Bacterial endophyte communities in *Pinus flexilis* are structured by host age, tissue type, and environmental factors

Dana L. Carper[1], Alyssa A. Carrell[2,3,4], Lara M. Kueppers[5,6], A. Carolin Frank[2,5]

1. Quantitative and Systems Biology Program, University of California Merced, Merced, USA

2. Life and Environmental Sciences, School of Natural Sciences, University of California Merced, Merced, USA

3. Bredesen Center for Interdisciplinary Research and Graduate Education, University of Tennessee, Knoxville, USA

4. Biosciences Division, Oak Ridge National Laboratory, Oak Ridge, USA

5. Sierra Nevada Research Institute, University of California Merced, Merced, USA

6. Energy and Resources Group, University of California, Berkeley, Berkeley, USA

Abstract

Background and aims: Forest tree microbiomes are important to forest dynamics, diversity, and ecosystem processes. Mature limber pines (*Pinus flexilis*) host a core microbiome of acetic acid bacteria in their foliage, but the bacterial endophyte community structure, variation, and assembly across tree ontogeny is unknown. The aims of this study were to test if the core microbiome observed in adult *P. flexilis* is established at the seedling stage, if seedlings host different endophyte communities in root and shoot tissues, and how environmental factors structure seedling endophyte communities. *Methods:* The 16S rRNA gene was sequenced to characterize the bacterial endophyte communities in roots and shoots of *P. flexilis* seedlings grown in plots at three elevations at Niwot Ridge, Colorado, subjected to experimental treatments (watering and heating). The data was compared to previously sequenced endophyte communities from adult tree foliage sampled in the same year and location. *Results:* Seedling shoots hosted a different core microbiome than adult tree foliage and were dominated by a few OTUs in the family *Oxalobacteraceae*, identical or closely related to strains with antifungal activity. Shoot and root communities significantly differed from each other but shared major OTUs. Watering but not warming restructured the seedling endophyte communities. *Conclusions:* The results suggest differences in assembly and ecological function across conifer life stages. Seedlings may recruit endophytes to protect against fungi under increased soil moisture.

Keywords: 16S rRNA, Endophytic bacteria, *Pinus flexilis*, Climate change, Conifers

Introduction

The plant microbiome, the collection of microorganisms that live on and within the tissues of plants, is emerging as a crucial component of plant health and resilience. The portion of the plant microbiome that colonizes the

interior of plant tissues as endophytes is of particular interest due to their position to influence the host plant from within. Endophytes can be commensals or pathogens, but some provide their host with nutrients that are not readily available in the environment, for instance via nitrogen fixation (Elbeltagy et al. 2001), phosphorus solubilization (Oteino et al. 2015), and bedrock weathering (He et al. 2017). Beneficial endophytes can also buffer their plant host against biotic and abiotic stress, for example by altering the expression of stress-inducible genes (Sziderics et al. 2007), secreting antimicrobial compounds (Stinson et al. 2003), or inducing systemic plant resistance (Kloepper and Ryu 2006). Much of our understanding of bacterial endophytic communities and their roles in plant physiology and ecology is derived from agricultural plants and herbaceous model species. The endophytic microbiomes of *Arabidopsis thaliana* and crops such as rice tend to be diverse, originate mainly from the soil, and be structured by the environment, predominantly soil type or origin (Bulgarelli et al. 2012; Edwards et al. 2015; Lundberg et al. 2012; Peiffer et al. 2013; Schlaeppi et al. 2014; Schlaeppi and Bulgarelli 2015; Yeoh et al. 2017). Studies on rice have demonstrated rapid colonization of the root endosphere from soil via the root surface (Edwards et al. 2015) and studies on *A. thaliana* show that microbiomes can be dynamically recruited and modulated via phytohormones, sometimes in response to stress (Castrillo et al. 2017; Lebels et al. 2015). As a consequence, changing environmental conditions such as nutrient limitation or drought often leads to restructuring of plant microbiomes (Ikeda et al. 2014; Marasco et al. 2012; Naveed et al. 2014; Santos-Medellín et al. 2017). In contrast, much less is known about the structure, diversity, and transmission dynamics of endophyte communities associated with large and long-lived plants in situ.

The few studies that examine endophyte communities in forest trees identify host species, geographic location, and soil type as factors structuring the leaf- and root endophyte communities. A study of leaf endophytes in maple and elm (*Acer negundo*, *Ulmus pumila*, and *Ulmus parvifolia*) growing in an urban environment showed that season was more important than species in structuring the communities (Shen and Fulthorpe 2015). Host genotype matters when distantly related species are compared, such as those in the roots of willow oak (*Quercus phellos*), loblolly pine (*Pinus taeda*) and eastern cottonwood (*Populus deltoides*), which were found to be structured more by host species than by soil origin (Bonito et al. 2014). Studies focusing only on *P. deltoides* show that geography is more important than host genotype in structuring microbial communities (Gottel et al. 2011; Shakya et al. 2013). A study in Poplar clones identified plant compartment (rhizosphere, root, stem and leaf endosphere) as important in structuring microbial communities (Beckers et al. 2017), suggesting active selection on the colonization of different compartments. Carrell and Frank (2015) characterized the bacterial communities in foliage of mature coast redwood (*Sequoia sempervirens*) in two locations and giant sequoia (*Sequoiadendron giganteum*) in one location,

and found differences between both site and species (though site and species effects could not be separated). In contrast, studies of subalpine conifers suggest some stability in foliar endophyte community across individuals, host species, sites, and time. Specifically, taxa in the Alphaproteobacterial family *Acetobacteraceae*, or acetic acid bacteria (AAB) appear to make up a core endophytic microbiome across host species, location, and year of sampling in limber pine (*Pinus flexilis*), lodgepole pine (*Pinus contorta*) and Engelmann spruce (*Picea engelmannii*), potentially reflecting a nitrogen-fixation partnership (Carrell et al. 2016; Carrell and Frank 2014; Moyes et al. 2016). Within the current scientific literature, a core microbiome at the level of operational taxonomic units (OTUs) has not been observed in other plants. The consistent recurrence of AAB taxa in the needles of adult pines may reflect a nitrogen–fixing partnership between tree and endophytes, as well as restriction in the colonization of new taxa. Some community turnover was observed, including changes in the relative abundance of dominant AAB OTUs between years, and the appearance of vagrant or temporary community members (Moyes et al. 2016). The mechanism by which pine trees acquire their bacterial endophytes is not known, nor is the timing of colonization. Endophytes may colonize trees early during the seed or seedling stage, or later throughout the lifetime of an individual tree, but studies on how ontogeny affects the microbiome composition of pines and other forest trees are lacking.

At the seedling stage, soil is likely the main transmission route for the tree microbiome, and the proximity to the diverse soil bacterial community is likely to result in a richer and more variable microbiome compared to the foliage of adult trees. In addition, the microbiome may be involved in buffering seedlings against biotic and abiotic environmental stress during this vulnerable life stage. Seedlings of subalpine conifers are exposed to extremes of temperature, humidity, radiation, and soil moisture (Germino et al. 2002; Germino and Smith 1999). In *P. contorta*, overall soil biota has been shown to have a strong effect on conifer seedling growth. Gundale et al. (2014) tested the effect of soil origin on growth of seedlings while controlling for differences in soil nutrient status, and found higher seedling growth in Swedish soil than in Canadian soil. The study did not characterize soil-bacterial communities or the plant microbiomes, but the results suggest that the fungi or bacteria available for recruitment to the rhizosphere and endosphere were different in the two soils. To our knowledge, the microbiomes of conifer seedlings have not been characterized, and it is not known to what extent they are sensitive to environmental change.

