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REGULAR ARTICLE

# *Frankia* bacteria in *Alnus rubra* forests: genetic diversity and determinants of assemblage structure

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**Abstract** To quantify the genetic diversity of *Frankia* bacteria associated with *Alnus rubra* in natural settings and to examine the relative importance of site age, management, and geographic location in structuring *Frankia* assemblages in *A. rubra* forests, root nodules from four *A. rubra* sites in the Pacific Northwest, USA were sampled. *Frankia* genetic diversity at each site was compared using sequence-based analyses of a 606 bp fragment of the *nif*H gene. At a 3% sequence similarity cutoff, a total of 5 *Frankia* genotypes were identified from 317 successfully sequenced nodules. Sites varied in the total number of genotypes present, but were typically dominated by only one or two genotypes. Phylogenetic analyses showed that all of the *A. rubra-Frankia* genotypes grouped with other *Alnus*-infective *Frankia*. Analysis of similarity (ANOSIM) and chi-square analyses indicated that *Frankia* assemblages were more strongly influenced by site age/management than geographic location. This study demonstrates that the *Frankia* assemblages in *A. rubra* forests have low genotype diversity, but that genotype abundance can differ significantly in forests of different age/management history.

**Keywords** *Frankia* · *Alnus* · Symbiosis · *nif*H · Site age · Management · Geography · Pacific Northwest

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## Introduction

Biological nitrogen fixation is widely considered to be one of earth's most important biotic processes (Zuberer 2005). Although the synthesis of modern fertilizers has lessened its importance in agricultural settings, biological nitrogen fixation continues to significantly influence natural systems, especially those where nitrogen is a primary limitation of plant productivity (Cleveland et al. 1999). In terrestrial environments, major sources of biological nitrogen fixation come from microbial symbioses with lichens (Nash 1996), mosses (Deluca et al. 2002), and

angiosperms (Graham 2005). When abundant, these symbioses can provide up to 20% of the annual nitrogen utilized by plant communities (Van der Heijden et al. 2008). Many studies have shown that biological nitrogen fixation has large effects on ecosystem productivity (Vogel and Gower 1998), plant diversity (Spehn et al. 2002), invasive species dynamics (Vitousek and Walker 1989) and patterns of vegetational succession (Chapin et al. 1994).

In temperate forests, the most widely studied nitrogen fixation symbiosis is that between *Frankia* and angiosperms (Benson and Silvester 1993). *Frankia* is a genus of Gram positive actinomycete bacteria that has a world-wide distribution and forms nodules on the roots of host plants from eight dicotyledonous families (Graham 2005). Phylogenetic analyses from multiple gene regions (e.g. 16s rRNA, *gln*, *nif*H) have consistently supported three major *Frankia* clusters that vary in host plant specificity, physiological properties, and symbiotic interactions (Jeong et al. 1999; Clawson et al. 2004; Benson and Dawson 2007). Several factors have been shown to influence the structure of *Frankia* assemblages, including host species richness (Huguet et al. 2001; Anderson et al. 2009), geographic location (Dai et al. 2004; Chen et al. 2008), elevation (Igual et al. 2006; Khan et al. 2007), and edaphic factors (Navarro et al. 1999; Huguet et al. 2004).

The genus *Alnus* is the dominant *Frankia* host in northern hemisphere temperate forests (Benson and Dawson 2007). Due to its nitrogen fixing symbiosis, *Alnus* plays an important role in the successional dynamics and nutrient cycling of many ecosystems in that region (Roy et al. 2007). For example, the presence of *Alnus* greatly increases the rate of soil development during primary stages of succession due to the annual deposition of large quantities of nutrient rich litter (Van Cleve et al. 1971). Although *Alnus* species may initially out compete co-occurring conifer species (Shainsky and Radosevich 1992), improved soil fertility can significantly increase long-term conifer growth in mixed stands (Miller and Murray 1978). Furthermore, the presence of *Alnus* has been shown to aid reforestation efforts in sites colonized by the fungal pathogen *Phellinus weirii* (Nelson et al. 1978) as well as to stimulate microbial lignin-cellulose degradation and mineralization of organic nitrogen, phosphorus, and sulfur compounds (Selmants et al. 2005).

