

# *Frankia* and *Alnus rubra* Canopy Roots: An Assessment of Genetic Diversity, Propagule Availability, and Effects on Soil Nitrogen

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Received: 9 July 2009 / Revised: 15 August 2009 / Accepted: 22 August 2009  
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**Abstract** The ecological importance of microbial symbioses in terrestrial soils is widely recognized, but their role in soils that accumulate in forest canopies is almost entirely unknown. To address this gap, this study investigated the *Frankia*–*Alnus rubra* symbiosis in canopy and forest floor roots at Olympic National Park, WA, USA. Sixteen mature *A. rubra* trees were surveyed and *Frankia* genetic diversity in canopy and forest floor nodules was assessed with sequence-based *nifH* analyses. A seedling bioassay experiment was conducted to determine *Frankia* propagule availability in canopy and forest floor soils. Total soil nitrogen from both environments was also quantified. Nodules were present in the canopies of nine of the 16 trees sampled. Across the study area, *Frankia* canopy and forest floor assemblages were similar, with both habitats containing the same two genotypes. The composition of forest floor and canopy genotypes on the same tree was not always identical, however, suggesting that dispersal was not a strictly local phenomenon. *Frankia* seedling colonization was similar in canopy soils regardless of the presence of nodules as well as in forest floor soils, indicating that dispersal was not likely to be a major limiting factor. The total soil nitrogen of canopy soils was higher than that of forest floor soils, but the presence of *Frankia* nodules in canopy soils did not significantly alter soil nitrogen levels.

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Overall, this study indicates that the *Frankia*–*A. rubra* symbiosis is similar in canopy and forest floor environments. Because canopy roots are exposed to different environmental conditions within very small spatial areas and because those areas can be easily manipulated (e.g., fertilizer or watering treatments), they present microbial ecologists with a unique arena to examine root–microbe interactions.

## Introduction

Many studies have documented that the structure of species assemblages in forest canopies can differ significantly from that of the forest floor [1, 10, 20, 30]. These differences in species richness and/or abundance are thought to be the result of dispersal-related issues (e.g., greater patchiness of suitable habitat in the canopy) and differences in abiotic conditions (e.g., temperature, relative humidity) between the two environments [10, 20]. There are also differences between the soils present in the canopy and on the forest floor [21]. Canopy soils are completely organic in origin and thus often have a much lower pH than forest floor soils [9]. Additionally, the exposed nature of canopy soils results in much greater fluctuations in moisture content in canopy versus forest floor soil [4]. Both of these factors may have significant effects on soil-dwelling organisms as well as the epiphytic plants present in forest canopies [22].

Certain tree species that live in wet environments can produce canopy roots which exploit the nutrient and water reservoirs present in canopy soils [19]. Canopy roots are anatomically indistinguishable from those produced below-ground and often support the same types of microbial symbioses [16, 19]. In addition to mycorrhizas, associations

with nitrogen-fixing bacteria have been documented in the canopy roots of multiple tree species [15, 19]. Leary et al. [15] surveyed the genetic diversity of *Bradyrhizobium* bacteria from canopy and forest floor nodules of *Acacia koa* in three Hawaiian forests and found that within a single tree, canopy and forest floor isolates showed no overlap in composition. However, there was considerable genetic similarity between canopy and forest floor isolates when samples were compared among different forest sites. Cluster analyses also revealed that multiple canopy isolates were most closely related to a single forest floor isolate, which suggested that the canopy isolates diversified after colonization by the adjacent forest floor isolate [15].

*Alnus rubra* is one of the dominant tree species in the coastal forests of the Pacific Northwest, USA [13]. This species readily forms root symbioses with *Frankia*, a cosmopolitan genus of Gram-positive actinomycete nitrogen-fixing bacteria [3]. *Frankia* nodules have been collected from *A. rubra* canopy roots as high as 20 m [19], and based on acetylene reduction assays, canopy nodules have been confirmed to actively fix nitrogen (N. Nadkarni, unpublished data). However, since their initial discovery, no research has further examined *Frankia* in *A. rubra* canopy roots. Thus, critical aspects of this *Frankia*–*Alnus* relationship remain unknown. Since canopy-based nutrient dynamics can have significant impacts on ecosystem nutrient cycling, a better understanding of this symbiosis is needed [7, 26].

