Diversity of *Frankia* Populations in Root Nodules of Geographically Isolated Arizona Alder Trees in Central Arizona (United States)\(^\dagger\)\(^\ddagger\)\(^\star\)

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The diversity of uncultured *Frankia* populations in root nodules of *Alnus oblongifolia* trees geographically isolated on mountaintops of central Arizona was analyzed by comparative sequence analyses of *nifH* gene fragments. Sequences were retrieved from *Frankia* populations in nodules of four trees from each of three mountaintops (\(n = 162\)) and their levels of diversity compared using spatial genetic clustering methods and single-nucleotide or 1, 3, or 5% sequence divergence thresholds. With the single-nucleotide threshold level, 45 different sequences with significant differences between the mountaintops were retrieved, with the southern site partitioning in a separate population from the two other sites. Some of these sequences were identical in nodules from different mountaintops and to those of strains isolated from around the world. A high level of diversity that resulted in the assignment of 14 clusters of sequences was also found on the 1% divergence level. Single-nucleotide and 1% divergence levels thus demonstrate microdiversity of frankiae in root nodules of *A. oblongifolia* trees and suggest a partitioning of diversity by site. At the 3 and 5% divergence levels, however, diversity was reduced to three clusters or one cluster, respectively, with no differentiation by mountaintop. Only at the 5% threshold level do all *Frankia* strains previously assigned to one genomic group cluster together.

*Frankia* spp. are nitrogen-fixing actinomycetes that form root nodules in symbiosis with more than 200 species of non-leguminous woody plants in 24 genera of angiosperms (5, 24, 43). These actinorhizal plants have an almost worldwide distribution and can live in soils with low nitrogen availability and thus exploit habitats not favorable for growth of many other plant species (12). *Alnus oblongifolia* Torr. (Arizona alder) is an actinorhizal plant that can be found in mountainous regions in northern Mexico and the southwestern United States. Within the southwestern United States, isolated populations of Arizona alder trees are frequently found along streams draining the southern edge of the Colorado Plateau and the scattered mountain ranges found throughout central Arizona. The alder sites are in mountains that are surrounded by deserts, grasslands, brush or woodland types, and forests and as such are home to many endemic species that have developed as a consequence of geographic isolation (47).

*Alnus oblongifolia* grows in unique moist environments in this desert region, specifically along perennial streams of canyons, primarily at elevations between 1,400 and 2,300 m, and has been shown to form effective root nodules in nature (13). Because mountainous sites inhabited by *A. oblongifolia* are geographically isolated, analyses of *Frankia* populations in nodules of *A. oblongifolia* trees growing on different mountaintops may provide an opportunity to get new insights into the diversity and biogeography of these *Frankia* populations.

Specific factors that drive *Frankia* diversification are presently unclear, even though there are preferences among *Frankia* strains for specific host plants, separating strains into host infection groups and subgroups (15, 22, 28). *Frankia* strains infecting *Casuarina* plants have been shown to have coevolved with their host plant, illustrating the importance of the host plant in shaping the diversity and evolution of these strains (44). However, for most *Frankia* strains, no simple pattern of coevolution is present (3). While phylogenetic analyses reveal three clades for each of the partners in this symbiosis, *Frankia* populations within one clade may form root nodules with plants in more than one clade (4). This lack of correlating phylogenies is likely due to *Frankia* populations occupying two distinct ecological niches, root nodules and soil, where symbiotic or saprophytic growth conditions may drive diversification of *Frankia* populations differently (3). Thus, the complex divergence patterns in *Frankia* phylogeny may best be explained in a geographic mosaic theory of coevolution in which multiple confounding factors, like geographic isolation, plant host preferences, and environmental factors, converge to shape the evolutionary patterns of *Frankia* (3, 46).

One aspect of the geographic mosaic theory of coevolution is allopatric speciation, the divergent evolution of geographically isolated populations, which may be a potential driver of *Frankia* diversification (38, 50). Comparative analysis of *Frankia* populations on isolated mountainous habitats may be a unique opportunity to test if geographic divergence is indeed driving *Frankia* evolution. The isolation of *Frankia* populations in root nodules of trees growing on different mountaintops may permit differentiation, as the effects of neutral drift, pop-

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lead to allopatry (38), as indicated for other bacteria (37, 51). However, Frankia populations are capable of forming spores that allow them to survive transport from one hospitable habitat to another (26). Additionally, Frankia strains, particularly those of the Alnus host infection group, seem to have a cosmopolitan distribution (4) because strains from the same species or genomic group have been isolated from all over the world (see references 1 and 19) and have been found in soils with no extant actinorhizal plants (9, 25, 30, 39).

