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Evidence for foliar endophytic nitrogen fixation in a widely distributed subalpine conifer

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Summary

- Coniferous forest nitrogen (N) budgets indicate unknown sources of N. A consistent association between limber pine (*Pinus flexilis*) and potential N_2-fixing acetic acid bacteria (AAB) indicates that native foliar endophytes may supply subalpine forests with N.
- To assess whether the *P. flexilis*–AAB association is consistent across years, we re-sampled *P. flexilis* twigs at Niwot Ridge, CO and characterized needle endophyte communities via 16S rRNA Illumina sequencing. To investigate whether endophytes have access to foliar N_2, we incubated twigs with $^{13}N_2$-enriched air and imaged radioisotope distribution in needles, the first experiment of its kind using ^{13}N. We used the acetylene reduction assay to test for nitrogenase activity within *P. flexilis* twigs four times from June to September.
- We found evidence for N_2 fixation in *P. flexilis* foliage. N_2 diffused readily into needles and nitrogenase activity was positive across sampling dates. We estimate that this association could provide 6.8–13.6 $\mu g\,N\,m^{-2}\,d^{-1}$ to *P. flexilis* stands. AAB dominated the *P. flexilis* needle endophyte community.
- We propose that foliar endophytes represent a low-cost, evolutionarily stable N_2-fixing strategy for long-lived conifers. This novel source of biological N_2 fixation has fundamental implications for understanding forest N budgets.

Introduction

Old-growth temperate and boreal coniferous forests accumulate more nitrogen (N) in soil and vegetation than can be explained by known sources of N, limiting our ability to understand and predict carbon (C) and N cycling across 15% (Wade *et al.*, 2003) of Earth's land surface (Dickson & Crocker, 1953; Richards & Bevege, 1967; Son & Gover, 1992; Bormann *et al.*, 1993, 2002; Yang *et al.*, 2011). While recently identified N input pathways may contribute N to some high-latitude forests (e.g. N_2 fixation by cyanobacteria in feather moss (DeLuca *et al.*, 2002) and canopy lichens (Antoine, 2004), and weathering of N-rich bedrock (Morford *et al.*, 2011)), these sources are not universally present. Woody actinorhizal species in the genus *Alnus* are widespread in cool climates, but are usually restricted to early succession in relatively wet habitats (Walker, 1993). Nodulated shrubs – both actinorhizal and legumes – represent other potential sources of N within temperate zones; however, actinorhizal genera such as *Ceanothus*, *Shepherdia* and *Cercocarpus*, as well as the majority of herbaceous legumes, are primarily found in open or recently disturbed areas (Schwintzer & Tjepkema, 1990; Kershaw *et al.*, 1998).

A resource-based evolutionary model predicts that N_2-fixing symbioses should evolve readily in old-growth temperate and boreal forest trees, which have a long leaf lifespan, high N use efficiency, and high litter recalcitrance (Menge *et al.*, 2008). However, beyond early forest succession, trees with N_2-fixing root nodules are rare or absent in these ecosystems (Vitousek *et al.*, 2002). This calls into question whether the traditional view – that N_2 fixation in association with plants depends on nodulating symbioses – captures the existing biodiversity of N_2-fixing mutualisms between bacteria and trees.

Awareness of the ubiquity and diversity of known N_2-fixing organisms has increased drastically in recent years, expanding the recognized pathways for ecosystem acquisition of new N (Reed *et al.*, 2011; Vitousek *et al.*, 2013). A relatively unexplored source of N in natural ecosystems is N_2 fixation by bacterial associates colonizing the interior of plants or their ectomycorrhizal fungi. N_2 fixation by endophytic bacteria has been studied in crop species (James, 2000; Reinhold-Hurek & Hurek, 2011), but beyond invasive grasses (Rout *et al.*, 2013), dune grasses (Dalton *et al.*, 2004), and *Populus* growing under nutrient-poor conditions (Knoth *et al.*, 2014), endophytic N_2 fixation is not typically considered a major N_2 fixation strategy or source of N in natural ecosystems. However, both endophytic N_2 fixation and N_2 fixation associated with tuberculate ectomycorrhizae have been reported in association with conifer trees. Significant nitrogenase activity was demonstrated in *Pinus contorta* (lodgepole pine) tuberculate ectomycorrhizas, especially in young stands (Paul *et al.*, 2007), and another study demonstrated the presence and expression of *nifH*, the gene encoding the dinitrogen reductase subunit of nitrogenase, within ectomycorrhizas of *Pinus nigra* (Corsican pine) (Izumi *et al.*, 2006). Chanway and colleagues isolated diazotrophs from *P. contorta* seedling stems and needles, and demonstrated N_2 fixation in re-inoculated conifer seedlings (Bal & Chanway, 2012; Anand & Chanway, 2013; Anand *et al.*, 2013).

Many coniferous species grow in low-nutrient environments or where low temperatures limit soil N turnover (Miller *et al.*, 1979; Weetman *et al.*, 1988), and have consequently evolved special strategies to cope with N limitation, including maximization of nutrient use efficiency (McGroddy *et al.*, 2004), and uptake of organic N from soil, both directly (Näsholm *et al.*, 2009) and through mycorrhizal fungi (Govindarajulu *et al.*, 2005; Albarracín *et al.*, 2013). Endophytic N_2 fixation may be an additional strategy that could enable conifers to thrive across broad gradients in N availability, including in very low fertility sites. However, it is not clear if native foliar endophytic bacteria fix N_2 in natural conifer stands, or whether the isolated bacteria used in re-inoculation experiments (Anand & Chanway, 2013; Anand *et al.*, 2013) are those most abundant or active in nature.

In a previous study, conducted in September 2009, we surveyed the endophytic communities in needles of adult *Pinus flexilis* (limber pine) and *Picea engelmannii* (Engelmann spruce), long-lived subalpine conifers widely distributed in western United States (US) mountains. Observations of 16S rRNA pyrosequences from needle samples taken in September 2009 at Niwot Ridge, CO demonstrated that a few taxa in the acetic acid bacteria (AAB) (*Acetobacteraceae*, a family in the Alphaproteobacteria) consistently made up 20–50% of the needle endophyte community (Carrell & Frank, 2014). By contrast, consistent associations with specific bacterial taxa or a high relative abundance of AAB has not been demonstrated in other plants and tissues, including rice (*Oryza sativa*) roots (Edwards *et al.*, 2015), Arabidopsis leaves and roots (Bulgarelli *et al.*, 2012; Lundberg *et al.*, 2013; Bodenhausen *et al.*, 2013), poplar roots (Gottel *et al.*, 2011; Shakya *et al.*, 2013), roots of the subalpine meadow plants *Pilosella aurantiaca*, *Leucanthemum vulgare* and *Trifolium hybridum* (Aleklett *et al.*, 2015), and needles of giant sequoia (*Sequoiadendron giganteum*) and coast redwood (*Sequoia sempervirens*) (Carrell & Frank, 2015). Given that the AAB have documented N_2-fixing functions as endophytes of crop plants (Gillis *et al.*, 1989; Fuentes-Ramirez *et al.*, 2001; Saravanan *et al.*, 2008), and consistently occur at high relative sequence abundance in the endophyte communities of high-elevation pines, we hypothesized an N_2-fixing role in subalpine conifer foliage (Carrell & Frank, 2014).