In this study, we examined four aspects of the ecology of the *Frankia*–*A. rubra* symbiosis. Our specific questions were: (1) What are the frequency and distribution of *Frankia* nodules in *A. rubra* canopies? (2) How do *Frankia* assemblages found in canopy soils compare with those found in the forest floor? (3) Is *Frankia* propagule dispersal limited to canopy soils where nodules are present? (4) Does the presence of *Frankia* nodules in canopy soils increase their nitrogen content? Based on previous studies, we hypothesized that genotypic diversity would be different in the two environments but that the number of canopy genotypes would be lower than that on the forest floor [15]. Furthermore, we speculated that *Frankia* propagules may be present in canopy soils without nodules based on their ubiquity in forest floor soils [17] and that their presence would positively influence canopy soil nitrogen levels [5].

## Methods

### Study Site and Sampling Design

The study was conducted in the Olympic National Rainforest, WA, USA along a 10-km-long area adjacent to the Quinalt River (47°33.215' N, 123°40.416' W). In May 2008, we

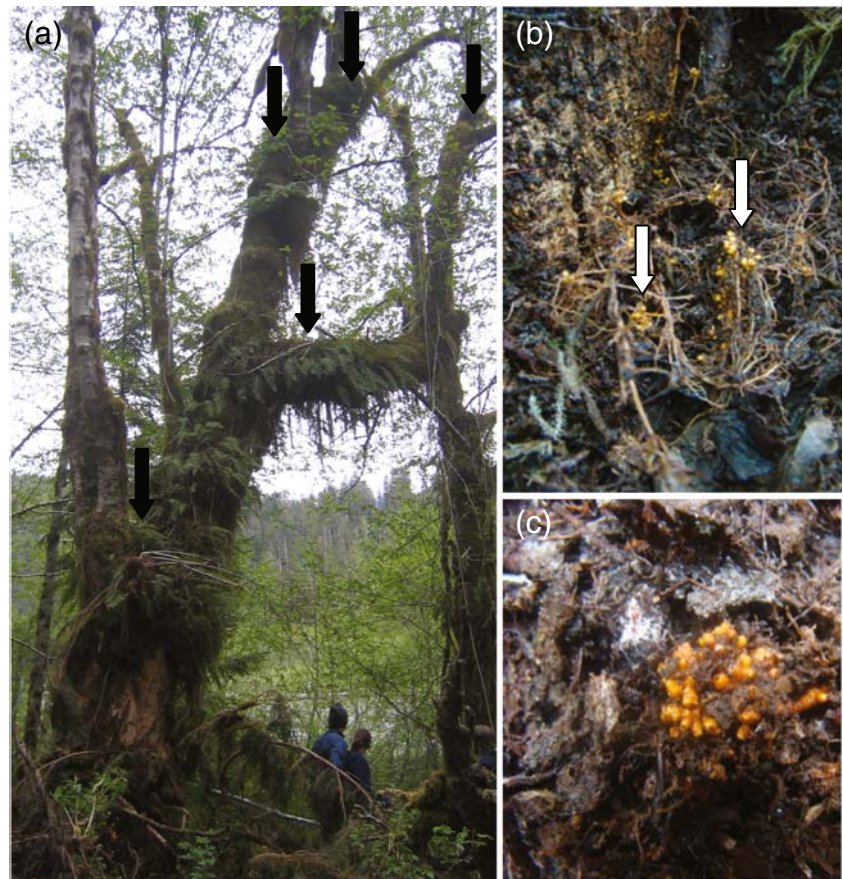
selected 16 mature *A. rubra* trees for sampling. The trees were chosen based on abundant epiphytic growth and the presence of multiple stem–branch junctions where canopy soils were present (Fig. 1a). Distance between trees ranged from 25 m and 10 km apart. The trees were relatively evenly spaced along the 10-km study area, except for trees 1–2 and 3–6 which were all located within a ~500 and ~1,000-m<sup>2</sup> area, respectively. At each tree, we used modified rope climbing techniques to ascend into the canopy. All reachable canopy soils were searched for *Frankia* by peeling back epiphyte mats and looking for nodules in the exposed soil (Fig. 1b). The number of locations surveyed varied among trees, ranging from one to ten (median=2). When present, samples of all nodules found were collected (range 1–5) as well as one to two 50-mL soil samples from each location. In trees where canopy nodules were found, we also attempted to find an equal number of forest floor nodules (Fig. 1c). Forest floor nodules and soils were all sampled within 5 m of the trunk to maximize the likelihood that the samples came from the same individual from which the canopy nodules were collected. Two trees were subjected to more extensive analysis because of the higher abundance of *Frankia* nodules in the canopy. At those trees (trees 1 and 3), 20 and 12 nodules were collected from the canopy, respectively, along with 23 and 21 forest floor nodules. Ten soil samples from the forest floor and nine from the canopy at both trees 1 and 3 were also collected. All samples were put on ice after collection and taken to the lab within 96 h of collection.

