown axenically in glass bottles filled with half-strength nutrient solution (Smolander and Sundman, 1987). Bottles containing 5-week-old seedlings were inoculated with soil suspensions and plants were grown for five weeks in a growth cabinet. A more detailed description of the nodulation capacity analysis is given in (Maunuksela et al., 1999).

In situ hybridization

Root nodules of *A. incana* and *A. glutinosa* capture plants as well as pure

cultures (AiPs1, AiBp5, AiPa1 and AgB32) (Maunuksela et al., 1999; Zepp et al., 1997a) were fixed in paraformaldehyde (Hahn et al., 1997). From the nodules, 52 lobes were homogenized per treatment, i.e. per capture plant species and per soil inoculum (Zepp et al., 1997a), and hybridized on slides with Cy3-labeled oligonucleotide probes targeting specific sequences on the 23S rRNA insertion of *Frankia* strains belonging to different groups of the *Alnus* host infection group (23Mut(II), 23AvC, 23AvN and 23B32(II)] as described in (Maunuksela et al., 1999; Zepp et al., 1997a). Specificity of hybridization conditions were always controlled with *Frankia* isolates. The DNA intercalating dye 4',6-diamidino-2-phenylindole (DAPI) was used as universal stain to detect all bacterial cells while probe EUB338 (Amann et al., 1990) targeting members of the Domain *Bacteria* was used to demonstrate the detectability of *Frankia* cells in all homogenates (Zarda et al., 1997). After hybridization and washing, samples were mounted with Citifluor solution (Citifluor, Canterbury, UK), and examined with an Olympus BX50 + BX-FLA reflected light fluorescence attachment microscope and filter sets U-MWU (DAPI) and U-M41007 (Cy3).

Rep-PCR fingerprinting

Fixed nodule lobe homogenates and *Frankia* strains AiBp5, AiPs1, and AiPa1 (Maunuksela et al., 1999) were washed in sterile, distilled water and 1 µl of nodule lobe homogenate or *Frankia* pure culture amplified directly using the BOX A1R primer (Versalovic et al., 1994). PCR amplification and analysis of PCR products was done as described in (Maunuksela et al., 1999) except that for analysis the images were scanned and analyzed using Fluor-STM MultiImager and Quantity One Quantification Software (Bio-Rad). Fingerprint patterns were compared and a dendrogram constructed based on the unweighed pair group method using arithmetic averages (UPGAMA).

3. Results and Discussion

Analysis of nodulation capacity of soils

All soils tested contained nodule-forming and nitrogen-fixing *Frankia* populations. The nodulated test plants grew faster, were larger, and developed greener leaves than non-inoculated control seedlings that did not harbor any nodules. Compared to results of the previous study analyzing nodulation capacities of fresh soils with *A. incana* as capture plant (Maunuksela et al., 1999), nodulation capacities on the same soils frozen and stored for 4 years were much lower with both *A. incana* and *A. glutinosa* as capture plants. Nodulation

Table 1. Nodulation capacity[a] (nodulation units [g soil dry wt.]$^{-1}$) (X ± SD) on seedlings of *A. incana* and *A. glutinosa* inoculated with soil collected from sites under birch, spruce or pine

	A. incana[b]	*A. incana*[c]	*A. glutinosa*[c]
"Pine" soil	2267 ± 13	40 ± 57	560 ± 226
"Spruce" soil	2747 ± 6	180 ± 28	180 ± 141
"Birch" soil	3160 ± 7	140 ± 28	1300 ± 424

[a]Calculated from bioassays using 0.025 g of soil as inoculum; [b]Nodulation units of fresh soils (Maunuksela et al., 1999); [c]Nodulation units of soils stored at –20°C for several years.

