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Soil Biology & Biochemistry 33 (2001) 931–941

Soil Biology & Biochemistry

www.elsevier.com/locate/soilbio

Population size and diversity of *Frankia* in soils of *Ceanothus velutinus* and Douglas-fir stands

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Received 11 April 2000; received in revised form 21 August 2000; accepted 21 August 2000

Abstract

The influence of host plants on *Frankia* populations was investigated using soils from *Ceanothus velutinus* (Dougl.) and Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) stands. Population sizes of *Ceanothus*-infective *Frankia* in the soils were measured using plant bioassays with *C. velutinus, C. sanguineus* (Pursh), and *C. integerrimus* (H. & A.) as trap plants. The *Frankia* population in soil from the *C. velutinus* stand soil was about 10 times higher than that from the Douglas-fir stand. This result supports previous reports that, although the presence of host plants increases *Frankia* populations, *Frankia* persist without host plants. Nodulation capacities of the three trap plants were not significantly different. All nodules showed N_2 fixation activity using the acetylene reduction assay. The diversity of *Frankia* that nodulated trap plants was examined using repetitive intergenic DNA and the polymerase chain reaction (rep-PCR). A newly designed, direct repeat sequence and a BOX sequence were used as rep-PCR primers. The results showed that infective *Frankia* in the two soils contained a common group of *Frankia* as well as some *Frankia* strains unique to each soil. The level of host specificity of the infective *Frankia* was low; however, one group of *Frankia* nodulated only *C. integerrimus* seedlings. Taken together, the results suggest that the higher populations in the soil from the *C. velutinus* stand may be due to preferential increases in particular groups of *Frankia*.

Keywords: *Frankia*; *Ceanothus*; Diversity; Populations

1. Introduction

The genus *Frankia* (Frankiaceae) can proliferate in root nodules and persist in soil, as demonstrated by its isolation from nodules (Callaham et al., 1978) and from soil (Baker and O'Keefe, 1984). Generally, large *Frankia* populations are found in soils near host plants. However, significant numbers of *Frankia* have also been observed in soils well outside the normal geographic range of host plants (Benecke, 1969), under non-host plant stands (Smolander and Sundman, 1987), or long after host plants have disappeared from a site (Wollum et al., 1968). In this study we examined soils under *C. velutinus* (Dougl.) and Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) stands to further address *Frankia* ecology in soil.

Ceanothus species are host plants of *Frankia*. A rich diversity of *Ceanothus* species exists in Oregon. They are early successional species at disturbed sites (burns, landslides, clear-cuts, etc.) and can fix significant amounts of N_2 (Hibbs and Cromack, 1990). Douglas-fir, a non-host plant, is one of the dominant forest tree species in Oregon. Stands of Douglas-fir often replace *Ceanothus* stands during plant succession.

Previous population studies of *Ceanothus*-infective *Frankia* included observations of *Ceanothus* stands in the field and greenhouse inoculation studies. Nodulation of seedlings decreased with higher soil N level (Youngberg and Wollum, 1976) and in response to soil amendments of a high N litter (Zavitkovski and Newton, 1968) or inorganic N (Thomas and Berry, 1989), indicating that nodulation and rates of N_2 fixation in the field may vary inversely with soil N content. There is some indication that the speed of *Ceanothus* nodulation during stand development is a function of soil Ca, with more rapid nodulation on sites with higher Ca concentrations (W. Scott, 1973, unpublished PhD thesis, Oregon State University, Corvallis, OR, USA). By the end of the 10-year observation period, most *Ceanothus* were nodulated, however. As a forest stand develops and *Ceanothus* drops out of the stand, the nodulation capacity of the soil decreases, although some *Frankia* seem to persist even in 300-year-old conifer stands (Wollum et al., 1968). Results

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0038-0717/01/$ - see front matter
PII: S0038-0717(00)00241-8

Table 1
Characteristics of soils sampled

Site	pH	C ($g\ kg^{-1}$ soil)	N ($g\ kg^{-1}$ soil)	P ($mg\ kg^{-1}$ soil)	K ($mg\ kg^{-1}$ soil)	Ca ($cmol_c\ kg^{-1}$ soil)	Mg ($cmol_c\ kg^{-1}$ soil)	Na ($cmol_c\ kg^{-1}$ soil)
C. velutinus stand	6.0	44	1.6	7	234	3.5	0.6	0.04
Douglas-fir stand	6.3	41	1.7	7	343	6.8	1.0	0.04

of a greenhouse experiment using *C. velutinus* germinated from different populations and soils collected under those stands (Nelson and Lopez, 1989) suggested that the rate of N_2 fixation in actinorhizal associations depended on the soil source rather than the host population.

Quantitative estimates of *Frankia* populations have used plant bioassay (e.g. C. van Dijk, 1984, unpublished PhD Thesis, State University, Leiden, the Netherlands; Paschke and Dawson, 1992; Myrold and Huss-Danell, 1994) and PCR–MPN (most-probable number) methods (e.g. Picard et al., 1990; Myrold and Huss-Danell, 1994) to measure the population size of *Frankia*. PCR–MPN methods use *Frankia*-specific primers and allow one to measure population size directly from soils; however, PCR–MPN methods still need improvement because PCR primers may amplify DNA of closely related bacteria (Normand and Chapelon, 1997). A widely used plant bioassay based on counting nodules of trap plants offers an indirect measure of *Frankia* population size in soils and is the only method for measuring population size of host-infective *Frankia*.

The diversity of *Ceanothus*-microsymbiont *Frankia* has been studied only recently because the microsymbiont is recalcitrant to isolation. Genomic fingerprints of microsymbiont *Frankia* assessed by RFLPs (Baker and Mullin, 1994) or rep-PCR (Murry et al., 1997) revealed some degree of diversity, which was not related to geographic origins or *Ceanothus* populations. A more comprehensive survey using PCR–RFLP analysis (Ritchie and Myrold, 1999a) suggested that diversity of *Ceanothus*-microsymbiont *Frankia* was associated more with geographical differences than host plant phylogeny. Phylogenetic studies using DNA sequences amplified directly from nodules showed that the *Ceanothus*-microsymbiont *Frankia* are closely related to *Elaeagnus*-infective (Murry et al., 1997) or *Purshia*-microsymbiont *Frankia* (Benson et al., 1996; Jeong et al., 1999; Ritchie and Myrold, 1999b) and distantly related to *Alnus*-infective *Frankia*. These diversity studies did not address how many *Ceanothus*-infective *Frankia* strains exist in a handful of soil, and such quantitative estimates are needed if the ecology of *Ceanothus*-microsymbiont *Frankia* is to be better understood.