Here, this study takes advantage of a warming experiment conducted on *P. flexilis* seedlings in forest, treeline, and alpine sites at Niwot Ridge, Colorado, a location where adult foliar communities from *P. flexilis* trees have been previously sampled and characterized. This experiment allowed several questions to be answered about the factors that structure endophyte communities of forest trees: First, since seedlings were sampled the same

year as the previously analyzed adult foliage (Carrell et al. 2016), is the *P. flexilis* seedling microbiome similar to the microbiome of adult needles from the surrounding forest, (i.e. if the AAB core microbiome is established early)? Second, are the seedling shoot and root communities different, as observed in other plants? Third, do environmental factors (including experimental heating and water addition, as well as site) shape seedling root and shoot communities?

Material and methods

Site and experimental climate treatments

The Alpine Treeline Warming Experiment (ATWE) was established at Niwot Ridge, Colorado to study effects of climate change on seedling establishment within and beyond the elevation range of subalpine forest (Castanha et al. 2013; Kueppers et al. 2017a). Common gardens were established at three sites: near the lower, warm edge of the current subalpine forest (forest, 3060 m), within the alpine-treeline ecotone (treeline, 3430 m), and above treeline (alpine, 3540 m). At each of the sites, 20 3-m diameter plots were assigned to one of four treatments: control (C), heated (H), watered (W), and heated and watered (HW). Heated plots were surrounded by six, 240 V, 1000 W, infrared (IR) heaters (Mor Electric Heating, Comstock Park, MI, USA) mounted on perimeter scaffolding at 1.2 m height. Heaters were supplied with constant power during the snow-free season, and a lower level (except in 2009–2010) of constant power during winter. To offset increased soil water evaporation by the heaters, watered plots received 2.5 mm of water weekly, beginning about 2–3 weeks following snowmelt. Experimental treatments began in October 2009. Seed was collected locally, processed as described in Kueppers et al. (2017a) and sown in the common gardens in the autumn of each year. Emergent seedlings were surveyed weekly in spring and summer, and individually tracked from one season to the next to quantify germination (Kueppers et al. 2017b) and recruitment up to 4 years (Kueppers et al. 2017a). The work was carried out with permission from the U.S. Forest Service via the University of Colorado Mountain Research Station.

Sample collection and sterilization

Thirty-three one-year-old seedlings were collected from the ATWE in July 2012. At the forest and alpine sites, three and five control samples were taken respectively, Within the treeline site, seedlings were sampled from all four experimental treatments; control (6), heated (6), watered (10), and heated and watered (3). Seedling replicates were limited by seedling availability for destructive harvest. Seedlings were placed in sterile tubes and shipped on ice to University of California, Merced for surface sterilization and DNA extraction. The seedlings were surface-sterilized by submersing in 30% hydrogen peroxide for 3 min followed by three rinses with sterile deionized water, and stored at −20°C. The final rinse after sterilization was saved to verify sterility by negative PCR amplification of the 16S rRNA gene.

DNA extraction

The seedlings were separated into shoot (stem and emerging needles) and root (the minimally branched root) tissues using sterile tweezers and razor blades and ground it to a fine powder in a Fisher Scientific™ PowerGen™ cryogenic homogenizer using sterile mortar and pestles with liquid nitrogen. DNA was extracted from varying amounts of tissue due to the small size of the seedlings using a modified CTAB extraction as previously described (Carrell and Frank 2014). For each sample, 800 µl of CTAB solution (1 ml of CTAB buffer, 0.04 g of polyvinylpyrollidone, 5 µl of 2-mercaptoethanol) was added to the ground tissue in a 2 ml screw cap tube. The tubes were then incubated in a dry bath at 60 °C for 1.5 h with intermittent vortexing. After incubation, 0.3 g of 0.11 mm sterile glass beads was added to the tube and the sample was homogenized using a bead beater for 1.5 min. To remove proteins, an equal amount of chloroform was added to the tube, which was then mixed and centrifuged for 10 min at 16,000 rcf. For precipitation of nucleic acids, the aqueous top phase was placed in a sterile 2 ml snap cap tube and 1/10 volume of cold 3 M sodium acetate and 1/2 volume of cold isopropanol were added and the tubes were placed in a − 20°C freezer for 12 h. The samples were then centrifuged for 30 min at 16,000 rcf, the supernatant was decanted, 700 µl of 70% ethanol was added, and the tubes were centrifuged again for 10 min. Finally, the air-dried pellet was resuspended with 30 µl of DNA resuspension fluid (1.0 M Tris-HCl, 0.1 M EDTA) and stored at −20°C.

DNA amplification

The 16S rRNA gene was amplified using the extracted DNA as template and a nested PCR approach. Chloroplast DNA amplification was reduced by primer pair 16S 799f (AACMGGATTAGATACCCKG) and 16S 1492r (TACGGHTACCTTGTTACGACT) in the first PCR reaction (PCR1). These primers were developed to suppress amplification of chloroplast DNA, and yield a mitochondrial amplicon approximately 1000 bp and a bacterial amplicon of 750 bp (Chelius and Triplett 2001). In the second round of PCR (PCR2) an appropriate amplicon length for Illumina sequencing was achieved with Golay-barcoded primer pair 799f and 1115r (AGGGTTGCGCTCGTTG), an optimized primer set for phylogenetic analysis of short reads (Redford et al. 2010). The number of cycles was reduced to reduce primer bias (Jiao et al. 2006), using the following thermocycler profile for PCR1 and PCR2: one cycle of 3 min at 95 °C; 20 cycles of 40 s at 95 °C, 40 s at 50 °C, 1.5 min at 72 °C; followed by a final elongation of 10 min at 72 °C. The 50 µl PCR1 reaction contained 5 µl of DNA extract, 20 µl of 5 PRIME Hot Master Mix (5 PRIME Inc., Hilden Germany), 0.5 µg/µl Bovine Serum Albumin (Thermo Fisher Scientific, Waltham, MA, USA), 21.5 µl PCR grade water (Fisher BioReagents, Waltham, MA, USA) and 0.2 µM of forward and reverse primers. The PCR2 reactions were performed in triplicate with each reaction containing 3 µl of PCR1, 10 µl of 5 PRIME Hot Master Mix, 0.5 µg/µl Bovine Serum Albumin, 8.75 µl of PCR grade water and 0.2 µM of forward and reverse primers. The barcoded DNA

was cleaned and pooled in equal amounts from each sample, and gel extracted (QIAquick gel extraction kit, Qiagen Inc. Valencia, CA, USA) to ensure the correct band size and remove most of the mitochondrial amplicons. The pooled sample was submitted for Illumina sequencing at the University of California, Davis Genome Center. The sequences have been submitted to the GenBank SRA under BioProject PRJNA307272.