capacities on *A. incana* decreased by more than one order of magnitude for all three soils and resembled only about 2, 7, and 4% of the nodulation capacities of fresh soils of pine-, spruce-, and birch-stands, respectively (Table 1). Using *A. glutinosa* as capture plant, nodulation capacities for soils kept frozen were still smaller than those in fresh soils obtained with *A. incana* as capture plant (Table 1). Differences, however, were less pronounced as with *A. incana* as capture plant since values of about 25, 7, and 41% of nodulation units for fresh soils were obtained for soils from pine-, spruce-, and birch-stands, respectively. Although the nodulation capacities of all three soils were still comparable to those found in other soils (Elo et al., 2000; Myrold et al., 1994; Myrold and Huss-Danell, 1994; Smolander and Sundman, 1987), the significantly lower nodulation capacities in soils kept frozen compared to those in fresh soils indicate a large impact of freezing and low temperature-storage.

It was suggested that the nodulation capacity of a soil was controlled largely by the physiological status of *Frankia*, as indicated by infectivity, rather than by the total population size (Myrold et al., 1994; Myrold and Huss-Danell, 1994). The physiological status of a specific *Frankia* population in soil might be triggered by environmental factors such as e.g. the vegetation favoring saprophytic growth of this population and increasing its competitive abilities with respect to root nodule formation (Maunuksela et al., 1999). This assumption was supported by studies in which only one population of *Frankia* was detected in nodules of the host plant at the respective site by *in situ* hybridization, though different *Frankia* populations were detected in soil by PCR (Zepp et al., 1997b). In our previous study we detected different *Frankia* populations in the nodules of the capture plant *A. incana*, depending on whether the soil originated from the birch-, pine- or spruce-stand (Maunuksela et al., 1999). These differences were not reflected in the *Frankia* populations

present in the respective soils because all *Frankia* populations analyzed were found to be present in all three soils (Maunuksela et al., 1999). These PCR-based results indicated a similar diversity of the total *Frankia* population in soils of the birch-, pine- and spruce-stands. However, since PCR products do not necessarily reflect the abundance of the target sequences in the original sample (Suzuki and Giovannoni, 1996) the size of the different *Frankia* populations detected might have differed significantly. Nevertheless, the results showed that the nodulation capacities of these soils were determined by a limited number of specific populations rather than a function of the size of all *Frankia* populations present. Therefore, our results on nodulation capacities of frozen soils suggest a large impact of freezing and storage at –20°C on physiologically active *Frankia* populations in soil, most likely decreasing the amount of infective frankiae in the soils.

Previous studies have demonstrated that results of bioassays depended on the cultivation conditions as well as on the capture plant used, with nodule numbers consistently being greatest on *A. rubra*, less on *A. incana*, and least on *A. glutinosa* (Huss-Danell and Myrold, 1994). In contrast to this study, nodulation capacities in soils from pine- and birch-stands in our study were about 14- and 9-fold higher, respectively, with *A. glutinosa* than with *A. incana*. Values for soil from the spruce-stand, however, were comparably low with 7% of the nodulation capacity of fresh soil (Table 1). These results suggested that studies on nodulation capacities of soils from birch-, pine- or spruce-stands using *A. incana* and *A. glutinosa* were impacted by variable compatibilities of the host plants to specific *Frankia* populations. Variable compatibilities of *A. incana* and *A. glutinosa* to different *Frankia* populations have been demonstrated (Van Dijk et al., 1988). In Finland, for example, nodules on *A. glutinosa* have been mostly of the spore (–) type and nodules on *A. incana* of the spore (+) type (Weber, 1986). Therefore, in order to evaluate possible variable compatibilities of *A. incana* and *A. glutinosa* we investigated the structure of the *Frankia* populations forming root nodules on both capture plant species inoculated with soils from pine-, spruce- and birch-stands, respectively, using *in situ* hybridization.

Analysis of Frankia populations in root nodules

All lobes analyzed contained vesicles that were permeable and hybridized to probe EUB338 demonstrating their general detectability by *in situ* hybridization (Table 2). Spores were not detected in any of the samples. This latter observation is in accordance with previous observations on the predominant occurrence of *Frankia* populations in areas devoid of actinorhizal plants inducing nodules of the spore (–) type on capture plants (Weber, 1986).

