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Diversity and Specificity of *Frankia* Strains in Nodules of Sympatric *Myrica gale, Alnus incana*, and *Shepherdia canadensis* Determined by *rrs* Gene Polymorphism

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The identity of *Frankia* strains from nodules of *Myrica gale, Alnus incana* subsp. *rugosa*, and *Shepherdia canadensis* was determined for a natural stand on a lake shore sand dune in Wisconsin, where the three actinorhizal plant species were growing in close proximity, and from two additional stands with *M. gale* as the sole actinorhizal component. Unisolated strains were compared by their 16S ribosomal DNA (rDNA) restriction patterns using a direct PCR amplification protocol on nodules. Phylogenetic relationships among nodular *Frankia* strains were analyzed by comparing complete 16S rDNA sequences of study and reference strains. Where the three actinorhizal species occurred together, each host species was nodulated by a different phylogenetic group of *Frankia* strains. *M. gale* strains from all three sites belonged to an *Alnus-Casuarina* group, closely related to *Frankia alni* representative strains, and were low in diversity for a host genus considered promiscuous with respect to *Frankia* microsymbiont genotype. *Frankia* strains from *A. incana* nodules were also within the *Alnus-Casuarina* cluster, distinct from *Frankia* strains of *M. gale* nodules at the mixed actinorhizal site but not from *Frankia* strains from two *M. gale* nodules at a second site in Wisconsin. *Frankia* strains from nodules of *S. canadensis* belonged to a divergent subset of a cluster of *Elaeagnaceae*-infective strains and exhibited a high degree of diversity. The three closely related local *Frankia* populations in *Myrica* nodules could be distinguished from one another using our approach. In addition to geographic separation and host selectivity for *Frankia* microsymbionts, edaphic factors such as soil moisture and organic matter content, which varied among locales, may account for differences in *Frankia* populations found in *Myrica* nodules.

The nitrogen-fixing actinomycete *Frankia* establishes a symbiosis with actinorhizal plants, leading to root nodule formation. The host plants, distributed among eight different families and more than 200 species (9), typically colonize N-deficient and disturbed environments such as bogs, sandy coastal dunes, arctic tundra, mine spoils, and volcanic soils. Many actinorhizal species occur early after disturbances and are pioneer species.

Among the actinorhizal genera, *Myrica*, belonging to the *Myricaceae* family, has the widest geographic distribution. This genus is divided into 35 species, with 28 reported to be actinorhizal (9). They are small trees or shrubs common to nearly all major landmasses and with species occurring from tropical to temperate areas. According to morphological features (17), fossil and pollen records (26, 39), and a recent molecular study based on the *rbcL* gene (35), the *Myricaceae* family is considered the most primitive actinorhizal family.

Since the first confirmed isolation of the microsymbiont genus *Frankia* (11), a variety of methods have been used to obtain a coherent classification of the bacterial symbiont at the species level: soluble protein patterns (5, 6, 25), isoenzyme patterns (24), fatty acids (58), serology (4), DNA-DNA relatedness (1, 2, 23), and genome (19) and plasmid restriction analyses (52). These studies concluded that *Frankia* strains were heterogeneous and clustered within two main groups: the *Alnus-Myrica-Casuarina* group and the *Elaeagnaceae* group. Strains that infect *Myrica* spp. often grouped with *Alnus*-infective strains. However, some studies based on proteins (25, 55), host specificity (3), and genetic characterization (13, 60) pointed out the existence of strains isolated from or identified on *Myrica* spp. nodules having close relationships with *Elaeagnaceae*-infective strains. These studies led researchers to consider *Myrica* spp. promiscuous. Maggia and Bousquet (35) supported this hypothesis and linked it to the evolutionary position of the host plant. These authors have proposed that host plant evolution has proceeded toward more specificity, and therefore the most primitive actinorhizal plants, such as *Myrica* spp., should have a broad host range.

However, studies of strains isolated from *Myrica* spp. have some experimental limitations: (i) pure cultures tested for their infectivity often appeared to be atypical because they nodulated *Elaeagnaceae* but not their original host plant, (ii) some of the pure cultures were obtained after passage through an intermediate host, generally an *Alnus* species, before isolation, and (iii) to avoid the bias due to the isolation and culture steps, some authors identified the strains using direct detection in plant nodules, but the approaches used, even when based on the *rrs* gene or on the 16S-23S intergenic spacer, never yielded total 16S sequences allowing precise phylogenetic positioning.

The purpose of this study was to further elucidate phylogenetic and ecological relationships of *Myrica*-infective strains, *Alnus*-infective strains, and *Elaeagnaceae*-infective strains. We

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TABLE 1. Nodules and isolates used in this study

Site no.	Nodule or strain[a]	Designation[b]	Original host plant	Geographic origin	Source or reference
1	*Alnus* nodules (An)[a]	1-1 to 1-7 2-1 to 2-9 3-1 to 3-8	*A. incana* subsp. *rugosa*	Door County, Wis.	This study
	Shepherdia nodules (Sn)	1-1 to 1-4 2 3-1 to 3-4 4-1 to 4-3 5-1 to 5-8	*S. canadensis*		This study
	Myrica nodules				
	MMn	1-1 to 1-13 2 3-1 to 3-6	*M. gale*		This study
2	MRn	1-1 to 1-4 2-1 to 2-5	*M. gale*		This study
3	MWn[c]	1-1 to 1-2	*M. gale*	Botanical Garden, Glasgow, U.K.	This study
	Myrica isolates[d]				
	M16386		*M. californica*	Westport-Legget, Calif.	31
	M16464		*M. pensylvanica*	Harvey Cedars, N.J.	7
	M16467		*M. pensylvanica*	Tinton Falls, N.J.	7
	MgI5		*M. gale*	Tupper Lake, N.Y.	31
	MpI1		*M. pensylvanica*	Nantucket, Mass.	33

