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# Microbes on the Mountain: Plant-Microbe Associations and Interactions on Mount St. Helens

Emily Rose Wolfe  
*Portland State University*

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Microbes on the Mountain:  
Plant–Microbe Associations and Interactions on Mount St. Helens

by  
Emily Rose Wolfe

A dissertation submitted in partial fulfillment of the  
requirements for the degree of

Doctor of Philosophy  
in  
Biology

Dissertation Committee:  
Daniel J. Ballhorn, Chair  
Mitchell Cruzan  
Sarah Eppley  
Suzanne Estes  
Robert Strongin

Portland State University  
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## Abstract

Plant–microbe associations and interactions provide critical context to studies in both community and ecosystem ecology, especially in systems that are relatively new and still undergoing early successional processes. Microbes can colonize the surfaces and interiors of all plant tissues, and these assemblages vary in composition both spatially and temporally, even within the same plant. Endophytes are bacteria or fungi that spend most of their lifecycles living within plant tissues asymptotically—typically, “endophyte” refers specifically to aboveground tissues such as leaves and stems, and therefore may have direct influences on defenses against herbivory, pathogen or pest tolerance, and even afterlife effects on litter decomposition. Similarly, root-associated symbionts such as mycorrhizae, *Frankia*, and Rhizobia are especially crucial to host plants’ abilities to survive in nutrient-poor environments, such as those found during primary succession. Within these new, harsh systems, advantageous microbial symbioses can be critical for plant establishment and survival, as is the case for pioneer plants with nitrogen-fixing symbionts—e.g. alder, an important native tree species in the Pacific Northwest that is nodulated by *Frankia* bacteria. Here, we chose red and Sitka alder (*Alnus rubra* and *A. viridis* ssp. *sinuata*, respectively) as plant systems to model multi-scale interactions and associations in the primary successional environment of the Pumice Plain of Mount St. Helens. In Chapter 2, we first surveyed culturable fungal endophytes within red alder to gain a baseline understanding of the structure and composition of endophytes in a closely related species within more established habitats. We found significant differences in community composition among sample sites that may have been driven by differences in

air quality, and also verified taxa common to red alder. We were able to use these results as a comparison for Chapter 3, where we surveyed culturable fungal endophytes of six woody species on the Pumice Plain of Mount St. Helens. We found much lower frequencies of isolation (<15%, compared to ~95%) and diversity, but still identified host specificity—in fact, Sitka alder had the highest OTU richness of the six species sampled. Our results suggest that culturable fungal endophyte communities still seem to be in the early stages of community development on the Pumice Plain, and that pioneer species like Sitka alder are potentially serving as important microbial reservoirs. Since microbial communities within plant tissues can interact with plant genotype to influence ecosystem processes like litter decomposition, we examined population genetics of Sitka alder around Mount St. Helens, in part to determine possible source populations of Pumice Plain colonists (Chapter 4). We found virtually no genetic differentiation among Sitka alders sampled from Castle Lake, June Lake, Norway Pass, the Pumice Plain, or Smith Creek. However, we did identify three subpopulation clusters that suggest that the colonists of the Pumice Plain either came from Norway Pass or from the same seed rain. Finally, to complement our study of Sitka alder genetic structure, we investigated the population structure of *Frankia* strains in sympatrically-occurring alder species on the Pumice Plain (Chapter 5). While we only found four genotypes (99.3% threshold), our results are consistent with other regional studies of *Frankia* and indicate that even small-scale spatial variation (<1.5 km) and microclimatic conditions can affect an important plant–microbe symbiosis. In Chapter 6, we discuss the implications of our findings within our representative model systems. Pioneer species like alder are important ecosystem

engineers that ameliorate harsh environments by increasing soil nitrogen and organic matter inputs. Our results can be used to calibrate models of early community assembly in plant–microbe symbioses, which can in turn be used to inform models of nutrient cycling—especially carbon and nitrogen. As global climate change increases the frequency of catastrophic natural disasters and generally expands the amount of land undergoing early succession, these models will be important for determining benchmarks of and projecting rates of recovery.

## Dedication

This dissertation is dedicated to my children Adelaide and Aidan, to my husband Tyler, and to my father who did not live to read this, but always believed I would write it.

## Acknowledgements

I have been debating how to start this section for nearly as long as it took me to write the pieces of this dissertation. I want it on the record for other parents out there that I ended up finally starting this section on my phone in the wee hours of the morning with my youngest child sprawled across my chest, head cocked at a seemingly uncomfortable angle and snoring gently. No, it is not how I imagined writing anything, let alone large sections of my final data chapters and sections like this one. No, this is not the section I thought I would write before the pandemic. But after the last year and a half, I feel like this section now needs to begin with an acknowledgement of just how impactful the COVID-19 pandemic has been (and still is)—on my research, my personal life, and everything in between. First and foremost, I am grateful to have made it through this pandemic with the health of my family and friends largely unaffected. Second, this is not the dissertation I intended to write, but it is the dissertation I finished, and I am proud of it and grateful for the many opportunities I have been given to make it this far.

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## Chapter 1 Introduction

Do foliar endophytes matter in litter decomposition?

Note: The contents of this chapter were published in the journal *Microorganisms* with the following citation: Wolfe, E.R.; Ballhorn, D.J. Do Foliar Endophytes Matter in Litter Decomposition? *Microorganisms* **2020**, *8*, 446.

### Abstract

Litter decomposition rates are affected by a variety of abiotic and biotic factors, including the presence of fungal endophytes in host plant tissues. This review broadly analyzes the findings of 67 studies on the roles of foliar endophytes in litter decomposition, and their effects on decomposition rates. From 29 studies and 1 review, we compiled a comprehensive table of 710 leaf-associated fungal taxa, including the type of tissue these taxa were associated with and isolated from, whether they were reported as endo- or epiphytic, and whether they had reported saprophytic abilities. Aquatic (i.e., in-stream) decomposition studies of endophyte-affected litter were significantly under-represented in the search results ( $P < 0.0001$ ). Indicator species analyses revealed that different groups of fungal endophytes were significantly associated with cool or tropical climates, as well as specific plant host genera ( $P < 0.05$ ). Finally, we argue that host plant and endophyte interactions can significantly influence litter decomposition rates and should be considered when interpreting results from both terrestrial and in-stream litter decomposition experiments.



Keywords: plant–microbe interactions, ecosystem processes, microbe–microbe interactions, fungi, bacteria

## Introduction

Litter decomposition is an essential ecosystem process that significantly contributes to the global carbon cycle. Numerous studies have identified several overarching controls on litter decomposition rates, including temperature, dissolved oxygen in aqueous environments, soil moisture, seasonality, quantity of litter pulses, and litter chemistry or quality [1–3]. However, litter chemistry is unique among these factors in that it may be mediated by both abiotic—e.g., drought and nutrient availability [4,5]—as well as biotic factors, such as herbivores [6], microbial symbionts [7], and pathogens [8].

Changes in litter chemistry primarily affect decomposition rates by influencing interactions with macroinvertebrate and microbial decomposer communities, which mechanically process litter and break down recalcitrant compounds, respectively. Chomel et al. [9] reviewed the generally recalcitrant properties of alkaloids, phenolic compounds, and terpenes in litter decomposition, but only briefly covered the roles of endophytes—ubiquitous microbes that mostly live asymptotically within host plant tissues—in regulating the production of these secondary metabolites in host plants. Both endophytic fungi and bacteria were isolated from healthy plant tissues, including stems, leaves, and roots [10]; their *in planta* functions are mostly unknown. Fungal endophytes were most extensively studied in agriculturally significant grass hosts (i.e., Class 1, or

clavicipitaceous endophytes; e.g., [11]), where these systemic endophytes were shown to produce toxic alkaloids (e.g., [8,12]), and even alter plant community assembly [13,14]. Furthermore, these systemic endophytes were reported to cause slower rates of litter decomposition in terrestrial systems [15,16]. However, few studies examined the role of foliar endophytes in the decomposition of litter from non-grass hosts (i.e., Class 2 or 3, or non-clavicipitaceous endophytes), and even fewer studies focused on the effects of endophytes on leaf litter decomposition in aquatic systems. Although ubiquitous within plant tissues, endophyte communities can vary spatially and temporally within host plants [17] and represent an important bridge between host plant characteristics that influence decomposition and the decomposer community.

Here, we aimed at distilling the available literature on the effects of foliar endophytes on leaf litter decomposition into an up-to-date review. We used 67 published studies and compiled a report analyzing the contributions of foliar endophytes to litter decomposition rates. We further collected information about the reported taxa of both endophytic and epiphytic fungi recovered from leaf tissue and constructed a comprehensive table for reference.

## Materials and Methods

In February 2019, we returned 77 results after searching the following keywords in Web of Science: “endophyte”, “litter”, and “decomposition”. Of the 77 total results, 10 studies were excluded due to irrelevancy, as they did not report foliar microbial community composition or decomposition rates. The remaining 67 studies—spanning 25 years—were subsequently included in this review. The search results are reported in

Table S1, and span from 1994 to 2019. A table of 710 leaf-associated taxa was compiled from 29 studies and 1 review (Table S2; raw data available in Table S3) and expands upon Table 2 presented by Osono [18]. “Taxa” refers to the particular taxonomic level identified by the original study authors; to simplify reporting, only classifications at the genus-level or above are included in figures. Reported taxonomic names of species were cross-referenced in MycoBank (export date: 31 July 2019), and updated for improved consistency. The fifteen taxa most frequently reported as having decomposition abilities from litter and as endophytes are shown in Figures 1, 2, and 3, respectively. We chose not to use meta-analysis techniques due to the small sample size of relevant experimental studies ( $n = 14$ ), which would have been further reduced by inconsistent reports of statistical data that would have limited the ability to calculate effect sizes. Finally, we classified the ecosystem climate type for each of the 30 publications mined for taxa, grouping studies into three broad categories: cool climate (e.g., boreal), temperate climate, and tropical climate. We conducted indicator species analyses (R v. 3.6.1, `indicspecies` package) for these climate types, as well as for grouping by host genera.

## Results

Our search yielded studies of endophytes in diverse climates, forest types, and in both grass and non-grass hosts, with varying effects on litter decomposition rates. Several studies (and one review) provided either brief overviews of endophyte effects on litter decomposition [19–21] or general contributions of endophytes to changes in soil microbial communities [22], particulate organic matter [23], and soil organic carbon [24,25], rather than directly referring to litter decomposition. Of the studies that reported

taxa, 14 were conducted in temperate forests [26–39], 7 in tropical or subtropical forests [40–46], and 3 in boreal or subboreal/subalpine forests [47–49], with the remaining studies conducted in various forest types (maritime–continental, old-growth, oak, and mountainous forests, respectively) in Europe [50–53] and a *Cinnamomum* plantation in China [54]. Of these, 13 studies focused primarily on litter microbial community, while 14 studies incorporated some measure of litter decomposition rates. However, out of 67 relevant results, only 14 studies directly tested decomposition of endophyte-affected litter, with about half reporting increased rates [28,55–61] and the other half decreased rates [15,29,38,49,62,63]. The numbers of studies that reported either increased rates or decreased rates were not statistically significant (binomial exact test,  $P > 0.05$ ). Mikola et al. [64] reported no effects on decomposition rate, even after swapping endophyte-infected litter into endophyte-free plots. Finally, three studies by LeRoy et al. [29], Grimmett et al. [38], and Wolfe et al. [28] were the only in-stream (aquatic) studies that were statistically significant (binomial exact test,  $P < 0.0001$ ). Only one study [28] reported the bacterial community composition, which was also statistically significant (binomial exact test,  $P < 0.0001$ ).

From the 30 publications that we mined, 710 taxa were reported from 25 different host species spanning cool, temperate, and tropical climates (Tables S2 and S3). We found that reports of *Peniophora* and *Zalerion* were indicative of studies conducted in cool forests (e.g., boreal or subalpine), while reports of *Fusarium*, *Phomopsis*, *Idriella*, *Dactylaria*, *Acremonium*, *Cryptophiale*, *Thozetella*, *Mycoleptodiscus*, *Volutella*, and *Verticillium* were specific to studies conducted in tropical forests (indicator species

analysis,  $P < 0.05$ ). Several taxa were significantly associated with particular host genera (Table 1). Overarching trends identified in the search results include endophyte effects on litter chemistry, interactions with detritivores and microbial decomposers, and fungal succession patterns in litter, which are addressed below.

Table 1.1. Taxa significantly associated with host genera.

Taxa	Associated Host Genus	Fungal Order	Indicator Value	P-value
<i>Rhytisma</i>	<i>Acer</i>	Rhytismatales	0.816497	0.005
<i>Boeremia</i>	<i>Alnus</i>	Pleosporales	0.912871	0.005
<i>Ophiognomonia</i>	<i>Alnus</i>	Diaporthales	0.5	0.015
<i>Pseudopithomyces</i>	<i>Alnus</i>	Pleosporales	0.5	0.03
Amphisphaeriaceae	<i>Fagus</i>	Xylariales	0.57735	0.025
<i>Apiognomonia</i>	<i>Fagus</i>	Diaporthales	0.57735	0.025
<i>Arthrinium</i>	<i>Fagus</i>	Sordariales	0.5	0.015
<i>Ascochyta</i>	<i>Fagus</i>	Pleosporales	0.745356	0.005
<i>Beauveria</i>	<i>Fagus</i>	Hypocreales	0.57735	0.025
<i>Cryptococcus</i>	<i>Fagus</i>	Tremellales	0.537484	0.045
<i>Cylindrium</i>	<i>Fagus</i>	Hypocreales	0.447214	0.05
<i>Discosia</i>	<i>Fagus</i>	Amphisphaeriales	0.547723	0.01
Dothideomycetes	<i>Fagus</i>	NA	0.471405	0.05
Fungal sp. (undetermined)	<i>Fagus</i>	NA	0.573026	0.025
<i>Geniculosporium</i>	<i>Fagus</i>	Xylariales	0.645497	0.005
<i>Hypoxyton</i>	<i>Fagus</i>	Xylariales	0.456436	0.035
<i>Mycosphaerella</i>	<i>Fagus</i>	Capnodiales	0.707107	0.005
Nectriaceae	<i>Fagus</i>	Hypocreales	0.544331	0.025
Pezizomycotina	<i>Fagus</i>	NA	0.57735	0.025
<i>Phaeosphaeria</i>	<i>Fagus</i>	Pleosporales	0.447214	0.04
<i>Phialemoniopsis</i>	<i>Fagus</i>	Cephalothecales	0.57735	0.025
<i>Hormonema</i>	<i>Picea</i>	Dothideales	0.632456	0.005
<i>Lachnum</i>	<i>Picea</i>	Helotiales	0.489898	0.03
<i>Lophodermium</i>	<i>Picea</i>	Rhytismatales	0.6	0.005
<i>Rhizoctonia</i>	<i>Picea</i>	Cantharellales	0.516398	0.02
<i>Thysanophora</i>	<i>Picea</i>	Eurotiales	0.632456	0.005
<i>Rhabdocline</i>	<i>Pseudotsuga</i>	Helotiales	0.894427	0.005
<i>Helminthosporium</i>	<i>Quercus</i>	Pleosporales	0.516398	0.02

## Discussion

### Endophytes and litter chemistry

Litter chemistry directly influences decomposition rates by altering interactions with detritivores and microbes [9]. Many Class 1 systemic endophytes produce toxic alkaloids in grass hosts, which are thought to contribute to slower decomposition rates. However, while alkaloid concentrations in live tissues are a major concern for grazing livestock, concentrations may decrease following senescence [65] and, therefore, may not directly affect decomposition. It was suggested, however, that N-rich alkaloids could act as a nutrient pulse and stimulate decomposer communities [16]. Conversely, changes in C:N ratios and phenolics—including those induced by plant defenses against microbial pathogens—do persist in litter with well-studied, recalcitrant effects when endophyte status is not considered [66,67]. High C:N ratios typically result in slower decomposition, but in Class 1 endophyte-infected (E+) *Schedonorus pratensis* litter, Gundel et al. [58] reported higher C:N ratios and faster overall decomposition rates compared to E- (endophyte-free) litter, suggesting that other factors may influence endophyte-mediated effects in host grasses. Gundel et al. [58] also measured lower N concentrations in E+ litter, but Soto-Barajas et al. [68] found that symptomatic endophyte infection (i.e., endophytes that have transitioned to a symptomatic infection of host plant tissues; [69]) increased N concentrations in *Lolium perenne*, indicating that the type of and structures associated with infection are important considerations in predicting decomposition effects. Similar results were reported by LeRoy et al. [29] for *Rhynchospora punctatum*-infected punches of bigleaf maple (*Acer macrophyllum*) litter, in which punches with

symptomatic Class 3 infections had significantly higher N content, but significantly lower C:N ratios compared to nearby or uninfected patches. Soto-Barajas et al. [68] did not measure decomposition rates, but LeRoy et al. [29] found that symptomatic Class 3 infection retarded in-stream decomposition. Additionally, phenolic compounds are produced as defenses against microbial pathogens; high concentrations in litter also typically slow decomposition. In poplar (*Populus* sp.) and Norway spruce (*Picea abies*) trees, increased concentrations of phenolics contributed to decreased foliar endophyte presence [50,70]. Bailey et al. [70] specifically reported differences in leaf chemistry among different genotypes of poplar (see [71]), while Korkama-Rajala et al. [49] found that clone origin affected fungal community composition. Finally, while widely recognized for vertically transmitted Class 1 endophytes, coevolutionary relationships between host taxa and specific Class 2 and 3 endophytes may further influence community composition, as reviewed by Sieber [72] and supported here by the results of our indicator species analyses in Table 1. Consequently, plant host genotypes, origin, and host–endophyte coevolutionary relationships may be considered alongside endophyte community composition to interpret patterns in litter decomposition rates.

Endophytes as decomposers (interactions with detritivores and microbial decomposers)

Seven of the studies from the search results specifically investigated the ability of isolated Class 2 and 3 endophytes to decompose leaf litter ([28,29,38,56,57,60]; Figure 1.1). While most species of endophytes may not persist into the later stages of decomposition, some endophytes are capable of directly participating in the process, in competition with persisting epiphytes and new colonizers [74]. Importantly, all of the

isolated strains used in the studies had some effect on litter decomposition and produced several extracellular enzymes, including cellulases, laccases, and  $\beta$ -glucosidases [43,51,57,75]. *Colletotrichum* sp. [57] and *Coccomyces* sp. [33] were dominant strains that stimulated litter decomposition when inoculated alone or as the initial colonizer, respectively. Sun et al. [60] also found *Phomopsis liquidambari* to be capable of increasing decomposition of straw from rice plants, but only under conditions of low to moderate nitrogen. Additionally, Chen et al. [56] found that the same endophyte increased the concentrations of phenolics in the soil, subsequently affecting the composition of the microbial decomposer community by reducing soil fungi and increasing bacteria during the early stages of decomposition. This shows that in addition to competing directly, persisting endophytes can also have allelopathic effects on other microbes, although the specific effects of phenolic compounds on microbes is context-dependent, in that it matters which compounds and microbes are present [76,77].



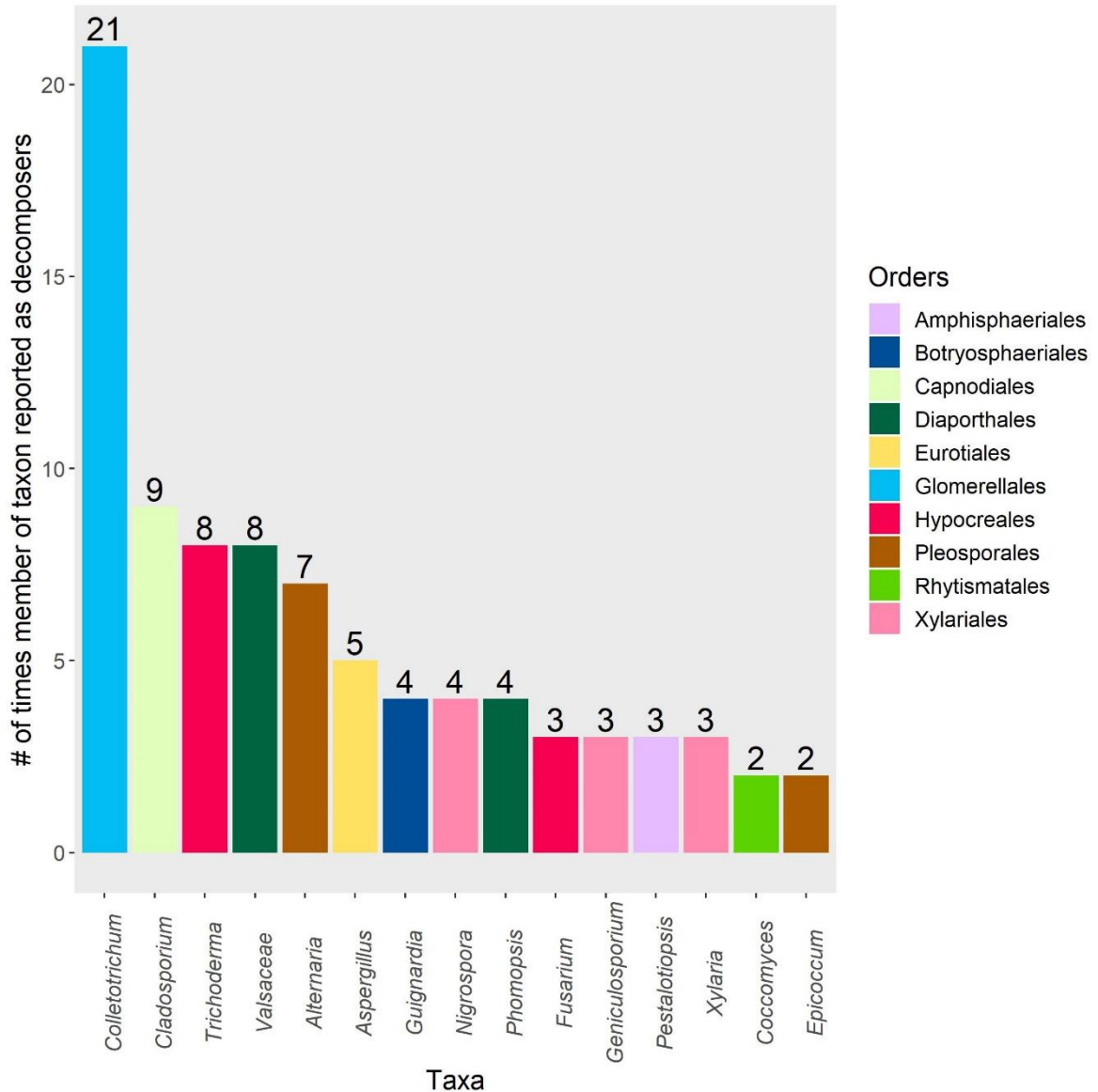


Figure 1.1. The 15 most frequently reported taxa that have measurable decomposition or saprophytic ability (e.g., cellulase secretion, etc.). Bars represent the number of times members of a taxon were reported. Taxa identified as “undetermined” or “Fungal sp.” are excluded.

Two in-stream studies also found negative effects of endophytes on litter decomposition, with one study specifically focusing on the fungal decomposer community [29,38]. Aquatic hyphomycetes are the primary in-stream decomposers of leaf litter, and their sporulation rates were significantly reduced by symptomatic Class 3

endophyte infection by *Rhytisma* sp. in *Acer* sp. litter [28,38]. However, Wolfe et al. [28] reported faster rates of decomposition for litter with symptomatic Class 3 infections and suggested macroinvertebrate presence as a contributing factor, given that Lemons et al. [15] had previously reported negative effects in Class 1 E+ *Lolium arundinaceum* litter in the absence of mesodetrivores. Detrivores also appear to play an important role in mediating at least Class 1 endophyte-produced secondary compounds; Mayer et al. [55] found that macrodetrivore abundance increased with the presence of *L. arundinaceum* in plots and contributed to increased decomposition rates of herbaceous litter. Increased arthropod abundance was also supported by Faeth and Shochat [78] in *Neotyphodium*-infected *Festuca arizonica*. Jackrel and Wootton [79] emphasized that changes in litter chemistry due to plant defense responses can decrease palatability for detritivores, although the effects on decomposition rates differed for aquatic and terrestrial groups.

#### Endophytes in litter decomposer assemblages

Taxa identified as endophytes in living to decaying leaves are included in Table S2 and Table S3 and summarize the results reported in the 29 studies and 1 review returned by our search (Figure 1.2 and Figure 1.3). The presence of endophytes and epiphytes was previously reported in leaf litter from various species, and fungal succession on decomposing leaves was reviewed by Osono [18]. However, the bacterial phyllosphere community during in-stream decomposition was only reported by one study in our search [28], which presents an opportunity for future work. Osono and Takeda [26] and Osono [27] identified xylariaceous endophytes as significant contributors to terrestrial litter decomposition due to their abilities to persist from living tissues and

decompose lignin. Reviews by Purahong and Hyde [80] and Saikkonen et al. [81] also recognized endophytes as potential saprophytes, which is a role governed by specific nutrient requirements and the capacity to produce certain classes of extracellular enzymes. Additionally, some endophyte species are considered to be latent pathogens and can switch lifestyles based on the presence of environmental stressors [82]. Hagiwara et al. [48] and Matsukura et al. [83] each surveyed ligninolytic endophytes that caused bleaching in up to 32% of measured leaf area. Switches to symptomatic endophyte infections are important considerations, since they were shown to affect decomposition rates differently than asymptomatic tissue [28,29,68]. Chauvet et al. [84] and Seena and Monroy [85] also reviewed the occurrence of aquatic hyphomycetes living as endophytes. This occurrence has interesting implications for in-stream decomposition, since it is largely mediated by aquatic hyphomycetes in the initial stages. However, Mustonen et al. [47] found that, while aquatic hyphomycete taxa were present, terrestrial endophytes dominated the sequenced litter decomposer communities under low-flow conditions and may have contributed to the greater mass loss observed. Tateno et al. [35] also isolated similar endophytes from twigs, leaves, and cupules in beech, suggesting that horizontally transmitted foliar endophytes communities are likely influenced by propagules in other plant parts. Importantly, Guerreiro et al. [30] found that endophyte communities are linked to and influenced by fungal communities in litter; endophytic fungi were still present and active in one-year-old litter.

