

AN ABSTRACT OF THE THESIS OF

Sarah J. Shaffar for the degree of Bachelor's of Science in Bioresource Research – Sustainable Ecosystems Option, Soils Minor on August 22, 2003.

Title: Genetic Diversity of *Frankia* in Three Different Oregon Soils with Co-occurring Actinorhizal Hosts

Abstract approved



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David D. Myrold

Little is known about *Frankia*, a gram-positive, nitrogen-fixing bacterium, because of the difficulty associated with isolating pure cultures. This nitrogen-fixing symbiosis involves *Frankia* and the roots of actinorhizal host plants belonging to eight plant families and 25 genera. These plants are important pioneer species traditionally found in nitrogen-poor soils and disturbed sites. Additionally, many of these plants have economic potential. Because isolation is difficult, the use of molecular biology methods can be quite helpful. This study uses the LH-PCR to study the intergenic spacer region of *Frankia* sampled both from nodules and the soil bacterial community. Three locations with co-occurring actinorhizal hosts were sampled across the state of Oregon. Three sets of nodules were sampled from each host and a soil sample was taken beneath each host along with an additional soil sample between the two co-occurring hosts.

DNA was extracted from these samples and used to perform the LH-PCR. It was determined that soil *Frankia* communities were more closely related based on geographic location rather than host plant. LH-PCR was able to distinguish between nodules obtained from *Ceanothus* and *Alnus* but not between *Ceanothus* and *Purshia*. Also, LH-PCR was not able to distinguish any differences between the two species of *Ceanothus* but did show that *Alnus* may be infected by two different strains of *Frankia*. The primers used in this study may have also amplified other bacteria belonging to *Actinomycetales*. Further work in cloning and sequencing of the soil samples is needed to determine what organisms were amplified.

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Genetic Diversity of *Frankia* in Three Different Oregon Soils with  
Co-occurring Actinorhizal Hosts

by  
Sarah J. Shaffar

A THESIS

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degree of

Bachelor's of Science

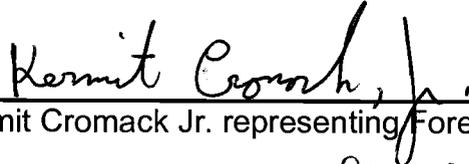
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## Introduction/Background

Actinorhizal plants are found on every continent except Antarctica (Schwintzer and Tjepkema 1990). These plants, usually considered pioneer species, are found in a number of different environments, including: nitrogen-poor soils, disturbed sites, sand dunes, gravels, shores of streams and lakes, wetlands, and exposed raw mineral soils (Schwintzer and Tjepkema 1990, Berry 1994). Many actinorhizal plants have economic potential. Some are important in timber and forestry, land reclamation, and horticulture. Wasteland reclamation and land stabilization are important in Western Europe, yet here in the Pacific Northwest, the primary interest is in forestry. Particular focus is on Red alder (*Alnus rubra*) that is managed for pulp and wood and is an important alternative crop in areas infected with conifer root diseases. Alder can also be used to improve the growth of associated species by increasing the amounts of nitrogen and organic matter in soils (Schwintzer and Tjepkema 1990).

*Frankia* are gram-positive, septate-filamentous, nitrogen-fixing bacteria that can live symbiotically in root nodules and saprophytically in soil (Paschke 1997). They are classified as *Actinomycetes*, based on morphological and biochemical characteristics, high DNA G+C content (approximately 70%), and 16S rRNA-DNA sequence analysis (Nazaret et al. 1991, Berry 1994). Figure 1 shows the major *Frankia* groups and the most closely related *Actinomycetes*. The nitrogen-fixing symbioses involves *Frankia* and the roots of actinorhizal host plants belonging to eight plant families and 25 genera (Schwintzer and Tjepkema 1990). There is no good explanation as to why they nodulate some

plants and not others within the different families. However, the efficiency of this symbiosis depends on the host plant and the associated symbiont; thus, the relationship seems to be host-species dependent (Chauhan and Misra 2002, Rouvier et al. 1996). This relationship can also build up soil organic matter and create a more favorable environment for other plants and soil organisms (Paschke 1997).