[a] The designation used in phylogenetic trees is shown.
[b] The first digit represents the plant number, and the second digit represents the nodule number on this plant.
[c] Kindly provided by C. T. Wheeler (University of Glasgow, Glasgow, U.K.).
[d] Kindly provided by D. Labeda (U.S. Department of Agriculture [USDA]). The accession numbers in the USDA collection are NRRL B-16386, NRRL B-16464, NRRL B-16467, NRRL B-16404, and NRRL B-16317, respectively, and the registration numbers are LLR 160401, RBR 162013, RBR 162021, DDB 16110210, and LLR 162001, respectively.

studied nodular *Frankia* strains from a site on which three different actinorhizal plants belonging to different infectivity groups (*Myrica gale, Alnus incana* subsp. *rugosa*, and *Shepherdia canadensis*) were growing in close proximity. Using PCR-restriction fragment length polymorphism (RFLP) analysis of the whole *rrs* gene, we analyzed the level of heterogeneity of *M. gale*-nodulating strains and the specificity of their genetic patterns compared with those of *Alnus*- and *Shepherdia*-infective strains. *M. gale* nodules were also sampled from two additional sites in order to detect relationships between site factors and *Frankia* strain diversity. The total *rrs* gene sequences from several *Myrica* isolates and field nodules were determined to assess the phylogenetic position of *Myrica*-infective strains with greater precision.

MATERIALS AND METHODS

Nodules and sites. A total of 75 nodules were sampled at three sites (Table 1). Most of the nodules were collected at sites 1 and 2 in Wisconsin. Site 1 had pH values of 7 to 7.5 and organic matter percentages of 0.7 to 2.8. Site 2 had a pH value of 6.1 and an organic matter percentage of 9.6. Site 3 was in Glasgow, United Kingdom (U.K.), and consisted of an upland acid peat from the Trossachs with a pH range of 3.5 to 4.0 transferred 7 years previously along with *M. gale* transplants to a pit in a loam soil derived from glacial boulder clay in the Glasgow Botanic Gardens. The nodules came from roots close to the root collar and were probably derived from the upland acid peat soil. Several *Alnus* species grew near this site in the Gardens but no *Myrica* spp. *M. gale* occurred on all the sites, while *S. canadensis* and *A. incana* subsp. *rugosa* occurred only on site 1. On site 1 there is a steep moisture gradient on a lake shore sand dune, and *M. gale, S. canadensis*, and *A. incana* subsp. *rugosa* grow in close proximity. *A. incana* subsp. *rugosa* and *M. gale* occupy the moist lower dunes near interdunal ponds, while *S. canadensis* occurs on the drier upper slopes. Entire plants were excavated, and their root systems were removed intact before cleaning and sampling in order to ascertain the host origin of nodules.

Bacterial strains. Five *Frankia* strains isolated from three different *Myrica* species were included in this study (Table 1). They were grown in BuCT medium (28) at 28°C in the dark with weekly manual agitation.

DNA extraction. DNA from root nodules was directly extracted according to Rouvier et al. (49). After washing the nodules with water, a single lobe was selected, and the outer layers were removed. Each lobe was crushed in 300 μl of TCP extraction buffer (100 mM Tris-HCl [pH 7], 0.5 M NaCl, 50 mM EDTA [pH 8], 2% [wt/vol] cethyltrimethylammonium bromide [Sigma, St. Louis, Mo.], 1% [wt/vol] polyvinylpolypyrrolidone [Sigma]). The homogenate was incubated at 65°C for 1 h and centrifuged twice at 6,000 × *g* for 5 min. The supernatant was extracted with an equal volume of chloroform-isoamyl alcohol (24:1, vol/vol) and centrifuged at 13,000 × *g* for 20 min. DNA from the aqueous phase was precipitated in ethanol for at least 2 h at −20°C. The sample was then centrifuged at 13,000 × *g* for 30 min, and the resulting DNA pellet was washed with 70% (vol/vol) ethanol, air dried, and dissolved in 10 μl of Tris-EDTA (TE) buffer (pH 7.5).

For isolated *Frankia* strains, hyphae were fragmented by repeatedly passing colonies through a 1.2-mm-diameter needle and were then washed three times in 500 μl of TE buffer (pH 7.5). DNA was extracted by sonication using a Cup Horn probe (Bioblock Scientific, Illkirch, France) for 1 to 2 min (level 2 and 50% active cycles). These samples were then kept at −20°C.

PCR amplification. Prior to the PCR, DNA extracted from nodules was digested with *Nru*I (Boehringer Mannheim, Meylan, France) to avoid amplification of the 16S ribosomal DNA (rDNA) of the chloroplasts present in the root cells (44). Amplification of the whole 16S rDNA was performed using primers FGPS-1509′-153 (5′-AAGGAGGGGATCCAGCCGCA-3′) and FGPS4-281bis (5′-ATGGA[G/A]AG[T/C]TTGATCCTGGCTCA-3′) (43) in a GeneAmp PCR system 2400 thermocycler (Perkin-Elmer Instruments, Norwalk, Conn.) under the following conditions: initial denaturation for 3 min at 94°C, 35 cycles of denaturation (1 min at 94°C), annealing (45 s at 60°C), and extension (1 min at 72°C), and a final extension for 7 min at 72°C. The reaction volume was 50 μl, containing 5 μl of diluted DNA, 1× PCR buffer (Gibco-BRL, Cergy-Pontoise, France), 1.5 mM $MgCl_2$, W-1% detergent (0.05%, vol/vol) (Gibco-BRL), 0.5 μM each primer, and 2.5 U of *Taq* polymerase (Gibco-BRL). When amplification was not sufficient, T4 gene 32 protein (Boehringer Mannheim) was used to improve its yield. The amplification of DNA was checked by agarose gel electrophoresis (1%, wt/vol) with 5 μl of PCR product, and the gel was stained with ethidium bromide (0.5 μg/ml) (Sigma).