There is some prior evidence for *in situ* N_2 fixation in association with conifer foliage. Coniferous forest studies found nitrogenase activity in the canopy of *Pseudotsuga menziesii* and *Pinus nigra* (Jones, 1970; Jones *et al.*, 1974; Favilli & Messini, 1990). Observations of ^{15}N natural abundance in foliage are lower than expected in some pines, raising the possibility of an atmospheric source of N (Virginia & Delwiche, 1982). In Bormann *et al.*'s (1993) 'sandbox' mesocosm study of *Pinus rigida* and *Pinus resinosa*, they sought to identify unknown sources of N. Their study detected substantial N accumulation in vegetation that was not explained by nodulating plants, N deposition, or rhizosphere N_2 fixation, raising the possibility that the trees hosted N_2-fixing bacteria (Bormann *et al.*, 1993). N_2 fixation activity was detected in pine roots with soil attached (Bormann *et al.*, 1993), but an additional unidentified N source was required to explain observed N accumulation (Barkmann & Schwintzer, 1998).

In this study, we explored the possibility that native endophytic bacteria inside conifer foliage represent an important source of N for subalpine forests. Specifically, we investigated whether native endophytes have access to N_2 inside *P. flexilis* foliage; whether nitrogenase, the enzyme responsible for N_2 fixation, is active within *P. flexilis* foliage; and whether the previously observed association between *P. flexilis* and AAB endophytes is still prevalent, with AAB sequences abundant in *P. flexilis* foliage at Niwot Ridge 5 yr after initial surveys. Previously, we detected differences in the relative abundance of specific AAB phylotypes between trees growing at the treeline and trees growing in the subalpine forest (Carrell & Frank, 2014). We hypothesized that these differences could reflect differences in plant available soil N between the cooler treeline and warmer forest sites; a plant response to low soil N could be recruitment or retention of endophytic microbes that have N-fixing capabilities. To investigate whether N_2 fixation activity in the foliage is sensitive to plant available soil N, as is common in legumes (Pearson & Vitousek, 2001; Fujikake *et al.*, 2002), or insensitive to plant available soil N, as is suspected for alders (Menge & Hedin, 2009; Menge *et al.*, 2009), we measured nitrogenase activity in twigs collected from treeline and forest sites, and measured concurrent plant available N in surface soil.

Materials and Methods

Experimental sites and sampling

All plant material was collected in 2014 from limber pine (*Pinus flexilis* James) at Niwot Ridge, in the Colorado Front Range, CO, USA. Limber pine, which can live to > 1000 yr, is widely distributed in western US mountain ranges and is dominant on dry, rocky, windswept sites, where birds preferentially cache its seeds (Schoettle & Rochelle, 2000). We chose two stands: a 'forest' site (3022 m) surrounded by mixed forest composed of *P. flexilis*, lodgepole pine (*Pinus contorta*), subalpine fir (*Abies lasiocarpa*), Engelmann spruce (*Picea engelmannii*), and aspen (*Populus tremuloides*); and a 'treeline' site within the alpine treeline ecotone (3430 m) with adjacent stands of mixed *P. flexilis*, *A. lasiocarpa*, and *P. engelmannii*.

Radioisotope labeling and imaging

To investigate whether native endophytes have access to and potentially fix N_2 in the foliage of *P. flexilis*, we exposed fresh and surface-sterilized twigs to air enriched in radioisotope-labeled $[^{13}N]N_2$, and analyzed needles by radioisotope counting and phosphor imaging methods. We sampled twigs of 10–15 cm by cutting branches with a sterile razor blade just below the terminal cluster of needles, where the branch diameter was *c.* 0.5 cm, packed them on ice and shipped them overnight for laboratory processing on the following day. We used three twigs per tree collected from six trees, nine collected in each of June and July ($n = 18$) for radioisotope counting, and three twigs per tree collected from two trees in September ($n = 6$) for phosphor imaging. For all time-points, we autoclaved (20 min at 12 psi/122°C) one twig per tree, surface-sterilized (30% hydrogen peroxide) another, and left the third fresh. We re-cut all twigs under water, set them in a 14-cm-diameter labeling chamber in *c.* 1 cm of water and exposed them three at a time to $^{13}N_2$-labeled air for 16 min.

We produced ^{13}N as $[^{13}N]NH_4^+$ in-target on the LBNL Biomedical Isotope Facility CTI RDS-111 cyclotron (Siemens Healthcare, Knoxville, TN, USA) by irradiating 5 mM ethanol. Following 30 min of irradiation, we transferred the $[^{13}N]NH_4^+$ solution by high-performance liquid chromatography (HPLC) pump to a lead-shielded hot-cell, equipped with radiation detectors (Powell & O'Neil, 2012) for monitoring radiochemistry processes. To maximize the radioactive concentration, we directed the nonlabeled prefraction to waste before the radioactive fraction was collected in a 20-ml crimp-top glass vial. To oxidize the $[^{13}N]NH_4^+$ to the desired $[^{13}N]N_2$ radiotracer, 3 min before collection we added 200 µl of fresh 1.5 M NaOBr (6 N NaOH and 1.0 ml Br_2 to 7 ml H_2O (Vaalburg *et al.*, 1981)) to the vial. We began purging the vial with a 25 ml min^{-1} stream of air as soon as $[^{13}N]NH_4^+$ arrived, displacing any $[^{13}N]N_2$ generated. The estimated radiochemical yield of $[^{13}N]N_2$ was *c.* 80%.