The aim of this study was to determine if uncultured Frankia populations in root nodules of A. oblongifolia trees isolated on mountaintops within different geologic regions of Arizona showed signs of endemism in a functional gene, nifH, and whether that unique diversity could be correlated with differences in Frankia populations from root nodules among mountaintops. Nodules were collected in June of 2008 from four trees at each of three mountaintop sampling sites, each separated from the nearest by 150 km, proceeding from north to south, within 1° longitude of each other along a 300-km latitudinal gradient in southern Arizona (Fig. 1). Site 1 (Oak Creek in the Coconino National Forest, 35°00.6′N, 111°44.3′W) was a sandy alluvial soil located near Oak Creek at an elevation of 1,703 m, site 2 (Workman Creek watershed in the Sierra Ancha Experimental Forest of Tonto National Forest, 33°49.1′N, 110°55.8′W) a streamside soil high in organic matter at an elevation of 2,073 m, and site 3 (Sabino Canyon in Coronado National Forest, 32°26.1′N, 110°45.5′W) a sandy loam soil adjacent to Sabino Creek at an elevation of 2,310 m. Each site was in a different geologic province: the Colorado Plateau Province was in the north, the Central Highlands Province in a transition zone, and the Basin and Range Province within the Madrean Archipelago in the south (8, 32). Nodules were stored in cold 95% ethanol until analyzed.

DNA was extracted from individual lobes of different root nodules, and a 606-bp fragment of nifH, the structural gene for nitrogenase reductase, was amplified using Frankia-specific primers nifHf1 and nifHr (34, 49) and sequenced as described previously (49). Initially, nifH gene sequences were obtained from 24 nodules from one tree from each mountaintop to determine the level of sampling required to capture the diversity present. A rarefaction curve was generated using DOTUR (41) with a threshold level of divergence set to 3%, which was found to group Frankia strains into appropriate genomic groups in a previous study (34). Based on rarefaction analyses, 10 nodules were sampled from the remaining three trees from each mountaintop, for a total of 54 nodules from each mountain. Sequences of amplified nifH gene fragments of uncultured Frankia populations from 54 A. oblongifolia nodules from each of three mountaintops (GenBank accession numbers FJ977167 to FJ977328) were aligned with those of the three pure cultures of Frankia populations (GenBank accession numbers FJ977329 to FJ977331) and sequences of 46 other strains or uncultured populations analyzed in previous studies (34, 49) or retrieved from public databases and analyzed using maximum likelihood, maximum parsimony, neighbor-joining, and Bayesian analyses as described previously (49).

Phylogenetic analyses of this data set of 211 sequences produced similar topologies independent of the methodology used (data not shown) and assigned all sequences in nodules of A. oblongifolia to frankiae of the Alnus host infection group (see Fig. S1 in the supplemental material). The analysis retrieved 45 different sequences in these nodules from A. oblongifolia, differing from each other by at least one nucleotide, with most of the changes being synonymous. For presentation purposes, to show the relationship of frankiae from root nodules of A. oblongifolia to available pure cultures, the complete data set was reduced to 51 representative sequences, including 21 sequences from frankiae in root nodules of A. oblongifolia, and was reanalyzed using the above-mentioned parameters (Fig. 2). Several of the sequences obtained from root nodules were identical or nearly identical (i.e., single-nucleotide differences) to those of strains or uncultured Frankia populations from other parts of the world (Fig. 2). For example, sequence AO3-14nodF was identical to nifH gene fragment sequences from
four *Frankia* strains isolated from around the world (CpI1 in Massachusetts [7], ArI3 in Oregon [6], AvsI4 in Washington [2], and Ai14a in Finland [48]). Identical sequence does not mean that these are identical strains but does suggest that certain genotypes may have a ubiquitous distribution (34).

Sequences retrieved from *Frankia* populations in root nodules of *A. oblongifolia* were organized into populations by the tree that they were isolated from and by mountaintop (18) for analysis of molecular variance (AMOVA) using Arlequin version 3.01 (17). The AMOVA settings included 16,000 permutations and a more conservative proportion of differences for matrix criteria. AMOVA includes both differences in sequences at the single-nucleotide level and differences in abundance of sequences present (40) and indicated significant differences in sequence diversity within trees ($P < 0.001$), with most of the variation in diversity (83.6%) found within populations of *Frankia* from each *A. oblongifolia* tree. Differences in sequence diversity among trees on each mountaintop, however, were not significant ($P = 0.165$). Significant differences in the levels of diversity of *Frankia* populations among mountaintops were also recovered, accounting for 14.6% of the variation in diversity, suggesting differences by site in the diversity of *Frankia* populations recovered at the single-nucleotide level of differences.

To explore this geographic component in more detail, spatial genetic clustering methods were used in GENELAND version 3.1.4, which utilizes a Bayesian algorithm to make population assignments and weights by using geographic coordinates (20). The analyses of 54 variant characters among the 162 root nodule sequences proceeded in two stages, similar to what was observed by Coulton et al. in 2006, except that the number of populations in the data set initially fluctuated between 1 and 24 and 1 million generations were run with a burn-in of 100,000 (10). Spatial and nonspatial settings were used, and uncertainty in coordinates was tested at 3 m and 10 m; however, all analyses yielded two populations in the data.
Frankia root nodule set and assigned individuals in the same way. Sequences of 108 Frankia clusters from uncultured DOTUR/SONS analysis was completed on the entire data set of 49) into the same cluster on the basis of comparative sequence species or genomic group (34) and uncultured root nodule thresholds previously used to assign strains were unambiguously assigned to the other population, corresponding to site 3. Regions encompassing both site 1 and site 2 have some geographic connectivity by forest along the Mongollon Rim and have been in the Central Highlands Floristic Subdivision and thus might have been expected to be more similar (8, 31). However, site 3 is isolated from the other two sites on account of being surrounded by desert on four sides and was placed in the Southeastern Arizona Floristic Subdivision, supporting the comparative uniqueness of site 3 revealed by our spatial clustering analysis (8, 31).