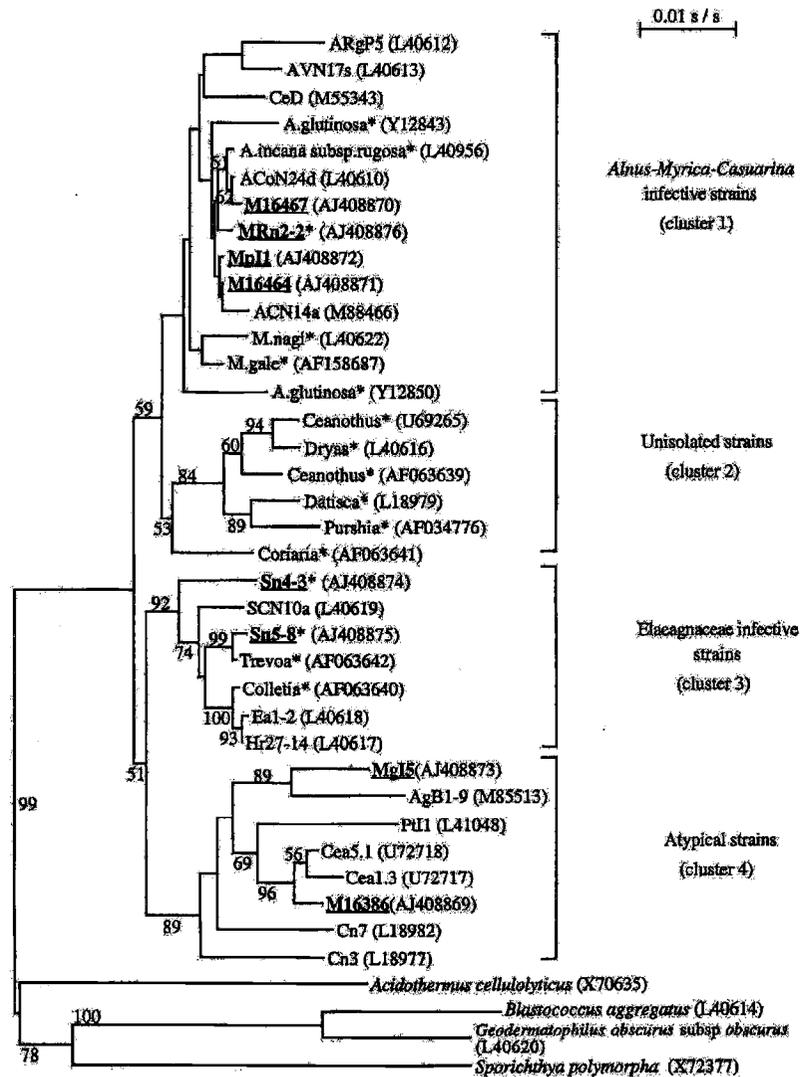


Fig. 1. Phylogenetic tree of major *Frankia* groups showing most closely related *Actinomycetes* based on *rrs* gene sequences obtained by the neighbor-joining method (Huguet et al. 2001).

Although much has been learned about *Frankia* since the first successful isolation in 1978 (Callaham et al. 1978), little is yet known about the actinomycete because of the difficulty of isolating pure cultures. Isolation difficulties include:

- 1) Very slow growth rate in relation to other actinomycetes and eubacteria
- 2) Contamination by fast growing eubacteria and actinomycetes associated with the soil
- 3) Inhibitory nature of phenolic compounds released from host plant cells during isolation procedures (Lal 2002)

Much of the previous research on the ecology of *Frankia* has focused on a specific strain of *Frankia* or on microsymbionts of a single host. Therefore, little is known about the ecology of entire *Frankia* populations in soils and what factors influence their dynamics. It is clear that the presence of one actinorhizal host does not eliminate *Frankia* that nodulate other host plants, and it is also known that the few cultured representatives of *Frankia* only represent a small fraction of the natural community (Wintzingerode et al. 1997). In the past, research concerning *Frankia* in soils has involved the use of plant trapping and genetic characterization on DNA extracted from nodules (Nalin et al. 1999). More and more, *Frankia* research is focusing on using available molecular biology techniques.