We transferred the $[^{13}N]N_2$ from the headspace of the reaction vial to a cylindrical 2-l exposure chamber through 50 cm of 1/16-inch tubing equipped with an in-line boric acid trap (for trapping unreacted $[^{13}N]NH_3$ gas). Delivery of $[^{13}N]N_2$ continued for 5 min to allow maximum $[^{13}N]N_2$ to build up in the exposure chamber. We allowed the $[^{13}N]N_2$ to incubate with the twigs for 14–20 min before flushing the chamber with $[^{13}N]N_2$-free air for 4 min. During the incubation period, we collected a 20-ml sample from the exposure chamber and determined the activity-volume concentration. We used this concentration to determine the total radioactivity in the chamber, and estimated the specific activity of the radiotracer. Separate analyses by gas chromatography of $[^{13}N]N_2$–air mixtures eluted from the reaction vial and measurement of the boric acid trap indicated 100% radiochemical purity for the $[^{13}N]N_2$. Half-life analyses showed 100% radionuclidic purity of ^{13}N.

We placed the exposure chamber under a 1000-W metal halide lamp (Digilux 1000 MH; Hydrofarm Inc., Petaluma, CA, USA) at a distance of 1.0 m and a 12° zenith angle to achieve a photon flux density of *c.* 300 µmol of photons m^{-2} s^{-1} to the top surface of the exposure chamber. Following the exposure period and flushing, we removed the twigs from the exposure chamber and measured June and July twigs for total radioactivity using a dose calibrator (CRC-15; Capintec Inc., Ramsey, NJ, USA). Based on a specific activity value (µCi ^{13}N/mol $^{13/14}N_2$) calculated from the activity-volume concentration of $[^{13}N]N_2$ gas in the exposure chamber, radioactivity measures were converted to moles of uptake of N_2. We normalized this uptake value to exposure time, and converted to a rate of uptake in µmol N_2 min^{-1}. We performed analysis of variance (ANOVA) to compare total µmol ^{13}N inside autoclaved, surface-sterilized, and unsterilized twigs after incubation with ^{13}N.

In September, following exposure to $[^{13}N]N_2$, we removed a subsample of needles from the base, mid-section, and tip of each twig. We fixed needles to paper, covered them in cellophane and placed them in contact with a phosphor screen (Knol *et al.*, 2008) for > 30 min to image the relative distribution of radioactivity among and within the needles.

Acetylene reduction assay

To determine whether the nitrogenase enzyme, responsible for breaking the N–N triple bond and reducing N_2 to NH_3, is active in *P. flexilis* foliage, we selected 15 healthy, mature trees from the forest and treeline, within an area of *c.* 1 ha, and sampled twigs as described for the radioisotope labeling. Up to three twigs from each tree were sampled each month between June and September for acetylene reduction assays (ARAs) (Hardy *et al.*, 1968) on the day following collection.

In the laboratory, we recut branch tips under water, 1–2 cm above the field-cut ends. To test for potentially confounding *epiphytic* (leaf surface) N_2 fixation, we surface-sterilized two branch tips from each of five randomly selected trees per site by immersing twigs in a 30% H_2O_2 solution for 2 min before rinsing three times with deionized water. We placed the surface-sterilized branch tips, plus two untreated branch tips from all 15 trees (80 samples in total), into separate 473-ml mason jars, with stems immersed in 20 ml of deionized water. We sealed jars with lids containing septa.

We assigned half of the samples to acetylene exposure and half to controls, and injected 50 ml of acetylene gas at ambient pressure into acetylene jars, and 50 ml of ambient air into control jars with a 60-ml syringe. We then immediately inserted a 25-ml gas-tight syringe, mixed headspace gas by pumping the plunger to 15 ml three times, and then removed a 15-ml sample (t_0) and placed it in an evacuated 12-ml vial (exetainer; Labco, Lampeter, UK). After 2 h of incubation, we mixed headspace gas with the syringe as above, and removed a second 15-ml sample ($t_{2\,h}$) from each jar. Headspace samples were analyzed on a gas chromatograph (6890; Hewlett Packard, Palo Alto, CA USA) equipped with a flame ionization detector using helium as the carrier gas.

We estimated conversion rates of acetylene to ethylene in each jar as the change in headspace moles of ethylene from t_0 to $t_{2\,h}$ per unit needle dry mass, using no-acetylene controls to correct for endogenous ethylene production and no-twig controls to correct for any ethylene in the acetylene. We used a one-tailed *t*-test, pooling all acetylene samples (combining sampling date, elevation, and surface-sterilized and unsterilized samples) to test for significant ethylene production. We then tested for differences between surface-sterilized and untreated branch tips using a two-tailed *t*-test. We used ANOVA to evaluate sampling date and elevation effects on ethylene production, pooling surface-sterilized and untreated samples (the groups did not differ ($P = 0.39$)). Inclusion of tree as a random effect did not improve the model (using the Aikake information criterion), and therefore source tree was omitted from the ANOVA.

Endophyte community 16S rRNA sequencing

To investigate if AAB were still present in high relative abundance in Niwot Ridge *P. flexilis* foliage in 2014, 5 yr after our initial study (Carrell & Frank, 2014), we used needles collected from three trees at each of the forest and treeline sites in June and September 2014 (12 samples in total). We sterilized the surface of needles by submersion in 30% hydrogen peroxide for 3 min, rinsed three times by shaking with sterile deionized water for 1 min (Izumi *et al.*, 2008), and stored them at −20°C. Sterility was confirmed by negative PCR amplification of the 16S rRNA gene from a DNA extraction performed on the final rinse.

We ground the needles to a fine powder in a sterile mortar in the presence of liquid N. We extracted DNA using a cetyl trimethylammonium bromide (CTAB) extraction method (Carrell & Frank, 2014). Briefly, we added 800 µl of CTAB solution (1 ml of CTAB buffer, 0.04 g of polyvinylpyrollidone and 5 µl of 2-mercaptoethanol) to 0.6 g of tissue, incubated for 2 h at 60°C, and homogenized with glass beads for 3 min. We removed proteins with the addition of an equal volume of chloroform, centrifuged for 10 min at 16 000 ***g***, and placed the top aqueous phase in a sterile tube. We precipitated nucleic acids with the addition of 1/10 volume of cold 3 M sodium acetate and 1/2 volume cold isopropanol, froze them at −20°C for 12 h, and centrifuged for 30 min at 16 000 ***g***. We removed the supernatant, added 700 µl of 70% ethanol, and centrifuged for 10 min. We resuspended the air-dried pellet with 30 µl of DNA resuspension fluid (1.0 M Tris-HCL and 0.1 M EDTA) and stored it at −20°C.

