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Amplification of 16S rRNA Genes from *Frankia* Strains in Root Nodules of *Ceanothus griseus*, *Coriaria arborea*, *Coriaria plumosa*, *Discaria toumatou*, and *Purshia tridentata*

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To study the global diversity of plant-symbiotic nitrogen-fixing *Frankia* strains, a rapid method was used to isolate DNA from these actinomycetes in root nodules. The procedure used involved dissecting the symbiont from nodule lobes; ascorbic acid was used to maintain plant phenolic compounds in the reduced state. Genes for the small-subunit rRNA (16S ribosomal DNA) were amplified by the PCR, and the amplicons were cycle sequenced. Less than 1 mg (fresh weight) of nodule tissue and fewer than 10 vesicle clusters could serve as the starting material for template preparation. Partial sequences were obtained from symbionts residing in nodules from *Ceanothus griseus*, *Coriaria arborea*, *Coriaria plumosa*, *Discaria toumatou*, and *Purshia tridentata*. The sequences obtained from *Ceonothus griseus* and *P. tridentata* nodules were identical to the sequence previously reported for the endophyte of *Dryas drummondii*. The sequences from *Frankia* strains in *Coriaria arborea* and *Coriaria plumosa* nodules were identical to one another and indicate a separate lineage for these strains. The *Frankia* strains in *Discaria toumatou* nodules yielded a unique sequence that places them in a lineage close to bacteria that infect members of the Elaeagnaceae.

Actinomycetes belonging to the genus *Frankia* form nitrogen-fixing root nodule symbioses with actinorhizal plants. The plants are woody angiosperms that generally inhabit soils with marginal fertility. Infective and effective (capable of fixing N_2) *Frankia* strains have been isolated from only 9 of the 24 known actinorhizal plant genera (4). Attempts to isolate *Frankia* strains from plants in the families Coriariaceae, Datiscaceae, Rhamnaceae, and Rosaceae have been generally unsuccessful (4), as have many informally reported attempts performed with plants that normally yield isolates. These observations have engendered the notion that *Frankia* strains in nodules have a degree of hidden diversity beyond the diversity displayed by the strains available in culture (1, 2, 5, 8, 17).

Several studies have addressed the issue of identifying *Frankia* strains in root nodules. Approaches have been developed for isolating DNA of sufficient purity for PCR amplification from nodules of *Coriaria* spp. (13, 16, 21) *Datisca* spp. (14, 15), *Dryas* spp. (23), *Alnus* spp., and *Myrica* spp. (17, 26, 27). With some exceptions, most procedures have required the use of relatively large quantities of nodule tissue for DNA isolation, and PCR amplification has usually been limited to short segments of 16S ribosomal DNA (rDNA). Two continuing problems are the abundance of plant-derived inhibitors that interfere with the PCR and the lack of a routine method for deriving template DNA that can be used for all nodule types.

In this study, we amplified DNA sequences directly from single nodule lobes by using as few as 10 hyphal clusters as the starting material. A hyphal cluster is the bacterial contents of a single plant cell. The 16S rRNA gene was chosen to address the issues of hidden diversity and coevolution of *Frankia* strains with actinorhizal plants because it is highly conserved and has utility for phylogenetic comparisons. We describe the first 16S rDNA sequences of *Frankia* strains found in *Ceanothus griseus*, *Coriaria arborea*, *Coriaria plumosa*, *Discaria toumatou*, and *Purshia tridentata* root nodules.

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MATERIALS AND METHODS

Root nodules. The sources of the root nodules used in this study are listed in Table 1. In all cases the nodules were frozen at −25°C after collection or receipt at the University of Waikato.

DNA isolation. Extreme care was used to avoid exogenous DNA in all solutions and during isolation of *Frankia* strains from nodules. All solutions were filtered and autoclaved, nylon mesh filters were exposed to UV light in a clean hood for 30 min, and dissecting implements were flamed frequently before and during use.

