

Low genetic diversity among *Frankia* spp. strains nodulating sympatric populations of actinorhizal species of Rosaceae, *Ceanothus* (Rhamnaceae) and *Datisca glomerata* (Datisceae) west of the Sierra Nevada (California)

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Abstract: *Frankia* spp. strains typically induce N₂-fixing root nodules on actinorhizal plants. The majority of host plant taxa associated with the uncultured Group 1 *Frankia* strains, i.e., *Ceanothus* of the Rhamnaceae, *Datisca glomerata* (Datisceae), and all actinorhizal members of the Rosaceae except *Dryas*, are found in California. A study was conducted to determine the distribution of *Frankia* strains among root nodules collected from both sympatric and solitary stands of hosts. Three DNA regions were examined, the 5' end of the 16S rRNA gene, the internal transcribed spacer region between the 16S and 23S rRNA genes, and a portion of the glutamine synthetase gene (*glnA*). The results suggest that a narrow range of Group 1 *Frankia* spp. strains dominate in root nodules collected over a large area of California west of the Sierra Nevada crest with no apparent host-specificity. Comparisons with Group 2 *Frankia* strain diversity from *Alnus* and *Myrica* within the study range suggest that the observed low diversity is peculiar to Group 1 *Frankia* strains only. Factors that may account for the observed lack of genetic variability and host specificity include strain dominance over a large geographical area, current environmental selection, and (or) a past evolutionary bottleneck.

Key words: actinorhizal Rosaceae, *Ceanothus*, *Frankia*, *Datisca*, strain diversity.

Résumé : Les souches de *Frankia* induisent typiquement des nodules fixateurs d'azote sur les racines des plantes actinorhiziennes. La majorité des taxons de plantes hôtes associées avec les souches de *Frankia* du groupe 1 non cultivées est retrouvée en Californie ; i.e. *Ceanothus* chez les Rhamnacées, *Datisca glomerata* (Dasticacées) et tous les membres actinorhiziens des Rosacées, à l'exception de *Dryas*. Une étude a été entreprise afin de déterminer la distribution des souches de *Frankia* parmi les nodules recueillis de racines d'hôtes solitaires ou sympatriques. Trois régions d'ADN ont été examinées ; i.e. l'extrémité 5' du gène de l'ARNr 16S, la région interne traduite de l'espaceur (ITS) localisé entre les gènes des ARNr 16S et 23S, ainsi qu'une portion du gène codant la glutamine synthase (*glnA*). Les résultats suggèrent qu'un nombre limité d'espèces de *Frankia* du groupe 1 domine dans les nodules recueillis sur un vaste territoire de la Californie, à l'ouest de la Sierra Nevada, sans apparente spécificité pour l'hôte. En comparaison avec les souches de *Frankia* du groupe 2 trouvées sur *Alnus* et *Myrica* à l'échelle de notre étude, il apparaît que la faible diversité observée est particulière aux souches de *Frankia* du groupe 1. Les facteurs qui peuvent expliquer le manque de variabilité génétique et de spécificité pour l'hôte incluent la dominance d'espèce sur un vaste territoire géographique, la sélection environnementale courante et/ou une pression évolutive ancestrale.

Mots clés : Rosacées actinorhiziennes, *Ceanothus*, *Frankia*, *Datisca*, diversité d'espèces.

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Introduction

Strains of nitrogen-fixing actinomycetes of the genus *Frankia* can form mutualistic, actinorhizal (*actino*- referring

to actinomycete, *-rhizal* referring to root) relations with at least 200 species of woody plants representing 24 genera in 8 plant families (Baker and Schwintzer 1990). *Frankia* spp. strains provide their hosts with a source of fixed nitrogen

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while living in root nodules; the host, in turn, provides fixed carbon to the *Frankia* strain. Actinorhizal plants typically colonize nitrogen-deficient soils and disturbed environments, such as arctic tundra, chaparral, sand dunes, bogs, and glacial till, and are important members of plant succession (Baker and Schwintzer 1990). Western North America has the most diverse complement of actinorhizal plants in the world, hosting species from 6 of the 8 families, including representatives of the widespread Datisceae, Betulaceae, Myricaceae, and Elaeagnaceae and providing the center of distribution for *Ceanothus* of the Rhamnaceae and all actinorhizal members of the Rosaceae except *Dryas* L. (Baker and Schwintzer 1990).

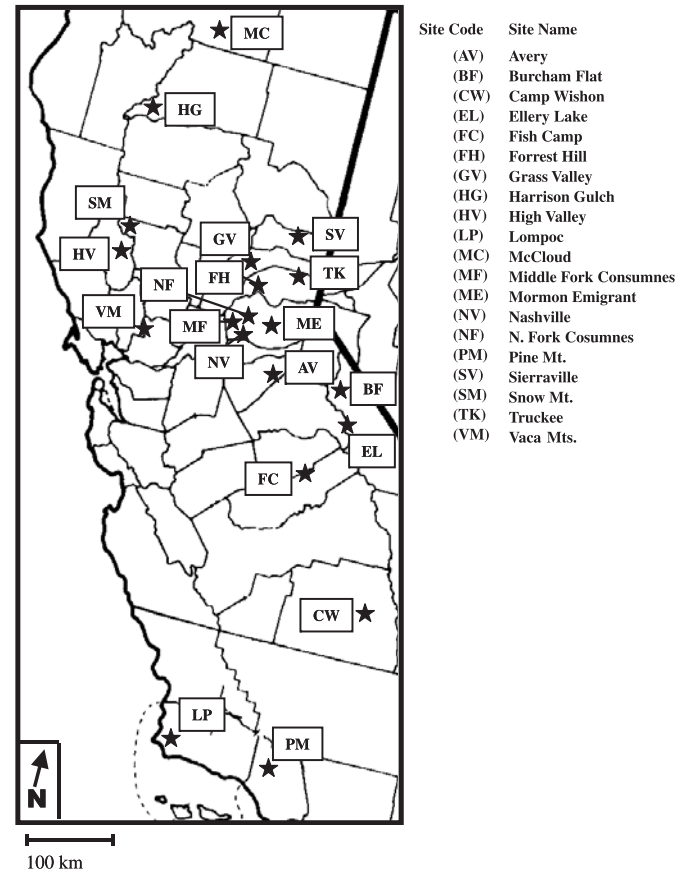
Phylogenetically, typical nitrogen-fixing *Frankia* strains fall into 3 major groups designated 1–3 (Benson et al. 2004). Group 2 and 3 strains have been well studied primarily because they can be isolated and used to reinfest plants. Group 1 strains are specifically associated with members of the Rosaceae, Datisceae, Coriariaceae, and *Ceanothus* of the Rhamnaceae (Clawson et al. 2003). Members of this group capable of reinfesting the host plants have proven refractory to isolation attempts (Mirza et al. 1992), although there is a report of recent success (Hu et al. 1997).

Our purposes in this work were 3-fold: (i) to investigate the diversity of Group 1 *Frankia* strains infecting actinorhizal taxa in California west of the Sierra Nevada crest, (ii) to investigate the diversity of *Frankia* strains infecting members of Rosaceae in the same range, and (iii) to examine Group 1 *Frankia* host specificity in sites where multiple actinorhizal species are growing sympatrically.