Table 2. Analysis of uncultured *Frankia* populations in homogenates of nodule lobes obtained on seedlings of *A. incana* and *A. glutinosa* used as capture plants in bioassays analyzing nodule forming capacities of soils under pine, spruce or birch

	Domain bacteria[a]	*Frankia* populations[b]				Unidentified[c]
		IIIa	IIIb	IVa	IVb	
A. incana[d]						
"Pine" soil	100	25	0	0	40	35
"Spruce" soil	100	0	0	35	25	40
"Birch" soil	100	0	0	100	0	0
A. incana[e]						
"Pine" soil	100	25	0	42	29	4
"Spruce" soil	100	0	0	63	27	10
"Birch" soil	100	0	0	71	12	17
A. glutinosa[e]						
"Pine" soil	100	0	0	40	48	12
"Spruce" soil	100	0	0	73	13	13
"Birch" soil	100	0	0	77	15	8

[a]% lobes in which *Frankia* vesicles strongly hybridized to probe EUB338 targeting all members of the Domain Bacteria; [b]% lobes in which *Frankia* vesicles hybridized to probes 23AvC (targeting *Frankia* of the *Alnus* host infection subgroup IIIa), 23B32(II) (*Frankia* subgroup IIIb), 23Mut(II) (*Frankia* subgroup IVa), and 23AvN (*Frankia* subgroup IVb); [c]% lobes in which *Frankia* vesicles did not hybridize to probes 23AvC, 23B32(II), 23Mut(II), or 23AvN; [d]bioassay with fresh soils (Maunuksela et al., 1999); [e]bioassay with soils stored at –20°C for several years.

Based on the observation that in Finland nodules on *A. glutinosa* have been mostly of the spore (–) type and nodules on *A. incana* of the spore (+) type (Weber, 1986), one might speculate that the higher nodulation capacities of soils with *A. glutinosa* as capture plant compared to *A. incana* in this study were caused by strong associations of the *Frankia* populations of the spore (–) type in these soils *to A. glutinosa. In situ* hybridization with *Frankia* group-specific probes allowed us to identify the *Frankia* population in about 90% of the lobes (Table 2). In the remaining 10% of the lobes, the *Frankia* populations did not hybridize to the *Frankia* probes used, and could therefore not be identified (Table 2). Freezing and storage at –20°C for several years induced only small differences in the structure of nodulating *Frankia* populations. On *A. incana* inoculated with fresh or frozen soils nodules usually did not contain

Frankia populations of groups IIIa and IIIb, except for "pine" soil that had 25% of the lobes analyzed formed by group IIIa frankiae (Table 2). In this soil a shift in *Frankia* populations from IVb and unidentified frankiae to IVa frankiae occurred after freezing and storage. No significant differences were observed in *Frankia* populations in nodules induced from "spruce" and "birch" soils, though slight changes were detected (Table 2). The large reduction in nodulation capacity of all three soils after freezing and storage on *A. incana* is therefore not correlated to the reduction or the increment of specific *Frankia* populations.

Comparison of *Frankia* populations in nodules formed on *A. incana* or *A. glutinosa* inoculated with frozen and stored "pine", "spruce" or "birch" soil showed also only small differences. In nodules obtained on both *A. incana* and *A. glutinosa* that were induced by frankiae from "spruce" and "birch" soil only *Frankia* groups IVa and IVb, but not IIIa and IIIb were detected (Table 2). *Frankia* group IVa was dominant inhabiting 3 to 4 times as many lobes as group IVb frankiae. In nodules induced by frankiae from "pine" soil, *Frankia* populations belonging to group IVa were also detected in comparable numbers of nodules on both plant species (42 versus 40%), however, nodules on *A. glutinosa* were much more often inhabited by group IVb frankiae than those on *A. incana* (48 versus 29%) (Table 2). On *A. glutinosa*, none of the nodules contained *Frankia* groups IIIa or IIIb. Group IIIb was also not detected in nodules on *A. incana*, however, frankiae of group IIIa were detected in 25% of the lobes. Although there are small differences in *Frankia* populations between both capture plant species that support the theory of variable compatibilities of the host plants to specific *Frankia* populations, the differences cannot be correlated to the large differences in nodulation capacities of the different soils obtained between both plant species.