We amplified community 16S rRNA using nested PCR to reduce the occurrence of plastid sequences and improve consistency. To suppress plant DNA amplification, we used the primer pair 16S 799f (AACMGGATTAGATACCCKG) and 16S 1492r (TACGGHTACCTTGTTACGACT) in the first PCR reaction (PCR1). Amplification with 16S 799f and 16S 1492r results in mitochondrial amplicon of *c.* 1000 bp and a bacterial amplicon of *c.* 750 bp (Chelius & Triplett, 2001). In the second round of PCR (PCR2), PCR1 amplicons were used as a template to amplify the bacterial fragment with the Illumina adapted, Golay-barcoded primer pair 799f and 1115r (AGGGTTGCGCTCG TTG) (Redford *et al.*, 2010). We decreased the number of cycles to reduce primer bias (Jiao *et al.*, 2006), with the following thermocycle profile used 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 and 1.5 min at 72°C; and a final 10 min of elongation 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 $\mu g\,\mu l^{-1}$ bovine serum albumin (Thermo Fisher Scientific, Waltham, MA, USA), 21.5 µl of PCR-grade water (Fisher Bioreagents, Waltham, MA, USA), and 0.2 µM of forward and reverse primers. The 25-µl PCR2 reaction contained 3 µl of PCR1 product, 10 µl of 5 Prime Hot Master Mix, 0.5 $\mu g\,\mu l^{-1}$ bovine serum albumin (Thermo Scientific), 8.75 µl of PCR-grade water (Fischer BioReagents), and 0.2 µM forward and reverse primers. We cleaned and pooled the barcoded PCR2 products, and extracted the bacterial band to the exclusion of the plant mitochondrial band (QIAquick Gel Extraction Kit, Qiagen Inc., Valencia, CA, USA). Pooled samples were sequenced on an Illumina MiSeq platform at the University of California, Davis Genome Center. The sequence data have been submitted to GenBank and can be accessed under project accession no. SRP058776.

We processed and analyzed the sequences using the QIIME (1.8.0) package (Caporaso *et al.*, 2010b), with the UPARSE method for clustering operational taxonomic units (OTUs) (Edgar, 2013). Briefly, we joined forward and reverse paired-end reads using fastq-join, with the barcode filtered from the data set if the forward and reverse reads did not overlap (Aronesty, 2011). We quality filtered joined paired-end reads with QIIME default settings (maximum number of consecutive low-quality base calls of three bases; minimum number of consecutive high-quality base calls as a fraction of the input read length of 0.5 total read length; maximum unacceptable Phred quality score of 3; no N characters) which have been found sufficient for community analysis (Bokulich *et al.*, 2012). We clustered the remaining sequences into OTUs at the 97% level using UPARSE (Edgar, 2013). We aligned representative sequences using PYNAST (Caporaso *et al.*, 2010a) against the Greengenes core set (DeSantis *et al.*, 2006). We assigned OTUs to taxonomic groups using UCLUST (Edgar, 2010) with the Greengenes representative set of sequences as reference. We removed sequences classified as 'Chloroplast' or 'Mitochondria', and 'Unassigned'. We constructed a maximum-likelihood tree from the aligned representative sequences using FASTTREE (Price *et al.*, 2009). To calculate the significance of clustering of samples by elevation and time-point, we used analysis of similarity (ANOSIM) and permutational multivariate analysis of variance (PERMANOVA), each with 999 permutations. For the phylogenetic analysis, we used sequences identified as Alphaproteobacteria and observed > 100 times in our data set and inferred a maximum likelihood tree using RAxML (Stamatakis, 2006). We used Perl Tk to plot the heatmaps of top OTUs from the OTU tables.

Soil nitrogen availability

We measured relative differences in plant-available inorganic soil nitrogen in our forest and treeline sites and across dates using Plant Root Simulator probes (Western Ag, Saskatoon, SK, Canada). We inserted two cation and two anion probes within 2 m of the base of 12 trees at each sample date with reactive

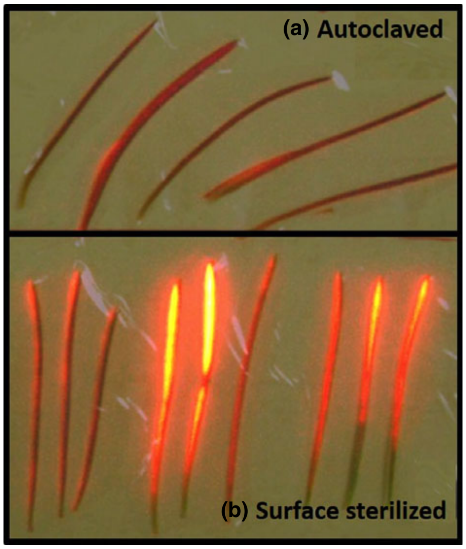

Fig. 1 Merged photograph and phosphor image of *Pinus flexilis* needles exposed to the radioisotope [^{13}N]N_2. Needles from three autoclaved twigs (a) and from three surface-sterilized twigs (b) are shown for comparison. Brighter (whiter) colors indicate greater uptake of radioactive N_2.

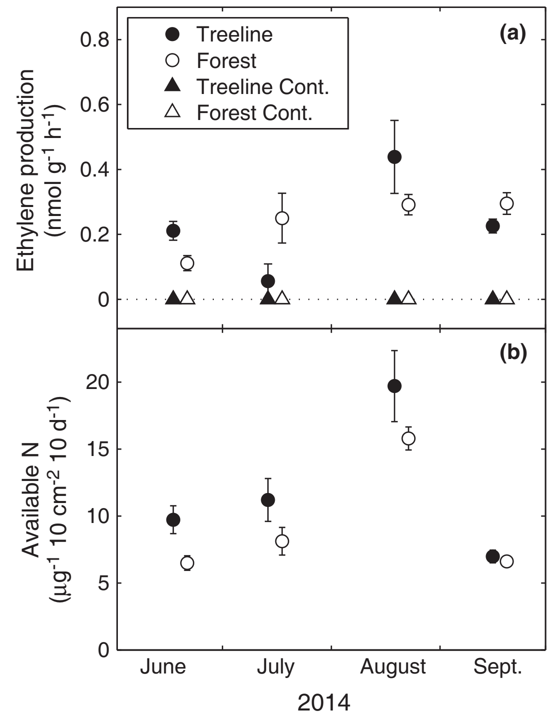

Fig. 2 (a) Rates of acetylene conversion to ethylene (mean ± SEM), indicating activity of the nitrogenase enzyme, from incubations of *Pinus flexilis* branch tips harvested from trees at alpine treeline and subalpine forest sites. Controls for endogenous ethylene production are shown with triangles. Sample sizes ranged from six to 19, after quality control. There were no significant differences between sites or among months. (b) Mean ± SEM of total plant available nitrogen (N) (ammonium + nitrate) as measured by PRS probes at the base of trees at treeline and forest sites. August total N availability was greater than that in other months ($P < 0.0001$), and total N availability at the treeline was greater than in the forest site ($P = 0.05$).

membranes exposed to the top 5 cm of mineral soil. We left probes in place for 8–10 d before collecting them and rinsing with distilled water. Ammonium and nitrate accumulated on probes were detected colorimetrically by the manufacturer and provide an index of integrated nutrient supply to plant roots (Qian & Schoenau, 2002; Harrison & Maynard, 2014). We used ANOVA to evaluate sampling date and elevation effects on ammonium, nitrate and total inorganic nitrogen (ammonium + nitrate) availability. To identify statistically significant differences ($P < 0.05$) between dates, we conducted Tukey honest significant difference pairwise comparisons, with Bonferroni corrections.