Along the Pacific coast of the United States and Canada, *Alnus rubra* (Red Alder) is a common deciduous tree species found in disturbed, riparian, and geologically young areas (Hibbs et al. 1994). Previous research on the *A. rubra-Frankia* symbiosis has examined various aspects of nodulation potential (Markham and Chanway 1996; Martin et al. 2003a; Kennedy et al. 2010), seedling growth (Martin et al. 2003b; Markham 2008), spore type distribution (Markham and Chanway 1998) and nitrogen fixation (Markham and Chanway 1999; Rojas et al. 2002). Little work has, however, examined the diversity of *Frankia* genotypes in *A. rubra* forests using molecular methods, which typically capture greater genotypic diversity than culture and spore type characterization methods (Welsh et al. 2009a). In a recent study, Kennedy et al. (2010) found only two *Frankia* genotypes associated with *A. rubra* in Olympic National Rainforest, WA, USA. Due to the limited spatial scale of that study (0–10 km), it is unclear if this low level of *Frankia* genotypic diversity is representative on *A. rubra* across other parts of its geographic range. Since *Frankia* genotypes vary significantly in their ability to promote *A. rubra* growth (Dillon and Baker 1982; Prat 1989) more information about their distribution and abundance is important for understanding the ecology and management of *A. rubra* forests.

In this study, we addressed three questions: 1) How many *Frankia* genotypes are associated with *A. rubra* in natural settings, 2) How are *A. rubra*-infective *Frankia* phylogenetically related to other *Frankia* genotypes? and 3) How are *Frankia* assemblages on *A. rubra* influenced by site age/management and geographic location? To answer these questions, we sampled four *A. rubra* forests varying in age, management, and geographic location and compared *Frankia* genotypic diversity using sequence-based analyses of the *nif*H gene.

## Methods

### Site characteristics

Four sites in western Oregon, U.S.A. were chosen for sampling. Two of the sites are part of a long-term research project examining the effects of different forest management practices on *A. rubra* growth (see

the Hardwood Silvicultural Cooperative (HSC) website for details, http://www.cof.orst.edu/coops/hsc). The HSC sites in this study, Toledo (HSC 2208) and Thompson Cat (HSC 5203), were established in 1992. Prior to the HSC project, the sites were second-growth coniferous forests that were clear-cut the year before *A. rubra* monocultures were planted. Thompson Cat was burned as part of the site planting preparation, Toledo was not. *A. rubra* seedlings were planted from nursery stock (Brooks Tree Farm, Brooks, OR) during the beginning of their second year of growth. Seedling nodule status at the time of planting was not assessed, but nursery fumigation practices indicate nodulation was very unlikely (Brooks Nursery, pers. com.). Aside from site preparation and tree density (see below), no other forest management treatments were applied. The two HSC sites were located on the west side of the coast range and Cascade mountains, respectively. The third site, Fox Creek, was located in the Tillamook State Forest on the west side of the coast range mountains. This site was an unmanaged naturally regenerating mature *A. rubra* stand approximately 70 years in age (Tillamook Ranger District, pers. com.). The fourth site, Mt. Hood, was located in Mt. Hood National Forest in the western Cascade mountains. Like Fox Creek, this site was an unmanaged naturally regenerating approximately 60 year old stand (Clackamas Ranger District, pers. com.). Additional climate and soil details for each site are listed in Table 1.

## Sampling design

At each of the younger managed sites, *Frankia* nodules were sampled from two plots (each 1,225 $m^2$) varying in tree density (planted at 568 trees/ha and 2,967 trees/ha). Within each plot, 50 individual nodules (~1–10 g fresh weight) were removed from the upper 5 cm of soil at haphazardly scattered distances across the plot (all collections were >1 m apart). The nodules were separated from surrounding roots with forceps, placed in 20 ml scintillation vials, and kept on ice until they were processed in the laboratory. At the older unmanaged sites, two plots (~1,250 $m^2$ each) were each sampled for 50 nodules using the same methods. All sites were sampled between February and August 2008.

