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Genotypic Diversity and Host-Specificity of Frankia Bacteria Associated with Sympatric Populations of *Alnus rubra* and *Alnus rhombifolia* in Oregon

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Genotypic Diversity and Host-Specificity of *Frankia* Bacteria Associated with Sympatric Populations of *Alnus rubra* and *Alnus rhombifolia* in Oregon

Abstract

Biological nitrogen fixation is one of the most critical processes contributing to ecosystem productivity and stability on a global scale. In temperate climates of the northern hemisphere, plant-root associated bacteria of the genus *Frankia* are the major nitrogen fixers in forest environments. Trees belonging to the genus *Alnus* are the most widespread hosts of *Frankia* in the Pacific Northwest, and a myriad of biotic and abiotic factors can influence the robustness of this symbiosis. Host identity and bacterial strain are important features that can impact *Alnus-Frankia* association, but little is known about the interplay of intrageneric hosts that co-occur in natural settings. In this study we investigated the genetic diversity and host specificity of *Frankia* bacteria associated with sympatrically occurring populations of *Alnus rubra* (red alder) and *Alnus rhombifolia* (white alder) in Oregon. Based on sequence analysis of the *nifH* gene recovered from root nodules we found low overall bacterial diversity. One dominant *Frankia* genotype was associated with both host species, indicating a lack of strong host specificity in this system. Our results suggest that certain intrageneric plant hosts with overlapping distributions show cross-compatibility with symbiotic actinorhizal bacteria, and that low strain diversity of these bacteria can persist across mixed host populations.

Keywords: plant-microbe interactions, genotype, nitrogen-fixation, red alder, white alder.

Introduction

Nitrogen-fixing bacteria associated with plant roots are one of the primary sources of biological nitrogen inputs in terrestrial ecosystems. In temperate climates, bacteria of the genus *Frankia* are the major group that forms such symbioses with 24 actinorhizal angiosperm genera worldwide (Schwintzer and Tjepkema 1990, Benson and Silvester 1993). Members of the genus *Alnus* (alders) are the only known trees that form associations with *Frankia* bacteria, and *Alnus rubra* Bong. (red alder) is one of the most common tree species in the northwest (Franklin and Dyrness 1973). Accordingly, *A. rubra* represents a critical host for actinorhizal bacteria in the Pacific Northwest owing to its widespread distribution and relatively long lifespan compared to non-tree actinorhizal hosts in this region. Alders are pioneer tree species that can not only enhance soil nitrogen content,

but can also lower soil pH (Lawrence 1958), significantly increase soil organic matter (Tarrant and Miller 1963, Franklin et al. 1968, Bormann and DeBell 1981, Binkley et al. 1982, Binkley 1983), and improve the retention of important anions such as PO_4^{3-} and SO_4^{2-} (Johnson et al. 1986). Furthermore, the soil enhancing capacity of *Frankia*-associated *Alnus* species can also benefit neighboring trees of economic importance such as *Pseudotsuga menziesii* (Mirb.) Franco (Douglas-fir) (Miller and Murray 1978) and *Pinus* species (Dai et al. 2004), with which they are sometimes interplanted for this purpose.

The performance of the actinorhizal symbiosis can vary significantly depending on host plant-bacterial genotype combinations (Wheeler et al. 1986, Weber et al. 1987). While a number of different factors can influence the genotypic diversity of *Frankia* including edaphic (Sheppard et al. 1989, Crannell et al. 1994, Nickel et al. 1999) and biogeographic (Khan et al. 2007, Pölme et al. 2014) factors, the effect of host identity is of particular interest and has been widely studied (van Dijk et

