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Host species and habitat affect nodulation by specific *Frankia* genotypes in two species of *Alnus* in interior Alaska

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Abstract Alders (*Alnus* spp.) are important components of northern ecosystems due to their ability to fix nitrogen (N) in symbiosis with *Frankia* bacteria. Availability of optimal *Frankia* may be a contributing factor in limiting the performance and ecological effects of *Alnus*, but the factors underlying distribution of *Alnus*-infective *Frankia* are not well understood. This study examined the genetic structure (*nif*D–K spacer RFLP haplotypes) of *Frankia* assemblages symbiotic with two species of *Alnus* (*A. tenuifolia* and *A. viridis*) in four successional habitats in interior Alaska. We used one habitat in which both hosts occurred to observe differences between host species independent of habitat, and we used replicate sites for each habitat and host to assess the consistency of symbiont structure related to both factors. We also measured leaf N content and specific N-fixation rate (SNF) of nodules (^{15}N uptake) to determine whether either covaried with *Frankia* structure, and whether *Frankia* genotypes differed in SNF in situ. *Frankia* structure differed between sympatric hosts and among habitats, particularly for *A. tenuifolia*, and was largely consistent among replicate sites representing both factors. Leaf N differed between host species and among habitats for both hosts. SNF did not differ among habitats or host species, and little evidence for differences in SNF among *Frankia* genotypes was found, due largely to high variation in SNF. Consistency of *Frankia* structure among replicate sites suggests a consistent relationship between both host species and habitat among these sites. Correlations with specific environmental variables and possible underlying mechanisms are discussed.

Keywords Actinorhizal · Boreal · Distribution · Nitrogen fixation · Symbiosis

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Nomenclature: Flora of North America (http://hua.huh.harvard.edu/FNA/).

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Introduction

In terrestrial ecosystems of the northern hemisphere, alders (*Alnus* spp., Betulaceae) are important early colonizing plants in both primary and secondary seral systems (e.g., Chapin et al. 1994; Van Cleve and Viereck 1981), due largely to their ability to form root nodule-based, nitrogen (N) fixing symbioses with actinomycete bacteria of the genus *Frankia*. This symbiosis allows alder to maintain high N content and growth rates in low nutrient and/or disturbed habitats, which can result in both strong competitive effects of alder growth and facilitative effects of alder-derived N on associated plant species throughout succession (e.g., Vogel and Gower 1998; Wurtz 1995; Chapin et al. 1994; Walker and Chapin 1986; Van Cleve and Viereck 1981). Controlled inoculation studies between *Alnus* species and *Frankia* strains indicate that specificity of associations is variable for both organisms, and that infection by different *Frankia* inocula can have large effects on host growth, N content, and N-fixation (Martin et al. 2003; Prat 1989; Hooker and Wheeler 1987; Sellstedt et al. 1986; Dillon and Baker 1982; Dawson and Sun 1981).

Moreover, isolates from a given host species are not necessarily the highest-performing on that host in the laboratory (Sellstedt et al. 1986; Dillon and Baker 1982; Dawson and Sun 1981), and the relative performance of specific *Alnus* spp.–*Frankia* combinations can vary widely based on soil conditions (Kurdali et al. 1990; Sheppard et al. 1988). Thus, availability of optimal symbiont genotypes due to host specificity and/or spatial heterogeneity of *Frankia* may contribute to factors limiting the establishment, performance, and ecological effects of alder in natural habitats. However, current knowledge of the distribution of alder-nodulating *Frankia* among habitats and host species, and associated patterns in host physiology, is limited.

Genetic variation in symbiotic *Frankia* is subject to a wide range of selective effects exerted by both environmental factors and host plants. *Frankia* appear to disperse more readily than their host plants and to maintain a free-living existence in soil (reviewed in Benson and Dawson 2007; Benson and Silvester 1993). Plant-trapping studies indicate that population size of soil-dwelling *Frankia* can covary with a number of environmental factors related to soil conditions (e.g., Batzli et al. 2004; Huguet et al. 2004a, b; Myrold and Huss-Danell 1994; Zitzer and Dawson 1992; Dawson et al. 1989) and non-host vegetative cover (e.g., Maunuksela et al. 1999; Paschke et al. 1994; Zitzer and Dawson 1992; Smolander and Sundman 1987), and that some of these factors appear to differentially affect *Frankia* genotypes (Huguet et al. 2004a, b; Zitzer and Dawson 1992). Given the evolutionary trade-off expected to occur between genetically-determined symbiotic and free-living bacterial lifestyles (Denison and Kiers 2004; West et al. 2002), such environmental selection is unlikely to favor optimal symbionts. However, host plants may also exert considerable influence over symbiont assemblages. Evolutionary models of root-nodule symbioses predict the existence of physiological mechanisms in host plants for selective allocation of resources to optimal symbionts and/or withholding of resources from poor mutualists (West et al. 2002), and both processes have been observed in legume hosts under greenhouse conditions (Simms et al. 2006; Kiers et al. 2003). Since bacteria inhabiting nodules can outnumber their soil-dwelling conspecifics in the same area by several orders of magnitude (West et al. 2002), such host selection has strong potential to feed back to soil-dwelling populations. Some evidence for such positive feedback has been reported for *Frankia* hosts; positive correlations between nodulation potential of soils on host seedlings in the greenhouse and presence of the same host in the field have been observed in *Alnus* (Myrold and Huss-Danell 1994), *Casuarina* (Zimpfer et al. 1999), and *Ceanothus* (Jeong and Myrold 2001), and in the latter two genera the positive effect of host presence differed among *Frankia* genotypes.

