

# Natural Diversity of *Frankia* Strains in Actinorhizal Root Nodules from Promiscuous Hosts in the Family Myricaceae

MICHAEL L. CLAWSON AND DAVID R. BENSON\*

Department of Molecular and Cell Biology, University of Connecticut, Storrs, Connecticut 06269-3044

Received 21 May 1999/Accepted 12 July 1999

**Actinorhizal plants invade nitrogen-poor soils because of their ability to form root nodule symbioses with N<sub>2</sub>-fixing actinomycetes known as *Frankia*. *Frankia* strains are difficult to isolate, so the diversity of strains inhabiting nodules in nature is not known. To address this problem, we have used the variability in bacterial 16S rRNA gene sequences amplified from root nodules as a means to estimate molecular diversity. Nodules were collected from 96 sites primarily in northeastern North America; each site contained one of three species of the family Myricaceae. Plants in this family are considered to be promiscuous hosts because several species are effectively nodulated by most isolated strains of *Frankia* in the greenhouse. We found that strain evenness varies greatly between the plant species so that estimating total strain richness of *Frankia* within myricaceous nodules with the sample size used was problematical. Nevertheless, *Myrica pensylvanica*, the common bayberry, was found to have sufficient diversity to serve as a reservoir host for *Frankia* strains that infect plants from other actinorhizal families. *Myrica gale*, sweet gale, yielded a few dominant sequences, indicating either symbiont specialization or niche selection of particular ecotypes. Strains in *Comptonia peregrina* nodules had an intermediate level of diversity and were all from a single major group of *Frankia*.**

Actinorhizal plants are defined by their ability to form N<sub>2</sub>-fixing root nodule symbioses with actinomycetes from the genus *Frankia* (2). The symbiosis uncouples the plants from a need for soil nitrogen. As a result, the plants and their infective symbionts have radiated into a remarkable variety of niches that include dry tropical soils, temperate wetlands, northern forests, sand dunes, chaparral and matorral, subarctic bogs and tundra, and glacial till (2).

Twenty-five genera of actinorhizal plants have been identified in eight families of angiosperms (2). One family, the Myricaceae in the subclass Hamamelidae, is considered the first actinorhizal family to emerge during the late Cretaceous (17, 19). Other actinorhizal families differentiated independently much later (29). Modern *Myrica* sp. have successfully adapted to habitats ranging from tropical and temperate dry soil and sand dunes to water-saturated bogs in northern temperate and subarctic regions.

Because of the wide variety of niches occupied by myricaceous plants, it is likely that some *Frankia* strains have adapted to symbiosis in the context of the prevailing conditions in specific niches. Such specialization can only be revealed by studying the diversity of strains that actually occupy nodules in nature. In greenhouse studies, *Myrica* spp. have the unusual ability to nodulate with virtually all isolated strains of *Frankia* tested. For this reason, they are considered to be “promiscuous” hosts, but the extent of their promiscuity in nature has not been addressed (1, 31).

Phenotypic diversity of *Frankia* strains has previously been studied primarily by isolating and characterizing representative strains from a handful of plant species (2). Nodules from many plants fail to yield isolates, and many isolates grow so slowly that a classical approach to diversity studies that includes isolation, cultivation, and physiological characterization is not practical. Developing an understanding of the broader issues

of diversity in actinorhizal nodules requires an approach that circumvents the need to isolate and grow strains in culture. In this study, we used the molecular ecological approach of amplifying partial 16S rRNA gene sequences (rDNAs) directly from root nodules of three species within the Myricaceae as a means to assess the richness and evenness of *Frankia* strains that inhabit root nodules (richness is the total number of unique sequences found in the nodules; evenness refers to the relative number of times that each unique sequence appears in the nodules sampled). *Comptonia peregrina* (L.) Coult. (sweet fern), *Myrica gale* L. (sweet gale, bog myrtle), and *Myrica pensylvanica* Loisel. (bayberry) were chosen as test plants because they overlap in their geographical distribution but occupy different niches in northeastern North America (24).

## MATERIALS AND METHODS

**Collection of root nodules.** To obtain a broad sample of *Frankia* strains and to avoid bias introduced by local strain dominance (10), root nodules were collected from 96 separate sites, including 30 with *C. peregrina*, 29 with *M. pensylvanica*, and 37 with *M. gale* (Table 1; Fig. 1). *C. peregrina* grows inland in open dry soil from Georgia and Minnesota to Nova Scotia. *M. pensylvanica* is distributed from North Carolina to Nova Scotia, particularly in coastal environments, and grows west to the Great Lakes. *M. gale* grows in bogs and borders lakes from northern New Jersey to Newfoundland and has a circumboreal distribution through Canada, Alaska, northwestern Europe, Scandinavia, and Asia (33). Most of the collection sites for this study were distributed from New Jersey to northern New York to Maine in the northeastern United States, but *M. gale* nodules were also received from Canada, Sweden, and Scotland and one *M. pensylvanica* plant was an introduced plant in Washington State (Fig. 1).

