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Genetic Diversity in *Frankia* -- Host Plant Relationships

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by

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Abstract

The genus *Frankia* is made up of actinomycetes, nitrogen-fixing bacteria that form symbioses with actinorhizal trees (Benson and Silvester, 1993). With the first isolation of *Frankia* not occurring until 1978 (Torrey et al., 1978), far less research has been conducted on these actinomycetes in comparison to the nitrogen-fixing bacteria of leguminous plants, rhizobia. Thus, there is still much to be learned regarding genetics and host plant specificity of *Frankia*. This study was performed to evaluate possible genetic diversity amongst samples of a sympatric population of Red Alder (*Alnus rubra*) and White Alder (*Alnus rhombifolia*). Sanger sequencing methods were used to target the nifH gene of *Frankia*. Results showed some genetic differences and grouping amongst the collected samples, however, none of these differences were directly correlated to the species of Alder, or the relative geographic location.

Introduction

In many environments, nitrogen is often a limiting nutrient for plants. Some plants are able to make use of atmospheric nitrogen (N_2) by forming mutualistic symbioses with nitrogen-fixing microbes. In exchange for sugars from the plant, nitrogen-fixing microbes use the enzyme nitrogenase to alter N_2 into forms the plant can readily use, such as ammonium (NH_4^+) and nitrate (NO_3^-). Nitrogen fixation is estimated to provide up to 20% of a plant's annual nitrogen intake (Van der Heijden et al., 2008) and largely affects 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).

Over the last 30 years, it has become apparent that nitrogen fixation properties can be found within nearly all phyla of bacteria as well as some *Archaea* (Young, 1992). *Frankia* are nitrogen-fixing bacteria characterized by multilocular sporangia, filamentous growth, and nitrogenase-containing vesicles enveloped in multilaminated hopanoid lipid envelopes (Benson & Silvester, 1993). *Frankia* can be found in over 200 species of host plants from 24 genera, eight families, and seven orders of Angiosperms (Schwintzer and Tjepkema, 1990; Benson and Silvester, 1993). Plants that are capable of forming such relationships are known as actinorhizal plants (Dawson, 1990). Thus far, *Frankia* strains have been organized into three broad groups based upon the family of their host plant (Pawlowski & Sirrenberg, 2003). Furthermore, several factors have been noted to influence *Frankia* assemblages, these include: host species richness (Huguet et al., 2001; Anderson et al., 2009), edaphic factors (Navarro et al., 1999; Huguet et al., 2004), elevation (Igual et al., 2006; Khan et al., 2007), and geographic location (Dai et al., 2004; Chen et al., 2008).

This study seeks to evaluate the potential genetic diversity of *Frankia* strains present in the root nodules of Red Alder (*Alnus rubra*) and White Alder (*Alnus rhombifolia*). Sequencing methods will specifically target the nifH gene, which is associated with the nitrogenase enzyme responsible for nitrogen-fixation. By sampling the roots of sympatrically occurring Red and White Alder, any evident diversity may provide further insight to the determinants of *Frankia* – host plant pairings.

Materials and Methods

Nodule-bearing roots were collected from both Red and White Alder from a site in western Oregon (Figure 1). In total, 28 samples of White Alder and 30 samples of Red Alder were collected, with each sample originating from a different tree.

Surface sterilization

First, the roots and nodules were hand washed with deionized water to eliminate excess soil and organic matter. Next, nodules were removed from the roots with a flamed scalpel and placed into individual 14mL sterile scintiliation vials. Each vial was then filled three quarters with a 10% bleach solution and placed in an incubated shaker at room temperature (21°C) for two minutes at 140 rpm. After shaking was completed, the bleach solution was decanted from each vial and the process was repeated once more. After the second bleach wash, the process was repeated three more times, but with deionized water instead of the bleach solution. After completing all five wash cycles, a single nodule lobe of each sample was removed with a flamed scalpel. All single lobes were transferred to 1.5mL Eppendorf tubes using a sterile toothpick. All remaining nodules were placed in individual 1.5mL Eppendorf tubes and stored in a freezer at -20°C.

Extraction

The extraction process was conducted using the Sigma Tissue Extract-N-Amp Kit. First, 40 µl of extraction buffer was pipetted into each 1.5 mL tube. Then, sterile micropestles were

used to crush the nodules and homogenize the solution. Samples were then spun down in the microfuge for 1 minute at 15,000 rpm. Next, 20 µl of liquid was pipetted off the surface of each sample and placed into individual 0.2 mL strip tubes. Then, samples were placed in the thermocycler. Once the cycle was completed, 25 µl of neutralization buffer was pipetted into each sample. All samples were briefly vortexed and stored in the refrigerator at 4°C.