The most direct approach used to study the diversity of Group 1 *Frankia* strains has been the PCR amplification of DNA fragments directly from root nodules. The DNA used includes 16S rRNA genes (Benson et al. 1996; Bosco et al. 1994; Clawson et al. 1997; Mirza et al. 1994a, 1994b, 1994c; Nick et al. 1992; Ramirez-Saad et al. 1998), internal transcribed spacer (ITS) regions between the 16S and 23S rRNA genes (Jeong and Myrold 1999; Ritchie and Myrold 1999), repetitive sequence PCR (rep-PCR) (Jeong 2001; Murry et al. 1997), RFLPs of *nif* genes (Baker and Mullin 1994) or ITS regions (Ritchie and Myrold 1999), and, more recently, the gene for glutamine synthetase, *glnA* (Clawson et al. 2003). Much of this work has focused on identifying the symbionts in nodules of individual plants containing Group 1 *Frankia*, some studies providing more information on their diversity and geographical distribution (Clawson et al. 1997; Jeong 2001; Murry et al. 1997; Ritchie and Myrold 1999).

In contrast to nodules from *Ceanothus* spp., which are abundant on plants in the field, nodules from the actinorhizal Rosaceae are rare (Klemmedson 1979; Kohls et al. 1994; Nelson 1983). Consequently, the Group 1 symbionts that inhabit nodules from the Rosaceae are poorly studied. Actinorhizal Rosaceae consists of 4 genera, *Dryas* L., *Chamaebatia* Benth., *Cercocarpus* H. B. & K., and *Purshia* DC. (including *Cowania* D. Don ex Tilloch & Taylor). All except *Dryas*, which is circumboreally distributed, are limited to western North America. Representative species from 3 of these 4 genera can be found in the Sierra Nevada range in eastern California and include *Cercocarpus ledifolius* Nutt., *Cercocarpus betuloides* Torr. & A. Gray, *Purshia*

Fig. 1. Map of the location of the 20 sites sampled in this study. The identity of the host species sampled at each site can be found in Table 1; site characteristics can be found in Table 2.



tridentata DC., and *Chamaebatia foliolosa* Benth. In many locations, 2 or more of these taxa grow next to each other and (or) with one or more species of *Ceanothus* or *Datisca glomerata* (C. Presl) Baill. The species composition of these sites permits a study of both the diversity and host specificity of *Frankia* strains infecting the actinorhizal Rosaceae in California.

Materials and methods

Nodule collection

Our sampling strategy was designed to collect root nodules as broadly as possible throughout California west of the Sierra Nevada crest (Fig. 1). Since nodulation in the actinorhizal Rosaceae is sparse it was necessary to focus on a limited sample of nodules from many sites rather than multiple samples from fewer sites. Our sampling within sites was exhaustive, in that we collected all nodules we found in a population. In most cases, this exhaustive sampling strategy still yielded only few nodules. We sampled 51 nodules from 20 sites in California distributed over an area of about 212 500 km² west of the Sierra Nevada mountain range (Table 1; Fig. 1). Plants encountered at these sites included all 4 species of actinorhizal Rosaceae native to this area, *Cercocarpus ledifolius* (2 nodules), *C. betuloides* (3 nodules), *Purshia tridentata* (9 nodules), and *Chamaebatia foliolosa* (7 nodules); 3 species of *Ceanothus*, *C. cuneatus* (Hook.) Nutt. (3

Table 1. A list of nodule and site information for the 20 California sites sampled.

Site name, code, and habitat type	Nodule code	Host taxon	GenBank acc. Nos.		
			16S	glnII	ITS
Mormon Emigrant Rd. (ME)	Cea.pro(ME1)	<i>Ceanothus prostratus</i>	AY644806	AY644857	AY644908
	Cea.int(ME2)	<i>Ceanothus integerrimus</i>	AY644807	AY644858	AY644909
	Cea.int(ME3)	<i>Ceanothus integerrimus</i>	AY644808	AY644859	AY644910
	Cea.int(ME4)	<i>Ceanothus integerrimus</i>	AY644809	AY644860	AY644911
	Chama.fol(ME5)	<i>Chamaebatia foliolosa</i>	AY644810	AY644861	AY644912
	Cea.pro(ME6)	<i>Ceanothus prostratus</i>	AY644811	AY644862	AY644913
	Chama.fol(ME7)	<i>Chamaebatia foliolosa</i>	AY644812	AY644863	AY644914
	Chama.fol(ME8)	<i>Chamaebatia foliolosa</i>	AY644813	AY644864	AY644915
Avery (AV)	Cea.int(AV1)	<i>Ceanothus integerrimus</i>	AY644814	AY644865	AY644916
	Chama.fol(AV2)	<i>Chamaebatia foliolosa</i>	AY644815	AY644866	AY644917
Forrest Hill (FH)	Cea.int(FH1)	<i>Ceanothus integerrimus</i>	AY644816	AY644867	AY644918
	Chama.fol(FH2)	<i>Chamaebatia foliolosa</i>	AY644817	AY644868	AY644919
Grass Valley (GV)	Cea.pro(GV1)	<i>Ceanothus prostratus</i>	AY644818	AY644869	AY644920
	Chama.fol(GV2)	<i>Chamaebatia foliolosa</i>	AY644819	AY644870	AY644921
Sierraville (SV)	Cea.pro(SV1)	<i>Ceanothus prostratus</i>	AY644820	AY644871	AY644922
	Pur.tri(SV2)	<i>Purshia tridentata</i>	AY644821	AY644872	AY644923
Fishcamp (FC)	Cea.int(FC1)	<i>Ceanothus integerrimus</i>	AY644822	AY644873	AY644924
	Chama.fol(FC2)	<i>Chamaebatia foliolosa</i>	AY644823	AY644874	AY644925
Ellery Lake (EL)	Cerc.led(EL1)	<i>Cercocarpus ledifolius</i>	AY644824	AY644875	AY644926
	Cerc.led(EL2)	<i>Cercocarpus ledifolius</i>	AY644825	AY644876	AY644927
	Pur.tri(EL3)	<i>Purshia tridentata</i>	AY644826	AY644877	AY644928
Truckee (TK)	Pur.tri(TK1)	<i>Purshia tridentata</i>	AY644827	AY644878	AY644929
	Pur.tri(TK2)	<i>Purshia tridentata</i>	AY644828	AY644879	AY644930
	Pur.tri(TK1)	<i>Purshia tridentata</i>	AY644829	AY644880	AY644931
Burcham Flat (BF)	Pur.tri(BF1)	<i>Purshia tridentata</i>	AY644829	AY644880	AY644931
	Pur.tri(BF2)	<i>Purshia tridentata</i>	AY644830	AY644881	AY644932
Snow Mt. (SM)	Cea.cun(SM1)	<i>Ceanothus cuneatus</i>	AY644831	AY644882	AY644933
High Valley (HV)	Cea.cun(HV1)	<i>Ceanothus cuneatus</i>	AY644832	AY644883	AY644934
	Cea.cun(HV2)	<i>Ceanothus cuneatus</i>	AY644833	AY644884	AY644935
	Cea.cun(HV2)	<i>Ceanothus cuneatus</i>	AY644833	AY644884	AY644935
Harrison Gulch (HG)	Cea.int(HG1)	<i>Ceanothus integerrimus</i>	AY644834	AY644885	AY644936
	Cea.int(HG2)	<i>Ceanothus integerrimus</i>	AY644835	AY644886	AY644937
	Cerc.bet(HG3)	<i>Cercocarpus betuloides</i>	AY644836	AY644887	AY644938
	Cea.int(HG4)	<i>Ceanothus integerrimus</i>	AY644837	AY644888	AY644939
	Cea.int(HG4)	<i>Ceanothus integerrimus</i>	AY644837	AY644888	AY644939
McCloud (MC)	Pur.tri(MC1)	<i>Purshia tridentata</i>	AY644838	AY644889	AY644940
	Pur.tri(MC2)	<i>Purshia tridentata</i>	AY644839	AY644890	AY644941
	Pur.tri(MC3)	<i>Purshia tridentata</i>	AY644840	AY644891	AY644942
Lompoc (LP)	Cerc.bet(LP1)	<i>Cercocarpus betuloides</i>	AY644841	AY644892	AY644943
Pine Mt. (PM)	Cerc.bet(PM1)	<i>Cercocarpus betuloides</i>	AY644842	AY644893	AY644944
Vaca Mts. (VM)	Dat.glo(VM1)	<i>Datisca glomerata</i>	AY644843	AY644894	AY644945
	Dat.glo(VM2)	<i>Datisca glomerata</i>	AY644844	AY644895	AY644946
	Dat.glo(VM3)	<i>Datisca glomerata</i>	AY644845	AY644896	AY644947
	Dat.glo(VM4)	<i>Datisca glomerata</i>	AY644846	AY644897	AY644948
	Aln.rhm(VM5)	<i>Alnus rhombifolia</i>	AY644851	AY644902	N/A
Nashville (NV)	Dat.glo(NV1)	<i>Datisca glomerata</i>	AY644847	AY644898	AY644949
	Dat.glo(NV2)	<i>Datisca glomerata</i>	AY644848	AY644899	AY644950
	Dat.glo(NV3)	<i>Datisca glomerata</i>	AY644849	AY644900	AY644951
North Fork Cosumnes River (NF)	Dat.glo(NF1)	<i>Datisca glomerata</i>	AY644850	AY644901	AY644952
	Aln.rhm(NF2)	<i>Alnus rhombifolia</i>	AY644852	AY644903	N/A
Middlefork Cosumnes River (MF)	Myr.har(MF1)	<i>Myrica hartwegii</i>	AY644853	AY644904	N/A
	Aln.rhm(MF2)	<i>Alnus rhombifolia</i>	AY644854	AY644905	N/A
	Aln.rhm(MF3)	<i>Alnus rhombifolia</i>	AY644855	AY644906	N/A
	Aln.rhm(MF3)	<i>Alnus rhombifolia</i>	AY644855	AY644906	N/A
Camp Wishon (CW)	Aln.rhm(CW1)	<i>Alnus rhombifolia</i>	AY644856	AY644907	N/A