RFLP. PCR products were digested for 2 h at the optimal conditions recommended by the manufacturer. The following restriction endonucleases were used: 10 U of *Cfo*I, *Hae*III, and *Msp*I and 5 U of *Nde*II (all from Boehringer Mannheim). Restriction fragments were separated by electrophoresis on a 2.5% (wt/vol) Metaphor agarose gel (FMC Bioproducts, Rockland, Maine) containing

ethidium bromide (0.5 μg. ml^{-1}) and photographed with Ilford FP4 film. The combination of the restriction patterns obtained with the four endonucleases used allowed the determination of PCR-RFLP groups (or types) and the assignment of each strain to one of these groups. Nine reference strains for which the *rrs* genes had already been sequenced were also used, and their RFLP patterns were simulated using the MacVector software (International Biotechnology Kodak, Rochester, N.Y.). These strains, from different actinorhizal families, allowed us to clearly identify the nonisolated strains. A dendrogram was obtained by calculating the Dice coefficient and by using the UPGMA algorithm (53).

Sequencing. The *rrs* gene of the five *Frankia* strains isolated from *Myrica* spp. and three nonisolated strains (nodules MRn2-2, Sn4-3, and Sn5-8) was sequenced by Génome Express S.A. (Grenoble, France). Before sequencing, the amplificon was purified with the QIAquick kit (Qiagen S.A., Les Ulis, France). Sequences were deposited in EMBL, and their accession numbers are given in Fig. 2. The sequences were aligned by using ClustalX software (57) and compared to reference sequences (from isolated and nonisolated *Frankia* strains and closely related bacteria) by using the MASE software (21). The distances were calculated according to Kimura's two-parameter method (30), and a dendrogram was obtained by using the neighbor-joining algorithm (50). The bootstrap values were determined from 1,000 replicates (22). The reference sequences were chosen in order to have a representative range of infectivity groups and the infectivity and effectivity capacities of the strains.

RESULTS

DNA extraction and PCR amplification. DNA extraction and *rrs* gene amplification were conducted for 40 nodules. The high concentration of plant molecules (tannins, polyphenols, polysaccharides, etc.) indicated by the dark color of the nodule extracts, together with the large size of the amplified fragment, may explain the low amplicon yields of some samples. The PCR products generated consisted of a unique amplicon of the expected size (approximatively 1,500 bp) for isolated and nonisolated strains.

RFLP analysis. Because of the detection threshold of the agarose gel electrophoresis, fragments shorter than 80 bp were not considered for the analysis. The restriction patterns obtained with the four enzymes used are shown in Table 2. Depending on the restriction enzyme used, from 2 (*Nde*II) to 11 (*Hae*III) different patterns were generated. Combining all the patterns obtained allowed the determination of 20 PCR-RFLP types. Seven types were obtained from amplified DNA of unisolated, nodular strains of *M. gale, A. incana* subsp. *rugosa*, and *S. canadensis*. Five types were from *Frankia* strains isolated from *Myrica* spp., and there were eight reference strain types. DNA from *Elaeagnaceae* strains, including both reference strains and unisolated (nodules) strains, was characterized by M2 and N2 patterns. Two *Myrica* spp. isolates together with two *Ceanothus* spp. isolates also harbored the N2 RFLP pattern. *Msp*I restriction pattern analysis appears to be a useful tool for distinguishing between the two groups, which include strains that nodulate the *Elaeagnaceae* on the one hand, and *Alnus, Myrica*, and *Casuarina* on the other hand. The dendrogram obtained by the UPGMA algorithm is presented in Fig. 1. *Frankia* strains studied are divided into two groups. Group 1 includes *Elaeagnaceae* strains (reference strains and *Shepherdia* unisolated strains), the atypical strains Cea5.1 and Cea1.3, and strains MgI5 and M16386, isolated from *M. gale* and *Myrica californica*, respectively. *Shepherdia* unisolated strains are grouped with the only *Shepherdia* isolate known to be clearly separated phylogenetically from the *Elaeagnus-Hippophaë* branch. Group 2 includes strains infective on *Alnus, Myrica*, and *Casuarina*. The DNA of *Frankia* strains in *Myrica*

TABLE 2. Restriction patterns of amplified *rrs* gene from nodular *Frankia* strains and reference strains