## Amplification, sequencing, and genotyping of *Frankia nif*H

Nodule DNA extraction, PCR, and sequencing methods were identical to those described in detail in Kennedy et al. (2010), so they are only briefly summarized here (see Appendix 1 for full description and experimental tests of sampling methodology). Total genomic DNA was extracted from individual root nodules using the DNeasy® Blood and Tissue Kit (Qiagen, Carlsbad, CA). A 606 bp *nifH* gene fragment was amplified with the *Frankia*-specific primers $nifHf_1$ and nifHr (Welsh et al. 2009a). For samples that initially did not amplify, PCR reactions were re-run at 1:20, 1:100, or 1:1000 dilutions of DNA extract. All successful PCR products were cleaned with ExoSAP IT (USB Corp., Cleveland, OH) and sent to the Genomic Analysis and Technology Core Facility, University of Arizona, USA, for sequencing on a 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA).

Sequence chromatograms were trimmed to 580 bp and manually corrected where necessary using

**Table 1** Geographic, soil, and climate information for the four study sites. Mean precipitation and average temperature data are based on the USDA Forest Service climate model (http://forest.moscowfsl.wsu.edu/climate/)

| Site | Location | Geographic region | Management status | Forest age (yr) | Soil series | Precipitation (mm) | Temperature (C) |
|---|---|---|---|---|---|---|---|
| Mt. Hood | N45′09.091″ W122′ 08.496″ | Cascade | Unmanaged | ~60 | Not available | 1973 | 9.0 |
| Fox Creek | N45′34.007″ W123′ 34.119″ | Coast Range | Unmanaged | ~70 | Alic-Hapludands complex | 2138 | 10.1 |
| Thompson Cat | N45′29.514″ W122′ 12.177″ | Cascade | Managed | 16 | Aschoff cobbly loam | 1983 | 9.7 |
| Toledo | N44′37.700″ W123′ 54.659″ | Coast Range | Managed | 16 | Toloyana-Reedsport complex | 2312 | 10.7 |

Sequencher 4.8 (Gene Codes Corp., Ann Arbor, MI). Sequences were grouped into operational taxonomic units (hereafter referred to as genotypes) using the furthest neighbor algorithm in MOTHUR (Schloss et al. 2009). Previous analyses of *Frankia nif*H assemblages have used a range of different sequence similarity cutoffs to determine genotypes (Welsh et al. 2009a, b; Mirza et al. 2009; Kennedy et al. 2010). For this dataset, 1%, 3%, and 5% cutoffs resulted 15, 5, and 3 genotypes, respectively. The cutoff of 3% was used in the analyses below because this level of similarity has been shown to place *Frankia* genotypes into their proper genomic groups (Mirza et al. 2009; Welsh et al. 2009a). Representatives of all non-identical sequences for each genotype were submitted to GenBank with the accession numbers GU810473-GU810477 and HM031938-HM031960. For all samples with identical sequences, the site locations of each sample is listed in Appendix 2.

## Phylogenetic analyses

Since *Alnus*-associated *Frankia* can associate with both *Alnus* and *Elaeagnus* hosts (Benson and Dawson 2007), a preliminary analysis was conducted to determine to which *Frankia* host cluster the *A. rubra* genotypes belonged. A second more detailed analysis was conducted to examine the relationships between the *A. rubra* genotypes and representatives of the five *Alnus* sub-groups designated by Welsh et al. (2009a). For that analysis, all non-identical sequences of each *A. rubra Frankia* genotype, three divergent representatives of each *Alnus* sub-group, and two additional sequences (one from a *Frankia* strain isolated from *A. rubra* and one from the genome sequence of *Frankia* ACN14a) were aligned using MUSCLE (Edgar 2004), along with an outgroup sequence from a *Datisca cannabina Frankia* nodule. Maximum likelihood (ML) and Bayesian analyses were conducted using PhyML 3.0 (Guindon and Gascuel 2003) and Mr. Bayes v3.1.2 (Ronquist and Huelsenbeck 2003), respectively. Modeltest (Posada and Crandell 1998) using the Akaike Information Criterion determined that GTR + I + G model of sequence evolution fit the data. ML analyses were conducted on the Montpellier bioinformatics platform (http://www.atgc-montpellier.fr/phyml/) using the following settings: substitution model GTR, proportion of invariable sites fixed, gamma parameter estimated, number of substitution categories = 4, starting tree BIONJ, type of tree improvement NNI, optimized topology and branch lengths, and SH-like aLRT scores. The Bayesian analyses each consisted of two simultaneous runs, each with four Markov Chain Monte Carlo chains. The default cold and heated chain parameters were used with a tree saved every 100 generations. The analyses were run until the average standard deviation of split frequencies was <0.01. The consensus tree was constructed following a visually determined burn-in of 10%.