nodules), *C. integerrimus* Hook. & Arn. (9 nodules), and *C. prostratus* Benth. (4 nodules); *Datisca glomerata* (8 nodules); *Alnus rhombifolia* (5 nodules); and *Myrica hartwegii* (1 nodule). The latter 2 species were included to compare the diversity obtained with Group 1 strains to that of the more extensively studied Group 2 strains in *Alnus* and *Myrica*.

Eleven of the 20 sites contained at least 2 different actinorhizal plant host species (Table 1; Fig. 1). At 7 of these sites, we collected nodules from sympatric actinorhizal species of Rosaceae and 1 or more species of *Ceanothus*. Nine of the 20 sites yielded nodules from only 1 actinorhizal taxon (Table 1), and in 3 of the 9 we were able to find nodules on only a single individual (site codes SM, LP, and PM) (Table 1). Although both *Chamaebatia foliolosa* and *Cercocarpus betuloides* were present at the Avery site, we were unable to find nodules from these taxa at that site. We did find nodules present on *Chamaebatia foliolosa* at the Grass Valley site, at the same elevation, same soil type, and parent rock material as the Avery site.

The 20 sites chosen represent a range of elevations, soil types, parent rock substrates, and plant communities (Table 2). Entire plants were removed, and their root systems were rinsed with water before nodule collection to ensure correct host identification. Field-collected nodules were removed and placed in 100% ethanol for transport back to the laboratory.

DNA extraction

The procedure for DNA extraction that we used was modified from previous descriptions (Benson et al. 1996). Single nodule lobes were excised from the nodule, washed with double distilled H₂O, and then washed in TEA buffer (10 mmol/L Tris-HCl, 1 mmol/L Na₂EDTA, 20 mmol/L ascorbic acid, pH 7.6). After washing, the periderm of the lobe was removed with a sterile probe and forceps. The contents of the nodule lobe were placed in a 1.5-mL tube and were subjected to a modified CTAB extraction (Doyle and Doyle 1987). Briefly, 600 µL of 2× CTAB (2% cetyltrimethylammonium bromide, 1.4 mol/L NaCl, 100 mmol/L Tris-HCl, and 20 mmol/L EDTA) (65 °C) was added to the tube containing the nodule tissue, macerated with a small pestle, and incubated at 65 °C for 10 min. Chloroform and isoamyl alcohol (400 µL; 24:1 v/v) were added and shaken to create an emulsion. The tube was centrifuged at 13 000g in a microcentrifuge for 1 min, and the aqueous layer was removed and transferred to a new tube where the chloroform/isoamyl alcohol extraction was repeated. The aqueous layer was placed in a clean, 1.5-mL microcentrifuge tube, and the DNA was precipitated by adding 500 µL of cold isopropanol and storing at -20 °C overnight. DNA was collected by centrifugation at 10 000g for 10 min, then washed with 76% ethanol and 10 mmol/L ammonium acetate and allowed to air dry. Finally, total DNA was redissolved in 20 µL of 10 mmol/L Tris-HCl.

Amplification and sequencing

Three DNA regions were targeted for amplification and sequencing for this study: a variable 16S rDNA region using the primers rDB1 and fD1 (Benson et al. 1996); a fragment of the ITS between the 16S and 23S rDNA using the primers 1649F and 23S13 (Ritchie and Myrold 1999); and a partial

sequence of the *glnA* gene using primers DB41 (5'-TTCTTCATCCACGACCCG-3') and DB44 (5'-GGCTTCGGCATGAAGGT-3') (Clawson et al. 2003). These regions were amplified separately using a *Taq* PCR Core Kit (Qiagen, Santa Clarita, Calif.) according to the manufacturer's protocols. Fifty microlitre PCR reactions (10 ng template DNA, each primer at 0.5 mol/L, 2 mmol/L MgCl₂, 1 unit of *Taq* DNA polymerase, 20 mol/L of each deoxynucleoside triphosphate, and 5 L of 10× buffer) were run on a Perkin Elmer Thermal Cycler (Perkin-Elmer manual, Perkin-Elmer Corp., Norwalk, Conn.) programmed for a hot start (95 °C, 2 min.) and 30 cycles of 94 °C for 30 s, 50 °C for 45 s, and 72 °C for 1 min with a final extension for 7 min. A negative control, lacking template DNA, was also run.