PCR-based methods can eliminate the need to isolate microorganisms in pure culture for studying the diversity and structure of microbial communities. An example is rep-PCR, a PCR technique with primers that correspond to ubiquitous repetitive DNA sequences (Versalovic et al., 1991). Rep-PCR is a highly specific genomic fingerprinting technique. Recently, rep-PCR has been utilized to identify cultured *Frankia* strains with resolution at the strain level (Murry et al., 1995) and to assess the diversity of the *Frankia* microsymbiont in nodules from *Ceanothus* species (Murry et al., 1997; Jeong and Myrold, 1999).

The objective of this study was to elucidate how host plants affect *Frankia* population size and diversity. We compared soils under a host plant, *C. velutinus*, and under a non-host plant, Douglas-fir. Population sizes of *Ceanothus*-infective *Frankia* in soils were measured using plant bioassays. Diversity of *Ceanothus*-microsymbiont *Frankia* was examined using rep-PCR and compared with standard ecological diversity indices.

2. Materials and methods

2.1. Soil collection

Soil samples were collected from a 20-year-old *C. velutinus* stand and an adjacent Douglas-fir stand more than 100 years old at the H.J. Andrews Experimental Forest (44°09′N; 122°22′W) in Oregon. Both stands had the same slope and aspect and the *C. velutinus* stand developed after a portion of the 100-year-old Douglas-fir stand was harvested. Three soil samples (each a composite of several cores to yield a total mass of 3–4 kg; 0–20 cm depth) taken near the roots of two plants were collected 10 m apart within the *C. velutinus* stand. Nodules were also collected to inoculate plants as a positive control. Three 3–4 kg composite soil samples were collected in a similar manner from the Douglas-fir stand. Different collection tools were used in each stand to avoid cross-contamination. Samples were transported in an ice box and stored at −20°C. The soil chemical properties (Table 1) were analyzed by the Central Analytical Laboratory at Oregon State University (Corvallis, OR) using standard methods (Sparks et al., 1996). All data are presented on the basis of dry weight of soil.

2.2. Plant material and growth conditions

Seeds of *C. velutinus*, *C. sanguineus* (Pursh), and *C. integerrimus* (H. & A.) were soaked for 1 h in 2.5% sodium hypochlorite solution and scarified by placing approximately 500 seeds in 200 ml boiling deionized water for 1 min, removing them from the heat, and slowly cooling to room temperature. Scarified seeds were stratified by wrapping them in several layers of moistened, sterile cheese cloth and holding at 4°C for 60 days. Stratified seeds were transferred to sterilized, moistened Perlite at 21°C under

25 μmol m^{-2} s^{-1} fluorescent lighting for germination. Seedlings were grown until one true leaf developed and then transferred to pots containing a Perlite:sand (3:1) mix.

Pots were designed to avoid cross-contamination in growth chamber or greenhouse. A pot consisted of two Square Grower Containers (57 × 60 mm, S-225, McConkey Co., Sumner, WA), one upon another so there was a 12-mm space between the two containers to prevent cross-contamination from dripping solution during watering. Two fiberglass nets (1.25-mm mesh) were inserted between the two containers to exclude Perlite or sand from flowing down. The pots were supported on a lattice.

Pots were left in the laboratory for 3 days before inoculation so that the transferred plants became accustomed to the pot environment. Pots of *C. velutinus* were moved to a greenhouse; *C. sanguineus* and *C. integerrimus* pots were moved to growth chambers. The greenhouse was held at a 21/16°C day/night regime. Plants were grown from December to March, therefore the day/night period was adjusted to 16/8 h using sodium lamps (approximately 65 μmol m^{-2} s^{-1}). Growth conditions for *C. sanguineus* were 26/16°C on a 16/8 h day/night regime. The photosynthetic photon flux density was 145 μmol m^{-2} s^{-1} for 3 h at the beginning and end of the day and 270 μmol m^{-2} s^{-1} for 10 h in the middle of the day. Growth conditions for *C. integerrimus* were the same as for *C. sanguineus* except that the temperature regime was 21/16°C. Plants were watered with sterilized tap water everyday and one-quarter strength, modified Hoagland's solution (Huss-Danell and Myrold, 1994) once a week.

2.3. Inoculation

The three soil samples from each stand were mixed thoroughly by shaking in a plastic bag and sieved to remove gravel and plant debris (>2 mm). Soil corresponding to 20 g of dry weight was suspended in quarter-strength Hoagland's solution at a 1:5 dilution. From this suspension, serial dilutions corresponding to 1:5, 1:50, 1:500, and 1:5000 were prepared. At each dilution step, the soil suspension was shaken vigorously by hand. The soil suspensions were added to the root system of test seedlings. One dilution series consisted of 16 seedlings, four of each dilution. There were three replicate dilution series for each species.

Negative controls were left uninoculated and served to detect any contaminants. Positive controls were inoculated with a crushed nodule suspension to verify that the growth conditions used would allow nodulation to take place. Additional pots, which contained 4 or 0.4 g soil, were set up to check nodulation over time.