Results

Accumulation of $^{13}N_2$ in *P. flexilis* needles

^{13}N accumulated in the interior of surface-sterilized *P. flexilis* needles (Fig. 1), indicating ready diffusion of N_2 into needles, with significantly less accumulation in an autoclaved control ($P = 0.0007$). Fresh and surface-sterilized twigs accumulated 1.58 and 1.45 µmol N_2 min^{-1} per twig, respectively, while autoclaved twigs accumulated 0.37 µmol N_2 min^{-1} $twig^{-1}$. Radioisotope analysis of six whole twigs indicated no significant difference in ^{13}N accumulation between surface-sterilized and fresh twigs ($P = 0.705$). Phosphor imaging also showed greater ^{13}N accumulation in green relative to brown needles, and in the tips of some green needles.

Nitrogenase activity

The ARA indicated nitrogenase activity (ethylene production) in *P. flexilis* twigs exposed to acetylene and no ethylene production in controls without acetylene (Fig. 2a). We found significant nitrogenase activity across all sample dates and both elevations ($P < 0.0001$), with no significant difference between surface-sterilized and fresh samples ($P = 0.39$), indicating that activity occurred within dry needle tissue, not on the surface. Average ethylene production rates (nmol ethylene g^{-1} needle h^{-1}) were 0.22 ± 0.02 SEM ($n = 64$) in the forest, 0.19 ± 0.04 ($n = 50$) at the treeline, and 0.21 ± 0.02 ($n = 114$) combined. Large variation across individual samples resulted in no net effects of elevation ($P = 0.28$), month ($P = 0.28$), or their interaction ($P = 0.54$) on nitrogenase activity. If we extrapolate an average ARA-derived estimate of 0.21 nmol ethylene g^{-1} needle h^{-1} using a theoretical ratio of 3 : 1, acetylene : N_2 (Hardy *et al.*, 1968; Bellenger *et al.*, 2014), *P. flexilis* specific leaf area of 143.96 cm^2 g^{-1} (Schoettle & Rochelle, 2000), and subalpine forest leaf area index of 4.2 m^2 m^{-2} at Niwot Ridge (Monson *et al.*, 2002), we estimate potential rates of N_2 fixation in needles of 6.8–13.6 µg N m^{-2} d^{-1} or 1–2 mg m^{-2} yr^{-1} in *P. flexilis* stands (Supporting Information Methods S1).

Endophyte community analysis

We found that OTUs belonging to the AAB family were dominant members of the endophyte community in the *P. flexilis* needles, with three AAB OTUs consistently present at high relative sequence abundance (Fig. 3). On average, 29% of the community belonged to these three OTUs, although the percentage ranged widely from 6.1% to 62.2%. The most dominant AAB OTUs were similar to those identified in trees sampled in 2009 (Carrell & Frank, 2014). For example, OTU 3 from this study shares 99% identity to OTU 1045 from the previous study, and OTU 5 from this study is identical to OTU 1516 from the previous study (Table 1). Among the top OTUs that do not belong to the AAB, only one (most similar to *Pandorea pnomenusa*) was common between the previous and current studies, suggesting high turnover of vagrant (outside of their normal range) or transient (temporary) endophytes in *P. flexilis* needles, or batch effects associated with sample processing, PCR, or sequencing (Leek *et al.*, 2010). In particular, an OTU with 100% identity to *Stenotrophomonas maltophila* (phylum Proteobacteria), a species that is often found in association with plants (Ryan *et al.*, 2009), was found in the highest relative sequence abundance in several samples, despite not having been a prominent community member in *P. flexilis* needles sampled in September 2009 (Carrell & Frank, 2014). In several samples, higher abundance of this OTU correlated with lower abundance in AAB OTU 3 and vice versa, potentially suggesting a negative or competitive interaction between the two taxa. Likewise, an OTU 100% identical to *Chitinophaga pinensis* (phylum Bacteroidetes), which was originally isolated from pine litter (Sangkhobol & Skerman, 1981), was found in all of the 2014 samples, sometimes at high relative sequence abundance (Fig. 3), but also was not observed in 2009.

Table 1 Pairwise per cent identity between top acetic acid bacteria (AAB) operational taxonomic units (OTUs) in the *Pinus flexilis* foliar endophyte community from the current study and the top AAB OTUs identified in the same host in our previous study (Carrell & Frank, 2014)

	2009 top OTUs		
2014 top OTUs	OTU 1045	OTU 1516	OTU 1644
OTU 3	99	93	97
OTU 5	93	100	95
OTU 147	97	92	94
OTU 13	96	92	94

AAB was the most diverse family in our samples (23 OTUs in total), and the most dominant AAB OTUs were distinct from one another (Fig. 4) (e.g. OTUs 5 and 3 shared only 93% identity over the sequenced region), suggesting that *P. flexilis* supports multiple dominant and distinct members of the AAB as foliar endophytes. The Rhizobiales, an order that also contains N_2-fixing bacteria, was as diverse as the Rhodospirillales in our samples (Fig. 4), but did not contain a single dominating family, and no OTU that was present at high relative abundance in all samples. The three most prominent OTUs in the Rhizobiales were OTU 7 (*Agrobacterium* sp.), 8 (*Methylobacterium* sp.), and 12 (*Rhizobium* sp.) (Fig. 4). These three OTUs made up on average 2.2%, 1.4%, and 0.8% of the sequences in our samples, respectively.

The endophyte community was not structured by elevation (PERMANOVA: pseudo-F statistic = 0.6567; P = 0.835; ANOSIM: R = −0.1204; P = 0.879) and did not differ between samples taken in June and September (PERMANOVA: pseudo-F statistic = 1.1207; P = 0.330; ANOSIM: R = 0.0593; P = 0.263).