Sequence analysis

The sequences were analyzed and processed using the QIIME (v1.9.1) package (Caporaso et al. 2010b) and UPARSE (v8.0.1517) package (Edgar 2013). Paired-end forward and reverse reads were joined with fastq-join, with the barcode filtered from the dataset if the forward and reverse reads did not overlap (Aronesty 2011). The joined reads were quality filtered as implemented in QIIME (Bokulich et al. 2013): maximum number of consecutive low quality base calls of 3 bases; maximum unacceptable Phred quality score of 3; no N characters; the minimum number of consecutive high quality base calls as a fraction of the input read length of 0.50 total read length. A previously published dataset of 16S rRNA sequences amplified with the same primer set from five *P. flexilis* and five *P. contorta* adult needle tissue samples taken in 2012 from Niwot Ridge (Carrell et al. 2016) was combined with the seedling sequences. Both *P. flexilis* and *P. contorta* adult samples were included as the previous analysis indicated no statistically significant differences in the communities (Carrell et al. 2016). UPARSE was used to dereplicate the remaining sequences, remove singletons, and cluster the remaining reads by 97% similarity into operational taxonomic units (OTUs). After removing chimeras using UPARSE, taxonomy was assigned to the OTUs via the UCLUST consensus taxonomy assigner implemented in QIIME against the SILVA database (Pruesse et al. 2007), and OTUs classified as ‘Chloroplast’, ‘Mitochondria’, and ‘Unassigned’ were removed. The sequences were aligned using PyNAST (Caporaso et al. 2010a) against the SILVA database, and an approximate maximum-likelihood tree was built using FastTree (Price et al. 2009). Sequence counts were normalized across samples using the cumulative sum scaling implemented in MetagenomeSeq (v.1.16.0) (Paulson et al. 2013, 2016) to overcome uneven sequencing depth.

Community structure analysis

A rarefaction analysis was performed as implemented in QIIME to check for adequate sequencing depth for each sample. While amplification and sequencing of 16S rRNA gene amplicons from shoot tissues generated 10–100 thousand sequences per sample, fewer reads were recovered from the root tissues, with many samples containing only a few hundred sequences. This may have been due to insufficient amounts of DNA obtained from the small unbranched 1-year seedling roots. Alternatively, the lack of sequences from the root tissues may have been the result of an excessively harsh surface sterilization method, given that seedling root surface has very few

cell layers. Two root samples (one heated and the other watered) had to be removed from the dataset due to low counts. Indeed, the rarefaction analysis indicated that the root communities were undersampled more than the shoot communities (Fig. S1). Alpha-diversity was compared across tissue types using multiple indices of diversity (Chao1, and Shannon diversity index), with statistical significance calculated with Wilcoxon Signed-Rank test using the MASS package in R (Venables and Ripley 2002). Bacterial taxa graphs were generated using the normalized count data to calculate relative abundances. A heat map for each tissue type (shoot and root) of the top 10 OTUs within the samples were generated using the normalized count data. The top 10 OTUs were then used to perform a BLAST (Altschul et al. 1990) search against the nr database. To test for significant differences between classes of bacteria in root and shoot tissues a nonparametric Kruskal-Wallis test was used and corrected for multiple comparisons using the Bonferroni correction as implemented in the group significance test in QIIME.

To test if the communities were significantly different between seedling tissue type (shoot vs root) and age group (seedling vs adult), the normalized counts were used to calculate unweighted and weighted Unifrac distances (Lozupone and Knight 2005) in phyloseq (v.1.19.1) (McMurdie and Holmes 2013). Unweighted UniFrac treats all taxa the same, regardless of their abundance, and weighted UniFrac takes taxon abundance into account. The unweighted and weighted UniFrac distances were visualized using principal coordinate analysis (PCoA). Adonis, a nonparametric statistical method with 999 permutations, was used to calculate the significance of sample clustering by tissue type and tissue age in R using the vegan package (v. 2.4–2) (Oksanen et al. 2017).

Next, generalized linear models (GLM) were used to test if tissue type and experimental treatments structured seedling endophyte communities. GLMs were used to test between tissue type and experimental treatments due to their higher sensitivity for detecting between group differences. Distance-based methods (as used above) make assumptions regarding the mean-variance relationship of samples which are often not representative of the actual data and can impair interpretation of the results (Warton et al. 2012). To overcome these biases, GLMs were used with a negative binomial distribution to overcome mean-variance assumptions and correctly model the overdispersion which is common with sequencing data. The non-normalized sequencing counts were used to construct GLMs in R using mvabund (v.3.12), an R package for modeling and analyzing multivariate abundance data in community ecology (Wang et al. 2012). This method treats each OTU as a variable and an individual GLM is fitted using a negative binomial distribution. Multivariate hypothesis testing was carried out by applying the ANOVA function in mvabund to the GLMs. The OTUs from control seedlings were used to test for site (forest, treeline, and alpine), tissue type (shoot, root), and their interaction (site and tissue type). The OTUs from seedlings sampled from the treeline site were used to examine

the effects of heating, watering and their interaction, as well as interactions of heating and watering with tissue type (shoot vs root). All graphs were produced in R using the ggplot2 package unless otherwise noted. Radial space-filling plots were generated using Krona (Ondov et al. 2011).

Results

Differences between bacterial communities in adult trees and seedlings

Tissue age (seedling vs adult) significantly structured endophytic communities both with only taxa presence (unweighted Unifrac; Adonis $R = 0.148$, $P = 0.001$) and when relative abundances were included (weighted Unifrac; Adonis $R = 0.250$, $P = 0.001$) (Fig. 1). Across all samples after removing mitochondrial (0.90%), plastid (0.023%) and unassigned sequences (3.69%), a total of 382 operational taxonomic units (OTUs) were recovered. Adult needle communities, both *P. flexilis* and *P. contorta*, were comprised mainly of Alphaproteobacteria (83%) with one family, the *Acetobacteraceae*, dominating primarily due to three OTUs (OTU2, OTU3 and OTU4) (Figs. 2 and 3). OTU2 and OTU3 were within the top 10 OTUs in seedling shoot tissue but were present at a much lower relative abundance than in adult tissues (Fig. 4a). In contrast, seedling endophyte communities were dominated by Betaproteobacteria (58%) comprised of the three families *Oxalobacteraceae*, *Comamonadaceae* and *Burkholderiaceae*, all within the order Burkholderiales (Figs. 2 and 3). All three Betaproteobacterial families were present in adult needle communities but in much lower relative abundance compared to the seedlings (1% *Oxalobacteraceae*, 0.4% *Comamonadaceae* and 1% *Burkholderiaceae*).