**Table 2** A) Distribution of the five *Frankia*genotypes across the four study sites. B) Number of successfully identified nodules, observed genotype richness, Jack2 estimated genotype richness (mean ±1s.d.), Simpson's D diversity, and Camargo's E evenness per site

| A) Site | Genotype | | | | |
|---|---|---|---|---|---|
| | KL1 | KL2 | KL3 | KL4 | KL5 |
| Fox Creek | 28 | 42 | 0 | 1 | 0 |
| Mount Hood | 32 | 57 | 0 | 0 | 1 |
| Toledo | 84 | 2 | 2 | 1 | 0 |
| Thompson Cat | 50 | 9 | 6 | 1 | 1 |
| B) | Nodule # | Genotypes | Jack2 | Simpson's D | Camargo's E |
| Fox Creek | 71 | 3 | 3.25 (1.40) | 0.49 | 0.62 |
| Mt Hood | 91 | 3 | 3.20 (1.39) | 0.48 | 0.59 |
| Toledo | 89 | 4 | 4.38 (2.18) | 0.11 | 0.30 |
| Thompson Cat | 66 | 5 | 5.18 (1.95) | 0.42 | 0.37 |

Statistical analyses

To estimate *Frankia* genotype richness at each site, we used EstimateS (Colwell 2005). The observed number of taxa is often an inaccurate estimator of true richness (Colwell and Coddington 1994), so the Jack2 estimator was chosen based on the recommendation of Brose et al. (2003). Estimates were based on 500 randomizations of sample order with replacement. Site genotype diversity (Simpson's D) and evenness (Camargo's E) indices were calculated as described in Krebs (1999). *Frankia* assemblages were compared among sites in PRIMER v5 (Clarke fand Warwick 2001). A standardized genotype abundance matrix was created using each plot at each site, with values being square root transformed to down weight the importance of highly abundant genotypes. From that abundance matrix, a Bray-Curtis similarity matrix was calculated and a non-metric multi-dimensional scaling (MDS) plot was used to visually examine similarities among plots (Clarke and Warwick 2001). Two one-way analyses of similarity (ANOSIM) were used to test for differences in assemblage structure based on site age/management (young/managed vs. old/unmanaged) and geographic location (coastal vs. Cascade). Differences in abundance based on site age/management and geographic location for the three most common genotypes were also determined using Yates corrected $\chi^2$ goodness-of-fit tests. To account for multiple tests, significance levels were