**Amplification of 16S rDNA from nodules.** The procedure for nodule dissection and DNA amplification has been described previously (3, 12). Field-collected nodules were frozen or stored in 95% ethanol. Single nodule lobes (1 to 10 mg of plant tissue) were excised from the nodules. After the lobes were washed, the periderm was removed in sterile TEA buffer (10 mM Tris-HCl, 1 mM Na<sub>2</sub>EDTA, 20 mM ascorbic acid [pH 7.6]). The nodule contents were macerated with a scalpel and rinsed in TEA buffer, and *Frankia* vesicle clusters (mycelial contents from a single infected cell) were washed and collected on nylon screens with Tris-EDTA buffer. Total DNA was extracted by using alkaline lysis and standard ammonium acetate-ethanol precipitation (26). A 380-bp fragment of *Frankia* 16S rDNA was PCR amplified with primers fD1 (32) and rDB1 (3) in quadruplicate reactions by using a Perkin-Elmer (Norwalk, Conn.) amplification kit. Amplicons were combined, purified on Qiagen (Santa Clarita, Calif.) columns, and cycle sequenced in both directions, and the sequencing reactions were

\* Corresponding author. Mailing address: University of Connecticut, 75 North Eagleville Rd., U-44, Storrs, CT 06269-3044. Phone: (860) 486-4258. Fax: (860) 486-1784. E-mail: dbenson@uconnvm.uconn.edu.

TABLE 1. Sources and accession numbers for 16S rDNA sequences

Sequence no. <sup>a</sup>	Source plants and strains, locations, and accession no. <sup>b</sup>	Reference
1	<i>Alnus glutinosa</i> nod, Chile (AF116171)	This study
2	<i>M. gale</i> nod, Sweden (AF116075)	This study
3	<i>M. pensylvanica</i> nod, Sandy Hook, N.J. (AF116076)	This study
4	<i>C. peregrina</i> nod, Mansfield, Conn. (AF116077)	This study
5	<i>Alnus acuminata</i> nod, Argentina (AF116172)	This study
6	<i>C. peregrina</i> nod, Windsor, Conn. (AF116078); <i>M. pensylvanica</i> nod, Nantucket, Mass. (AF116080), Middlebury, Conn. (AF116081), Manchester, Conn. (AF116083), Enfield, Conn. (AF116082); <i>M. gale</i> nod, Lake George, Maine (AF116079)	This study
7	<i>C. peregrina</i> nod, Storrs, Conn. (AF116084), Willington, Conn. (AF116085), Waterbury, Conn. (AF116086); <i>M. pensylvanica</i> nod, Bolton, Conn. (AF116087), Pomfret, Conn. (AF116088)	This study
8	<i>C. peregrina</i> nod, Sturbridge, Mass. (AF116089)	This study
9	<i>C. peregrina</i> nod, Union, Conn. (AF116090)	This study
10	<i>C. peregrina</i> nod, Bangor, Maine (AF116091)	This study
11	<i>M. gale</i> nod, Newfoundland (AF116092)	This study
12	<i>A. cordata</i> AcoN24d, Canada (L40610); <i>M. pensylvanica</i> nod, Nantucket, Mass. (AF116146)	21; This study
13	<i>M. pensylvanica</i> nod, Hartford, Conn. (AF116093)	This study
14	<i>M. pensylvanica</i> nod, Manchester, Conn. (AF116094)	This study
15	<i>C. peregrina</i> nod, Willington, Conn. (AF116095), Columbia, Conn. (AF116096), Ashford, Conn. (AF116097), Glastonbury, Conn. (AF116098); <i>M. pensylvanica</i> nod, Manchester, Conn. (AF116099)	This study
16	<i>A. glutinosa</i> nod, New Zealand (U82101); <i>M. gale</i> nod, Grand Lake, Maine (AF116147); Granby, Mass. (AF116148)	11; This study
17	<i>Myrica nagi</i> nod, India (L40622)	21
18	<i>C. peregrina</i> nod, Manchester, Conn. (AF116100)	This study
19	<i>C. peregrina</i> nod, Coventry, Conn. (AF116101)	This study
20	<i>C. peregrina</i> nod, Nantucket, Mass. (AF116102)	This study
21	<i>C. peregrina</i> nod, Mansfield, Conn. (AF116104), Storrs, Conn. (AF116103), Coventry, Conn. (AF116108), Nantucket, Mass. (AF116105), Colchester, Conn. (AF116106), Walpole, Mass. (AF116107)	This study
22	<i>C. peregrina</i> nod, Colchester, Conn. (AF116109)	This study
23	<i>Casuarina equisetifolia</i> nod, New Zealand (U82104)	11
24	<i>M. pensylvanica</i> nod, Andover, Conn. (AF116110)	This study
25	<i>A. incana</i> nod, Storrs, Conn. (AF116173); <i>C. peregrina</i> nod, Manchester, Conn. (AF116149); <i>M. pensylvanica</i> nod, Storrs, Conn. (AF116150), Danielson, Conn. (AF116152), Nantucket, Mass. (AF116151)	This study
26	<i>A. incana</i> strain 57-2, Storrs, Conn. (AF116174); <i>M. pensylvanica</i> nod, Southington, Conn. (AF116153)	10; This study
27	<i>A. rugosa</i> ARgP5, Quebec City, Canada	21
28	<i>C. peregrina</i> nod, Griswold, Conn. (AF116111), Lisbon, Conn. (AF116112)	This study
29	<i>M. pensylvanica</i> nod, Waterbury, Conn. (AF116113), Columbia, Conn. (AF116114)	This study
30	<i>M. gale</i> nod, Storrs, Conn. (AF116115), Old Town, Maine (AF116116), Peecham, Mass. (AF116117), Speculator, N.Y. (AF116118), White Lake, N.Y. (AF116119), Raquet Lake, N.Y. (AF116120), Newcomb, N.Y. (AF116121), Alexander, Maine (AF116122, AF116136), Baskhegan Lake, Maine (AF116123), Parks Pond, Maine (AF116124), Amherst, Maine (AF116125), Orange, Mass. (AF116126), Otis, Mass. (AF116127), Pelham, Mass. (AF116128), Shutesbury, Mass. (AF116129)	This study
31	<i>M. gale</i> nod, Mercer Bog, Maine (AF116130)	
32	<i>A. viridis</i> nod, Christchurch, New Zealand (U82103); <i>M. gale</i> nod, Mercer Bog, Maine (AF116156), Gilead, Maine (AF116155, AF116157), Great Pond, Maine (AF116158), Aurora, Maine (AF116159), Scotland, United Kingdom (AF116160), Algonquin National Park, Canada (AF116161), Sweden (AF116154)	11; This study
33	<i>A. cordata</i> nod, Hamilton, New Zealand (U82102); <i>M. gale</i> , White Lake, N.Y. (AF116162), unknown location, N.H. (AF116163), Woodland, Maine (AF116164), Houlton, Maine (AF116165), Lake Mattawamkeg, Maine (AF116166), Bangor, Maine (AF116167), Becket, Mass. (AF116168)	11; This study
34	<i>C. peregrina</i> nod, Storrs, Conn. (AF116131), Putnam, Conn. (AF116132), Phoenixville, Conn. (AF116133), Gilman, Conn. (AF116134), Bolton, Conn. (AF116135); <i>M. pensylvanica</i> nod, Nantucket, Mass. (AF116137)	This study
35	<i>M. pensylvanica</i> nod, Washington State (AF116138)	This study
36	<i>Trevoa trinervis</i> nod, Chile (AF063642)	12
37	<i>Talguenea quinquinervis</i> nod, Chile (AF063643)	12
38	<i>M. pensylvanica</i> nod, Nantucket, Mass. (AF116139)	This study
39	<i>M. pensylvanica</i> nod, Nantucket, Mass. (AF116140), Manchester, Conn. (AF116141)	This study
40	<i>M. pensylvanica</i> nod, Nantucket, Mass. (AF116142), Waterbury, Conn. (AF116143)	This study
41	<i>Colletia hystrix</i> nod, Chile (AF063640); <i>M. pensylvanica</i> nod, Southington, Conn. (AF116169)	12; This study
42	<i>Elaeagnus angustifolia</i> nod, Willington, Conn. (AF064144); <i>M. pensylvanica</i> nod, Phoenixville, Conn. (AF116170)	12; This study
43	<i>E. angustifolia</i> strain EA 1-2, France (L40618)	16