Four genera of actinorhizal plants, which occur in Oregon, were sampled in this study: *Purshia*, *Alnus*, *Ceanothus*, and *Myrica*. *Purshia tridentata* is found east of the Cascade Mountains. Red alder is found on the coast and partially up the Cascades, where it mixes with and is eventually replaced by Sitka alder (*Alnus viridis*) as elevation increases. Snowbrush (*Ceanothus velutinus*) inhabits middle-elevation sites in the Cascades and higher elevations in the Coast Range. California lilac or Blueblossom (*Ceanothus thyrsiflorus*) occurs in the southern coastal regions. Pacific Waxmyrtle (*Myrica californica*) is found along the Pacific Coast and in coastal valleys.

Using primers specific to the intergenic spacer region (IGS) between the 16S rRNA gene and the 23S rRNA gene (Ritchie-Posavatz and Myrold, unpublished data), DNA was extracted directly from the soil and the polymerase chain reaction (PCR) was used with *Frankia*-specific primers to rapidly compare *Frankia* communities in the soil. Sequencing of the IGS region shows high sequence conservation in *Frankia* found in the *Casuarina*-infectivity group (Rouvier 1996) but this has not been shown to be true among the different infectivity groups. Therefore, the IGS region should prove useful in differentiating *Frankia* among various host plants.

Study objectives were:

- 1) Characterize *Frankia* strains infecting nodules
- 2) Compare *Frankia* communities in soils sampled under and between co-occurring plant hosts
- 3) Compare *Frankia* communities in soils sampled under the same species of host plant at different sites

The working hypotheses were: (1) that nodules of different hosts are infected by distinct strains of *Frankia* and (2) *Frankia* strains found in nodules will also be dominant members of the *Frankia* community in soil beneath the host.

Given the economic and ecological potential, it is important to gain a better understanding of these actinorhizal associations.

## Materials and Methods

### Sample Collection

Three locations, with two sites per location, were sampled (Fig. 2). The first location was found on the Southern Oregon Coast. Representative actinorhizal hosts were *Ceanothus thyrsiflorus*, *Alnus rubrus*, and *Myrica californica*. The second location was found in the H.J. Andrews Experimental Forest near the McKenzie River in the Cascades. Representative actinorhizal hosts were *Ceanothus velutinus* and *Alnus rubrus*. The third location was found in Central Oregon near Sisters, Oregon on Highway 20 near milepost 96. Representative actinorhizal hosts were *Ceanothus velutinus* and *Purshia tridentata*.

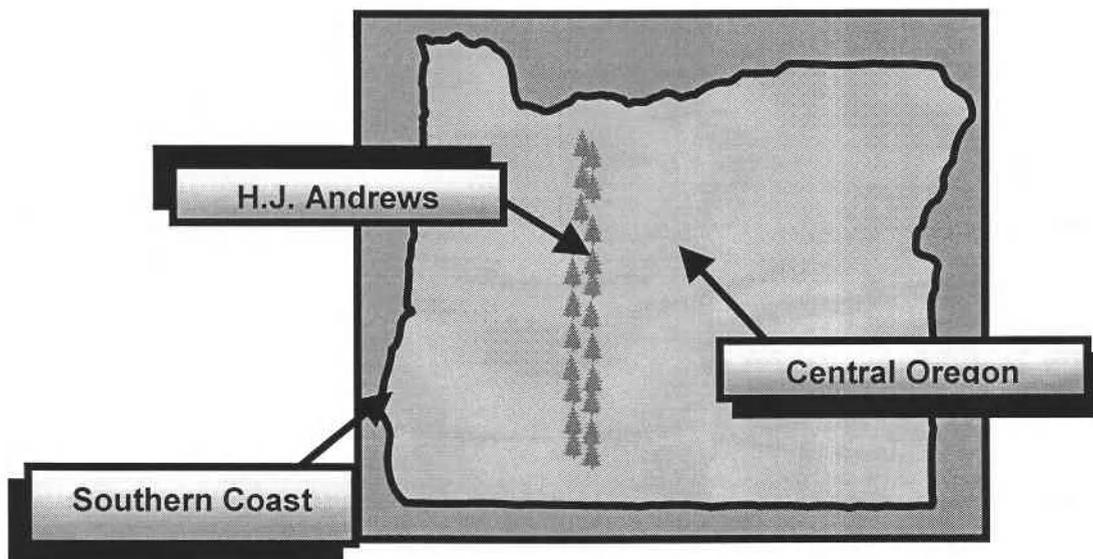


Fig. 2. Sampling sites across the state: Southern Oregon Coast (includes Sites 1 and 2), H.J. Andrews Experimental Forest (includes Sites 3 and 4), and Central Oregon (includes Sites 5 and 6).