2.4. Nodulation and measurement of root and shoot dry weight

Each week, three of the extra pots were destructively sampled and plant roots inspected to determine when nodulation leveled off. *Frankia* populations, or nodulation units (NUs) g^{-1} soil, were calculated from the number of plants with nodules according to the most probable number (MPN) method (Koch, 1994). *Frankia* populations also were calculated from the number of nodules per plant according to a nodulation capacity method based on Van Dijk (1984, unpublished PhD Thesis, State University, Leiden, the Netherlands). For measurement of root and shoot weight of seedlings, nodules were first excised from roots. Nodule lobes of some nodules were excised and tested for acetylene reduction activity. Remaining nodules were stored at −20°C for later DNA extraction. Roots and shoots were separated approximately at the Casparian strip, dried in an oven at 70°C for 2 days and weighed separately. Statistical analyses were performed with multifactor ANOVA in Statgraphics plus 2.0 (Statistical Graphics Corp., Princeton, NJ).

2.5. Acetylene reduction activity assay

Commercially purified C_2H_2 (0.3 ml) was injected into a 3.0-ml tube containing one nodule lobe. After 2 h a 0.1-ml gaseous sample from each tube was removed and analyzed for C_2H_4 and C_2H_2 with a Hewlett-Packard 5830A gas chromatograph (Walnut Park, CA) fitted with a 2 m × 2.1 mm, 80–100 mesh, Porapak R column; oven temperature 70°C. Injection temperature and flame ionization detector temperature were adjusted to 100°C. Flow rate of the N_2 carrier gas was adjusted to 40 ml min^{-1}. After subtraction of appropriate controls, acetylene reduction activity was calculated as the rate of ethylene production per nodule weight (Hardy et al., 1968).

2.6. DNA extraction

For DNA extraction, bacterial cells from liquid cultures of *Frankia* were concentrated by centrifugation and washed using TNE (10 mM Tris–Cl (pH 8.0), 100 mM NaCl, 1 mM EDTA). Nodule lobes were excised and surface-sterilized using 2.5% v/v sodium hypochlorite solution. Genomic DNA was extracted following a modified protocol of the cetyltrimethylammonium bromide (CTAB) procedure (Jeong and Myrold, 1999). Basically, nodule lobes were frozen, ground, extracted with CTAB, and DNA was purified by chloroform:phenol extraction, and precipitated twice with isopropyl alcohol and once with PEG/NaCl. This method yields sufficient high-quality DNA for PCR amplification.

DNA was quantified using Hoechst dye 33258 and a DNA fluorometer (Model TKO 100; Hoefer Scientific Instruments, San Francisco, CA) according to the manufacturer's instructions.

2.7. Rep-PCR

Total nodule DNA was amplified using the BOX A1R primer and the DR1R primer (Jeong and Myrold, 1999).

Each PCR experiment included a control lacking template DNA. For each nodule sample amplified, a control utilizing DNA isolated from root sections adjacent to that nodule was included. The amplification products were separated by agarose gel electrophoresis and stained with ethidium bromide. The band patterns were converted into a binary matrix (presence or absence) for subsequent statistical analysis.

We used nonmetric multidimensional scaling (NMS) to ordinate nodule rep-PCR banding patterns because NMS is more appropriate for discrete data than the more commonly used principal components analysis (Mather, 1976). The NMS analysis was done with PC-ORD software (MjM Software Design, Gleneden Beach, OR). The Sørensen distance was used as the measure of the percent dissimilarity among banding patterns and 150 iterations were done. We present only the results of a two-dimensional NMS because it best described the data (58.8% of the total variance was explained) and higher dimensions gave less stable solutions. We constructed probability ellipses to help visualize the relationships among rep-PCR banding patterns arising from nodules from different soils or trap plants (Jackson, 1991). These relationships were examined further using the multiresponse permutation procedures (MRPP; Mielke, 1984) of PC-ORD. We also used UPGMA (unweighted pair group method with arithmetic mean) clustering to determine OTU (operational taxonomic unit). This was done with NTSYS software (Applied Biostatistics, Inc., Exeter Software, Setauket, NY), using the Jaccard coefficient as the measure of percent similarity among rep-PCR banding patterns. The OTUs identified by the clustering analysis were used to determine several univariate diversity indices (Magurran, 1988).

3. Results

3.1. Measurement of Frankia populations by plant nodulation

It was necessary to do preliminary experiments to design appropriate nodulation systems, because no previous bioassay had been reported using *Ceanothus* species. For example, we grew *C. velutinus* in a hydroponic culture system for 12 months as in a bioassay system used for *Alnus* species (Huss-Danell and Myrold, 1994); however, we did not observe any nodulation. On the basis of a previous inoculation study with red alder (*Alnus rubra* Bong.), in which almost no nodules were observed on plants in negative control pots (Crannell et al., 1994), we evaluated the growth of *Ceanothus* plants in Perlite:sand (3:1) pots in a greenhouse and a growth chamber. Sixteen plants each of three *Ceanothus* species were grown in pots, eight were inoculated with 4 g of soil and eight with 20 g of soil from the *C. velutinus* stand. Half of the plants were grown in the greenhouse and half in the growth chamber. The pots were maintained for 11 weeks. In the greenhouse, all three species grew well. Only *C. velutinus* plants were nodulated in both 4 g and 20 g soil treatment plants, however, and no *C. integerrimus* or *C. sanguineus* plants were nodulated. In the growth chamber, all *C. sanguineus* plants grew well, whereas all *C. velutinus* plants and half of the *C. integerrimus* plants died. All *C. sanguineus* plants and some healthy *C. integerrimus* plants were nodulated. These results may have been caused by different moisture and temperature requirements of the three *Ceanothus* species. Thus, we used three different environmental systems for the three *Ceanothus* species in subsequent plant bioassay experiments.

The first nodules appeared on the three species at different times. Nodules were observed on *C. sanguineus* and *C. integerrimus* in the growth chambers 8 weeks after inoculation and after 10 weeks on *C. velutinus* in the greenhouse. Nodulation leveled off 3–4 weeks after first observing nodules. Thus, we counted nodule numbers of *C. sanguineus* and *C. integerrimus* 12 weeks after inoculation and those of *C. velutinus* 14 weeks after inoculation. We observed nodulation of all positive control pots after 14 weeks; however, we did not observe any nodulation of negative controls or the highest diluted soil pots even after 6 months. Nodulation occurred for at least one dilution within every bioassay replicate. In this study, the highest frequency of nodulated plants and the highest number of nodules per plant were found with the least dilute inoculum. Thus, our bioassays reflect the *Frankia* populations in these soils.