Field surveys of *Frankia* variation in alder nodules point to the importance of both environmental factors and host species in the distribution of particular *Frankia* types (e.g., Dai et al. 2005; Weber 1990; van Dijk et al. 1988). The patterns revealed by the studies to date, however, are ambiguous in a number of respects. First, the majority of such studies have not been concerned with genetic variation of symbionts per se, but with geographic patterns in the presence of *Frankia* spores in host nodules. However, in addition to the low resolution of this distinction, which sorts symbionts only into 'spore-positive' (Sp+) and 'spore-negative' (Sp−) categories, the genetic basis of this dichotomy is considered unresolved (Benson and Silvester 1993), so it is unclear whether the observed patterns represent distribution of symbiont genotypes or phenotypic plasticity of symbionts or hosts. Second, in the handful of studies that include more than one *Alnus* species, the contributions of host and habitat to the observed patterns are confounded due to non-overlapping distributions of host species at regional scales (Dai et al. 2005) or among different microhabitats within a region (e.g., Weber 1990; Weber et al. 1987; Weber 1986). Third, the consistency of *Frankia* structure on single host species in specific habitats is unclear, since most studies aim to describe variation across relatively large areas, and do not include pre hoc replicate sites for each habitat examined (e.g., Igual et al. 2006; Dai et al. 2004; Huguet et al. 2004b; Markham and Chanway 1998). Exceptions are provided by Holman and Schwintzer (1987), who report significant differences between replicated 'streamside' and 'disturbed' habitats in the frequency of Sp+ *Frankia* in *A. incana* ssp. *rugosa* nodules in the state of Maine, and Kahn et al. (2007), who found that *Frankia* structure in Himalayan *A. nepalensis* nodules was correlated with elevation in replicate sampling locales. And fourth, it does not appear that any survey of alder symbiont variation has examined correlations between observed *Frankia* variation and host physiology in the field.

The Tanana River floodplain in the boreal forest of interior Alaska provides a 'natural laboratory' in which some of these ambiguities can be addressed. Low regional plant diversity and repetition of ecosystem-controlling factors such as soil parent material, topography, and cycles of flood and fire across the landscape (Van Cleve et al. 1996) create a mosaic of intermixed patches of successional habitats, making for relatively straightforward habitat replication. The sympatric occurrence of two alder species—*Alnus viridis* ssp. *fruticosa* (Ruprecht) Regel (formerly *Alnus crispa*; hereafter, *Alnus viridis*) and *Alnus incana* ssp. *tenuifolia* (Nuttall) Breitung (hereafter, *Alnus tenuifolia*)—in some habitats also allows for examination of host-based differences without the confounding effects of habitat or location. Additionally, the presence of sites belonging to the Long Term Ecological Research (LTER) program allows access

to environmental data continuously collected for each of the sites that are included in the program. The present study sought to characterize genetic variation in symbiotic *Frankia* and host physiology among habitats and host species within a restricted area in the Bonanza Creek LTER. Specifically, we sought to determine whether: (1) genetic structure of *Frankia* is consistently associated with habitat for either host species, (2) genetic structure of *Frankia* is consistently associated with host species between sympatric hosts in one of these habitats, (3) variation among *Frankia* assemblages parallels variation in host physiology among habitats and host species, and (4) different *Frankia* genotypes differ in specific N_2 fixation rate (SNF) in situ in either host species. For objective (3) we chose to measure SNF and leaf N content because both are ecologically important, readily measured in the field, and known to vary among host species and *Frankia* genotypes.

Materials and methods

Study sites

The Bonanza Creek Long Term Ecological Research area (BNZ LTER) is located approximately 30 km south-west of Fairbanks, Alaska (64°48′N, 147°52′W). The dominant feature of the region is the active floodplain of the Tanana River, but upland forests occur adjacent to the north bank. Soil development and successional dynamics differ between floodplain (FP) and upland (UP) forests. On the floodplain, primary succession begins with the formation of alluvial silt bars, which are rapidly colonized by *Salix* spp., balsam poplar (*Populus balsamifera* L.), and *A. tenuifolia*. *A. tenuifolia* grows rapidly and forms a dense closed canopy approximately 5–10 years after initial colonization which persists for 25–30 years. Nitrogen fixed during this alder-dominated stage may account for 60–70% of the N accumulated during 200 years of progressive succession (Van Cleve et al. 1993, 1971). Approximately 50 years after bar formation, balsam poplar overtops the alder canopy. Alder abundance declines as the poplar canopy matures, and the poplar is eventually replaced by white spruce (*Picea glauca* (Moench) Voss) ~125 years after substrate formation. *A. tenuifolia* persists in the understory of these spruce forests and, in some floodplain spruce stands, *A. viridis* also occurs.

In secondary successional ecosystems on south-facing slopes adjacent to the Tanana floodplain *A. viridis* appears shortly after fire, along with Alaska paper birch [*Betula neoalaskana* (Sarg.)], trembling aspen (*Populus tremuloides* Michx) and a few species of *Salix*. Approximately 25–50 years post-fire, an overstory of paper birch and/or aspen develops, which yields to white spruce dominance 100–200 years post-fire. *A. viridis* persists throughout this sequence, and continues to provide significant amounts of fixed N (Mitchell 2006; Van Cleve and Viereck 1981).

Experimental design and sample collection

In June 2002, 12 study sites were selected; 3 replicate sites representing each of 4 habitat types: (1) early succession floodplain with dense (~1,200 stems/ha) *A. tenuifolia* canopy (FPE sites 1, 2 and 3), (2) late succession floodplain with white spruce canopy and moderately dense understory (~400 stems/ha) of *A. tenuifolia* and *A. viridis* (FPL sites 1, 2 and 3), (3) early succession upland with scattered (~200 stems/ha) *A. viridis* individuals (UPE sites 1, 2 and 3), and (4) late succession upland with white spruce canopy and moderately dense (~400 stems/ha) *A. viridis* understory (UPL sites 1, 2 and 3). Where possible, these sites were selected to avoid geographic clustering with respect to habitat (Table 1). This was not possible for UPE sites, which were all located in the same burn area (Table 1). In the sympatric sites (FPL1-3), individuals of the two species were intermixed and generally within 5 m of each other. All but four of these sites—the three UPE sites and FPL3—are established long-term monitoring sites defined and maintained by the BNZ LTER project (http://www.lter.uaf.edu/).