### Molecular Analyses

Nodules were surface-sterilized by manual agitation in a 10% bleach solution for 2 min. The nodules were then rinsed three times with deionized water and stored at –20°C prior to DNA extraction. To extract total genomic DNA, one to two lobes from individual nodule samples were macerated in 180 µL of buffer ATL from the Qiagen DNA tissue 200 kit (Qiagen, Carlsbad, CA, USA). One hundred eighty microliters of 20 mg/mL lysozyme was added to the homogenized tissue solution and incubated at 37°C for 30 min. The remainder of the DNA extraction was performed according to the manufacturer's instructions.

We used PCR to amplify a 606-bp fragment of the *nifH* gene with the *Frankia*-specific primer pair *nifHf1* (5'-GGC AAG TCC ACC ACC CAG C-3') and *nifHr* (5'-CTC GAT GAC CGT CAT CCG GC-3'). This region was chosen because it has previously been demonstrated to differentiate among closely related *Frankia* genotypes occurring on the same host plant species [18, 31]. PCR amplifications were performed in 20-µL reactions containing 0.5 µL bulk DNA, 0.4 µL of each primer (10 µM), 10 µL MasterAmp F PCR buffer (Epicenter, Madison, WI, USA), and 0.15 µM Taq Polymerase (New England Biolabs, Ipswich, MA, USA).

**Figure 1** *Frankia* on canopy and forest floor roots of *A. rubra*. **a** Tree 1, with arrows indicating the junctions surveyed for canopy nodules. **b** *Frankia* nodules on canopy roots in a junction of tree 3. **c** One of the *Frankia* nodules collected from the forest floor at tree 3



Samples that did not successfully amplify initially were rerun using 1:20 dilutions of the DNA template. PCR cycling conditions were as follows: 96°C for 5 min; 35 cycles at 96°C for 30 s, 64°C for 30 s, and 72°C for 45 s; and a final 7-min 72°C extension. Amplification was checked with electrophoresis on 1.5% agarose gels (GenePure LE). Gels were stained with ethidium bromide and visualized under UV fluorescence. All successful PCR products were cleaned using 1.5  $\mu$ L of ExoSAP IT (USB Corp., Cleveland, OH, USA) with 7.5  $\mu$ L of DNA and cycled at 37°C for 45 min, followed by 80°C for 15 min. Sequencing was performed on a 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA) at the Genomic Analysis and Technology Core Facility at the University of Arizona, USA. Representative sequences of the two genotypes (see below) were submitted to GenBank with the accession numbers GQ387372 and GQ387373.