PCR amplifications were analyzed in a 1.5% agarose gel run in 0.5× Tris-borate-EDTA buffer and visualized with ethidium bromide under UV light. Fragments were purified using a QIAquick Gel Extraction kit (Qiagen manual, Qiagen, Santa Clarita, Calif.). PCR products were cycle sequenced in both directions using Big Dye reagents (Applied Biosystems, Foster City, Calif.) and analyzed on an ABI Prism 3100 automated sequencer (Applied Biosystems) according to the manufacturers' protocols. Sequences for each region were proofread and assembled using SeqMan (1998, DNASTar, Madison, Wis.) and then Blast searched at the National Center for Biotechnology Information Home page (www.ncbi.nlm.nih.gov/BLAST/) to ensure that *Frankia* sequences were being amplified from nodule DNA. Sequences generated were then aligned for each region using ClustalW in MegAlign (DNASTar Inc., Madison, Wis., 1998).

Phylogenetic analysis

Two analyses were conducted on the aligned sequences. The first analysis examined the diversity of the sequences generated from the 45 Group 1 *Frankia* strains that we obtained from the actinorhizal Rosaceae, *Ceanothus* spp., and *Datisca glomerata* listed in Table 1. Alignments for each region were examined for the identification of residue substitutions and were imported into PAUP 4.0b810 (Swofford 2002), where unrooted neighbor-joining dendrograms using total sequence differences were constructed.

The second analysis placed the 16S rDNA and *glnA* sequences from the first analysis into a larger dataset created from sequences generated from nodules and pure cultured *Frankia* from sources shown in Table 3, sampling the known range of *Frankia* diversity. The sequences retrieved from nodules in this study were combined with a dataset from Clawson and Benson (Clawson et al. 2003) to place them in a broader phylogenetic context. The combined dataset included the 51 nodule samples from Table 1 and the 25 sequences from Table 3, for a total of 76 sequences. The sequences from the 6 Group 2 *Frankia* in nodules from *Alnus rhombifolia* and *Myrica hartwegii* were included in this larger dataset. The 16S rDNA and *glnA* sequences from *Acidothermus cellulolyticus* (Table 3) served as the outgroup. A single, aligned dataset was created from the 2 regions and imported into PAUP, where a neighbor-joining phenogram was constructed. Support for groups was estimated using 1000 bootstrap replicates (Felsenstein 1985) as implemented in PAUP. We did not include ITS sequences in this larger

dataset for 2 reasons. First, there is not a large inventory of ITS sequences in GenBank to represent the known range of *Frankia* diversity and, second, ITS sequences from strains from Group 2 and Group 1 are very divergent, yielding suspect alignments not suitable for phylogenetic reconstruction.

Sequence distances

Average uncorrected p-distances (proportion of differences between sequences) were computed for each region, 16S, *glnA*, and ITS within PAUP 4.0b810 (Swofford 2002) for the 45 nodules we obtained from the actinorhizal Rosaceae, *Ceanothus* spp., and *Datisca glomerata*. For a comparison, the average uncorrected p-distances for the 16S and *glnA* were also computed for 6 nodules we obtained from *Alnus* and *Myrica*, as well as for all of the Group 2 and Group 3 strains present in the second analysis (Fig. 2).

Given the divergence between ITS sequences from *Frankia* from Group 2 as compared with those from Group 1 and the lack of a good sample of ITS sequences from a full range of *Frankia* diversity, we computed and compared average uncorrected p-distances for ITS sequences from our 45 nodules to ITS sequences generated from *Frankia* strains from *Ceanothus* spp., which are known to be in Group 1, published by Ritchie and Myrold (1999) and Jeong and Myrold (1999). Distances for the *Ceanothus* spp. ITS sequences were computed with and without the inclusion of the nodule from *Ceanothus americanus* because it was collected far outside our study area (Tennessee), and its considerable divergence inflated the average p-distance.

Results

Nodule abundance

Nodulation frequency varied from very low (<3% of roots examined) in the case of members of the Rosaceae to moderately abundant (>20%) in the case of members of *Ceanothus*, to abundant (100%) in the case of members of the genera *Alnus*, *Datisca*, and *Myrica*. Soil characterization for each site can be found in Table 2.

Sequence diversity within collected nodules

PCR products for the 3 DNA regions (16S, *glnA*, and ITS) were successfully amplified and sequenced from all 45 nodules collected from *Ceanothus*, *Datisca*, and the actinorhizal Rosaceae. The length of the aligned dataset for the hypervariable region of the 16S rDNA was 377 base pairs (bp), for the *glnA* fragment it was 430 bp, and for the fragment of the ITS region between the 16S and 23S rDNA it was 397 bp, for a total of 1204 aligned bp. After alignment and analysis, we found that all of the 16S sequences generated from the 45 nodules from the actinorhizal Rosaceae, *Ceanothus* spp., and *Datisca glomerata* were identical with each other (results not shown). Alignments of both the *glnA* and ITS regions did show variation. The sequence types obtained from each nodule are given in Table 4.

The analysis of the *glnA* region yielded 9 distinct sequence types (A–I), and only 5 ITS types (A–E) were found in the 45 nodule samples (Table 4). Fourteen allele combinations were detected (Fig. 3). Three combinations, BC, AB, and AA, predominated and were found in 10, 9, and 7 nodules, respectively, distributed among representatives of all

the Group 1 families examined. The remaining combinations were found in 3 (BD, BH, EB), 2 (AG, BE) or 1 nodule (BA, BB, CC, BF, BI, DF) each. No *glnA* type was more than 6-bp substitutions different from any other (Fig. 3), although 4 DNA substitutions found in the *glnA* alignment resulted in changes in the amino acid sequence. The 5 ITS types differed from one another by at most 3-bp substitutions (Fig. 3). Except for 2 instances, all allele combinations could be separated by a single step (Fig. 3). No correlation was found between plant of origin or geographical region and sequence type (Figs. 1 and 3; Table 1).

Phylogenetic position of *Frankia* in collected nodules compared with total *Frankia* diversity

The 16S rDNA aligned dataset that included *Frankia* strains from other studies and from this study contained 380 bp, 31 of which were potentially phylogenetically informative, and the *glnA* aligned dataset contained 457 bp, 129 of which were informative. In the neighbor-joining dendrogram produced from the combined data set of the concatenated 16S and *glnA* regions, 3 main clusters are resolved (Fig. 2). These correspond to the 3 groups of *Frankia* identified by earlier studies (Normand et al. 1996): Group 2, the *Alnus* Mill, *Casuarina* L., and *Myrica* L.-infective cluster; Group 1, the uncultured cluster; and Group 3, the *Elaeagnus* L.-infective cluster, including strains R2 and PtI6 isolated from *Ceanothus* and *Purshia* nodules, respectively. The latter do not reinfect their hosts of origin but do infect *Elaeagnus* sp. (Clawson et al. 2003). All 45 nodule samples we collected from the Rosaceae, *Ceanothus* sp., and *Datisca glomerata* are deeply nested within Group 1 or uncultured cluster, with strong support from bootstrap evidence along with previously published sequences from nodules collected from *Chamaebatia foliolosa* Benth., *Purshia glandulosa* Curran, and *Datisca cannabina* L. (Fig. 2).