To isolate vesicle clusters, whole nodules were washed in a stream of cold water, and obviously young, light-colored lobes were excised with a scalpel into 0.5 ml of freshly prepared filtered-sterilized TEA buffer (10 mM Tris-HCl, 1 mM Na_4 EDTA, 20 mM ascorbic acid; pH 7.6) in a petri dish. The nodule lobes weighed between 0.5 and 7 mg (fresh weight) depending on the plant and soil type. The nodule periderm was carefully removed with forceps while the preparation was viewed through a dissecting microscope, and each peeled lobe was rinsed in three 0.5-ml drops of TEA buffer. With a scalpel and forceps, hyphal clusters were teased from the cortical cells of the nodule lobe while it was immersed in 0.5 ml of TEA buffer.

Hyphal clusters were purified by passing them through a 150-μm-mesh nylon screen and were collected on a 25-μm-mesh nylon screen; the screens were heat annealed and epoxy glued to the barrels of 5- and 10-ml plastic syringes, respectively (3). Clusters were washed on the 25-μm-mesh screen with eight successive 1-ml portions of TEA buffer. The residual ascorbate was removed by washing each preparation with three 1-ml portions of TE (10 mM Tris-HCl, 1 mM Na_4 EDTA; pH 7.6). The hyphal clusters were collected from the 25-μm-mesh nylon screen in TE and transferred to 1.5-ml microcentrifuge tubes. Samples (50 μl) were plated onto Luria-Bertani agar (25) and R2A agar (Difco Laboratories, Detroit, Mich.) to estimate the number of contaminating bacteria.

After centrifugation at 12,500 rpm (13,500 × *g*) for 10 min, the hyphal clusters were resuspended in 0.1 ml of TE, and then 0.1 ml of 0.2 N NaOH–1% sodium dodecyl sulfate (SDS) was added and the solution was briefly vortexed. The sample tubes were placed in a boiling water bath for 7 min, cooled rapidly on ice, and centrifuged at 12,500 rpm for 5 min. After centrifugation, 190 μl of each preparation was removed and placed in a fresh, sterile, 1.5-ml microcentrifuge tube, and 0.33 volume of 7.5 M ammonium acetate was added, followed by 2 volumes of ice-cold absolute ethanol.

After incubation for 1 h on ice, the precipitate was collected by centrifugation at 12,500 rpm for 10 min, the pellet was washed twice with cold 70% ethanol, and the preparation was centrifuged at 12,500 rpm for 5 min. The precipitate was

TABLE 1. Sources of nodules used in this study

Root nodule source	Family	Source	Native or introduced[a]
Alnus cordata	Betulaceae	University of Waikato, Hamilton, New Zealand	−
Alnus glutinosa	Betulaceae	Waikato River and Heritage Horticulture, Hamilton, New Zealand	−
Casuarina equisetifolia	Casuarinaceae	University of Waikato, Hamilton, New Zealand	−
Coriaria arborea	Coriariaceae	Mt. Tarawera soil, University of Waikato	+
Coriaria plumosa	Coriariaceae	Mt. Hikurangi, East Cape, New Zealand	+
Ceanothus griseus	Rhamnaceae	Palmerston North, New Zealand, greenhouse	−
Discaria toumatou	Rhamnaceae	Porter's Pass, Canterbury, New Zealand	+
Elaeagnus pungens	Elaeagnaceae	Ruakura, Hamilton, New Zealand	−
Purshia tridentata	Rosaceae	Palmerston North, New Zealand, greenhouse	−

[a] +, plant associated with the indigenous flora of New Zealand; −, introduced species.

dried at 37°C and finally resuspended in 50 μl of TE. The crude DNA sample was diluted as described below and was used as the template for PCR amplification.

PCR. The 16S rRNA genes of the *Frankia* strains in the nodules were amplified by using universal bacterial primers fD1 and rD1 (30). These primers are designed to yield nearly full-length 16S rDNA from most bacteria. For the initial amplification, 4-μl portions of 10^0, 10^{-1}, and 10^{-2} dilutions of the DNA in distilled water prepared as described above were added to 96-μl portions of a reaction mixture which contained (final concentrations) 10 mM Tris-HCl (pH 8.3); 3 mM $MgCl_2$, 50 mM KCl; 200 μM (each) dATP, dCTP, dGTP, and dTTP (Boehringer Mannheim); 0.5 μM primer fD1; 0.5 μM primer rD1; and 2.5 U of *Taq* DNA polymerase (Boehringer Mannheim). DNA was amplified by using an Easycycler apparatus (Ericomp, Inc., San Diego, Calif.) and the following temperature profile: denaturation for 2 min at 95°C, 35 cycles consisting of denaturation at 94°C for 1 min, annealing for 1 min at 55°C, and extension for 2 min at 72°C, and a final extension step at 72°C for 6 min. Products were examined by horizontal agarose gel electrophoresis in 1.0% agarose (Agarose MP; Boehringer Mannheim).