#### *Frankia* Soil Bioassays

To examine if *Frankia* colonization of canopy roots was limited by propagule availability, we conducted bioassays of soils collected from canopy and forest floor locations at the study site. *A. rubra* seeds from a nearby coastal location (seed

lot 030-05, Silva Seed Co., Roy, WA, USA) were germinated in sterilized potting medium (Black Gold Seedling Mix, Sun Gro Horticulture Distribution, Vancouver, Canada) and grown in a growth chamber. The growth chamber was set to a 14:10-h light/dark cycle, with temperatures ranging between 18°C and 20°C and ambient humidity levels. After 2 months, seedling root systems were rinsed in deionized water, checked for nodulation (none was found), and transplanted into modified 9-cm diameter Petri dishes containing soils from either canopy or forest floor environments. Twelve seedlings were planted into independent canopy soil samples where we had found *Frankia* nodules, 12 seedlings into independent canopy soil samples where we had found no *Frankia* nodules, and 12 seedlings into independent soil samples collected from the forest floor. We also planted a series of controls in which soils from each location were autoclaved to eliminate any potential inoculum. Seedlings were grown for 6 weeks and then assessed for nodulation (all controls remained uncolonized).

#### Soil Analyses

Soils from the same locations as those used in the bioassay experiment were analyzed for total nitrogen



content on a Leco CNS Macro Analyzer at the Oregon State University Central Analytical Laboratory. We analyzed nine canopy samples with *Frankia* present, nine canopy samples with *Frankia* absent, and 12 samples from the forest floor.

### Phylogenetic Analyses

Sequences from a total of 92 *nifH* fragments from canopy and forest floor nodules were trimmed to 590 bp using Sequencher 4.8 (Gene Codes Corp., Ann Arbor, MI, USA). They were then grouped at 99% similarity, a threshold based on previous studies showing that *Frankia* from different genomic groups can have 98.8% similarity in this portion of the *nifH* gene [11, 18]. Consensus sequences of each group (hereafter referred to as genotypes) were then aligned with 13 additional *Frankia* genotype sequences using MUSCLE [8]. Each of the additional sequences came from distinct phylogenetic *Frankia* clusters as well as diverse geographic regions [31].

To examine the phylogenetic relationships among *Frankia* genotypes, we used maximum parsimony and Bayesian analyses. Maximum parsimony analysis was performed in MEGA 4.0 [29]. Maximum parsimony trees were obtained using the Close-Neighbor-Interchange algorithm [23] with search level 4 [29] in which the initial trees were obtained with the random addition of sequences (20 replicates). All positions containing gaps and missing data were eliminated from the dataset. There were a total of 455 positions in the final dataset, out of which 54 were parsimony informative. The Bayesian analysis was performed in MRBAYES v3.1.2 [27]. It consisted of two simultaneous runs, each with four Markov chain Monte Carlo chains. The current tree was incrementally saved to a file every 100 generations. We used default cold and heated chain parameters and compared the separate runs every 100 generations. The analysis was run for 100,000 generations, which we determined to be adequate based on the average standard deviation of split frequencies being <0.01. The tree presented in Fig. 2 was constructed following a visually determined burn-in of 10%.

### Statistical Analyses

A chi-square test was used to assess differences in the number of seedlings colonized by *Frankia* among the bioassay treatments (canopy with nodules, canopy without nodules, and forest floor). A one-way analysis of variance (ANOVA) was used to compare soil total nitrogen levels among the same treatments. Because the variances among treatments were heterogeneous even after various transformations, a non-parametric Kruskal–