Mean numbers of *Frankia* NUs ranged from 0.2 to 5.2 g^{-1} soil when calculated by the nodulation capacity method. Using three replicates of each soil sample resulted in adequate precision, with coefficients of variation ranging from 2 to 21%. Mean numbers of *Frankia* NUs ranged from 3.6 to 5.2 among the three bioassay species for soil under *C. velutinus* stand and from 0.2 to 0.4 for soil under Douglas-fir stand. Within each bioassay species, the mean numbers of *Frankia* NUs for soil under the *C. velutinus* stand was 8- to 19-fold higher than those for soil under the Douglas-fir stand. Mean numbers of *Frankia* NUs calculated by the MPN method ranged from 0.9 to 2.4 for soil under the *C. velutinus* stand and from 0.2 to 0.3 for soil under the Douglas-fir stand. Within each bioassay species, the mean *Frankia* NUs calculated by the MPN method for soil under the *C. velutinus* stand was 5- to 11-fold higher than those for soil under the Douglas-fir stand. The overall *Frankia* NUs calculated by nodulation capacity were significantly higher than those by MPN methods (Fig. 1). A two-way ANOVA of *Frankia* NUs for plant species and soil type was done (Table 2). On this basis the *Frankia* NUs were significantly different between the two soils, but not significantly different among the three species and the interaction between the soil and plant species was not significant. The MPN method and the nodulation capacity method led to the same conclusion: The population size of *Ceanothus*-infective *Frankia* of

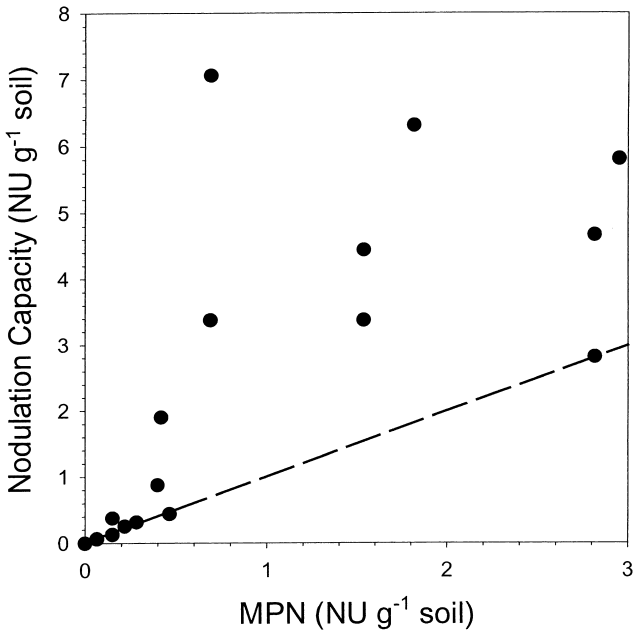

Fig. 1. Relationship between NUs determined by the nodulation capacity and the MPN methods from *C. velutinus* and Douglas-fir stand soils using three *Ceanothus* species. Data are from all replicates ($n = 18$) of the three *Ceanothus* species treated with soil from *C. velutinus* and Douglas-fir stands. Dashed line is 1:1 line.

soil under the *C. velutinus* stand was several-fold higher than that of *Ceanothus*-infective *Frankia* of soil under the Douglas-fir stand.

The shoots of all nodulated plants were larger and darker green than those of non-nodulated plants. Root and shoot weights of all plants were measured (Table 3). A completely randomized three-way ANOVA found none of the interactions significant ($P > 0.05$) but some main effects ($P < 0.05$) were significant. There was no significant difference in shoot weight between the soils, however, there were significant differences in shoot weight among *Ceanothus* species and among soil dilution treatments. There was a significant difference in root weight between the soils and among *Ceanothus* species but not among soil dilution treatments.

Table 2
Mean NUs calculated using nodulation capacity and MPN methods for three *Ceanothus* species using soil from *Ceanothus velutinus* and Douglas-fir stands. Within a factor and a column, populations followed by different letters are significantly different based on Fisher's LSD ($P < 0.05$)

Factor	Nodulation capacity (NUs g^{-1} soil)	MPN (NUs g^{-1} soil)
Species		
C. integerrimus ($n = 6$)[a]	2.2a	0.5a
C. sanguineus ($n = 6$)	2.9a	1.2a
C. velutinus ($n = 6$)	2.0a	1.1a
Soil		
C. velutinus stand ($n = 9$)	4.4a	1.7a
Douglas-fir stand ($n = 9$)	0.3b	0.2b

[a] Number of replicates.

Table 3
Comparison of shoot and root dry weight in terms of bioassay species, soil source, and soil dilution. Within a factor and a column, weights followed by different letters are significantly different based on Fisher's LSD ($P < 0.05$)

Factor	Shoot dry weight (mg)	Root dry weight (mg)
Species		
C. integerrimus($n = 24$)[a]	26b	28a
C. sanguineus($n = 24$)	36a	28a
C. velutinus($n = 24$)	13c	14b
Soil		
C. velutinus stand ($n = 36$)	25a	21b
Douglas-fir stand ($n = 36$)	25a	25a
Soil dilution		
1:5 ($n = 18$)	31a	24a
1:50 ($n = 18$)	26b	23a
1:500 ($n = 18$)	21c	22a
1:5000 ($n = 18$)	22c	24a

[a] Number of replicates.

We estimated N_2-fixation ability of individual nodule lobes separated from nodules of selected plants by the acetylene reduction assay. Some level of acetylene reduction activity was detected in 34 out of 37 nodule lobes tested, indicating that the *Ceanothus* nodules obtained from our bioassay system could fix N_2. Mean acetylene reduction rates for nodules from the three *Ceanothus* species ranged from 2.8 to 6.6 μmol g^{-1} h^{-1} (Table 4). Because of high variability (coefficients of variation from 23 to 36%), no significant difference among *Ceanothus* species or between two soils was found with two-way ANOVA (Table 4).