Polymerase Chain Reaction (PCR)

A 1:10 dilution was made for each sample using 36 µl of nuclease-free water and 4 µl of DNA template. Following dilution, samples were briefly vortexed and spun down in microfuge. Next, a master solution was made from PCR water, GoTaq PCR Master Mix, bovine serum albumin (BSA), and nifH forward and reverse primer. In a new set of strip tubes, 23 µl of the master solution was combined with 1 microliter of each 1:10 dilution of sample DNA. Samples were spun down in the microfuge for a few seconds and then placed in the thermocycler for a duration of approximately 2.5 hrs.

Gel Electrophoresis

After PCR was completed, all samples were subjected to gel electrophoresis. To begin, 2 µl of loading dye was pipetted onto a strip of parafilm for each sample. Then, 4 µl of each PCR product was pipetted onto the 2 µl of loading dye. From there, 4 µl of each PCR product and loading dye mixture was drawn up and loaded into the wells of the 1% agarose gel submerged in 1x lithium borate. Additionally, 4 µl of Generuler express ladder was loaded into the well at the

end of each lane. Using a Bio-Rad meter, 200 volts was ran through the gel for a total of 15 minutes.

Sequencing

All samples that were proved viable by gel electrophoresis were sent off to be sequenced. Returned sequence data was assessed using Geneious software version 10.0.5 (2016).

Results

The nifH sequences showed no apparent genetic differences between the *Frankia* of *A. rubra* and the *Frankia* of *A. rhombifolia*. Comparison of the sequencing consensuses from *A. rubra* and *A. rhombifolia* yielded a genetic congruency of 99%. The genetic congruency within the *A. rubra* samples and the *A. rhombifolia* samples were 98.1% and 97.8%, respectively.

Despite the lack of genetic diversity between the two *Alnus* species, phylogenetic trees generated by the Geneious software did show some distinction between several groups (Figure 2). The trees were produced in conjunction with an outgroup (rhizobia) retrieved from the National Center for Biotechnology Information (NCBI). A statistical bootstrapping feature was also used to improve the accuracy of the tree. Within the tree appears one larger group consisting of 29 samples with 100% genetic congruency. In addition to the one large group, 4 other smaller groups are also present showing individual genetic similarities of 80.5%, 90.6%, 99%, and 100%. The large grouping was shown to have 98.2% genetic similarity compared to all other samples as a whole.

Discussion

This research intended to explore the genetic diversity between *Frankia* living in symbiosis with *A. rubra* and *A. rhombifolia* in order to investigate what factors influence or dictate the specifics of *Frankia* – host plant pairings. With an overall genetic similarity of 99%, the nifH gene sequences showed no consistent genetic difference between the *Frankia* associated with *A. rubra* and those associated with *A. rhombifolia*. Furthermore, a larger proportion of genetic differences were actually found to be within the host plant – specific groups. The Frankia of *A. rubra* had a similarity of 98.1% amongst one another, while those of A. rhombifolia were even less similar (97.8% similar). These results do not support the hypothesis of Frankia – host plant pairings being driven by the host plant species themselves.

Species of the host plant is only one of the potential driving forces that has been proposed in recent literature (Kiers et al. 2003), however, along with edaphic factors (Navarro et al.1999; Huguet et al. 2004), geographical location (Dai et al. 2004; Chen et al. 2008), age/site management (Kennedy et al. 2010), and host species richness (Huguet et al. 2001; Anderson et al. 2009). The phylogenetic tree generated from this study (fig. 2) displays several distinct groupings of Frankia. The largest group is genetically identical, but is composed of Frankia collected from both *A. rubra* and *A. rhombifolia.* These samples did not group together geographically, though. As seen in figure 3, the samples in this group were collected from trees dispersed throughout the entire sample site.

The overall low genetic diversity amongst these Frankia samples does not differ drastically from other findings. *A. rubra* in particular, is known to associate with only 1-2 different genotypes of *Frankia* independent of geographic location (Kennedy et al. 2010). Different cut-offs have been used for differentiating between genotypes of Frankia, such as 1%,

3%, and 5%. A genetic difference of 3% has been shown to most accurately assign Frankia into their genomic groups (Mirza et al. 2009; Welsh et al. 2009a). The groups evaluated in this study barely show a 3% difference in the base pair sequence, and no significant group was as much as 5% dissimilar.

