Trees
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ORIGINAL ARTICLE

Frankia diversity in sympatrically occurring red alder (*Alnus rubra*) and Sitka alder (*Alnus viridis*) trees in an early successional environment

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Abstract

Key Message ***Frankia*** **diversity on Mount St. Helens has recovered to regionally-observed levels, but small-scale geographic heterogeneity affects host-specificity of** ***Frankia*** **communities in red and Sitka alder.**

Abstract Alders (*Alnus* sp.) are key pioneer tree species in disturbed and nutrient-poor ecosystems. Due to their association with nitrogen-fixing *Frankia* bacteria they have a disproportionate impact on soil quality and successional processes. However, surprisingly little information exists on the host-specificity and colonization patterns of *Frankia* communities among sympatrically occurring alder hosts in nature. We analyzed variation in *Frankia* community composition in sympatric red alder (*Alnus rubra*) and Sitka alder (*Alnus viridis*) root nodules from the Pumice Plain of Mount St. Helens, WA (Lawetlat'la in the Cowlitz language; USA). Five 2500 m^2 plots containing both red ($n = 11$) and Sitka alder ($n = 12$) trees were sampled along a 1.5-km transect. Five root nodules were collected from each tree, and *Frankia* genotypes were assessed by sequencing both *nifH* and *16S* rRNA genes. In addition to root nodules, soil samples were collected from the rhizosphere of each tree for chemical analyses. We did not observe within-tree variation as only one *Frankia* genotype was detected per host tree, and the overall observed *Frankia* diversity was low and comparable to other studies of *Alnus–Frankia* symbioses. The most abundant *nifH* genotype was observed in both alder host species, in all plots, and occurred in 70.8% of all samples (69.6% of all trees). However, community composition was significantly different among plots (PERMANOVA, $p = 0.002$). Comparisons of communities among plots revealed modest correlations between geographic distance and community similarity (Mantel test, $p = 0.001$). Our findings suggest that even small-scale spatial variation and microenvironment conditions can affect an important plant–microbe symbiosis, which may have consequences for host local adaptation.

Keywords Plant–microbe interactions · Biodiversity · Symbiosis · Nitrogen-fixation · Succession · Mount St. Helens · Lawetlat'la

Introduction

Alders (Betulaceae: *Alnus* sp.) are important pioneer species in disturbed ecosystems with an extensive distribution throughout the northern hemisphere. In the Pacific Northwest of North America (PNW), red alder (*Alnus rubra* Bong.) and Sitka alder (*Alnus viridis* (Chaix) DC. ssp. *sinuata* (Regel) A. Löve & D. Löve) are native trees that show a wide and broadly overlapping distribution range, although they tend to occupy different elevations within that range. The more common red alder occurs from southeast Alaska to southern California, with some isolated communities in northern Idaho (Favorite and Immel 2006), while Sitka alder occurs naturally from central Alaska south to northern California and east to Alberta, northwest Wyoming, and western Montana at higher elevations (Deal and Harrington 2006; Darris and Gonzalves 2009). While a number of alder species occur sympatrically (Balkan et al. 2020), red and Sitka alder are the only species to co-occur within the Mount St. Helens National Volcanic Monument (WA, USA). This area—particularly the Pumice Plain and debris avalanche—is still undergoing early successional processes following the 1980 eruption as it returns to its natural state without human

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interference. Consequently, Mount St. Helens (Lawetlat'la in the Cowlitz language) has been studied extensively as a successional model system and, in the context of our work presented here, offers a unique opportunity to study natural community assembly processes in alder and their microbial symbionts, *Frankia*.

As with all other alders, red and Sitka alder form a symbiotic association with nitrogen-fixing, gram-positive filamentous *Frankia* bacteria (actinomycetes), which is critical to their role as pioneer species. In alders, microbially fixed nitrogen can account for 70 to 100% of the host plant's nitrogen requirement, enabling colonization even of extremely nutrient-poor environments (Binkley 1983; Mallet and Roy 2014) including habitats disturbed by wildfires, landslides, erosion, or volcanism. Consequently, alders play a disproportionate role in successional processes as they enrich the soil with plant-available nitrogen and promote the establishment of subsequent species (Binkley 1983; Dawson 1990; Wall 2000; Rhoades et al. 2001; Hiltbrunner et al. 2014). *Alnus–Frankia* symbioses have been reported to fix from 1 to 320 kg N ha^{-1} $year^{-1}$ depending on age and species (Tobita et al. 2016), which is comparable to the 200 to 300 kg of N ha^{-1} $year^{-1}$ fixed by *Rhizobium*-associated legume crops alone (Zahran 1999). Additionally, as a common riparian species in the Pacific Northwest, alders can also stimulate nutrient-cycling rates and provide important sources of nitrogen for streams through flow paths (Callahan et al. 2017) and nitrogen-rich litter inputs (Perakis et al. 2012).