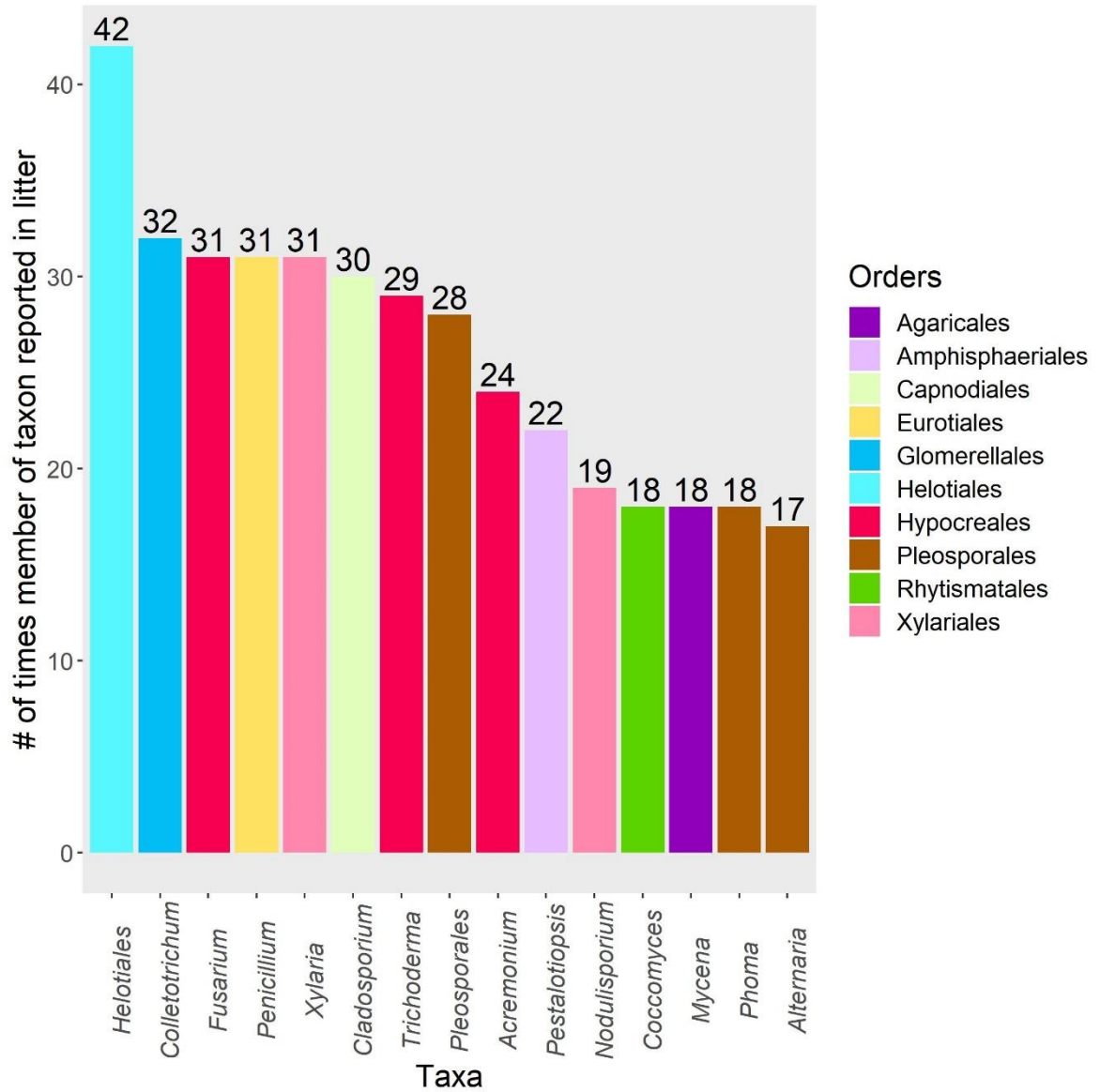


Figure 1.2. The 15 most frequently reported taxa from leaf litter (e.g., senescent tissue, dead leaves, dried leaves, fresh litter, and decaying litter). Bars represent the number of times members of a taxon were reported. Taxa identified as “undetermined” or “Fungal sp.” are excluded.

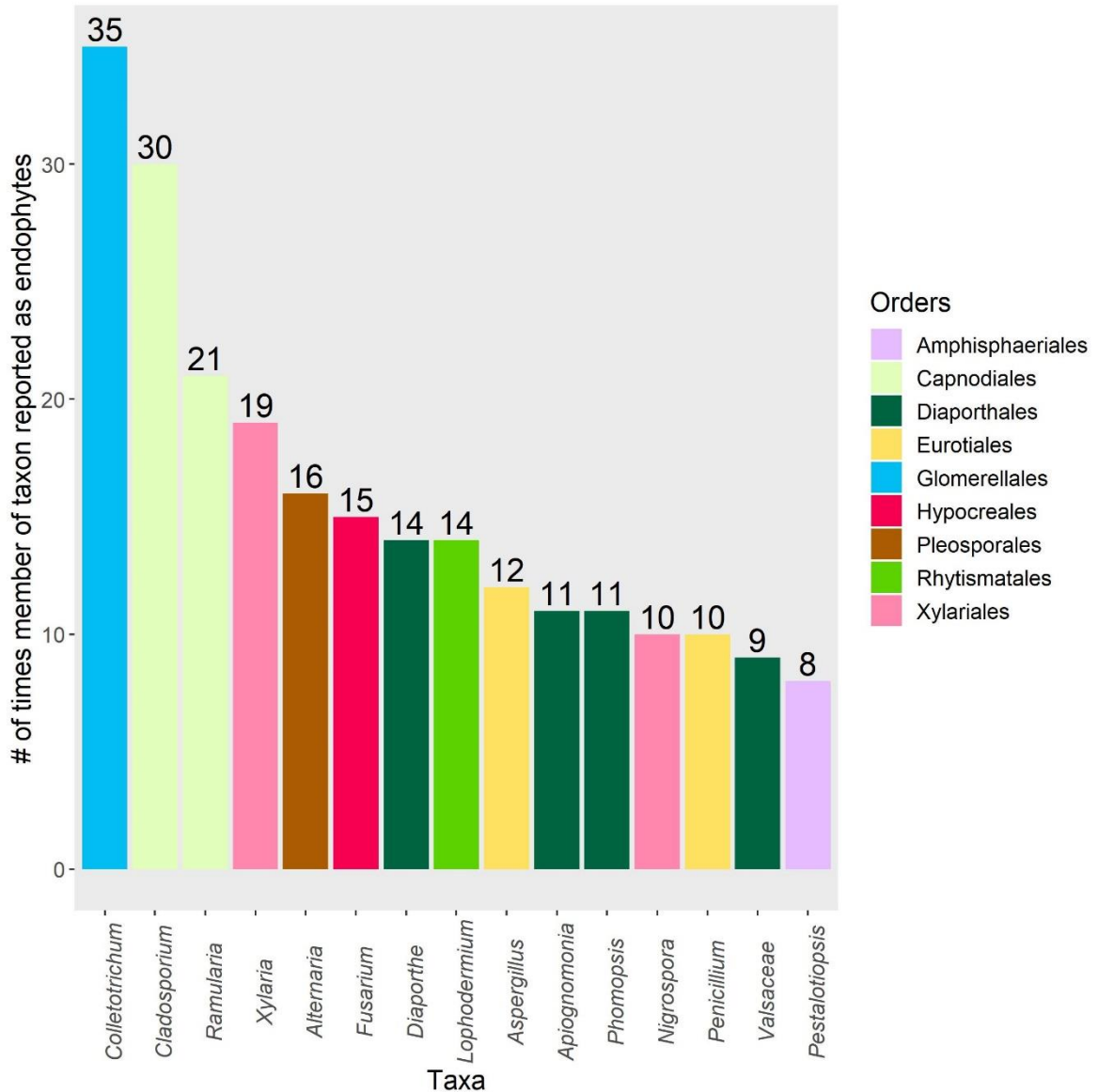


Figure 1.3. These are the 15 taxa most frequently reported as endophytic (leaves were surface-sterilized). Bars represent the number of times members of a taxon were reported. Taxa identified as “undetermined” or “Fungal sp.” are excluded.

## Conclusions

Do foliar endophytes matter in litter decomposition? Our review of the current literature suggests that it depends, given the complexity of abiotic and biotic factors influencing ecosystem processes. While it is important to point out that there were only 12 studies that directly tested foliar endophyte effects on litter decomposition—half of

which focused exclusively on Class 1 grass–endophyte interactions—our synthesis suggests that there is an overarching theme of mismatched focus among studies of litter decomposition. Endophytes are a hyperdiverse group of organisms that includes both bacteria and fungi, colonizing a wide range of host plants and plant tissues from tropical to boreal ecosystems. These microbes exist within the phyllosphere of their host plants and emphasize just one example from the tangled web of plant–microbe interactions. A host plant can represent a patch of occupiable habitat to an endophytic colonizer. Within that patch, there is competition with other endophytes and parasites, but also specific host–endophyte interactions. These specific interactions are, in turn, complicated by variation in the endophyte community composition and the host responses to abiotic factors. However, the resolution of the “patch” unit matters. For example, endophytes can vary spatially within the same host plant, shifting the occupiable patch unit to leaf. Similarly, endophytes within the local litter community can colonize neighboring host plants, shifting the occupiable patch unit to a localized area. Because endophyte–host interactions span more than a single level within the scale of an ecosystem—and ultimately represent just one of many poorly defined mechanisms and interactions between different scales within an ecosystem—endophyte communities present special challenges to predicting and understanding ecosystem processes like litter decomposition.

Several key contributing factors should be considered in litter decomposition studies when designing experiments or interpreting results (Figure 1.4). First, host–endophyte interactions are context-dependent, and may be influenced by the host’s genotype or origin and both abiotic and biotic factors that affect leaf chemistry (e.g.,

drought or herbivores). The presence of secondary compounds—whether produced as host defenses or induced by systemic Class 1 endophyte infection (in grasses)—tend to slow litter decomposition overall in terrestrial habitats. Similarly, symptomatic endophyte infections in litter typically slow decomposition dynamics by inhibiting subsequent colonization or directly breaking down recalcitrant compounds. However, saprotrophic endophytes can both exert priority effects on new colonizers and ameliorate available nutrients on litter; as common members of the foliar endophyte communities, their presence should be considered, especially in studies of microbial decomposer succession or community assembly on litter. Finally, access by detritivores is important in mediating litter decomposition rates, particularly when unpalatable or recalcitrant compounds are present and would otherwise retard microbial decomposition.

Most importantly, affecting all of these contributing factors is the type of ecosystem itself (e.g., riparian versus terrestrial, or grassland versus forest). Decomposition proceeds faster in aquatic environments due to a combination of factors including constant moisture and mechanical breakdown from moving water. Likewise, warm ambient temperatures and high humidity tend to accelerate decomposition in tropical forests. In grass systems, other factors, such as variable precipitation regimes and agricultural land use, must be considered, especially since the presence of toxic alkaloids can harm grazing livestock. Grasses also harbor Class 1 endophytes, which are vertically transmitted, as opposed to the horizontal transmission of Class 2 and 3 endophytes that are more prevalent in non-grasses. We found that climate type—even broadly categorized—resulted in significantly different groups of specialized taxa. Different plant

genera also tended to host specialist fungal taxa, in addition to many well-known generalist endophytes (e.g., *Phoma* and *Xylaria*). In summary, host plant and endophyte interactions can be significant factors in both terrestrial and aquatic litter decomposition rates and should be taken into consideration when interpreting results, but more studies specifically exploring foliar endophyte effects on litter decomposition are clearly needed.

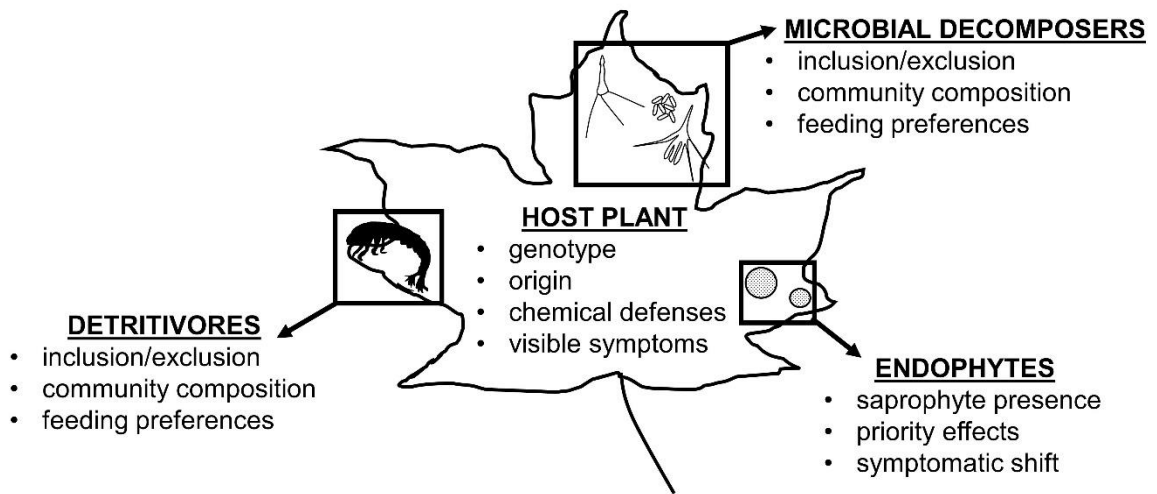


Figure 1.4. Infographic of factors that influence endophyte-mediated litter decomposition.

Supplementary Materials: The following are available online at [www.mdpi.com/2076-2607/8/3/446/s1](http://www.mdpi.com/2076-2607/8/3/446/s1), Table S1: Exported citation report generated from Web of Science for the keyword search: endophyte litter decomposition, Table S2: Leaf-associated taxa compiled from 29 studies and 1 review, and Table S3: Raw data collection sheet.

Author Contributions: DJB came up with the concept for this manuscript and critically revised it. ERW conducted the literature review and analysis. All authors have read and agreed to the published version of the manuscript.



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## Chapter 2

Differences in foliar endophyte communities of red alder (*Alnus rubra*) exposed to varying air pollutant levels

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### Abstract

In the Pacific Northwest, *Alnus rubra* (red alder) is a common deciduous tree species especially prevalent in riparian corridors and disturbed sites, including metropolitan areas undergoing land use changes and development. Importantly, red alder is also considered a bioindicator for ozone pollution, and, like all plants, harbors a diverse endophyte community that may interact with aerial pollutants. In this study, we surveyed foliar fungal endophyte communities (microfungi) in red alder leaves from the metropolitan area of Portland, Oregon, USA using culture-based techniques, and found that communities differed significantly by site. Our results suggest that fungal endophyte community composition in red alder leaves may be influenced in part by local air pollution sources, likely in conjunction with other site characteristics. As urban areas expand, more studies should focus on how the urban environment affects plant-microbe community ecology and endophyte-host interactions, as well as if there are long-term consequences for other ecosystem processes such as leaf litter decomposition.

Keywords: microbial community, diversity, air pollution, microfungi, urban ecosystems

## Introduction

Plant and microbial communities are sensitive to local environmental conditions, especially those resulting from anthropogenic sources. Elevated atmospheric CO<sub>2</sub> has been shown to interact with nitrogen availability and plant community species richness to influence community biomass (He et al. 2002), while warming negatively affected plant productivity in a grassland community (De Boeck et al. 2007). Soil microbial community composition in grassland plots have exhibited similar responses to warming (Frey et al. 2008), and were further affected by drought conditions (Sheik et al. 2011). Likewise, soil pollution has been found to negatively affect mycorrhizae—common root-associated plant symbionts (Cairney and Meharg 1999). The plant-microbe holobiont—the sum of a plant and all of its microbial symbionts—has been shown to critically determine the performance and resistance of plants to a variety of stresses including environmental pollutants (Li et al. 2012), yet still little information exists on interaction between pollutants and plant and microbial communities.

Plant responses to air pollution can vary with species, exposure duration, pollutant concentration, or a combination of factors, but may include the production of reactive oxygen species (Schützendübel and Polle 2002; Wohlgemuth et al. 2002) and phytoalexins (Rakwal et al. 2003). Further, beyond affecting the plants themselves, pollutants may have effects on the microbial communities associated with every plant which further can reduce plant growth and fitness (Porter and Sheridan 1981). Well-studied symbionts determining plant performance include nitrogen-fixing bacteria and mycorrhizal fungi that inhabit plant roots. However, microbial colonization can occur in

any part of the plant, and communities may vary temporally and by plant tissue type (Ek-Ramos et al. 2013; Younginger and Ballhorn 2017). A particularly diverse group of plant-associated microbes are foliar endophytic fungi.

Fungal endophytes are ubiquitous, asymptomatic plant symbionts (Stone et al. 2004; Rodriguez et al. 2009) that can affect host plant chemistry (Arnold 2007; Suryanarayanan 2013), competition and survival (Rudgers and Clay 2007; Aschehoug et al. 2012; McCulley et al. 2014), and as pioneer colonizers directly contribute to leaf litter decomposition (Lemons et al. 2005; LeRoy et al. 2011; Grimmer et al. 2012). Importantly, fungal endophytes have been shown to increase drought tolerance (Hesse et al. 2003; Khan et al. 2016), pathogen resistance (Waller et al. 2005; Lin et al. 2015; Busby et al. 2016), and metal tolerance in a variety of host plants (Ren et al. 2011; Li et al. 2012). Although endophytes may colonize any plant tissue, we focus here on foliar endophytes due to eventual contribution to ecosystem processes like litter decomposition and nutrient cycling.

In this study we studied the taxonomic diversity and frequency of endophytic fungi in red alder (*Alnus rubra*) in urban and rural sites that previously have been identified as differently exposed to various air pollutants including sulfur, lead, iron, and nickel (Gatziolis et al. 2016). Red alder is a common deciduous tree species occurring throughout the Pacific Northwest in riparian areas and moist forests mostly within 200 km of the Pacific coast. The species is of significant economic importance within the timber industry, exceeding the annual value of the dominant softwood species Douglas-fir (*Pseudotsuga menziesii*) (Warren 2009). Ecologically, red alder is a key species in the

Pacific Northwest. As an early pioneer tree, red alder colonizes disturbed sites and stabilizes eroded soils as well as river banks. Like other members of its genus, red alder is especially important in riparian corridors and disturbed sites (Harrington et al. 1994) due to its association with N<sub>2</sub>-fixing *Frankia* bacteria. Red alder in particular also serves as a bioindicator of ozone damage (Campbell et al. 2000; Bennett and Tkacz 2008) that may be useful in impacted urban areas, while other members of its genus have been shown to tolerate metal-polluted soils at other sites (Lee et al. 2009; Printz et al. 2013).

Here, we surveyed fungal endophyte communities in red alder leaves from the metropolitan area of Portland, Oregon, USA using culture-based techniques, and add to the findings of another culture-based study of red alder foliar endophytes by Sieber et al. (1991). The Portland-Metro area has poor air quality that has recently garnered media attention for a variety of industrial point sources of pollutants (Department of Environmental Quality 2017). The region's air pollution has previously been found to contribute to reduced diversity in lichen communities (Geiser and Neitlich 2007), and a recent study by Gatziolis et al. (2016) revealed possible pollution hotspots in several areas by measuring the concentrations of 22 elements in mosses sampled from across Portland. In addition to our culture-based survey, we used several publicly-available datasets to characterize the environments surrounding our metropolitan sample sites, including data provided by Gatziolis et al. (2016). By sequencing culturable endophytes in red alder exposed to varying influences of air pollutants, we draw connections between host growing environments and foliar fungal community composition. We hypothesized that the sampling sites closer to these pollution hotspots would have lower biodiversity,



and that fungal communities would differ among sites based on the local point sources of pollutants.

## Methods

Between 9-15 November 2016, red alder (*Alnus rubra*) leaves were sampled from three sites around the metropolitan area of Portland, Oregon (see Figure 2.1), and one site located in the Tillamook State Forest, approximately 65 kilometers west of downtown Portland (see Table 1.1). The metropolitan sites included Errol Heights Park (referred to as “Flavel”), Delta Park/Vanport Park & Ride (referred to as “Raceway”), and the Overlook neighborhood (“Overlook”), and were chosen according to their relative proximity to possible sources of air pollution suggested by several recent studies (Geiser and Neitlich 2007; United States Environmental Protection Agency 2011; Gatzolis et al. 2016; Donovan et al. 2016). The Tillamook State Forest site (“Tillamook”) served as a rural site. For Overlook we used the Portland Tree Inventory Project (Parks & Recreation 2017) to locate street trees, and then averaged the GPS locations of the trees for the overall site coordinates to maintain privacy of property owners.



Figure 2.1. Map of the four study sites in the Portland metropolitan area and part of the Oregon Coast Range; service layer credits: Esri, HERE, Garmin, USGS, Intermap, INCREMENT P, NRCan, Esri Japan, METI, Esri China (Hong Kong), Esri (Thailand), NGCC, OpenStreetMap contributors.

Table 2.1. GPS coordinates and descriptions of study sites across the Portland metropolitan area.

Site	Coordinates	Elevation (m)	Description
Flavel	45°27'55.1"N, 122°36'36.0"W	59	Errol Heights Park is a small, wooded preserve between an industrial plant near SE Flavel Street and a busy intersection (near SE Johnson Creek Boulevard).
Raceway	45°35'49.6"N, 122°41'13.6"W	5	The Delta Park/Vanport Park & Ride in North Portland is located at the entrance to the Portland International Raceway, and adjacent to Interstate 5. This site is within a few kilometers of the Columbia River Crossing and sees heavy traffic volumes daily.
Overlook	45°33'33.8"N, 122°41'14.2"W	63	Overlook is a North Portland neighborhood bordered by the Willamette River, and busy portions of Interstate 5 and Interstate 405. The neighborhood is adjacent to shipyards and industrial areas along the river.

Tillamook	45°37'25.4"N, 123°22'35.2"W	483	The Tillamook State Forest is part of the Coast Range and surrounded by rural communities.
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At each site, five trees were selected and three leaves were collected from each using clean gloves doused with 95% ethanol, and stored in paper envelopes. We randomly selected leaves without visible lesions or other defects when possible on branches between 1 and 2.5 m from the ground. Leaves were no more than 15 cm long from apex to petiole and all trees had DBH < 17.5 cm. Samples were returned to the lab and surface-sterilized in a laminar flow cabinet using the following procedure: dip in 0.1% Tween 20, 30-second rinse in DI water, 10-second bath in 95% ethanol, 2-minute bath in 0.5% bleach (8.25%), and 2-minute bath in 70% ethanol, followed by air-drying in the cabinet (Younginger and Ballhorn 2017). Leaves were then sectioned into approximately 5 mm diameter pieces with flame-sterilized scalpels; four pieces were plated on malt extract agar (MEA) and sealed with Parafilm, while the remaining leaf parts were placed into Eppendorf tubes and stored at -80°C until further analysis by direct sequencing of the ITS region. All samples were plated or processed for storage less than 24 hours after collection.

Once leaves were plated, they were checked every few days for new fungal growth. These isolates were transferred to new MEA plates until they were axenic (i.e. no new morphotypes appeared), at which point DNA was extracted using the Sigma REDExtract-N-Amp™ Tissue Kit (St. Louis, MO). DNA was amplified using the primer pair ITS1F and ITS4, and 34 cycles of 94°C, 50°C, and 72°C for 1 minute each, followed

by a final extension at 72°C for 10 minutes. PCR products were sent to Functional Biosciences (Milwaukee, WI) for Sanger sequencing, after which the sequencing data were cleaned and analyzed using Geneious 10.2.3. Cleaned sequences were checked against both NCBI GenBank and UNITE to determine identity at the lowest possible taxon, using 97% similarity as the assignment threshold. If GenBank accession numbers differed between NCBI and UNITE, the UNITE accession and taxon were used.

Gaziolis et al. (2016) overlaid a grid system on the Portland metropolitan area, sampled mosses from within these sites, and measured the concentrations of 22 elements that had accumulated in the moss biomass. We used coordinates from those sampling locations to first determine the ten closest locations to each of our metropolitan alder-sampling sites, averaged the concentrations for each of the 22 elements measured in moss biomass collected from those locations, and then examined between-site differences in elemental concentrations previously measured in moss in the context of our alder sites. Pollution point-sources identified by Donovan et al. (2016) in a similar study of epiphytic mosses were also considered during site selection. We attempted to use air quality data provided by the Environmental Protection Agency (EPA) to find monitoring stations adjacent to our sampling sites, although comparable data between those three stations (EPA sites 0080, 2001, and 0246 in Multnomah County, OR) were limited (United States Environmental Protection Agency 2017). Traffic volume tables produced by the Oregon Department of Transportation provided average daily traffic on each of the major roadways neighboring our sites (Transportation Data Section 2016). Comparisons of elemental concentrations previously measured in moss and traffic volume were made to

further interpret fungal community data against the background of pollution and to identify possible trends for future studies.