Nodule or strain	Restriction enzyme patterns[a]				Types[b]
	*Cfo*I	*Hae*III	*Msp*I	*Nde*II	
Alnus nodules					
An 1-1	C1	H1	M1	N1	I
An 1-2	C1	H1	M1	N1	I
An 1-3	C1	H1	M1	N1	I
An 1-4	C1	H1	M1	N1	I
An 1-5	C1	H1	M1	N1	I
An 1-7	C1	H1	M1	N1	I
An 2-3	C1	H1	M1	N1	I
An 2-4	C1	H1	M1	N1	I
An 2-5	C1	H1	M1	N1	I
An 2-6	C1	H1	M1	N1	I
An 2-8	C1	H1	M1	N1	I
An 2-9	C1	H1	M1	N1	I
An 3-4	C2	H1	M1	N1	II
An 3-5	C2	H1	M1	N1	II
An 3-8	C2	H1	M1	N1	II
Shepherdia nodules					
Sn 3-1	C3	H2	M2	N2	III
Sn 4-2	C3	H1	M2	N2	IV
Sn 4-3	C3	H2	M2	N2	III
Sn 5-2	C3	H1	M2	N2	IV
Sn 5-4	C3	H1	M2	N2	IV
Sn 5-8	C3	H1	M2	N2	IV
Myrica nodules					
MMn 1-2	C2	H3	M3	N1	V
MMn 1-3	C2	H3	M3	N1	V
MMn 1-6	C1	H1	M4	N1	VI
MMn 1-7	C2	H3	M3	N1	V
MMn 1-11	C2	H3	M3	N1	V
MMn 3-1	C2	H3	M3	N1	V
MMn 3-2	C2	H3	M3	N1	V
MMn 3-3	C2	H3	M3	N1	V
MMn 3-5	C2	H3	M3	N1	V
MRn 1-2	C1	H4	M1	N1	VII
MRn 1-3	C1	H4	M1	N1	VII
MRn 1-4	C1	H4	M1	N1	VII
MRn 2-1	C1	H4	M1	N1	VII
MRn 2-2	C1	H1	M1	N1	I
MRn 2-3	C1	H1	M1	N1	I
MRn 2-4	C1	H4	M1	N1	VII
MRn 2-5	C1	H4	M1	N1	VII
MWn 1-1	C1	H1	M4	N1	VI
MWn 1-2	C1	H1	M4	N1	VI
Myrica isolates					
M16386	C3	H2	M5	N2	VIII
M16464	C4	H5	M6	N1	IX
M16467	C4	H6	M4	N1	X
MgI5	C5	H7	M7	N2	XI
MpI1	C4	H8	M6	N1	XII
Alnus strains[c]					
ACoN24d	C4	H5	M1	N1	XIII
ARgP5	C6	H5	M8	N1	XIV
Elaeagnaceae strains[c]					
SCN10a	C3	H5	M2	N2	XV
Hr27-14	C6	H5	M2	N2	XVI
Ea1-2	C6	H9	M2	N2	XVII
Other strains[c]					
Cea1.3	C7	H10	M9	N2	XVIII
Cea5.1	C8	H10	M9	N2	IXX
CeD	C6	H11	M6	N1	XX

[a] The letters designate the restriction enzyme used, and the number designates the pattern obtained with this enzyme.

[b] The types represent the combination of patterns obtained with the four enzymes tested on the *rrs* gene.

[c] Reference strains. Theoretical restriction patterns of the *rrs* gene using MacVector software.

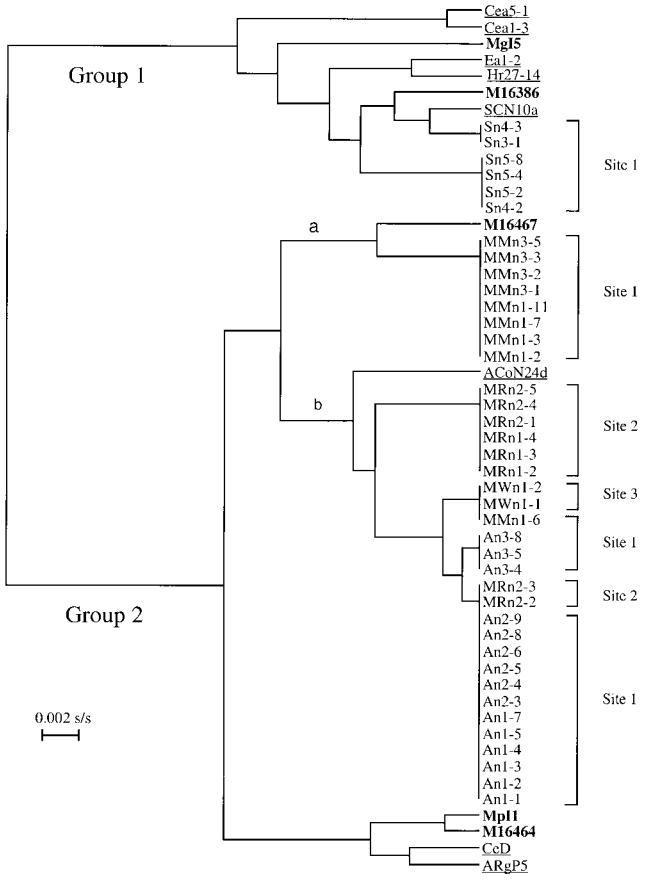

FIG. 1. Phylogenetic tree based on PCR-RFLP patterns analyzed with the UPGMA algorithm (53). Strains isolated from *Myrica* spp. are in bold. Reference strains (GenBank accession numbers, from top to bottom: U72718, U72717, L40618, L40617, L40619, L40610, M55343, and L40612) are underlined.

nodules and *Frankia* DNA detected in *Alnus* nodules differed with the exception of *Frankia* DNA in nodules MRn2-2 and MRn2-3. The *Frankia* strains detected in *Myrica* nodules are phylogenetically related but clearly separated according to their site of origin.

Sequencing. The phylogenetic tree based on the 16S rDNA sequencing results and obtained by the neighbor–joining algorithm is shown in Fig. 2. In agreement with PCR-RFLP results, strains infective on *Myrica* spp. are divided into two groups. Isolated strains MpI1, M16464, and M16467 and unisolated strain MRn2-2 have as the closest phylogenetic neighbor strain ACN14a and belong to the *Alnus-Myrica-Casuarina* infectivity group. Isolates MgI5 and M16386, previously grouped with the *Elaeagnaceae*-infective strains (Fig. 1), have for their closest phylogenetic neighbor strain Cea5.1, isolated from a *Ceanothus* sp. (*Rhamnaceae* family), and form a coherent cluster, according to bootstrap values, with strains known to be atypical for their infectivity and/or effectivity phenotype. Unisolated *Shepherdia* strains Sn4-3 and Sn5-8 are closely related to strain FE39, isolated from *Trevoa trinervis* (*Rhamnaceae* family) and belong to the group of strains infective on members of the *Elaeagnaceae* and *Rhamnaceae* families.