Continued on following page

TABLE 1—Continued

Sequence no. <sup>a</sup>	Source plants and strains, locations, and accession no. <sup>b</sup>	Reference
44	<i>Discaria toumatou</i> nod, New Zealand (U54609)	11
45	<i>M. pennsylvanica</i> nod, Nantucket, Mass. (AF116144)	This study
46	<i>Coriaria arborea</i> nod, New Zealand (U54610)	3
47	<i>Coriaria plumosa</i> nod, New Zealand (U82100)	11
48	<i>Purshia tridentata</i> nod, New Zealand (U54611)	3
49	<i>Ceanothus americanus</i> nod, Storrs, Conn. (AF063639)	12
50	<i>Ceanothus caeruleus</i> strain Cea1.3, Mexico (U72717)	25
51	<i>C. caeruleus</i> strain Cea5.1, Mexico (U72718)	25
52	<i>M. pennsylvanica</i> nod, Nantucket, Mass. (AF116145)	This study

<sup>a</sup> Numbers correspond to those shown in Fig. 2.

<sup>b</sup> Accession numbers are from GenBank. nod, sequences obtained from root nodules.

analyzed with an Applied Biosystems (Perkin-Elmer) Prism sequencer, all according to manufacturers' protocols.

**Mathematical estimates of diversity.** Diversity indices were from Magurran (18). The Alpha and Shannon indices give particular weight to species richness within a sample, with higher values reflecting greater richness. The Berger-Parker (reciprocal) and Simpson (reciprocal) indices are weighted towards dominance, with a lower number representing greater dominance in the sample.

Estimates of total strain abundance were obtained by using the abundance-based coverage estimator (ACE) (9) and second-order jackknife (jackknife) (7, 8, 13, 23); these are nonparametric estimates of the true richness of *Frankia* strains (as defined by 16S rDNA sequence variation) found in *C. peregrina*, *M. gale*, and *M. pennsylvanica*. The effect of sample size on richness estimates was analyzed by using the EstimateS program (13) with 100 randomizations at each sample accumulation level. In this analysis,  $S_{obs}$  is the mean number of strains observed in the pooled randomized samples. The ACE is calculated from each sample, and the mean value for each estimator is computed for each sample accumulation level (9). As the samples increase, the mean ACE value increases and reaches a maximum that corresponds to the best estimate of the total number of unique strains (13).

**Dendrogram construction.** To construct trees, 16S rDNA sequences were initially aligned by using Pileup in the Genetics Computer Group package (14). The alignments were reformatted in ClustalW (30) and checked by eye. All sites in the 380-bp region were included in the analysis. Dendrograms were constructed by using PHYLIP (15). A distance matrix was made by using the maximum likelihood option in DNADIST with a transition/transversion ratio of 2. A neighbor-joining tree was then constructed in NEIGHBOR. The tree was viewed as a cladogram in TREEVIEW (22).