Although various plant–*Frankia* symbioses—including *Alnus–Frankia* systems—have been studied regarding their species specificity (Bosco et al. 1992; Huguet et al. 2001; Anderson et al. 2009; Lipus and Kennedy 2011; Balkan et al. 2020) there is still little known about the degree of variation in *Frankia* assemblages among sympatric alder host species in nature, as well as the potential impact of small-scale geographic variability within sampling sites. Microclimatic soil conditions are known to affect microbial community structure (Hugoni et al. 2021), but also pathogen infectivity (Donald et al. 2020). Here, we used *nifH* gene and *16S* rRNA sequences to elucidate *Frankia* genotype diversity present within co-occurring red and Sitka alder trees on the Pumice Plain of Mount St. Helens. The *nifH* gene that encodes for one of the two subunits of the nitrogenase enzyme complex is commonly used for studies on variation within nitrogen-fixing bacteria (Gaby and Buckley 2014). The highly conserved *16S* rRNA gene is useful as a general marker to identify more broadly comparable variation in *Frankia* phylogenetics (Clawson et al. 1999; Normand et al. 2017).

According to molecular data, *Frankia* spp. specific to *Alnus* appear to be cosmopolitan and seem to be strikingly homogeneous over large distances (Kennedy et al. 2010; Higgins and Kennedy 2012). However, few studies have evaluated *Frankia* genetic diversity using more than one molecular marker while also comparing host specificity and soil chemical features. The concentration of specific nutrients such as nitrogen (N) and phosphorus (P) in soil has been shown to affect the development of and nitrogenase activity within nodules. According to Gentili and Huss-Danell (2003), N quantitatively inhibits nodulation and nitrogenase activity, whereas increasing concentrations of P serve to increase nodule development. Specifically, in the presence of moderate N and P and high N and P, the availability of P counteracted inhibiting effects of N. Given these known effects of soil chemistry on *Frankia* nodulation, we used the heterogeneous landscape created by the 1980 eruption of Mount St. Helens to study possible interactions between host plant specificity and small-scale environmental variation on a naturally occurring *Alnus–Frankia* symbiosis. Consequently, we examined the following five questions in our study: (1) How diverse are nodulating *Frankia* genotypes found on the Mount St. Helens Pumice Plain? (2) Do species-specific interactions exist between the sympatric red and Sitka alder and *Frankia* genotypes? (3) In addition to inter-host plant variation do colonizing *Frankia* strains show variation within the individual host trees? (4) Does spatial distance influence host-*Frankia* pairings? and (5) Is the presence and diversity of alder-*Frankia* symbiosis correlated to variation in soil chemical characteristics at the study site?

Materials and methods

Study site

We sampled root nodules from sympatric red (*Alnus rubra*) and Sitka alder (*A. viridis* ssp. *sinuata*) trees on the Pumice Plain (i.e., sites were distributed across both the pyroclastic and debris flows), south of Spirit Lake along the northern flank of Mount St. Helens (Washington State, USA; 46.25861° N, − 122.17319° W; Fig. 1). The Pumice Plain resulted from the catastrophic lateral blast of the 1980 eruption that buried the area in deposits of debris up to 200 m deep. Sitka alder and Sitka willow (*Salix sitchensis*) are the dominant shrubs on the Pumice Plain, although occurrences of red alder have been increasing in recent years. Twenty-three alder trees were sampled ($n = 11$ of red alder and $n = 12$ of Sitka alder; $n = 53$ root samples of each sp.) in five plots (50×50 m), which were selected based upon proximity to an existing transect (Che-Castaldo et al. 2015), as well as the limiting factor of red alder individuals on the Pumice Plain (Supplemental Table 1). In order for an area to be considered a potential plot, at least one individual of each alder species had to be present.