## Statistics

All statistical analyses were performed using R version 3.4.1. Community data were analyzed with the “vegan” and “indicspecies” packages (Cáceres and Legendre 2009; Oksanen et al. 2017). All analyses were performed after operational taxonomic units (OTUs) were combined by common species assignments (e.g. all *Alternaria alternata* OTUs with similarity  $\geq 97\%$  were combined into a single *Alternaria alternata* column within the community matrix, while OTUs with similarity  $<97\%$  were combined as *Alternaria* sp.). Two extreme outliers (i.e. two leaf samples), both from the Overlook site, were removed because they were singletons that prevented examination of beta diversity. Non-metric multidimensional scaling (NMDS) with Bray-Curtis distances was used to model community composition overlap between sites, and permutational multivariate analyses of variance (PERMANOVA) were used to examine statistical differences, also with Bray-Curtis distances and using 999 permutations. A Bray-Curtis dissimilarity matrix represents the differences in community composition among sites (0 = identical species composition, and 1 = no shared species). PERMANOVA then assumes that random permutation of the values in the matrix (e.g. 999 times) results in the sites having the same calculated centroids, meaning that community composition does not differ significantly among sites. To determine pairwise differences, PERMANOVAs were run for each site pair and the p-values adjusted with Bonferroni correction. Shannon diversity indices were also calculated by site after determining relative abundance with

the “phyloseq” package (McMurdie and Holmes 2013). Indicator species analysis was performed to determine if particular taxa were significantly associated with specific sites or site combinations (Dufrière and Legendre 1997; De Cáceres et al. 2010). Analysis of variance (ANOVA) and Tukey HSD post-hoc tests were used to assess differences in 22 elemental concentrations among our study sites, which was done by using measurements from the ten closest moss locations to our alder study sites (i.e. each of our Portland sites were comprised of  $n = 10$  locations from Gatziolis (2016). If data did not meet the assumptions for ANOVA even after transformation, nonparametric Kruskal-Wallis tests and Dunn post-hoc tests were used.

#### Maps and Venn Diagram

Figure 2.1 was generated with ArcGIS Desktop 10.5, and Figure 2.6 was generated in R with the ggmap package (Kahle and Wickham 2013). Figure 2.4 was generated in R with the VennDiagram package (Chen and Boutros 2011).

#### Results

Fungal endophyte communities isolated from *Alnus rubra* leaves differed significantly across sites in the Portland metropolitan area during November 2016. A total of 267 OTUs representing 56 assigned species and 36 assigned genera were isolated (Supplementary Table 2.), averaging  $3.14 \pm 1.67$  species per leaf, with varying relative abundances by site (Supplementary Figure 2.). The number of different assigned species isolated from each tree varied from 1 (Tillamook; Figure 2.2) to as many as 8 (Raceway) while the average frequency of isolation for all assigned species across all sites was  $5.6 \pm$

5.37%. *Epicoccum nigrum* (15 leaves), *Asteroma alneum* (12 leaves), and *Cladosporium allicinum* (10 leaves) were the three most frequently isolated assigned species but occurred unevenly among sites. *Epicoccum nigrum* occurred mostly in leaves from Raceway (7/15 leaves or 46.7% of all occurrences), *A. alneum* occurred frequently in leaves from Tillamook (41.7%) and also Flavel (33.3%), and *C. allicinum* was found in leaves from Raceway (40.0%) and Overlook (60.0%). The species accumulation curve did not level off, which suggests that sampling more leaves, trees, and/or sites would reveal new species (Figure 2.3).

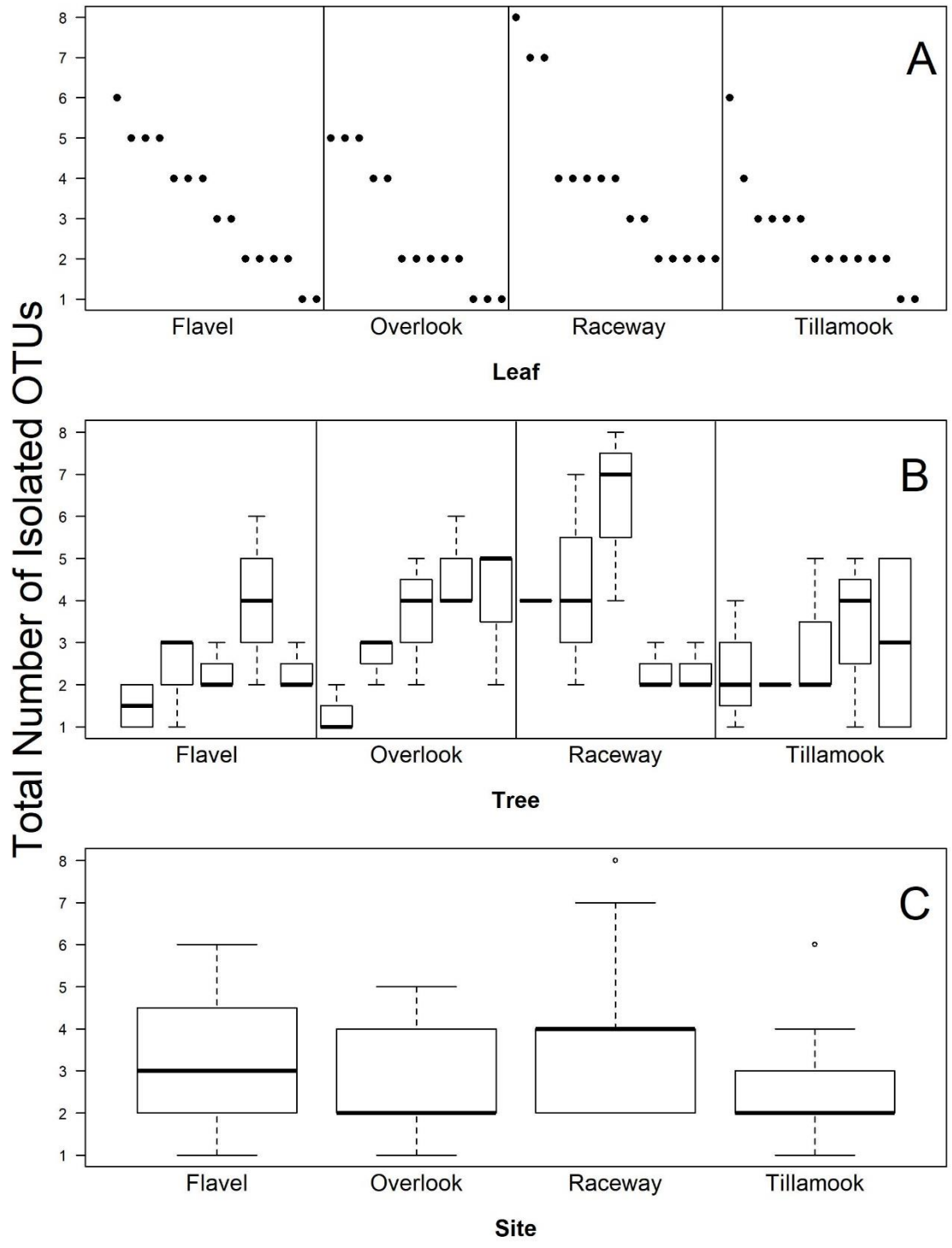


Figure 2.2. Numbers of assigned species (OTUs) cultured at the leaf (A), tree (B), and site (C) level demonstrate variability between sampling units.



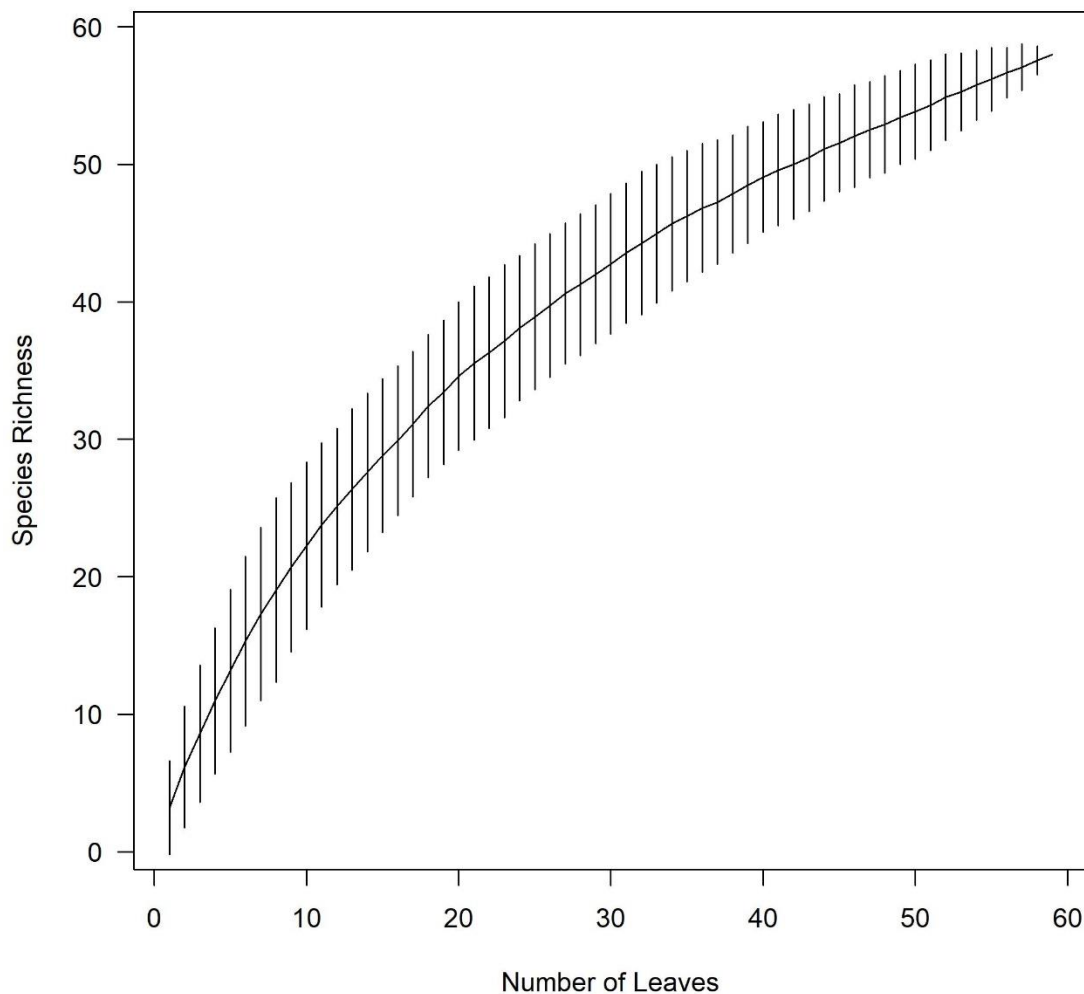


Figure 2.3. Species (OTUs) accumulation curve showing that a larger sample of leaves would have yielded additional fungal taxa.

Shannon diversity indices were only marginally different among sampling sites (Kruskal-Wallis,  $\chi^2=7.35$ ,  $df=3$ ,  $p=0.06$ ); 19, 27, 28, and 23 species were isolated from leaves from Tillamook, Flavel, Raceway, and Overlook, respectively. Of these species, four were isolated exclusively from Tillamook samples: *Diaporthe cotoneastri*, *Cladosporium macrocarpum*, *Colletrotrichum gloeosporioides*, and *Trichoderma harzianum*; seven exclusively from Overlook samples: *Chaetomium* sp., *Boeremia*

*exigua*, *Cryptostroma corticale*, *Lophiostoma* sp., *Penicillium glabrum*, and *Podospora curvicolla*; nine exclusively from Raceway samples: *Sordaria humana*, *Colletotrichum salicis*, *Colletotrichum* sp., *Pyrenophora erythrospila* (as *Drechslera erythrospila*), *Cryptodiaporthe pulchella*, *Curvularia inaequalis*, *Ophiognomonium ibarakiensis*, *Ophiognomonium* sp., and *Preussia* sp.; and eleven exclusively from Flavel samples: *Diaporthe viticola*, *Diaporthe nobilis*, *Phoma* sp., *Botryosphaeria stevensii* (as *Diplodia mutila*), *Glomerella acutata* (as *Colletotrichum acutatum*), *Gnomoniopsis idaeicola*, *Leptosphaeria* sp., *Nigrospora oryzae*, *Phaeosphaeriaceae* sp., *Pseudopithomyces chartarum* (as *Pithomyces chartarum*), and *Ramularia archangelicae* (Figure 2.4).

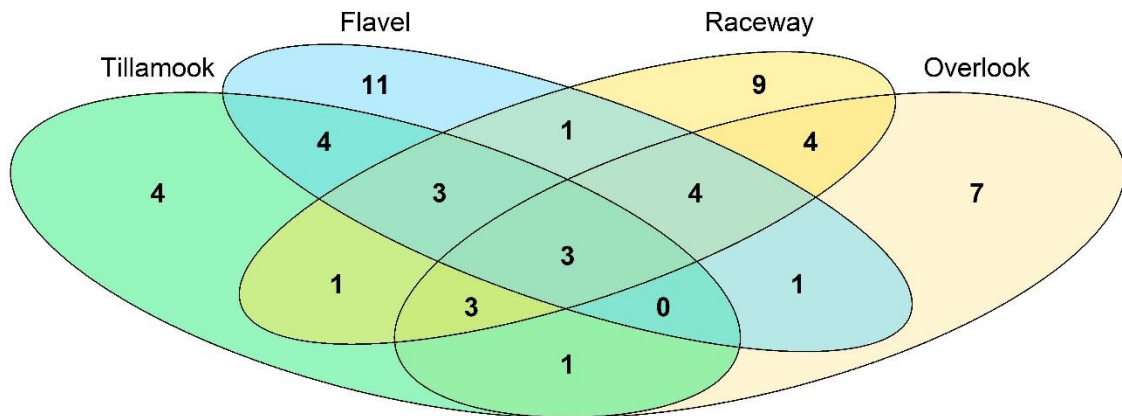


Figure 2.4. Venn diagram of number of taxa shared among and between our four study sites.

Fungal endophyte community composition differed significantly by site (PERMANOVA,  $F_{3,56}=2.86$ ,  $p=0.001$ ), illustrated in part by NMDS ordination that demonstrates the high variation within samples and some overlap among sites (Figure 5). All pairwise site comparisons of endophyte communities were significantly different from one another, except between Tillamook and Flavel which were marginally different ( $p=0.06$ ; Table 2.2). Tillamook and Flavel also shared indicator species within the genus

*Diaporthe* (Table 2.3) and Raceway and Overlook shared *C. allicinum* as an indicator species, while two *Alternaria* species were positively correlated with Raceway only. Several species that approached significance as indicators for Overlook, Flavel, and Raceway are included in Table 2.3.

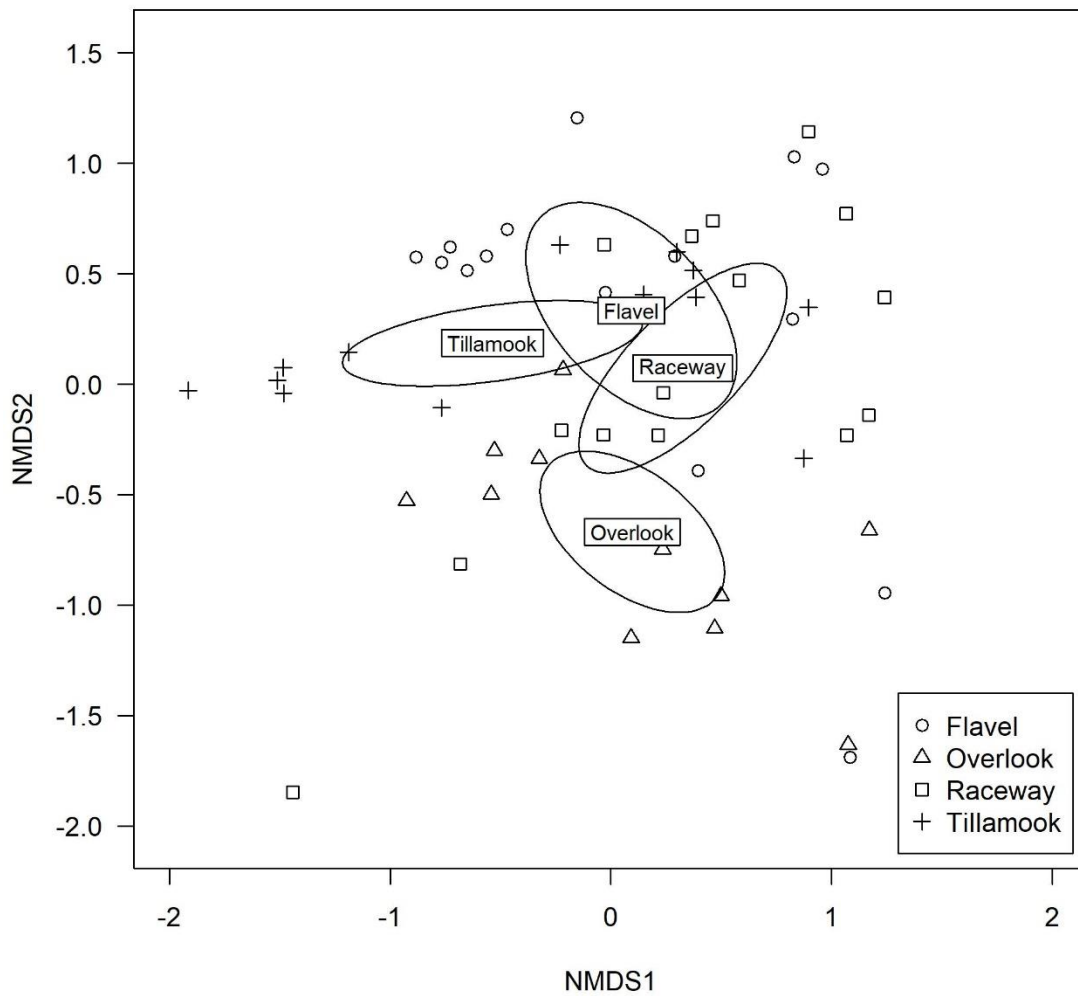


Figure 2.5. Non-metric multidimensional scaling ordination of fungal endophyte communities of *Alnus rubra* across the Portland metropolitan area; ellipses represent 95% confidence intervals based on the standard error.

Table 2.2. perMANOVA results of pairwise site comparisons of fungal endophyte communities, following Bonferroni adjustment.

Site Pair	F	R <sup>2</sup>	p-value
Tillamook-Flavel	2.41	0.082	0.060
Tillamook-Raceway	3.43	0.113	0.006
Tillamook-Overlook	3.59	0.126	0.006
Flavel-Raceway	2.46	0.081	0.024
Flavel-Overlook	2.81	0.098	0.012
Overlook-Raceway	2.54	0.089	0.030

Table 2.3. Results of indicator species analysis by study site, showing the dominant members of site and site-combination communities.

Species	Site	Indicator Value	p-value
<i>Alternaria alternata</i>	Raceway	0.545	0.005
<i>Alternaria arborescens</i>	Raceway	0.520	0.020
<i>Chaetomium</i> sp.	Overlook	0.392	0.065
<i>Cladosporium allicinum</i>	Raceway & Overlook	0.598	0.005
<i>Cladosporium</i> sp.	Overlook & Flavel	0.423	0.095
<i>Diaporthe cotoneastri</i>	Tillamook	0.598	0.005
<i>Diaporthe rudis</i>	Flavel	0.514	0.035
<i>Diaporthe viticola</i>	Flavel	0.447	0.070
<i>Ophiognomonium intermedia</i>	Tillamook	0.505	0.035
<i>Sordaria humana</i>	Raceway	0.447	0.060

After clustering sites from the Gatzolis et al. (2016) dataset to coincide with our three metropolitan sampling sites, we found differences in several previously measured elemental concentrations in moss biomass among our study sites. All 22 elemental concentrations were tested, but only significant results are shown (Table 2.4; P and K are included in Figure 2.6 as well). In general, mosses from areas clustered around Raceway and Overlook had accumulated insignificantly different amounts of S (%), Mn (mg/kg), Ni (mg/kg), and Zn (mg/kg) (Tukey HSD,  $p > 0.5$ ). Mosses from areas clustered around Flavel had insignificantly different amounts of S and Mn content compared to Overlook (Tukey HSD,  $p > 0.06$ ), significantly lower amounts of S and Mn content compared to

mosses from areas clustered around Raceway (Tukey HSD,  $p < 0.05$ ), and significantly higher Ni content than either mosses from areas clustered around Overlook or Raceway (Tukey HSD,  $p < 0.0001$ ). Mosses from areas around Overlook had significantly higher levels of Zn compared to mosses from around Flavel. Both Pb and Cu concentrations in mosses were significantly lower in areas clustered around Flavel compared to mosses clustered around Overlook (Dunn test,  $p < 0.01$ ). Areas clustered around Raceway had significantly higher concentrations of Cu in mosses compared to mosses clustered around either Overlook or Flavel (Dunn test,  $p < 0.01$ ). Areas clustered around Flavel had significantly lower concentrations of Fe in mosses than areas clustered around either Raceway or Overlook (Tukey HSD,  $p < 0.01$ ), which had insignificantly different concentrations.

Table 2.4. ANOVA results for previously measured elemental concentrations in moss biomass, showing differences between the current study's sites.

Element	$F_{2,26}$	p-value
S	8.64	0.00133
Fe	5.78	0.00841
Mn	4.11	0.028
Ni	42.1	<0.0001
Zn	6.11	0.00668

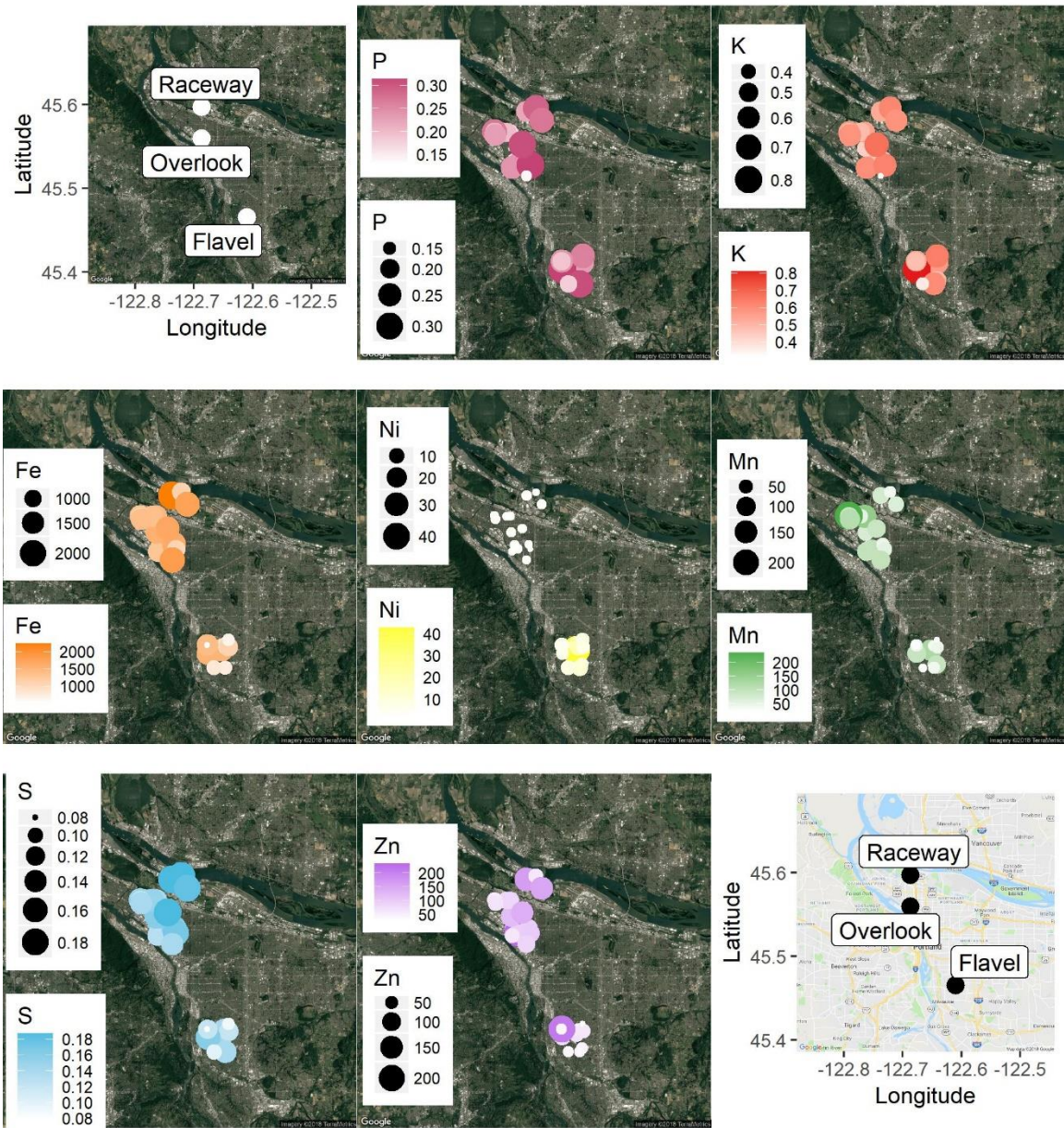


Figure 2.6. Maps of the Portland area with our three urban sites labelled (top-left: satellite image; bottom-right: street map), followed by seven panels of selected elemental concentrations (P, K, Fe, Ni, Mn, S, Zn) measured in moss biomass collected from the ten closest locations to each of our metropolitan alder-sampling sites.

## Discussion

In this study, we determined that foliar fungal endophyte communities may be structured in part by proximity to specific point sources of air pollution in sites across a metropolitan area. We did observe high variability in isolation frequency among both

trees and sampling sites, underscoring the dynamic nature of endophyte communities, which can vary temporally and spatially within host plant tissues (Ek-Ramos et al. 2013; Younginger and Ballhorn 2017). While overall community composition varied by site, the most abundant species were recovered from multiple sites and represent a considerable population of generalist endophytes (Stone et al. 2004), including several species in *Alternaria* and *Cladosporium* as well as *Diaporthe*, another common endophyte genus that contains many pathogenic species. Our findings corroborate many of the fungi identified in the earlier survey of red alder by Sieber et al. (1991), but also suggest that a large component of the foliar fungal endophyte community widely occurs among hosts and also within different host tissues, lending support to the concept of a “core microbiome” (Shade and Handelsman 2012) at least within plants although next-generation sequencing analyses will better identify core OTUs. A majority of the fungal genera, if not species, isolated in this study were also found in stem tissues of Spanish olive trees (Fisher et al. 1992), in twigs from ash, oak, and beech (Griffith and Boddy 1990), in various tissues of *Arabidopsis thaliana* (Junker et al. 2012), and in nine different tree species in urban and rural sites in Japan (Matsumura and Fukuda 2013). Shared genera include *Aureobasidium*, *Botrytis*, *Cytospora*, *Epicoccum*, *Phoma*, *Phomopsis*, and *Sordaria*. Additionally, this study may be the first to report the presence of *Pyrenophora erythrospila* in *Alnus*.