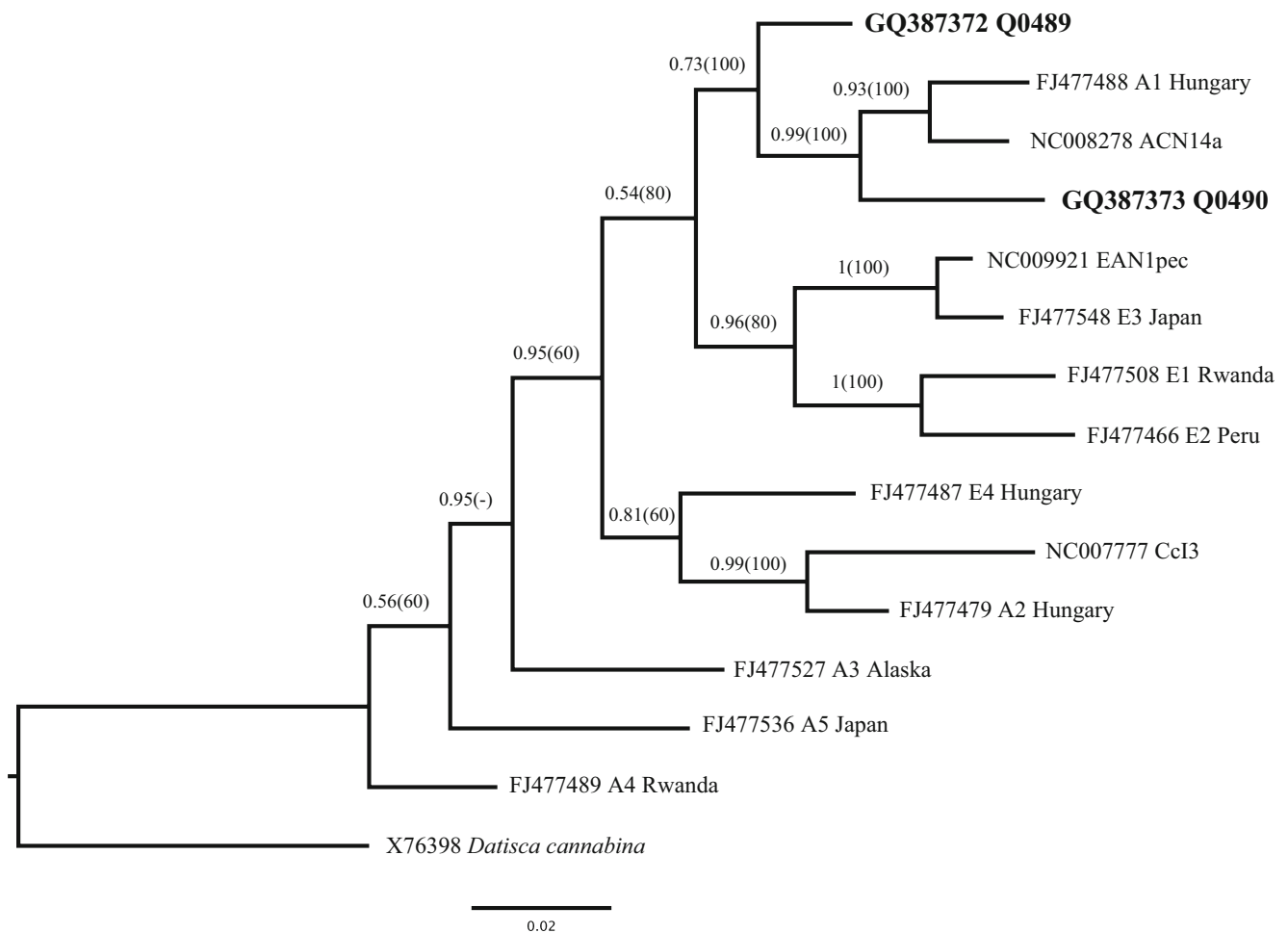
Wallis ANOVA was selected. To determine specific differences in nitrogen levels among soil types, we used a post hoc Dunnett's test. All inferential statistics were performed in JMP 5.0 (Cary, NC, USA) and considered significant at  $P < 0.05$ .

### Results

Canopy nodules were present in nine of the 16 trees sampled. In the nine canopy colonized trees, an average of 52% of the locations surveyed contained nodules. The number of canopy nodules varied from 1 to 20 per tree (median=3) and were collected from heights ranging from 1.4 to 13.5 m (median=3.3 m). In total, 47 canopy and 57 forest floor nodules were collected. Of those, 92 (89%) were successfully sequenced and identified. The sequences grouped into two genotypes, here named *Frankia* Q0489 and Q0490, which were present in both the forest floor and canopy. Bayesian and maximum parsimony trees had similar topologies, and most clades were supported by high posterior probabilities and bootstrap values in both analyses (Fig. 2). Both of the genotypes encountered in this study were closely related to a previously identified group of *Alnus*-infective *Frankia* strains.