Each time a site was sampled, ten mature plants (*A. tenuifolia* in FPE, and *A. viridis* in UPE and UPL sites) were haphazardly chosen for measurement of SNF and collection of nodule and leaf tissue. At the sympatric (FPL) sites, ten plants of each species were sampled. In general, one site was sampled per field day (between 0900 and 1400 hours Alaska Daylight Time). In order to account for the known effect of seasonality on SNF (Anderson et al. 2004; Uliassi and Ruess 2002) in our statistical model, we used a Latin Square sampling design (Neter et al. 1996) in which each site was sampled at three separate time periods over the growing season—period 1 extended from 19 June to 2 July, period 2 from 22 July to 10 August and period 3 from 19 August to 1 September—for a total of 30 plants of each species at each site. At each sampling period, we selected plants not chosen during the previous sampling period(s) in

Table 1 Mean geographic distances (km ± 1 SD) among study sites in the Bonanza Creek Experimental Forest

Landscape	Stage	Habitat	Floodplain		Upland	
			Early	Late	Early	Late
			FPE	FPL	UPE	UPL
Floodplain	Early	FPE	8.7 ± 6.1	5.7 ± 4.4	7.5 ± 3.7	8.5 ± 2.9
	Late	FPL		3.9 ± 2.1	7.0 ± 0.7	8.5 ± 1.7
Upland	Early	UPE			1.2 ± 0.6	3.2 ± 1.2
	Late	UPL				4.1 ± 2.3

order to avoid any effect of previous sampling disturbance on SNF. At each site the combined area sampled for all three periods was approximately 2,000 m^2.

From each plant, approximately 5–10 g (fresh weight) of nodule tissue was harvested for field measurement of SNF. In the sympatric (FPL) sites, roots were traced to a host stem prior to nodule harvest in order to identify host species. Leaf tissue samples, consisting of five 13-mm-diameter leaf punches, were taken from fully-emerged leaves located at the periphery of the canopy of each plant. Leaf punches were dried at 40°C for at least 48 h, weighed for measurement of specific leaf weight (SLW = g/cm^2 dry leaf tissue), then ball-milled and analyzed via mass spectrometry for N content on a dual-inlet isotope ratio mass spectrometer (PDZ Europa Scientific Instruments, Crewe, Chesire, UK). Soil temperature and moisture data were also collected for each plant in order to include these variables in the statistical model for SNF. One soil core (5 cm diameter × 10 cm depth) per plant was taken within 1 m of each sampled nodule for determination of soil water content. Cores were dried to constant weight at 65°C, and moisture content determined as the difference between fresh and dry weight, expressed as a percentage of fresh weight. Soil temperatures at 1-cm and 5-cm depths were recorded at each plant using a hand-held digital thermometer (Taylor Thermometers, Oak Brook, Illinois, USA).

From each nodule sample (1 per plant) two subsamples consisting of 2–3 nodule lobes were collected in the laboratory. Mass spectrometry was performed on one subsample for measurement of SNF, and the other subsample was used for PCR-RFLP analysis. When possible, both subsamples were taken from the same nodule cluster to minimize the chance that they contained different strains of *Frankia*. For 51 of the 177 plants included in the final SNF-RFLP analysis, small nodule cluster size prevented the use of this strategy. For these, a subsample was chosen from a short section of root, thoroughly mixed, and split in half. SNF was then examined on one half, and RFLP analysis was performed on the other. All samples which appeared to contain more than one RF pattern were excluded from the final analysis, providing an additional barrier against the possibility that SNF and RFLP analyses included different *Frankia* strains.

Difficulties in locating plants at some field sites, and unsuccessful sample processing for some samples, particularly during PCR-RFLP analysis, resulted in unequal amounts of data among the assays performed and the sites sampled (Table 2b).

SNF measurement

To avoid uncertainties related to the commonly-used acetylene reduction assay (Anderson et al. 2004), we measured SNF using a $^{15}N_2$ uptake assay we developed previously. This method is described in detail in Anderson et al. (2004). Briefly, half of each field nodule sample was exposed to an atmosphere of ~15% (atom %) $^{15}N_2$ for 10 min immediately after harvest. $^{15}N_2$ fixation rate was determined via mass spectrometry by comparing ^{15}N enrichment in assayed nodules to natural abundance of ^{15}N in non-assayed nodules, and dividing by elapsed time for the assay. $^{15}N_2$ fixation rate was converted to N_2 fixation rate by adjusting for the initial ^{15}N concentration (15%) in the assay atmosphere, which was determined via mass spectrometry.

PCR-RFLP analysis

Prior to DNA extraction, nodule subsamples were surface sterilized in 10% H_2O_2 for 1 min, rinsed continuously for 5 min with RO water, and stored at −80°C. Nodules were ground on a shaker mill (Retsch, Newtown, PA) in 200 µL of AP1 extraction buffer from the Plant DNeasy 96 kit (Qiagen, Carlsbad, CA) in the plates provided. The remainder of the extraction was performed according to the manufacturer's instructions, with the addition of 20 mg/mL lysozyme followed by a 30 min incubation at 37°C, as recommended for Gram-positive bacteria.

The *nif*D–K intergenic spacer (IGS) locus was selectively amplified via polymerase chain reaction (PCR) (Mullis et al. 1986) using newly designed primers: forward, *nif* D1310fr-CAGATGCACTCCTGGGACTACTC; reverse, *nif* KR331fr –CGGGCGAAGTGGCTGC. Maximum PCR success rate was achieved for each species using different reaction compositions: *A. tenuifolia* samples: 25 µL final volume (18 µL ultrapure H_2O, 1 µL each primer (10 µM) and 5 µL undiluted DNA extract added to dehydrated PCR "beads" (Amersham Biosciences, Piscataway, NJ)); *A. viridis* samples: 25 µL final volume (2.5 µL of AmpliTaq 10X Buffer II (Applied Biosystems, Foster City, CA), 0.25 µL AmpliTaq DNA polymerase (Applied Biosystems), 0.2 mM each dNTP, 2.0 mM $MgCl_2$, 0.4 µM of each primer, and 4.0 µL of 0.4% bovine serum albumin. The possibility of bias introduced by this PCR difference was examined on 19 samples (eight *A. tenuifolia* and 11 *A. viridis*) that yielded successful PCR reactions with both compositions. All of these samples yielded the same restriction fragment patterns for both PCR reaction compositions. For all samples, initial denaturation was carried out at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 45 s, annealing at 58°C for 45 s, and extension at 72°C for 90 s. A final 5 min extension at 72°C was also included. Product yield and length were checked by electrophoresis on 1.5% SeaKem agarose (Cambrex, Rockland, ME) and visualized by ethidium bromide/UV fluorescence.