Figure 1. Principal coordinate analysis showing differentiation between tissue age, shoot and root communities. (**a**) unweighted and (**b**) weighted UniFrac distance matrix. Points that are closer together have more similar communities. Each point corresponds to a sample with the tissue type of each sample indicated by color (blue = shoots/needles, green = root) and tissue age indicated by shape (circle = adult, triangle = seedling)

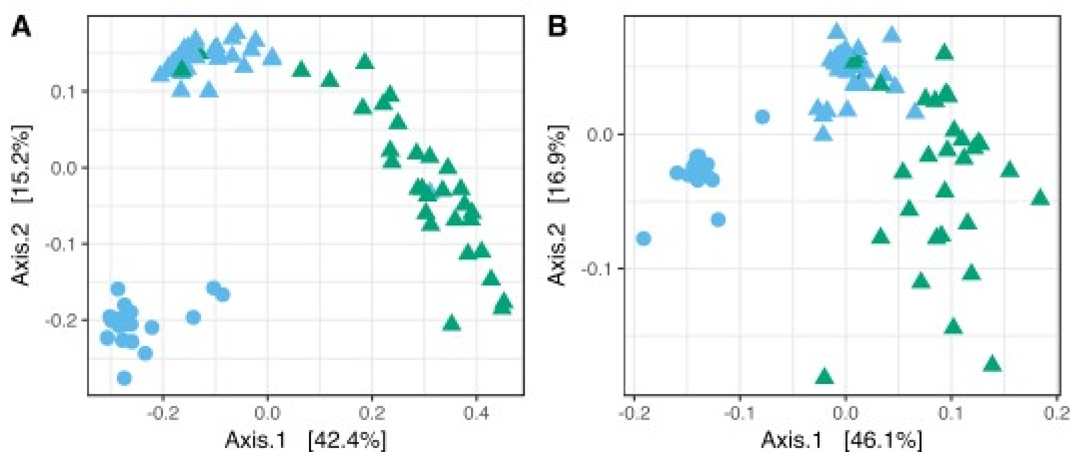


Figure. 2. Relative abundance and taxonomic hierarchy using Krona radial space-filling. (a) Bacterial endophyte community of limber pine seedling shoot tissue, (b) seedling roots, and (c) mature conifer foliage from a previous study of mature limber pine foliage at Niwot Ridge collected in 2012 with the seedlings (Carrell et al. 2016).

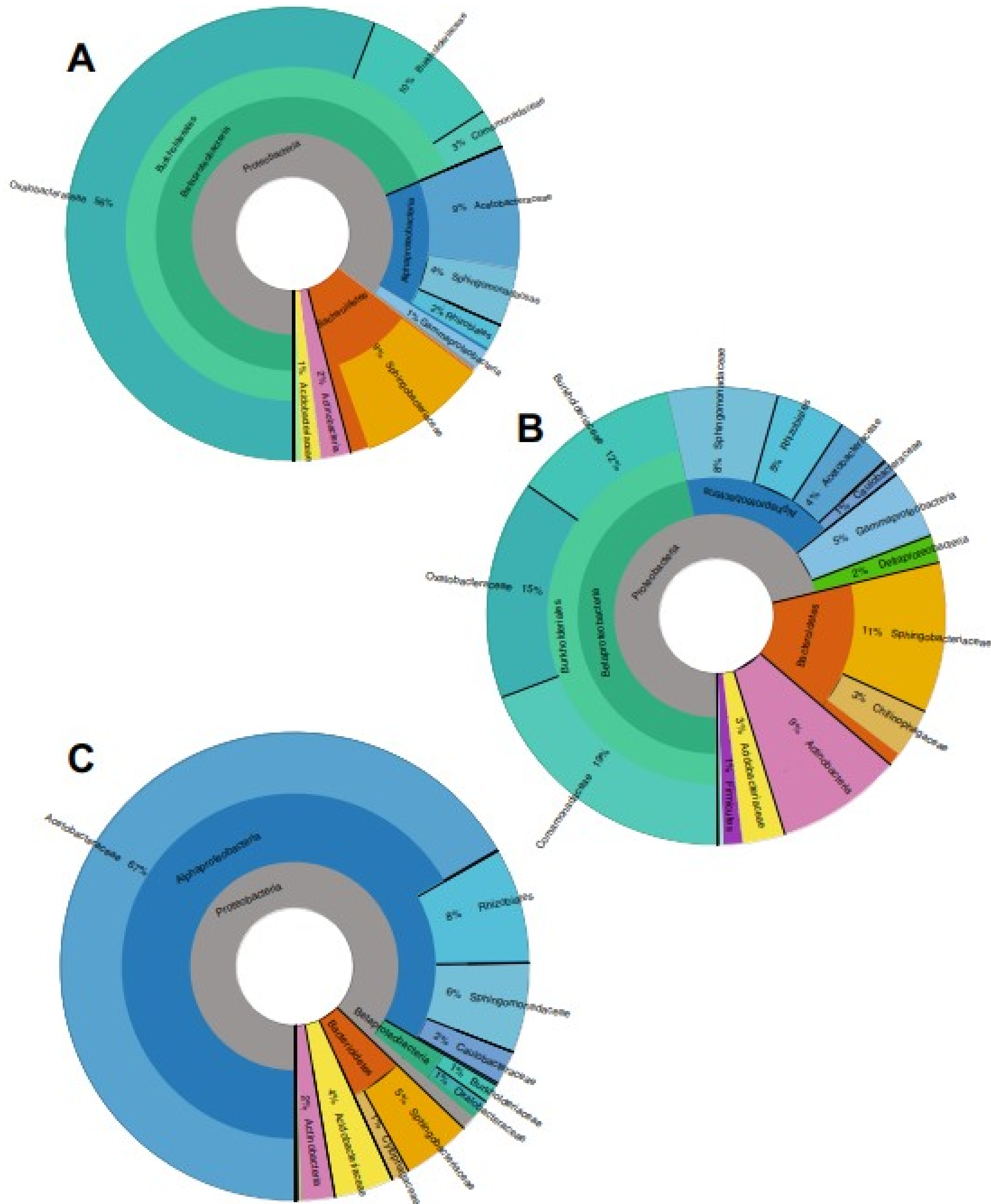
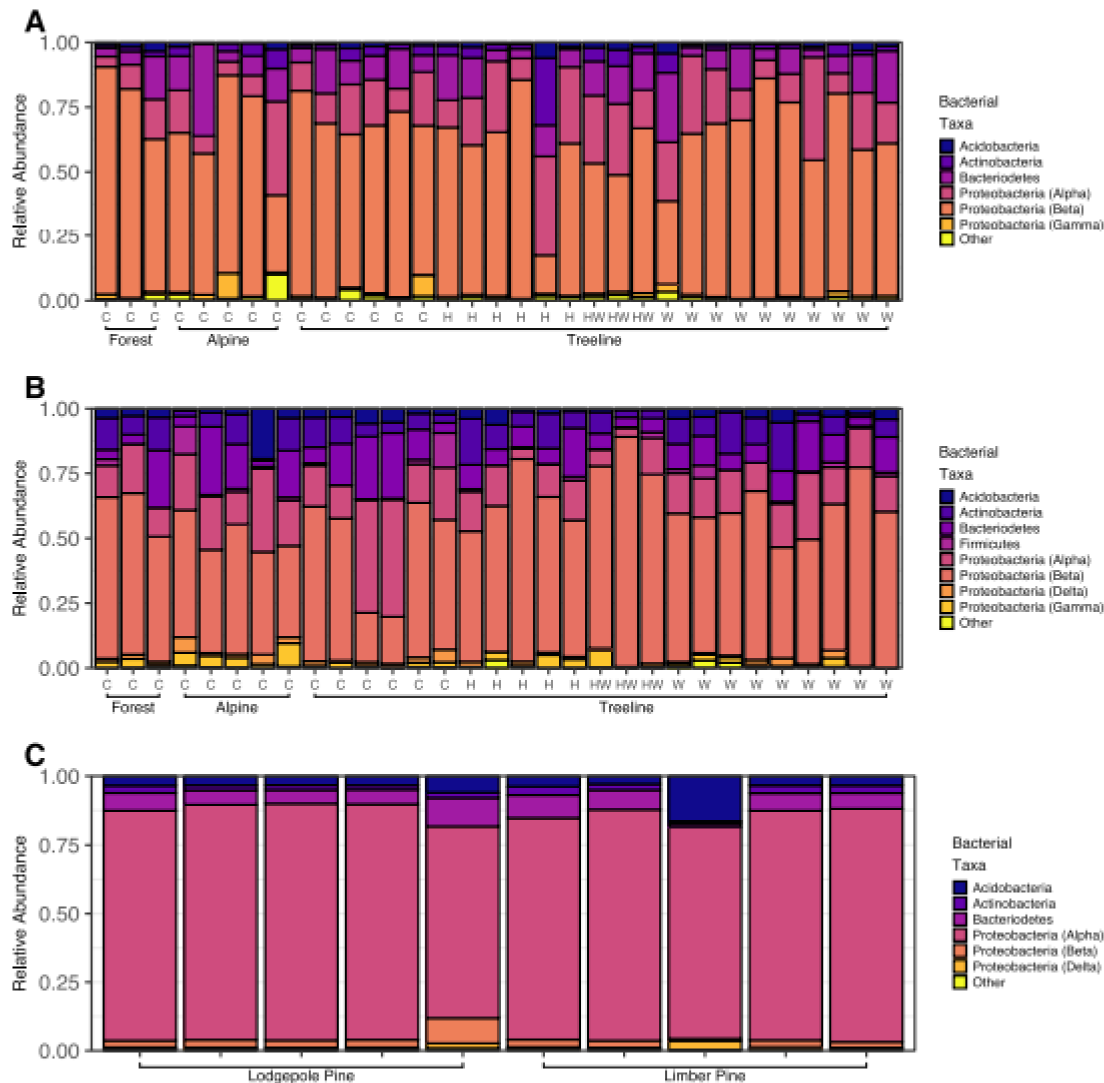