Plant-available soil nitrogen

Plant-available soil N as estimated by inorganic N accumulation on PRS probes averaged 8.64 ± 0.63 (SEM) μg N probe^{-1} wk^{-1} across the two elevations and 4 months (Fig. 2b). Nitrate and ammonium constituted 39 and 61 ± 4% of available inorganic N. Nitrogen accumulation by the probes was highest in August (P < 0.0001 for ammonium, nitrate, and total N) and ammonium accumulation was lowest in September (P < 0.0001). Contrary to expectations based on temperature differences, the high-elevation treeline site had greater ammonium (P = 0.019) and total inorganic N (P = 0.05) availability than the lower elevation forest, but nitrate availability was more similar between the two sites (P = 0.093), and there were no date by elevation interactions (P > 0.388). Ethylene production was not correlated with total plant-available soil N across months and sites (R^2 = 0.344; P = 0.126; n = 8).

Discussion

Our results provide the first evidence for endophytic N_2 fixation in *P. flexilis* foliage. N_2 diffused readily into needles and

	June						September						GenBank no.	% ID	Match
	treeline			forest			treeline			forest					
OTU_2	15.3	40.1	27.0	1.0	10.4	5.2	2.9	47.3	7.2	7.8	51.2	33.4	KP100336.1	100	*Stenotrophomonas maltophila*
OTU_3*	17.1	7.4	13.0	7.4	25.6	35.6	35.3	3.7	17.9	24.2	4.5	11.0	NR_074292.1	97	*Gluconacetobacter diazotrophicus*
OTU_6	6.7	3.6	15.8	4.0	12.1	14.0	13.0	1.0	6.3	8.8	2.0	20.2	CP009553.1	100	*Pandoraea pnomenusa*
OTU_4	2.3	9.6	6.8	0.7	2.5	1.3	0.7	11.4	2.3	2.0	15.5	8.6	AB681009.1	100	*Chitinophaga pinensis*
OTU_5*	6.6	2.6	2.9	10.0	8.9	13.9	14.0	1.3	6.7	8.9	1.7	1.7	NR_113406.1	95	*Gluconacetobacter liquefaciens*
OTU_7	1.1	3.6	2.7	0.8	1.1	0.4	0.3	7.2	1.2	0.8	4.6	3.2	LC015594.1	100	*Agrobacterium tumefaciens*
OTU_147*	3.3	1.7	2.4	2.0	7.2	12.5	12.4	1.1	3.6	4.5	0.9	1.6	NR_118181.1	95	*Gluconacetobacter johannae*
OTU_8	0.8	2.3	1.6	0.0	0.7	0.3	0.3	5.5	0.3	0.5	2.7	1.8	LC026013.1	100	*Methylobacterium radiotolerans*
OTU_9	5.1	1.8	0.9	3.0	0.9	0.3	0.2	0.0	4.7	7.7	1.4	0.7	NR_043726.1	100	*Brevundimonas terrae*
OTU_11	3.0	1.2	1.5	1.5	3.1	1.7	1.6	0.1	3.0	4.5	0.8	1.4	NR_028731.1	90	*Desulfofrigus ocanense*
total AAB	27.0	11.7	18.3	19.4	41.1	62.0	61.7	6.1	25.7	37.6	7.1	14.3			

>15% >10% >5% >2% <2%

Fig. 3 Heatmap showing the ten most common operational taxonomic units (OTUs) in the *Pinus flexilis* endophyte community data set and their relative abundances in each sample as the percentage of all sequences in that sample. GenBank identifiers (nonredundant and 16S rRNA databases), per cent identity, and names of the most similar database sequences are shown along with each OTU. OTUs belonging to the acetic acid bacteria (AAB) are indicated with an asterisk. The total percentage of sequences in each sample that belong to the top three AAB OTUs (3, 5 and 147) are shown below the heatmap.