Sequence distances

As mentioned, the 16S sequences from the 45 nodules of the actinorhizal Rosaceae, *Ceanothus* spp., and *Datisca glomerata* were identical, yielding an average uncorrected p-distance of 0.0. The 16S sequence variation as estimated by the average uncorrected p-distance was nearly identical between the 6 Group 2 *Frankia* we collected from *Alnus* and *Myrica* ($p = 0.01267$) and the 13 more widely collected sequences we used to represent the full range of Group 2 diversity ($p = 0.01713$) (Table 3). The 16S sequence variation representing Group 3 *Frankia* diversity was intermediate ($p = 0.00733$).

Sequence variation for the *glnA* region of the 45 nodules was small ($p = 0.00454$) compared with both the full Group 2 diversity ($p = 0.06681$) and the Group 2 nodules collected in California ($p = 0.03962$). Group 3 *glnA* sequence variation was once again intermediate ($p = 0.04358$).

The 11 ITS sequences published by Ritchie and Myrold (1999) and Jeong and Myrold (1999) obtained from *Frankia* strains inhabiting nodules of *Ceanothus* spp. yielded an uncorrected p-distance of $p = 0.01535$, almost 10-fold greater than the average distance from our 45 nodules obtained from the actinorhizal Rosaceae, *Ceanothus* spp., and *Datisca glomerata* ($p = 0.00223$). Correcting for the 1 divergent ITS

Table 2. Site information for the 7 California locations examined.

Site name (code)	Latitude/longitude	Elevation (m)	Plant community ^a	Soil type	Soil classification (reference)
Mormon Emigrant Rd. (ME)	38°43'N 120°32'W	1000	lower montane ponderosa pine	well-drained stony loam	Cohasset-Aiken-McCarthy; volcanic conglomerate (Rogers 1974)
Avery (AV)	38°12'N 120°22'W	1000	lower montane ponderosa pine	well-drained stony loam	Aiken Cohasset; andesitic conglomerate, metabasic (Sketchley 1993)
Forrest Hill (FH)	39°01'N 120°53'W	800	blue oak and foothill pine woodland	well-drained stony loam	Cohasset-Aiken-McCarthy; volcanic conglomerate (Rogerw 1980)
Grass Valley (GV)	39°16'N 120°58'W	1000	lower montane ponderosa pine	well-drained stony loam	Aiken Cohasset; andesitic conglomerate, metabasic (Brittan 1975)
Sierraville (SV)	39°33'N 120°20'W	1700	coniferous forest	well-drained stony, sandy loam	Trojan-Delleka-Portola; volcanic (Sketchley 1975)
Fishcamp (FC)	37°28'N 119°38'W	1500	lower montane ponderosa pine	well-drained sandy loam	Holland-Chawanakee; granitic (Giger and Schmitt 1984)
Ellery Lake (EL)	37°56'N 119°14'W	2800	high sagebrush steppe	well-drained, shallow stony sand	Ordovician Palmetto; metamorphic, parent rock, soil classification unknown (Newlan et al. 1989)
Truckee (TK)	39°21'N 120°11'W	2000	coniferous forest	well-drained course sandy loam	Beckwourth-Loyalton-Orasby; granitic (Sketchley 1975)
Burcham Flat (BF)	38°21'N 119°26'W	2200	high sagebrush steppe	well-drained course sandy loam	Sedimentary glacial deposits; granitic (Jennings 2000)
Snow Mt. (SM)	39°22'N 122°38'W	600	chaparral pine and oak woodland	well-drained gravelly, sandy loam	Neuns-Sheetiron-Deadwood; sedimentary (Smith and Broderson 1989)
High Valley (HV)	39°03'N 122°43'W	600	chaparral pine and oak woodland	well-drained gravelly, sandy loam	Maymen-Hopland-Etsel; sedimentary (Smith and Broderson 1989)
Harrison Gulch (HG)	40°24'N 122°57'W	800	chaparral pine and oak woodland	well-drained stony loam	Goulding stony loam; greenstone (Klaseen and Ellison 1974)
McCloud (MC)	41°15'N 121°59'W	1000	lower montane ponderosa pine	well-drained sandy loam	Delaney sandy loam; igneous/ash (Newlan et al. 1989)
Lompoc (LP)	34°42'N 120°23'W	200	chaparral and coastal scrub	clay	Diablo clay; shale (Shipman 1981)
Pine Mt. (PM)	34°39'N 119°22'W	1300	southern oak and dry montane	well-drained sandy loam	Upper Eocene marine sediment; sedimentary (Jennings 2000)
Vaca Mts. (VM)	38°35'N 120°51'W	500	oak and pine woodland	well-drained sandy loam	Riverwash-Rincon; sedimentary (Bates 1977)
Nashville (NV)	38°35'N 120°51'W	400	blue oak and foothill pine woodland	well-drained gravelly, silt loam	Whiterock silt loam; slate (Rogers 1974)
North Fork Cosumnes River (NF)	38°39'N 120°40'W	400	blue oak and foothill pine woodland	well-drained coarse sand loam	Chawanakee coarse loam; granitic (Rogers 1980)
Middle Fork Cosumnes River (MF)	38°37'N 120°42'W	500	blue oak and foothill pine woodland	well-drained coarse sand loam	Chawanakee coarse loam; granitic (Rogers 1980)
Camp Wishon (CW)	36°11'N 118°40'W	1300	coniferous forest	well-drained course sandy loam	Sedimentary glacial deposits; granitic (Jennings 2000)

^aPlant community types from the Jepson *Manual of Higher Plants of California*.

Table 3. Nodule names and strain sources, locations, and accession numbers of *glnII* and 16S sequences used in this study.