Figure 3. Relative abundances of the major phyla in seedlings and adult needles as percentages of all of the 16S rRNA gene sequences for (**a**) shoot tissues, (**b**) root tissues and (**c**) mature conifer foliage (both limber pine and lodgepole pine collected in 2012 at Niwot Ridge). Each bar represents a sample and the letter under the bar represents the treatments— control (C), heated (H), watered (W) and heated and watered (HW)



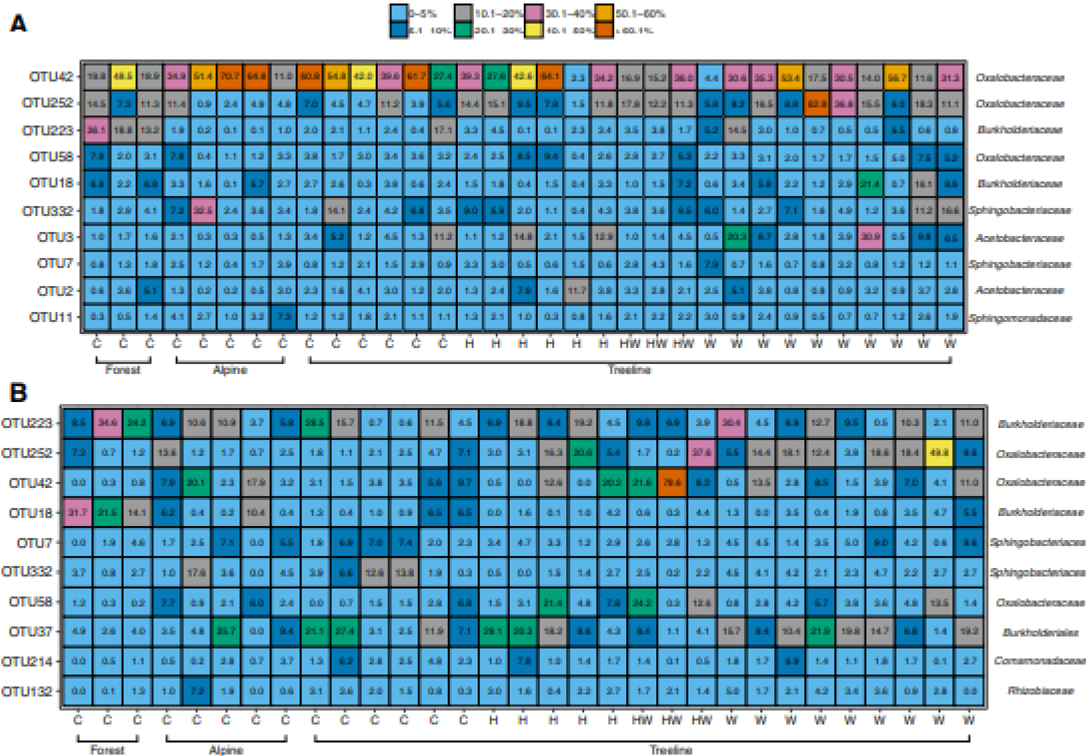
Bacterial communities in seedling root and shoot tissues

Overall, tissue type (shoot vs root) significantly structured the endophyte community, both when considering only taxa presence (unweighted Unifrac; Adonis $R = 0.359$, $P = 0.001$) and when including relative abundances (weighted Unifrac; Adonis $R = 0.262$, $P = 0.001$) (Fig. 1). The majority of OTUs (287) were present in both shoot and root tissues, with 86 OTUs unique to shoot tissues and 9 unique to root tissues. The abundance of shoot- and root-specific OTUs represented only a small fraction of the community (0.06% of the shoot and 0.03% of the root). It was found that α diversity was significantly higher in shoot samples than in root samples ($P < 0.05$) when

measured by Chao1, and that root sample values were significantly higher than shoot samples ($P < 0.05$), when measured by the Shannon index, which accounts for both abundance and evenness of OTUs.

Seedling shoot samples were dominated by the phyla Proteobacteria (85.3% ±9.9%) and Bacteriodetes (10.6%±7.2%), and root samples were dominated by the phyla Proteobacteria (71.2%±8.3%), Bacteriodetes (15.1%±7.2%) and Actinobacteria (8.9%±4.7%) (Figs. 3 and 4). Within the Proteobacteria, both shoot and root samples were dominated by the order Burkholderiales (Betaproteobacteria) (Figs. 2 and 3). In shoot samples one family in the order Burkholderiales, the *Oxalobacteraceae*, made up more than half of the community on average (Figs. 2 and 3), largely due to the consistent presence of two single OTUs that together made up 47% on average (OTUs 42 and OTU 252 in Fig. 4). In root samples, the Betaproteobacteria were split across three families: *Comamonadaceae* (19%) *Oxalobacteraceae* (15%), and *Burkholderiaceae* (12%) (Figs. 2 and 3). Both shoot and root communities had roughly equal proportions of Alphaproteobacteria (15 and 17%, respectively), both with the families *Acetobacteraceae* and *Sphingomonadaceae* dominating, but with a higher relative abundance of *Rhizobiaceae* in roots than shoots (2 and 0.8% respectively). Gamma- (2.2 vs 1.4%) and Deltaproteobacterial taxa (1.5 vs 0.4%) were present at higher relative abundances in root than shoot samples.