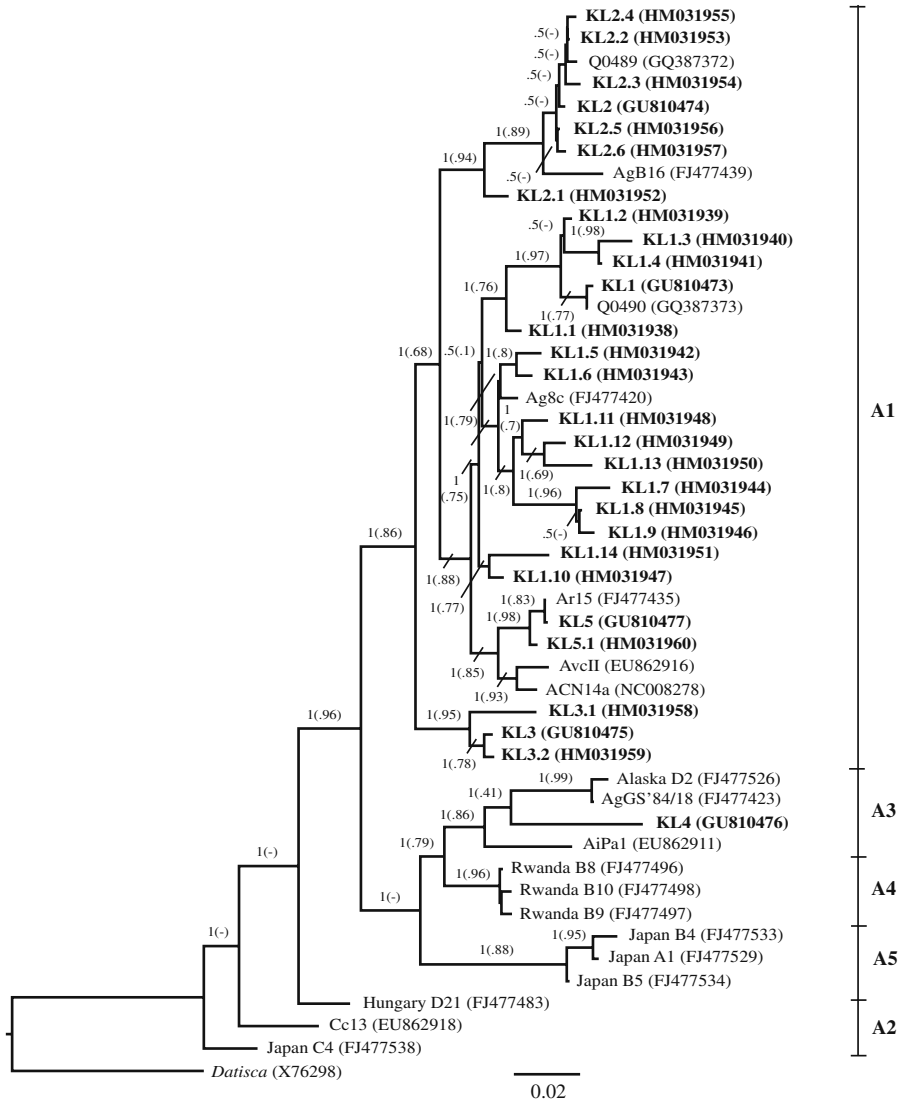


**Fig. 1** Phylogenetic reconstruction of 48 *Frankia* taxa based on 518 bp of the *nif*H gene. Non-identical sequences of each KL genotype encountered in this study are present in bold. Other sequences include multiple representatives of the five *Frankia Alnus* sub-groups designated by Welsh et al. (2009a). The different *Alnus* sub-groups are designated on the right. Nodes are labeled with posterior probabilities from Bayesian analysis and, in parentheses, aLRT scores from Maximum Likelihood (ML) analysis. Dashes indicates that branch was not present in the ML analysis. Taxa are labeled by strain or genotype name, with GenBank accession number in parentheses. A *Frankia* sequence from *Datisca cannabina* was designated as the outgroup following Welsh et al. (2009a)

adjusted to $P$=0.025 for the ANOSIM and $P$=0.0085 for the $\chi^2$ tests using the Dunn-Sidak method (Sokal and Rohlf 1995).

## Results

From the 400 nodules sampled, 317 (79%) were successfully identified. A total of five *Frankia* genotypes were identified across all sites, hereafter designated as KL1-KL5 (Table 2). Jack2 richness estimates indicated that our sampling likely captured the full genetic diversity present at the four sites. All sites had similar Simpson's diversity values except for Toledo, which was dominated by *Frankia* genotype KL1 (Table 2). Camargo's E evenness values were consistently lower at the younger/managed than the other older/unmanaged sites. Genotypes KL1 and KL2 were by far the most abundant, representing 96% (304/317) of all the nodules identified. The dominant two genotypes were present at all four sites, while the other three genotypes were present at two or three sites, respectively (Table 2).

Maximum likelihood and Bayesian phylogenetic trees had similar overall topologies with high aLRT scores and posterior probabilities, respectively (Fig. 1, Figure S1). The host cluster analysis clearly indicated that all of the genotypes encountered in this study belonged to the *Alnus* host cluster (Figure S1). Within that cluster, all non-identical sequences from each of the five *A. rubra Frankia* genotypes formed monophyletic clades (Fig. 1). The genotypes belonged to two of the five *Alnus* subgroups designated by Welsh et al. (2009a), with KL1, KL2, KL3, and KL5 belonging to sub-group 1 and KL4 belonging to sub-group 3. All of the KL genotype clades contained *Frankia* genotypes or strains from previous studies except for KL3. The dominant genotypes encountered in Kennedy et al. (2010), Q0489 and Q0490, were closely related to sequences from KL2 and KL1, respectively (Fig. 1).