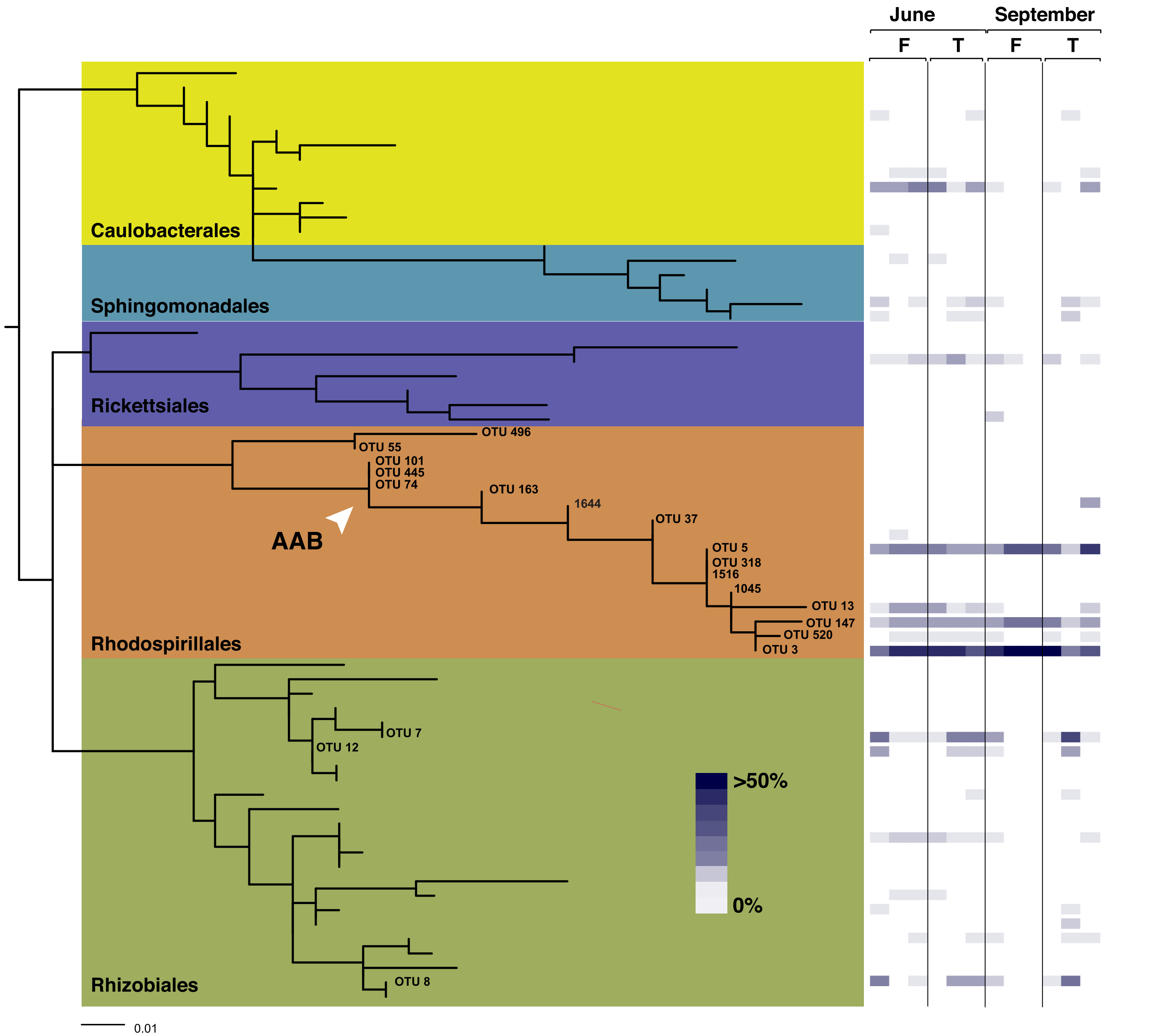

Fig. 4 Maximum likelihood phylogeny of the Alphaproteobacterial operational taxonomic units (OTUs) that occurred > 100 times in our sequences, along with a heatmap that shows the relative abundance of each OTU as the percentage of Alphaproteobacterial sequences belonging to that OTU in each sample (from forest (F) and treeline (T) locations). Dominant acetic acid bacteria (AAB) OTUs are distinct from each other, and AAB is the most diverse Alphaproteobacterial family represented in our samples.

nitrogenase activity was positive across sampling dates. This evidence challenges the widely held notion that conifers do not harbor N_2-fixing symbionts. Even though an N_2-fixing strategy should be favored in N-limited temperate and boreal coniferous forests, previous studies have reasoned that the costs of N_2 fixation by nodulating symbioses, which tend to have low C : N ratios and relatively high rates of mortality, are too high for these species to persist past early succession (Menge *et al.*, 2008). We propose that foliar endophytes represent a lower cost for the plant, and may be a more competitive and evolutionarily stable N_2-fixing strategy in established temperate and boreal forests. We found that AAB dominated the *P. flexilis* needle endophyte community in all samples and suggest that members of this group could be responsible for the N_2-fixing activity. Further, *P. engelmannii*, a dominant conifer in the subalpine forest at Niwot Ridge, hosts a high relative abundance of the same endophytic taxa as hosted by *P. flexilis* (Carrell & Frank, 2014), indicating that endophytic N_2 fixation may occur in other pine family species. Thus, we propose that endophytic N_2 fixation is a potentially widespread source of N for temperate and boreal forests that could partly explain the gap in N sources observed in these ecosystems.

Using the well-established ARA, we found ethylene build-up comparable to what was observed in the *P. resinosa* rhizosphere in Bormann *et al.*'s sandbox experiment (1993), and in rhizomes of *Sorghum halpense*, an invasive grass shown to alter soil N content via endophytic N_2 fixation (Rout *et al.*, 2013). Acetylene reduction rates did not vary between surface-sterilized and fresh tissue, suggesting that nitrogenase is active in the endosphere (interior) rather than in the phyllosphere (leaf surface), which is a site of N_2 fixation in some tropical plants. Thus, our results support the hypothesis that endophytic bacteria fix N_2 inside *P. flexilis* foliage. Substantial between-sample and month-to-month variability in nitrogenase activity could be attributable in part to measurement 'noise' resulting from the multi-step technique, or to real variability between samples, or both.

While our method cannot rule out the possibility of acetylene inhibiting bacterial oxidation of endogenous ethylene that might have been produced by the plant (Debont, 1976), ARA has been successfully used as an indicator of nitrogenase activity in many studies of both above- and belowground tissues and in bacterial isolates from plant tissues (DeLuca *et al.*, 2002; Doty *et al.*, 2009; Bal & Chanway, 2012; Jean *et al.*, 2012; Rout *et al.*, 2013). Endogenous ethylene oxidation, which could potentially be inhibited by acetylene, and therefore confound ARA, has been demonstrated in soil, but only after 8 d of incubation (Debont, 1976), and in wheat (*Triticum aestivum*) roots, but only in samples with soil attached, not in surface-sterilized roots (Sloger & van Berkum, 1988). Given the short incubation time used here (2 h), the lack of soil in our samples, and the complimentary sequencing data, we think that there is strong evidence for the presence of N_2-fixing endophytes in *P. flexilis*. However, more work – in particular tissue incubation with $^{15}N_2$ free of $^{15}NH_3$ contamination (Dabundo *et al.*, 2014) – is needed to quantify *in situ* rates of N_2 fixation.

Using radioisotope labeling, we showed that N_2 readily diffuses into fresh and surface-sterilized needles on short timescales (minutes). Because the half-life of $^{13}N_2$ is short (*c.* 10 min), measurements of radioactivity occurred quickly. Our technique could not distinguish $^{13}N_2$ within needle air-space from that fixed by bacteria, but confirms that N_2 readily diffuses into needles, where it could potentially be fixed, and that accumulation (and thus potential fixation) is higher in fresh green needles than in brown needles and stems. There was significantly less accumulation of ^{13}N in autoclaved tissue; however, this could be a consequence of greater resistance to diffusion in autoclaved needles resulting from stomatal closure, cell/tissue damage or saturation with steam water. Radioisotopes have been used as a tool to study N_2 fixation in bacterial isolates and root nodules for decades, and more recently for root endophytic bacteria (Campbell *et al.*, 1967; Ishii *et al.*, 2009; Pankievicz *et al.*, 2015), but to our knowledge this is the first application of $[^{13}N]N_2$ in aboveground (foliar) tissue studies. While limited in its utility *in situ*, radioisotope labeling offers the potential for rapid and sensitive measurement of N_2 fixation rates in the laboratory. To realize this potential requires further development of the technique to distinguish biological N_2 fixation from accumulation of $^{13}N_2$ in needle air-space.

Our 16S rRNA community analysis points to AAB as the bacterial group responsible for endophytic N_2 fixation in *P. flexilis* twigs, as no other N_2-fixing bacterial lineage was consistently found in comparable relative abundance within and across years. We found that AAB were dominant endophyte community members from samples taken in both 2009 (Carrell & Frank, 2014) and in this study, suggesting that the association is not only consistent across individuals and species, but also over time. If AAB are responsible for N_2 fixation, the variation in their relative abundance (6.1–62% in the current study) could potentially explain some of between-sample and month-to-month variability in nitrogenase activity; however we cannot make direct comparisons because bacterial sequences and nitrogenase activity were characterized on different twigs.