Restriction fragments were generated via separate digests of PCR product with *Cfo*I and *Hae*III (5 U/rxn) (Promega, Madison, WI). All digests included 10–12 µL of PCR product, and were carried out at 37°C for at least 5 h. Digestion products were electrophoresed at 10 V/cm on 3% (1% SeaKem/2% NuSieve; Cambrex) agarose gels and visualized by ethidium bromide/UV fluorescence. Restriction fragment patterns obtained for each enzyme were compared visually within a gel and scored as unique based on the presence or absence of bands of particular length. Fragments shorter than 80 bp were excluded from the analysis, since this is the shortest length measurable with the standard used (MassRuler^{TM} DNA Ladder, Low Range; Fermentas Life Sciences, Burlington, Ontario). Restriction patterns occurring on more than one gel were verified on a second gel, and all RFLP patterns were checked against PCR product length to ensure additivity of fragments. Each unique combination of restriction patterns across the two enzymes was given a single numerical restriction fragment pattern (RF) designation.

In order to check that our PCR was selective for *Frankia*, and to examine whether sequence variation existed within our RF groups, we performed DNA sequencing on a subset of nodules which included multiple representatives of the most frequent RF groups. Cycle sequencing was performed on cleaned (QiaQuick PCR purification kit; Qiagen) PCR product using 2.0 µl BigDye and 3.0 µl 10X buffer (Applied Biosystems) together with 3.2 µl of 1.0 µM PCR primer and 40–60 ng of DNA per 20 µl reaction, which were subjected to 98°C for 1 min followed by 25 cycles of 98°C for 10 s, 58°C for 5 s, and 60°C for 4 min. Cycle sequencing products were cleaned using Centri-Sep sephadex columns (Princeton Separations, Adelphia, NJ), and capillary sequencing was performed on an ABI3100 analyzer (Applied Biosystems, Foster City, CA). Raw sequences were edited using CodonCode Aligner (CodonCode, Dedham, MA), aligned in BioEdit (Hall 1999) and compared with the NCBI nucleotide database using the BLASTn utility (Altschul et al. 1997).

Data analysis

Because the two host species were unequally distributed among our landscapes and stages, and our desired comparisons were between sampling blocks in which a given host species occurred in a given habitat (floodplain/upland landscapes and early/late succession), we consolidated landscape, stage and host species into a single variable, "HABSPEC" (habitat||species), with five levels: *A. tenuifolia* in early and late succession on the floodplain (FPE AT and FPL AT, respectively), *A. viridis* in late succession on the floodplain (FPL AV) and in early and late succession in the uplands (UPE AV and UPL AV, respectively). Composition of symbiotic *Frankia* assemblages was examined using correspondence analysis (PROC CORRESP; SAS Institute 2001) to estimate the relative contributions of habitat and host species, and to visually assess whether *Frankia* composition was more similar among replicate sites within or among HABSPEC levels.

Continuous variables (SNF, leaf N, soil moisture, and soil temperature) were analyzed using general linear models (PROC GLM; SAS Institute 2001) with post hoc comparisons performed using Tukey's HSD on unadjusted or least-square adjusted means, as appropriate. When necessary, raw data were square-root or log transformed to meet the assumptions of the GLM. For soil variables, sampling period and HABSPEC were included as class variables, with replicate site nested within HABSPEC and the interaction between HABSPEC and sampling period also included. For SNF and leaf N models were built according to a backward elimination protocol (Neter et al. 1996) which initially included HABSPEC, sampling period, and replicate site nested within HABSPEC as class variables, soil moisture, and soil temperature (quadratic) as covariates, and interaction terms for HABSPEC and sampling period, and among all class and continuous variables. At each step of the elimination, the independent variable with the smallest F value was dropped from the model, until $P < 0.05$ for all remaining variables. All F and P values used were based on type III sums of squares due to the presence of covariates, and empty cells for SNF. For SNF, sample losses resulted in low sample sizes for sites within each sampling period and unbalanced distribution of RF groups with respect to sampling period. RF effects on SNF were therefore only examined using data from the peak sampling period of SNF for each site.

We examined environmental variation among the habitats included in this study via principal-components-analysis (PCA) (PROC PRINCOMP; SAS Institute 2001) using unpublished data publicly available on the BNZ-LTER website (http://www.lter.uaf.edu/). These data included measurements of 13 physical and chemical soil characteristics in sites representing the same (FPE, FPL, and UPL), or very similar (UPE), stand types as our sites within the BNZ-LTER. The soil characteristics included in this analysis were total carbon, organic matter content, cation exchange capacity, pH, Kjeldahl N, total P, Mg, K, Ca, bulk density, percent sand, percent silt, and percent clay. Information on the specific methods used for collecting these data is available on the BNZ-LTER website. Site scores for the first two principal components were regressed against site scores from the correspondence analysis of RF pattern distribution using simple linear regression in JMP version (1989) 7.0.

Results

Frankia distribution, host specificity and diversity

RFLP analysis was successful for 216 of the 444 nodule samples collected, and yielded ten RF patterns (Table 2). DNA sequence data was obtained from 18 nodules which included all RF groups except RF2 and RF5, and >1 nodule for RF1, RF4, RF7, RF8, and RF9. In BLASTn searches, the closest match for all sequences was to *Frankia alni* strain ACN14A (accession #CT573213.2); *E* values for this match ranged from 10^{-48} for RF7 to 0.0 for RF8 and RF9. All RF groups for which >1 nodule was sequenced yielded identical sequences. All sequences are available on the NCBI website under accession numbers FJ655072–FJ655080, FJ655082–FJ655090.