Seminested PCR and sequence analysis. PCR products were precipitated with ammonium acetate-ethanol as described above. For PCR amplification, pellets were dissolved in 20 μl of distilled H_2O. Bacterial sequences corresponding to *Escherichia coli* positions 8 to 436 were specifically amplified by adding 1 μl of template DNA to a PCR mixture (GeneAmp PCR reagent kit; Perkin-Elmer, Norwalk, Conn.) containing 2 mM $MgCl_2$, 0.1 μM primer fD1, and 0.1 μM primer rDB1 (5′-CCAAGCTTGAGGTTTACAACCCGAA-3′). Amplification was done with a Perkin-Elmer model 2400 thermal cycler by using the following temperature profile: 94°C for 1 min, 30 cycles consisting of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, and a final extension step at 72°C for 2 min. The PCR products were purified by using Wizard Prep columns (Fisher Scientific Co., Springfield, N.J.) and were cycle sequenced in both directions by using ^{35}S-labeled dATP and an AmpliCycle kit (Perkin-Elmer) according to the manufacturer's protocols. The sequencing primers used were fD1 and rDB1 and two internal primers, DB14 (5′-GTGGAAAGATTTATCGGCTCGGG-3′) and DB15 (5′-CCGAGCCGATAAATCTTTCCACACC-3′).

Restriction analysis. Samples of amplified DNA were digested without further purification by using a series of restriction endonucleases. Usually, 10-μl portions were digested with 10 U of enzyme for 1 h according to the manufacturers' protocols. Digested samples were analyzed on 2.5% agarose MP gels.

RESULTS

DNA isolation. Individual nodule lobes were used as a starting material for isolating template DNA. Lobe morphology was quite variable, with the lobes ranging from 0.4 to 2 mm in cross section and from 1 to 3 mm in length; the fresh weights ranged from less than 1 mg to about 7 mg.

Two impediments to isolating DNA from actinorhizal root nodules for PCR are the presence of contaminating bacteria that grow on or in the nodules and inhibitory plant phenolics. To minimize both of these problems, the periderm was peeled away, and each naked lobe was washed in several changes of sterile TEA buffer. The hyphal clusters of the actinomycete were teased from the remaining tissue and were fractionated through nylon screens (3). Pieces of unmacerated plant tissue containing vascular elements and uninfected cells were retained on the large-mesh filter (pore size, 150 μm), and hyphal clusters that were between about 25 and 150 μm in size were retained on the 25-μm-mesh filter. Contaminating bacteria passed through the second filter. The viable contaminant load ranged from 0 to 5 CFU on LB agar and R2A agar per 10^4 hyphal clusters. Each hyphal cluster contained between 10^2 and 10^3 *Frankia* genomes, so the measurable proportion of contaminating bacterial genomes was quite low.

Phenolic compounds present in actinorhizal nodule homogenates normally turn bright red or orange because of spontaneous oxidation in air and can complicate the purification of proteins or DNA. Adding ascorbate to the TE buffer and washing the hyphal clusters thoroughly on the 25-μm-mesh nylon screen eliminated color development. Subsequent digestion by SDS-NaOH treatment, which normally intensifies phenolic compound coloration, yielded a colorless solution.

DNA prepared as described above was sufficiently pure to allow PCR amplification of 16S rDNA in one of the dilutions tested from each nodule. In some cases, amplification was achieved from the equivalent of one hyphal cluster.