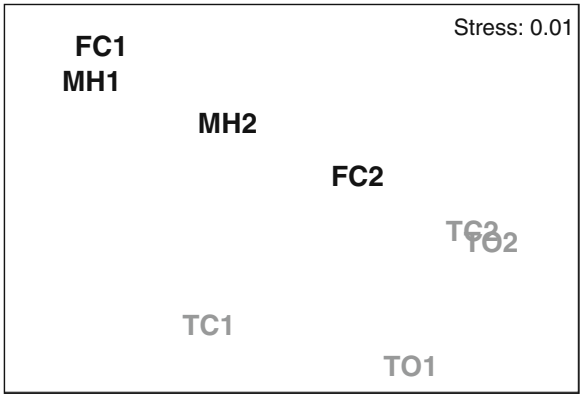


**Fig. 2** Non-metric multidimensional scaling (MDS) diagram of the plots at each of the five sites. Plots located closer together have greater similarity in assemblage structure than those located further apart. Site names are abbreviated as: FC = Fox Creek, MH = Mount Hood, TO = Toledo, TC = Thompson Cat. Letter color corresponds with age/management status: younger/managed (*gray*), older/managed (*black*). TO1 and TC2 were planted at low density (568 trees/ha); TO2 and TC1 were planted at high density (2,967 trees /ha)

The MDS plot of all the *Frankia* assemblages had a low stress score indicating good two-dimensional representation (Fig. 2). While there was considerable within-site variation, the *Frankia* assemblages generally grouped more closely based on site age/management than geographic location (Fig. 2). The ANOSIM analyses also suggested that site age/management better explained the observed assemblage structure than geographic location (age/management: $r$=0.563, $p$=0.057; location: $r$=−0.094, $p$=0.60), although neither factor had a statistically significant effect. For the younger/managed sites, tree density had no clear effect, as evidenced by the high assemblage similarity between the low and high density plots at Toledo and Thompson Cat, respectively (Fig. 2). At the individual genotype level, however, there were significant differences in abundance based on site age/management and geographic location. The two most abundant genotypes, KL1 and KL2, differed significantly by site age/management but not by geographic location ($\chi^2$ tests, $P$<0.005). Genotype KL3 was only encountered at the younger managed sites, but due to its low overall abundance, differences based on both site age/management and geographic location were not statistically significant ($\chi^2$ tests, $P$>0.01). KL4 and KL5 were not tested due to low abundance.

## Discussion

We found that *A. rubra* associates with a relatively limited number of *Frankia* genotypes in natural settings. The two dominant genotypes in this study were the same as those encountered in Kennedy et al. (2010), indicating that *A. rubra* associates with the same *Frankia* genotypes throughout much of its geographic range. The total number of *Frankia* genotypes observed in this study was also similar to that encountered on other *Alnus* hosts using *nif* based-

analyses in both Alaska and Arizona (Anderson et al. 2009; Welsh et al. 2009b). Reasons why natural *Frankia* assemblages seem to have limited genetic diversity are not clear, but may reflect selective pressure by the host to maintain only beneficial genotypes (Kiers et al. 2003). Although *Alnus* can host *Frankia* genotypes from both the *Alnus*-infective and *Elaeagnus*-infective clusters (Benson and Dawson 2007), all the genotypes associated with *A. rubra* belonged to the *Alnus*-infective cluster (Figure S1). Within the *Alnus* cluster, most of the *A. rubra* genotypes encountered in this study were similar to *Frankia* identified in other studies. KL3, however, appeared to represent a novel *Frankia* lineage based on the absence of related sequences in Fig. 1. KL5 was the only genotype that clustered closely with strains previously isolated from *A. rubra*, suggesting the majority of *A. rubra* associated-*Frankia* likely also colonize other *Alnus* hosts. While greater sampling is needed to make a conclusive generalization, our data indicate that *A. rubra Frankia* assemblages have low genetic (i.e. the number of genotypes) and phylogenetic (i.e. the number of *Alnus* sub-group lineages) diversity.

The MDS and ANOSIM analyses indicated that site age/management had a much greater effect on *Frankia* assemblage structure than geographic location. Given the confounded sampling design of this study (site age and management are not independent) we were unable to determine which factor is more significant in driving the observed pattern. In a previous study of the same sites, however, we found that *A. rubra* ectomycorrhizal fungal assemblages were also more significantly influenced by site age/management than geographic location (Kennedy and Hill 2010). This general response indicates younger managed and older unmanaged stands harbor different microbial communities, which may be specifically adapted to their differing environmental conditions (Martin et al. 2003a). A similar age-related result was reported by Markham and Chanway (1998), who found no spore + *Frankia* genotypes present in young *A. rubra* forests. Interestingly, the two most abundant genotypes in this study, KL1 and KL2, showed contrasting patterns of abundance in the younger/managed and older/unmanaged forests. Additional research examining the symbiotic performance of these two genotypes would be useful in determining if they are better adapted to promoting younger versus older *Alnus* growth, respectively.