Figure 4. Heatmap showing the 10 most abundant OTUs and their relative abundances as percentages of all the 16S rRNA gene sequences in each sample. (**a**) Shoot and (**b**) root tissues types. Color tones range from warm (orange) to cool (blue) to indicate the highest and lowest abundances. The value in each square is the percentage of the sample that is made up of that OTU. The lineage on the right side is the taxonomic order for which each OTU has been classified. Each column is a single sample, the letters underneath each column represent the treatments, control (C), heated (H), watered (W) and heated and watered (HW).



The relative abundance of eight classes of bacteria differed significantly between the shoot and root samples (Bonferroni-corrected $P < 0.05$) (Table 1). Three of those classes—Acidobacteria, Deltaproteobacteria and Actinobacteria—made up substantial portions of the communities in both shoot and root samples, while the others were low-abundance classes comprising less than 1% of the community. All three classes are commonly found in soil microbial communities (Table 1). Figure 4 shows heatmaps with the relative abundance of the 10 most abundant OTUs in our shoot and root datasets across all samples, and Table 2 shows the closest database matches from published studies to the same OTUs. There was substantial overlap between the most dominant OTUs in shoot and root samples (Fig. 4a & b). However, whereas a single OTU (OTU_42) dominated most of the shoot samples, the identity of the most abundant OTU varied across root samples. OTU_42, which is identical to a sequence of an endophyte from the arctic tundra plant *Diapensia lapponica* (Nissinen et al. 2012) (Table 2), made up over 10% of the community in all except two aboveground samples, and as much as 71% in one of the samples. In contrast, the relative abundance of OTU_42 was much lower in all but one belowground sample (Fig. 4b). The majority of the abundant OTUs in this dataset were closely related to sequences from cold environments, including Arctic and Antarctic habitats (Table 2). In addition, a few OTUs were closely related to bacteria found on the skin of amphibians (OTU_252 and OTU_214).

Effect of site and climate treatments on seedling bacterial communities

Climate treatments (heat, water) and site (forest, treeline, alpine) did not consistently structure the bacterial communities, but environmental effects were contingent on tissue type. (Tables 3 and 4). In particular, site and watering treatment had tissue type–specific effects on the endophyte community structure (Tables 3 and 4).

Table 1. Bacterial classes with significantly different relative abundance in shoot and root tissues

Lineage	Shoot mean	Root mean	*P*-Value
Phycsipaere	0.03%	0%	6.86E-09
Thermoleophilia	0.20%	1.44%	5.68E-05
Cytophagia	1.08%	0.32%	0.00001
Deltaproteobacteria	0.40%	1.57%	0.00001
Deinococci	0.01%	0%	0.0007
Actinobacteria	3.38%	6.48%	0.001
Acidobacteria	1.58%	3.40%	0.007
Ktedonobacteria	0%	0.01%	0.024

Table 2. Database matches of the top 10 seedling OTUs

OTU	Tissue	GenBank ID	% ID	Lineage	Species	Source
OTU_42	Both	HE815088	100	*Oxalobacteraceae*	Uncultured clone	*Diapensia lapponica*, Arctic tundra[a]
		HE815064	100%	*Oxalobacteraceae*	Uncultured clone	*Diapensia lapponica*, Arctic tundra[a]
		HE814645	100%	*Oxalobacteraceae*	*Massilia* sp. M1 U34	*Diapensia lapponica*, Arctic tundra[a]
OTU_252	Both	KP067134	99%	*Oxalobacteraceae*	*Janthinobacterium* sp. R86	Potato, Rhizosphere, Switzerland[b]
		AB991043	99%	*Oxalobacteraceae*	Uncultured clone	Surface of glacier, Byron Glacier, Alaska, USA[c]
		KM817575	99%	*Oxalobacteraceae*	Uncultured *Janthinobacterium* sp.	*Lissotriton vulgaris*, Amphibian skin, Germany[d]
OTU_7	Both	EU136864	100%	*Sphingobacteriaceae*	Uncultured bacterium	Rainwater with coniferous forest canopy, Wisconsin, USA[e]
		HE814987	100%	*Sphingobacteriaceae*	Uncultured endophytic bacterium	*Diapensia lapponica*, Arctic tundra[a]
OTU_3	Shoot	KJ606803	100%	*Acetobacteraceae*	Unclassified Acetobacteraceae	Antarctic lichen[f]
OTU_58	Both	JN367235	98%	*Oxalobacteraceae*	Uncultured bacterium	Maize Rhizosphere Soil, Granada Spain[g]
		EU636047	98%	*Oxalobacteraceae*	Antarctic bacterium	Surface of glacier, Collins Glacier, Antarctica[h]
OTU_2	Shoot	HE815061	99%	*Acetobacteraceae*	Uncultured Clone	*Diapensia lapponica*, Arctic tundra[a]
		GU300331	99%	*Acetobacteraceae*	Uncultured bacterium	Forest soil under *Pseudotsuga menziesii*, Canada[i]
OTU_11	Shoot	HE814903	100%	*Sphingomonadaceae*	Uncultured endophytic bacterium	*Diapensia lapponica*, Arctic tundra[a]
		NR_137233	99%	*Sphingomonadaceae*	*Sphingomonas psychrolutea* str. MDB1-A	Ice, Midui glacier, Tibet[j]
OTU_18	Both	HE814630	100%	*Burkholderiaceae*	*Burkholderia* sp. M1 U23	*Diapensia lapponica*, Arctic tundra[a]
OTU_37	Root	JQ291763	100%	Burkholderiales	Uncultured Bacterium	Apple roots, Italy[k]
		HE815395	100%	Burkholderiales	Uncultured Bacterium	*Juncus trifidus*, Arctic tundra[a]
OTU_132	Root	HE814711	100%	*Rhizobiaceae*	*Rhizobium* sp. J5H16a	*Oxyria digyna*, Arctic tundra[a]
OTU_214	Root	KM187606	100%	*Comamonadaceae*	Comamonadaceae bacterium PRE22F	*Notophthalmus viridescens*, Amphibian skin, Virginia, USA[l]
OTU_223	Both	KJ529010	100%	*Burkholderiaceae*	*Burkholderia* sp. FL97–1	*Deschampsia flexuosa*, Sub-Arctic sand dune[m]
OTU_332	Both	KR181805	100%	*Sphingobacteriaceae*	*Mucilaginibacter* sp. L356	Forest Litter, Czech Republic[n]
		NR_134093	100%	*Sphingobacteriaceae*	*Mucilaginibacter pineti* str. M47C3B	*Pinus pinaster* wood, Portugal[o]

[a] Nissinen et al. 2012, [b] Hunziker et al. 2015, [c] Murakami et al. 2015, [d] Vences et al. 2015, [e] Jones et al. 2008, [f] (Lee et al. 2014), [g] GarcíaSalamanca et al. 2013, [h] García-Echauri et al. 2011, [i] Brooks et al. 2011, [j] Liu et al. 2015, [k] Bulgari et al. 2012, [l] Walke et al. 2015, [m] Poosakkannu et al. 2015, [n] López-Mondéjar et al. 2016, [o] Paiva et al. 2014

Table 3. Generalized linear model (GLM) and summary statistics for tissue type and experimental climate treatment (heat, water)