Contrary to earlier studies of urban versus rural endophyte communities (Jumpponen and Jones 2009, 2010; Matsumura and Fukuda 2013), we only found a marginally significant difference between fungal community diversity between our study

sites, which included three metropolitan locations and one forested location. Previous work anticipates lower fungal endophyte diversity in plants growing in polluted soils at former industrial sites (Lappalainen et al. 1999; Renker et al. 2005; Likar and Regvar 2009). However, while this effect may be due to small sample size, the trends in the number of species isolated by site appear to be opposite of our expectations, given that about 30% more species were cultured from the Raceway and Flavel sites compared to the Tillamook site. Given that Wężowicz et al. (2014) also reported higher diversity in more polluted sites, these trends may be driven in part by local pollution sources or dispersal limitation; additionally, priority effects may be at play in compositional differences, as well as other environmental factors such as elevation. If pollution is a major contributor, an obvious source of pollutants in our sampling sites is vehicle traffic; all of our sites were located along roadways, and thus represent an impact gradient for vehicle traffic, with Raceway and Overlook receiving considerably more emissions than Flavel or Tillamook because of their proximity to Interstate 5 (< 1000 m). Additionally, dominance effects may have influenced diversity patterns for Tillamook samples. For example, indicator species characterized the dominant members of their respective site communities (Table 2.2), and three out of the five trees sampled at the Tillamook site had assemblages that were dominated by pathogenic *Diaporthe* species (Figure 2.2) that may have inhibited other species from colonizing leaf tissue; members of the genus have been shown to produce antimicrobial compounds (Tanney et al. 2016). Fewer species were also isolated from the Overlook site, where assemblages were similarly dominated by other pathogenic fungi in *Cladosporium*, of which species have been documented as



antagonistic towards other pathogens (Busby et al. 2016). Considering the high variability among leaves from the same tree, and trees in the same site, a larger survey may clarify patterns in both alpha and beta diversity.

While we did not directly measure air pollutants in our study, the publicly-available dataset provided by Gatziolis et al. (2016) uses mosses as bioindicators of air pollution in the same metropolitan area as our study. We acknowledge that the moss bioaccumulation data were collected several years before our sample collection period—however, we contend that the trends identified by Gatziolis (2016) remain applicable to our sites, and merely suggest historical environmental differences among our sites that may be underlying variation in fungal endophyte community composition, in addition to other environmental factors. Raceway and Overlook are adjacent to Interstate 5 (a major highway in the region), and mosses collected from near these sites had accumulated elevated levels of sulfur, iron, and lead relative to Flavel, the only other metropolitan site in our study. *Cladosporium* and *Alternaria* species were most common in these sites, which may suggest that these fungi are more tolerant of pollution from vehicle emissions. Likar and Regvar (2009) observed the same fungal endophytes in *Salix caprea* L. growing in both polluted and non-polluted sites, although they hypothesized that the relative tolerances of species likely differed according to the local environment. Our study was not designed to test metal tolerances of isolated fungi, but Li et al. (2012) found that an *Alternaria* isolate was not inhibited by zinc-supplemented media, which may coincide with elevated levels of zinc in mosses collected near Overlook where *Alternaria* was a dominant genus.

## Conclusion

Fungal endophyte communities are often dynamic and are known to show seasonal, spatial, and functional variation in their host plants (Arnold 2007; Scholtysik et al. 2013; Younginger and Ballhorn 2017). Red alder is an economically and ecologically important tree species in the Pacific Northwest but beyond its root associated microbes (*Frankia* bacteria and endomycorrhizal fungi) little is known about the microbial communities it harbors and their responses to environmental conditions. Our results suggest proximity to different point sources of air pollution—potentially in conjunction with other site characteristics—partially influences fungal endophyte community composition in red alder leaves. Future studies should quantitatively examine relationships between endophyte diversity in red alder and sources of air pollution.

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### Chapter 3

#### Culturable fungal endophyte communities of primary successional plants on Mount St. Helens, WA, USA

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#### Abstract

**Background:** While a considerable amount of research has explored plant community composition in primary successional systems, little is known about the microbial communities inhabiting these pioneer plant species. Fungal endophytes are ubiquitous within plants, and may play major roles in early successional ecosystems. Specifically, endophytes have been shown to affect successional processes, as well as alter host stress tolerance and litter decomposition dynamics—both of which are important components in harsh environments where soil organic matter is still scarce.

**Results:** In order to determine possible contributions of fungal endophytes to plant colonization patterns, we surveyed six of the most common woody species on the Pumice Plain of Mount St. Helens (WA, USA; created during the 1980 eruption)—a model primary successional ecosystem—and found low colonization rates (<15%), low species richness, and low diversity. Furthermore, while endophyte community composition did differ among woody species, we found only marginal evidence of temporal changes in community composition over a single field season (June–September).

Conclusions: Our results indicate that even after a post-eruption period of 40 years, foliar endophyte communities still seem to be in the early stages of community development, and that dominant pioneer riparian species Sitka alder (*Alnus viridis* ssp. *sinuata*) and Sitka willow (*Salix sitchensis*) may be serving as important microbial reservoirs for incoming plant colonizers.

Keywords: plant-microbe interactions, ecosystem processes, microbe-microbe interactions, fungi, bacteria



## Background

Endophytes are ubiquitous plant symbionts most simply defined as microbes spending the majority of their lifecycles living asymptotically within host plant tissues [1, 2]. Fungal endophytes specifically have been most extensively studied in agriculturally-significant grass hosts, but have also been reported in important forest trees such as red alder [3, 4], maple [5, 6], oak [7, 8], and various conifers [9, 10]. Like other types of symbionts—e.g. root-associated mycorrhizal fungi or nitrogen-fixing *Frankia* and rhizobia—foliar endophytes can play critical roles in host plant fitness by affecting disease or pest resistance [11], drought tolerance [12], and overall competitive ability [13]. In addition to direct effects on host plants, foliar endophytes are also capable of altering plant community assembly [14, 15] and afterlife effects (reviewed in Wolfe and Ballhorn 2020) that can eventually affect ecosystem processes and nutrient cycling. These host–endophyte dynamics are especially important to consider in early successional ecosystems where effects may be disproportionate due to harsh environmental conditions, limited propagules, and sparse host plant communities.

While there have been many studies of primary succession of vascular plants (e.g. Chapin et al. 1994; Grishin et al. 1996; del Moral 1998; Titus et al. 1998) and even their associated mycorrhizae [21–23], virtually no information exists on the diversity, temporal variation, and turnover of foliar endophyte communities colonizing these plants. Primary successional ecosystems are characterized by barren substrate produced by natural disasters or climate change, such as volcanic eruptions or receding glaciers [24, 25]. These ecosystems are harsh and nutrient-limited, and may take centuries to develop into

forests [26]. In these sparse environments, fewer propagules exist for microbial colonization of pioneering plant species [27]. Since microbial communities are critical to ecosystem function, a slow development thereof has significant implications for succession [28]. Dispersal limitation and environmental filters further reduce the pool of successful colonists in successional landscapes [29]. However, community assembly rules suggest that priority effects may alter successional trajectories of some species, which may contribute to changes in other communities or biogeochemical processes within an ecosystem. In a culture-based experiment that consisted of inoculating wooden disks with fungi and varying the initial colonizers to study how arrival order affected community composition and decomposition rates, Fukami et al. [30] emphasized the importance of assembly history in connecting community and ecosystem ecology. Consequently, a primary successional system is ideal for understanding how initial endophyte communities become established.

The Pumice Plain of Mount St. Helens has served as a model for studying primary succession since its catastrophic eruption in 1980 (Wood & del Moral 1987; del Moral 1999). Old growth forest was obliterated and replaced with a 20 km<sup>2</sup> layer of sterile pumice up to 200 m deep, creating a landscape that resembled the surface of the moon. A few years following the eruption, del Moral [33] observed just 3% vegetation cover on an exposed ridge affected by the pyroclastic flows, and by 1995, Lawrence and Ripple [34] estimated that vegetation cover on the Pumice Plain remained at 0-10% cover, with some areas of 11-20% cover. Even after forty years following the eruption, the area remains largely barren—especially of woody species, except for sparse, stunted conifers and

thickets of shrubs along riparian corridors [32, 35]. Given that the establishment and prevalence of plant–endophyte interactions are still widely unknown in these types of new, anthropogenically-undisturbed ecosystems, we surveyed six major woody species on the Pumice Plain and characterized their culturable fungal endophyte communities over a three-month field season to answer the following questions: 1. How diverse are culturable foliar endophyte communities in a successional ecosystem after four decades of recovery? 2. Do these communities exhibit host specificity? and 3. Do endophyte communities in woody deciduous and coniferous host plants show temporal variation in this successional environment?

## Results

We isolated foliar fungal endophytes from the six most abundant woody species on the Pumice Plain of Mt. St. Helens throughout the 2017 summer field season, and found species-specific endophyte community composition despite overall low colonization rates. A total of 40 OTUs were isolated from 113 leaf samples (14.1% of total  $n=801$  leaves; 19.4% of surveyed trees), representing 18 families and 23 genera. The highest number of OTUs were isolated from Sitka alder (22), followed by Sitka willow (14), cottonwood (10), Douglas fir (11), western hemlock (7), and noble fir (1). All of the OTUs belonged to Ascomycota except for one member of Basidiomycota, and included a total of four classes: Dothidiomycetes (16 OTUs), Pezizomycetes (13 OTUs), Sordariomycetes (9 OTUs), Eurotiomycetes (1 OTU), and Agaricomycetes (1 OTU). Frequencies of isolation ranged from 45.13% (OTU01; *Arthrinium* sp.) to 0.885% for singletons (18 OTUs), averaging  $3.12 \pm 7.12\%$  (mean  $\pm$  SD) over all cultured OTUs. As

anticipated, the species accumulation curve showed that sampling more leaves would have revealed additional taxa (Figure 3.1).

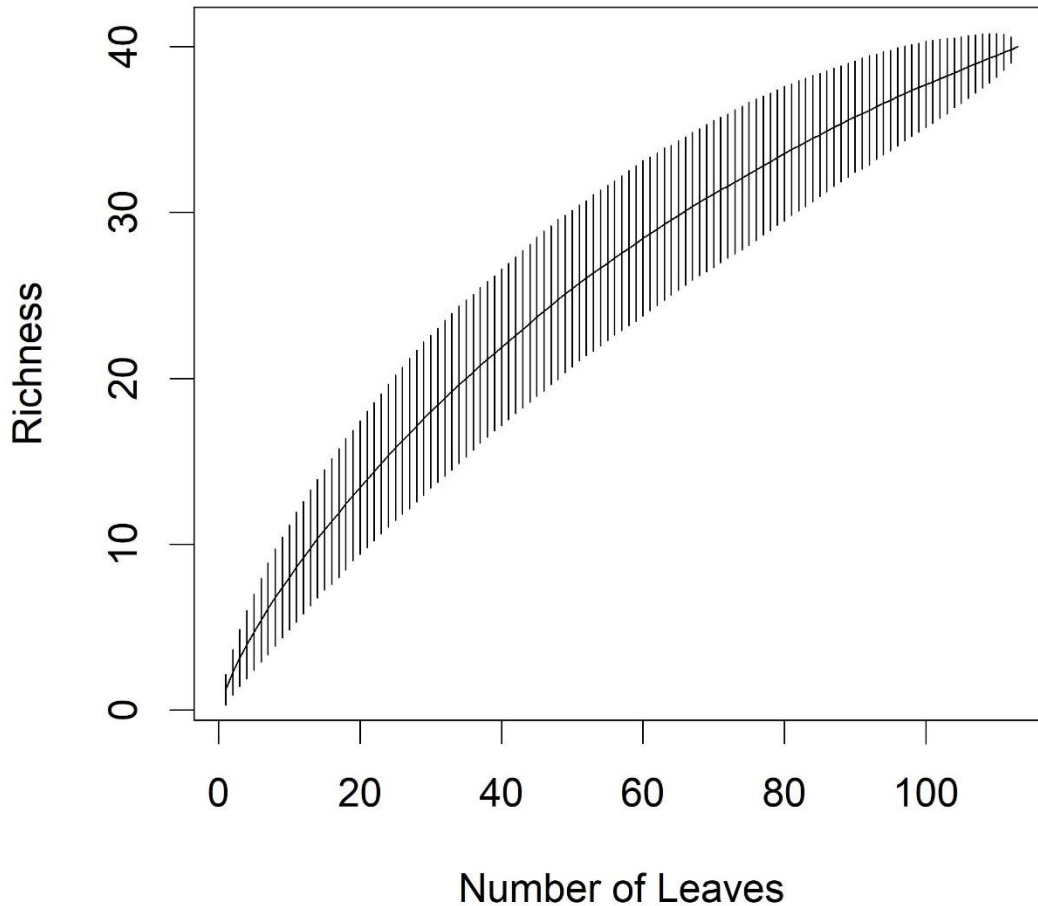


Figure 3.1. Species (i.e., OTU) accumulation curve indicating that sampling more leaves would have detected additional culturable taxa.

While the fungal endophyte community composition differed significantly among species (PERMANOVA,  $F_{5,112}=1.7781$ ,  $p=0.005$ ; Figure 3.2), it did not vary over across months during a single summer (PERMANOVA,  $p=0.091$ ; Figure 3.3A). Tree type (i.e. conifer vs. deciduous) however did have a significant effect on community composition

(PERMANOVA,  $F_{1,112}=1.9764$ ,  $p=0.035$ ; Figure 3.3B). The endophyte community composition also varied significantly between deciduous host species (PERMANOVA,  $F_{2,89}=2.4337$ ,  $p=0.004$ ), but only marginally over time (PERMANOVA,  $F_{2,89}=1.4523$ ,  $p=0.088$ ). Overall OTU richness ( $1.248 \pm 0.492$ ) and Shannon diversity indices ( $0.166 \pm 0.311$ ) were low, and no significant differences existed either among species or over time for either the entire community or the deciduous subset. Over three-quarters of leaves yielded a single OTU, while the remaining leaves had at least two, including one cottonwood leaf (Site 62, harvested in September) that had the highest species richness of 4.

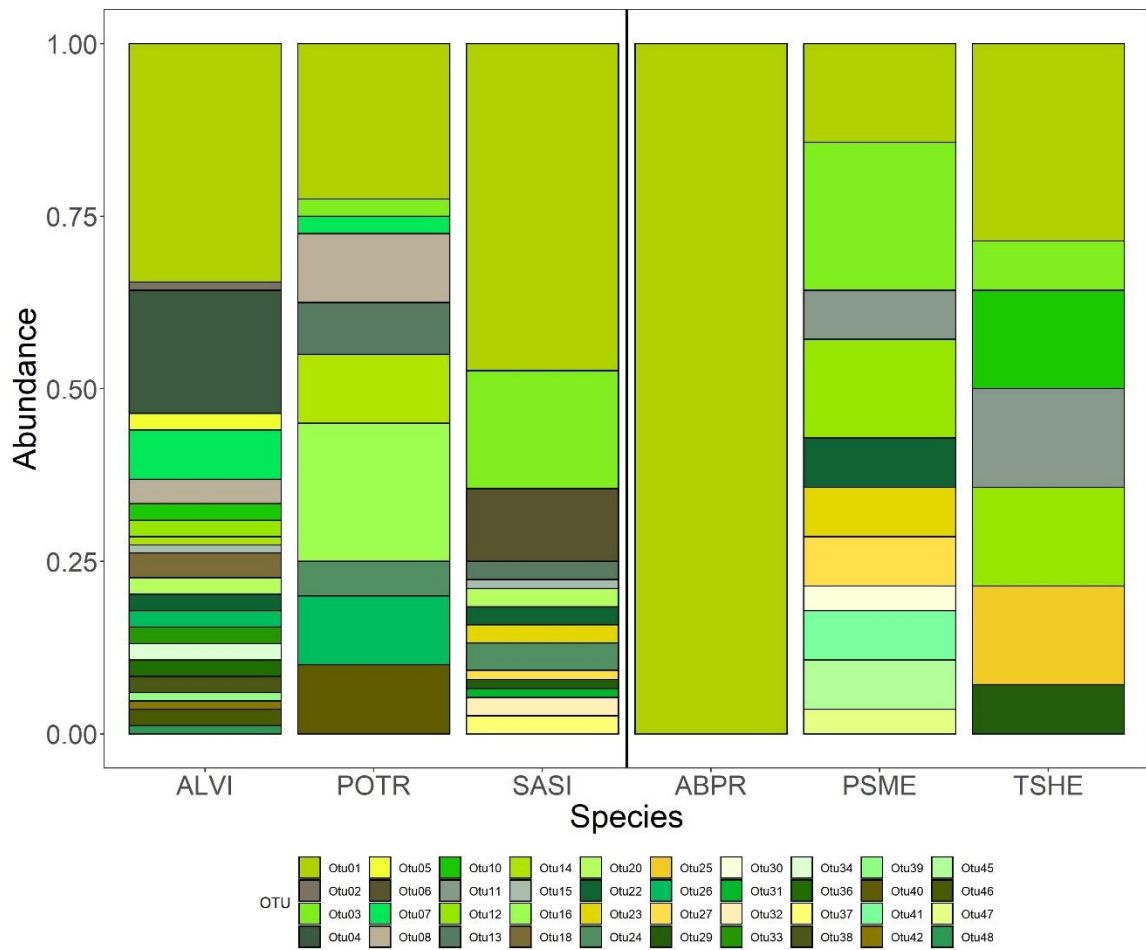


Figure 3.2. Relative abundance of culturable OTUs among the six prominent woody species colonizing the Pumice Plain.

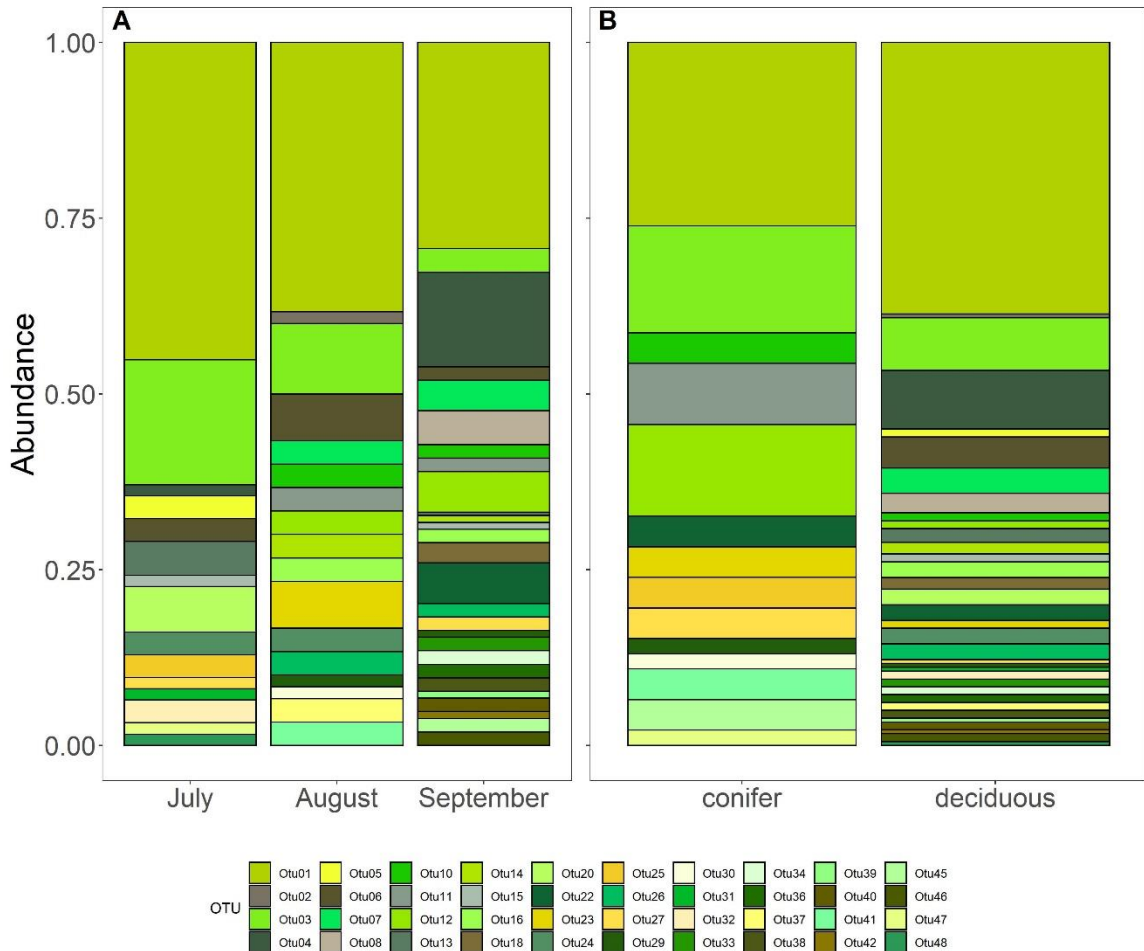


Figure 3.3. Relative abundance of culturable OTUs (A) over the three-month field season, and (B) between host tree type.

Only OTU16 (*Tricharina praecox*) was significantly associated with a particular host plant species (cottonwood; indicator species analysis,  $IV=0.447$ ,  $p=0.040$ ), while OTU11 (unclassified Pleosporales sp.;  $IV=0.295$ ,  $p=0.045$ ) and OTU12 (*Sporormiella intermedia*;  $IV=0.347$ ,  $p=0.035$ ) were associated with conifer hosts. OTU04 (*Melanconis italica*) was almost exclusively isolated from samples collected in September ( $IV=0.357$ ,  $p=0.045$ ). Within the alder-willow subset, OTU03 (*Pseudoplectania episphagnum*) and OTU06 (Pyronemataceae sp.) were associated with Sitka willow as a host species

(IV=0.459, p=0.005; IV=0.324, p=0.035, respectively), while OTU04 (*Melanconis italica*) was associated with Sitka alder hosts (IV=0.463, p=0.010).

## Discussion

Our study revealed that after nearly four decades, culturable foliar fungal endophytes have been slow to colonize major woody host species on the early primary successional Pumice Plain of Mount St. Helens. In contrast, vegetation communities stabilized on the nearby Plains of Abraham after just two decades [36]. Overall, on the Pumice Plain we observed colonization rates that were considerably lower than those that have been recorded for both deciduous and coniferous trees in the region using similar or identical methods [3, 4, 9]. For example, in red alder trees in the Portland Metro area and Tillamook State Forest (both in Oregon), we found significantly higher numbers of OTUs and a smaller proportion of singletons from over 95% of leaves sampled, compared to less than 15% of leaves sampled here (Wolfe et al. 2018). In conifers throughout the Pacific Northwest, Carroll and Carroll [9] found that the percentage of infected needles ranged from 20-100% depending on the conifer host species, while we observed an overall colonization rate of 14.1% on the Pumice Plain. This colonization rate is consistent with the colonization rate in needles of *Pinus taeda* seedlings (14.0%; Oono et al. 2015).

The richness and diversity of the endophyte community are an order of magnitude lower than values measured in a nearby non-successional (i.e. significantly later stage) site [3], suggesting a sizeable lag between the establishment of host plants and eventual foliar colonists on the Pumice Plain. Similar patterns have been observed in root-

associated symbionts (i.e. mycorrhizae, dark septate endophytes) at the forefront of a receding glacier [23, 38], as well as in mycorrhizal and *Alnus*-associated *Frankia* communities also on the Pumice Plain [39, 40], although soil microbial communities appear to be structured by different factors during early succession [41]. While plant community richness may have peaked in some areas of the Pumice Plain [42], plant cover is largely patchy and separated by stretches with inhospitable conditions. Conifers are especially sparse on the Pumice Plain, while substantial thickets of Sitka willow and alder are present along riparian corridors and within seeps, and clusters of the shrubs are interspersed throughout the upland areas. Propagules of endophytes may be present but unable to effectively disperse or establish, as was the case for early mycorrhizae just a few years following the eruption [40]. Consequently, given that the presence and proximity of host plants were important factors for foliar endophyte colonization of beachgrasses in coastal sand dunes [1], the infrequent distribution of host plants on the Pumice Plain compared to surrounding, less impacted areas likely contributed to the poverty of the culturable endophyte community. Additionally, microclimatic conditions and local soil chemical features play particularly significant roles in determining plant community establishment and composition on the Pumice Plain [42–44], and may act as additional filters for endophyte colonization.

Endophyte community compositions were host-specific and included several generalist taxa—a pattern consistent with other studies on oak, cypress, ash, and maple [45, 46]. The most commonly isolated OTU (*Arthrinium* sp.) was found in all six species, and members of the genus have been previously reported in the leaves and stems of *Alnus*



sp. [47], *Salix* sp. [48], and *Populus* sp. [49], as well as in a variety of both temperate and tropical plants [50–53]. To our knowledge, we are the first to report *Arthrinium* sp. in Douglas fir, western hemlock, and noble fir, and *Pseudoplectania* sp. in Sitka willow. Notably, *Arthrinium* sp. was isolated from *Salix alba* infested with the woodwasp *Xiphydria* that disperses its fungal symbionts between trees [48], and both willow and cottonwood shrubs are affected by stem-boring, poplar-willow weevils on the Pumice Plain [54]. A number of taxa isolated from these particular weevils can also occur as generalist endophytes [55], and demonstrate a possible dispersal vector that should be further investigated with molecular techniques.

Surprisingly, we did not find a significant temporal effect on foliar endophyte community composition throughout the vegetation period, contrary to other studies [56, 57], including one that was conducted in the same geographical region [58]. We did obtain nearly four times as many cultures from deciduous compared to conifer hosts—reflecting the significant effect of tree type on community composition—but even the deciduous community that excluded conifers only marginally varied over the three-month field season. This pattern was largely driven by the presence of OTU01 (*Arthrinium* sp.) in nearly every harvest for each host species and a considerable number of singletons. The three most abundant genera that were isolated are broadly classified as either saprotrophs or pathogens in the FUNGuild database [59]. Initial colonists also have significant priority effects on the trajectories of endophyte community composition [60], and may explain some of the shared colonization patterns on the Pumice Plain. However, culture-based methods are inherently more biased than culture-independent methods due

to variation in surface-sterilization effectiveness, media used, and the low percentage of microbes that are capable of growing in culture. Consequently, overrepresentation of fast-growing, possibly aggressive competitors should not be unexpected.