Host plant or strain source	Abbreviation	GenBank acc. <i>glnA</i>	GenBank acc. 16S
<i>Acidothermus cellulolyticus</i>	Acid.cell	AY027649	AJ007290
<i>Alnus incana</i> subsp. <i>rugosa</i> nodule	Alnus.incana	AF156755	AF131893
<i>Casuarina cunninghamiana</i> strain CcI3	Cas.cunn	AF156756	AF149268
<i>Ceanothus americanus</i> R2	Cea.amer.R2	AY027647	AY027645
<i>C. americanus</i> nodule	Cea.amer	AF156762	AF063639
<i>Cercocarpus ledifolius</i> nodule	Cerc.led	AF156763	AF149275
<i>Chamaebatia foliolosa</i> nodule	Chama.fol	AF156758	AF149271
<i>Colletia hystrix</i> nodule	Coll.hyr	AF156741	AF063640
<i>Comptonia peregrina</i> nodule	Comp.per	AF156747	AF116103
<i>Comptonia peregrina</i> strain CpI1	Comp.per.CpI1	L10631	AF130811
<i>Coriaria arborea</i> nodule	Cor.arb	AF156764	AF063641
<i>Cowania stansburiana</i> nodule	Cow.stans	AF156760	AF149276
<i>Datisca cannabina</i> nodule	Dat.cann	AF156761	AF158686
<i>Discaria serratifolia</i> strain Ds12B	Dis.serr.Ds12B	AF156743	AF064149
<i>Elaeagnus angustifolia</i> nodule	Ela.ang	AF156742	AF064144
<i>Hippophae rhamnoides</i> nodule	Hipp.rham	AF156745	AF149269
<i>Myrica gale</i> nodule 2	Myr.gale.2	AF156748	AF158684
<i>Myrica gale</i> nodule 1	Myr.gale.1	AF156757	AF116162
<i>Myrica pensylvanica</i> nodule	Myr.pen	AF156754	AF116076
<i>Purshia glandulosa</i> nodule	Pur.gland	AF156759	AF149273
<i>P. tridentata</i> strain PtI6	Pur.tri.PtI6	AY027648	AY027646
<i>Retanilla ephedra</i> strain ReI6	Ret.eph.ReI6	AF156740	AF064150
<i>Talguenea quinquinervia</i> nodule	Tal.quin	AF156746	AF063643
<i>Trevoa trinervis</i> nodule	Tre.trin	AF156744	AF063642
<i>Talguenea quinquinervia</i> strain TqI5	Tal.quin.TqI5	AF156739	AF064147

sequence from a nodule of *C. americanus* collected in Tennessee gave a smaller average distance ($p = 0.00845$).

Discussion

We report a surprisingly shallow level of diversity among *Frankia* strains inhabiting root nodules from plant species of 3 families nodulated by Group 1 *Frankia* growing in a large area of western North America.

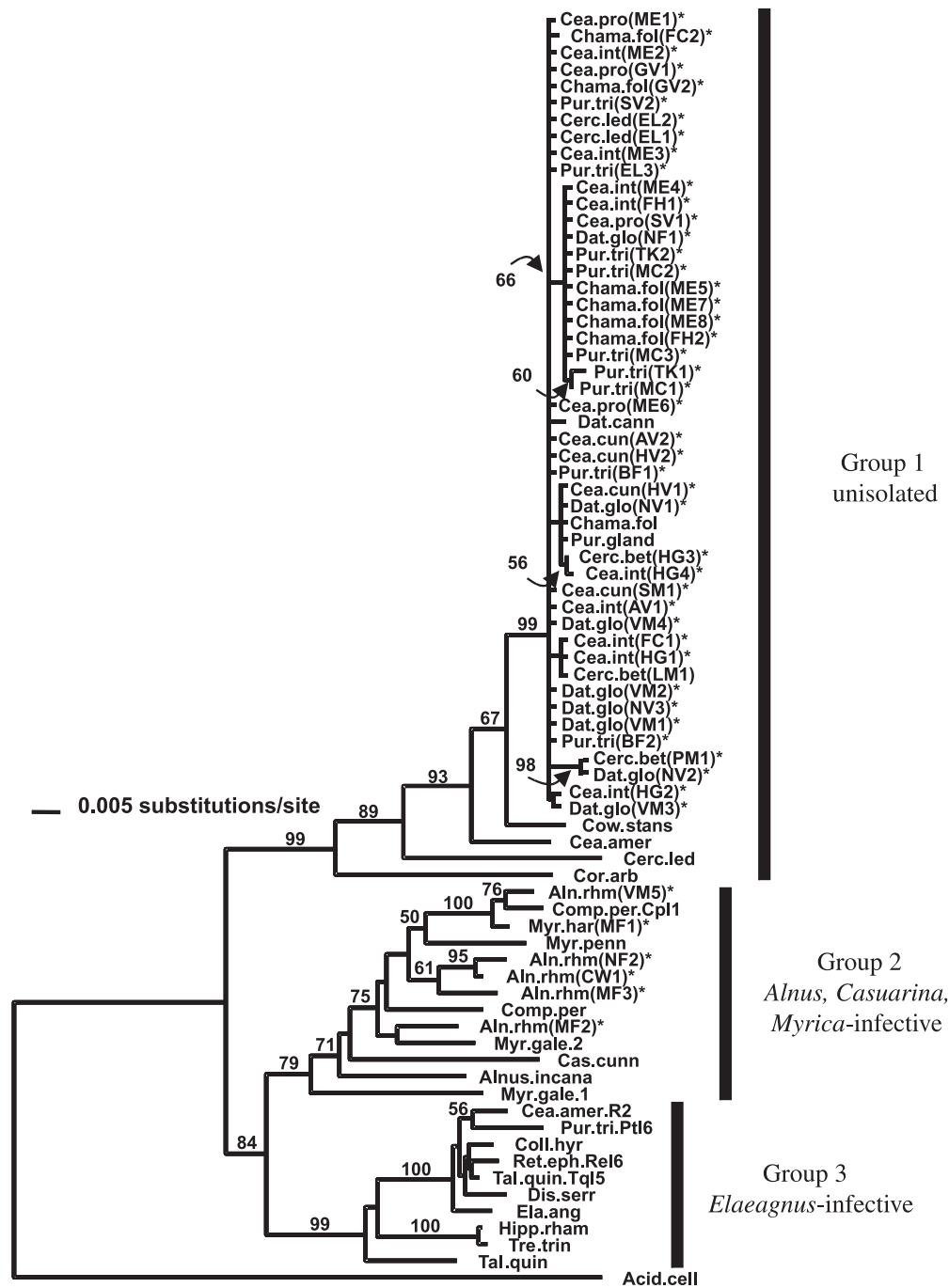
This work is the first to report the diversity of *Frankia* strains from Rosaceae nodules collected in the field. Previous work on this family has been limited to classifying frankia in 1 or a few nodules using 16S rRNA genes (Benson et al. 1996; Bosco et al. 1994; Clawson et al. 2003). The diversity of *Frankia* in the Rosaceae has not been examined previously because root nodules are difficult to collect and because the nodulating strains, like other Group 1 strains, have proven refractory to isolation. The scarcity of nodulation in the Rosaceae that we observed parallels previous work describing nodulation in these plants as sporadic (Klemmedson 1979; Paschke 1997). One study reported nodulation rates of *Cercocarpus*, *Cowania*, and *Purshia* between 8.3% and 32.2% in the field (Nelson 1983). Given that these plants occur in mostly dry habitats, the lack of observed nodulation may be due to water stress or simply the ease with which nodules become detached from the roots during harvest.

We also report, for the first time, the phylogenetic position of *Frankia* living in *Datisca glomerata* root nodules.

DNA sequences from *Frankia* associated with the nodules of the only other species of *Datisca*, *D. cannabina*, have been obtained in previous studies and have clustered within Group 1 (Mirza et al. 1994a, 1994b, 1994c). The 2 species of *Datisca* have markedly disjunct distributions, with *D. cannabina* present in the Mediterranean basin and *D. glomerata* found only on the west slope of the Sierra Nevada from northern to Baja California. Although their geographical ranges are widely separated, we confirm here that *D. glomerata* associates with Group 1 *Frankia* as well. To date, no studies have assessed the geographic diversity of *Datisca* strains.