The two genotypes had similar abundance in the canopy, but Q0489 was nearly twice as abundant as Q0490 on the forest floor (Table 1). For the two trees sampled most extensively, trees 1 and 3, the *Frankia* genotype most abundant in the canopy was also most common on the forest floor (Table 1). However, the genotype composition on canopy and forest floor roots of the same tree was not identical. On tree 1, for example, the canopy had a *Frankia* genotype not present on the forest floor, while the opposite was true for tree 3. Similar mismatches in patterns of genotype composition were also seen at multiple other trees where canopy and forest floor nodules were sampled (Table 1).

Seedlings planted into canopy soils with and without *Frankia* nodules did not differ significantly in *Frankia* colonization (with nodules=6/12 (50%), without nodules=4/12 (33%),  $\chi^2=1.58$ ,  $df=2$ ,  $33$ ,  $P=0.45$ ). The number of seedlings colonized by *Frankia* in forest floor soils was also not significantly different from that in both types of canopy soils (7/12 (58%)). Total nitrogen levels of canopy soils was significantly higher than that of forest floor soils (canopy with *Frankia* nodules=2.55±0.15% canopy without *Frankia* nodules=2.40±0.19%, forest floor=1.03±0.24% (mean±1 SE);  $F=19.9$ ,  $df=2$ ,  $28$ ,  $P<0.001$ ), but there was no significant difference between the total nitrogen levels of canopy soils with and without *Frankia* nodules (Dunnett's test,  $P>0.05$ ).



**Figure 2** Phylogenetic reconstruction of 15 *Frankia nifH* fragment 590-bp sequences. Nodes are labeled with posterior probabilities from Bayesian analysis and, in parentheses, bootstrap support values from maximum parsimony analysis. A dash indicates that branch was not present in the maximum parsimony analysis. Genotypes are coded by

GenBank number, infection group of Welsh et al. [31], and the geographic location of the soil from which the sequence was obtained. Nodes with *A. rubra*-associated genotypes from this study are designated in bold. A *Frankia nifH* fragment from *Datisca cannabina* was designated as the outgroup following Welsh et al. [31]

## Discussion

We found that over half of the *A. rubra* individuals sampled had *Frankia* nodules present on canopy roots. However, the distribution of nodules in those trees was patchy and the total number of nodules per tree was relatively low compared to forest floor sampling. We believe that there are multiple factors responsible for this pattern. Although we did not quantify canopy root distributions independent of *Frankia* distributions, there is clearly considerable variation in their presence. In many locations, we observed no roots in junctions with well-developed canopy soils. Since the absence of canopy roots precludes the presence of *Frankia* nodules, the patchy nature of canopy root production places a fundamental limit on *Frankia* canopy nodule distribution. At the same time, we also observed multiple junctions with canopy roots that had no *Frankia* nodules present. In these

locations, one possibility is that soil environmental conditions may have been unfavorable for nodule formation. Previous studies have found that nodulation is negatively affected by low soil moisture and low pH [12, 28], both of which are characteristic of canopy soils, at least on a seasonal basis [4, 9]. Alternatively, the absence of nodules in those locations with canopy roots may reflect an absence of *Frankia* propagules. The results of the bioassay, however, suggest that this explanation is less likely because canopy soils with and without resident nodules had similar levels of seedling colonization. Given that a multitude of factors are involved and that they are not mutually exclusive, it appears that this canopy root symbiosis may only occur under a limited set of ecological conditions.

The results of the soil bioassay suggest that canopy root nodulation is not dispersal limited in our study system. *Frankia* propagules have been found to be transported

**Table 1** Abundance of *Frankia* genotypes at each of 16 *A. rubra* trees where nodules were present

Tree	Canopy		Forest floor	
	Genotype		Genotype	
	Q0489	Q0490	Q0489	Q0490
1	10	9	20	0
2	1	0	0	0
3	0	12	7	13
6	4	0	1	1
9	2	0	1	3
11	1	0	0	0
13	2	1	2	0
14	2	0	0	0
Total	22	22	31	17