Furthermore, host plant age can be an important factor determining endophyte presence and diversity [37]. The trees sampled in this study ranged between 1–3 m in height and likely were younger than trees sampled in comparable studies, but the true variation in ages and any effects it may have had on our results are unknown [3, 9, 37, 61]. In addition, leaf age can significantly affect the foliar endophyte community [62, 63], and may have influenced the taxa that we were able to isolate, at least in regard to the conifer hosts. Needles in particular were sampled near the ends of branches, and new growth that had not been colonized yet may have been overrepresented. Carroll and Carroll [9] also cite high elevation as a factor in affecting the quality of a plant host—particularly for *Tsuga heterophylla*, also sampled here—which may also apply to our field site (~1000 m a.s.l.). Still, the strikingly low diversity of endophytes at our study site across multiple tree species suggests slow succession of these microbial foliar communities.

## Conclusions

Our findings are an important first step in further characterizing the plant-associated microbial community of the Pumice Plain, a particularly well-studied primary model ecosystem, which has so far only included studies of mycorrhizae [21, 40] and *Frankia* symbionts [39, 64]. We found that culturable endophyte communities are dominated by saprotrophs and pathogens, and overall show high species-specificity.

However, at the same time, endophyte communities exhibited remarkably little diversity—in particular in comparison to relatively closely located control sites—and no statistically significant temporal variation. However, dominant pioneer riparian species like Sitka willow and alder are likely serving as important microbial reservoirs and facilitators for subsequent plant colonization. Primary successional systems like the Pumice Plain are unique models that allow critical insight into how communities and ecosystems reestablish after catastrophic destruction. While mock communities and experimental manipulation can help clarify the underlying mechanisms within and effects of plant–microbe interactions, the well-documented eruption and initial stages of recovery on Mount St. Helens are especially crucial for observing the way that plant–microbe interactions develop after catastrophic events.

## Methods

Between July and September 2017, we sampled six common woody species found on the Pumice Plain of Mount St. Helens, WA: Sitka alder, Sitka willow, cottonwood, Douglas fir, noble fir, and western hemlock (Table 3.1). Sites were identified by traversing previously-established horizontal transects [65, 66] and collecting GPS coordinates for over 300 trees. Using GPS coordinates and the R package *Imap*, trees less than 100 meters from one another were clustered into sites; however, any prospective sites with fewer than four of the six species of interest present were excluded from final consideration. Twenty sampling sites (n=371 trees) were identified based on the number of species, whether there were more than two individuals of a given species present, and if the sites were reasonably accessible (Figure 3.4). Except in the case of no remaining

leaves, the same trees were sampled every thirty days during the three-month field season (July–September). Either three deciduous leaves or 5-10 cm branch pieces (conifers) were randomly collected from each tree using gloves sterilized with 95% ethanol. Leaves were transported to the lab in a cooler (<15° C), and stored at 4° C for less than 48 hours before surface-sterilization. The following minor modifications were made to the methods detailed in Wolfe et al. [3] to accommodate bulk surface-sterilization of leaves: custom sheets of mesh sleeves were constructed from window-screen and sterilized prior to use, and then used to transfer leaves between washes in sterilized polypropylene bins. Sheets of leaves were air-dried in a laminar-flow hood and then processed aseptically onto malt extract agar (MEA) plates. We verified the efficacy of these modifications by re-sampling previously studied red alder (*Alnus rubra*) trees in the Portland Metropolitan area and finding similar colonization rates as Wolfe et al. [3].

Table 3.1. Dominant woody species of the Pumice Plain on Mount St. Helens, WA, including the three most common species for both riparian and upland areas.

Scientific name	Common name
<i>Alnus viridis</i> ssp. <i>sinuata</i>	Sitka alder
<i>Salix sitchensis</i>	Sitka willow
<i>Populus balsamifera</i> ssp. <i>trichocarpa</i>	Black cottonwood
<i>Pseudotsuga menziesii</i>	Douglas fir
<i>Abies procera</i>	Noble fir
<i>Tsuga heterophylla</i>	Western hemlock

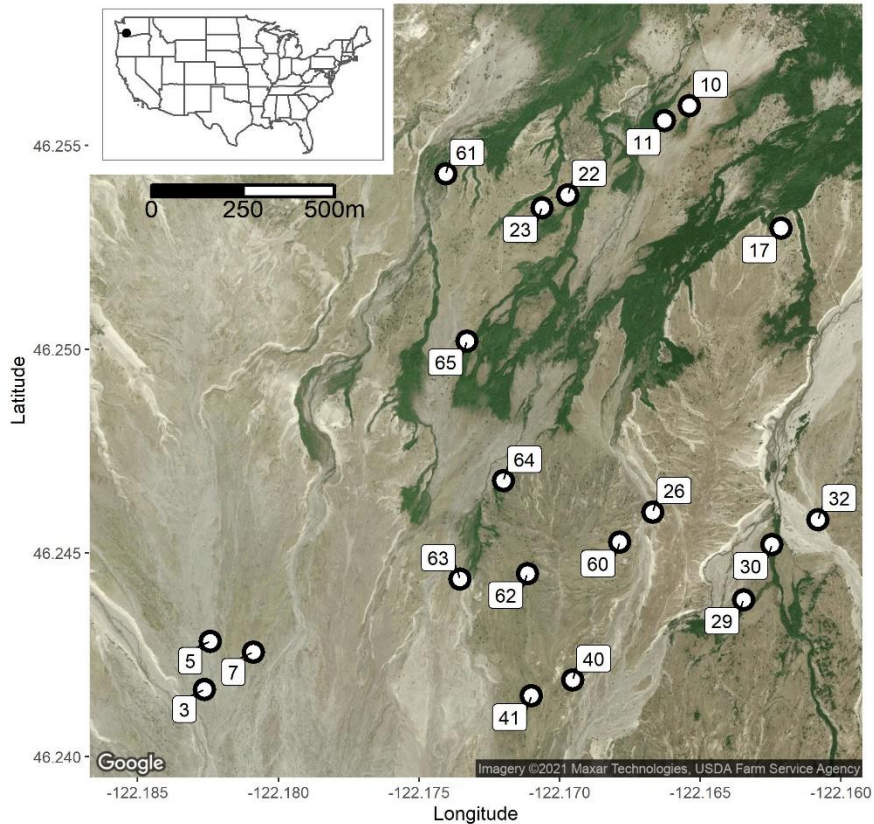


Figure 3.4. Map of the twenty sampling sites on the primary successional Pumice Plain of Mount St. Helens, WA, USA.

Plates were checked every three days for fungal growth, and any new isolates were transferred to new MEA plates until axenic. Fungal isolates from MEA plates were processed with the Sigma Tissue Extract-N-Amp Kit (St. Louis, MO). We used primers ITS1F and ITS4 to amplify DNA with the following thermocycler settings: 34 cycles of 94° C, 50° C, and 72° C for 1 minute each, followed by a final extension at 72° C for 10 minutes. Products were checked with gel electrophoresis before being sent to Function Biosciences (Milwaukee, WI) for Sanger sequencing. Raw sequencing data were manually inspected and cleaned in Geneious 10.2.3. The output was then aligned with MAFFT on XSEDE v7.427 [67, 68] and imported into mothur v.1.42.3 [69] to generate

both putative taxonomic assignments [70] and operational taxonomic unit (OTU) tables. OTUs were clustered at 99% similarity using the furthest-neighbor algorithm [61].

Data were analyzed with R v. 3.6.3 with `vegan`, `car`, and `indicspecies` [71, 72], and abundance figures were generated with `phyloseq` [73], while the map in Figure 1 was created with `ggmap` [74]. Differences in community compositions were compared with permutational multivariate analysis of variance (PERMANOVA; Bray-Curtis distance, and 999 permutations) for all host species, tree type (conifer vs. deciduous), and collection month (July, August, or September). Since over three-quarters of the isolates came from deciduous hosts, we created a subset of the community for those species (alder, willow, and cottonwood), and used PERMANOVA tests to compare differences in species composition and collection month after verifying dispersion was homogeneous within and among all groupings (i.e. by species or over time). Non-metric multidimensional scaling ordinations (Bray-Curtis distances,  $k=3$  determined by screeplot) were used to visualize community compositions. Richness and Shannon diversity indices were calculated using `vegan`. If assumptions could not be met, nonparametric Kruskal-Wallis tests were used instead.

#### Declarations

Ethics approval: not applicable

Consent to participate: not applicable

Consent for publication: not applicable

Availability of data and material: The raw data are available in the following GitHub repository: [https://github.com/emwolfe/MSH\\_endophytes](https://github.com/emwolfe/MSH_endophytes).

Conflicts of interest/Competing interests: The authors declare that they have no conflicts of interest.

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Authors' contributions: ERW designed the study, collected samples in the field, processed samples, and conducted the analyses. RD and CW assisted in processing samples. ERW, RD, and DJB wrote the manuscript. All authors edited and reviewed the manuscript.

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## Chapter 4

### Genetic diversity and population structure of (*Alnus viridis* ssp. *sinuata*) on Mount St. Helens

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#### Abstract

Alders (*Alnus* sp.) are common pioneers in disturbed landscapes, ranging from naturally-occurring destruction such as volcanic eruptions to anthropogenic-mediated land-use changes like mine tailings. Sitka alder specifically is common in northwestern North America at early successional sites and plays a critical role in increasing soil nitrogen and organic matter inputs. However, population genetics of Sitka alder have not been investigated despite influencing plant–microbial and plant–ecosystem interactions. We sampled Sitka alder from sites around Mount St. Helens (Washington, USA) to determine 1. genetic diversity and underlying structure following the 1980 eruption, and 2. possible source populations for the shrubs colonizing the blast zone. We found no evidence of genetic differentiation among five sites from around the mountain ( $F_{ST}=0.000$ ), although three subpopulations ( $k=3$ ; Clusters 1–3) were identified by Bayesian clustering in STRUCTURE. The Pumice Plain—youngest and most impacted of the sites—was likely colonized by Sitka alder genotypes originating from either the Norway Pass area, or from the same seed rain that colonized Norway Pass. Follow-up studies should assess population genetics of Sitka alder across a volcanic chronosequence

in the Pacific Northwest to better clarify patterns in initial colonization after disturbance and consequences for genetic diversity of *Frankia* symbionts.

## Introduction

Pioneer plant species are critical to recovery following disturbance and as global climate change subjects more land to early successional processes, the colonization patterns and population dynamics of such species are becoming increasingly important. These plants are the first to establish on new substrates and help to ameliorate harsh habitats for subsequent colonizers—for example, leguminous prairie lupine was one of the first plants to colonize the sterile Pumice Plain of Mount St. Helens (Morris and Wood 1989; Halvorson et al. 1991), and dwarf willow on Mount Fuji facilitated establishment of ectomycorrhizal fungal communities (Nara 2006). Alders (*Alnus* sp.) in particular frequently dominate early successional landscapes as pioneers due to a wide global distribution, and are often found at the forefronts of glaciers (Chapin et al. 1994), on floodplains (Anderson et al. 2009), and at reclamation sites (Meiman et al. 2012). However, contrary to other woody pioneers like willow and poplar, we present alders as model systems of multi-scale interactions and processes that span plant–microbial symbioses to biogeochemical cycles and offer valuable opportunities to study a commonly-occurring species with subsequently broad applications.

Green alder (*Alnus viridis*) and its subspecies have a circumpolar distribution and represent one example of these valuable alder model systems. In northwestern North America, Sitka alder (*Alnus viridis* (Chaix) DC. ssp. *sinuata* (Regel) A. Löve & D. Löve) readily colonizes open, nutrient-poor environments following disturbance, and has played

an important role as a pioneer species in deglaciaded areas of Glacier Bay National Park, AK (Chapin et al. 1994; Fastie 1995). Like other members of its genus, it forms a critical symbiosis with nitrogen-fixing *Frankia* bacteria, facilitating colonization in harsh environments and functioning in an ecosystem engineering role by contributing to higher soil nitrogen and organic matter accumulation (Anderson et al. 2009). Evidence of genetic adaptation to local environmental conditions has been reported in Sitka alder specifically (Benowicz et al. 2000), and several silviculture studies have focused on the role of *Frankia* genotypes in local adaptation of alder hosts (Teissier du Cros et al. 1984; Carpenter et al. 1984).

However, the population genetic structure of Sitka alder and related subspecies during early succession is not well understood or defined despite potentially large impacts of colonization dynamics on community assembly trajectories. Notably, multiple common names (e.g. green alder, mountain alder, slide alder, and wavy-leaf alder) can refer to the same shrub. In the Alps, green alder has caused biodiversity to decrease due to aggressive colonization of grassland (Anthelme et al. 2003), and in Alaska, alders have historically been correlated with substantial expansion into newly deglaciaded land due to warming. Given that tree genotypes can affect plant–microbial symbioses and even afterlife effects in leaf litter decomposition (LeRoy et al. 2007; Paaso et al. 2017), it is worth determining population structure to understand possible patterns in founder effects, dispersal mechanisms, and colonization success of important pioneer species.

Following the 1980 eruption of Mount St. Helens (WA, USA), Sitka alder and Sitka willow have been the dominant woody species in the blast zone, particularly in

riparian areas where dense thickets tend to form. Subsequent land management decisions resulted in the creation of the Mount St. Helens National Volcanic Monument, a unique natural laboratory and model system for studying successional processes in the absence of anthropogenic influences. During the eruption, the entire north face of the mountain was altered as massive amounts of material were expelled and deposited in a 20-km<sup>2</sup> area during the lateral blast and pyroclastic flows, resulting in the sterile Pumice Plain and expansion of Spirit Lake. Trees on neighboring ridges were flattened while lahars flooded down the North Fork Toutle and the Muddy Rivers. This variation in ecosystem destruction resulted in entirely new substrate, new refugia, and new connections and avenues of dispersal between formerly disparate areas around the mountain. However, while physical barriers to dispersal were removed, environmental heterogeneity and exposure to harsh conditions increased. Previously, only colonization genetics of herbivore-dispersed *Vaccinium membranaceum* have been studied on Mount St. Helens (Yang et al. 2008), but alder is water- or wind-dispersed and wind-pollinated. In this study, we investigated the likely source populations of the blast zone Sitka alder colonists, as well as the overall genetic diversity in Sitka alder on and around Mount St. Helens.

## Methods

### Sampling and DNA extraction

We sampled five sites around Mount St. Helens, WA to characterize population structure of the pioneer species Sitka alder: Castle Lake, June Lake, Norway Pass, the Pumice Plain, and Smith Creek. These sites were chosen based on the variation in impact

from the catastrophic eruption in 1980: the northeastern sites at Norway Pass and on the Pumice Plain were located in the blast zone and subjected to the lateral blast and, in the case of the Pumice Plain, pyroclastic flows. Smith Creek is situated at the edge of the lateral blast in the blowdown zone. Castle Lake, located to the northwest of the crater, is part of the debris avalanche that flowed down the North Fork Toutle River, while June Lake to the south was comparatively untouched. In August 2018, we collected unblemished leaves from shrubs at least 50m apart (Table 4.1) and stored individually-bagged samples in coolers ( $<15^{\circ}$  C) in the field before transporting to refrigeration in the lab ( $4^{\circ}$  C). For the fifth site, we used samples previously collected from 43 shrubs on the Pumice Plain of Mount St. Helens during the 2017 field season (Wolfe et al., in review). All leaves were surface-sterilized after collection and processed for storage at  $-80^{\circ}$ C, then lyophilized for 48 hours at  $-50^{\circ}$ C and at most 0.133 mBar prior to DNA extraction (Wolfe et al. 2018). Leaf tissue was loaded into 1.2 mL 96-well plates with one steel ball bearing per well (Royal Steel Ball Products, Sterling, IL), and finely powdered with alternating 1 min runs at 30 Hz on a TissueLyser II (Qiagen, Germantown, MD). Total genomic DNA was then extracted using a modified version of the CTAB protocol described by Inglis et al. (2018) (Supplementary Information 4.1). We quantified extraction products using a QuBit 3.0 fluorometer (ThermoFisher Scientific, Waltham, MA) and verified quality through gel electrophoresis prior to preparation for genotyping-by-sequencing (GBS).

#### Construction and Sequencing of GBS libraries

DNA was submitted to the University of Wisconsin-Madison Biotechnology Center. DNA concentration was verified using the Quant-iT™ PicoGreen® dsDNA kit

(Life Technologies, Grand Island, NY). Libraries were prepared as in Elshire et al. (2011) with minimal modification; in short, 50 ng of DNA was digested using the 5-bp cutter ApeKI (New England Biolabs, Ipswich, MA) after which barcoded adapters amenable to Illumina sequencing were added by ligation with T4 ligase (New England Biolabs, Ipswich, MA). Ninety-six adapter-ligated samples were pooled and amplified to provide library quantities amenable for sequencing, and adapter dimers were removed by SPRI bead purification. Quality and quantity of the finished libraries were assessed using the Agilent Bioanalyzer High Sensitivity Chip (Agilent Technologies, Inc., Santa Clara, CA) and Qubit® dsDNA HS Assay Kit (Life Technologies, Grand Island, NY), respectively. Libraries were sequenced targeting 2 million reads on a NovaSeq6000 (Illumina Inc.). Images were analyzed using the standard Illumina Pipeline, version 1.8.2.

Table 4.1. List of sample sizes and averaged coordinates by field site.

Site	Shrubs (n)	Latitude	Longitude
Castle Lake	11	46.233493	-122.21412
June Lake	12	46.138635	-122.15703
Norway Pass	17	46.31033667	-122.1053133
Pumice Plain	43	46.24786683	-122.1692317
Smith Creek	11	46.24411636	-122.1028636

#### GBS data processing

Given that a reference genome is not currently available for Sitka alder, we used the GBS SNP CROP pipeline to process raw data from the sequencing core and call SNPs (Melo et al. 2016). The default settings were kept throughout the pipeline, which resulted in 2657 SNPs from 331940969 raw reads. We further filtered these data in R v. 4.0.5 by keeping loci with less than 10% missing data and minor allele frequency of 5% or greater (Linck and Battey 2019; Pavan et al. 2020) with packages adegenet (Jombart

2008; Jombart and Ahmed 2011) and poppr v. 2.9.1 (Kamvar et al. 2014, 2015), resulting in 230 informative SNPs.

### Population structure & genetic diversity analyses

We used both Bayesian clustering in STRUCTURE v. 2.3.4 and discriminant analysis of principal components (DAPC) with adegenet in R to assess underlying genetic structure (Pritchard et al. 2000; Porras-Hurtado et al. 2013). STRUCTURE was initially run with both burn-in and Markov Chain Monte Carlo model (MCMC) replications set to 50000 for 10 iterations each of  $k=1-8$  (without location information), and the results passed through STRUCTURE Harvester (Earl and vonHoldt 2012) to identify the appropriate  $k$  value based on the Evanno delta  $k$  method. After determining  $k=3$ , we set both burn-in and MCMC replications to 100000 and ran 30 iterations. The cluster assignments generated from these runs were merged into plots with the R package pophelper (Francis 2017). Finally, population genetics statistics including  $F_{ST}$  and pairwise  $F_{ST}$  (95% CI; 999 bootstraps) were calculated with hierfstat (Goudet 2005), while a neighbor-joining tree (1000 bootstraps) and analyses of molecular variance (AMOVA) were carried out with poppr (999 permutations). The phylogenetic tree and maps were created with ggtree (Yu et al. 2017) and ggmap (Kahle and Wickham 2013), respectively. Raw data and R scripts are available in the following GitHub repository: [https://github.com/emwolfe/MSH\\_GBS](https://github.com/emwolfe/MSH_GBS).

### Results

STRUCTURE identified three subpopulations ( $k=3$ ) within our samples that did not distinctly cluster with any one of the five *a priori* sampling sites (Castle Lake, June

Lake, Norway Pass, Pumice Plain, Smith Creek). Instead, most Cluster 1 assignments were split between shrubs sampled from Castle Lake and Smith Creek, while most Cluster 2 and 3 assignments belonged to shrubs sampled near June Lake and Norway Pass, respectively (Table 4.2; Figure 4.1). Every individual contained some combination of all three clusters (Figure 4.2), and approximately half (45.5-66.7%) of all shrubs from each site were considered admixed (i.e. <70% membership to any one cluster)—including the Pumice Plain, which otherwise had nearly equal distributions among the three clusters. This pattern is also reflected in the DAPC results (Figure 4.3), which show the Pumice Plain at the crux of the distribution, overlapped by all other sites.

Table 4.2. STRUCTURE cluster assignments as a proportion of sample size for each site; “admixture” denotes <70% membership to any one cluster.

Cluster	Castle Lake	June Lake	Norway Pass	Pumice Plain	Smith Creek
1	0.273	0.083	0.118	0.140	0.273
2	0.091	0.250	0.118	0.186	0.091
3	0.091	0.000	0.235	0.186	0.182
admixed	0.545	0.667	0.529	0.488	0.455



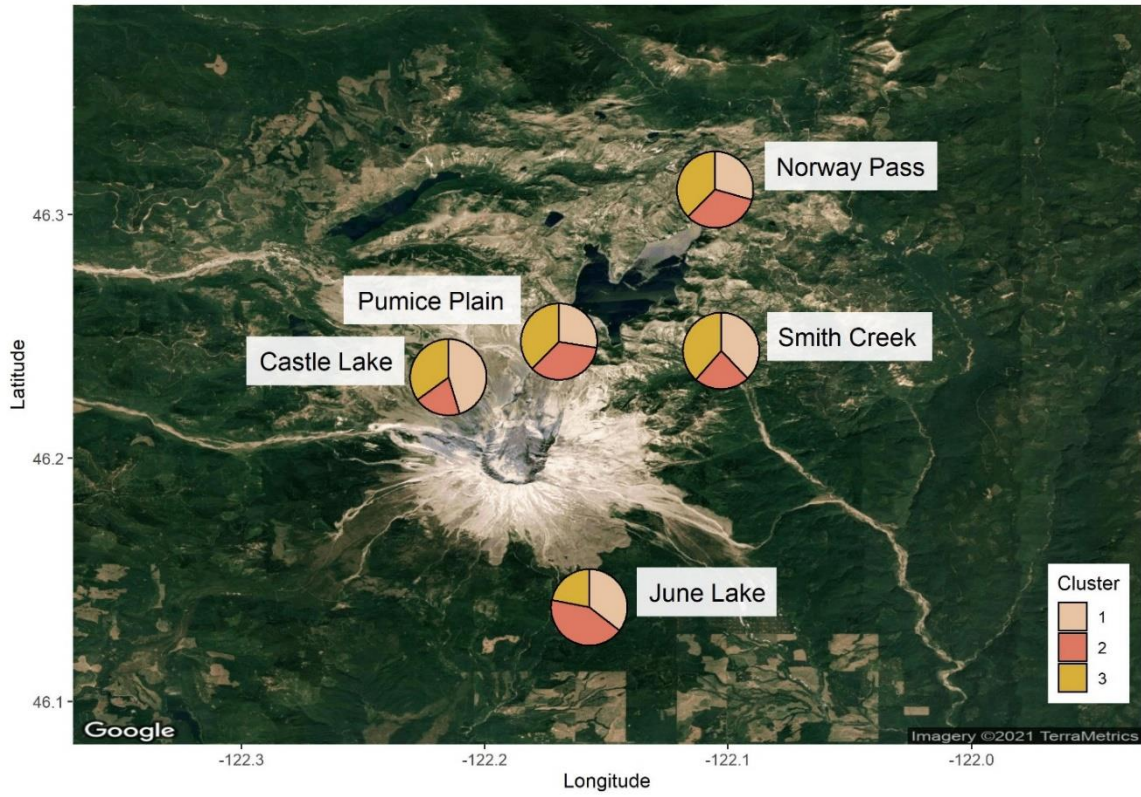


Figure 4.1. Map of sampling sites showing relative cluster membership assigned by STRUCTURE.