Seven RF patterns were found in *A. tenuifolia* nodules on the floodplain, three of which were unique to a particular successional stage: RF5 to early succession; RF4 and RF6 to late succession. RF5 and RF6 were rare, each occurring in only one *A. tenuifolia* nodule in a given site, but RF4 occurred in all three FPL sites, at moderate frequency in two sites and co-dominant in the third. RF7 was dominant in *A. tenuifolia* nodules in all FPE sites, whereas in all three FPL sites RF1 was the most frequent pattern, though it occurred at much lower frequency than RF7 in FPE sites. Within a successional stage, the most common RF pattern was the same in all sites, but the most common in FPE sites, RF7, was present in only two FPL sites, while the most common RF group in FPL sites, RF1, was only found in one FPE site. In all FPE sites the most common RF pattern occurred with at least three times the frequency of the second most frequent pattern, but this level of dominance only occurred in one FPL site.

The majority of *A. viridis* nodules yielded either RF8 or RF9, and these patterns were mostly evenly distributed. Over 90% of all nodules collected from this host yielded one of these two RF patterns and, in six of the nine sites in which this host occurred, the two patterns were co-dominant. The exceptions were two FPL sites, in which RF9 was dominant, and one UPE site, in which RF4 was dominant. However, the latter site was one of two sites—both UPE3 and UPL2—yielding very low sample success, <10 samples. Thus, while the occurrence of RF4 in this site appears unique among upland sites, the proportion of this genotype may be overrepresented in such a small sample, and the occurrence of RF4 in other upland sites may have been missed due to the generally low sample size in upland sites (Table 2).

In the sympatric (FPL) sites, three of the nine RF patterns found in the two host species occurred on both hosts (RF2, RF4, RF6). The remaining six patterns were unique to a particular host species: three to *A. tenuifolia* (RF1, RF3, RF7) and three to *A. viridis* (RF8, RF9, RF10). The shared patterns together accounted for 26% (27 of 103) of the nodules collected in the sympatric sites. RF patterns from these three groups occurred in 22 of 59 (37%) of the *A. tenuifolia* nodules sampled in these sites, but only 5 of 44 (11%) of the sympatric *A. viridis* nodules. In all sites, the most frequent pattern occurring on each host species was unique to the host.

Correspondence analysis yielded three distinct clusters along the first two dimensions (Fig. 1), which account for nearly 75% of the total χ^2 in the analysis. Dimension 1 accounts for 44% of the overall χ^2, and is clearly associated with host species; all points associated with *A. tenuifolia* lie to the right of the origin, while all but one *A. viridis* point

Table 2 a. Number of nodules from each site and host species yielding each PCR-RFLP pattern (*nif*D-K spacer) observed in the Bonanza Creek Experimental Forest, b. Number of nodule samples, out of 30 included in the experimental design, yielding data for PCR-RFLP (*RF*) (*nif*D-K spacer), specific N fixation (*SNF*), and leaf N content for each experimental site in the Bonanza Creek Experimental Forest

Site	Host species	a. Restriction fragment (RF) pattern										b. Totals		
		RF1	RF2	RF3	RF4	RF5	RF6	RF7	RF8	RF9	RF10	RF	SNF	Leaf N
FPE1	*A. tenuifolia*	4	2					12				18	30	30
FPE2						1		17				18	17	30
FPE3				1		1		13				15	27	28
FPL1	*A. viridis*		1		1				6	7	1	16	29	29
	A. tenuifolia	8	4		8			3				23	30	30
FPL2	*A. viridis*				2				2	16		20	30	30
	A. tenuifolia	7	4	1	1							13	20	30
FPL3	*A. viridis*						1		3	14		18	30	30
	A. tenuifolia	13		3	4		1	2				23	19	30
UPE1	*A. viridis*								6	6		12	30	30
UPE2									4	6		10	26	27
UPE3					3				1			4	30	30
UPL1									7	4		11	30	30
UPL2									2	3		5	20	25
UPL3									5	5		10	30	30

Site designations include landscape (*FP* floodplain, *UP* upland), successional stage (*E* early, *L* late), and replicate site number 1–3

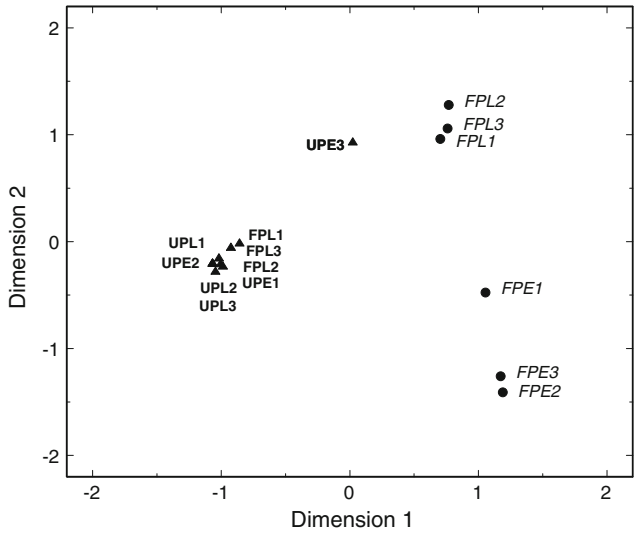

Fig. 1 Graph of the first 2 dimensions of correspondence analysis performed on RFLP pattern (*nif*D–K spacer) abundance data from *Alnus tenuifolia* (*circle symbols*) and *A. viridis* (*triangle symbols*) nodules collected from three replicate sites (1–3) representing early (*E*) and late (*L*) successional habitats in floodplain (*FP*) and upland (*UP*) landscapes in the BNZ-LTER. Dimension 1 accounts for 44.3% and dimension 2 for 30.4% of the total χ^2 for the analysis

lie to the left. The exception is site UPE3, which was dominated by RF4, a pattern more common in *A. tenuifolia* nodules. Dimension 2 accounts for another 30% of the overall χ^2, and is primarily associated with floodplain successional stage: all points associated with *A. tenuifolia* in FPE sites fall below the origin and all those in FPL sites fall above, with UP sites in the center. With the exception of UPE3, very little variation is evident among *A. viridis* points, which form a single small cluster.

Continuous variables

Analysis of variance indicated significant effects of sampling period and replicate site nested within HABSPEC on SNF, leaf N, soil moisture, and soil temperature. Additionally there was a small but significant interaction between HABSPEC and sampling period for all dependent variables. A significant main effect of HABSPEC was observed for all variables except SNF (Tables 3, 4), which was highly variable (coefficient of variation = 122.8) and mostly correlated with sampling period (Table 4).