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al. 1988, Rouvier et al. 1996, Navarro et al. 1999, Vanden Heuvel et al. 2004, Hugué et al. 2004, Lipus and Kennedy 2011, Pölme et al. 2014). Four major host infection groups have been identified for *Frankia* based on their ability to form nodules within actinorhizal plant families (Baker 1987), though it has since become clear that strains do not always remain within the boundaries of this host-specific classification (Torrey 1990). Much of the work examining *Frankia* host specificity in *Alnus* has focused on strain compatibility across allopatric populations of different (Anderson et al. 2009, Lipus and Kennedy 2011, Pölme et al. 2014) or the same (Welsh et al. 2009) host species. Few studies have investigated genotypic diversity and host specificity of the bacterial symbiont in sympatric populations of different *Alnus* species (Anderson et al. 2009, Pokharel et al. 2011), beckoning the questions of how proximity, shared soil environment, and exposure to the same *Frankia* strain pool act to influence host colonization and specificity. Pokharel et al. (2011) conducted a comprehensive assessment of *Frankia* associated with 12 *Alnus* taxa—most of which do not typically co-occur in their natural habitats—growing sympatrically in an arboretum setting. Using Rep-PCR (repetitive element polymerase chain reaction) fingerprinting, Pokharel and colleagues (2011) generated genetic strain profiles for nodules collected from multiple individuals of each taxon. Strikingly, only three distinct strain groupings were observed, with *Frankia* associated with nine of the *Alnus* taxa comprising a single strain (I), associates of two other taxa comprising a second strain (II), and a single taxon associate comprising the third group (III), suggesting low genetic diversity and high intrageneric host cross-compatibility. In contrast, Anderson et al. (2009) observed a total of nine unique RFLP (restriction fragment length polymorphism) profiles generated from *Frankia* nodules from *A. viridis* and *A. tenuifolia* populations growing sympatrically in interior Alaska, three of which occurred on both hosts and three of each of the remaining six profiles occurring on either host. Interestingly, the most abundant genotypes in each of three sites were consistently those that were unique to each host suggesting a greater degree of host specificity for those profiles.

Given such mixed results, the paucity of more accurate and replicable molecular approaches (e.g., sequence-based genotyping), and the underrepresentation of some *Alnus* species in previous studies, there is a need to further investigate the diversity and host specificity of actinobacteria among sympatrically distributed populations of alders in natural settings. Of the four *Alnus* species that are native to the Pacific Northwest (including northern California), red alder (*Alnus rubra*) and white alder (*Alnus rhombifolia* Nutt.) are the most common (Little 1976) and have overlap in their distribution. Here we assessed the genetic diversity and host specificity of *Frankia* bacteria associated with the roots of sympatric populations of *A. rubra* and *A. rhombifolia* in western Oregon.

Methods

Study Area

Nodule-bearing roots were collected from both red and white alder from a site in Sherwood, western Oregon (Heaton Creek, Figure 1). Alder species were identified considering a set of traits including variation in leaf shape, flowers, and extent and speed of oxidation processes resulting in red coloration of scratched inner tree bark. Red alder periderm (in line with its common name) shows a faster and much more intense coloration in response to physical damage compared to white alder. In total, 28 white alder and 30 red alder samples were collected, with each sample originating from a different tree. Trees had diameters at breast height between 10 and 25 cm. Both species of trees showed similar variation in diameter (red alder: 16.4 ± 4.0 , white alder: 15.9 ± 4.7 [mean \pm SD]). Nodule bearing roots were collected from the upper soil level (15 cm) at a radius of 50 cm around each tree. Nodules were only collected from larger roots (> 1 cm in diameter) easily trackable to the specific sample tree or from finer roots that were clearly attached to these larger roots. After collection, the roots with nodules were stored in sealed zip lock bags and transported to the lab in a cooler (4 °C). Nodules were processed within 48 hours.