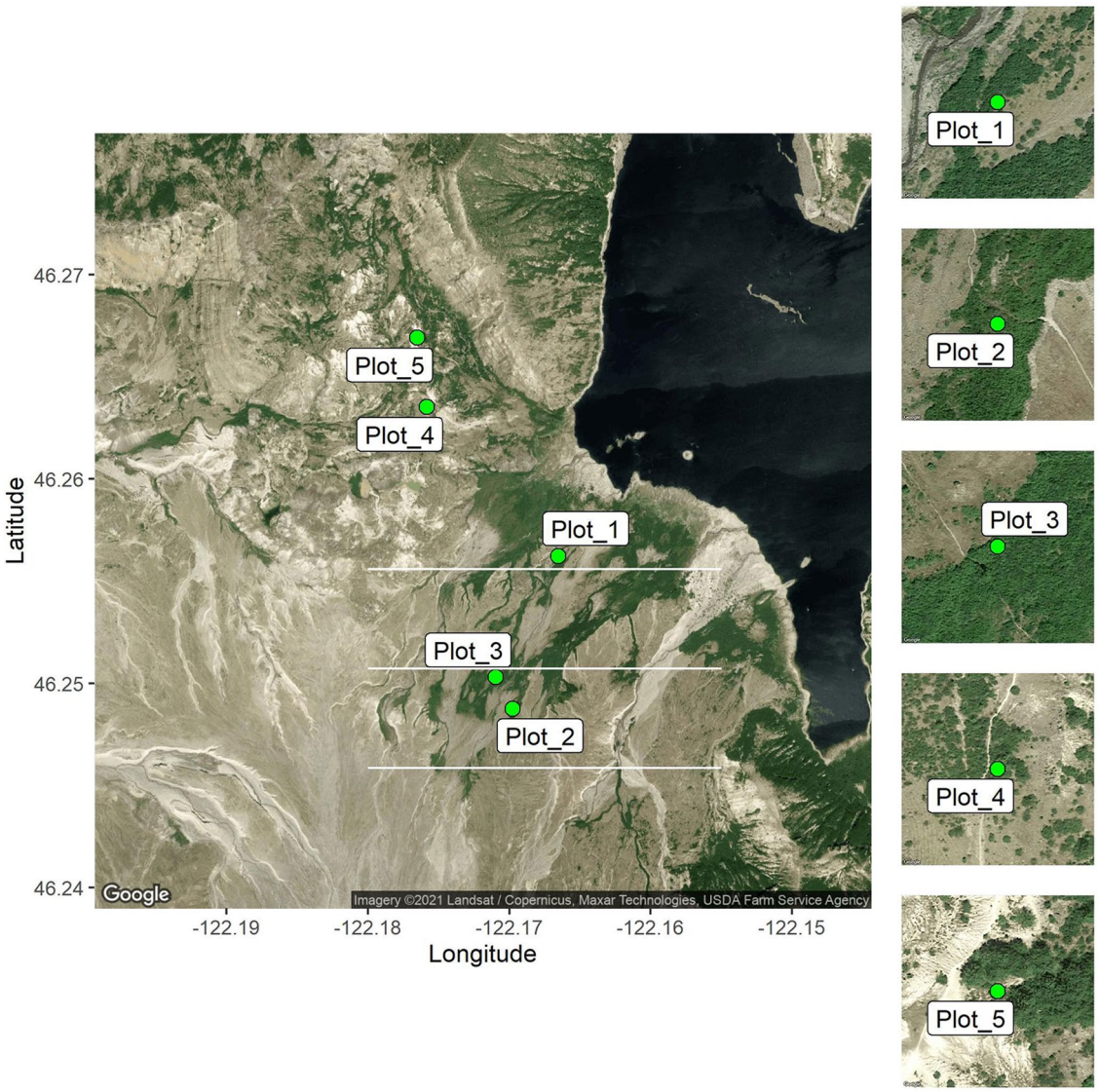

Fig. 1 Map of the study sites on the Pumice Plain of Mount St. Helens, with Spirit Lake featured along the right side of the main panel. Plot-level panels show the differences in vegetation coverage and, to a lesser degree, surrounding substrate

Soil sampling

Because of the significant impact of soil chemistry on the alder–*Frankia* symbiosis, we analyzed soil from the rhizosphere of each sampled tree in all plots. One-hundred grams of soil were collected from the base of each tree, on the downhill slope in an attempt to capture the greatest amount of organic material and hydrophilic nutrients that had been washed out by draining water. The trowels used were washed in 90% EtOH in between samples to prevent cross-contamination. The samples were stored at 4° C until they were processed and analyzed for moisture, ammonium, phosphorous, magnesium, sulfur, calcium, copper, boron content, cation exchange capacity, and electrical conductivity by the Oregon State University Crop and Soil Science Central Analytical Laboratory (Corvallis, OR, USA).

Nodule sampling & processing

Five root nodules were collected between 2 and 10 cm under the base of each tree with hand trowels and pruning scissors. Roots were removed with nodules attached, and tweezers were used to collect and transfer the nodules into Ziploc® bags. In between samples, the pruning scissors and tweezers were rinsed with 90% EtOH to prevent cross-contamination. Nodule samples were individually packed in Ziploc® bags and transported to the lab in a cooler (4 °C).

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 two min 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 individual, sterile 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) according to the manufacturer's instructions. Forty µl of extraction buffer was 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 min at 15,000 rpm, and 20 µl of supernatant from each tube was transferred into an individual 0.2 mL PCR strip-tube. Strip-tubes containing extractions were placed in a thermocycler and incubated at 65 °C for 10 min, followed by 95 °C for 10 min. Following incubation, 25 µl of neutralization buffer was pipetted into each sample which were then briefly vortexed and stored at 4 °C until use.

PCR and sequencing

A 606 bp portion of the *nifH* gene was amplified by PCR using *Frankia*-specific primers nifHf1 (5′-GGC AAG TCC ACC ACC CAG C-3′) and nifHr (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 thermocycling conditions: 96 °C for 5 min, 35 cycles at 95 °C for 30 s, 60 °C for 30 s, 72 °C for 45 s, and a final extension at 72 °C for 7 min. 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). Raw sequences were manually inspected and trimmed in Geneious 10.2.3. Sequences were then trimmed to 604 bp, aligned using MAFFT (v. 7), and binned into operational taxonomic units (OTUs) using a cutoff of 99.3% and the default opticlust algorithm in MOTHUR version 1.42.0 (Schloss et al. 2009; Põlme et al. 2014).

A 423 bp portion of the 16S rRNA gene spanning variable regions V1-V2 was amplified using the primers fD1 (5′-AGA GTT TGA TCC TGG CTC AG-3′) and rDB1 (5′-CCA AGC TTG AGG TTT ACA ACC CGA A-3′). PCR was carried out using the same reaction mixtures as for *nifH*, with the following thermocycling conditions: 95 °C for 2 min, 35 cycles at 91 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min, and a final extension at 72 °C for 6 min. Sequencing and sequence processing were performed as described for the *nifH* gene, with the exception that sequences were trimmed to 410 bp and were not clustered into OTUs prior to tree building.

Phylogenetics

Representative sequences of each *nifH* OTU were aligned with other *Frankia* sequences obtained from NCBI using MAFFT as implemented in Geneious 10.2.3, and the resulting alignment was manually improved where necessary and trimmed to obtain approximately equal coverage. Tree building was performed using RAxML version 8.2.11 (Stamatakis 2014) using a general time-reversible nucleotide model with an estimation of invariant sites, gamma-distributed rate heterogeneity across sites, and a maximum-likelihood estimation of base frequencies (GTR + I + G + X), and 500 bootstrap replicates using the rapid bootstrapping algorithm. 16S sequences were treated in the same manner with the exception that sequences were not clustered into OTUs prior to alignment and tree building.