DISCUSSION

M. gale-nodulating strains from three sites on two continents were genetically uniform (Fig. 1, group 2), grouping together with strains infecting *Alnus* and clustering with strain ACoN24d, representative of *Frankia alni* species. Nevertheless, specific genotypes apparently occurred at each site with few exceptions, leading to the conclusion that low diversity is present in each stand. This local dominance has been reported previously for *A. incana* and *Myrica pensylvanica* (15). Soil composition parameters that frequently correlated with the level of *Frankia* population diversity include soil moisture (18, 46, 48), pH (27, 29), and organic matter content (10). In this study, site 1 (neutral, low in organic matter, not flooded) and sites 2 and 3 (acidic, high in organic matter, flooded or moist) represent contrasting ecological environments that possibly support different types of *Myrica*-infective *Frankia* strains. It will be necessary to study more sites before drawing general conclusions.

All *Elaeagnaceae* strains, including *Shepherdia*-infective strains of site 1, were grouped in a distinct and deeply diverging branch of the phylogenetic tree (Fig. 1, group 1). In our study, all of the *M. gale* nodules sampled contained *Alnus*-like geno-

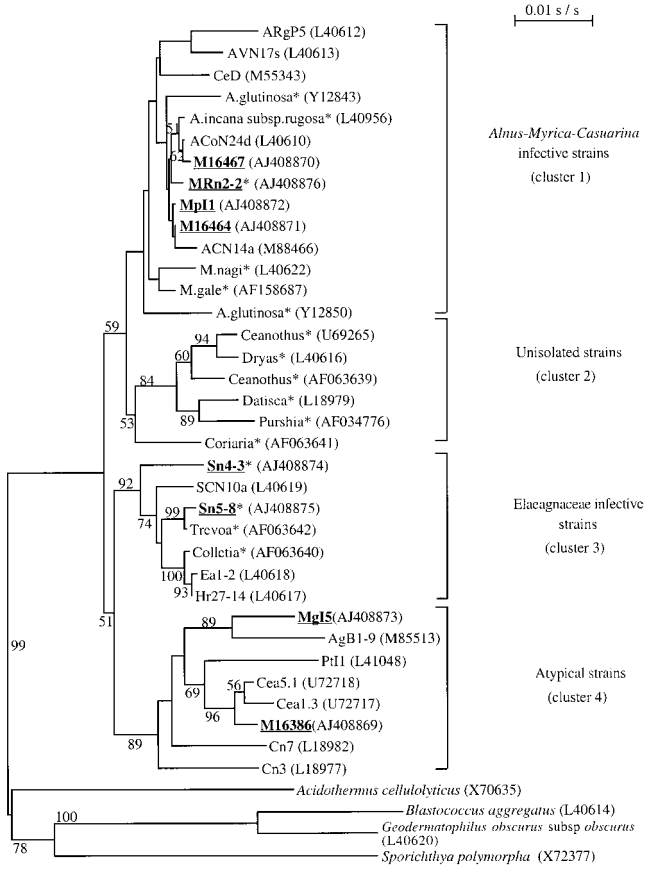

FIG. 2. Phylogenetic tree based on *rrs* gene sequences obtained by the neighbor-joining algorithm (50). The numbers on the tree branches are bootstrap values. Only values larger than 50% are shown. GenBank accession numbers are shown in parentheses. Strains characterized in the present study are underlined and in bold. *, unisolated strains. Clusters are defined in reference 44.

types, and none contained *Elaeagnaceae* (or closely related)-infective *Frankia* strains. Other authors (13, 60) found "*Elaeagnaceae*-like genotypes" but on two other *Myrica* species nodules, *M. cerifera* and *M. pensylvanica*. Among the isolated strains tested, strain Mg15 isolated from *M. gale* was found to group with *Elaeagnaceae* strains by PCR-RFLP but not with the complete *rrs* gene sequence. However, this strain is atypical, since no nodulation was obtained on its original host plant, *M. gale*, but was obtained on *Elaeagnaceae* species (3, 31). Thus it can be concluded that even if *Myrica* is often considered a promiscuous actinorhizal genus, our results show that *M. gale* does not exhibit such promiscuity in its natural environment. The limited diversity of strains infective on *M. gale* could be correlated with the fact that this *Myrica* species typically grows in a narrow and possibly restrictive range of wet acidic and organic soils. Such a possibility has also been proposed by Clawson and Benson (13).

Results from site 1, where the three actinorhizal genera grow in close proximity, showed that each plant species is nodulated by a distinct *Frankia* genotype. Although *Myrica*- and *Alnus*-infective strains belong to the same host specificity group (HSG), distinct sets of closely related genotypes were associated with these species with only two exceptions. Surprisingly, in spite of the genetic proximity of *Alnus*- and *Myrica*-infective strains, *Myrica* strains from site 2 (Fig. 1, group 2b) were more closely related to *Alnus* strains from site 1 (Fig. 1, group 2b) than are *Myrica* strains from the same site 1 (Fig. 1, group 2a). Thus, strain selection in the soil by the host plant may entail a complex interaction between elements in the soil ecosystem and root-*Frankia* molecular dialog.