Evidence for differences in SNF among RF groups was equivocal, and was hampered by large variation in SNF and small sample sizes for RF groups within peak sampling periods. At peak sampling period, only replicate site within HABSPEC was significant in the GLM, both with all data included ($n = 143$, $r^2 = 0.38$, $F = 5.5$, $P < 0.0001$) and with only samples for which ≥5 RF data points were available ($n = 53$, $df = 13$ $r^2 = 0.45$, $F = 2.5$, $P = 0.015$). When RF was included in the latter model it was the only other class variable retained ($df = 16$, $F = 3.1$, $P = 0.037$) by backward elimination and explained a further 12% of the variance in SNF ($df = 3$, $r^2 = 0.57$, $F = 2.9$, $P = 0.004$). *T* tests on unadjusted means indicated significant differences between RF7 and RFs 4, 8 and 9, and between RF1 and RF8, but when Tukey's HSD was used only RF7 and RF8 were significantly different.

LTER soil data

The clustering pattern yielded by the PCA of the LTER soil data (Fig. 2) was very similar to the pattern yielded by correspondence analysis of RF data (Fig. 1), with three clusters separated by the first two principal components (PC). FPE and FPL sites occupy opposite ends of PC1 and, with the exception of site FPL3, upland sites form a smaller cluster separate from floodplain sites. PC1 accounts for over half the variance in the PCA, and correlates strongly with exchangeable cations (loading value (LV) = 0.37), N (LV = 0.36), Mg (LV = 0.35), C (LV = 0.34) and organic matter content (LV = 0.35), and negatively with pH (LV = −0.34) and bulk density (LV = −0.35). PC2, which separates floodplain from upland sites, accounts for nearly 22% of the variance in the data and is correlated with sand (LV = 0.51) and P content (LV = 0.35), and negatively with silt (LV = −0.47) and clay

Table 3 Mean values of continuous variables (mean ± SEM) measured in the present study for plant physiological and environmental factors in the Bonanza Creek Experimental Forest

Habitat/host species			Physiological variables			Environmental variables		
Landscape	Stage	Species	SNF (μmol N_2 g noddwt^{-1} × h^{-1})	Leaf N (% by mass)	SLW (g dwt leaf tissue/cm^2)	Soil moisture (% by mass)	Soil temperature (ºC)	Canopy position
Floodplain	Early	*A. tenuifolia*	28.3 ± 5.5^{a}	2.64 ± 0.05^{a}	6.21 ± 0.19^{a}	$28.7 \pm 0.5^{b,c}$	9.5 ± 0.3^{a}	C
	Late		22.4 ± 3.2^{a}	2.46 ± 0.03^{b}	5.06 ± 0.12^{b}	33.4 ± 1.2^{a}	7.5 ± 0.2^{b}	U
		A. viridis	30.3 ± 3.3^{a}	2.26 ± 0.03^{c}	4.79 ± 0.11^{b}	$32.0 \pm 1.2^{a,b}$	$8.2 \pm 0.2^{b,d}$	U
Upland	Early		33.9 ± 4.5^{a}	2.50 ± 0.04^{b}	7.17 ± 0.80^{c}	$25.8 \pm 0.7^{d,e}$	$9.3 \pm 0.2^{a,c}$	C
	Late		34.0 ± 3.9^{a}	2.49 ± 0.03^{b}	4.24 ± 0.09^{d}	$26.5 \pm 0.6^{c,e}$	$8.8 \pm 0.2^{c,d}$	U

Common superscripts indicate homogeneous subsets (Tukey HSD, $P > 0.05$) (PROC GLM; SAS Institute 2001). C = alder canopy; U = alder in understory

Table 4 Analysis of variance (PROC GLM; SAS Institute 2001) table for selected continuous variables measured in the experiment

Independent variables	Dependent variables															
	SNF (μmol N_2 g nod $dwt^{-1} \times h^{-1}$)				Leaf N by mass (%)				Soil moisture (% by mass)				Soil temperature (ºC, 5 cm depth)			
	r^2	*P*	*F*	*df*	r^2	*P*	*F*	*df*	r^2	*P*	*F*	*df*	r^2	*P*	*F*	*df*
Whole model	0.65	***	25.6	27	0.47	***	15.0	24	0.41	***	12.3	24	0.60	***	26.5	24
Sampling period		***	63.1	2		***	48.9	2		***	46.1	2		***	70.3	2
HABSPEC		NS	–	–		***	21.6	4		***	16.4	4		***	20.8	4
Replicate site (HABSPEC)		***	4.9	10		***	14.8	10		***	11.4	10		***	37.7	10
HABSPEC × Sampling period		***	7.6	8		**	3.7	8		*	2.6	8		***	4.3	8
Soil temperature (quadratic)		*	9.5	1		–				–				–		

Independent variables were retained by backward elimination starting with a model which contained all class and continuous variables and interactions

NS Not significant

*** $P < 0.0001$, ** $P < 0.001$, * $P < 0.01$

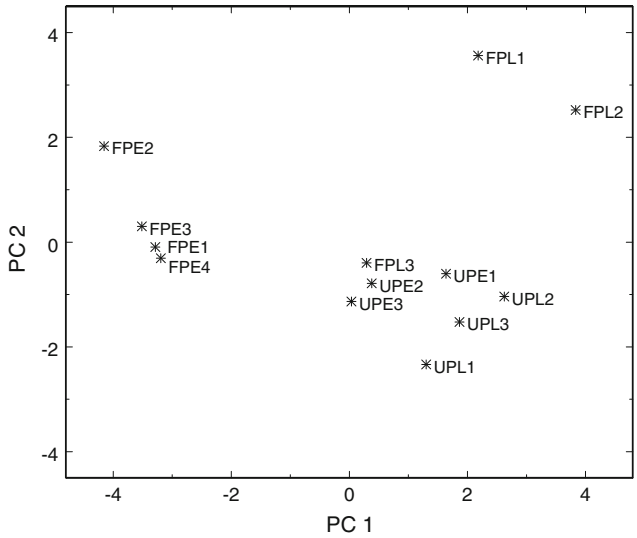

Fig. 2 Graph of the first 2 principal components of a principal components analysis performed on public domain data (http://www.lter.uaf.edu/) collected for 13 edaphic characteristics in the same habitat types sampled in the present study in the Bonanza Creek Experimental Forest. *FP* and *UP* indicate floodplain and upland sites, respectively and *E* and *L* indicate early and late succession habitats. *Numbers* refer to replicate sites for each habitat. Principal component 1 accounts for 54.5%, and principal component 2 for 21.7% of the total variance in the analysis