Statistical analysis

Statistical analyses were conducted in R version 3.6.1. The OTU tables generated in MOTHUR were imported into R with the "phyloseq" package, and resulting community analyses were conducted using the "vegan" and "ecodist" packages (McMurdie and Holmes 2013; Oksanen et al. 2017). If environmental variables did not meet the assumptions for ANOVA even after transformation (ln + 1), nonparametric Kruskal–Wallis and Dunn post hoc tests were used instead. Environmental data were analyzed with "PMCMRplus" for nonparametric post hoc tests (Pohlert 2018). We used permutational multivariate analyses of variance (PERMANOVA; adonis; 999 permutations and Bray–Curtis distances) to resolve differences in community composition between host tree species and among plots. We used partial Mantel tests to determine the relationships between community composition, environmental variables, and geographic

distance between plots (999 permutations and Euclidean distances; Zhang et al. 2017). Finally, the species accumulation curve was generated in "vegan," while Chao1 was calculated in "fossil", and indicator species analysis from "indicspecies" was used to identify associations between OTUs and soil chemical features (Cáceres and Legendre 2009; Vavrek 2011). Data and scripts are available in the following GitHub repository: https://github.com/emwolfe/MSH_Frankia.

Results

The *nifH* and *16S* rRNA genes were sequenced in *Frankia* root nodules collected from red and Sitka alder on the Pumice Plain of Mount St. Helens. However, due to poor sequence coverage, only *nifH* sequence data are discussed here; all *16S*-related data are available in the supplemental materials. When clustered at the 97%, 99%, and 99.3% similarity thresholds, we found 3, 4, and 4 genotypes (used here synonymously with operational taxonomic units), respectively. The results reported below are based on the OTU tables binned at the 99.3% cutoff, as it was optimal for the *nifH* gene of interest (Põlme et al. 2014). Based on *nifH* sequence data, the most abundant genotype (Genotype 1) was found in all plots while the three remaining genotypes (Genotypes 2–4) only occurred in Plot 5. Dispersion within plots was tested prior to PERMANOVA with the betadisper function, and showed that samples were not dispersed homogeneously among plots. Although the species accumulation curve did not level off (Fig. 2)—suggesting that sampling more trees would have revealed additional taxa—calculations of Chao1 indices matched our observed number of genotypes (4), indicating a low probability of missing OTUs.

Within the *nifH* phylogeny (Fig. 3), representative sequences of the four genotypes (Supplemental Table 2) did not form a monophyletic clade, but instead were dispersed across the tree. The genotypes (2–4) that were found only in Plot 5 also did not form a monophyletic clade. The *16S* phylogeny showed a similar pattern, though branch support was generally lower across the entire tree (Supplemental Fig. 1).

We did not find evidence of host tree specificity (PERMANOVA, $p >> 0.05$). All genotypes were represented in both host species except for Genotype 4, which was isolated from only red alder. The highest richness occurred in Plot 5; four genotypes occurred here, while only one genotype (Genotype 1) occurred elsewhere. We did not observe within-tree variation (i.e., variation in the number of genotypes identified from all nodule samples taken from the same tree) of *Frankia* genotypes as among all tree samples only one *Frankia* genotype was detected per host tree.