Phylogenetic positioning of the different groups of strains studied was achieved by sequencing the complete *rrs* gene of representative strains. This approach was chosen instead of the more rapid partial *rrs* sequencing. Indeed, the variable and conserved zones of the *rrs* gene, even if generally located in the same regions, were shown to exhibit variable positioning within a genus, which could result in misclassifications (12, 34, 45, 59). In the case of *Frankia*, the use of partial sequences leads to the separation of *Casuarina*- from *Alnus*-infective strains, while strains nodulating *Elaeagnaceae* become closer to strains nodulating *Alnus* (data not shown). This grouping is not consistent with most of the results reported on *Frankia* strains, including biochemical, physiological, and phylogenetic approaches (for a review, see reference 32). On the contrary, the phylogenetic tree based on complete *rrs* sequences (Fig. 2) confirms the results obtained using RFLP data (Fig. 1), demonstrating that the data used in the RFLP approach were sufficiently informative and discriminating even if only a few restriction enzymes were used. Some of them, such as *Nde*II, produced banding patterns that are highly specific for the different phylogenetic groups and are phylogenetically and taxonomically useful (37, 38). The bootstrap values shown in Fig. 2, even if not very high, appear to be significant. The topology of this tree is congruent with various phenotypic data, such as infection pathways (36), serology (4), cell wall 2-*O*-methyl-D-mannose abundance (54), cellular fatty acid composition (51), susceptibility to antibiotics (20), and more recent phylogenetic studies using the *gln*II gene (16).

For *Myrica* spp., *rrs* genes of five reference strains and one unisolated strain were sequenced. The isolates MpI1, M16464, and M16467, all isolated from *M. pensylvanica*, and the unisolated strain MRn2-2, from *M. gale*, are closely related to *F. alni* species and to unisolated strains nodulating *Myrica* and *Alnus* according to PCR-RFLP and sequencing data. This result confirms previous studies including these three isolates (5, 7, 8, 31, 42), where they belong to the cluster of *Alnus*-infective strains (cluster 1 [44]) and appear to be Nod^+ Fix^+ on their original host plant. According to Bloom and al. (7, 8), the *Frankia* strains isolated from *M. pensylvanica* are heterogeneous, which was confirmed by Clawson and Benson (13), who described this species as promiscuous in the field. The isolates MgI5 and M16386 clustered with isolated strains from various host plants. These strains have a common feature, lack of infectivity and/or effectivity on their original host plant, and group with the atypical strains in the phylogenetic tree (cluster 4 [44]). This positioning confirms what is already known about MgI5. Indeed, this strain is Nod^- Fix^- on its original host plant, *M. gale* (31), belongs to HSG 4, which includes strains infective only on the *Eleaegnaceae* species according to Baker (3), and grouped together with atypical strains in the study of Nittayajarn and al. (42). We therefore agree with the proposal that these marginal strains are not the real symbionts of the actinorhizal plants (47) but were present in their rhizosphere or at the nodule surface and were selected by the culture method. This highlights the necessity of working directly with nodular *Frankia* strains to avoid biases of isolation and culture.

Few studies examined the diversity and phylogenetic positioning of *Shepherdia*-infective strains. This study shows that *Shepherdia*-infective strains present more diversity than strains that nodulate *Alnus* or *Myrica* at site 1. In the phylogenetic tree based on *rrs* sequences, unisolated strains Sn4-3 and Sn5-8 grouped with SCN10a, close to a strain isolated from *Trevoa trinervis* (*Rhamnaceae* family) in the *Elaeagnaceae*-infective strain cluster (cluster 3 [44]). All the *Shepherdia* nodular DNA identified by the PCR-RFLP approach belongs to this group. The phylogenetic proximity of *Elaeagnaceae*- and *Rhamnaceae*-infective strains has been attributed to the common phyletic origin of these families (14, 56). Thus, the set of *Shepherdia*-infective strains identified in this study provides additional evidence for the congruence of host plant taxa and associated *Frankia* strain phylogenies. The group of strains that nodulate *Ceanothus* (*Rhamnaceae* family) appear to be an exception because they cluster with *Rosaceae*-infective strains in comparisons of nearly full length *rrs* sequences (14).

It now seems obvious that the apparent heterogeneity of *Myrica* isolates is partly due to the consideration of atypical isolated strains that probably originate from the nodule surface as an artifact of incomplete nodule surface sterilization during the isolation process. Such an isolation bias observed on *Casuarina*-infective strains has led to erroneous classification of strains and still hinders biodiversity studies (23, 41, 49). Because the *Myricaceae* family is the most primitive actinorhizal family, widely distributed and universally nodulated in its natural environment, it was suspected to harbor a large diversity of strains able to nodulate other introduced actinorhizal plants. Our study indicates that this is not the case for *M. gale*. Genetic differences in nodular *Frankia* strains among *Myrica* host species, soil types, and geographic sources remain to be more fully elucidated in the context of evolution, ecology, and conserva-

tion (40), particularly by sampling more sites with varied ecological conditions.