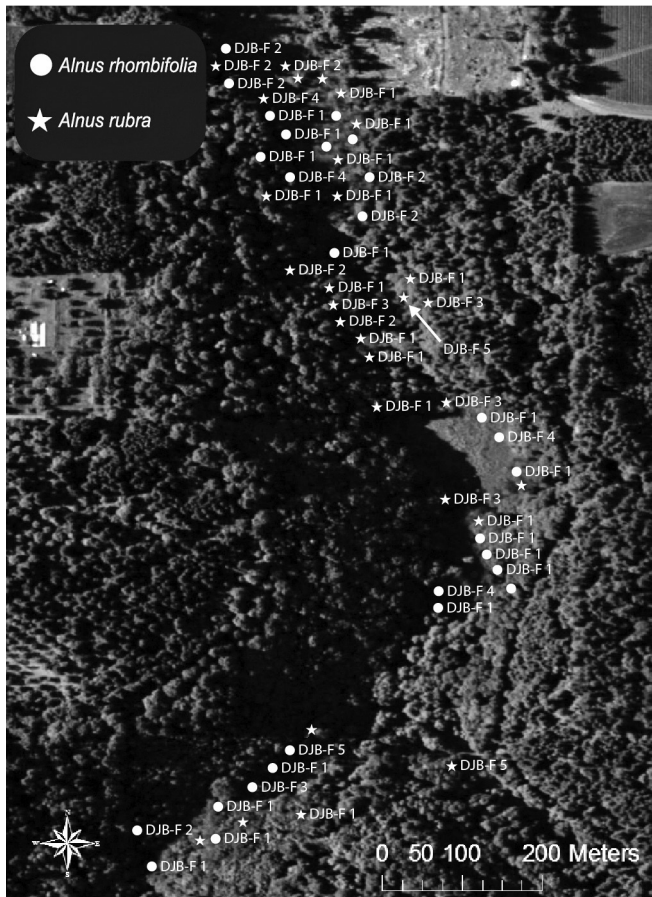


Figure 1. Map of collection site in western Oregon displaying locations of individual *Alnus rubra* and *A. rhombifolia* trees. *Alnus rubra* trees are labelled with star icons, *A. rhombifolia* trees with circles. Information on the associated *Frankia* genotype recovered from root nodules is provided for each tree. Icons without corresponding labels indicate trees from which *nifH* sequences were not obtained.

Surface Sterilization

Roots and attached nodules were washed with deionized water to remove soil and organic matter. Nodules were then removed from roots with a sterile scalpel and placed into individual 14 mL sterile scintillation vials. Each vial was then filled with 10 mL of 10% bleach (0.6% hypochlorite) solution and agitated in an orbital shaker at room temperature (21 °C) for 2 minutes at 140 rpm. After agitation, the bleach solution was decanted from each vial and the bleach washing process was

repeated once more. After the second bleach wash, the washing process was repeated three more times with deionized water instead of bleach. After surface sterilization, a single nodule lobe of each sample was removed with a sterile scalpel. All single lobes were then transferred to 1.5 mL microcentrifuge tubes for DNA extraction using a sterile toothpick.

DNA Extraction

DNA was extracted from individual nodule lobes using the Sigma Tissue Extract-N-Amp Kit (Sigma-Aldrich, St. Louis, MO). Forty μL of extraction buffer were added into each 1.5 mL microcentrifuge tube containing an individual nodule lobe, which was then homogenized using a sterile micropestle. Lobe homogenates were centrifuged for 1 minute at 15,000 rpm (21,130 \times g), and 20 μL of supernatant from each tube transferred into an individual 0.2 mL PCR strip-tube. Strip-tubes containing extractions were placed in a thermal cycler and incubated at 65 °C for 10 minutes, followed by 95 °C for 10 minutes. Following incubation, 25 μL of neutralization buffer were pipetted into each sample which were then briefly vortexed and stored at 4 °C until use.

PCR and Sequencing

A 606 bp (base pair) portion of the *nifH* gene was amplified by PCR using *Frankia*-specific primers *nifH*f1 (5'-GGCAAG TCC ACC ACC CAG C-3') and *nifH*r (5'-CTC GAT GAC CGT CAT CCG GC-3'). PCR reactions were set up in 24 μL volumes containing 8.45 μL PCR water, 12.5 μL GoTaq Master Mix, 1.25 μL BSA (1 mg/1 mL), 0.4 μL of each primer (10 μM), and 1 μL of 1:10 diluted template. Reaction mixtures were then subjected to the following thermal cycling conditions: 96 °C for 5 minutes, 35 cycles at 95 °C for 30 seconds, 60 °C for 30 seconds, 72 °C for 45 seconds, and

a final extension at 72 °C for 7 minutes. Following PCR, all reaction products were visualized by gel electrophoresis. Successful PCR reactions were cleaned up using ExoSAP IT (USB Corp., Cleveland, OH) according to the manufacturer's instructions. Sanger Sequencing was performed in both directions using the same primers used in PCR on an ABI 3730xl (Applied Biosystems, Foster City, CA) at Functional Biosystems, Inc. (Madison, WI).