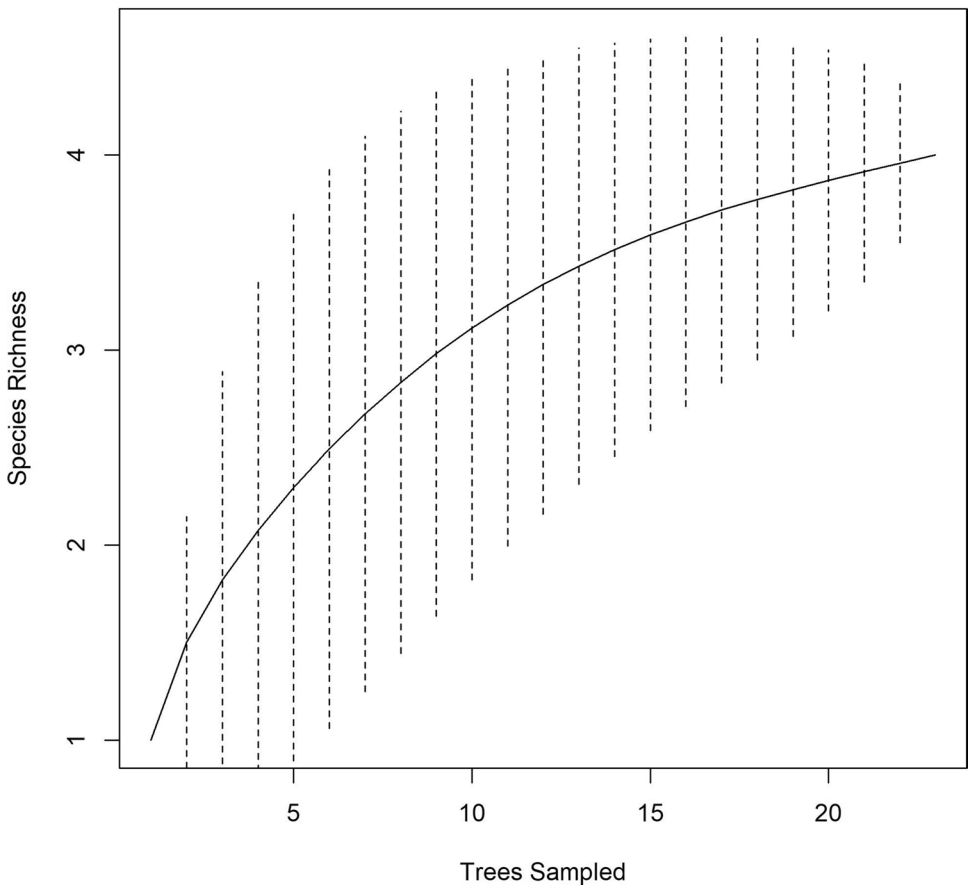

Fig. 2 Species accumulation curve for *Frankia* genotypes identified at the 99.3% similarity threshold. Vertical lines are 95% confidence intervals

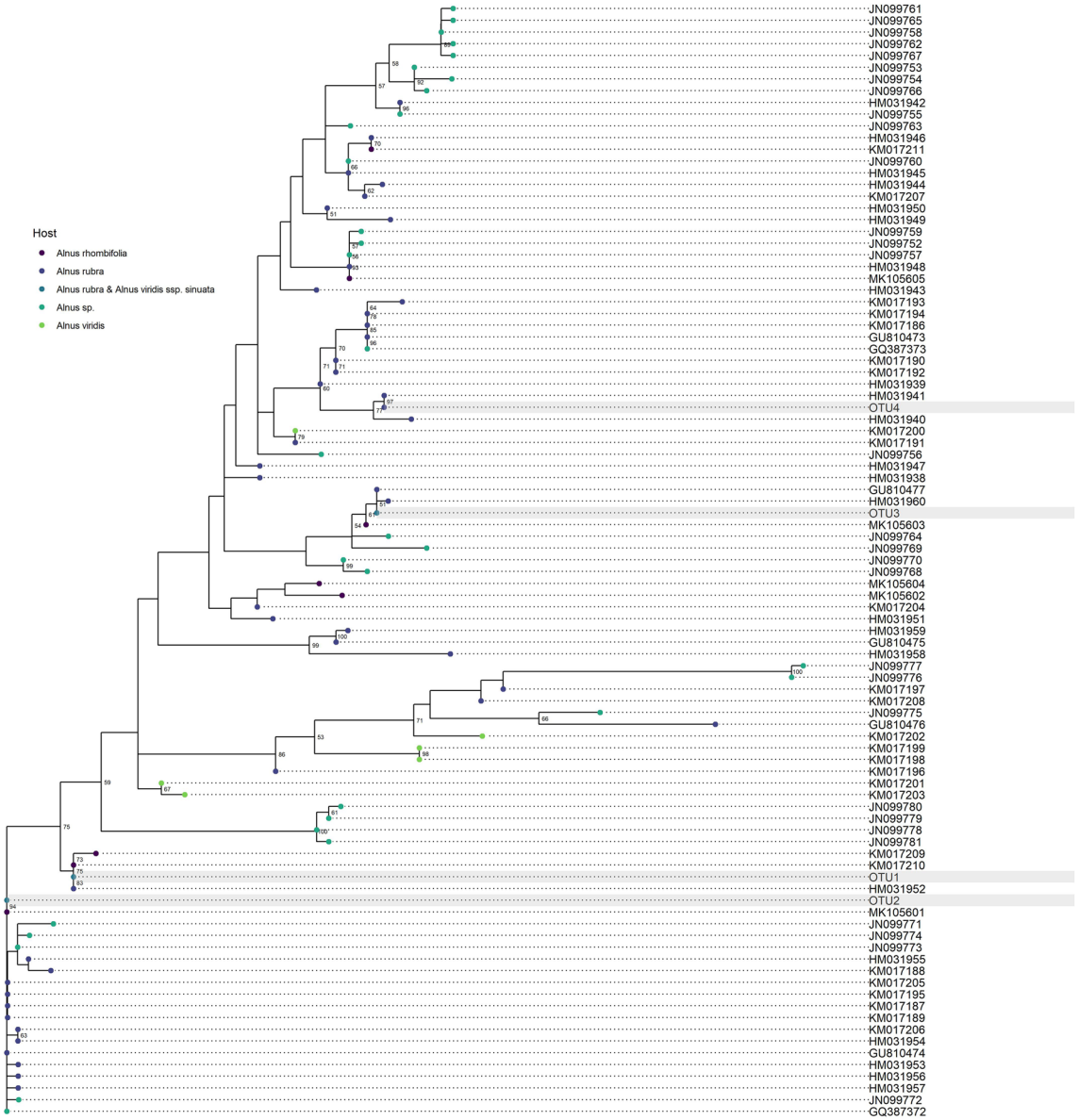

Fig. 3 Phylogenetic tree of the *nifH* sequences isolated from Sitka and red alder on the Pumice Plain, as well as additional sequences from other alder species curated from NCBI. Bootstrap values (> 50) are printed at the branch tips. The OTUs identified in the present studied are enclosed in shaded boxes. KL1 and KL2 from Lipus and Kennedy (2011) are accessions GU810473 and GU810474