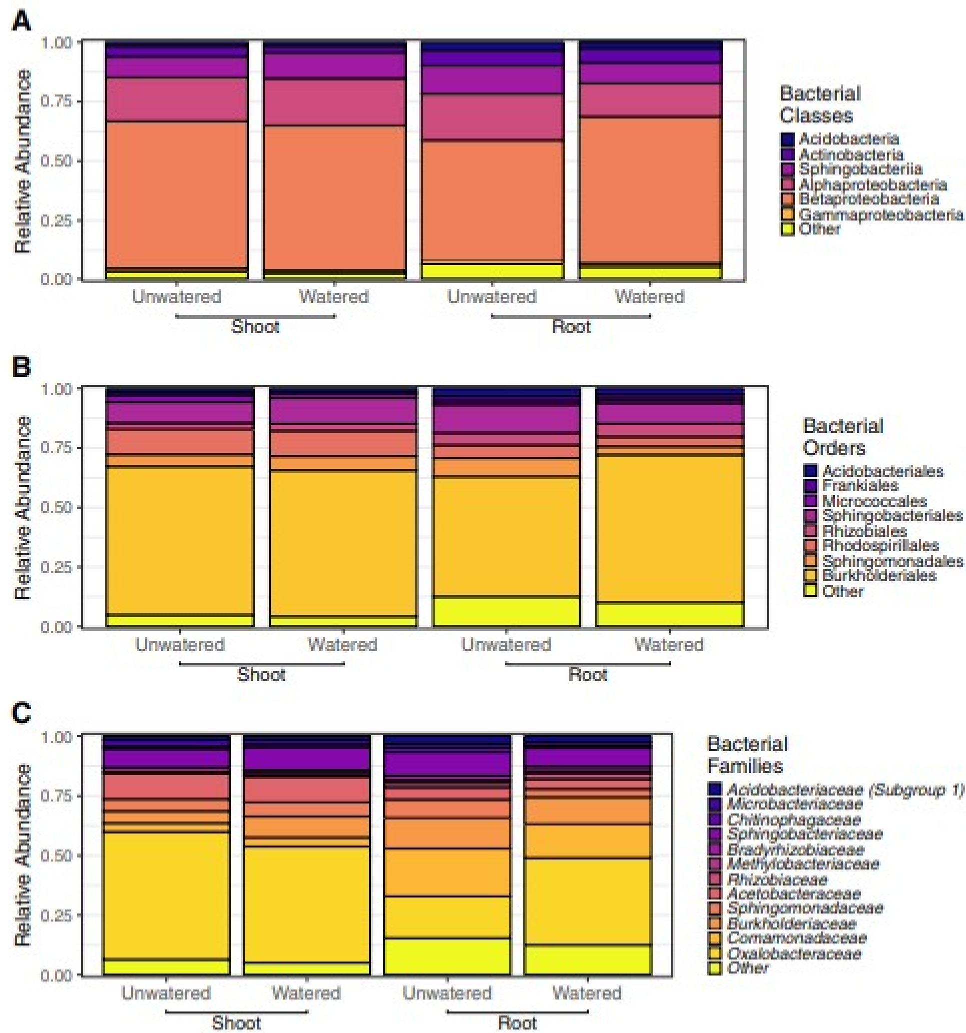
Model: Taxa abundance versus heated, watered and tissue type		
Source	Deviance	P
Heated	336	0.566
Watered	326	0.496
Tissue Type	5477	0.001
Heated x Watered	720	0.115
Heated x Tissue Type	392	0.202
Watered x Tissue Type	1557	0.001
Heated x Watered x Tissue Type	785	0.011

Table 4. Generalized linear model (GLM) and summary statistics for tissue type and experimental site

Model: Taxa abundance versus site and tissue type		
Source	Deviance	P
Tissue type	1269	0.010
Site	1305	0.128
Site x Tissue type	1544	0.031

Unwatered shoot samples (C and H) were dominated by Betaproteobacteria (62%) made up mainly by one order and family (Burkholderiales and *Oxalobacteraceae*, respectively). Watered shoot samples had similar percentages of Betaproteobacteria but increases in Alphaproteobacteria and Sphingobacteriia with decreases in minor groups (Fig. 5). Unwatered root samples were also dominated by Betaproteobacteria (51%) that were divided into roughly equal proportions of three bacterial families (*Oxalobacteraceae*, *Comamonadaceae*, and *Burkholderiaceae*) (Fig. 5). Watered roots showed an increase in the proportion of Betaproteobacteria (62%) with a drastic reduction in overall evenness of the bacterial families favoring a single family *Oxalobacteraceae*, which shifted from 18% in unwatered samples to 37% in watered samples (Fig. 5).

Figure 5. Bar charts of relative abundances of the major phyla as percentages of the all the 16S rRNA gene sequences grouped by tissue type and watered treatment. (**a**) Bacterial classes, (**b**) bacterial orders, and (**c**) bacterial families. Each bar represents the average of all samples within that tissue type and watering treatment



Discussion

A number of recent studies on the plant microbiome have focused on how host genotype and environment, particularly soil type, structure communities of plant-associated microbes. However, this work has largely been done with model- and agricultural plants, with less focus on factors structuring endophyte communities of forest trees. Climate change is predicted to shift the distributions of forest trees upward, moving the current forest boundary into the alpine zone. In the alpine-treeline ecotone, climate change could enhance establishment by reducing cold stress, while at lower elevations, climate change could exacerbate heat and drought stress impairing seedling recruitment (Moyes et al. 2013; Reinhardt et al. 2011; Smith et al. 2003). With climate change, new and existing seedling-microbe associations could

contribute to establishment beyond the current range but little research to date has explored forest seedlings microbiomes. Here, as a first step towards incorporating microbiomes in research on how climate change influences forest trees, this study characterized the root- and shoot endophytic communities of *P. flexilis* seedlings in a common garden warming experiment. This work shows that the bacterial endophyte communities of 1-year old pine seedling tissues are influenced by site differences and moisture addition and are different from the communities in co-occurring adult pines.

Previous studies of leaves from mature subalpine conifers have identified a core of endophytes belonging to the *Acetobacteraceae*, or AAB, making up 59% of the OTUs in the most recent study (Carrell et al. 2016). In contrast, the endophyte communities of seedlings were more diverse and varied more across individual seedlings. Higher diversity in younger tissues has been shown previously for fungal endophyte communities of *P. taeda* (Oono et al. 2015). Although the same AAB OTUs found in adults were detected in both shoot and root samples (9 and 4% respectively), seedlings were dominated by bacteria in the family *Oxalobacteraceae* (Betaproteobacteria). Different acquisition routes could be a major driver of the large discrepancies observed between adult and seedling endophyte communities. The major AAB OTUs in adult needles overlap with conifer leaf surface communities and have been found in air samples (Carrell et al. 2016), suggesting that the dominant route for foliar microbiome acquisition in mature trees is horizontal via air or rain, similar to what has been described for fungal endophytes (Rodriguez et al. 2009). In seedlings on the other hand, soil is likely the dominant source of endophytes.