Sequence Processing

Sequence data were visually inspected for quality, trimmed to 615 bp, and contigs generated from forward and reverse reads for each sample in Geneious version 10.2.3 (Kearse et al. 2012). Operational taxonomic units (OTUs) were generated by clustering contigs with 97% sequence similarity. This similarity cutoff was chosen because previous work has shown that *Frankia* genotypes are accurately placed in appropriate genomic groups at this threshold (Welsh et al. 2009, Mirza et al. 2009, Lipus and Kennedy 2011, Higgins and Kennedy 2012, Rodriguez et al. 2016). Representative sequences for each OTU group (i.e., genotype) were submitted to Genbank under the accession numbers MK105601-MK105605. The OTUs were then aligned using MAFFT version 7.309 (Katoh and Standley 2013). The *nifH* sequence of *Frankia caesusarum* strain Ccl3 (Normand et al. 2007) was downloaded from GenBank and included in the alignment as an outgroup. The ends of the alignments were trimmed to ensure each sequence was of the same length.

Phylogenetic Analysis and Statistics

To determine the optimal nucleotide substitution model for use in phylogenetic analysis, the alignment of OTUs created from both *Alnus* spp. at the 97% similarity threshold was analyzed using PartitionFinder version 2.1.1 (Lanfear et al. 2016). The entire alignment was treated as a single data block, "models" and "schemes" were set to all, and model selection was done based on the corrected Akaike Information Criterion. This analysis indicated that a general time reversible model with an estimation of invariant sites (GTR+I)

was the best fit, and this model was used in both maximum likelihood and Bayesian tree building.

Maximum likelihood trees were built in Geneious using RAxML version 8.2.11 (Stamatakis 2014) with the "GTR CAT I" model setting and the Rapid Bootstrapping algorithm; 10,000 bootstrap replicates were performed, and the consensus trees were built with a 25% bootstrap support minimum. Bayesian trees were built using BEAST version 1.8.4 (Drummond et al. 2012) with the GTR+I nucleotide model. A strict clock model was used, along with a coalescent constant-population tree prior. The program was run with chain lengths of 10,000,000 states, with sampling every 1,000 states. Maximum clade credibility trees were generated in Tree Annotator with a 10% burn-in.

The software EstimateS 9.1.0 (Colwell 2013) was used to estimate the true richness of *Frankia* genotypes associated with *A. rubra* and *A. rhombifolia*. The Chao2 estimator was computed on the basis of 500 randomizations of sample order without replacement. To assess whether the host identity significantly influenced the co-occurrence of the two most frequently observed *Frankia* genotypes, Fisher's Exact Probability Test was performed.

Results

nifH Sequence Analysis

We sequenced a portion of the *nifH* gene from *Frankia* nodules associated with the roots of 28 *Alnus rhombifolia* and 30 *Alnus rubra* individuals in order to assess the genetic diversity and level of host specificity of these actinobacteria in sympatric populations of their hosts. Of the 58 nodules processed we successfully generated *nifH* sequences for 47, which were comprised of five OTUs (herein referred to as genotypes) at 97% sequence similarity across both tree species. Sequences derived from both *A. rubra* and *A. rhombifolia* associated nodules clustered into each of five genotypes at varying proportions with *A. rubra* derived sequences outnumbering *A. rhombifolia* derived sequences in four out of the five genotype groupings (Table 1). The calculated Chao2 richness estimator was identical to the observed richness of

TABLE 1. Abundance of *Frankia* genotypes associated with host *Alnus* species.

Host Species	<i>Frankia</i> Genotype				
	DJB-F1 ¹	DJB-F2	DJB-F3	DJB-F4	DJB-F5 ²
<i>Alnus rubra</i>	12	3	5	1	2
<i>Alnus rhombifolia</i>	13	6	1	3	1

¹ Identical to genotype KL2 (GenBank accession GU810474.1) described in Kennedy et al. 2010.