The general absence of a geographic location effect was surprising given that many studies have found *Frankia* composition and abundance often varies along temperature/moisture gradients (Igual et al. 2006; Chen et al. 2008; Welsh et al. 2009b). Previously, Markham and Chanway (1998) found that the distribution of *Frankia* spore types on *A. rubra* was significantly influenced by geographic location, with no spore + types encountered in the submaritime climatic zone. The coastal and Cascade sites in this study did differ in mean annual precipitation and temperature, with the coastal sites being, on average, both wetter and warmer (Table 1). These differences, however, are relatively small and may not be enough to significantly influence the structure of entire *Frankia* assemblages. The geographic range of *A. rubra* is almost completely restricted to coastal western North America, however, there are a very small number of inland populations in Idaho, USA (Johnson 1995). To better understand the effects of abiotic conditions on *A. rubra*-associated *Frankia*, sampling those disjunct Idaho populations could be particularly helpful, as they experience a much drier and more seasonally extreme climate compared with the four locations studied here.

In summary, this study indicates that *A. rubra* associates with a limited array of *Frankia* genotypes in natural settings and that forest age/management appears to be a more significant factor determining assemblage structure than geographic location. Future research examining the effectiveness of different *nif*H genotypes in promoting host growth will help in determining how genetic and functional diversity are related. Similarly, studies relating data based on molecular identification with that based on spore-type (e.g. Markham and Chanway 1998) will help in connecting past and future research of *A. rubra-Frankia* interactions. Finally, additional experiments looking at factors such as genotype competition (Martin et al. 2003b) and specific abiotic factors (e.g. soil moisture, pH) will aid in further understanding the processes driving the patterns of *Frankia* distribution in *A. rubra* forests.

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## Appendix 1

Molecular analyses of *Frankia* nodules and experimental tests of sampling methodology

*Amplification and sequencing of Frankia nifH*

Within 48 h of field collection, individual nodules were surface sterilized by manual agitation in a 10% bleach solution for 2 min. The nodules were then rinsed three times with deionized water and stored at −20°C prior to DNA extraction. To extract total genomic DNA, 1–2 lobes from single individual nodule samples were macerated in 180 μL of buffer ATL from the Qiagen DNA Tissue 200 Kit (Qiagen, Carlsbad, CA). The periderm of each nodule sample was not removed prior to maceration (see below for justification). One hundred eighty μL of 20 mg/mL lysozyme was added to the homogenized tissue solution and incubated at 37°C for 30 min, as recommended for Gram-positive bacteria. The remainder of the DNA extraction was performed according to the manufacturer's instructions.

The polymerase chain reaction (PCR) was used to amplify a 606 bp fragment of the *nif*H gene with the *Frankia*-specific primer pair nifHf1 (5′-GGC AAG TCC ACC ACC CAG C-3′) and *nif*Hr (5′-CTC GAT GAC CGT CAT CCG GC-3′). This region was chosen because it has previously been demonstrated to differentiate among closely related *Frankia* genotypes occurring on the same host plant species (Welsh et al 2009a; Mirza et al. 2009; Kennedy et al. 2010). PCR amplifications were performed in 20 μL reactions containing: 0.5 μL bulk DNA, 0.4 μL of each primer (10 μM), 10 μL MasterAmp F PCR buffer (Epicenter, Madison, WI) and 0.75 U Taq Polymerase (New England Biolabs, Ipswich, MA). Samples that did not successfully amplify initially were re-run using 1:20 dilutions of the DNA template. PCR cycling conditions were as follows: 96°C for 5 min; 35 cycles at 96°C for 30 s, 64°C for 30 s, and 72°C for 45 s; and a final 7 min 72°C extension. Amplification was checked with electrophoresis on 1.5% agarose gels (GenePure LE, ISC BioExpress, Kaysville, UT). Gels were stained with ethidium bromide and visualized under UV fluorescence. All successful PCR products were cleaned using 1.5 uL of ExoSAP IT (USB Corp., Cleveland, OH) with 7.5 uL of DNA and incubated at 37°C for 45 min, followed by 80°C for 15 min. Sequencing was performed on a 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA) at the Genomic Analysis and Technology Core Facility at the University of Arizona, USA.