RESULTS

**Reproducibility and amplification.** By using only healthy nodule lobes, all attempts to amplify DNA from nodules were successful in this study. We avoided problems with errors due to mispriming and *Taq* misincorporation by using quadruplicate amplifications from the template DNA. The consistency of the data was supported by the observation that random

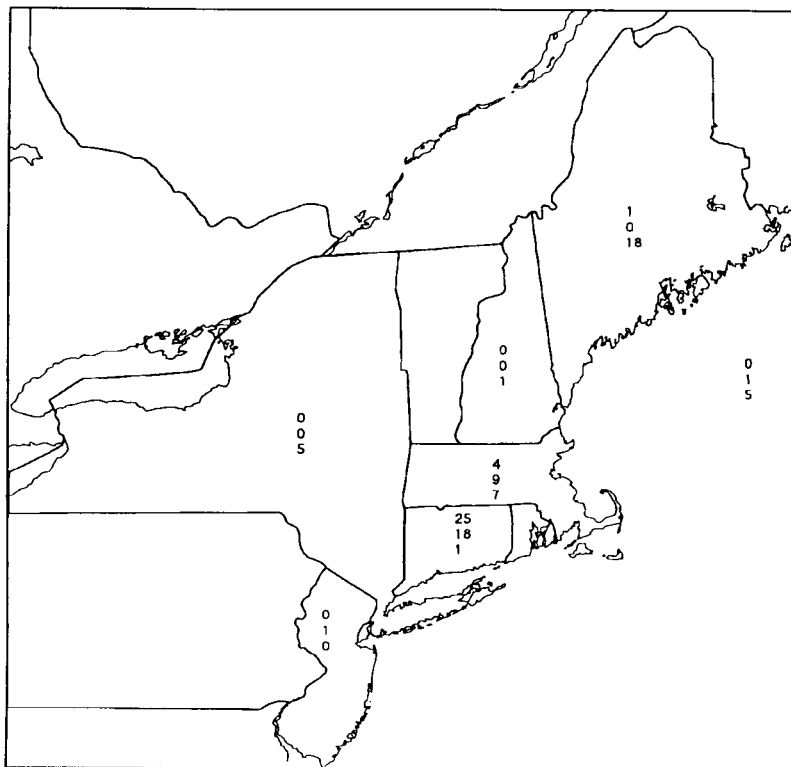


FIG. 1. Geographical distribution of collection sites for root nodules from the Myricaceae. Numbers indicate the number of nodules taken from each state. The first, second, and third numbers correspond to the number of samples obtained from each state for *C. peregrina*, *M. pennsylvanica* and *M. gale*, respectively. Numbers to the right correspond to nodules collected from beyond the geographical range shown.

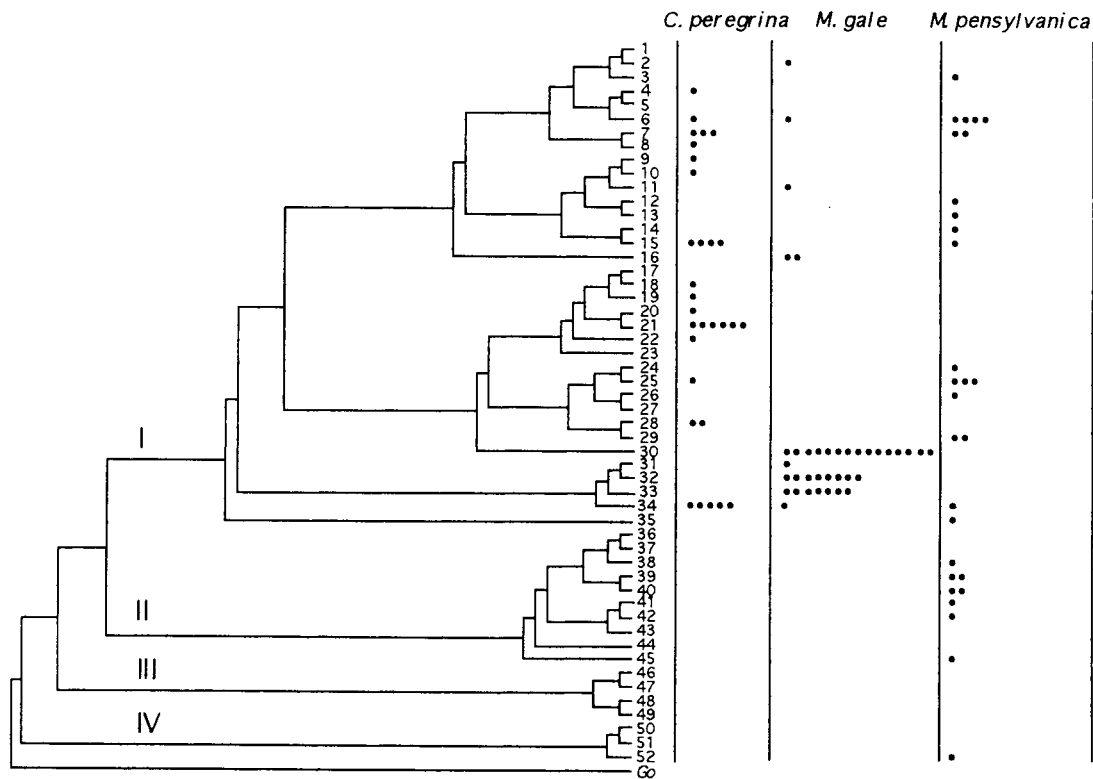


FIG. 2. Neighbor-joining tree of partial 16S rDNA from *Frankia* strains. The outer taxonomic unit numbers correspond to the sequence numbers in Table 1, and the frequency of occurrence of a sequence in *C. peregrina*, *M. gale*, or *M. pensylvanica* is shown by the number of bullets in the columns to the right of the tree. Groups I to IV correspond to the major lineages of *Frankia* identified by Normand et al. (21). Group I *Frankia* strains nodulate actinorhizal plants from the hamamelid families Betulaceae, Casuarinaceae and Myricaceae; Group II *Frankia* are typical inhabitants of nodules from the families Elaeagnaceae and Rhamnaceae and some hamamelid genera; group III *Frankia* strains occupy nodules of the families Rosaceae, Coriariaceae, and Datisceae plus *Ceanothus* of the family Rhamnaceae; group IV *Frankia* strains are a loosely associated group of Nod<sup>-</sup> and Fix<sup>-</sup> actinomycetes. The tree was rooted with the 16S rDNA sequence from *Geodermatophilus obscurus* subsp. *obscurus* (accession no. L40620).

substitutions in conserved regions of the 16S rDNA molecule were not found.