We found that *Frankia* communities were significantly different among plots (PERMANOVA, $F_{4,22} = 4.6375$, $p = 0.002$), which had varying microenvironmental conditions and showed differences in soil features including differences in moisture, ammonium, phosphorous, magnesium, sulfur, calcium, copper, boron, cation exchange capacity, and electrical conductivity (Table 2). PC axes 1 and 2 cumulatively explained 87.6% of the variance (Fig. 4). However, these differences were largely driven by Plot 5, both in richness and different soil microenvironment, and were likely additionally influenced by uneven sampling depth since Plot 5 also had the greatest number of trees sampled (8, compared to 5 in Plot 1, 4 in Plot 2, 4 in Plot 3, and 2 in Plot 4). Dominant genotypes were significantly associated with particular levels of soil chemical features aggregated from beneath individual trees (indicator species analysis; $p < 0.05$; Table 1). The most abundant genotype was associated with higher levels of sulfur and carbon to nitrogen ratio (C:N) while Genotype 2—found only in Plot 5—was indicative of intermediate soil moisture and low C:N. However, while soil chemistry alone did not account for *Frankia* community composition differences, there was a weak positive correlation with geographic distance between plots (Mantel test; Pearson's $r = 0.4030$; $p = 0.001$).

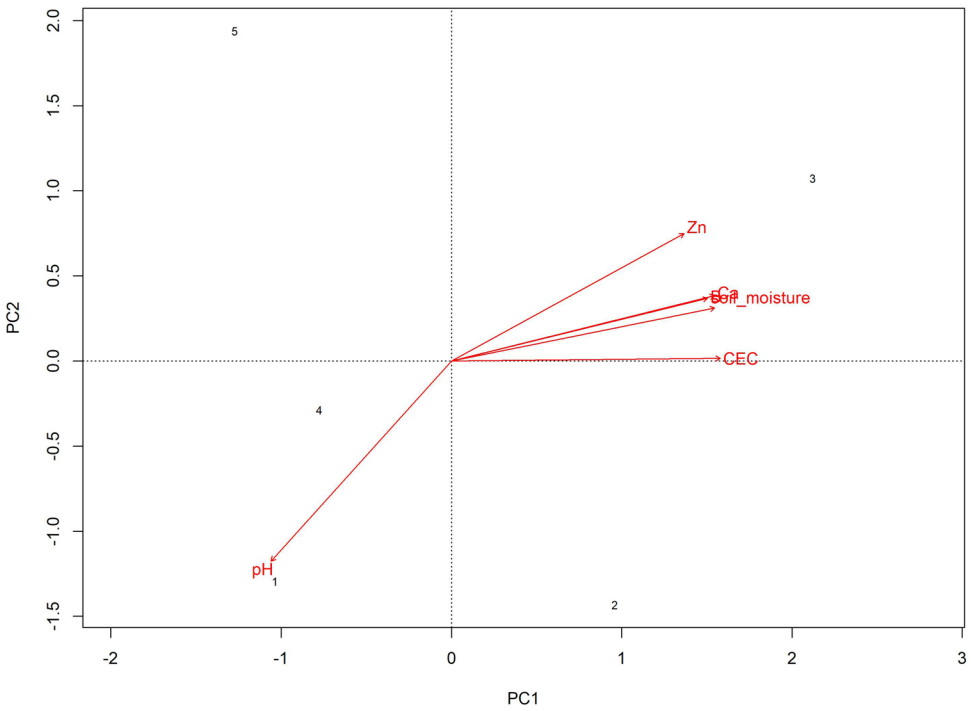

Fig. 4 Principal components analysis of significant soil chemical features among Plots 1–4

Table 1 Indicator analyses of plot soil chemical features

Soil chemical feature	Feature category	Genotype	Indicator value statistic	*p* value
NH4.N	High + med	Genotype 1	0.843	0.037
S	High + med	Genotype 1	0.857	0.023
moisture	High + low	Genotype 1	0.885	0.008
C:N	High + med	Genotype 1	0.885	0.006
moisture	Med	Genotype 2	0.655	0.026
C:N	Low	Genotype 2	0.655	0.022

Feature categories were calculated based on the following quantiles: 0–33% (low), 34–66% (medium), and 67–100% (high), relative to the measurement values for a given soil chemical feature

Discussion

After almost four decades of primary succession following a catastrophic volcanic eruption, we found low diversity of *Frankia* genotypes in red and Sitka alder root nodules. Nevertheless, this low diversity was similar to the results reported by another study on and around Mount St. Helens, including sites less impacted by the blast (Lipus and Kennedy 2011), and a study in the Portland Metropolitan area (Balkan et al. 2020). In a global study of *Alnus* sp. in a variety of natural habitats, Põlme et al. (2014) uncovered the same number of genotypes in *A. viridis* (5; 99.3% similarity; *nifH*) but over twice as many genotypes in *A. rubra*. However, at the 99.0% sequence similarity threshold, Kennedy et al. (2010) found 15 genotypes of *Frankia* in *A. rubra*—over three times what we recovered. This is likely partially reflective of the differences in the site characteristics, given that the stands sampled by Kennedy et al. (2010) were either managed or much older than the trees we sampled, and would likely have more propagules available for dispersal. At the 97% sequence similarity threshold, we recovered three genotypes, which is slightly more than the number of genotypes reported by Lipus and Kennedy (2011) in the same area (Pumice Plain; two genotypes; 97% similarity; *nifH*). The most abundant genotypes KL1 and KL2 previously reported by Lipus and Kennedy (2011) across the Pacific Northwest cluster with our less abundant OTU4 and OTU2, respectively (Fig. 3), which confirms that there appear to be few genotypes of *Frankia* across a broad range of the region (Kennedy et al. 2010; Balkan et al. 2020). It further indicates that successful colonization of *Frankia* genotypes is susceptible to heterogeneity even at small scales, since our plots are only a few hundred meters from the Mount St. Helens sites sampled by Lipus and Kennedy (2011).