*Experimental tests of sampling methodology*

To ensure our results were not biased by sampling methodology, two other analyses were performed. Previous *Frankia* studies on other hosts have found multiple strains per nodule (Reddell and Bowen 1985), so we tested whether multiple samples (i.e. different lobes) from the same nodule belonged to the same genotype. Five nodules were collected from multiple *A. rubra* individuals at Tryon Creek State Park in Portland, Oregon (N 45′27.139″, W 122′ 40.153″). Four lobes per nodule were removed and independently identified using the same methods described above. In all cases, the same genotype was recovered from the individual lobes of each nodule, indicating our nodule sampling method accurately captured *Frankia* genotype nodule richness. We also compared the interior versus periderm tissues of nine nodules from the same location to determine if periderm removal influenced genotype richness. In eight of the nine nodules, the genotype composition was identical between tissues. For the one nodule with non-matching genotypes, we believe this result was most likely caused by incomplete surface sterilization. Both of these analyses make us confident that the nodule sampling methodology did not significantly influence the observed patterns of genotype diversity.

## References

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## Appendix 2

Site information for samples from which a clear *nif*H sequence was obtained. Samples sharing the same GenBank accession number were identical across the 518 base pairs analyzed. Site abbreviations are as follows: FC = Fox Creek, M = Mt. Hood, T = Toledo, TC = Thompson Cat.

| Genotype | Site | Sample | GenBank Accession # |
|---|---|---|---|
| KL1 | FC | 3 | GU810473 |
| KL1 | FC | 26 | GU810473 |
| KL1 | FC | 28 | GU810473 |
| KL1 | FC | 32 | GU810473 |
| KL1 | FC | 42 | GU810473 |
| KL1 | FC | 49 | GU810473 |
| KL1 | FC | 55 | GU810473 |
| KL1 | FC | 62 | GU810473 |
| KL1 | FC | 66 | GU810473 |
| KL1 | FC | 67 | GU810473 |
| KL1 | FC | 68 | GU810473 |
| KL1 | FC | 76 | GU810473 |
| KL1 | FC | 77 | GU810473 |
| KL1 | FC | 78 | GU810473 |
| KL1 | FC | 81 | GU810473 |
| KL1 | FC | 82 | GU810473 |
| KL1 | FC | 89 | GU810473 |
| KL1 | FC | 92 | GU810473 |
| KL1 | FC | 94 | GU810473 |
| KL1 | FC | 95 | GU810473 |
| KL1 | FC | 96 | GU810473 |
| KL1 | FC | 98 | GU810473 |
| KL1 | FC | 99 | GU810473 |
| KL1 | FC | 100 | GU810473 |
| KL1 | FC | 101 | GU810473 |
| KL1 | T | 1 | GU810473 |
| KL1 | T | 2 | GU810473 |
| KL1 | T | 3 | GU810473 |
| KL1 | T | 5 | GU810473 |
| KL1 | T | 6 | GU810473 |
| KL1 | T | 7 | GU810473 |
| KL1 | T | 8 | GU810473 |
| KL1 | T | 9 | GU810473 |
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| KL2.2 | M | 54 | HM031953 |
| KL2.2 | M | 26 | HM031953 |
| KL2.3 | TC | 99 | HM031954 |
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| KL2.3 | M | 21 | HM031954 |
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| KL2.5 | M | 22 | HM031956 |
| KL2.5 | TC | 16 | HM031956 |
| KL2.6 | FC | 14 | HM031957 |
| KL3 | T | 4 | GU810475 |
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| KL3 | TC | 42 | GU810475 |
| KL3.1 | TC | 12 | HM031958 |
| KL3.1 | T | 50 | HM031958 |
| KL3.2 | TC | 46 | HM031959 |
| KL4 | TC | 98 | GU810476 |
| KL4 | T | 76 | GU810476 |
| KL4 | FC | 52 | GU810476 |
| KL5 | M | 24 | GU810477 |
| KL5.1 | TC | 36 | HM031959 |

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