Trees 4, 5, 7, 8, 10, 12, 15, and 16 had no nodules present in their canopies

readily by wind and water [14] and persist in areas where suitable host plants have been absent for long periods [17]. *Frankia* propagules have also been found to survive passage through the digestive tracts of birds [6], which may be an important vector for moving *Frankia* propagules from the forest floor into the canopy. Interestingly, following gut passage, seedling nodulation by *Frankia* was found to be higher when inoculum was not air-dried [6]. These results further support the idea that *Frankia* distributions in *A. rubra* canopies are more limited by environmental conditions than by dispersal. Our results also suggest that the movement of propagules into the canopy is not strictly associated with *Frankia* from adjacent terrestrial roots. The mismatch in genotype composition of adjacent canopy and forest floor assemblages indicates that although the regional propagule pool for canopy and forest floor roots is similar, dispersal is not necessarily a local phenomenon. This result corresponds with that of Leary et al. [15] who found that canopy and forest floor isolates of *Bradyrhizobium* were similar at regional but not local scales.

Our phylogenetic analysis corresponded well with previous *Frankia* analyses, which have consistently shown three major host infection groups (see [2] for a recent review). The genotypes present in our study area grouped closely with known *Alnus*-infective genotypes, including *Frankia* ACN14a, a ubiquitous cosmopolitan strain [24]. Using the same *nifH* gene fragment, Welsh et al. [31] observed five distinct clusters within the larger *Alnus*-infective group. Both of our *Frankia* genotypes grouped with the same *Alnus* cluster (A1), which in the study of Welsh et al. [31] included *Frankia* populations from a number of geographically distant areas. Given the limited

spatial scale of this study, additional sampling of *A. rubra*-associated *Frankia* nodules across a larger geographic range will help determine patterns of genetic diversity in *A. rubra* canopy and forest floor assemblages.

The mean total nitrogen content of canopy soils with *Frankia* present was 6% higher than in canopy soils with *Frankia* absent. However, this difference is not statistically significant and may reflect low statistical power rather than a lack of significant biological pattern. To investigate this possibility, we performed a power test and found that we would have needed 38 samples (compared to the nine we had from each location) to detect a significant difference among the observed means. Although our results indicate that the presence of *Frankia* does not strongly affect canopy soil nitrogen levels, a larger sample size is needed to more confidently address this question. The observed differences in soil nitrogen between the canopy and forest floor soils are similar to those found in previous studies [21, 25] and appear to be related to the higher organic matter content and slower decomposition rates of canopy soils [7].

In summary, we have shown that *Frankia* canopy and forest floor assemblages have similar genotypes at the regional (i.e., forest) scale but often somewhat different assemblages at the local (i.e., individual tree) scale. The distribution of *Frankia* in canopy soils appears to be limited by multiple factors, including canopy root production and environmental conditions. The bioassay results, however, indicate that the presence of *Frankia* in the canopy is not strongly dispersal-limited. Despite the important role of this symbiosis in increasing nitrogen levels in forest floor soils [5], *Frankia* may not have the same major impact on the canopy soils. Instead, canopy nodules may be more beneficial for *A. rubra* in terms of local nutrient support for leaf and/or stem growth. Future studies explicitly examining how *Frankia* is dispersed and the environmental factors controlling both canopy root and nodule formation will help in further understanding this interaction. Because canopy roots are exposed to different environmental conditions within very small spatial areas and because those areas can be easily manipulated (e.g., fertilizer or watering treatments), they present microbial ecologists with a unique arena to examine root–microbe interactions.

**Acknowledgments** We thank Holly Fautisch, David Hooper, August Longino, and Ralph Riley for assistance with climbing and nodule collection, Ingrid Gordon ([www.gearforgood.org](http://www.gearforgood.org)) for help with climbing gear, Jerry Franklin at Olympic National Park for access to the study site (permit no. OLYM-00234), Dittmar Hahn for advice about *Frankia* primers and molecular protocols, and L. Higgins, T. Hill, A. Lundgren, M. Palomino, K. Peay, J. Yayha, and J. Fierstein for constructive comments on previous versions of this manuscript. Funding for this project was provided by the Lewis & Clark College Rogers Research Fellowship, the Beckman Foundation, the Murdock Charitable Trust, and the National Science Foundation to N.M.N. (OPUS grant DEB 05-42130 and DBI 04-1731).

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