**Analysis of diversity.** Variability between *Frankia* 16S rDNA sequences was used as a measure of diversity. A neighbor-joining tree relating the sequences from this study plus published reference sequences was constructed and is shown in Fig. 2. The four groups designated I to IV in Fig. 2 have been identified as four clades in previous studies on the phylogenetic relationships of *Frankia* (21).

*M. gale* nodules yielded a collection of sequences characterized by low diversity. Nine different sequences were found, and all grouped in group I (Fig. 2). Three sequences clearly dominated in *M. gale* as they were found in 81% of the nodules. The most abundant sequence (15 nodules) was found only in *M. gale* nodules. The other dominant sequences were identical to ones previously reported from *Alnus cordata* and *Alnus viridis* nodules, both collected from wet sites in New Zealand (11). Of the remaining six sequences, three were unique to *M. gale*, two were also found in nodules from *C. peregrina* and *M. pensylvanica*, and one was reported previously in nodules from *Alnus glutinosa*, also growing in New Zealand (11).

The number of different *Frankia* sequences found in *C. peregrina* nodules was relatively higher than that found in nodules from *M. gale*; 15 different sequences from 30 nodules all grouped in group 1. Ten sequences are unique to *C. peregrina*, and five are identical to sequences also found in either *M. gale* or *M. pensylvanica*, or both. In addition, one sequence was the

same as one found in *Alnus incana* nodules growing in Connecticut. *C. peregrina* is therefore nodulated primarily by diverse strains from group 1. Although three of the 15 sequences account for 50% of the total, the dominance of *Frankia* strains is less pronounced in *C. peregrina* than in *M. gale*.

The number of *Frankia* strains found within *M. pensylvanica* nodules was clearly higher than that seen in nodules of the other two species. Twenty sequences that spanned three of the four groups were found among the 29 nodules examined (Fig. 2). Twenty nodules contained 13 sequences that group with symbionts in group I, eight nodules yielded six sequences that clustered with symbionts typical of the families Elaeagnaceae and Rhamnaceae in group II, and one nodule yielded a sequence that groups with those of Nod<sup>-</sup> and Fix<sup>-</sup> actinomycetes that define group IV. In addition to five sequences also found in nodules from *M. gale* and/or *C. peregrina*, three were the same as one from an *A. incana* nodule in Connecticut, one was identical to another from an *A. incana* nodule in Connecticut, one was also found in an *A. cordata* nodule from Canada, and two from group II had been reported previously from *Colletia hystrix* nodules in Chile and an *Elaeagnus angustifolia* nodule in Connecticut. Thus, *M. pensylvanica* seems to have the ability to associate with many *Frankia* strains, and very similar or identical *Frankia* strains are geographically widespread.

**Estimates of diversity.** To gain additional insight into the diversity of 16S rRNA sequences in these root nodules, several

TABLE 2. Diversity indices and estimates<sup>a</sup>

Species	Richness <sup>b</sup> (no. of nodules)	Richness indices		Evenness indices <sup>c</sup>		Abundance estimates <sup>d</sup>	
		Alpha	Shannon	Berger-Parker (1/d)	Simpson (1/D)	ACE	Jackknife
<i>C. peregrina</i>	15 (30)	11.94	2.43	5.00	12.43	34.66	33.10
<i>M. gale</i>	9 (37)	3.79	1.66	2.47	4.30	19.38	17.67
<i>M. pensylvanica</i>	20 (29)	28.47	2.87	7.25	31.23	45.11	42.96

<sup>a</sup> Indices are from reference 18. Abundance estimates are from reference 13.

<sup>b</sup> Richness is the total number of unique sequences found in the nodules.

<sup>c</sup> Evenness reflects the relative number of each unique sequence found in the samples. 1/d and 1/D are reciprocal forms of the indices.

<sup>d</sup> Abundance estimates refers to the total number of unique sequences that might be expected based on the characteristics of the sample used.

indices were computed by using the information shown in Fig. 2 (Table 2); each unique sequence was treated as a strain of *Frankia*. Indices that were weighted towards strain richness (as assessed by the total number of unique sequences) consistently reflected the higher diversity of strains in *M. pensylvanica* nodules relative to *C. peregrina* and especially *M. gale* nodules. Indices that weighted evenness (as assessed by the relative number of each unique sequence) reflected the dominance of a few strains in *M. gale*, the lower dominance of strains seen in *C. peregrina*, and the relatively greater evenness found in *M. pensylvanica* nodules.

The estimates of true species richness (ACE and second-order jackknife), in this case the estimated total number of unique sequences that might be expected to be found in the nodules from each species, yielded very similar estimates. Given the limited number of environments sampled in relation to the large number of microenvironments in which the plant and microorganism might encounter each other, these estimates can be considered to be the minimum number of strains actually able to infect the plants. To test the relationship between sample size and estimates of total strain richness, ACE values were analyzed by using the EstimateS program with 100 randomizations of sample order (13). In this analysis,  $S_{\text{obs}}$  is the mean number of different sequences observed on average in the pooled randomized samples at each sample accumulation level. ACE is the mean estimator calculated for each randomized sample at each accumulation level (9). As sample size increases, the ACE value should increase to a maximum and then stabilize if the estimates are robust. If more samples are required for an accurate estimate, ACE continues to increase. As illustrated in Fig. 3, the estimates for *M. pensylvanica* appear to stabilize whereas those for *C. peregrina* and *M. gale* continue to increase slowly with sample addition. This analysis suggests that for hosts that have dominant strains (low

evenness), sample sizes should be much larger for estimating total species richness.