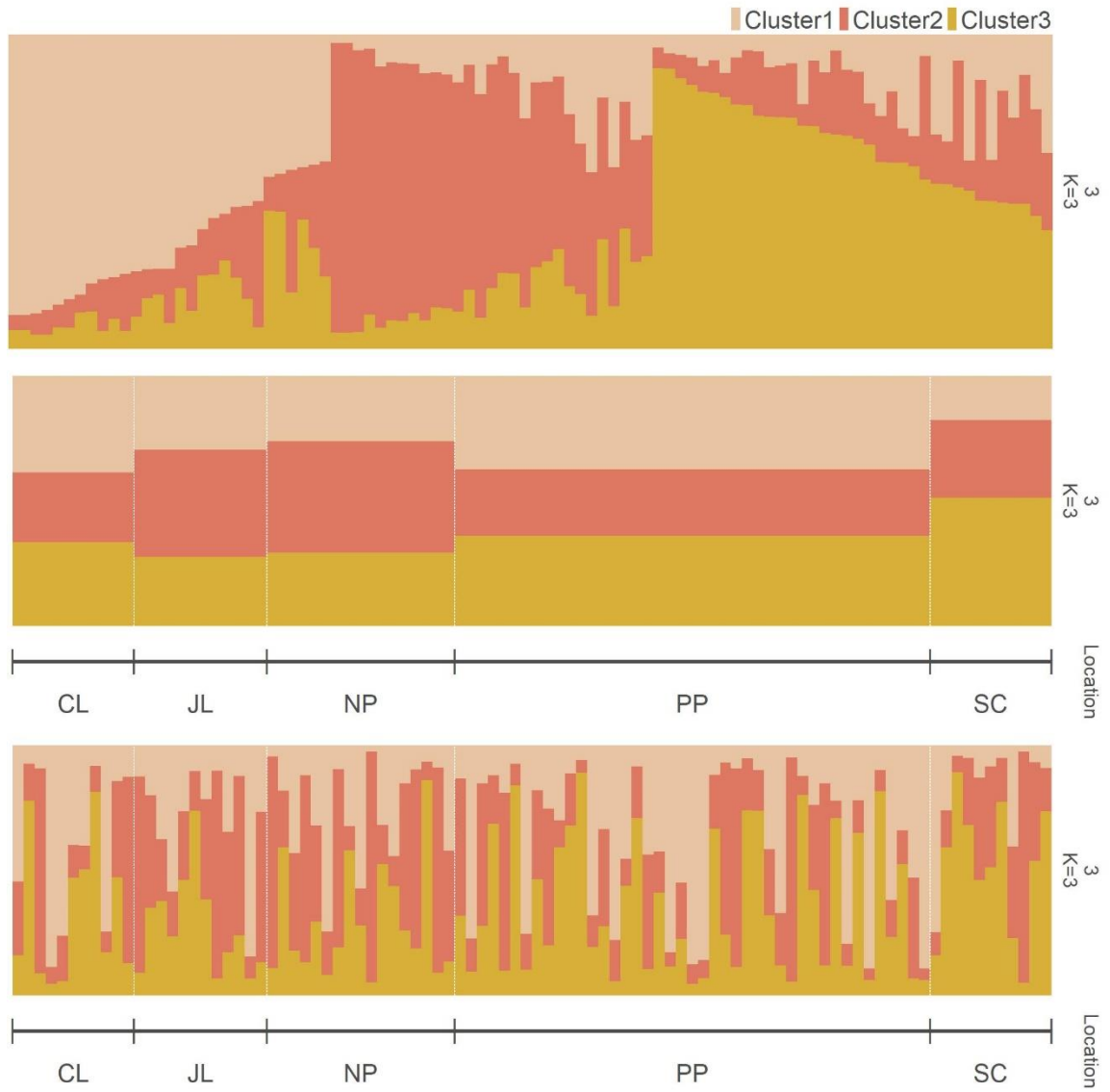


Figure 4.2. Proportions of STRUCTURE cluster assignments sorted by cluster (top), the mean of sampling sites (middle), and by sampling sites (bottom).

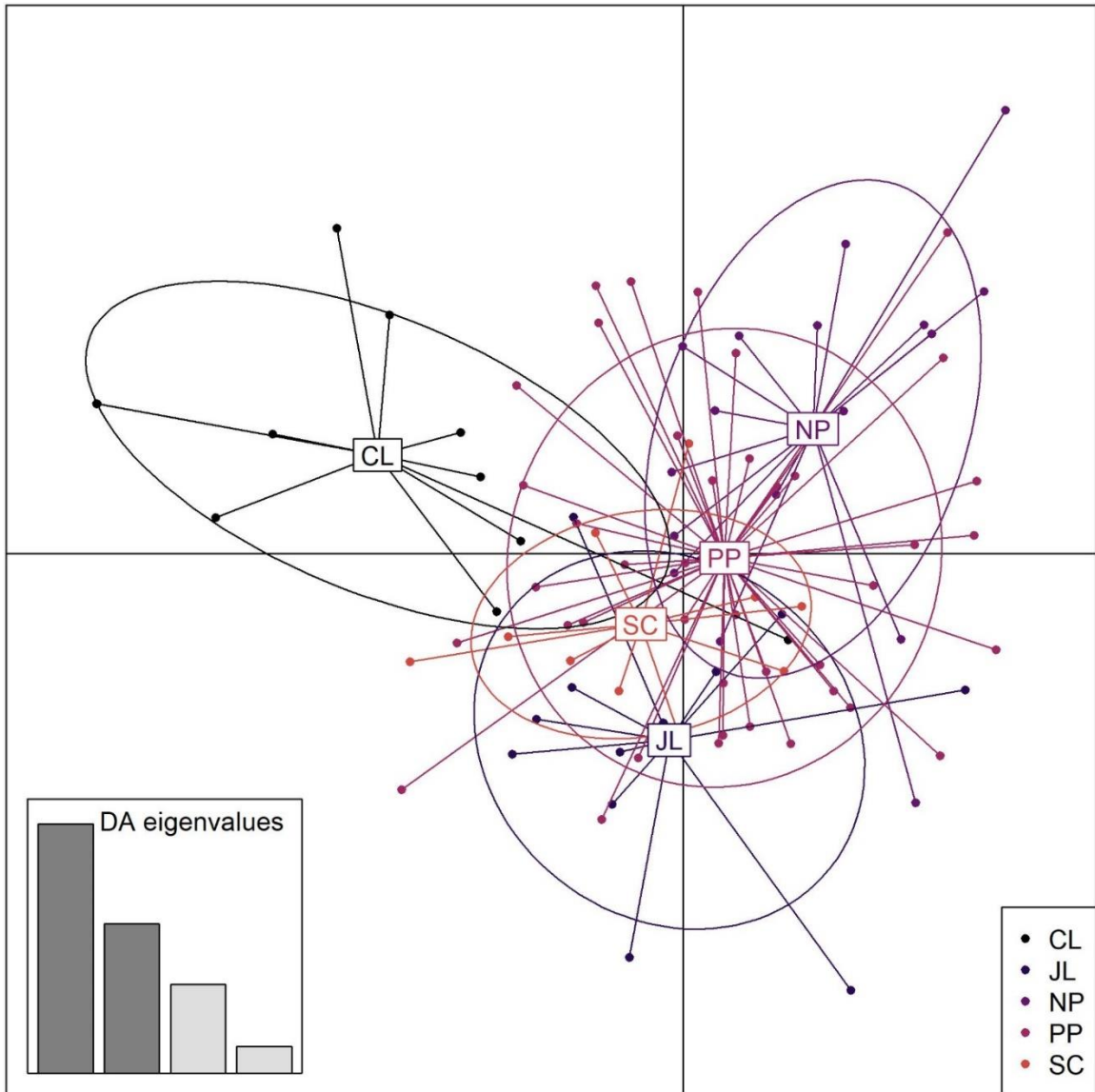


Figure 4.3. Discriminant analysis of principal components (DAPC) of shrub genotypes by sampling site: Castle Lake (CL), June Lake (JL), Norway Pass (NP), Pumice Plain (PP), and Smith Creek (SC).

$F_{ST}$  estimates support that these subpopulations are not genetically distinct, with considerable gene flow occurring among sampling sites. We found no overall genetic differentiation ( $F_{ST}=0.000$ ), significantly lower heterozygosity than expected (t-test,  $t=3.0805$ ,  $df=228$ ,  $p=0.002$ ;  $H_o=239$ ;  $H_e=0.243$ ), and an inbreeding coefficient of 0.043. The Pumice Plain had the highest number of alleles (458), followed by Norway Pass (451), Castle Lake (434), June Lake (433), and finally Smith Creek (430). Pairwise  $F_{ST}$

calculations between sampling sites were similarly low, with the only hints of differentiation found between June Lake and the Pumice Plain (95% CI = 0.000-0.010), and Norway Pass and the Pumice Plain (95% CI = 0.000-0.018). This lack of genetic differentiation among sites is also supported by AMOVA, which revealed that most of the variation (94.36%) existed within individuals ( $p \gg 0.05$ ,  $\Phi = 0.058$ ; Supplementary Figure 4.). Furthermore, we found no association of genetic variation with latitude, longitude, or elevation either (Mantel tests,  $p \gg 0.05$ ; Supplementary Figure 4.). Finally, a neighbor-joining tree confirms that the Pumice Plain is the youngest site, and indicates that Castle Lake and June Lake are the oldest sites, and that the northeastern sites (NP, PP, SC) that were in the blast zone cluster together (70.2% bootstrap support; Figure 4.4).

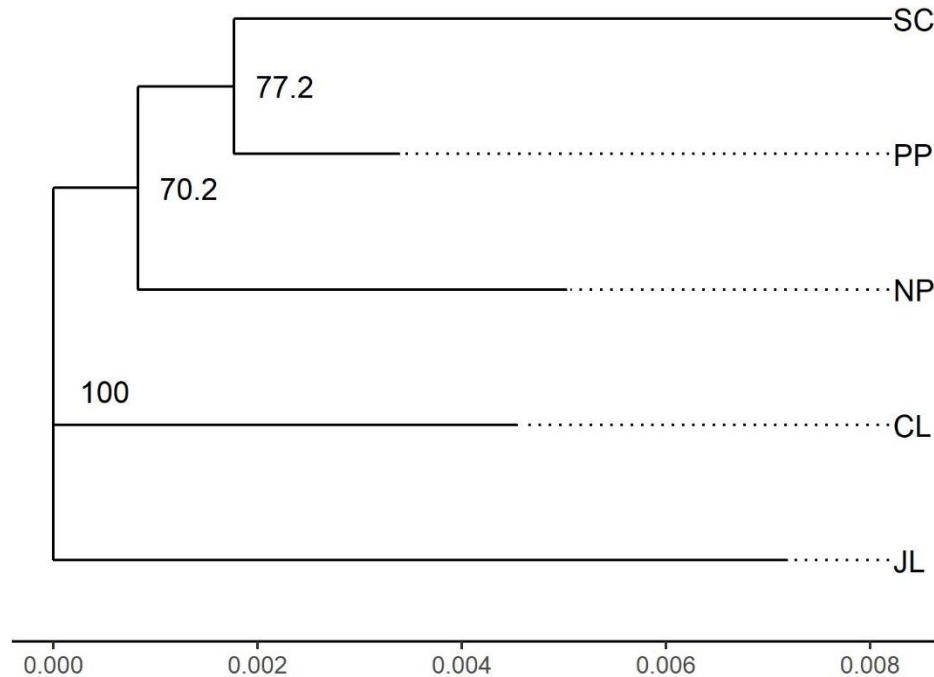


Figure 4.4. Neighbor-joining tree of the five sites sampled around Mt. St. Helens.

## Discussion

We sampled Sitka alder shrubs from around Mount St. Helens and found three subpopulations, high gene flow, and probable source populations for blast zone colonists. The relatively small spatial scale for this study emphasizes the outsized role that environmental factors play during primary succession and recovery following disturbances, as well as the dispersal mechanisms for pioneer plants. The devastating eruption in 1980 resulted in considerable environmental heterogeneity due to different types of deposits (e.g. pumice, tephra, etc.), the obliteration of communities, the formation of refugia, and the creation of microclimatic and microsite effects as springs and streams were buried or rerouted through new material (Swanson and Major 2005). The sites sampled in this study represent this heterogeneity in level of destruction that would have affected both the availability and success of propagules during recolonization—ranging from the pyroclastic flows that resulted in the creation of the barren Pumice Plain, to the untouched southern site near June Lake. The three subpopulations identified by STRUCTURE and represented by Clusters 1, 2, and 3 roughly correspond to site-specific impacts of the eruption: Cluster 1 is the defining cluster for Castle Lake which was part of the debris avalanche, and also Smith Creek, which was impacted by both the lateral blast and debris flows (Major et al. 2005); Cluster 2 dominates the site near June Lake, which was unscathed by the eruption; and Cluster 3 forms the technical majority of Norway Pass and the Pumice Plain, which were directly in the path of the lateral blast and pyroclastic flows, respectively. Consequently, the associated cluster assignments (i.e. majority Cluster 1 and 2, respectively), as well as the

site near Smith Creek (also majority Cluster 1), possibly represent the local allele frequencies of those survivors and colonists from nearby refugia in the form of sheltered ridges, mudflow deposits, and adjacent intact forest (Halpern and Harmon 1983). The dominant cluster assignment for both Norway Pass and the Pumice Plain, however, was Cluster 3; additionally, of the sites, these two had nearly identical cluster distributions. This suggests that colonists on the Pumice Plain most likely either came directly from the Norway Pass area or from the same seed rain.

Half of all shrubs sampled were considered admixed, containing some combination of all three STRUCTURE-identified clusters but less than 70% of any one cluster (Figure 4.2). Intraspecific admixture, as reviewed by Rius and Darling (2014), can aid in colonization success through heterosis and eventual adaptation. However, while we observed significantly lower heterozygosity than expected in these populations, local propagule pressure and multiple introductions may explain the compositional differences between the dominant clusters, especially given the similar successional stages in all sites except June Lake. We would expect to see higher diversity in blast zone colonists if the source populations had been differentiated and gene flow was limited (Banks et al. 2013), but the opposite is true here, suggesting multiple source populations and high gene flow among them. Similar results were observed in coastal tailed frog populations also in the blast zone (Spear et al. 2012), and a pattern of initial long-distance dispersal followed by short-distance dispersal has been observed in *Salix reinii* colonizing Mount Fuji (Lian et al. 2003). In a study of another pioneer species and wind-pollinated shrub, *Juniperus communis*, Oostermeijer and De Knegt (2004) also found evidence of high gene flow

during initial colonization consistent with our results—Sitka alder is still clearly in the midst of its initial colonization of the new substrate. Previously limited to pre-eruption avalanche chutes (Swanson et al. 2005), it is currently expanding across the blast zone along with *Salix sitchensis*, another pioneering shrub. Given the high admixture observed in our samples, it is probable that Sitka alder is colonizing these areas both from multiple sources and through multiple introductions, and that short-distance dispersal is contributing to compositional differences within sites following initial colonization.

The nonexistent population substructuring across our sites further supports high gene flow occurring among disparate areas around the mountain. Neither sites nor clusters were structured here—most of the variation partitioned by AMOVA was within individuals rather than between sites or clusters. However, wind-pollinated trees like Sitka alder typically have less population structure due to the large effective scale of pollen dispersal (Robledo-Arnuncio 2011; Llorens et al. 2017) particularly in the absence of potential barriers (Ashley et al. 2015), as is the case on the exposed north side of Mount St. Helens. It should be noted that Sitka alder may also sprout from damaged stumps which, as documented for sympatric red alder, may have been moved and partially buried by volcanic activity (Haeussler et al. 1990). Studies of other *Alnus* species have generally found low intraspecific  $F_{ST}$  values ranging from 0.01 (De Kort et al. 2014) to 0.021 (Guo et al. 2019) but at much greater spatial scales than the present study. For example, Mingeot et al. (2016) also detected low differentiation ( $F_{ST}= 0.014$ ) over long distances (i.e. hundreds to thousands of kilometers) and a very similar  $F_{IS}$  value (0.040) in black alder, as well as considerably higher pollen flow compared to seed flow.

Future investigations should determine genetic structure and pollen/seed flow in Sitka alder on Mount St. Helens and reference sites on nearby Mount Rainier or Mount Hood.

Our results support that the populations of the three sites within the lateral blast area are younger than Castle Lake and June Lake—consistent with how the eruption progressed—and that these latter two sites had the most readily available propagules (**Error! Reference source not found.**). Given the differences in site history, the exact ages of the sampled shrubs are unknown (and were untested here due to study limitations), although all of the shrubs sampled in this study can be estimated to have been at least 6-8 years old at the time of sampling due to the presence of catkins (Fastie 1995). However, the low overall  $F_{ST}$  indicates that age likely is not contributing to any differences in allele frequencies among sites. The higher numbers of alleles in both the Pumice Plain and Norway Pass sites are also consistent with populations having more alleles during early succession compared to those in later stages (Céspedes et al. 2003). Interestingly, Smith Creek had the lowest number of alleles despite also being in the blast zone and clustering with the Pumice Plain. We suspect that this may be due to a combination of propagules carried by the mudflows down Smith Creek and seed rain from the same sources as Norway Pass and the Pumice Plain. Cluster 3 is prevalent in sites on the north side of the mountain including Smith Creek, suggesting that those allele frequencies are potentially more common in populations along the bordering ridges in the blowdown zone, which are also colonized extensively by Sitka alder. It is also worth noting that Seeds and Bishop (2009) found low inoculation potentials for *Frankia* in sites located on the Pumice Plain; *Alnus* sp. form nitrogen-fixing symbioses with *Frankia*,



which are especially critical in nutrient-poor environments like the primary successional sites on Mount St. Helens. The lack of *Frankia* within early successional soils may be further filtering clusters that are able to establish in these areas. Consequently, as soil microbial community becomes more diverse, we would expect to see increases in Sitka alder population differentiation as time continues to pass since the eruption (Hahn et al. 2017).

## Conclusions

Mount St. Helens provides a unique opportunity to study plant community succession and assembly in an area permitted to recover naturally after a catastrophic natural disaster. In this study, we determined that probable source populations of Sitka alder are dispersing onto and colonizing the Pumice Plain from the Norway Pass area. We also found that June Lake and Castle Lake exhibit more ancestral allele frequencies that may be more representative of propagules that survived the eruption. Future work within the Mount St. Helens model system should consider using chloroplast markers for a finer-scale study of source populations, as well as using a chronosequence of volcanic disturbance within the Pacific Northwest to gain additional insight into colonization dynamics of pioneer plant species.

## Declarations

## Funding:

Conflicts of interest/Competing interests: The authors declare no conflicts of interest.

Availability of data and material: Raw data and R scripts are available in the following GitHub repository: [https://github.com/emwolfe/MSH\\_GBS](https://github.com/emwolfe/MSH_GBS).

Authors' contributions:

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## Chapter 5

### *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

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 despite the broad global distribution and critical role of *Alnus*–*Frankia* symbioses. 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 (USA). Five 2,500 m<sup>2</sup> 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 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 microclimatic 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, Mt. St. Helens

Declarations

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Consent for publication: Not applicable.

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Code availability: Not applicable.

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.

## 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. 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 unique in their co-occurrence 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 interference. Consequently, Mount St. Helens 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<sup>-1</sup> year<sup>-1</sup> depending on age and species (Tobita et al. 2016), which is comparable to the 200 to 300 kg of N ha<sup>-1</sup> year<sup>-1</sup> fixed by *Rhizobium*-associated legume crops alone (Zahran 1999). Additionally, as a common riparian species in the PNW, 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 potential larger scale 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. Consequently, we examined the following five questions in our study: 1) How diverse are nodulating *Frankia* genotypes found on the Mt. 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, south of Spirit Lake along the northern flank of Mount St. Helens (Washington State, USA; 46.25861°N, -122.17319°W; Figure 5.1). The Pumice Plain resulted from the catastrophic lateral blast of the 1980 eruption that buried the area in deposits of sterile pumice 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 x 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. In order for an area to be considered a potential plot, at least one individual of each alder species had to be present.

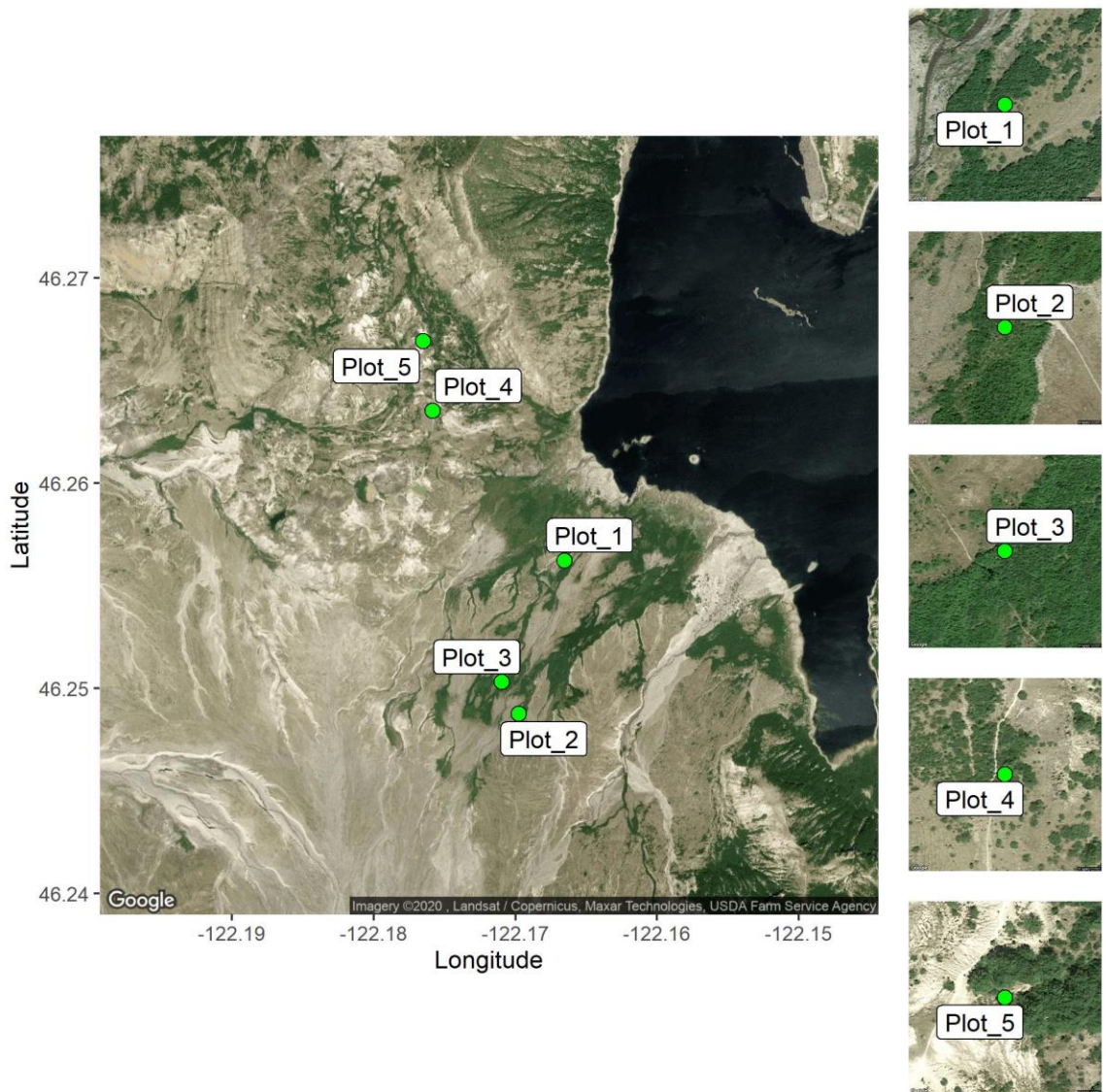


Figure 5.1. Map of the study sites on the Pumice Plain of Mount St. Helens, with Spirit Lake featured along the right side of the main panel.

### 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 2-10 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  $\mu$ l 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  $\mu$ l 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  $\mu$ l 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- $\mu$ l volumes containing 8.45  $\mu$ l PCR water, 12.5  $\mu$ l GoTaq Master Mix, 1.25  $\mu$ l BSA (1 mg/1 mL), 0.4  $\mu$ l of each primer (10  $\mu$ M), and 1  $\mu$ l 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 seconds, 60° C for 30 seconds, 72° C for 45 seconds, 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 optclust algorithm in MOTHUR version 1.42.0 (Schloss et al. 2009; Pölme et al. 2014).

Partial 16S rRNA gene 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.

### 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 done 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 done 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; 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.

### 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. 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 (Table 5.1). 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 (Pölme 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 groups was tested prior to PERMANOVA with the betadisper function, and showed that samples were not dispersed homogeneously among groups. Although the species accumulation curve did not level off (Figure 5.2)—suggesting that sampling more trees would have revealed additional taxa—calculations of Chao1 indices matched our observed number of genotypes (4).

Table 5.1. Three cutoff thresholds and the respective number of OTUs isolated from root nodules sampled from *Alnus rubra* and *Alnus viridis* individuals colonizing the Pumice Plain of Mount St. Helens.

Gene	99.3% cutoff	99% cutoff	97% cutoff
<i>nifH</i>	4	4	3

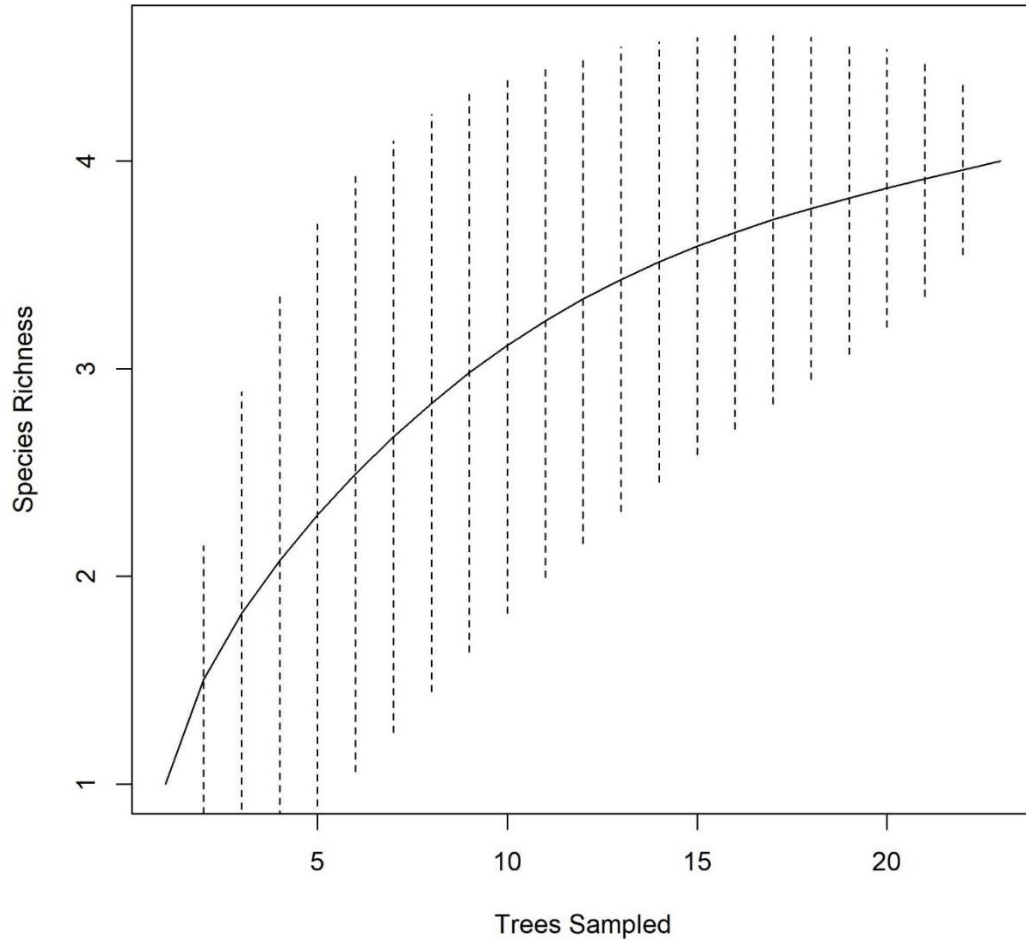


Figure 5.2. Species accumulation curve for Frankia genotypes identified at the 99.3% similarity threshold.