One of the major limitations of this study was the single sample site from which all *Frankia* nodules were collected. However, in more than one study, Kennedy (2010) has found the impact of geographic location not to be significant in influencing *Frankia* assemblages. Rather, it has been found that the age and management of given sites has a greater impact (Kennedy et al. 2010; Martin et al. 2003a). From this point, additional studies taking into account host species richness and site age and management, would be beneficial. Furthermore, utilizing more than one sample site would prove useful, not only statistically, but for comparative purposes as well. This would be particularly useful for a study looking further into the influence of site age and management, comparing Frankia assemblages found in newer, well-maintained sites to those found in older, natural areas.

In conclusion, the nifH gene sequence data did not show a significant difference between the two species of Alder. Thus, the species of Alder (host plant) does not appear to heavily influence the assemblages of *Frankia* strains. Amongst all of the samples collectively, significant genetic differences and groupings were seen, however. Further analysis of these particular genetic differences, as well as a study with multiple sample sites, designed to target other potential driving forces of Frankia – host plant relationships.

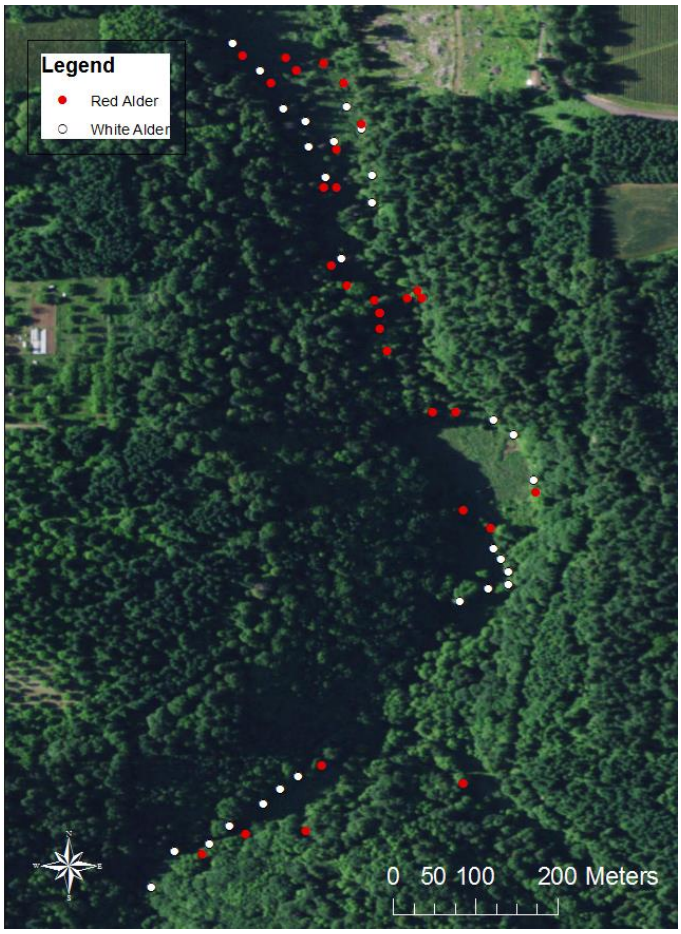

Figure 1: Map of Red and White Alder sample collection site.

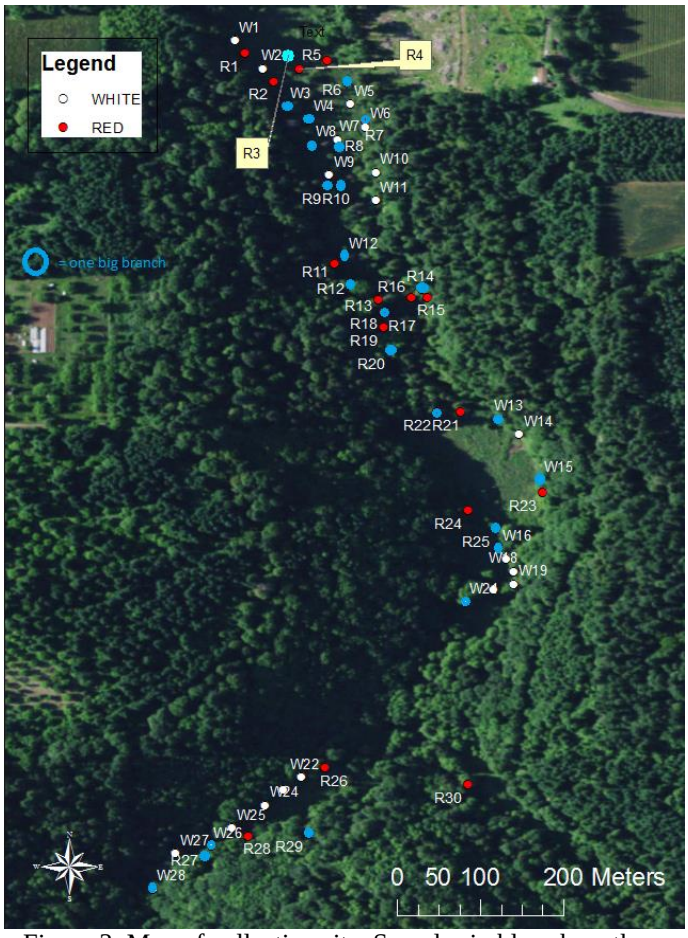

Figure 3: Map of collection site. Samples in blue show the dispersal of those within the genetically identical group depicted in the phylogenetic tree (see fig. 2).