Contrary to other studies, we did not find evidence of host specificity in *Frankia* communities of red and Sitka alder. Anderson et al. (2009) previously observed host-specific *Frankia* communities (*nifD-K* intergenic spacer) among sympatric *A. tenufolia* and *A. viridis* hosts in an Alaskan field site, as well as patterns in community composition based on habitat (floodplain vs. upland). Within our sites, sympatric hosts yielded similar community compositions

Table 2 Differences in soil chemical features by plot (mean ± SD), with superscript letters indicating pairwise differences among plots for a given feature (i.e., row; Dunn post hoc test)

Soil chemical feature	Plot 1 ($n=6$)	Plot 2 ($n=4$)	Plot 3 ($n=4$)	Plot 4 ($n=2$)	Plot 5 ($n=8$)
Moisture (%)	1.80 ± 1.15^{c}	67.16 ± 32.57^{a}	109.7 ± 166.5^{a}	3.17 ± 1.45^{bc}	12.28 ± 4.66^{b}
NH4-N (ppm)	6.63 ± 4.44^{ab}	14.34 ± 13.11^{a}	7.51 ± 6.71^{ab}	6.25 ± 5.14^{ab}	1.32 ± 0.92^{b}
P (ppm)	12.94 ± 3.37^{b}	17.35 ± 5.58^{ab}	34.36 ± 19.25^{b}	31.56 ± 10.35^{b}	23.43 ± 4.65^{b}
S (ppm)	681.7 ± 81.34^{ab}	1282 ± 552.0^{b}	820 ± 304.0^{ab}	435 ± 35.36^{a}	480 ± 35.86^{a}
Ca (ppm)	225.9 ± 54.13^{a}	697.4 ± 302.8^{b}	1204 ± 1593^{ab}	287.6 ± 25.63^{ab}	340.5 ± 51.5^{ab}
Mg (ppm)	58.56 ± 11.86^{b}	113.3 ± 39.76^{ab}	152.3 ± 123.2^{a}	96.11 ± 2.36^{ab}	117.6 ± 10.44^{a}
Cu (ppm)	1.27 ± 0.57^{a}	4.28 ± 2.85^{ab}	14.43 ± 14^{ab}	4.84 ± 0.24^{ab}	5.57 ± 1.15^{b}
B (ppm)	0.12 ± 0.11^{ab}	0.61 ± 0.54^{a}	1.77 ± 3.11^{ab}	0.07 ± 0^{ab}	0.07 ± 0.03^{b}
CEC (meq/100 g)	1.92 ± 0.36^{a}	4.79 ± 1.91^{b}	7.73 ± 9.32^{ab}	2.46 ± 0.14^{ab}	2.94 ± 0.36^{ab}
EC (dS/m)	0.04 ± 0.02^{ab}	0.62 ± 0.37^{a}	0.13 ± 0.15^{ab}	0.02 ± 0.01^{b}	0.03 ± 0.02^{b}

CEC cation exchange capacity, *EC* electrical conductivity; moisture (%) is the gravimetric moisture content of the soil sample

Letters are used to indicate that pairs that do not share letters are significantly different. For example, moisture (%) in Plot 1 is significantly different from Plots 2, 3, and 5

except for Plot 5, which may support a larger trend in habitat specificity. Our study is unique in that our sites are exclusively in the early stages of succession—i.e., mostly exposed, dominated by lupine (*Lupinus lepidus*) and sporadic shrubs—and located in much closer proximity to one another (< 1 km) than similar studies, which ranged from 1.2 to 630 km (Anderson et al. 2009; Lipus and Kennedy 2011). While Anderson et al. (2009) also sampled in mixed stands as we did, Lipus and Kennedy (2011) sampled in single-species stands and did not compare nodules collected from stands of both species in the same field sites. Additionally, the sites Lipus and Kennedy (2011) used for the high-elevation bioassay soil collection are in vastly different areas of the Pumice Plain; one single-species stand is just a few hundred meters from our Plot 1, near the saturated shore of Spirit Lake, while the other single-species site is located upland closer to the refugia along Windy Ridge. By contrast, our sites are located on a mixture of substrates, ranging from pyroclastic flow to debris avalanche deposits.