## DISCUSSION

Many *Frankia* strains have not been grown in culture, and others grow slowly once isolated (2). Therefore, the diversity of strains that actually live in root nodules in natural systems has been difficult to address. Several studies have used 16S rDNAs of *Frankia* as a way to group strains for phylogenetic analyses (21). The 380-bp portion used here is located at the 5' end of the gene and contains about 45% of the known variable sites found among available *Frankia* 16S rRNA genes (12). Trees constructed by using this region place *Frankia* strains into the same major groups as those found with longer 16S rDNA sequences, albeit with lower resolution (12). Strains differing at two or more positions in the region have recognizably different total protein profiles, colony morphologies, and carbon source utilization patterns (10). While the portion of the 16S rDNA used here has phylogenetic information, we were primarily interested in using it as a relatively rapid means to estimate the natural diversity of *Frankia* populations. In that context, two nodule inhabitants that have the same sequence can be viewed as being the same, or very closely related, strains. This view is supported by the observation that the inhabitants of five nodules from *M. gale*, growing in separate locations, that had identical short 16S rDNA sequences also had identical glutamine synthetase I gene (*glnA*) sequences (data not shown).

The molecular ecological approach used here has allowed us to estimate the strain richness and evenness of *Frankia* strains in the root nodules of three species from the plant family Myricaceae. We found that species (or strain) evenness differs dramatically in naturally occurring root nodules. The major groups I to IV in Fig. 2 relate closely to the major groups of

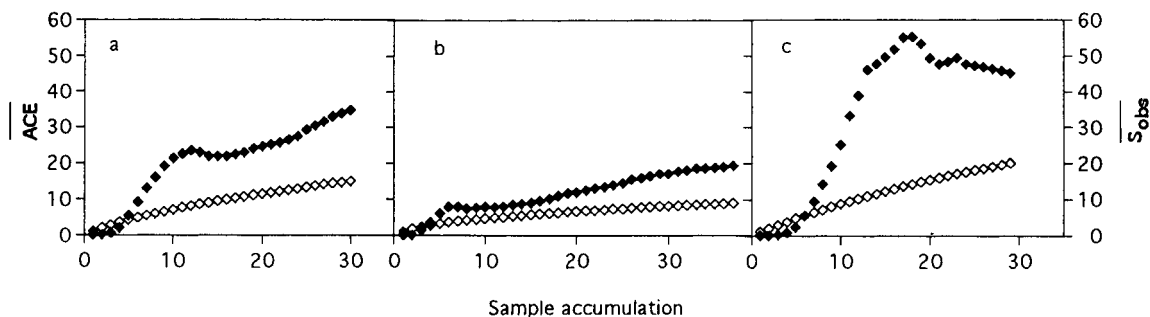


FIG. 3. Variability in abundance-based coverage estimates (ACE values) as samples accumulate. Data are from Fig. 2.  $S_{\text{obs}}$  is the mean total number of strains observed in the pooled randomized samples at each accumulation level (open diamonds). ACE (closed diamonds) values are the mean estimators computed by using the  $S_{\text{obs}}$  values at each accumulation level. Sample accumulation refers to the number of nodules used. As samples are added, the estimated total richness increases to a maximum. (a) *C. peregrina*; (b) *M. gale*; (c) *M. pensylvanica*.

plants that participate in the actinorhizal symbiosis. Group I includes strains that are typically found in, and are isolated from, nodules of plants in the hamamelid families Betulaceae, Casuarinaceae, and Myricaceae and is composed of more than one major subgroup (21, 34). Group II includes symbionts and isolates from plants in the families Elaeagnaceae and Rhamnaceae, as well as some hamamelid families (20). Group III contains symbionts from plants in the families Coriariaceae, Datisceae, Rosaceae and from *Ceanothus* of the Rhamnaceae (3, 12, 25). Group IV contains *Frankia*-like organisms whose role in symbiosis is unclear (25); the latter have been isolated from a variety of nodules but are noninfective when tested on actinorhizal plants. Isolates that represent groups I, II, and IV but not group III have been obtained.

*M. pensylvanica* was unusual in the sense that *Frankia* from groups I, II, and IV were found in its nodules. The species can thus be considered to be promiscuous in nature (31) and possibly a reservoir for different major lineages of *Frankia*. The existence of such reservoir plants may explain why certain actinorhizal species nodulate wherever they are planted, regardless of whether plants from the same group have previously grown in the vicinity (2). Previous studies on *Frankia* strains isolated from *M. pensylvanica* nodules have also indicated that many strains were present although the relationship of one strain with another could not be determined at the time (4, 5).

In contrast to *M. pensylvanica*, we found widely collected *M. gale* nodules to be primarily occupied by a limited set of strains from group I despite the observation that *M. gale* seedlings are infected by *Frankia* strains or crushed nodule inocula originating from many actinorhizal plants in the greenhouse (31). The major strain was not found in other myricaceous nodules examined despite its apparent abundance in *M. gale*. Thus, it seems likely that this strain has specialized in infecting *M. gale* in the context of its environment. The low diversity of strains may explain why isolating *Frankia* from *M. gale* nodules is often very difficult. In a previous study, only 30 of 3,000 (1%) *M. gale* nodule lobes prepared yielded isolated *Frankia* strains (28). Interestingly, one of the isolates obtained in that study had characteristics of group II strains. Therefore, *M. gale* exhibits promiscuity but at a very low frequency in nature.