Three additional threshold levels, i.e., the 1, 3, and 5% divergence levels, were subsequently used to compare the levels of diversity of nodule populations among mountaintops. While the 1% level, corresponding to ~5-nucleotide differences, was arbitrarily chosen, the 3 and 5% levels represented thresholds previously used to assign Frankia strains of the same species or genomic group (34) and uncultured root nodule frankiae of the Alnus or Elaeagnus host infection groups (34, 49) into the same cluster on the basis of comparative sequence analyses of nifH gene fragments. To formulate the assignment of clusters at these three levels of differentiation, the complete data set was reduced by removing all sequences but those representing frankiae in nodules of A. oblongifolia. This data set of 162 nifH gene fragments was executed using PAUP* 4.05b, where an uncorrected distance matrix was created and analyzed using DOTUR to assign taxa to clusters at various thresholds of diversity and then in SONS to compare memberships in these clusters by mountaintop (42). A similar DOTUR/SONS analysis was completed on the entire data set of 211 sequences to describe the groupings of uncultured nodule populations with pure cultures representing various genomic groups. Pie charts displaying the clusters of frankiae in root nodules of A. oblongifolia at 1%, 3%, and 5% differences were generated (Fig. 3).

At the 1% threshold level, analyses using the SONS program demonstrated the presence of 14 clusters of sequences (Fig. 3). Half of these clusters were represented by three or fewer sequences. Seven sequence clusters were found only in nodules of trees from one mountaintop, five were present in nodules from trees of two mountaintops, and two were detected on all three mountaintops (identified as A and B in Fig. 3). Cluster A was dominant overall (n = 73; 44% of all nodules recovered) and was found on all three mountaintops at various frequencies (Fig. 3). Cluster B was also found on all three mountaintops (n = 23; 14% of all nodules recovered) but was dominant on one site (site 3, Sabino Canyon) and barely detected on the other mountaintops, supporting the geographic uniqueness of this site (Fig. 3). The number of clusters decreased to three when a 3% divergence threshold was used. All three clusters were present on all three mountaintops, but in various frequencies (Fig. 3). At this threshold, however, pure cultures belonging to the same genomic group still did not cluster in the same group. Only when the threshold was set to 5% did all the pure cultures from Frankia genomic group 1 (see reference 21 for a summary) cluster together. At a threshold of 5%, all Frankia populations in nodules of A. oblongifolia were placed in one group, suggesting no differentiation by mountaintop and a limited overall level of diversity, with one cluster present, compared to the potential presence of at least six clusters of frankiae within the Alnus host infection group described in previous studies (34, 49). Low overall diversity has also been described to occur in other studies of natural Frankia diversity in root nodules of various alder species (11, 23, 27, 29).

Differences in nitrogenase activity and nodulation capacity have been reported for Frankia strains of the same genomic group on the same alder species in the same soils (14, 15). These differences in effectiveness and infectivity of strains in the same species group have been suggested to be evidence of the effects of plant host shaping symbiotic Frankia diversity under different environmental conditions (3). Thus, the variations in diversity and abundance seen at the single-nucleotide or 1% diversity level may reflect preferences by A. oblongifolia for one strain over another in the different environmental conditions on each mountaintop and microscale differences among trees on the same mountaintop. Genetic differences among Arizona alder populations are unknown, as is the extent to which seed and pollen dispersals occur among these isolated populations. Nonetheless, there were no morphological differences among trees in populations sampled, and the trees were all the same species. In contrast, Frankia diversity in root nodules has been shown to be affected by different edaphic conditions, like soil type and pH (35, 45), or environmental effects, like elevation (27, 29), which are different on each mountaintop. However, these variations in diversity may also reflect random chance and small sample size (16), because rarefaction analysis at the 1% diversity level did not indicate saturation of sampling for any mountaintop (data not shown).

Determination of reasons for selective nodulation by specific strains of Frankia becomes highly speculative. Some evidence suggests that active Frankia populations in the soil may be preferentially selected by the host for nodulation (33, 36). Previous research in our laboratory has confirmed the importance of the plant host in selecting Frankia strains for symbiosis when the same soil was inoculated into six different actinorhizal plant species and resulted in six different diversity profiles (34). Additionally, we have shown that the same actinorhizal plant species inoculated with soils from five different conti-
ents resulted in five different diversity profiles, demonstrating the effects of soil type and history on root nodule Frankia diversity (49).

Frankia microdiversity in root nodules of A. oblongifolia recovered in this study shows a clear geographic pattern, but the reasons for these patterns are unclear. The limited Frankia diversity in A. oblongifolia root nodules is likely due to a combination of factors, including saprophytic growth capabilities, host plant preferences, and edaphic conditions acting at the microecosystem level on Frankia populations.

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