3.2. Frankia diversity determined by the Rep-PCR

Representative fingerprint patterns of nodule and bacterial DNAs generated by BOX and DR primers are shown in Fig. 2. BOX- and DR-PCR yielded eight to 15 distinct products, ranging in size from approximately 100 bp to over 2 kbp. Differences among nodules were assessed visually on the basis of the banding patterns.

The BOX- and DR-PCR profiles of *Ceanothus* nodule

Table 4
Comparison of acetylene reduction rate of nodule lobes from three *Ceanothus* species inoculated with soil from two sources

Factor	Acetylene reduction rate[a] (μmol g^{-1} h^{-1})
Species	
C. integerrimus	6.6 ± 1.5 ($n = 11$)
C. sanguineus	2.8 ± 1.0 ($n = 19$)
C. velutinus	6.4 ± 1.7 ($n = 7$)
Soil	
C. velutinus stand	4.8 ± 1.1 ($n = 22$)
Douglas-fir stand	5.7 ± 1.3 ($n = 15$)

[a] Mean ± standard deviation (number of replicates).

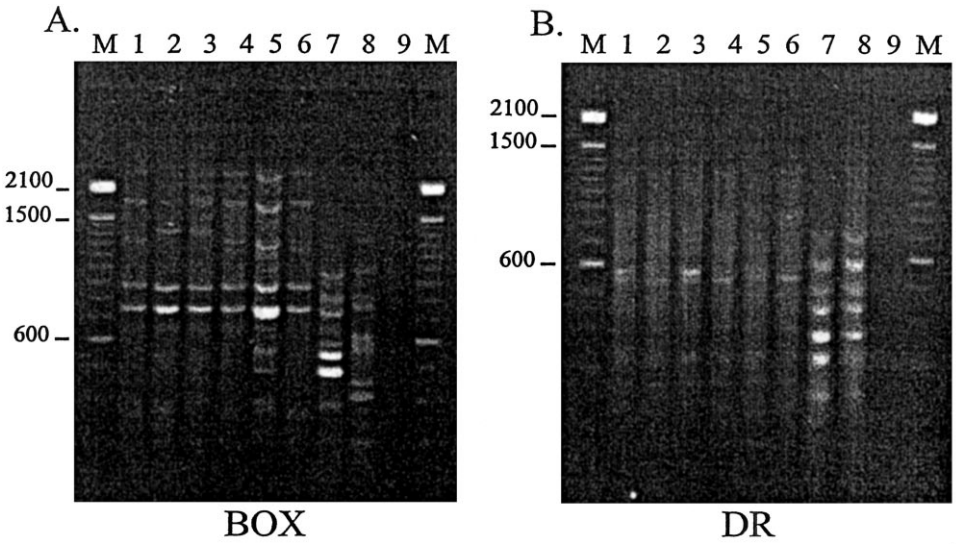

Fig. 2. Representative ethidium bromide-stained agarose gel patterns of BOX- (panel A) and DR-PCR (panel B) products from nodules of *C. velutinus*, *C. sanguineus*, and *C. integerrimus* inoculated with soils from *C. velutinus* and Douglas-fir stands and pure culture *Frankia* strains. Panel A: BOX-PCR products obtained from nodules of *C. velutinus* (lane 1), *C. sanguineus* (lane 3), and *C. integerrimus* (lane 5) inoculated with soils from the *C. velutinus* stand; nodules of *C. velutinus* (lane 2), *C. sanguineus* (lane 4), and *C. integerrimus* (lane 6) obtained with soils from the Douglas-fir stand; and pure cultures of EaI12 (lane 7) and CpI1 (lane 8). Panel B: DR-PCR products obtained from nodules of *C. velutinus* (lane 1), *C. sanguineus* (lane 3), and *C. integerrimus* (lane 5) inoculated with soils from the *C. velutinus* stand; nodules of *C. velutinus* (lane 2), *C. sanguineus* (lane 4), and *C. integerrimus* (lane 6) obtained with soils from the Douglas-fir stand; and pure cultures of EaI12 (lanes 7 and 8). In both panels, lane M shows the DNA molecular size marker (100 bp-ladder, Gibco-BRL) and lane 9 is a negative control lacking template DNA.

DNAs were found to be completely different from those of two reference strains, *Frankia* AvcI1 and EaI12. The presence of several comigrating bands of nodule DNA suggested that *Ceanothus* microsymbionts were closely related. At least four BOX-PCR products from nodule DNA comigrated on the agarose gel. DR-PCR also gave multiple bands of equal mobility. Nevertheless, the overall patterns from many nodule DNAs were distinct, therefore the relationship among *Ceanothus*-infective *Frankia* could be surmised from the BOX and DR-PCR fingerprint patterns. In total, 48 distinct bands were used for the cluster analysis. The 13 bands common to all 69 nodules were eliminated from the NMS and MRPP analyses.

Ordination of the nodule rep-PCR fingerprints by NMS did not produce independent groupings based on soil type or trap plant species: There was overlap between the soil groups and among the trap plant groups (Fig. 3). Nevertheless, MRPP analysis showed that each grouping was significantly different from a random collection of fingerprints ($P < 0.001$), which suggests that there is some structure to these data. In particular, there appeared to be fingerprints that were unique to nodules arising from Douglas-fir soil (Fig. 3).

We also assessed the structure of the rep-PCR fingerprints using cluster analysis, which identified eight different OTUs at the level of 65% similarity (Table 5). OTU 1, which was the largest cluster, was found in abundance in both soils and using all trap plant species. OTU 2 was associated almost exclusively with the Douglas-fir soil (see also Fig. 3) and OTU 4 was found only in nodules of *C. integerrimus* trap plants. The remaining OTUs had few representatives and were often quite different from other fingerprints (Fig. 4). Although each of these minor OTUs was restricted to single species of trap plants, the small numbers of representatives of each OTUs preclude any conclusions about their host specificity.

Univariate measures of diversity based on OTUs defined at several levels of similarity showed little difference between nodules from the two soil types or among nodules of the three trap plants (Table 6). This is also shown by the similar sizes of the 90% probability ellipses (Fig. 3).