In contrast to the Rosaceae and *Datisca*, a substantial amount of work has been done on *Frankia* spp. strains in *Ceanothus* nodules. Biogeographic diversity has been assessed primarily using fingerprinting techniques such as rep-PCR (Jeong 2001; Jeong and Myrold 1999; Murry et al. 1997), RFLP and sequencing of the ITS regions between 16S and 23S rRNA genes (Ritchie and Myrold 1999), and RFLP of *nif* genes (Baker and Mullin 1994). These previous studies have reported variable levels of diversity but have generally agreed that strains show no obvious host specificity for different *Ceanothus* spp. Ritchie and Myrold (1999) used RFLP patterns from a fragment of DNA spanning the 16S–23S ITS region and found only 4 related patterns that correlated with geographical region rather than with plant species. Murry et al. (1997) used rep-PCR to examine the diversity of *Frankia* in 6 *Ceanothus* spp. at 7 sites in southern California. Twelve groups were identified in the 60 nodules

Fig. 2. A neighbor-joining phenogram produced from the combined analysis of the *glnA* and 16S rDNA regions for the 51 nodules sampled (Table 1) and the 25 DNA accessions found in Table 3. Branch length refers to the number of substitutions per site. Numbers below branches are a measure of bootstrap support, and only numbers greater than 50 are shown. Names of *Frankia* spp. strains are abbreviated as in Tables 1 (with asterisks) and 3. Groupings are as defined in Normand et al. (1996).



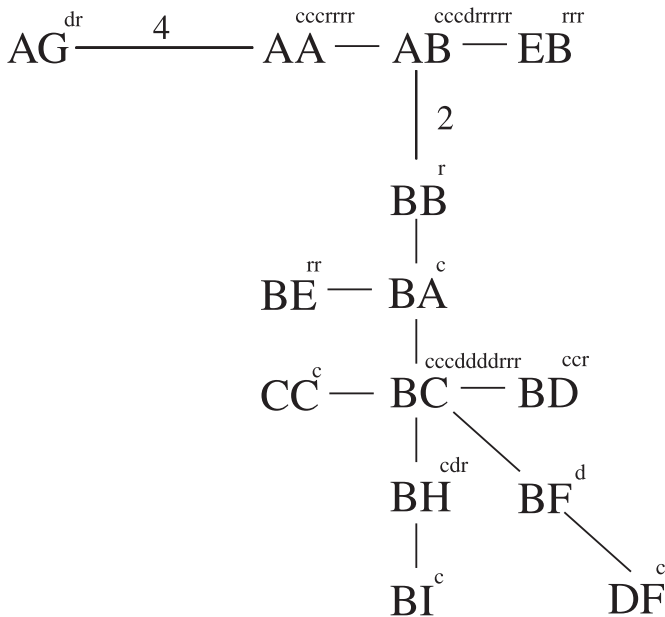
examined. Jeong (2001) also used rep-PCR to investigate diversity in 69 nodules induced from soil trapping experiments in the greenhouse and found little evidence of host specificity for the strains, although 1 of the 8 rep-PCR operational taxonomic units identified tended to dominate.

We chose not to use rep-PCR or RFLP fingerprinting primarily because the results are not amenable to phylogenetic analysis but also because it is difficult to control for contaminating plant or bacterial DNA. To put the Group 1 *Frankia*

in the context of overall *Frankia* diversity we instead chose to sequence 3 different DNA regions: a variable portion of the 16S rRNA gene, the more variable ITS region, and the glutamine synthetase I gene, *glnA*.

The 5' 380-bp fragment of the 16S rRNA gene has been estimated to contain approximately 45% of the total variation in the whole gene among *Frankia* strains (Clawson et al. 1998). Past studies have shown that *Frankia* spp. strains that have identical sequences in this region also have the same

Fig. 3. A network of ITS and *glnA* DNA sequence types based on unrooted neighbor-joining analysis. Nine *glnA* types, type A through I, and 5 ITS sequence types, type A through E, were found. The first letter in the pair refers to ITS sequence type and the second refers to the *glnA* type. All branches correspond to single nucleotide changes unless noted on the branches. Superscripts correspond to occurrences on a particular type in nodules from *Ceanothus* – ^c, *Datisca* – ^d, Rosaceae – ^r.



morphology, carbon utilization, and protein profiles and have been viewed as the same, or very closely related, strains (Clawson et al. 1999). In this study, all 45 16S rRNA gene sequences obtained from the Group 1 strains were identical. Thus, we conclude that closely related *Frankia* strains were sampled at all sites and on all host species surveyed in this study. Strains from this group of *Frankia* have not yet been isolated in pure culture so confirmation of their physiological similarity is currently not possible. Group 1 strains remain accessible only through morphological studies and molecular studies based on DNA amplification.

Since the 16S rRNA gene is not optimal for distinguishing diversity among strains we also used the more variable ITS and *glnA* regions. When comparing sequences from the known diversity of *Frankia* (Fig. 2), the *glnA* alignment had about 4 times as much variability as the 16S alignment (129 versus 31 parsimony informative characters). These observations suggest that the *glnA* and ITS regions are more useful markers for studying *Frankia* spp. strain diversity in the field. This confirms previous reports for the ITS region (Varghese et al. 2003), at least when one wishes to view the results in a phylogenetic context, although the ITS region is difficult to align for more distantly related strains.

The *glnA* and ITS types obtained did not correlate with host species, genus, or even family, indicating a general lack of host specificity in these strains, although not enough nodules were sampled from any 1 species to draw strong conclusions about varying degrees of preference between particular variants and host species. The results also show no correlation between sequence types and geographic sites. The limited diversity we detected in these DNA regions may

Table 4. *GlnA* and ITS sequence types for each nodule sampled.

Nodule code	ITS DNA type	<i>glnA</i> DNA type
Cea.pro(ME1)	A	A
Cea.int(ME2)	B	A
Cea.int(ME3)	A	A
Cea.int(ME4)	A	B
Chama.fol(ME5)	B	B
Cea.pro(ME6)	B	C
Chama.fol(ME7)	A	B
Chama.fol(ME8)	A	B
Cea.int(AV1)	B	C
Chama.fol(AV2)	B	C
Cea.int(FH1)	A	B
Chama.fol(FH2)	A	B
Cea.pro(GV1)	A	A
Chama.fol(GV2)	A	A
Cea.pro(SV1)	A	B
Pur.tri(SV2)	A	A
Cea.int(FC1)	B	D
Chama.fol(FC2)	B	E
Cerc.led(EL1)	A	A
Cerc.led(EL2)	A	A
Pur.tri(EL3)	A	A
Pur.tri(TK1)	A	B
Pur.tri(TK2)	A	B
Pur.tri(BF1)	B	C
Pur.tri(BF2)	B	C
Cea.cun(SM1)	C	C
Cea.cun(HV1)	B	H
Cea.cun(HV2)	B	C
Cea.int(HG1)	B	D
Cea.int(HG2)	D	F
Cerc.bet(HG3)	B	H
Cea.int(HG4)	B	I
Pur.tri(MC1)	E	B
Pur.tri(MC2)	E	B
Pur.tri(MC3)	E	B
Cerc.bet(LP1)	B	D
Cerc.bet(PM1)	A	G
Dat.glo(VM1)	B	C
Dat.glo(VM2)	B	C
Dat.glo(VM3)	B	F
Dat.glo(VM4)	B	C
Dat.glo(NV1)	B	H
Dat.glo(NV2)	A	G
Dat.glo(NV3)	B	C
Dat.glo(NF1)	A	B

therefore reflect intrastain variation resulting from genetic drift within what began as a single strain. In support of this notion, we found that most of the sequence variation for the combined *glnA* and ITS alleles could be accounted for by

progressive single nucleotide changes (Fig. 3). Undoubtedly, more changes would be detected if more genes were sequenced, but the results obtained support the idea that most of the strains sampled share a relatively recent common ancestor, as compared with the rest of the *Frankia* clade.