The tendency for AAB taxa to recur in endophyte communities probably reflects selective uptake on the part of the host, bacteria, or both, potentially reflecting mutualism. However, it is important to point out that the link between specific bacteria and nitrogenase activity still needs to be made using methods suitable for a low-abundance, uncultured community, such as sequencing of the *nifH* gene after enrichment of bacteria from plant tissue (Bragina *et al.*, 2011, 2013) combined with single-cell genome sequencing (Clingenpeel *et al.*, 2015), and fluorescence *in situ* hybridization (FISH).

Other potential N_2-fixing bacteria (in the Rhizobiales order of the Alphaproteobacteria) were present at substantially lower relative abundance; however, these taxa could still contribute to or be fully responsible for the observed nitrogenase activity. The remaining dominant OTUs (those not belonging to the AAB) were largely different between 2009 and 2014, suggesting that, despite being present at high relative abundance within a year, they are vagrant or temporary community members. Two new OTUs not belonging to the AAB were found in high relative abundance in the current study; one identical to *S. maltophila* and another identical to *C. pinensis*. *Stenotrophomonas maltophila* is commonly associated with plants, including as an endophyte, and has been isolated from the genera *Populus* and *Salix*, from rice, and maize (*Zea mays*) among others (Chelius & Triplett, 2000; Sturz *et al.*, 2001; Taghavi *et al.*, 2009; Hardoim *et al.*, 2012; Zhu *et al.*, 2012). N_2 fixation has been demonstrated for the genus *Stenotrophomonas* (Ramos *et al.*, 2011); thus, we cannot exclude the possibility that this taxon is responsible for or at least contributes to the nitrogenase activity observed in this study. The genus *Chitinophaga* was first discovered when *C. pinensis* was isolated from pine litter (Sangkhobol & Skerman, 1981); another species, *Chitinophaga costaii*, has been isolated from the trunk of *Pinus pinaster* (Proença *et al.*, 2014). This taxon may also contribute the observed nitrogenase activity; *Chitinophaga* spp. have been found to be the most active diazotrophs in carnivorous plants of the genus *Utricularia* (Sirová *et al.*, 2014).

Interestingly, while there was no significant seasonal pattern or elevation difference in nitrogenase activity, soil N availability did vary between elevations and months (Fig. 2), resulting in no significant correlation between nitrogenase activity and plant-available soil N. This runs counter to what has been observed in association with most legumes, where N_2 fixation rates are often inversely correlated with soil N availability (Andrews *et al.*, 2011; Barron *et al.*, 2011), but is consistent with studies on alder and some invasive legumes, which have found little relationship between N availability and N_2 fixation rates (Menge *et al.*, 2008; Andrews *et al.*, 2011; Drake, 2011). The PRS probes record inorganic N present in soil solution over the time of deployment, reflecting integrated effects of soil moisture, soil temperature, and organic substrate availability. While bacterial nitrogenase activity could respond to the same factors, it also could be mediated by variation in plant metabolic activity and plant N demand, which would decouple it from plant-available soil N in any given place and time. Additional, *in situ* measurements and controlled experiments are required to fully understand environmental and host-plant controls on nitrogenase activity.

The amount of N_2 fixed via aboveground endophytes per unit of plant biomass is probably smaller than for root nodulated plants, which host an abundance of microbes in specific symbiotic organs. Based on the ARA rates we measured and the calculations reported above (6.8–13.6 $\mu g\,N\,m^{-2}\,d^{-1}$), we estimate an annual N input of 1.02–2.04 $mg\,N\,m^{-2}\,yr^{-1}$ (or 10.2–20.4 $g\,N\,ha^{-1}\,yr^{-1}$) to a *P. flexilis* stand (assuming a 150-d growing season; Methods S1). Numerous assumptions, including the ratio of acetylene reduction to N_2 fixation, lend considerable uncertainty to these estimates, but this amounts to $<0.1\%$ of annual root-zone N mineralization in subalpine forests in this region (2.7 $g\,m^{-2}\,yr^{-1}$; Arthur & Fahey, 1992). Despite relatively low rates of N_2 fixation compared with nodulated plants, this pathway for N accumulation may still be biologically important. First, the low turnover of conifer foliage – up to 10 yr for individual needles – and high leaf C : N ratios (e.g. 48 : 1 in needles from our 30 trees; A. B. Moyes & L. M. Kueppers, unpublished data) suggest a modest annual need for N to support conifer biomass. Published estimates of aboveground net primary productivity (ANPP) per unit N absorbed in subalpine forests in the Colorado Rocky Mountains showed *c.* 250 $g\,ANPP\,g^{-1}\,N$ (Arthur & Fahey, 1992; Binkley *et al.*, 2003). This means that N uptake via N_2 fixation could add 2.6–5.1 $kg\,ANPP\,ha^{-1}\,yr^{-1}$ to a limber pine stand's productivity, based on our estimated rates. Second, sources of soil N are limited in this high-elevation ecosystem, with a short growing season, acidic soils and lack of nodulating N_2 fixers in the mature forest. A full accounting of the importance of this pathway, including rigorous calibration of the relationship between nitrogenase activity and N_2 fixation, for the N economy of the plant and subalpine forests more generally awaits more extensive measurements of N cycling.

Our results indicate that a pine that is widely distributed in the western USA can acquire N through associations with native N_2-fixing bacteria. We have identified a bacterial group that may be responsible: taxa in the family *Acetobacteraceae* consistently dominate the endophyte community in *P. flexilis* needles, and the association appears to be stable across time. However, to determine the significance of this association and its ubiquity across conifer species and ecosystems, it will be important to link the N_2-fixing activity to specific bacterial strains, to understand how the symbiosis is established, and to identify the factors that regulate its presence and activity.

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Author contributions

A.B.M., L.M.K., J.P-R., and A.C.F. designed the research. A.B.M., L.M.K., D.L.C., N.V., J.O. and A.C.F. performed experiments and analyzed the data. A.B.M., L.M.K., J.P-R., D.L.C., N.V., J.O. and A.C.F. interpreted the results and wrote the paper.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Methods S1 Explanation of calculations to extrapolate measured nitrogenase activity to potential annual endophytic N_2 fixation per unit area of a limber pine stand.

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