4. Discussion

Previous studies have shown that *Frankia* NUs were not affected significantly by the presence of a host plant, suggesting that *Frankia* persist as saprophytes without a host plant. For example, in a study of Swedish soils, Myrold and Huss-Danell (1994) found that alder (a host plant species) and lupine soils were lower in infective *Frankia* population size than a no-tree control. In fact, they found that the greatest change in *Frankia* population size occurred seasonally. Smolander and Sundman (1987) found that there was higher *Frankia* population size in birch soils, but lower in pine soils, than in alder soils on Finnish forests. In this study, the *Frankia* population size of the *C. velutinus* stand soil was about 10 times higher than that of the Douglas-fir stand soil, indicating that *C. velutinus* may have increased the size of soil *Frankia* population. This result supports an

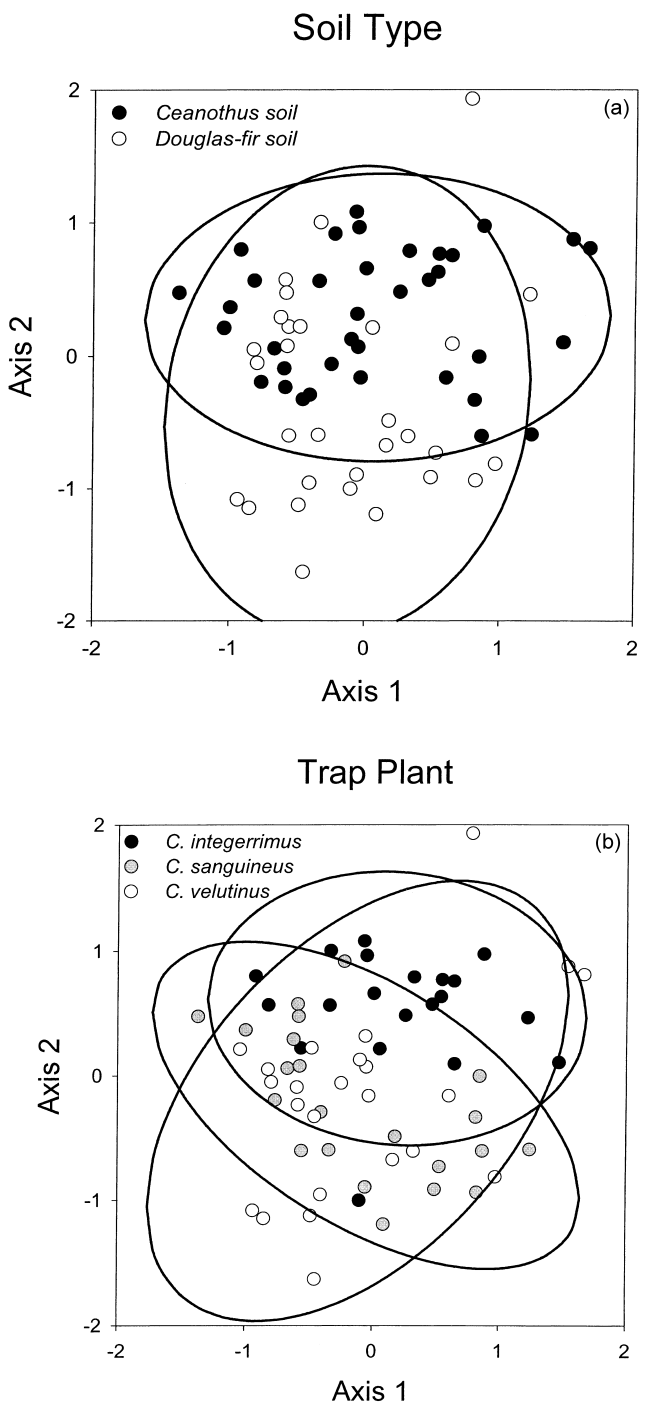

Fig. 3. Two-dimensional plots of rep-PCR fingerprint patterns of *C. integerrimus*, *C. sanguineus*, and *C. velutinus* nodules by nonmetric multidimensional scaling analysis. Axis 1 explained 29.8% of the total variance and Axis 2 explained an additional 28.9% of the total variance. Probability ellipses (P = 0.10) are included for soil type or trap plant species.

observation by Wollum et al. (1968) that the nodulation capacity of a soil was lower 300 years after *Ceanothus* dropped out during the development of a timber stand. Thus it appears that *Frankia* populations are often lower in soils occupied by coniferous trees.

Ceanothus velutinus seedlings, which were grown in the

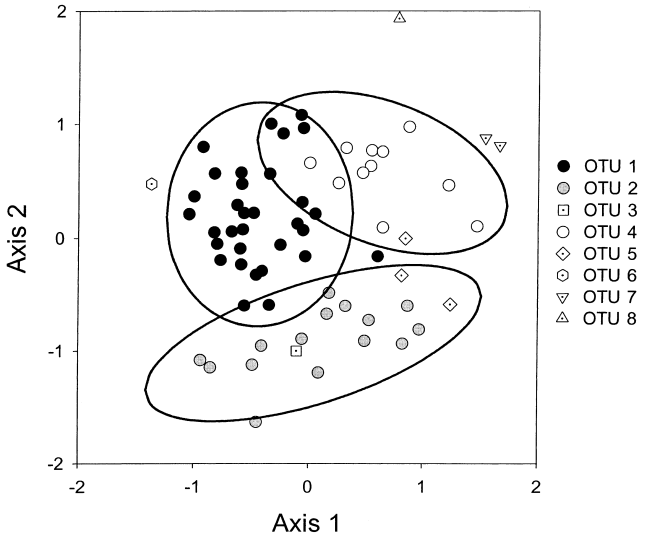

Fig. 4. Two-dimensional plot of rep-PCR fingerprint patterns of the eight OTUs obtained from cluster analysis at the level of 65% similarity. Axis 1 explained 29.8% of the total variance and Axis 2 explained an additional 28.9% of the total variance. Probability ellipses ($P = 0.10$) are included for the three major OTUs.

greenhouse, were significantly smaller (both root and shoot dry weight) than the other two *Ceanothus* species, which were grown in growth chambers. The size differences observed among *Ceanothus* species may represent inherent differences among them or may reflect different growth environments (e.g. the greenhouse had much lower light intensity than the growth chambers). Although there was no significant effect of soil dilution on root weight, there was a significant inverse relationship between shoot weight and soil dilution: the lower the dilution, the larger the seedling. This likely reflects the effect of nodulation, with greater nodulation (and presumably greater N_2 fixation) at the lower dilution, and suggests that plant nodulation increases shoot weight to a much greater degree than root weight. Such trends have been seen in inoculation trials with bare-root *Alnus rubra* seedlings (Hilger et al., 1991). The source of soil used as inoculum had no effect on shoot weight and only a small, but significant, effect on root weight.