Three nodules per host plant were collected. Soil samples were also collected directly beneath each host plant and approximately halfway between co-occurring hosts. All samples were kept on ice until returned to the laboratory. Upon return to the laboratory, soil samples were sieved through a 1-mm sieve and stored at -20°C. Nodules were cleaned with tap water to remove as much soil and debris as possible. Dilute Tween-80 and de-ionized water were added. Nodules were shaken in this solution and then rinsed. After thoroughly rinsing off the Tween-80 solution, nodules were surface sterilized in 80% ethanol and allowed to air dry. Once dry, nodules were stored at -20°C.

### **DNA Extraction**

DNA was extracted from the soil using the Fast DNA® Spin Kit for soils (Qbiogene, Inc., Carlsbad, CA). Soil ( $0.25 \pm 0.05$  g) was weighed into the provided Lysing Matrix E tube along with 978  $\mu$ l of sodium phosphate buffer and 122  $\mu$ l of the MT buffer. The tube was then processed in the FastPrep™ (Carlsbad, CA) machine for 30 seconds at a setting of 5.5. After centrifugation for 15 min, the supernatant was transferred to a new tube along with 250  $\mu$ l of PPS and mixed by inversion 10 times. After centrifuging again for an additional 5 min, the supernatant was transferred and 1 ml of binding matrix was added. The binding matrix was suspended for 2 min and allowed to settle for 3 min. Then, 500  $\mu$ l of the supernatant was removed and discarded. The settled matrix was resuspended and transferred in two aliquots of 600  $\mu$ l into a spin filter. Centrifugation removed the liquid and the remaining binding matrix

was washed with 500  $\mu$ l of SEWS-M and centrifuged for 1 min. An additional centrifugation of 2 min was done to remove any liquid remaining in the binding matrix. The matrix was allowed to dry for 5 min. After drying, 50  $\mu$ l of DES water was added, the binding matrix was gently stirred, and then centrifuged for 1 min. This was the final DNA extract used for the PCR.

Nodule DNA was extracted using a modified version of the Fast DNA<sup>®</sup> Spin Kit for soils. First, a lobe tip was excised from a frozen nodule. The excised lobe tip was ground using a sterile mortar and pestle in 489  $\mu$ l of the sodium phosphate buffer. This solution was then transferred to the Lysing Matrix tube and an additional 489  $\mu$ l of the sodium phosphate buffer was used to rinse the mortar and pestle. This rinsing solution was also added to the Lysing Matrix tube. The rest of the procedure follows the DNA extraction procedure for soils as previously described.

### **PCR Amplification**

Extracted DNA was used to amplify the intergenic spacer region (IGS) between the 16S rRNA gene and the 23S rRNA gene using the PCR. All reactions were performed in 50  $\mu$ l (final volume) containing: 5  $\mu$ l GeneAmp 10X PCR Buffer II (Applied Biosystems, Branchburg, NJ), 4  $\mu$ l 25 mM MgCl<sub>2</sub>, 4  $\mu$ l 2.5 mM dNTPs, 1  $\mu$ l 10 uM each of fluorescently labeled forward primer IGS-F (5'TTCTAAGGAGCGTCTGGC) and reverse primer IGS-R (5'TAACTTGGCCACAAAGAT), 8  $\mu$ l 0.4% BSA, and 0.5  $\mu$ l AmpliTaq<sup>®</sup> DNA polymerase (Applied Biosystems, Branchburg, NJ). An initial denaturation

temperature of 94°C for 3 min was followed by 30 cycles with a denaturation temperature of 94°C (45 s), an annealing temperature of 57°C (45 s) and an extension at 72°C (2 min) using a GeneAmp PCR system 2400 (Perkin Elmer Corp., Branchburg, NJ) or a PTC-100™ (MJ Research, Inc., Waltham, MA). There was a final extension at 72°C for 10 min. Products were run on a 2% agarose gel to determine if amplification had occurred. A low mass DNA ladder was used along with the Kodak 1D Image Analysis Software (Kodak Scientific Imaging Systems, Rochester, NY) to determine the concentration of DNA. Samples were diluted to approximately 0.5 ng/μl concentrations and 1 μl of the sample was submitted to the Center for Gene Research and Biotechnology Central Services Laboratory for length-heterogeneity (LH-PCR) analysis. Lengths of the amplified IGS region were then determined from data produced in GeneScan and from electropherograms generated by Genotyper.