² Identical to genotype KL1.16 (GenBank accession GU810479.1) described in Kennedy et al. 2010.

five *Frankia* genotypes shared by the two *Alnus* populations, illustrating that sampling of nodules was adequate to represent true genotypic richness. More than 50% of sequences derived from both *Alnus* host species clustered into one dominant genotype (DJB-F1), which was associated with trees that were heterogeneously distributed across the site (Figure 1). Similarly, trees associated with the other four genotypes (DJB-F2–DJB-F5) also exhibited heterogeneous spatial distribution. Upon comparing the *Alnus*-associated *Frankia* genotypes in our study to those characterized in other studies conducted in our region (Kennedy et al. 2010, Lipus and Kennedy 2011), we discovered that two of our genotypes (DJB-F1 and DJB-F5) were identical to two described previously (KL2 and KL1.6 respectively). The results of the Fisher's Exact Probability Test ($P = 0.618$) indicate that no systematic association exists between *Alnus* species and occurrence of *Frankia* genotype in at this site.

Phylogenetic Analysis

Both maximum likelihood and Bayesian phylogenetic analyses of the five *Frankia* genotypes recovered from *A. rubra* and *A. rhombifolia* yielded trees with identical topologies (Figure 2). The genotypes are clustered into two major clades, with one clade consisting of DJB-F3, and the other consisting of two monophyletic subclades containing two of each of the remaining four genotypes (DJB-F1, DJB-F5; and DJB-F2, DJB-F4, respectively). Relatively high bootstrap support values and posterior probabilities support the stability of these groupings.

Discussion

The goal of the current study was to assess genetic diversity and host specificity of actinorhizal *Frankia* bacteria associated with the roots of two co-occurring *Alnus* taxa. Our findings suggest that host identity is not a strong driver of *Frankia* genotype abundance associated with sympatric populations of *Alnus rubra* and *A. rhombifolia*. Whether or not these findings are applicable on a larger scale remains elusive as we were limited to a single location in western Oregon. Still, to the best of our knowledge, this is the first study comparing host-*Frankia* associations of these species at a natural site.

Similar to other studies we found low overall diversity of *Frankia* (Benson et al. 1996, Clawson et al. 1997, Oakley et al. 2004, Vanden Heuvel et al. 2004, Lipus and Kennedy 2011, Pokharel et al. 2011) with a single dominant genotype across the sampling site. Interestingly, two of the genotypes recovered in the current study were identical to two described in previous studies (Table 1) that were found at sites between 50 km and 115 km from the current study site. Remarkably these matching genotypes correspond to the most abundant genotype in our study and the two most abundant in those of Kennedy et al. (2010) and Lipus and Kennedy (2011), further underscoring the regional low diversity of *Alnus*-associated *Frankia*. There were no genotypes that were unique to either host, and no significant association between the abundance of genotypes and host species was observed indicating a lack of host specificity in this system. A similar pattern was observed by Pokharel et al. (2011) who found that Rep-PCR profiles (i.e. genotypes) of *Frankia* associated with nine out of 12 *Alnus* taxa in a common garden setting were identical. Likewise, Oakley et al. (2004) performed a sequence-based analysis of *Frankia* associated with several co-occurring *Ceanothus* species and reported a high level of sequence homogeneity across hosts despite not having clustered similar sequences into OTUs, which would have likely further reduced the observed diversity. Interest-

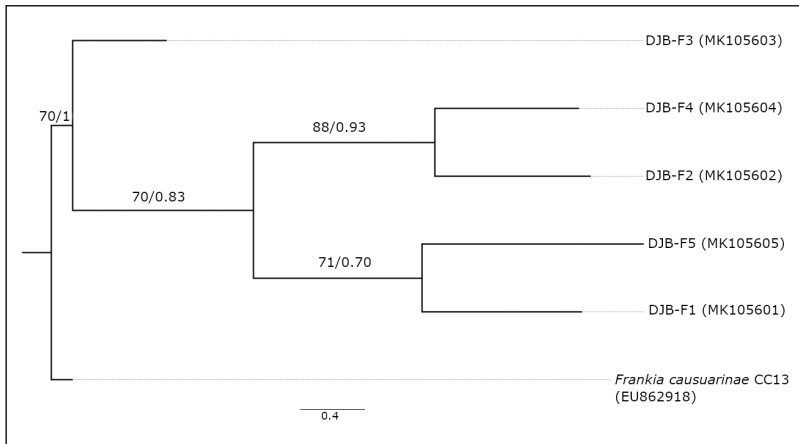


Figure 2. Maximum likelihood tree based on *nifH* gene sequences generated from *Frankia* nodules associated with *Alnus rubra* and *A. rhombifolia*. Branch labels reflect bootstrap support values for ML (maximum likelihood), followed by posterior probabilities for Bayesian analyses. A sequence for *Frankia causuarianae* Cc13 was used as an outgroup.