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REFERENCES

1. **Akimov, V. N., and S. V. Dobritsa.** 1992. Grouping of *Frankia* strains on the basis of DNA relatedness. Syst. Appl. Microbiol. **15:**372–379.
2. **An, S. C., J. W. Wills, W. S. Riggsby, and B. C. Mullin.** 1985. Relationships of *Frankia* isolates based on deoxyribonucleic acid homology studies. Int. J. Syst. Bacteriol. **35:**140–146.
3. **Baker, D.** 1987. Relationships among pure cultured strains of *Frankia* based on host specificity. Physiol. Plant. **70:**245–248.
4. **Baker, D., W. L. Pengelly, and J. G. Torrey.** 1981. Immunochemichal analysis of relationships among the isolated frankiae (*Actinomycetales*). Int. J. Syst. Bacteriol. **31:**148–151.
5. **Benson, D. R., S. E. Buchholz, and D. G. Hanna.** 1984. Identification of *Frankia* strains by two-dimensional polyacrylamide gel electrophoresis. Appl. Environ. Microbiol. **47:**489–494.
6. **Benson, D. R., and D. Hanna.** 1983. *Frankia* diversity in an alder stand as estimated by SDS-PAGE of whole cell protein. Can. J. Bot. **61:**2919–2923.
7. **Bloom, R. A., M. P. Lechevalier, and R. L. Tate.** 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.
8. **Bloom, R. A., B. C. Mullin, and R. L. Tate.** 1989. DNA restriction patterns and DNA-DNA solution hybridization studies of *Frankia* isolates from *Myrica pensylvanica* (bayberry). Appl. Environ. Microbiol. **55:**2155–2160.
9. **Bond, G.** 1983. Taxonomy and distribution of non-legume nitrogen-fixing systems, p. 55–87. *In* J. C. Gordon and C. T. Wheeler (ed.), Biological nitrogen fixation in forest ecosystems: foundations and applications. Martinus Nijhoff, The Hague, the Netherlands.
10. **Burleigh, S. H., and J. O. Dawson.** 1994. Occurrence of *Myrica*-nodulating *Frankia* in Hawaiian volcanic soils. Plant Soil. **164:**283–289.
11. **Callaham, D., P. Del Tredici, and J. G. Torrey.** 1978. Isolation and cultivation in vitro of the actinomycete causing root nodulation in *Comptonia*. Science **199:**899–902.
12. **Cilia, V., B. Lafay, and R. Christen.** 1996. Sequence heterogeneity among 16S ribosomal RNA sequences, and their effect on phylogenetic analyses at the species level. Mol. Biol. Evol. **13:**451–461.
13. **Clawson, M. L., and D. R. Benson.** 1999. Natural diversity of *Frankia* in actinorhizal root nodules from promiscuous hosts in the Myricaceae. Appl. Environ. Microbiol. **65:**4521–4527.
14. **Clawson, M. L., M. Carù, 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.
15. **Clawson, M. L., J. Gawronski, and D. R. Benson.** 1999. Dominance of *Frankia* strains in stands of *Alnus incana* subsp *rugosa* and *Myrica pensylvanica*. Can. J. Bot. **77:**1203–1207.
16. **Cournoyer, B., and C. Lavire.** 1999. Analysis of *Frankia* evolutionnary radiation using *glnII* sequences. FEMS Microbiol. Lett. **177:**29–34.
17. **Cronquist, A.** 1988. The evolution and classification of flowering plants, 2nd ed. The New York Botanical Garden, New York, N.Y.
18. **Dawson, J. O., D. G. Kowalski, and P. J. Dart.** 1989. Variation with soil depth, topographic position and host species in the capacity of soils from an Australian locale to nodulate *Casuarina* and *Allocasuarina* seedlings. Plant Soil **118:**1–13.
19. **Dobritsa, S. V.** 1985. Restriction analysis of the *Frankia* spp. genome. FEMS Microbiol. Lett. **29:**123–128.
20. **Dobritsa, S. V.** 1998. Grouping of *Frankia* strains on the basis of susceptibility to antibiotics, pigment production, and host specificity. Int. J. Syst. Bacteriol. **48:**1265–1275.
21. **Faulkner, D. V., and J. Jurka.** 1988. Multiple aligned sequence editor (MASE). Trends Biochem. Sci. **13:**321–322.
22. **Felsenstein, J.** 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution **39:**783–791.
23. **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.
24. **Gardes, M., J. Bousquet, and M. Lalonde.** 1987. Isozyme variation among 40 *Frankia* strains. Appl. Environ. Microbiol. **53:**1596–1603.
25. **Gardes, M., and M. Lalonde.** 1987. Identification and subgrouping of *Frankia* strains using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Physiol. Plant. **70:**237–244.
26. **Gladkova, A. N.** 1962. Fragments of the history of the Myricaceae family. Pollen Spores **4:**345.
27. **Griffiths, A. P., and L. H. McCormick.** 1984. Effects of soil acidity on nodulation of *Alnus glutinosa* and viability of *Frankia*. Plant Soil **79:**429–434.
28. **Hooker, J. E., and C. T. Wheeler.** 1987. The effectivity of *Frankia* for nodulation and nitrogen fixation in *Alnus rubra* and *A. glutinosa*. Physiol. Plant. **70:**333–341.
29. **Jamann, S., M. P. Fernandez, and A. Moiroud.** 1992. Genetic diversity of Elaeagnaceae-infective *Frankia* strains isolated from various soils. Acta Oecol. **13:**395–405.
30. **Kimura, M.** 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. **16:**111–120.
31. **Lechevalier, M. P.** 1986. Catalog of *Frankia* strains. Actinomycete **19:**131–162.
32. **Lechevalier, M. P.** 1994. Minireview: taxonomy of the genus *Frankia* (*Actinomycetales*). Int. J. Syst. Bacteriol. **44:**1–8.
33. **Lechevalier, M. P., and H. A. Lechevalier.** 1979. The taxonomic position of the actinomycetic endophytes, p. 111–122. *In* J. C. Gordon, C. T. Wheeler, and D. A. Perry (ed.), Symbiotic nitrogen fixation in the managment of temperate forests. Forest Research Laboratory, Oregon State University, Corvallis, Oreg.
34. **Ludwig, W., and K. H. Schleifer.** 1994. Bacterial phylogeny based on 16S and 23S rRNA sequence analysis. FEMS Microbiol. Rev. **15:**155–173.
35. **Maggia, L., and J. Bousquet.** 1994. Molecular phylogeny of the actinorhizal *Hamamelidae* and relationships with host promiscuity towards *Frankia*. Mol. Ecol. **3:**459–467.
36. **Miller, I. M., and D. D. Baker.** 1986. Nodulation of actinorhizal plants by *Frankia* strains capable of both root hair infection and intercellular penetration. Protoplasma **131:**82–91.
37. **Moyer, C. L., F. C. Dobbs, and D. M. Karl.** 1994. Estimation of diversity and community structure through restriction fragment length polymorphism distribution: analysis of bacterial 16S rRNA genes from a microbial mat at an active, hydrothermal vent system, Loihi Seamount, Hawaii. Appl. Environ. Microbiol. **60:**871–879.
38. **Moyer, C. L., J. M. Tiedje, F. C. Dobbs, and D. M. Karl.** 1996. A computer-simulated restriction fragment length polymorphism analysis of bacterial small-subunit rRNA genes: efficacy of selected tetrameric restriction enzymes for studies of microbial diversity in nature. Appl. Environ. Microbiol. **62:**2501–2507.
39. **Müller, J.** 1981. Fossil pollen records of extant angiosperms. Bot. Rev. **47:**1–142.
40. **Navarro, E., T. Jaffre, D. Gauthier, F. Gourbiere, G. Rinaudo, P. Simonet, and P. Normand.** 1999. Distribution of *Gymnostoma* spp. microsymbiotic *Frankia* strains in New Caledonia is related to soil type and to host-plant species. Mol. Ecol. **8:**1781–1788.
41. **Nazaret, S., P. Simonet, P. Normand, and R. Bardin.** 1989. Genetic diversity among *Frankia* isolated from *Casuarina* nodules. Plant Soil **118:**241–247.
42. **Nittayajarn, A., B. C. Mullin, and D. D. Baker.** 1990. Screening of symbiotic *Frankiae* for host specificity by restriction fragment length polymorphism analysis. Appl. Environ. Microbiol. **56:**1172–1174.
43. **Normand, P., and C. Chapelon.** 1997. Direct characterization of *Frankia* and of close phyletic neighbors from an *Alnus viridis* rhizosphere. Physiol. Plant. **99:**722–731.
44. **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 the family *Frankiaceae*. Int. J. Syst. Bacteriol. **46:**1–9.
45. **Nour, S. M., J. C. Cleyet-Marel, P. Normand, and M. P. Fernandez.** 1995. Genomic heterogeneity of strains nodulating chickpeas (*Cicer arietinum* L.) and description of *Rhizobium mediterraneum* sp. nov. Int. J. Syst. Bacteriol. **45:**640–648.
46. **Pratt, S. D., A. S. Konopka, M. A. Murry, F. W. Ewers, and S. D. Davis.** 1997. Influence of soil moisture on the nodulation of post-fire seedlings of *Ceanothus* spp. growing in the Santa Monica Mountains of Southern California. Physiol. Plant. **99:**673–679.
47. **Ramirez-Saad, H., J. D. Janse, and A. D. L. Akkermans.** 1998. Root nodules of *Ceanothus caerulus* contain both the N_2-fixing *Frankia* endophyte and a phylogenetically related Nod^-/Fix^- actinomycete. Can. J. Microbiol. **44:**140–148.
48. **Righetti, T. L., C. H. Chard, and R. A. Backhause.** 1986. Soil and environmental factors related to nodulation in *Cowania* and *Purshia*. Plant Soil **91:**147–160.
49. **Rouvier, C., Y. Prin, P. Reddell, P. Normand, and P. Simonet.** 1996. Genetic diversity among *Frankia* strains nodulating members of the family *Casuarinaceae* in Australia revealed by PCR and fragment length polymorphism analysis with crushed root nodules. Appl. Environ. Microbiol. **62:**979–985.
50. **Saitou, N., and M. Nei.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **2:**112–118.
51. **Simon, L., S. Jabaji-Hare, J. Bousquet, and M. Lalonde.** 1989. Confirmation of *Frankia* species using cellular fatty acids analysis. Syst. Appl. Microbiol. **11:**229–235.
52. **Simonet, P., P. Normand, A. Moiroud, and M. Lalonde.** 1985. Restriction enzyme digestion patterns of *Frankia* plasmids. Plant Soil **87:**49–60.
53. **Sokal, R. R., and P. H. A. Sneath.** 1963. Principles of numerical taxonomy.