Within the *nifH* phylogeny (Figure 5.3), representative sequences of the four genotypes did not form a monophyletic clade, but instead were dispersed across the tree. The four genotypes (2-5) 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 (Supplementary Figure 5.1).



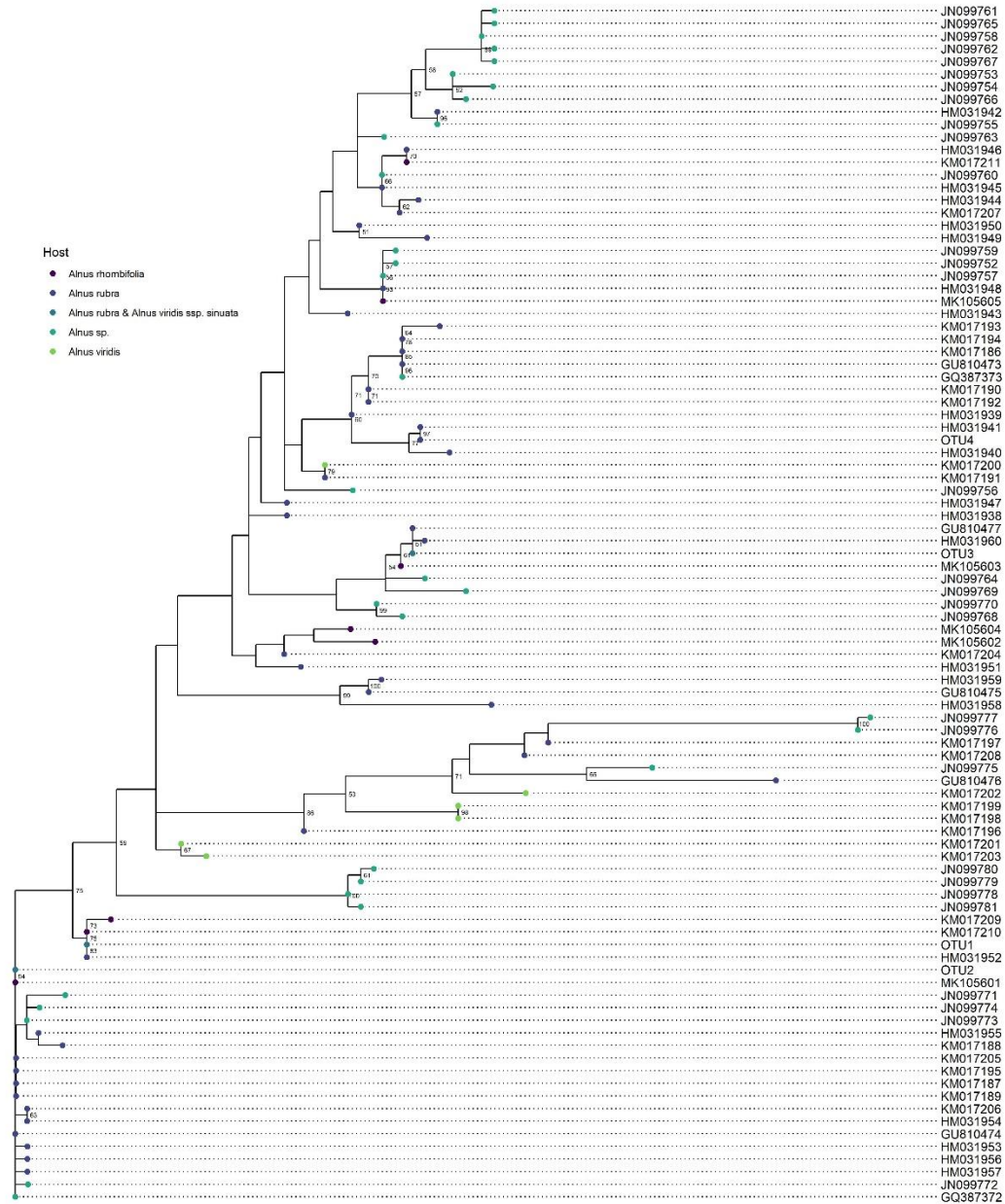


Figure 5.3. Phylogenetic tree of the *nifH* sequences isolated from Sitka and red alder on the Pumice Plain, as well as additional sequences from other alder species curated from NCBI. Bootstrap values (>50) are printed at the branch tips.

We did not find evidence of host tree specificity (PERMANOVA,  $p \gg 0.05$ ). 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, with only one genotype (Genotype 1) almost exclusively occurring 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.

We found that *Frankia* communities were significantly different among plots (PERMANOVA,  $F_{4,22}=4.6375$ ,  $p=0.002$ ), which had varying microclimatic conditions and showed differences in soil features including differences in moisture, ammonium, phosphorous, magnesium, sulfur, calcium, copper, boron, cation exchange capacity, and electrical conductivity (Figure 5.4). PC axes 1 and 2 cumulatively explained 87.6% of the variance (Figure 5.5). Dominant genotypes were significantly associated with particular levels of soil chemical features (indicator species analysis;  $p < 0.05$ ; Table 5.2). The most abundant genotype was associated with higher levels of sulfur and 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  $r=0.4030$ ;  $p=0.001$ ).

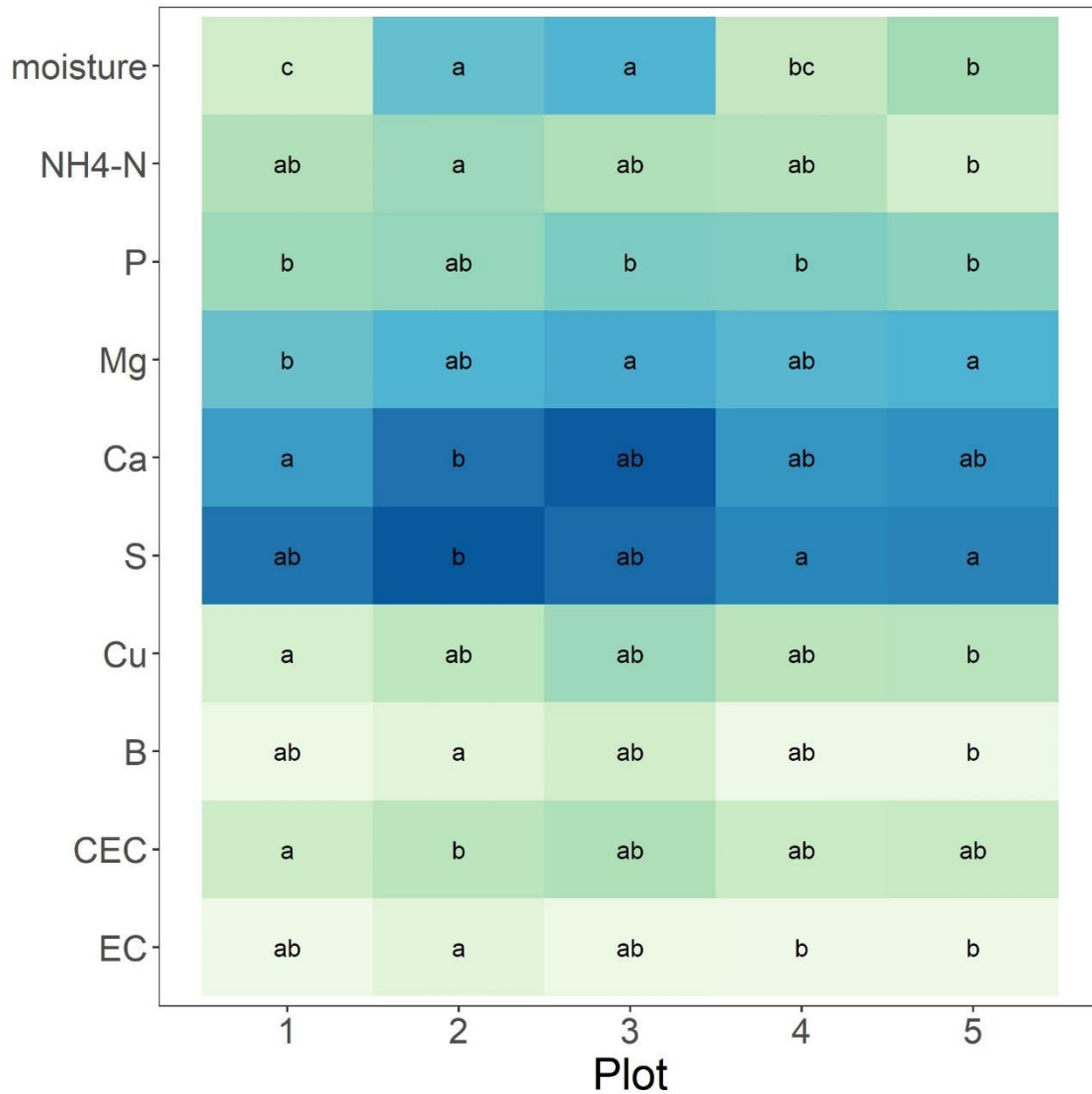


Figure 5.4. Differences in soil chemical features by plot, with lighter colors indicating lower levels of a given feature, and darker colors indicating higher levels of a given feature. Letters indicate pairwise differences among plots for a given feature (i.e., row). CEC = cation exchange capacity and EC = electrical conductivity.

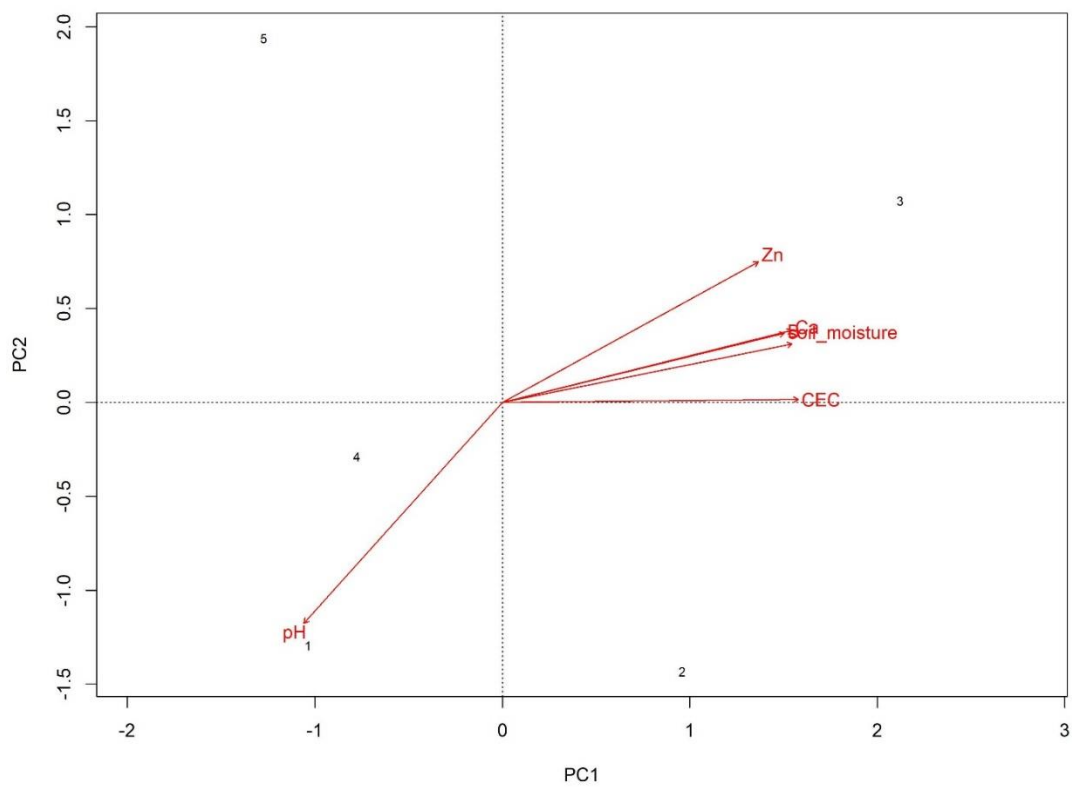


Figure 5.5. Principal components analysis of soil chemical features among plots.

Table 5.2. Indicator analyses of plot soil chemical features.

Soil chemical feature	Feature Category	Genotype	Indicator Value Statistic	p-value
NH <sub>4</sub> .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

## 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, Pölme et al. (Pölme 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 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. At the 97% sequence similarity threshold, we recovered 3 genotypes, which is slightly less than the number of genotypes reported by Lipus and Kennedy (2011) in the same area (Pumice Plain; 5 genotypes; 97% similarity), and other studies (99.0% and 97% similarity, respectively) of older, more established *Alnus–Frankia* symbioses in the Pacific Northwest, USA (Kennedy et al. 2010; Balkan et al. 2020).

Contrary to other studies, we did not find evidence of host specificity in *Frankia* communities of red and Sitka alder (Anderson et al. 2009; Lipus and Kennedy 2011). However, 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, 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 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.

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. For example, 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. 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 Mt. 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 (Figure 3), 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, 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 (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; 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. Finally, at the host-plant scale, chemical defenses within the host plant can directly alter relationships with symbionts (Godschalx et al. 2017). 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.



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## Chapter 6

### Conclusions and Future Directions

Plant function relies on the sum of its parts: genotypes, environment, microbial symbionts, and the interactions among these components. We chose Mount St. Helens as a model for early successional processes because the area within the National Volcanic Monument was permitted to return to its natural state without human interference—it is both a unique and fragile system due to this, and offers invaluable insights into community assembly trajectories and processes right here in the Pacific Northwest. In addition to our model ecosystem, we chose native alder (*Alnus rubra* and *A. viridis* ssp. *sinuata*) as a model plant system due to its association with nitrogen-fixing *Frankia* bacteria. Nearly all plants are colonized by mycorrhizae and endophytes (Wang and Qiu 2006; Rodriguez et al. 2009), but specialized nitrogen-fixing symbioses like that of alder are crucial components in plant recolonization of disturbed ecosystems (Morris and Wood 1989; Chapin et al. 1994), which are often harsh and nutrient-poor, as is the case on the Pumice Plain of Mount St. Helens. Nitrogen-fixation is also critical to agriculture, although that occurs primarily through Rhizobia symbioses that associate almost exclusively with legumes (Fabaceae; the genus *Parasponia* in Cannabaceae is the exception). The alder species studied here are excellent models for natural interactions and processes that span multiple scales: host–microbe, microbe–microbe, afterlife effects on litter decomposition, microbe–microbial decomposer, and numerous genotype–genotype and genotype  $\times$  environment interactions. Additionally, alder is widely distributed genus within the northern hemisphere and commonly occurs in a range of disturbed habitats, including many that are increasing in frequency and area due to global

climate change (Cortini et al. 2012). Consequently, the systems we have chosen for these studies provide insight into successional processes and microbial community assembly with very broad applications for climate change-mediated land-use changes and agriculture.

Within this dissertation, we present new data on culturable foliar endophytes during primary succession (Chapter 3), calibrated by a comparable study in a nearby metropolitan area (Chapter 2). We found that—at least for culturable endophytes—community assembly was still in the early stages of development despite nearly four decades after the 1980 eruption. Obviously, there are limitations to culture-dependent methods, and culture-independent methods that utilize direct sequencing will generally give more accurate estimations of the complete endophyte community. The sampling scheme described in Chapter 3 was used to collect additional samples for direct extraction and sequencing, which we were able to attempt prior to the completion of this document. However, the sequencing run was ultimately unsuccessful; the report suggests that there could be much lower reads than anticipated (~3.2 million compared to ~15 million reads, Illumina MiSeq V3 chemistry) for the sequenced community, but faulty index primers prevented demultiplexing and any concrete conclusions are not possible. We ultimately expect the sequenced community to have higher diversity metrics than the culturable community (which was subject to biases in media selection and growth requirements) and to exhibit a temporal shift due to an anticipated increase in statistical power. Sequencing has its own biases and limitations (e.g. primer bias), but the

community should include more taxa that were not amenable to culture-dependent methods.

If the composition of the sequenced community is still dominated by saprotrophs and pathogens, there may be stronger than anticipated effects on interactions with microbial decomposers and on leaf litter decomposition rates during early succession. Resource competition between dominant guild members (Fargione et al. 2003; Zee and Fukami 2018) should be clearer to identify in amplicon sequencing data due to greater rates of recovery—especially when compared to the <15% frequency of isolation we achieved through culture-dependent methods (Chapter 3). However, cultures will still be needed for more targeted work to identify specific endophyte community assembly dynamics and interactions, especially for potential applications in symbiosis optimization. Future work should utilize greenhouse inoculation experiments and focus on transcriptomics under common stressors (e.g., salt, heat, herbivory) to tease apart potential genes or genotypes involved in advantageous traits.

In Chapter 1, we introduced endophytes as ubiquitous members of the phytobiome with the ability to alter afterlife effects of host plant tissues. Microbial symbionts have formed complex, intimate interactions with plants since early plants first transitioned into terrestrial habitats over 400 mya, and these symbionts and other plant traits are influenced by plant genotype. We characterized population genetic structure of Sitka alder around Mount St. Helens to gain an initial understanding of colonization patterns (Chapter 4), and then examined the diversity of *Frankia* genotypes on the Pumice Plain specifically (Chapter 5) for comparison to more established sites. There is

little genetic differentiation across the sites sampled, including the Pumice Plain, which suggests that local adaptation has probably not occurred yet between particular Sitka alder and *Frankia* genotypes, even though *Frankia* diversity now matches regional studies (Brown et al. 2020). It would be worth surveying additional sites around Mount St. Helens and other reference sites in the Pacific Northwest to fully characterize regional *Frankia* diversity and how that may relate to the population genetics of its host, especially for inland populations of red alder (Loveless 2021). Alder is wind-pollinated, and other studies in Canada and across Europe have found low genetic differentiation between populations (De Kort et al. 2014; Mingeot et al. 2016), except where species ranges overlap and hybridization can occur (Bousquet et al. 1987; Villani et al. 2021). In the Pacific Northwest, red and white alder definitively hybridize (Harrington et al. 2008; Stanton et al. 2020), but ploidy should be investigated for sympatrically-occurring alder particularly on the Pumice Plain as well (J. Loveless, personal communication).

The work included in this dissertation represents a comprehensive initial study of microbial symbionts and population genetics of Sitka alder in a model early successional ecosystem. Ideally, the genome of Sitka or red alder will be sequenced, and subsequent studies will use common garden experiments for genome-wide association studies, in conjunction with transcriptomics to tease apart host–microbe interactions. Red alder is predicted to expand its range within the Pacific Northwest under global climate change, and despite having been seen as a weed species by foresters, it has become a desirable timber product in its own right (Deal and Harrington 2006). Sitka alder has been proposed as an alternative to red alder in conifer stands due to its smaller, more compact

size and comparable nitrogen-fixation rates (Harrington and Deal 1982). Studying a native alder system thus has economical implications for the forestry industry—i.e. provenance optimization through combinations of *Frankia* strains and beneficial foliar endophytes—as well as significant consequences for ecosystem processes in habitats converted by changing climate regimes. Our results can also be used to calibrate models of early community assembly in plant–microbe symbioses, which can in turn be used to inform models of nutrient cycling—especially carbon and nitrogen. As global climate change increases the frequency of catastrophic natural disasters and generally expands the amount of land undergoing early succession, these models will be important for determining benchmarks of and projecting rates of recovery.

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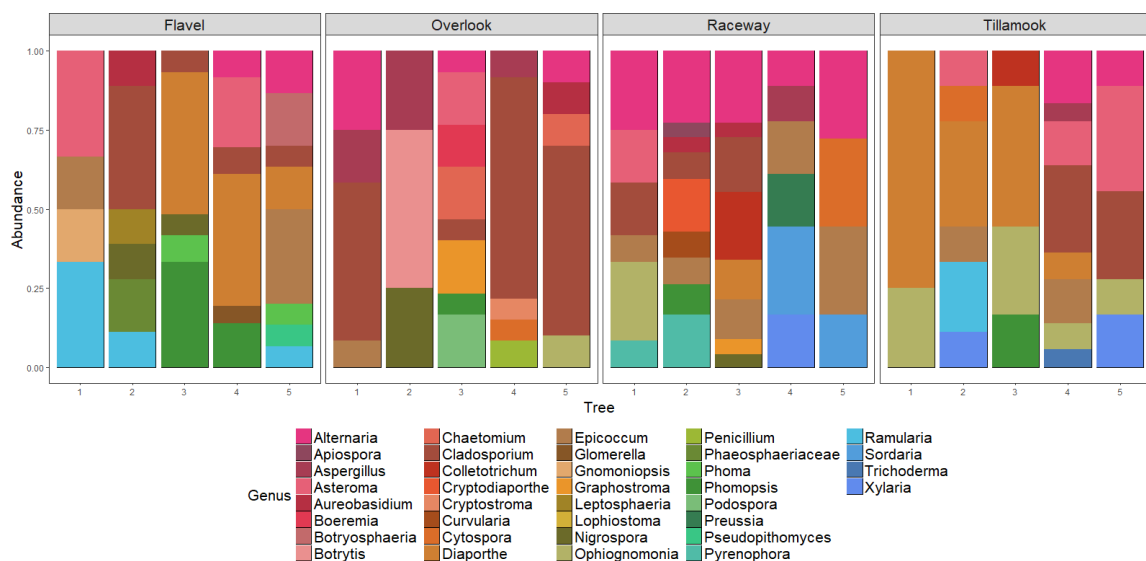
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## Appendix A: Supplementary Materials for Chapter 2



Supplementary Figure 2.1. Relative abundances of isolated taxa organized by tree (numbered 1-5) sampled within sites.

Supplementary Table 2.1. Sanger sequencing identities of axenic cultures as determined via UNITE. For analysis purposes, taxa were clustered into OTUs of the same name if identity was greater than 97% — otherwise, they were given the same genus name but only designated as “sp.” to indicate the uncertainty in that assignment.

Study Accession	GenBank Accession	Bit score	Identity	Taxonomic Identity
ERW_00461	KX768144	1049	100%	<i>Alternaria alternata</i>
ERW_00779	LT603038	976	100%	<i>Alternaria alternata</i>
ERW_00740	KX768145	812	91%	<i>Alternaria alternata</i>
ERW_00791	LC134323	1041	100%	<i>Alternaria alternata</i>
ERW_00232	KX768144	1043	100%	<i>Alternaria alternata</i>
ERW_00903	KX816024	958	100%	<i>Alternaria alternata</i>
ERW_00813	KY587323	996	100%	<i>Alternaria alternata</i>
ERW_00802	Y17071	872	100%	<i>Alternaria alternata</i>
ERW_00369	KX768144	1041	100%	<i>Alternaria arborescens</i>
ERW_00695	KX768144	1038	100%	<i>Alternaria arborescens</i>
ERW_00778	KX768144	1045	100%	<i>Alternaria arborescens</i>
ERW_00781	KX768144	1025	100%	<i>Alternaria arborescens</i>
ERW_00784	KX768144	1045	100%	<i>Alternaria arborescens</i>
ERW_00277	KX768144	1052	100%	<i>Alternaria arborescens</i>
ERW_00723	KR094444	1095	99%	<i>Alternaria infectoria</i>
ERW_00642	JX454532	1092	100%	<i>Alternaria infectoria</i>

ERW_00515	JX454532	1110	100%	<i>Alternaria infectoria</i>
ERW_00672	JX454532	1088	100%	<i>Alternaria infectoria</i>
ERW_00665	JX454532	1094	100%	<i>Alternaria infectoria</i>
ERW_00881	JX454532	1083	100%	<i>Alternaria infectoria</i>
ERW_00793	HG936537	1101	100%	<i>Alternaria infectoria</i>
ERW_00899	JX454532	1061	100%	<i>Alternaria infectoria</i>
ERW_00745	KT269034	1090	100%	<i>Alternaria</i> sp.
ERW_00641	KT268913	1101	100%	<i>Alternaria</i> sp.
ERW_00181	JQ910882	1070	100%	<i>Alternaria</i> sp.
ERW_00794	KX533933	1063	100%	<i>Apiospora montagnei</i> (as <i>Arthrinium arundinis</i> )
ERW_00904	LT719147	917	100%	<i>Apiospora montagnei</i> (as <i>Arthrinium arundinis</i> )
ERW_00084	KR909166	935	100%	<i>Aspergillus hiratsukae</i>
ERW_00164	KJ863526	939	100%	<i>Aspergillus hiratsukae</i>
ERW_00142	ISHAM:MIT244-GQ461906	1101	100%	<i>Aspergillus hiratsukae</i>
ERW_00601	KR909166	969	100%	<i>Aspergillus hiratsukae</i>
ERW_00530	ISHAM:MIT244-GQ461906	1104	100%	<i>Aspergillus hiratsukae</i>
ERW_00278	EU167609	1005	97%	<i>Asteroma alneum</i>
ERW_00288	EU167609	962	97%	<i>Asteroma alneum</i>
ERW_00262	EU167609	957	97%	<i>Asteroma alneum</i>
ERW_00207	EU167609	942	97%	<i>Asteroma alneum</i>
ERW_00487	EU167609	991	97%	<i>Asteroma alneum</i>
ERW_00097	EU167609	838	97%	<i>Asteroma alneum</i>
ERW_00443	EU167609	953	94%	<i>Asteroma alneum</i>
ERW_00882	EU167609	1016	99%	<i>Asteroma alneum</i>
ERW_00718	EU167609	1088	99%	<i>Asteroma alneum</i>
ERW_00101	EU167609	1047	99%	<i>Asteroma alneum</i>
ERW_00325	EU167609	1005	97%	<i>Asteroma alneum</i>
ERW_00797	EU167609	1097	99%	<i>Asteroma alneum</i>
ERW_00328	KX515494	1067	100%	<i>Aureobasidium pullulans</i>
ERW_00536	KX515494	1070	100%	<i>Aureobasidium pullulans</i>
ERW_00358	LN906592	892	100%	<i>Aureobasidium pullulans</i>
ERW_00668	KX515494	1047	100%	<i>Aureobasidium pullulans</i>
ERW_00116	LT158236	699	100%	<i>Boeremia exigua</i>
ERW_00231	KT268393	994	100%	<i>Boeremia exigua</i> (as <i>Boeremia exigua</i> var. <i>exigua</i> )
ERW_00093	KY465920	1036	100%	<i>Botryosphaeria stevensii</i> (as <i>Diplodia mutila</i> )
ERW_00112	Z99664	619	95%	<i>Botrytis cinerea</i>
ERW_00818	KM268718	1059	100%	<i>Chaetomium</i> sp.
ERW_00799	KM268718	1040	100%	<i>Chaetomium</i> sp.