ingly, allopatric populations have also exhibited intrageneric host cross-compatibility with symbionts in *Alnus* (Welsh et al. 2009, Lipus and Kennedy 2011, Pölme et al. 2014). In contrast, the findings of Anderson et al. (2009) revealed that the most abundant *Frankia* strains (based on RFLP profiles) recovered from overlapping *A. viridis* and *A. tenuifolia* were unique to each host, in that case indicating a high degree of host specificity in their study site.

Whereas strict host specificity appears to be more the exception than the rule within intrageneric hosts (especially those that are sympatrically distributed), variation in this phenomenon is likely to be influenced by host phylogeny and not simply taxonomic identity. This was more recently highlighted in a large-scale global assessment of *Frankia* associated with 22 *Alnus* species on four continents by Pölme et al. (2014). From their investigation, Pölme and associates discovered that host phylogeny was the primary driver of *Frankia* assemblages worldwide and that the majority of recovered genotypes were generalists, with a few that were strongly host specific. This may explain the mixed results obtained across other studies comparing the *Frankia* assemblages of co-occurring hosts of the same genus, whereby different host species combinations may produce different

outcomes in respect to symbiont affinity. The *Alnus* species in our study are more closely related compared to other similar studies (Chen and Li 2004) in which a high degree of host preference (Lipus and Kennedy 2011) or host specificity (Anderson et al. 2009) were found, supporting the idea that host phylogeny may reflect the degree of homogeneity in *Frankia* populations associated with co-occurring host species. It is also noteworthy that Pölme et

al. (2014) found that *Frankia* phylogeny did not significantly influence the identity of associated hosts, reinforcing the idea that the *Alnus* host is selecting the symbiont and not vice versa. This may also explain the oddity of the dominant genotype in our study being most closely related to the rarest in our study (Figure 2). In other words as suggested above, perhaps selective initiation of the actinorhizal mutualism depends on factors not bound to symbiont phylogeny, or at least not to the degree that is revealed by variation in the *nifH* gene.

While certain key features such as low overall diversity with few dominant genotypes appear to be consistent across studies, the reason for this phenomenon is still elusive. One possible explanation for the low diversity of host-associated *Frankia* are potential limitations in resolution of using single standard marker genes like *nifH*. However, the *nifH* gene currently represents by far the most utilized marker for studying genotypic variation in nitrogen-fixing bacteria—including *Frankia*—and many key studies on *Frankia* diversity available today have used this marker (Welsh et al. 2009, Mirza et al. 2009, Kennedy et al. 2010, Pölme et al. 2014, Rodriguez et al. 2016). Other new markers (or combinations thereof) such as the *pgk* region for example (Pozzi et al.

2018) are promising to provide greater analytical depth but are little tested so far or in the case of multiple markers often yield conflicting results. While *nifH* has limitations it still is useful to provide data with the particular advantage of being easily comparable to existing studies databases and allowing the specific targeting of *Frankia* bacteria in environmental samples. Ultimately, full genomic comparisons will eventually yield the best metric for deriving genotypic grouping of *Frankia* strains.

Another potential explanation for the relatively low diversity of host-associated *Frankia* is that only few *Frankia* genotypes provide significant adaptive advantages to host plants in nature. At this point, the question why and under which conditions host associations with few dominant *Frankia* genotypes—as opposed to diverse consortia—provide an adaptive advantage to host fitness remains unanswered. Bioassays with specific host-*Frankia* associations under controlled conditions would provide useful tools to study host-*Frankia* specificity (Lipus and Kennedy

2011), but whether or not different symbioses provide actual advantages or disadvantages in nature requires bioassays at natural sites. Thus, future efforts would benefit from the inclusion of multiple combinations of co-occurring host species, and detailed studies on the prevalence of specific host-*Frankia* combinations under variable micro-climatic conditions in nature. Combining such outdoor studies with measuring phenotypic responses of host plants in combinatorial experiments under controlled conditions will help illuminate the functional underpinnings of these unique associations.

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