*C. peregrina* nodules have an intermediate level of diversity with all strains grouping within group I. Some sequences are common since three accounted for half of the total number of sequences obtained. It is possible that additional sampling would show a distinct preference for one or a few sequences within *Comptonia* nodules. Although *M. pensylvanica* and *C. peregrina* share a predilection for colonizing dry sites, *C. peregrina* apparently does not commonly associate with *Frankia* strains from group II, at least at a sufficiently high frequency to be detected in our sample. This latter observation suggests that *C. peregrina* is genetically disposed to maintain a more restrictive level of symbiont specificity than *M. pensylvanica*.

Estimates of the total strain richness shown in Table 2 suggest that dozens of *Frankia* strains can inhabit myricaceous root nodules. Although that conclusion is likely to be correct, the estimates for individual species must be interpreted with caution since they depend on having a sufficient sample for the analysis. Calculating the ACE values by using random addition of samples (Fig. 3) indicates that the total number of strains that might be found to infect *M. gale* and *C. peregrina* in the field is likely to be higher than that shown in Table 2 since the ACE continues to increase with sample addition. On the other hand, the ACE for *M. pensylvanica* stabilizes, suggesting that the diversity of 16S sequences for these nodules is reflected in the sample used. The low evenness (dominance) observed in

*M. gale* and *C. peregrina* requires that a much larger sample set be used to estimate total species richness. It is likely that new strains would continue to emerge as more samples were analyzed to the extent that, if enough nodules could be examined, the total species richness in these plants may approach that of *M. pensylvanica*.

The characteristics of the niches occupied by the plants may largely explain the differences in diversity observed. *C. peregrina* and *M. pensylvanica* inhabit sandy soils or sand dunes that experience prolonged periods of dryness. Such conditions may be more conducive to the accumulation of frankia spores rather than vegetative hyphae whose growth would be limited by lack of moisture and organic matter. Thus, bayberry and sweet fern roots may be exposed to a high diversity but low overall populations of infective cells with the result that symbioses are formed with a greater variety of strains. In contrast, *M. gale* typically grows in and around wet habitats such as bogs and ponds, where the soil is water saturated and often acidic (27). Such factors are known to affect the ability of some *Frankia* strains to reinfect their host plants (6). Thus, the dominant *Frankia* strains in *M. gale* nodules may be those capable of living in an environment characterized by low oxygen and acidic pH. It would be interesting to determine if those strains have specialized in nodulating *M. gale* to the exclusion of other actinorhizal species.

The extent to which the observed levels of diversity stem from the ecology of *Frankia* populations in the soil in addition to plant and microbial genetic factors is unknown. Nevertheless, the variability we have observed shows that the diversity of *Frankia* strains that occupy field-grown nodules in the Myricaceae differs at the plant species level rather than at the genus or family levels. The common occurrence of identical sequences in nodules from different plants at widely separated sites indicates that the host ranges of some groups of *Frankia* strains in the field can be quite broad and that some strains are cosmopolitan (Table 1). Their distribution may best be described in terms of a geographical mosaic, and their interactions with their hosts take place within the constraints of each part of the mosaic.

#### ACKNOWLEDGMENTS

The following are gratefully acknowledged for sending root nodules for this study: D. Baker, C. Benson, M. Carú, J. Clawson, D. Eveleigh, O. Harriott, K. Huss-Danell, T. Hurd, R. Parsons, and C. Schwintzer. We thank Robert K. Colwell for reviewing the manuscript.

This project was supported by the U.S. Department of Agriculture, National Research Initiative Competitive Grants Program.

#### REFERENCES

- Baker, D. D. 1987. Relationships among pure cultured strains of *Frankia* based on host specificity. *Physiol. Plant* **70**:245-248.
- Benson, D. R., and W. B. Silvester. 1993. Biology of *Frankia* strains, actinomycete symbionts of actinorhizal plants. *Microbiol. Rev.* **57**:293-319.
- Benson, D. R., D. W. Stephens, M. L. Clawson, and W. B. Silvester. 1996. Amplification of 16S rRNA genes from *Frankia* in root nodules of *Ceanothus griseus*, *Coriaria arborea*, *Coriaria plumosa*, *Discaria toumatou*, and *Purshiatridentata*. *Appl. Environ. Microbiol.* **62**:2904-2909.
- Bloom, R. A., M. P. Lechevalier, and R. L. Tate III. 1989. Physiological, chemical, morphological, and plant infectivity characteristics of *Frankia* isolates from *Myrica pensylvanica*: correlation to DNA restriction patterns. *Appl. Environ. Microbiol.* **55**:2161-2166.
- Bloom, R. A., B. C. Mullin, and R. L. Tate III. 1989. DNA restriction patterns and DNA-DNA solution hybridization studies of *Frankia* isolates from *Myrica pensylvanica* (bayberry). *Appl. Environ. Microbiol.* **55**:2155-2160.
- Bond, G. 1951. The fixation of nitrogen associated with the root nodules of *Myrica gale* L., with special reference to its pH relation and ecological significance. *Ann. Bot. (London)* **15**:447-459.
- Burnham, K. P., and W. S. Overton. 1978. Estimation of the size of a closed population when capture probabilities vary among animals. *Biometrika* **65**: 623-633.