content (LV = −0.39). Site scores for PC1 were significantly correlated with dimension 1 ($P = 0.021$) in the correspondence analysis, which separates *Frankia* assemblages by both host species and landscape (FP vs UP), and with dimension 2 ($P = 0.011$) which separates assemblages by floodplain habitat. Both PC1 and dimension 2 yield similar site ordinations, with FPE and FPL sites at opposite ends and UP sites between them. PC2 scores did not correlate with either dimension in the correspondence analysis.

Discussion

Frankia genetic structure

In the present study, we used a surface-sterilization procedure in order to minimize the chance of contamination in our molecular analysis. Such sterilization has been suggested to be ineffective in removing *Frankia* and non-*Frankia* organisms (Valdés et al. 2005) from the nodule surface, and peeling of the nodule periderm is favored by a number of workers (e.g., Rouvier et al. 1996). While we cannot definitively discount the possibility of surface contamination in our study, the low occurrence of multiple RF patterns in single nodules (2 out of 218) suggests that most nodules were occupied by a single genotype, and our BLAST results closely match alder-infective *Frankia*. We therefore think that the possibility of contamination in our study is minimal.

This study confirms the results of previous field studies reporting differences in genetic structure of symbiotic *Frankia* assemblages associated with differences in alder host species and habitat conditions. Our study complements previous studies by examining sympatric host species and replicated examples of habitat conditions. Our most significant findings in this respect are: (1) intermixed sympatric hosts differed in *Frankia* structure to essentially the same degree as different host species in different habitats (Fig. 1; Table 2), demonstrating significant influence of host species over symbiotic *Frankia* structure independent of habitat, (2) the two host species differed in the degree to which habitat appeared to influence *Frankia* structure, with relatively large differences evident in *A. tenuifolia* compared to *A. viridis* nodules from different habitats, and (3) *Frankia* structure was largely consistent among replicate sites representing host species and habitat sampling blocks, suggesting

consistent relationships between both factors and *Frankia* structure among our study sites.

Differences in *Frankia* structure between sympatric hosts were the largest of any of the comparisons we examined, and were consistent among replicate sites. These differences were largely due to apparent reciprocal specificity between hosts and symbionts; i.e., the most common RF pattern found on each sympatric host species was unique to the host. Since the two species were largely intermixed within these sites, this difference cannot be wholly attributed to differences in microhabitat. While we cannot discount the possible interactive effect of habitat—that one or both hosts may associate with the dominant RF group from the other host under different environmental conditions (Simonet et al. 1999)—the fact that this was not observed in any of the other habitats in this study, nor in a wider survey of *A. viridis* in Alaskan tundra habitats of the Brooks Range and Seward Peninsula (Taylor, MacFarland and Ruess, unpublished data), casts doubt on this possibility. It is nevertheless possible that the specificity we observed in the field does not reflect potential associations that may occur under cross-inoculation conditions, which can result in broader associations between host species and *Frankia* than those observed in the field (Huguet et al. 2005; Simonet et al. 1999), and which typically indicate relative promiscuity of both *Alnus* and *Frankia* (Prat 1989; Du and Baker 1992; Sheppard et al. 1988; Dillon and Baker 1982; Dawson and Sun 1981). Alternatively, this apparent reciprocal specificity may represent genetic differences between alder species and/or among *Frankia* genotypes in the range of symbiotic partners with which they are compatible. Large such differences between the host species in this study would be congruent with the large phylogenetic distance between them (Chen and Li 2004; Navarro et al. 2003). Interspecific host-symbiont specificity in *Alnus* is apparent in some cross-inoculation studies (Du and Baker 1992; Weber 1990; van Dijk et al. 1988; Weber et al. 1987), and genetic variation even within a host species may affect the level of infection by specific symbiont genotypes (van Dijk and Sluimer 1994). Comparison of the potential and field specificities between our host species and *Frankia* groups will require a controlled inoculation experiment.

Frankia structure and soil conditions exhibited similar patterns of variation among habitats in this study. Large differences in both occurred between floodplain successional stages for *A. tenuifolia* and, with the exception of site UPE3, the largest differences in both for *A. viridis* occurred between upland and floodplain habitats (Figs. 1, 2; Tables 2, 3). The consistency of *Frankia* structure among replicate sites for most habitats suggests a close relationship between symbiont structure in planta and habitat conditions. Soil moisture and temperature patterns among habitats are similar to patterns in *Frankia* structure, and the correlation between dimension 2 in the correspondence analysis and PC1 in the PCA suggests that soil organic matter, N, exchangeable cations and pH are also important correlates. Many of these factors have been suggested to affect the size of infective *Frankia* populations in soil (e.g., Huguet et al. 2004a; Martin et al. 2003; Zitzer and Dawson 1992; Smolander 1990), and some appear to differentially affect *Frankia* genotypes (Huguet et al. 2004a, b; Zitzer and Dawson 1992). However, the mechanisms underlying such correlations are likely to be complex, involving covariation (e.g., soil N and organic matter), or synergistic interactions among soil variables (e.g., pH and nutrient content), as well as direct effects on soil bacteria and/or indirect effects acting through either host growth and nodulation (Uliassi and Ruess 2002; Wall 2000; Crannell et al. 1994), or non-host plant cover (Maunuksela et al. 1999; Paschke et al. 1994; Zitzer and Dawson 1992; Smolander and Sundman 1987). Nevertheless, such mechanisms are probably limited in their action to one or more points along the sequence of events which must occur for *Frankia* to colonize a host in a new site, a process which requires essentially three steps: (1) dispersal to the site and, within the site, to the rhizosphere of a potential host plant and/or, (2) maintenance of viability in the soil until the opportunity for a host interaction occurs, and (3) securing and maintaining a host interaction. In the following discussion, we envision these steps as a series of 'filters' acting on an initially random *Frankia* assemblage, and present hypotheses for our results based on this scenario.