### **Analysis of Soils Data**

For soil samples, which had multiple fragments, all fragments with a value of less than 60 relative fluorescence units and less than 2% of the total fluorescence for a sample were removed from the data set. Samples with fragments that were doublets, triplets, or quadruplets were treated as one fragment with the highest relative fluorescence being reported. The following were considered to be single fragments: 355-356, 357-362, 363-367, 370-373, 374-376, 377-379, 380-382, 383-386, 387-393, 394-396, 398-400, 404-408,

409-413, 420-424, and 427-431. This allowed for better comparison among the different samples.

This data was analyzed, according to Rich (2003), using PC-ORD v4.01 (McCune and Mefford 1999). Any fragment that occurred in less than 5% of the samples was considered a rare species and removed from the data set. Values for each fragment were relativized by sample. Differences were assessed graphically using the ordination method of Non-metric Multidimensional Scaling (NMS), based on Sørensen's distance, a relative distance measure (Kruskal 1964, McCune and Grace 2002). NMS was constrained to two ordination axes with a random starting configuration, 200 iterations, instability criterion of 0.0001, 40 runs with the real data and 50 runs with randomized data; Monte Carlo test of the real data vs. the randomized data were used to assess axes significance. A Multi-response Permutation Procedure (MRPP) (McCune and Grace 2002), with Sørensen's distance and rank transformation, was used to test for significant differences in community composition among groups defined by geographic region. The MRPP *A*-statistic describes the within and between group relatedness relative to that expected by chance (McCune and Grace 2002). It is somewhat analogous to an  $r^2$ -value (McCune and Grace 2002). To determine fragments that differentiated groups by geographic region, Indicator Species Analysis (Dufrêne and Legendre 1997) was run with 1000 randomizations in a Monte Carlo test (Rich 2003).

## Results

### Nodule Samples

Below is an example of the electropherograms received after LH-PCR. Data from the electropherograms suggest there is a difference in the IGS region between *Frankia* sampled from *Alnus* and *Ceanothus* (Fig. 3). It is also clear that differentiating between *Ceanothus* and *Purshia* is not possible using LH-PCR.

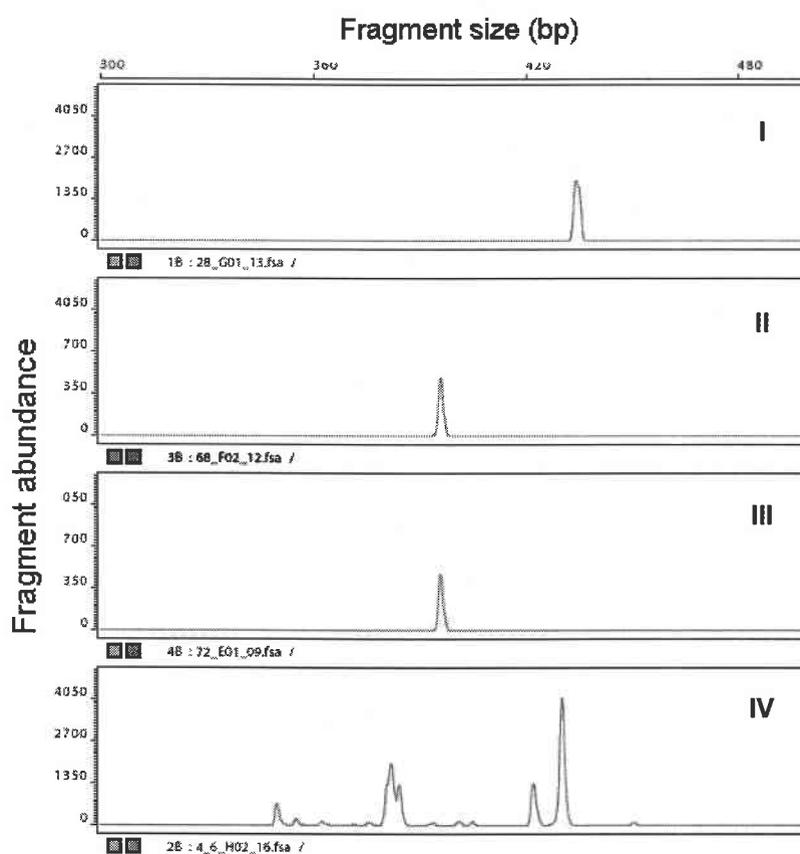


Fig. 3. Electropherogram representing I) *Alnus/Myrica*, II) *Ceanothus*, III) *Purshia*, and IV) soil samples.