8. **Burnham, K. P., and W. S. Overton.** 1979. Robust estimation of population size when capture probabilities vary among animals. *Ecology* **60**:927–936.
9. **Chazdon, R. L., R. K. Colwell, J. S. Denslow, and M. R. Guariguata.** 1998. Statistical methods for estimating species richness of woody regeneration in primary and secondary rain forests of NE Costa Rica, p. 285–309. *In* F. Dallmeier and J. A. Comiskey (ed.), *Forest biodiversity research, monitoring and modeling: conceptual background and Old World case studies*. Parthenon Publishing, Paris, France.
10. **Clawson, M. L., and D. R. Benson.** Dominance of *Frankia* strains in stands of *Alnus incana* subsp. *rugosa* and *Myrica pensylvanica*. *Can. J. Bot.*, in press.
11. **Clawson, M. L., D. R. Benson, D. W. Stephens, S. C. Resch, and W. B. Silvester.** 1997. Typical *Frankia* infect actinorhizal plants exotic to New Zealand. *N. Z. J. Bot.* **35**:361–367.
12. **Clawson, M. L., M. Cará, and D. R. Benson.** 1998. Diversity of *Frankia* strains in root nodules of plants from the families Elaeagnaceae and Rhamnaceae. *Appl. Environ. Microbiol.* **64**:3539–3543.
13. **Colwell, R. K.** 1997. EstimateS: statistical estimation of species richness and shared species from samples, version 5. 26 February 1999, posting date. User's guide and application. [Online.] <http://viceroy.eeb.uconn.edu/estimates>. [26 August 1999, last date accessed.]
14. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* **12**:387–395.
15. **Felsenstein, J.** 1993. PHYLIP (Phylogeny Inference Package), version 3.5c. Distributed by the author. Department of Genetics, University of Washington, Seattle.
16. **Fernandez, M. P., H. Meugnier, P. A. D. Grimont, and R. Bardin.** 1989. Deoxyribonucleic acid relatedness among members of the genus *Frankia*. *Int. J. Syst. Bacteriol.* **39**:424–429.
17. **Maggia, L., and J. Bousquet.** 1994. Molecular phylogeny of the actinorhizal Hamamelidae and relationships with host promiscuity towards *Frankia*. *Mol. Ecol.* **3**:459–467.
18. **Magurran, A. E.** 1988. *Ecological diversity and its measurement*. Princeton University Press, Princeton, N.J.
19. **Muller, J.** 1981. Fossil pollen records of extant angiosperms. *Bot. Rev.* **47**:1–143.
20. **Navarro, E., R. Nalin, D. Gauthier, and P. Normand.** 1997. The nodular microsymbionts of *Gymnostoma* spp. are *Elaeagnus*-infective *Frankia* strains. *Appl. Environ. Microbiol.* **63**:1610–1616.
21. **Normand, P., S. Orso, B. Cournoyer, P. Jeannin, C. Chapelon, J. Dawson, L. Evtushenko, and A. K. Misra.** 1996. Molecular phylogeny of the genus *Frankia* and related genera and emendation of family *Frankiaceae*. *Int. J. Syst. Bacteriol.* **46**:1–9.
22. **Page, R. D. M.** 1996. TREEVIEW: an application to display phylogenetic trees on personal computers. *Comput. Appl. Biosci.* **12**:357–358.
23. **Palmer, M. W.** 1991. Estimating species richness: the second-order jackknife reconsidered. *Ecology* **72**:1512–1513.
24. **Petrides, G. A.** 1958. *A field guide to trees and shrubs*. The Riverside Press Cambridge, Boston, Mass.
25. **Ramirez-Saad, H., J. D. Janse, and A. D. L. Akkermans.** 1998. Root nodules of *Ceanothus caeruleus* contain both the N<sub>2</sub>-fixing *Frankia* endophyte and a phylogenetically related Nod<sup>+</sup>/Fix<sup>-</sup> actinomycete. *Can. J. Microbiol.* **44**:140–148.
26. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
27. **Schwintzer, C. R.** 1990. Spore-positive and spore-negative nodules, p. 177–193. *In* C. R. Schwintzer and J. D. Tjepkema (ed.), *The biology of Frankia and actinorhizal plants*. Academic Press, Inc., New York, N.Y.
28. **St-Laurent, L., and M. Lalonde.** 1986. Isolation and characterization of *Frankia* strains isolated from *Myrica gale*. *Can. J. Bot.* **65**:1356–1363.
29. **Swensen, S. M.** 1996. The evolution of actinorhizal symbioses: evidence for multiple origins of the symbiotic association. *Am. J. Bot.* **83**:1503–1512.
30. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**:4673–4680.
31. **Torrey, J. G.** 1990. Cross-inoculation groups within *Frankia*, p. 83–106. *In* C. R. Schwintzer and J. D. Tjepkema (ed.), *The biology of Frankia and actinorhizal plants*. Academic Press, Inc., New York, N.Y.
32. **Weisburg, W. G., S. M. Barns, D. A. Pelletier, and D. J. Lane.** 1991. 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* **173**:697–703.
33. **Wheeler, C. T., and I. M. Miller.** 1990. Current and potential uses of actinorhizal plants in Europe, p. 365–389. *In* C. R. Schwintzer and J. D. Tjepkema (ed.), *The biology of Frankia and actinorhizal plants*. Academic Press, Inc., New York, N.Y.
34. **Wolters, D. J., C. Van Dijk, E. G. Zoetendal, and A. D. L. Akkermans.** 1997. Phylogenetic characterization of ineffective *Frankia* in *Alnus glutinosa* (L.) Gaertn. nodules from wetland soil inoculants. *Mol. Ecol.* **6**:971–981.