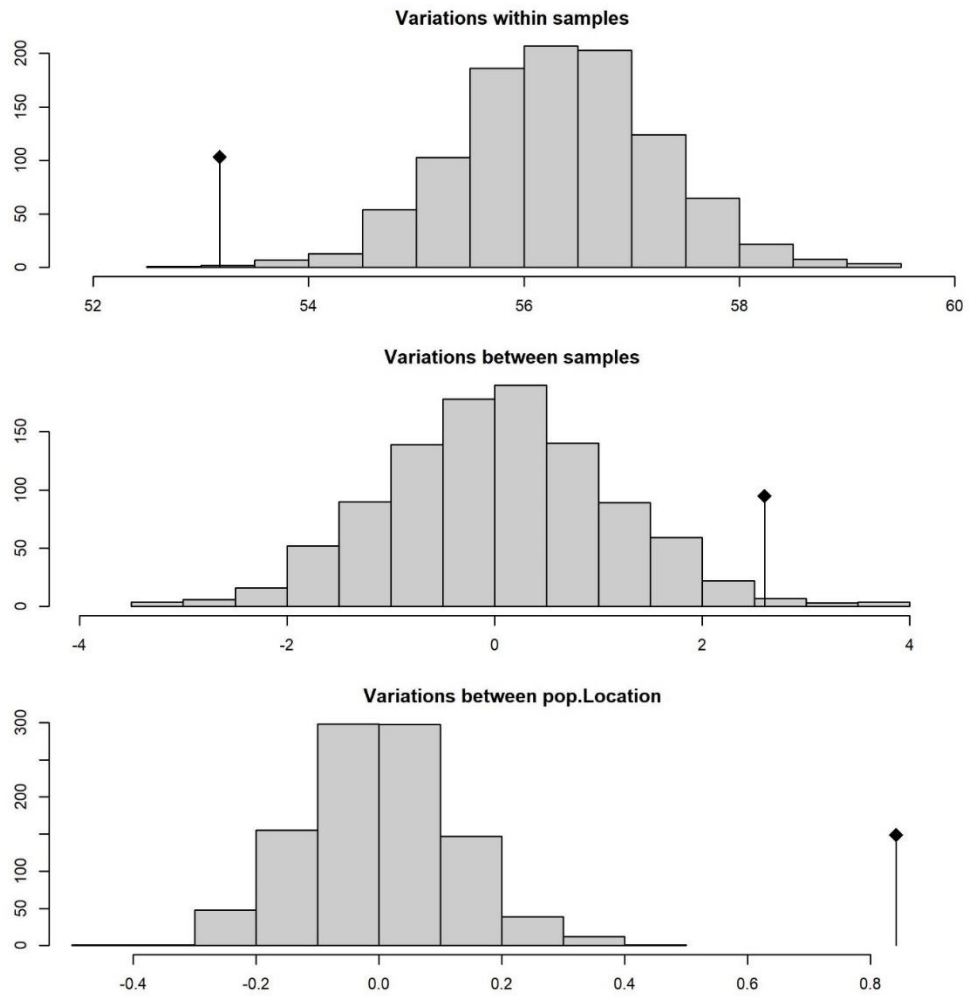
ERW_00291	KU515971	1011	100%	<i>Cladosporium allicinum</i>
ERW_00508	KU515971	1003	100%	<i>Cladosporium allicinum</i>
ERW_00796	LN834378	1002	100%	<i>Cladosporium allicinum</i>
ERW_00324	LN834378	1007	100%	<i>Cladosporium allicinum</i>
ERW_00660	LN834378	993	100%	<i>Cladosporium allicinum</i>
ERW_00215	LN834378	1009	100%	<i>Cladosporium allicinum</i>
ERW_00124	LN834378	1005	100%	<i>Cladosporium allicinum</i>
ERW_00198	KU515971	1011	100%	<i>Cladosporium allicinum</i>
ERW_00254	KU515971	1014	100%	<i>Cladosporium allicinum</i>
ERW_00454	KU515971	1011	100%	<i>Cladosporium allicinum</i>
ERW_00089	KX839305	1016	100%	<i>Cladosporium cladosporioides</i>
ERW_00127	KX515853	1012	100%	<i>Cladosporium cladosporioides</i>
ERW_00433	KX515853	1018	100%	<i>Cladosporium cladosporioides</i>
ERW_00479	LN809019	890	100%	<i>Cladosporium cladosporioides</i>
ERW_00256	LN834378	1005	100%	<i>Cladosporium herbarum</i>
ERW_00168	KU515971	1012	100%	<i>Cladosporium herbarum</i>
ERW_00310	LN834419	962	100%	<i>Cladosporium macrocarpum</i>
ERW_00625	KX674646	1005	100%	<i>Cladosporium ramotenellum</i>
ERW_00661	KX674646	1007	100%	<i>Cladosporium ramotenellum</i>
ERW_00437	KX674646	1018	100%	<i>Cladosporium ramotenellum</i>
ERW_00667	LN834387	966	100%	<i>Cladosporium ramotenellum</i>
ERW_00531	KX674647	1012	100%	<i>Cladosporium</i> sp.
ERW_00167	KX674647	1007	100%	<i>Cladosporium</i> sp.
ERW_00132	KP701932	1007	100%	<i>Cladosporium</i> sp.
ERW_00107	KC884371	181	100%	<i>Cladosporium</i> sp.
ERW_00279	KY096655	1016	100%	<i>Cladosporium</i> sp.
ERW_00578	LN850753	996	100%	<i>Cladosporium subuliforme</i>
ERW_00603	LN850753	856	100%	<i>Cladosporium subuliforme</i>
ERW_00421	AJ301972	1065	100%	<i>Colletotrichum gloeosporioides</i>
ERW_00751	KU498278	1059	100%	<i>Colletotrichum salicis</i>
ERW_00673	KT963798	1065	100%	<i>Colletotrichum salicis</i>
ERW_00675	KT963798	1063	100%	<i>Colletotrichum</i> sp.
ERW_00611	KT963798	1054	100%	<i>Colletotrichum</i> sp.
ERW_00832	GU367061	908	98%	<i>Cryptodiaporthe pulchella</i>
ERW_00906	KP114070	989	99%	<i>Cryptostroma corticale</i>
ERW_00329	EU490103	1040	100%	<i>Curvularia inaequalis</i>
ERW_00792	KY417726	969	100%	<i>Cytospora</i> sp.
ERW_00759	KP114086	899	100%	<i>Cytospora</i> sp.
ERW_00647	KP114086	886	100%	<i>Cytospora</i> sp.
ERW_00341	KF294010	1043	99%	<i>Cytospora</i> sp.
ERW_00064	KR094451	1074	99%	<i>Diaporthe cotoneastri</i>

ERW_00105	KR094451	1079	100%	<i>Diaporthe cotoneastri</i>
ERW_00283	KR094451	1065	100%	<i>Diaporthe cotoneastri</i>
ERW_00226	KR094451	1074	99%	<i>Diaporthe cotoneastri</i>
ERW_00330	KR094451	1083	100%	<i>Diaporthe cotoneastri</i>
ERW_00150	KX866898	1050	99%	<i>Diaporthe nobilis</i>
ERW_00702	KX866898	884	99%	<i>Diaporthe nobilis</i>
ERW_00825	KC145904	1088	100%	<i>Diaporthe rudis</i>
ERW_00365	KC145904	1085	100%	<i>Diaporthe rudis</i>
ERW_00271	KC145904	1086	100%	<i>Diaporthe rudis</i>
ERW_00537	KC145907	1081	100%	<i>Diaporthe rudis</i>
ERW_00752	KC145907	1072	100%	<i>Diaporthe rudis</i>
ERW_00398	KJ482538	1076	100%	<i>Diaporthe rudis</i>
ERW_00260	KJ482539	1038	100%	<i>Diaporthe rudis</i>
ERW_00504	KJ482538	1077	100%	<i>Diaporthe rudis</i>
ERW_00339	KJ482538	1083	100%	<i>Diaporthe rudis</i>
ERW_00742	KC145848	989	99%	<i>Diaporthe</i> sp.
ERW_00499	KC145901	1054	99%	<i>Diaporthe</i> sp.
ERW_00367	KU375726	897	100%	<i>Diaporthe</i> sp.
ERW_00411	NR_103702	998	100%	<i>Diaporthe viticola</i>
ERW_00498	NR_103702	630	100%	<i>Diaporthe viticola</i>
ERW_00438	NR_103702	915	100%	<i>Diaporthe viticola</i>
ERW_00252	KR094461	989	100%	<i>Epicoccum nigrum</i>
ERW_00210	KY430456	991	100%	<i>Epicoccum nigrum</i>
ERW_00257	KR094461	998	100%	<i>Epicoccum nigrum</i>
ERW_00115	KR094461	1002	100%	<i>Epicoccum nigrum</i>
ERW_00239	KR094461	998	100%	<i>Epicoccum nigrum</i>
ERW_00691	KR094461	1000	100%	<i>Epicoccum nigrum</i>
ERW_00247	KR094461	1000	100%	<i>Epicoccum nigrum</i>
ERW_00535	KY430456	877	100%	<i>Epicoccum nigrum</i>
ERW_00426	KR094452	996	100%	<i>Epicoccum nigrum</i>
ERW_00773	KY430456	989	100%	<i>Epicoccum nigrum</i>
ERW_00143	KX516492	998	100%	<i>Epicoccum nigrum</i>
ERW_00693	KX516492	996	100%	<i>Epicoccum nigrum</i>
ERW_00068	KX516492	975	100%	<i>Epicoccum nigrum</i>
ERW_00171	KR094452	1002	100%	<i>Epicoccum nigrum</i>
ERW_00453	KR094452	1000	100%	<i>Epicoccum nigrum</i>
ERW_00883	KU612886	1052	100%	<i>Glomerella acutata</i> (as <i>Colletotrichum acutatum</i> )
ERW_00732	KC145891	1124	100%	<i>Gnomoniopsis idaicola</i>
ERW_00687	EU715682	931	99%	<i>Graphostroma</i> sp.
ERW_00653	EU715682	1034	98%	<i>Graphostroma</i> sp.
ERW_00762	KC867873	948	99%	<i>Leptosphaeria</i> sp.

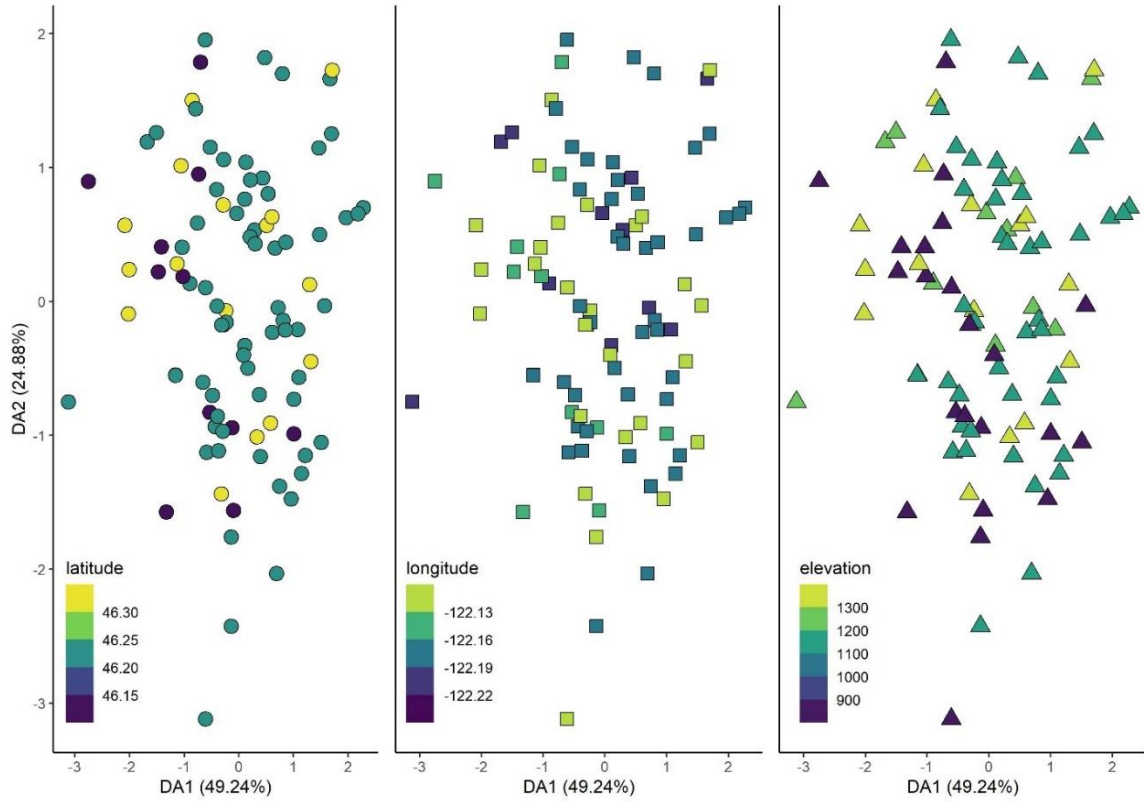
ERW_00148	FJ820764	971	99%	<i>Lophiostoma</i> sp.
ERW_00764	KT224862	928	100%	<i>Nigrospora oryzae</i>
ERW_00189	GQ120972	1018	100%	<i>Nigrospora</i> sp.
ERW_00630	GQ120972	1014	100%	<i>Nigrospora</i> sp.
ERW_00897	GQ120972	998	100%	<i>Nigrospora</i> sp.
ERW_00507	JQ414257	726	98%	<i>Ophiognomonium ibarakiensis</i>
ERW_00471	DQ323543	897	97%	<i>Ophiognomonium intermedia</i>
ERW_00864	DQ323543	868	98%	<i>Ophiognomonium intermedia</i>
ERW_00149	DQ323543	906	97%	<i>Ophiognomonium intermedia</i>
ERW_00713	DQ323543	863	98%	<i>Ophiognomonium intermedia</i>
ERW_00344	DQ323543	774	97%	<i>Ophiognomonium intermedia</i>
ERW_00179	DQ323543	937	97%	<i>Ophiognomonium intermedia</i>
ERW_00275	JQ414267	807	97%	<i>Ophiognomonium intermedia</i>
ERW_00888	EU254984	749	99%	<i>Ophiognomonium</i> sp.
ERW_00973	KX664349	1003	99%	<i>Penicillium glabrum</i>
ERW_00545	KX243327	1058	100%	<i>Penicillium</i> sp.
ERW_00217	EF505549	926	96%	<i>Phaeosphaeriaceae</i> sp.
ERW_00701	KT768220	985	100%	<i>Phoma</i> sp.
ERW_00705	JN225888	964	99%	<i>Phoma</i> sp.
ERW_00747	KX065017	836	100%	<i>Phomopsis vaccinii</i> (as <i>Diaporthe vaccinii</i> )
ERW_00834	KX065017	901	100%	<i>Phomopsis vaccinii</i> (as <i>Diaporthe vaccinii</i> )
ERW_00495	FJ478132	1065	100%	<i>Phomopsis velata</i> (as <i>Diaporthe eres</i> )
ERW_00823	FJ478132	1076	100%	<i>Phomopsis velata</i> (as <i>Diaporthe eres</i> )
ERW_00424	FJ478132	1065	100%	<i>Phomopsis velata</i> (as <i>Diaporthe eres</i> )
ERW_00721	FJ478132	1052	100%	<i>Phomopsis velata</i> (as <i>Diaporthe eres</i> )
ERW_00219	FJ478132	1072	100%	<i>Phomopsis velata</i> (as <i>Diaporthe eres</i> )
ERW_00377	FJ478132	1070	100%	<i>Phomopsis velata</i> (as <i>Diaporthe eres</i> )
ERW_00476	KC867961	955	100%	<i>Phomopsis velata</i> (as <i>Diaporthe eres</i> )
ERW_00200	AY999122	1045	100%	<i>Podospira curvicolla</i>
ERW_00634	KF752699	991	100%	<i>Preussia</i> sp.
ERW_00493	GU566269	1097	100%	<i>Pseudopithomyces chartarum</i> (as <i>Pithomyces chartarum</i> )
ERW_00722	FN386271	937	100%	<i>Pyrenophora erythrospila</i> (as <i>Drechslera erythrospila</i> )
ERW_00893	AY004783	976	99%	<i>Pyrenophora erythrospila</i> (as <i>Drechslera erythrospila</i> )
ERW_00170	KX287331	922	100%	<i>Ramularia archangelicae</i>
ERW_00616	KC867933	913	99%	<i>Ramularia</i> sp.
ERW_00696	KX287410	978	100%	<i>Ramularia</i> sp.
ERW_00760	KX287410	964	100%	<i>Ramularia</i> sp.
ERW_00623	KX287410	966	99%	<i>Ramularia</i> sp.

ERW_00205	KP698327	1070	100%	<i>Sordaria humana</i>
ERW_00664	KP698327	998	100%	<i>Sordaria humana</i>
ERW_00211	KP698327	1074	100%	<i>Sordaria humana</i>
ERW_00866	HQ630960	1121	100%	<i>Trichoderma harzianum</i>
ERW_00183	GU300096	1018	99%	<i>Xylaria hypoxylon</i>
ERW_00244	KY610407	1067	99%	<i>Xylaria hypoxylon</i>
ERW_00608	KY610407	1068	100%	<i>Xylaria hypoxylon</i>

## Appendix B: Supplementary Materials for Chapter 4



Supplementary Figure 4.1. Plots of variation within samples, between samples, and between sites generated from AMOVA.



Supplementary Figure 4.2. Discriminant analysis of principal components (DAPC) showing distribution of samples by latitude, longitude, and elevation.



Supplementary Information 4.1. Modified CTAB protocol

To calculate amt of buffer:		samples	94	468.75	ul per sample to acct for error
(Use orig Excel sheet to calculate)		blanks	2	937.5	ul sorbitol per sample to acct for error

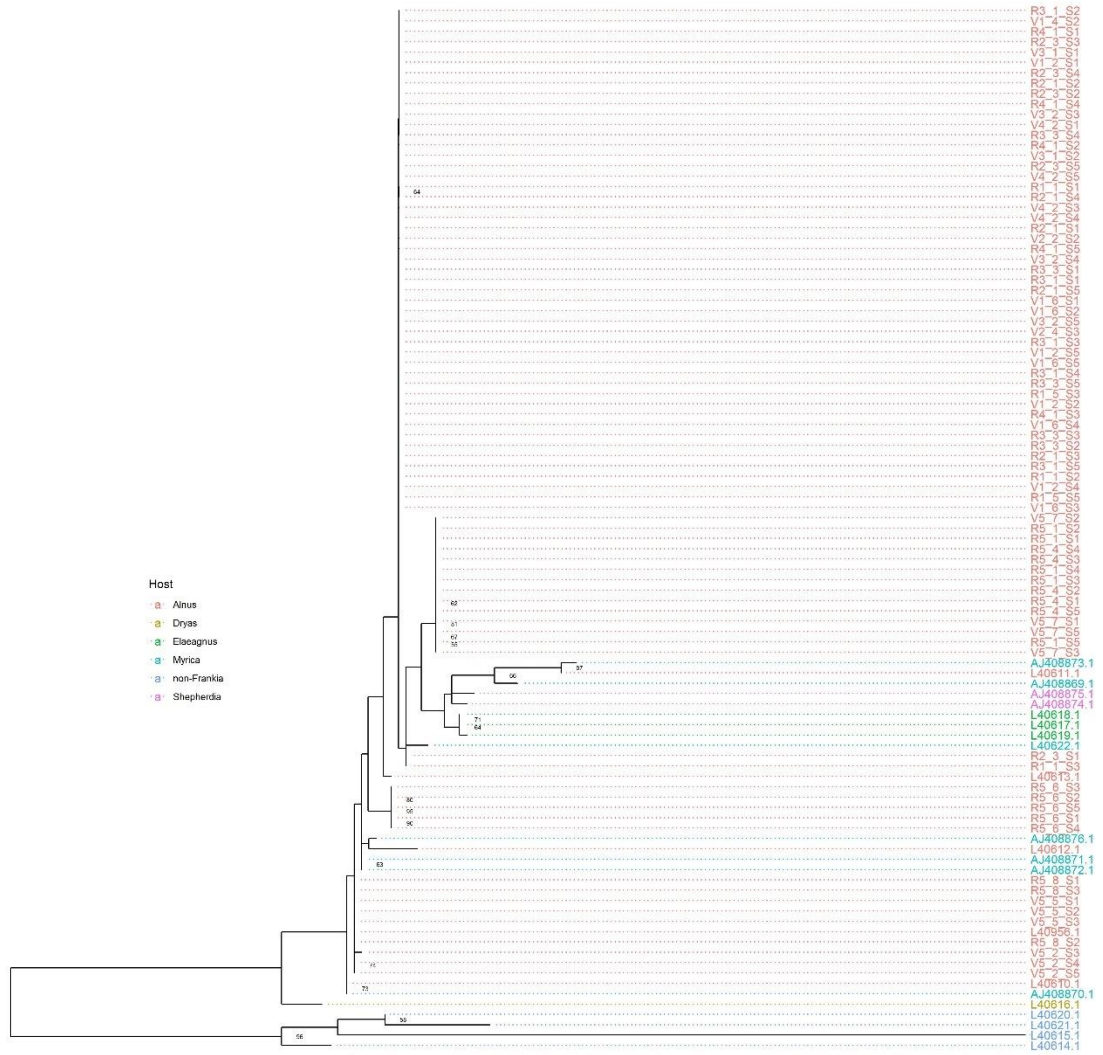
Step	Step notes	Reagent	Amt/ sample	# samples	Total vol (µl)	Total amt (mL)	Notes
1	Prepare CTAB buffer	3% (high-salt) CTAB buffer	468.75	96	45000	45	mL; accounts for 10% error & rounds to next whole number
		BME (0.03% v/v)			135	135	µl; 15 µl/5mL
		PVP (1% w/v)			(0 g)		mg; 50 mg/5mL
2	Prepare sorbitol wash buffer	sorbitol buffer	937.5	96	90000	90	mL; accounts for 10% error & rounds to next whole number
		BME (1% v/v)			450	900	µl; 1000 µl/100mL
		PVP (1% w/v)			(0 g)		mg; 50 mg/5mL

Step	Step notes	Reagent	Layer vol	Step vol	Final vol	Notes
3	Heat CTAB to 65C in incubator.					takes about 20 min if little oven starts cold
4	Put isopropanol aliquots in -20 freezer					
5	Add steel bead to each well, add samples to wells, add second steel bead, cap with strip tubes, and beat 4 x 1 min at 30 Hz or until fine powder					Powder will fly out, so centrifuge plates for ~1 min, and carefully peel the lids off sideways, to avoid cross-contam. Also check bottoms of plates for cracks and if caps are seated.
6	Add sorbitol wash buffer and beat 2 x 30 s at 20 Hz to mix. Centrifuge to pellet leaf material and decant supernatant. Repeat.			400 * 2		do 2 x 400 ul washes; 5 min at 4000 is plenty
7	Add RNase to CTAB buffer. Add CTAB buffer to each well.	prepared CTAB		400		float a trough in warm water to help with pipetting

Step	Step notes	Reagent	Layer vol	Step vol	Final vol	Notes
8	Beat again for 2 x 30 sec at 20 Hz					
9	Incubate for 1 hour at 65C. At ~10 minute intervals, beat 1 x 30 sec at 20 Hz to mix. Centrifuge 10 min at 4000 x g.					<b>SWITCH TO NITRILE GLOVES.</b> Can centrifuge at 1000 to avoid imbalance.
10	Add 400 µl of C:1 to each well and mix by beating 1 x 30 sec at 20 Hz!!!!!! Make sure caps are seated first and plate tape is applied to top.	C:1		400	800	Cut a piece of parafilm and unpeel the backing. Use it to keep the dish from sliding forward, and then wrap the bottle with it.
11	Seal with tape and centrifuge 10 minutes at 4000 x g.					Can centrifuge at 1000 to avoid imbalance.
12	Remove 150 µl of the aqueous phase from each well and move to 500 µl well plate. Repeat for replicates.	aqueous layer	150		150	Be extremely careful to avoid aqueous layer! Stop when about 1 cm of lip (before lip with filler inside) is level with the plate.
13	Add 15 µl of 3M sodium acetate and 150 µl of ice-cold isopropanol to each well. Mix 10 times. Incubate one hour at -20.	sodium acetate & isopropanol		150	315	go get ice from downstairs and chill the EtOH
14	Seal with tape and centrifuge 20 minutes at 4000 x g					
15	Carefully remove isopropanol by dumping onto clean paper towel. Wash 3x with 100 µl 70% EtOH, spin down, and dump on towel.	70% ethanol, chilled		100	100	5 min at 4000 is good but <b>don't</b> wait too long after the centrifuge step to do this, or the pellets will detach and fall out...
16	Speedvac to dry the pellet					~5 min with medium drying rate
17	Add 50-60 µl TE buffer and resuspend. Seal and put in fridge overnight (min. 20 minutes @ 37C with shaking) before measuring on QuBit.	TE buffer		50	50	check to see if pellets should be resuspended in PCR water vs TE buffer

## Appendix C: Supplementary Materials for Chapter 5



Supplementary Figure 5.1. Phylogenetic tree of partial 16S sequences from Mount St. Helens and several accessions from NCBI GenBank.