The variation in the *glnA* region among the Group 1 strains was very small when compared with variation among the Group 2 *Frankia* strains infecting *Alnus* and *Myrica* in our study region. Even though we sampled only 6 nodules from *Alnus* and *Myrica* in 4 sites, we found much greater sequence diversity for both the 16S and *glnA* regions when compared with the average sequence distance in the 45 nodules we sampled from the actinorhizal Rosaceae, *Ceanothus* spp., and *Datisca*, reinforcing the interpretation that the Group 1 *Frankia* strains sampled at all sites are very closely related.

Our sequences were identical or nearly identical to those previously published for species of Rosaceae from western North America, as is evident from their inclusion in a single, tight cluster in Fig. 2 (Clawson et al. 2003). Also included in this cluster is a strain from a nodule from *Datisca cannabina*, a species native to the Mediterranean basin. In this case, however, the nodule was induced following inoculation with crushed nodules from a *Ceanothus* sp. (J. Tjepkema, Univ. Maine, Orono, personal communication). Thus, it may be that in its native range this plant species is nodulated by a different *Frankia* strain or strains. Nonetheless, this result suggests that the lack of host specificity among some Group 1 strains may extend beyond Rosaceae, *Ceanothus*, and *Datisca* in California.

Our results tend to support the idea that this cluster of strains is shared among hosts from the 3 plant families. The results suggest 3 possibilities: (i) the lack of diversity reflects a numerical or biological dominance of 1 or a few closely related strains that infect multiple host species in the area sampled; (ii) there is currently strong selection limiting diversity, presumably due to some environmental factor such as soil type or moisture; or (iii) some past evolutionary bottleneck has reduced *Frankia* Group 1 diversity in our study area. These 3 hypotheses are not mutually exclusive, and it is difficult to differentiate among them with the evidence at hand. If the first hypothesis is correct, more diverged Group 1 strains may be present at low frequency in these soils and may come to dominance in root nodules on these plant taxa in specific but currently unidentified environmental conditions or geographical areas. The second and third hypotheses would predict that highly divergent Group 1 strains are absent from these soils such that further sampling would reveal little or no variation from the strains already identified. Proving the first scenario is only possible if additional sampling reveals more diverged strains; proving the alternatives that more diverged strains are absent, is not feasible in such a large geographical area.

Only 2 previous studies have examined *Frankia* diversity in sympatric actinorhizal species; both involved *Alnus*-infective *Frankia*, and the results of the 2 studies led to different conclusions. Clawson et al. (1999) found that *Myrica pensylvanica* Mirb. was nodulated with the same, or a closely related, strain, one that dominated nodules in an adjacent *Alnus incana* (L.) Moench subsp. *rugosa* (Du Roi) R.T. Clausen stand, leading to the conclusion that a strain can come to

dominance on more than 1 host species in a particular area. On the other hand, Hugué et al. (2001) found that, in a sympatric stand of *Alnus incana*, *Myrica gale* L., and *Shepherdia canadensis* (L.) Nutt., each plant species was nodulated by a distinct *Frankia* genotype. Group 3 *Frankia* inhabit *Shepherdia* nodules, but *Myrica gale* and *Alnus incana*, which are capable of hosting the same strains, apparently do not do so in the field. Our results suggest that a closely related group of strains of *Frankia* has come to dominance on all actinorhizal species that associate with Group 1 *Frankia* strains in the locations we examined. As mentioned, however, further study is needed to determine the extent to which our observations reflect lack of available diversity as opposed to dominance of 1 of several diverse strains. In other words, if this is a dominance effect, from how diverse a pool of possible strains is the dominant strain being selected? In this regard, the strains from *Cercocarpus ledifolius* and *Cowania stansburiana* Torr., which fall outside the cluster of strains collected here (Fig. 2), are intriguing. These strains were detected in nodules on greenhouse-grown plants that had been inoculated with nodule pieces and soil collected from beneath *Cowania stansburiana* (Spring Mountain, Nev.) and *Chamaebatia foliolosa* (Foresthill, Calif.), respectively, (D. Nelson, personal communication), suggesting that more diversity can be found in a broader geographical context, or that the artificial environment of a greenhouse may provide opportunities for different strains to nodulate host plants.

It has been noted previously that the environment in which the plants grow may determine which strains are available for nodulation. For example, although *Myrica gale* is nodulated by many strains of *Frankia* in the greenhouse, its predilection for growing in wet sites may limit the diversity of strains that inhabit nodules in the field (Clawson and Benson 1999; Clawson et al. 1999; Hugué et al. 2001). Similarly, particular strains that infect *Gymnostoma* L. A.S. Johnson spp. (Casuarinaceae) tend to appear in nodules of host species according to the kinds of soil in which the plants grow (Navarro et al. 1999). In the present case, the soils at sites varied considerably in both parent rock formation and soil moisture. Nodules collected from *Datisca* were found streamside in very wet soil, whereas nodules collected from the Ellery Lake site were dry. Sites also differed in elevation and plant community (Table 2). Our sites ranged from 700 m to 2800 m in altitude and were distributed over a geographical area of 212 500 km². Our results suggest that, in the case of *Frankia* associating with the Rosaceae, *Ceanothus*, and *Datisca*, site-specific environmental conditions are probably not as important in determining *Frankia* diversity.

Ritchie and Myrold (1999) also found limited diversity and lack of host specificity among strains of *Frankia* infecting 8 species of *Ceanothus* in Oregon, as assessed by PCR-RFLP and sequencing of the ITS. They did not examine all the genomic regions that we did, so their strains are not included in Fig. 2, but the ITS sequences of those strains suggest that they are very closely related to the ones examined here. However, we know that low strain richness is not a characteristic of Group 1 *Frankia* strains in general because of divergent 16S rDNA sequences collected from other members of this group, including *Coriaria arborea* Lindsay from New

Zealand and *Ceanothus americanus* L. from eastern North America (Fig. 2). The low diversity has been localized, so far, to the west coast of North America and may indicate that an as yet unknown factor has reduced the genetic variability of *Frankia* in this geographical location, perhaps through some past evolutionary bottleneck. It is important to point out, however, that this putative bottleneck would have affected only Group 1 *Frankia* strain diversity. Comparison of strain diversity in Group 2 strains within our study area found levels nearly identical to total Group 2 diversity, suggesting that reduced *Frankia* strain diversity is restricted only to Group 1 *Frankia* on the west coast.

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