W. H. Freeman, San Francisco, Calif.
54. **St.-Laurent, L., J. Bousquet, L. Simon, and M. Lalonde.** 1987. Separation of various *Frankia* strains in the *Alnus* and *Elaeagnus* host specificity group using sugar analysis. Can. J. Microbiol. **33:**764–772.
55. **St-Laurent, L., and M. Lalonde.** 1987. Isolation and characterization of *Frankia* strains isolated from *Myrica gale*. Can. J. Bot. **65:**1356–1363.
56. **Swensen, S.** 1996. The evolution of actinorhizal symbioses: evidence for multiple origins of the symbiotic association. Am. J. Bot. **83:**1503–1512.
57. **Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins.** 1997. The ClustalX-Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. **25:**4876–4882.
58. **Wheeler, C. T., J. E. Hooker, A. Crose, and A. M. M. Berry.** 1986. The improvement and utilization in forestry of nitrogen fixation by actinorhizal plants with special reference to *Alnus* in Scotland. Plant Soil **90:**393–406.
59. **Young, J. P. W., and K. E. Haukka.** 1996. Diversity and phylogeny of rhizobia. New Phytol. **133:**87–94.
60. **Zimpfer, J. F., G. J. Kennedy, C. A. Smyth, J. Hamelin, E. Navarro, and J. O. Dawson.** 1999. Localization of *Casuarina*-infective *Frankia* near *Casuarina cunninghamiana* trees in Jamaica. Can. J. Bot. **77:**1248–1256.