The limited number of oligonucleotide probes used in this study restricted the detection of *Frankia* populations to four groups within the *Alnus* host infection group. Since several *Frankia* strains have already been isolated that require different probes for detection (Hahn et al., 1999; Zepp et al., 1997a), it is not surprising that *Frankia* populations in a number of nodules were not identified. These *Frankia* populations were found in every treatment, the amount varying slightly depending on the soil and the host plant. On *A. incana*, *Frankia* populations in 4, 10 and 17% of the nodules induced by "pine", "spruce" or "birch" soil, respectively, were not identified, while unidentified populations in nodules on *A. glutinosa* accounted for 12, 13 and 8% for the respective soil inoculums (Table 2). Since it had been demonstrated that comparative 16S or 23S rRNA sequence analysis of *Frankia* strains showed a good correlation between degrees of relatedness with fingerprinting techniques such as rep-PCR fingerprinting (Maunuksela, et al., 1999; Murry et al., 1995), we tried to investigate the relatedness of unidentified *Frankia* populations in these

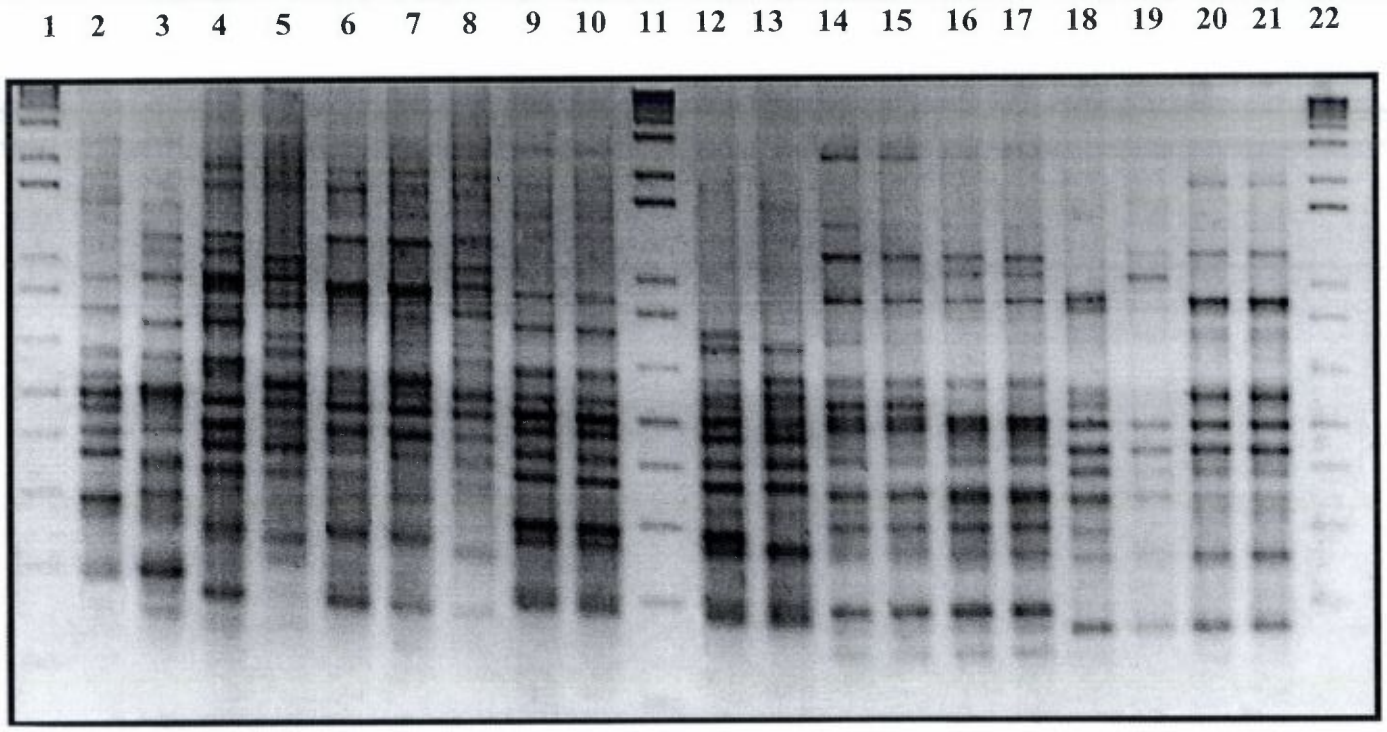

Figure 1. Rep-PCR fingerprint patterns generated using BOX-primer of total DNA from *Frankia* isolates and *A. incana* and *A. glutinosa* nodule lobes induced by soil from a birch, spruce and pine stand. Lanes 1, 11 and 22, 1-kb marker; lane 2, isolate AiPa1; lane 3, isolate AiPs1; lane 4, isolate AiBp5; lane 5, *A. glutinosa* nodule lobe induced by soil from a spruce stand; lane 6, *A. glutinosa* nodule lobe induced by soil from a pine stand; lane 7, *A. incana* nodule lobe induced by soil from a pine stand; lane 8, *A. incana* nodule lobe induced by soil from a birch stand; lanes 9–10, *A. incana* nodule lobes induced by soil from a spruce stand; lanes 12–13, *A. glutinosa* nodule lobes induced by soil from a pine stand; lanes 14–17, *A. incana* nodule lobes induced by soil from a pine stand; lanes 18–19, *A. glutinosa* nodule lobes induced by soil from a birch stand; lanes 20–21, *A. incana* nodule lobes induced by soil from a pine stand.