Differences in the endophytic communities in adult and seedlings tissues could also reflect different functional interactions with the host. The AAB are potentially responsible for the nitrogenase activity detected in the foliage of adult trees (Moyes et al. 2016). To our knowledge, nitrogenase activity in wild conifer seedlings has not been examined, although it is known from inoculation studies that *P. contorta* seedlings can host nitrogen fixing endophytes (Anand et al. 2013; Bal et al. 2012). Several of the most abundant OTUs in the seedling dataset were identical to sequences from arctic and sub-arctic plants (Nissinen et al. 2012; Poosakkannu et al. 2015), indicating that the seedling microbiome consists of plant-adapted bacteria rather than opportunistic soil bacteria. The most prominent group in seedlings, the *Oxalobacteraceae*, is known for the ability to metabolize oxalic acid (DeLeon-Rodriguez et al. 2013), weather minerals (Leveau et al. 2010), and promote plant growth (Baldani et al. 2014; Ofek et al. 2012). The high proportion of *Oxalobacteraceae* and *Burkholderia* species in seedlings could reflect oxalic acid content, the use of oxalic acid as a carbon source is associated with plant-beneficial microbes, and oxalic acid may be involved in recruiting plant-beneficial members from complex bacterial communities (Kost et al. 2013). Alternatively, the high proportion of *Oxalobacteraceae* could reflect some beneficial function like protection against fungal and

oomycete pathogens. The top OTUs in the *Oxalobacteracea*e belong to the genus *Janthinobacterium*, which is recognized for antifungal activity (Haack et al. 2016; Kueneman et al. 2016). The most common shoot OTU 42 is similar to strains with antifungal activity, while OTU252, the second most common in shoots, was similar to a potato rhizosphere strain antagonistic against the oomycete *Phytophthora infestans* (Hunziker et al. 2015), and to a strain from the disease-protective newt skin microbiome (Vences et al. 2015).

Although seedling roots and shoots shared many taxa and were both dominated by Betaproteobacteria, their overall bacterial endophyte communities were different, as reported for other plant species (Bodenhausen et al. 2013; Coleman-Derr et al. 2016; Mishra et al. 2012; Robinson et al. 2016; Wagner et al. 2016). These differences likely reflect a combination of factors including proximity to soil, acquisition routes (soil vs atmosphere), functional relationships with the plant, filtering imposed by the plant, or composition of nutrients and other phytochemicals. Above-ground tissues of plants can produce tannins, phenolics and terpenes to reduce herbivory (War et al. 2012), which have the potential to structure the endophytic communities. In *Pinus monticola* (Western white pine) the amount of sulfur, nitrate and calcium within the needles influenced the fungal communities between stands of trees (Larkin et al. 2012). In addition, the level of host genetic control might also differ between these two tissues, as suggested by a study of *Boechera stricta,* a perennial wild mustard that showed that host genotype shaped leaf but not root microbiomes (Wagner et al. 2016). In this study, two of the top 10 OTUs in shoots (but not root) communities were AAB. The higher relative abundance of AAB OTUs in shoot compared to root is consistent with an atmospheric source of these bacteria. At the same time, a large proportion of bacterial groups were shared between roots and shoots (i.e. 7 of the 10 major OTUs), suggesting colonization from soil followed by distribution to aerial tissues. A soil-to-shoot acquisition path for seedling endophytes seems more likely than the opposite, although migration in both directions has been reported (e.g. Lòpez-Fernàndez et al. 2017). Alternately, some of the endophyte taxa identified here could be seed-borne, which would also explain the dominance of a few OTUs across the seedlings. Bacteria in the family *Oxalobacteraceae*, which dominated the seedlings in our study, have been found in and on seed, radicle, and root of cucumber (*Cucumis sativus*) (Green et al. 2007; Ofek et al. 2012). Examination of seed, new germinants and established seedlings is needed to evaluate the possibility of vertical endophyte transfer in pines.

The bacterial endophyte communities in the seedlings were highly variable among individuals and treatments indicating that stochastic or environmental factors are important for structuring the communities. Watering treatment had a larger effect on root than shoot communities, with a reduction in the overall evenness, a moderate decrease in the relative

abundances of Acidobacteria, Actinobacteria and Alphaproteobacteria, and a large increase in relative abundance of Betaproteobacteria in the family *Oxalobacteraceae*. Alterations of precipitation regimes is expected with climate change, with shifts towards the extremes of flooding and drought (Xie et al. 2015). The results of this study show that seedlings establishing during a high moisture period could have an altered microbiome, although the long-term effects of this change remain unknown. This water-induced restructuring of the endophytic community could also reflect changes in the source pool (assumed to be soil). Several studies report an increase in Betaproteobacteria, specifically the *Oxalobacteraceae* with precipitation or watering (Yao et al. 2017; Zhang et al. 2013).

Alternatively, restructuring of root communities could reflect recruitment of specific bacterial in response to watering. For example, OTU_42 increased from an average of 5.5 to 13.5% and OTU_252 from an average of 6.2 to 15.8% with watering. These two OTUs were among the most abundant in both shoots and roots, and as mentioned above, similar to strains known for activity against fungi and oomycetes. The fungi: bacteria ratio in soil has been found to increase in response to increased precipitation (Bell et al. 2014; Bi et al. 2012) and it is possible that the increase in these endophytic OTUs in shoots reflects recruitment of strains that protect the seedlings against fungal pathogens. Increased fungal invasion in forest trees is predicted with climate change (La Porta et al. 2008), and the results here suggest that fungal invasion may be increasing with added moisture, or that fungi already inhabiting the roots (Vasiliauskas et al. 2007) shift from endophytic to pathogenic or saprotrophic (Fesel and Zuccaro 2016).

Warming had no effect on the seedling endophyte communities, potentially because warming had no or little effect on the soil community although this was not investigated in this study. Results from other experiments suggest no response (Schindlbacher et al. 2011) or a very slow response of soil microbial communities to experimental warming, starting only after extended periods of experimental treatments (>5 years) (DeAngelis et al. 2015; Zhang et al. 2013). Experimental treatments began just 2 years prior to the seedling sampling. Alternatively, endophyte communities may be insensitive to heating if the seedlings themselves are robust to it. While first-year limber pine seedling recruitment was reduced with warming, survival of one-year-old limber pine seedlings at the treeline site was insensitive to heating (Kueppers et al. 2017a), suggesting that the older seedlings sampled were robust to heating. Thus, the results do not suggest that warming alters bacterial endophyte communities in the seedlings' first year, but it cannot be determined if longer warming treatments would have an effect or if the lack of significance of the warming treatment is due to the limited number of seedlings sampled.

Conclusions

The results of this study show that the bacterial endophyte communities in subalpine conifer seedlings establishing across an elevation gradient are significantly different from the communities in the foliage of mature trees, potentially reflecting different transmission routes or endophyte functions between seedlings and adults. Shoots and roots hosted significantly different communities, with a few OTUs in the *Oxalobacteraceae* dominating shoots, and with the community in roots being more diverse. At the same time, there was a large overlap in OTUs between root and shoot tissues, suggesting inoculation from soil is the main acquisition route for seedling endophytes. It is possible, given that seedlings and adult trees share the same AAB OTUs, that some endophytes in seedlings originate from surrounding parent trees, either vertically via seed or pollen, or horizontally via the atmosphere or soil. The major OTUs were similar to endophytic *Oxalobacteraceae* in arctic and subarctic plants, and to strains with antifungal activity. Watering but not warming restructured the endophyte communities in a tissue-specific manner, increasing the abundance of *Oxalobacteraceae* in roots but not shoots. Seedlings, especially those under the watering treatment, may be under increased stress from fungal invasion compared to adult trees, which would be reflected in the recruitment of microbiomes with antifungal potentials. Further studies are required to determine if the community differences observed here reflect neutral processes such as different transmission routes and source communities, or selective processes, such as plant selection for endophyte function.

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