Analysis of the rep-PCR fingerprints suggested that the two different soils had a similar degree of diversity (Table 6) and contained a common collection of *Frankia* strains. However, the Douglas-fir soil also contained some unique *Frankia* strains, which clustered in OTU 2 (Table 5). Others have also found different types of *Ceanothus*-microsymbiont *Frankia* associated with different sites and soils (Baker and Mullin, 1994; Murry et al., 1997; Ritchie and Myrold, 1999a).

Cluster analysis and NMS ordination of BOX- and DR-PCR fingerprints indicated that *Frankia* from our soil samples did not show a high degree of host specificity to the three *Ceanothus* species. One possible exception was OTU 4, which was only found in nodules of *C. integerrimus*

Table 5
Distribution of operational taxonomic units (OTU) based on UPGMA dendrogram of BOX- and DR-PCR bands clustered at 65% similarity. Values are the number of nodules of each OTU by soil and species

Soil	Species	Nodules in each OTU								Total nodules
		1	2	3	4	5	6	7	8	
C. velutinus	*C. integerrimus*	5	0	0	9	0	0	0	0	14
	C. sanguineus	5	1	0	0	4	1	0	0	11
	C. velutinus	11	0	0	0	0	0	2	0	13
Douglas-fir	*C. integerrimus*	3	0	1	2	0	0	0	0	6
	C. sanguineus	6	6	0	0	0	0	0	0	12
	C. velutinus	3	9	0	0	0	0	0	1	13
C. velutinus		21	1	0	9	4	1	2	0	38
Douglas-fir		12	15	1	2	0	0	0	1	31
	C. integerrimus	8	0	1	11	0	0	0	0	20
	C. sanguineus	11	7	0	0	4	1	0	0	23
	C. velutinus	14	9	0	0	0	0	3	1	26

Table 6
Diversity indices calculated from UPGMA dendrogram at three levels of similarity

Similarity	Factor	OTUs[a]	Richness[b]	Evenness[c]	Diversity[d]
65%	Total	8	1.65	0.69	1.44 ± 0.13
	Species				
	Ceanothus integerrimus	3	0.67	0.77	0.85 ± 0.11
	Ceanothus sanguineus	4	0.96	0.83	1.16 ± 0.11
	Ceanothus velutinus	4	0.92	0.74	1.02 ± 0.13
	Soil				
	Ceanothus velutinus	6	1.37	0.70	1.25 ± 0.14
	Douglas-fir	5	1.16	0.70	1.12 ± 0.14
70%	Total	14	3.07	0.84	2.21 ± 0.18
	Species				
	Ceanothus integerrimus	6	1.67	0.89	1.60 ± 0.11
	Ceanothus sanguineus	7	1.91	0.76	1.47 ± 0.16
	Ceanothus velutinus	7	1.84	0.86	1.67 ± 0.14
	Soil				
	Ceanothus velutinus	9	2.20	0.87	1.91 ± 0.11
	Douglas-fir	8	2.04	0.85	1.77 ± 0.12
75%	Total	21	4.72	0.92	2.81 ± 0.37
	Species				
	Ceanothus integerrimus	7	2.00	0.90	1.73 ± 0.13
	Ceanothus sanguineus	10	2.87	0.94	2.16 ± 0.08
	Ceanothus velutinus	10	2.76	0.91	2.11 ± 0.11
	Soil				
	Ceanothus velutinus	12	3.02	0.90	2.24 ± 0.10
	Douglas-fir	11	2.91	0.93	2.23 ± 0.09

[a] Operational taxonomic units.
[b] Margalef index $= (S - 1)/\ln(N)$, where S is the number of OTUs and N is the total number of nodules from that factor assayed.
[c] Pielou index $= H'/\ln(S)$, where S is the number of OTUs and H' is the Shannon index.
[d] Shannon index $(H') = -\sum p_i \ln(p_i)$, where p_i is the proportional abundance of the ith OTU. Mean ± estimated standard deviation (Magurran, 1988).

(Table 5). This result is consistent with past research that had shown that diversity of *Ceanothus*-microsymbiont *Frankia* was not related to differences of *Ceanothus* species (Baker and Mullin, 1994; Murry et al., 1997; Ritchie and Myrold, 1999a).

Two additional lines of evidence indicate that specific associations between the three *Ceanothus* species and their infective *Frankia* were weak. First, we found no significant intrageneric variation in the quantification of NUs from within a soil sample. This is interesting because a significant intrageneric variation in nodulation of *Alnus* has been found (Huss-Danell and Myrold, 1994). Second, we found no significant differences among *Ceanothus* species or between two soils in acetylene reduction activity of nodules. Dillon and Baker (1982) found that both microsymbiont and host influenced the acetylene reduction rate,

but they compared host-microsymbiont sources from different species and genera. Nelson and Lopez (1989) found that variability in the N_2 fixation rate was more a function of the soil source than the *C. velutinus* source population in actinorhizal associations.