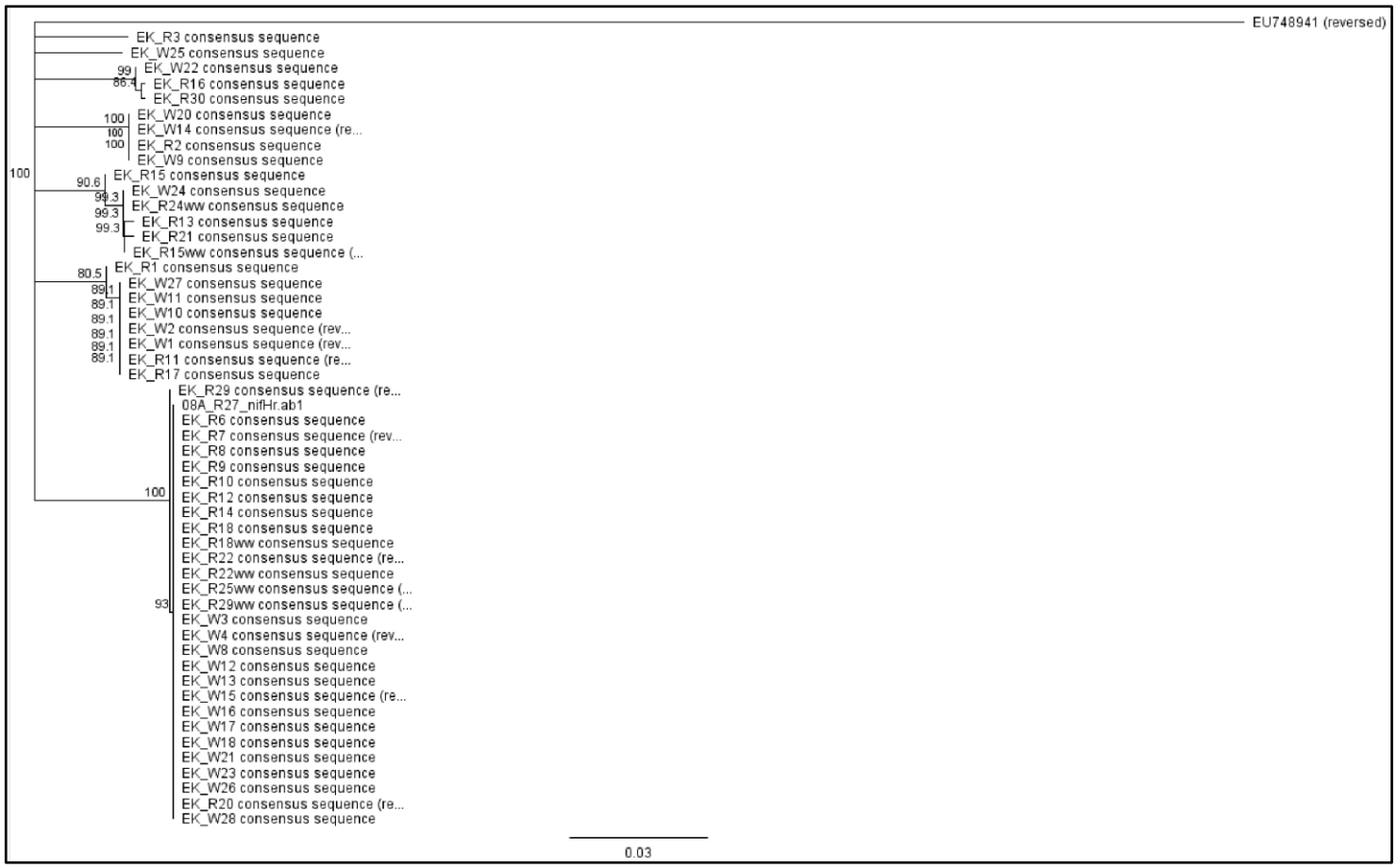

Figure 2: Phylogenetic *Frankia* tree with rhizobia outgroup. Generated with Geneious software version 10.0.5 (2016).

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