The devastated landscape of the Pumice Plain is a critical consideration when comparing studies in the area; the 1980 eruption of Mount St. Helens was catastrophic, completely obliterating existing forest and burying streams in deep, sterile, nutrient-poor substrate. Reemerging springs and seeps have facilitated the establishment of several densely thicketed riparian corridors and the development of vegetation cover and organic matter (LeRoy et al. 2020), but the Pumice Plain remains a largely barren, patchy landscape. Seeds and Bishop (2009) previously found low *Frankia* density based on bioassays using soil samples from the Pumice Plain, and identified thickets as important reservoirs of inoculum, thus emphasizing the importance that local ecosystem factors play in determining symbiont presence and composition. In our study, host trees harbored just one genotype each, and the dispersion of genotypes among plots was uneven. Consequently, sampling more transects of the Pumice Plain would further clarify patterns in true community composition. Nevertheless, the fact that we identified only one *Frankia* genotype per tree in all trees sampled is in contrast to the general assumption—based on other intimate host plant-symbionts interactions such as Fabaceae and their nitrogen-fixing rhizobial symbionts—that the diversity of nodulating microbial symbiont community in a plant population reflects the diversity found within individual host plants. At this point, we only can speculate what the reason might be for the lack of within host variability. Biases and limitations of Sanger sequencing leave room for undetected within-host variability that might be otherwise detected by next-generation sequencing techniques. Alternatively, priority effects from early colonization during seedling stages may lead to predominant presence of a single, first colonizing genotype. Alternatively, ecological filters such as pressure by herbivores or pathogens may select for or against the symbiosis with specific *Frankia* genotypes (Ballhorn et al. 2017). Our preliminary findings (unpubl. data) suggest that different *Frankia* genotypes have variable effects on the chemical phenotype of their host (C:N ratios and tannins in leaf tissue) which may affect susceptibility to attackers.

On the Mount St. Helens Pumice Plain, we found that even small-scale geographical distance (< 1 km) between plots played a larger role than soil chemistry and physical characteristics in affecting *Frankia* community composition. However, especially in the context of the Pumice Plain, the effect of distance may be confounded by unmeasured, local differences such as plant community cover and composition (Welsh et al. 2009), which may be reflected in our uneven distribution of genotypes among plots. Although measured soil chemistry and physical characteristics varied significantly (Table 2), the PCA only explained 87.6% of variation among plots. Battenberg et al. (2017) found that host

plant presence rather than identity or edaphic factors was important for predicting presence of cluster II-associated *Frankia*; however, host plant presence (i.e., proximity) and soil chemical features might be interlinked. Sitka alder has been established longer on the Pumice Plain (and in the immediate surrounding areas) than red alder (Titus et al. 1998; Titus 2009), indicating that there may eventually be different patterns of dispersal for host-specific *Frankia* strains. We observed the highest genotype diversity in the only plot that was semi-sheltered on the debris avalanche (Plot 5). The surroundings of this specific site were more colonized by plants compared to the completely exposed plots on the pyroclastic flow (Plots 1–3) and transition zone (Plot 4), consistent with Batzli et al. (2004) and Battenberg et al. (2017). Additionally, this plot may have received more propagules from runoff through surrounding thickets or from alders colonizing the blow-down zone of the neighboring ridges (Lawrence and Ripple 1999; Batzli et al. 2004; Seeds and Bishop 2009). This is further supported by the clustering pattern within the *nifH* phylogeny. Had the four genotypes observed in Plot 5 arisen from a smaller number of colonizing genotypes followed by subsequent diversification, we would expect to see samples from this plot clustering within a smaller number of clades with genotypic diversity within one or more clades. Instead, the four Plot 5 genotypes cluster within four separate clades, bolstering the hypothesis that Plot 5 is receiving a greater number of *Frankia* propagules—or propagules containing greater *Frankia* diversity—than Plots 1–4. Plots 1–4 are dominated by pumice and forbs, and thus have lower expected *Frankia* Inoculation Units. These differences in substrate and available propagules may be indicative of larger trends that should be explored with more comprehensive sampling designs that incorporate less impacted sites around Mount St. Helens.

Nitrogen-fixing plants and their symbionts such as *Frankia* spp. are critical to nutrient cycling during recovery after disturbance. On the Pumice Plain, nitrogen-fixing prairie lupine (*Lupinus lepidus*) and Sitka alder have been crucial to soil development and improving soil conditions for later colonizers (Morris and Wood 1989; Halvorson et al. 1991; Titus 2009). In agricultural systems, nitrogen-fixing cover crops can reduce the use of fertilizer and the subsequent detrimental effects on surrounding ecosystems, especially when symbioses are optimized for specific environmental conditions (Ballhorn et al. 2018; Wittwer and van der Heijden 2020). We found that even small-scale, plot-level variation was sufficient to affect the compositions of *Frankia* communities along a short transect (< 1.5 km) in a model primary successional ecosystem. These plot-level differences represent an important lesson in patch heterogeneity and differing scales within a metacommunity. On the Pumice Plain, patch heterogeneity is affected at the regional scale by the various types of deposits from the eruption that impacted substrate, drainage, and refugia status; at the plot-level scale, regional-scale deposits and geography further influence plant community composition, seed rain, herbivore (i.e., dispersal vector) presence, and edaphic conditions. The (in)hospitability of the between-patch matrix is also important to consider (Miller et al. 2018; Miller and Bohannan 2019) in such a devastated landscape, as large tracts of the Pumice Plain are still dry and barren, while other sections are inundated with moisture and regularly occupied by elk. Our findings underscore the critical role that local variation can play in determining microbial community composition, and emphasize the need to consider dynamics at multiple scales to fully understand factors affecting plant-associated symbioses.

Author contribution statement DJB devised the study, SS conducted sampling in the field and benchwork, ERW conducted the statistical analyses, NUS conducted the phylogenetics analyses, and MAB assisted in phylogenetic interpretation. ERW and DJB wrote the manuscript. All authors edited and reviewed the manuscript.

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Data availability Data and scripts are available in the following GitHub repository: https://github.com/emwolfe/MSH_Frankia.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare no conflicts of interest.

Ethical approval Not applicable.

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