Nodulation units estimated by the nodulation capacity method were consistently higher than those estimated by MPN plant bioassay methods even though they gave a similar trend in NUs between *C. velutinus* stand and Douglas-fir stand. A study by Huss-Danell and Myrold (1994) showed that agreement between the two methods was especially good between 30 and 300 NUs g^{-1} soil, although the methods are based on different assumptions. Huss-Danell and Myrold (1994) also suggest that *Frankia* NUs may be underestimated above this population level by the nodulation capacity method perhaps because of saturation of nodulation sites and, conversely, at lower population levels by the MPN method, perhaps because of difficulty in accurately diluting potentially infective *Frankia*. Thus, in this study, the discrepancy between the two estimates may come from low population levels in our soil samples, ranging from 0.2 to 5.2 NUs g^{-1} soil.

Frankia NUs calculated by both nodulation capacity and MPN methods in this study were at the low end of the range obtained by previous studies in which alders were used as trap plants. For example, the NUs varied from 4 to 400 NUs g^{-1} soil for the soil from the no-tree control plot (Huss-Danell and Myrold, 1994) or 0 to 900 NUs cm^{-3} of soil for the soil under some coniferous species (Smolander, 1990). Interestingly, Markham and Chanway (1996) observed a similar range of NUs, which varied from 0 to 18.9 NUs cm^{-3} of soil when using red alder as the trap plant, in a study of soils from harvested forest sites apparently because of the low population levels in the soils.

Many actinorhizal plants have been used for inoculation with isolated *Frankia* or for trapping soil *Frankia* (e.g. Baker, 1987). However, most studies on *Frankia* populations in soils have used *Alnus* species as a trap plant to quantify population size (e.g. Smolander and Sundman, 1987; Huss-Danell and Myrold, 1994). *Alnus* species grow well in hydroponic culture so that nodulation incidence can be easily observed. Here we demonstrated that *Ceanothus* species can be used as trap plants to assay plant nodulation using a pot system with solid media, as others have done with *Alnus*, *Casuarina*, and *Myrica* (Cranell et al., 1994; Zimpfer et al., 1997).

The difference of *Frankia* population size between two soils was probably not due to differences in soil chemical properties, because the soil properties from the soil chemical analysis were quite similar between the two soils except for Ca and K levels (Table 1). It has been suggested that nodulation rate or numbers of infective *Frankia* is significantly correlated to soil Ca levels or increase in response to the addition of Ca to soils (W. Scott, 1973, unpublished PhD thesis, Oregon State University, Corvallis, OR, USA; Crannell et al., 1994). In this study, however, the host plant seemed to play a more important role in regulating *Frankia* population size, because the soil from the Douglas-fir stand had two-fold higher Ca levels but was about 10 times lower in *Frankia* NUs than that from the *C. velutinus* stand. Although previous studies have suggested possible regulators of *Frankia* population in soil, there is no general agreement about what soil chemical factor is the major regulator. For example, Smolander and Sundman (1987) found that soil pH was the major regulator of the size of infective *Frankia* populations on Finnish forest soils with a range in pH 3.5–4.4. However, Myrold and Huss-Danell (1994) found that C, rather than pH, was regulating the size of the infective *Frankia* populations on Swedish soils with a narrower range of pH (4.66–4.93).

Most previous ecological studies of actinorhizal symbiosis were based on analysis of *Frankia* isolates from *Alnus* (Benson and Hanna, 1983), *Myrica* (Bloom et al., 1989), and *Elaeagnus* (Jamann et al., 1992), from which *Frankia* can be easily isolated. The lack of infective *Ceanothus* isolates of *Frankia* has hindered ecological studies of this particular actinorhizal symbiosis, particularly those focused on *Frankia*. Recent applications of molecular techniques have allowed circumvention of *Frankia* isolation. The first study used strain-specific *nif*H sequences to probe nodule DNA extracts (Simonet et al., 1990). The use of RFLP analysis of nodule DNA extracts (Baker and Mullin, 1994) was used to examine the population structure of *Frankia* without isolation, as can a newer variant of this, PCR–RFLP (Jamann et al., 1993; Rouvier et al., 1996). The PCR has been utilized to amplify specific regions of the *Frankia* genome for phylogenetic analysis based on nucleic acid sequence comparisons (e.g. Nazaret et al., 1991; Clawson and Benson, 1999). Here, we demonstrated that BOX- and DR-PCR can be utilized to quantify the diversity of *Ceanothus*-infective *Frankia* trapped from soils without *Frankia* isolation, supporting and expanding the previous report by Murry et al. (1997) that there was considerable diversity in the BOX-PCR patterns among nodules sampled between species, between individuals of the same species and between sites.

DNA sequences of the intergenic spacer region between 16S rRNA and 23S rRNA genes suggested that *Ceanothus*-microsymbiont *Frankia* from several sites in Oregon are related at the intraspecific level (Jeong and Myrold, 1999). Although several comigrating bands among rep-PCR patterns indicated a close relationship among *Ceanothus*-infective *Frankia* studied here, we observed 48 distinct bands from rep-PCR fingerprints of 69 nodules. This observation is not surprising according to previous studies that rep-PCRs revealed high genetic polymorphisms at the pathovar or genotype level (e.g. Louws et al., 1994; Frey et al., 1997). For example, Frey et al. (1997) found 16 different rep-PCR fingerprints from one 16S rDNA type identified by RFLP.

In conclusion, the plant bioassay results suggest that *Ceanothus*, a host plant of *Frankia*, supports *Frankia* persistence better than Douglas-fir, a non-host plant. Despite the

large differences in *Frankia* populations between the two sites, the overall diversity of *Frankia* at each site was similar, although each site harbored some unique rep-PCR types. It is interesting that *Frankia* strains from both soils can nodulate three *Ceanothus* species equally well, as evidenced by the quantification of *Frankia* population sizes, acetylene reduction activity, genomic fingerprinting using rep-PCRs.

Acknowledgements

We thank Arthur McKee for helping locate the appropriate sampling sites at H.J. Andrews Experimental Forest; C.-Y. Li for help with the gas chromatography analysis; Peter J. Bottomley for discussions; Nancy J. Ritchie for technical assistance; Mary E. Schutter for help with the multivariate statistical analyses; David Martin for comments on an early draft of this paper. This research was funded by a grant from the USDA National Research Initiative Competitive Grants Program. Contribution from the Oregon Agricultural Experiment Station; Technical paper no. 11713.

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