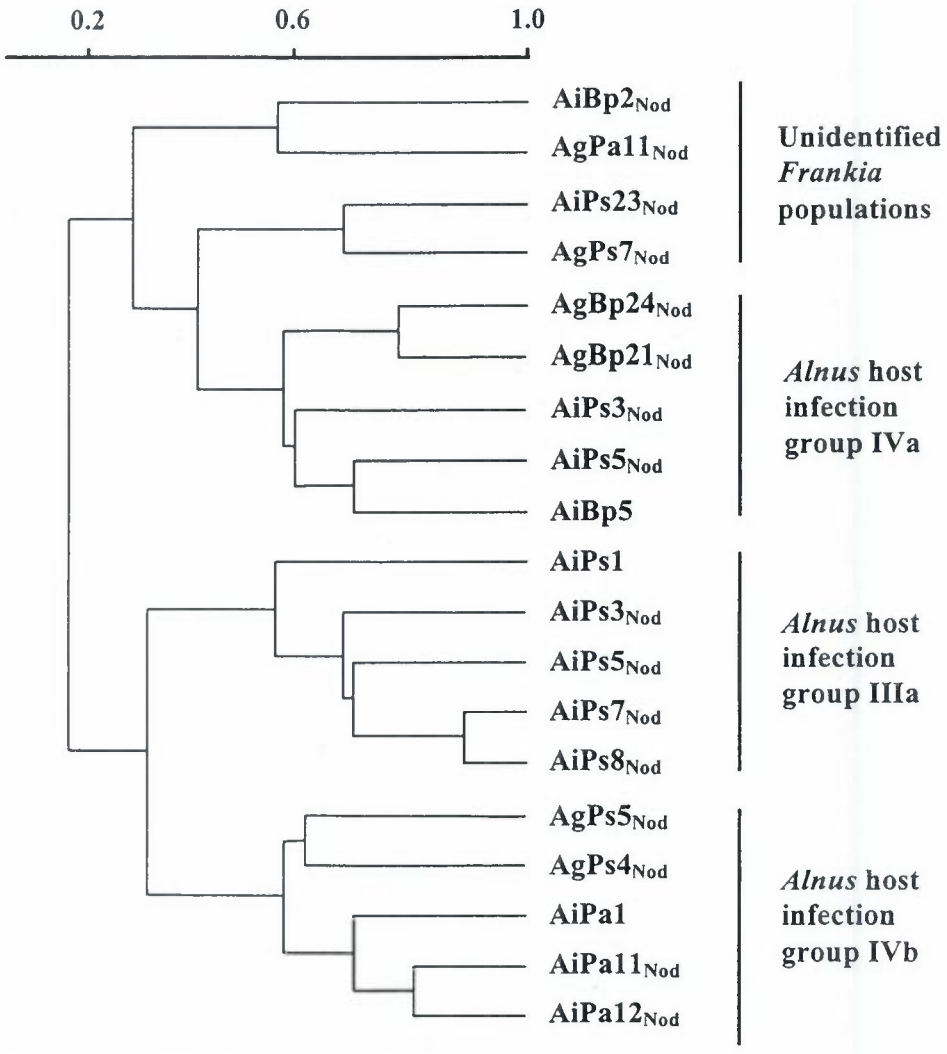

Figure 2. An UPGAMA dendogram obtained from DNA fingerprint patterns of *Frankia* isolates and nodule lobe homogenates.

nodules to identified *Frankia* populations and previously isolated strains by rep-PCR.

Analysis of unidentified Frankia populations in root nodules

Analysis of rep-PCR fingerprint patterns of 16 nodule lobe homogenates showed on average 13 bands (Fig. 1). The complexity of the fingerprint patterns

obtained were similar to the patterns obtained using DNA from *Frankia* isolates (Maunuksela et al., 1999). They were also comparable to those obtained from nodules in other studies (Jeong and Myrold, 1999). The limited complexity and the good clustering of frankiae in nodule homogenates and pure cultures indicates that there was no significant impact of plant or additional microbial DNA on the generation of rep-PCR fingerprint patterns. The UPGAMA dendrogram of the fingerprint patterns allowed us to cluster the *Frankia* populations in the root nodules into 5 groups (Fig. 2): All the patterns from the identified nodules clustered with the pure cultures. Four *Frankia* populations in nodules on *A. incana* (Ai) induced by "pine" soil (*Pinus sylvestris*, Ps) with the acronym AiPs clustered to *Frankia* strain AiPs1, isolated previously from *A. incana* nodules induced by "pine" soil (Maunuksela et al., 1999). Other populations in AiPs nodules, however, clustered to *Frankia* strain AiBp5 or fell into a separate group. In addition to two AiPs populations, two populations