*Alnus* samples generally produced fragments that were between 426-428 bp or 434-435 bp whereas *Ceanothus* samples produced fragments that were 396-397 bp (Table 1). Previous work has shown that 2 or 3 bp differences are probably due to drift and do not truly represent different fragment lengths (Kaplan and Kitts 2002).

Table 1. Fragment lengths in base pairs (bp) obtained by LH-PCR on individual nodules from actinorhizal hosts sampled at three different locations.

Host	Nodule	Location					
		Southern Oregon Coast		H.J. Andrews		Central Oregon	
		1	2	3	4	5	6
<i>Alnus</i> (1)*	A	434	434	428	ND	-	-
	B	428	434	426	428	-	-
	C	435	435	427	424	-	-
<i>Alnus</i> (2)	A	435	434	428	428	-	-
	B	428	434	427	428	-	-
	C	428	434	427	428	-	-
<i>Myrica</i>	A	-	426	-	-	-	-
	B	-	426	-	-	-	-
	C	-	ND	-	-	-	-
<i>Ceanothus</i> (1)	A	396	396,410	397	396	396	396
	B	397	410	397	397	396	397
	C	397	396,410	397	396	396	396
<i>Ceanothus</i> (2)	A	396	-	397	ND	397	396
	B	396	-	396	ND	396	396
	C	396	-	397	396	396	ND
<i>Purshia</i> (1)	A	-	-	-	-	396	396
	B	-	-	-	-	396	397
	C	-	-	-	-	396	396
<i>Purshia</i> (2)	A	-	-	-	-	396	396
	B	-	-	-	-	396	396
	C	-	-	-	-	396	396

\*Numbers (1) and (2) denote different plants sampled at the same site

ND= not determined

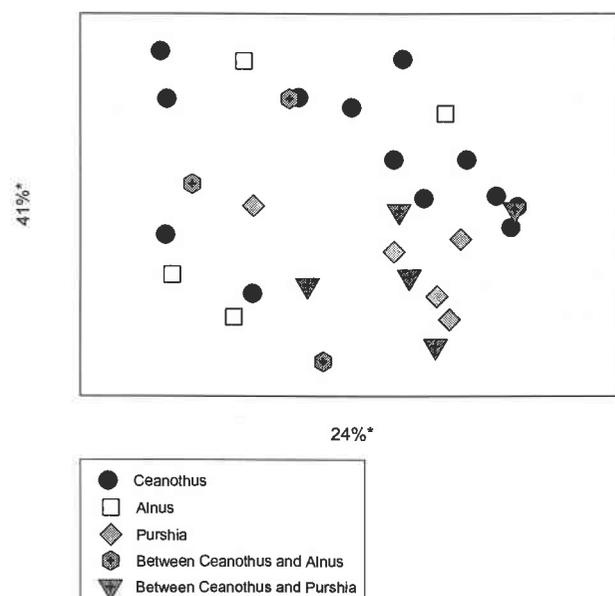
- = not sampled or not available at that site

*Alnus* samples showed some variability at the Southern Oregon sites and looking at *Alnus* (1) from Site 1 there are two different strains infecting one

plant (Table 1). This can also be seen in *Alnus*(2) from Site 1. This co-infection of the plant is not seen in *Alnus* at Site 2. In general, one nodule was infected by one strain of *Frankia*. This does not appear to be the case with *Ceanothus*(1) at Site 2, where it appears the host plant was infected by one strain with an IGS region length of 396 bp and another with a length of 410 bp. *Myrica* sampled at Site 2 exhibited a fragment length similar to that found in nodules sampled from *Alnus* at Site 1.