The largest difference in *Frankia* structure within a single host species in this study occurred between *A. tenuifolia* habitats, which also differed in environmental conditions to the largest degree of all habitats in the study. Given that these sites are not geographically clustered (Table 1), and the fact that the Tanana River is known to transport *Alnus*-infective *Frankia* propagules (Huss-Danell et al. 1997), the contribution of dispersal to this difference does not seem likely to be important, although the greater age of FPL ($\geq$150 years) versus FPE ($\sim$25 years) soils may contribute to the larger number of RF patterns found in the former habitat (Burleigh and Dawson 1994b; Huston 1994).

The large differences in soil characteristics between *A. tenuifolia* habitats in this study, and the reported effects of such factors on soil-dwelling *Frankia*, suggest a significant contribution of differential soil viability among bacterial genotypes to differences in symbiotic *Frankia* structure between these habitats. Higher richness in FPL sites may result from these sites supporting larger numbers of infective soil *Frankia* due to their lower pH (Zitzer and Dawson 1992) and salt content (Van Cleve et al. 1993; Young et al. 1992), higher organic matter (Burleigh and Dawson 1994b) and exchangeable cation content (Smolander 1990), and greater moisture and aeration (Dawson et al. 1989) than

FPE sites. Soil pH has been suggested as a particularly important factor affecting soil-dwelling *Frankia*. While opposite correlations to the one suggested here between pH and number of *Alnus*-infective *Frankia* units in soil have been reported (Martin et al. 2003; Smolander 1990; Smolander and Sundman 1987), soils in these studies were more acidic than in the present study, in which site means ranged from 5.0 to 7.4. In this range, Zitzer and Dawson (1992) observed negative correlation with number of *Alnus*-infective *Frankia* units in soil. Interestingly, these authors also report a positive correlation with *Frankia* infective on *Elaeagnus angustifolia* across the same pH range, indicating the effects of pH may be strain-specific. Soil temperature and moisture have also been observed to differentially affect host-infection groups of *Frankia* (Sayed et al. 1997), and structure of *Frankia* within a host infection group has been observed in relation to soil depth (Nalin et al. 1997). Considering the difference in soil temperature and moisture between our *A. tenuifolia* habitats, and the greater vertical soil development and more complex vertical structure of FPL than FPE soils, these factors may also have contributed to the differences we observed in symbiotic *Frankia* structure.

In addition to direct effects on soil *Frankia*, it is also possible that differences in environmental conditions affect host genetic structure and/or physiology, and that compatibility with/selection of specific symbionts by the host in response to genetic constraints or physiological demands contributes to genetic structure of symbionts in the nodules. While we cannot discount the possibility of genetic structure in alder hosts among our habitats, such structure seems unlikely given the restricted geographic range of this study and fact that alder is pollinated and dispersed by wind. Host physiology may differ greatly between habitats, however. This is suggested by the parallel differences observed in leaf N and canopy position in *A. tenuifolia* between FPE and FPL sites, which probably allows greater peak photosynthetic rates in canopy versus understory leaves (Dawson and Gordon 1979). If alder is able to select genotypes based on optimal physiological benefit in a given environment, such host choice would be a strong contributing 'filter' on the genetic structure of the symbionts. Both positive and negative selection of symbionts have been observed in legume–rhizobia interactions (Simms et al. 2006; Kiers et al. 2003) and *A. glutinosa* appears to exert negative selection on ineffective (non-N-fixing) *Frankia* genotypes (Wolters et al. 1997; van Dijk and Sluimer 1994). Positive symbiont selection by *Alnus* does not appear to have been investigated. The basis of such choice—e.g., differences in rate or cost of N-fixation among symbiont genotypes—may be difficult to detect in the field. The present study was hampered in this regard by high variability of SNF, low sample size within a site, and lack of prior knowledge of *Frankia* distribution patterns, but we hope that the information we collected will help to design more powerful field inquiries into host-relevant differences among *Frankia* genotypes in the region.

Both *Frankia* structure and soil conditions exhibited less variation among habitats for *A.viridis* than for *A. tenuifolia* (Table 2; Fig. 2). Among-habitat comparisons for *A. viridis* are hampered by low sample size in upland sites. However, reasonable, comparably-sized samples were collected for each landscape (UP and FP) and appear to indicate landscape-level differences in *Frankia* richness. This difference may be related to host plant distribution; RF groups not detected in UP sites were generally more frequently associated with *A. tenuifolia* than *A. viridis*, and may depend on the former host to maintain appreciable soil populations. Alternatively, this difference may be due to restricted survival of genotypes in uplands related to topography or lower soil moisture (Dawson et al. 1989), or to restricted dispersal of genotypes between landscapes. Both wind and birds have been suggested as *Frankia* vectors and both appear able to differentially affect *Frankia* strains (Burleigh and Dawson 1994a, b; Paschke and Dawson 1993; Burleigh and Torrey 1990), so it is possible that the lower diversity of upland sites is due in part to such dispersal limitations on some genotypes. Given the generally low variation in *Frankia* structure observed in *A. viridis* nodules and the low frequency with which RF2, RF4 and RF6 occur in FPL sites, host selection seems unlikely to be an important contributor to symbiont structure for this host.

This study was motivated by the possibility that limitations in availability of optimal *Frankia* genotypes due to host specificity and/or heterogeneous distribution of bacteria among habitats, together with differences among bacterial genotypes in host performance, may represent a limitation on performance and ecological effects of alder in boreal habitats of interior Alaska. We found evidence for strong and consistent field specificity between alder species and *Frankia* genotypes and, in *A. tenuifolia*, for differences in distribution of symbiont genotypes among habitats. In the latter host leaf N covaried with *Frankia* structure, but we found little evidence for variation in SNF among symbiont genotypes for either host. Further studies are needed to determine whether the distribution patterns we observed in symbiotic *Frankia* are primarily due to differences in corresponding soil populations or to non-random associations with hosts, and whether the *Frankia* genotypes observed in this study vary in physiological benefit to their hosts.

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