in nodules of *A. glutinosa* induced by "birch" soil (AgBp) clustered with *Frankia* strain AiBp5, while two *Frankia* populations each in nodules of *A. glutinosa* induced by "pine" soil (AgPs) and in nodules of *A. incana* induced by "spruce" soil (AiPa) clustered with *Frankia* strain AiPa1. The unidentified *Frankia* populations (AiBp2Nod, AgPa11Nod, AiPs23Nod and AgPs7Nod) in nodules did not cluster with the pure cultures (Fig. 2).

The rep-PCR results correlate with the *in situ* hybridization results and allow us to assign *Frankia* populations in root nodules with similar fingerprints to the same group within the *Alnus* host infection group. The fingerprint patterns from nodules harboring unidentified *Frankia* populations clustered into two groups outside our analyzed subgroups within the Alnus host infection group (Fig. 2). To further characterize these populations, however, additional studies on the identity of the uncultured *Frankia* populations are needed. Comparative sequence analysis of phylogenetically important macromolecules such as rRNA of *Frankia* populations in these nodules may then lead to the detection of other *Frankia* populations or also result in the discovery of new *Frankia* populations. Based on this sequence information, additional probes might be designed, used to analyze uncultured populations in root nodules or in soil, and then provide more information on the diversity of *Frankia* populations in soils devoid of actinorhizal plants.

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