### Soil Samples

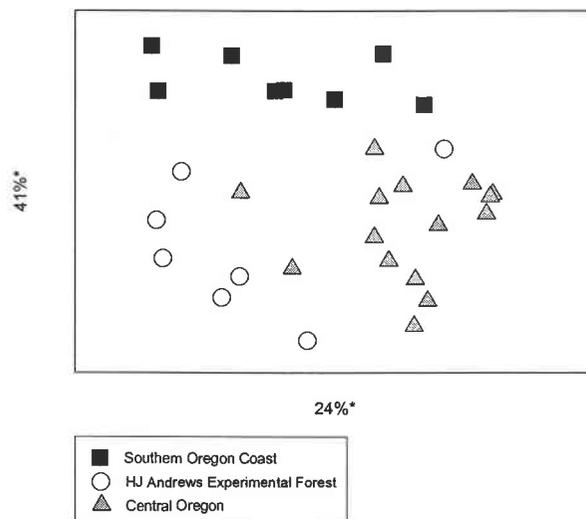
Soil samples exhibited multiple fragment lengths (Fig. 3). NMS on soil samples did not group regardless if the samples were taken below a particular plant host or between two co-occurring hosts (Fig. 4).



\* Coefficients of determination for the correlations between ordination distances and distances in the original matrix.

Fig. 4. Ordination plot generated by NMS, showing no separation of soil samples based on plant type.

There was grouping, however, based on the geographic locations of the samples (Fig. 5). These differences were statistically significant ( $p < 0.001$ ). Also, the  $A$ -stastic=0.315 indicated that there were strong differences between groups (Rich, 2003).



\* Coefficients of determination for the correlations between ordination distances and distances in the original matrix.

Fig.5. Ordination plot generated by NMS, showing separation of soil samples based on geographic location ( $p < 0.001$ ).

Indicator Species Analysis identified six fragment lengths that were statistically significant in contributing to the differences among the three geographic locations (Fig. 6). These six lengths were 171, 295, 341, 383-386, 409-413, and 420-424. Fragments 171 and 295 only occurred at Southern Oregon Coast Sites. Fragments 383-385 and 420-424 were much more abundant in the Central Oregon Sites. Fragment 341 was found in the highest abundance at the H.J. Andrews Experimental Forest sites. The 409-413

fragment found in the same abundance at both the H. J. Andrews Experimental Forest and Central Oregon Sites.

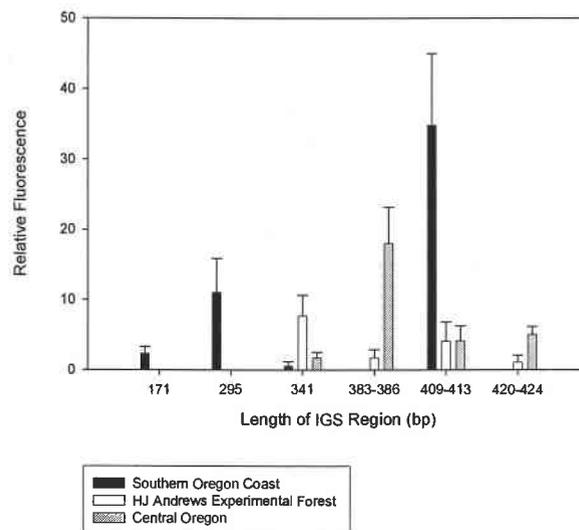


Fig. 6. Lengths of fragments, which contribute significantly to the separation of samples by geographic region according to Indicator Species Analysis ( $p \leq 0.003$ ).

The primers used may have amplified organisms in the soils other than just *Frankia* species. By using the BLAST program found on the website for the National Center for Biotechnology Information (NCBI) (Altschul 1990) sequences were found for the IGS region between the 16S and 23S rRNA genes which match with a known *Frankia* IGS region sequence. These sequences were then visually scanned to determine if the primers used would match at 7 bp or more. Table 2 contains *Actinomycetes* whose IGS regions could have been produced by our primers, all of which would have fragment lengths corresponding to lengths produced by the LH-PCR of the soil samples.

Table 2. *Actinomycetes*, which may have been amplified by IGS-F and IGS-R in soil samples.

Name of Organism	Length of IGS Region (bp)	NCBI Accession Numbers
<i>Rhodococcus erythropolis</i>	348	AF001265
<i>Nocardioides jensenii</i>	354	AF017491
<i>Micromonospora echinospora</i>	371	AF004005
<i>Micromonospora inositola</i>	376	AF004003
<i>Micromonospora halophytica</i>	389	AF004002
<i>Micromonospora carbonacea</i>	392	AF004001
<i>Micromonospora pallida</i>	393	AF004006
<i>Terrabacter tumescens</i>	397	AF017510
<i>Curtobacterium citreum</i>	403	AY191507
<i>Micromonospora olivasterospora</i>	408	AF004004

## Discussion

Using the IGS region from DNA extracted from nodules has proved to be a much easier and quicker method of studying *Frankia* than previous plant trapping methods (Nalin et al. 1999). Using *Frankia*-specific primers, rather than general 16S primers, was important because many *Frankia* strains in nodules belonging to the same host infection group exhibit identical rRNA sequences (Hönerlage et al. 1994). The IGS region is more variable, according to Hönerlage et al. (1994), than either the 16S or 23S rRNA regions. In addition, by using *Frankia*-specific primers, contaminating DNA from the host plant and possible contaminating DNA from bacteria not removed during surface sterilization of nodules were minimized (Rouvier et al. 1996).

This study showed that there is some variation in the length of the IGS region of *Frankia* strains infecting various actinorhizal hosts across the state of Oregon. There were no differences in the length of the IGS region between *Ceanothus* and *Purshia*, however, differences in the sequences would be expected (Jeong et al. 1999). These primers also did not distinguish any differences between the IGS region of nodules sampled from two different host species of *Ceanothus*. It is interesting that the *Myrica* sampled in this study contained a strain of *Frankia* with a fragment length identical to that of some *Alnus* found at the Southern Oregon Coast sites and the *Alnus* found at the H.J. Andrews Experimental Forest sites. This is not too surprising though because previous studies have suggested that perhaps *Myrica* is a

“promiscuous” host, citing its ability to nodulate with virtually all isolated strains of *Frankia* in greenhouse studies (Clawson and Benson 1999).

Although previous work has generally shown that the *Frankia* strain occurring in nodules seems to be host species dependent (Rouvier et al. 1996), it does not seem to be related to the populations found in the soil. It is also unknown whether one nodule contains only a single strain or if coinfection is possible (Reddel and Bowen 1985). Most of the nodules in this study seemed to have been infected by one strain. More samples would have to be included along with cloning and sequencing to have a more accurate picture of exactly how many strains are infecting one nodule. Additionally, these results show it may be possible for one host to be infected by different strains in different nodules. This is demonstrated in *Alnus* (1) and *Alnus* (2) from the Southern Oregon Site 1 (Table 1). Again, it would be necessary to have a larger data set to gain a more complete understanding.

Geographic region seems to play a more important role in determining the composition of the free-living *Frankia* community in the soil than the presence of an actinorrhizal host. This is supported by the work of Ritchie and Myrold (1999), where it was found that grouping of *Ceanothus*-infective *Frankia* followed a geographical pattern rather than the taxonomic lines of the *Ceanothus* host species. The lengths of the IGS regions amplified in the soil samples were not correlated to the lengths of the IGS regions from *Frankia* found in the nodules. Although the lengths and relative abundances of fragments varied among the three sites, there were six lengths associated with

each site that were statistically significant in contributing to the geographic separation of the samples (Fig. 6).

There was an abundance of data provided by the LH-PCR of the soil samples (Fig. 3). Because of the large amount of fragments generated, the specificity of the primers was further explored and it was determined that non-specific amplification may have taken place. Previous work has shown that primers as short as 7 bp can, under certain conditions, initiate amplification from the template-annealed 3' end of the primer (Rychlik 1995). Although it is not certain that the conditions used in this study would cause amplification to occur, it is a possibility.

It is important to remember that there is still relatively little known about *Frankia* strains and their symbiotic relationship with actinorhizal host plants. This study has shown that the IGS region can be a useful tool for studying some types of *Frankia* but that it cannot differentiate between the *Ceanothus* and *Purshia* and the two species of *Ceanothus* used in this study.

Future work using the IGS region should focus on cloning and sequencing of samples obtained from soil to determine if fragments generated by the LH-PCR are truly *Frankia* or if they are other members of *Actinomycetales*. In addition to cloning and sequencing soil samples, using T-RFLP's or cloning and sequencing would be an important way to determine differences between *Frankia* sampled from *Ceanothus* and *Purshia*.

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