Amplification of *Frankia* DNA from *Coriaria arborea*. To test the reliability of PCR amplification of rDNA from actinorhizal nodules, 20 individual nodule lobes from separate *Coriaria arborea* nodules were prepared, and amplification was carried out as described above. The nodules had developed in a series of soils collected in a transect across volcanic Mt. Tarawera, which last erupted in 1886. Thus, the strains originated from diverse soils, ranging from rich organic soils at the base of the volcano to gravelly soils at the rim of the caldera, and the nodules ranged from small and dispersed to large and robust. In each case, a band at about 1,500 bp that corresponded to the expected size for the 16S rRNA gene was obtained.

Restriction enzyme digestion with *Alu*I, *Cfo*I, *Hin*fI, *Rsa*I, and *Taq*I gave identical restriction patterns for each amplicon, demonstrating that amplification of contaminants was not occurring. In each case, the sum of the fragment sizes in the restriction pattern was greater than the value expected from a single band of 16S rDNA (1,500 bp), suggesting that more than one gene was being amplified. Given the lack of bacterial contamination in the preparations, it seemed likely that plant organellar DNA, specifically, plastid DNA which has sequences complementary to primers fD1 and rD1, was the origin of the second product. By using the Mapsort program in the Genetics Computer Group programs (6), a search for restriction enzymes that cut plastid rDNA but not most bacterial rDNA was performed. Thus, the amplified DNA was digested with *Pvu*II, which cleaves plastid 16S rDNA once but does not cut bacterial 16S rDNA. As shown in Fig. 1, the expected products, products about 1,330 and 140 bp long, were obtained from about one-half of the DNA, and the rest remained uncut. Isolation of the uncut band from the gel and subsequent digestion yielded a restriction pattern consistent with *Frankia*

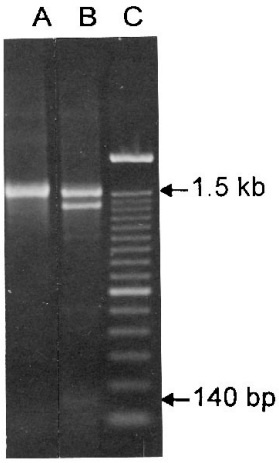

FIG. 1. *Pvu*II restriction digest of a *Coriaria arborea* nodule 16S rDNA amplicon. About one-half of the uncut amplicon from a *Coriaria arborea* root nodule lobe (lane A) was cut once with *Pvu*II (lane B). Lane C contained a 100-bp molecular weight ladder.

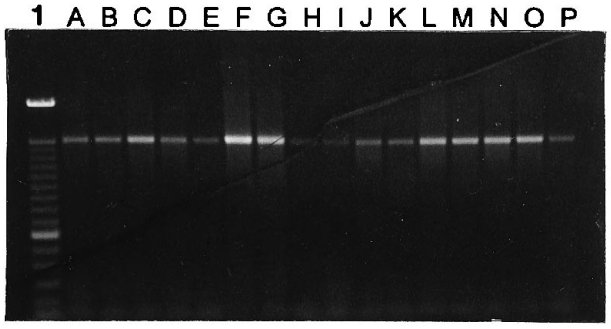

FIG. 2. PCR amplification of nearly full-length 16S rDNAs from diverse root nodules. Two nodule lobes were prepared from each species. Lane 1 contained a 100-bp molecular weight maker. Amplification mixtures were obtained from *Alnus cordata* (lanes A and B), *Alnus glutinosa* (lanes C and D), *Casuarina equisetifolia* (lanes E and F), *Coriaria arborea* (lanes G and H), *Ceanothus griseus* (lanes I and J), *Discaria toumatou* (lanes K and L), *Elaeagnus pungens* (lanes M and N), and *Purshia tridentata* (lanes O and P).

DNA (data not shown). Amplification of the plastid 16S rDNA from nonnodule plant roots yielded a single band that, when digested, gave a restriction pattern consistent with the pattern of the initial amplification minus *Frankia*-specific bands. Because the plastid DNA amplified from plant tissue accounted for all of the additional bands seen in the initial digestion preparations, we concluded (i) that multiple 16S rRNA genes from *Frankia* strains did not give rise to the complex restriction patterns, (ii) that both *Frankia* and plastid rDNAs were amplified, and (iii) that plastid DNA was present in hyphal clusters, even after nodule cells were disrupted. Transmission electron microscopy of infected cells from several actinorhizal plants, including *Coriaria* spp., revealed an abundance of plastids embedded in the hyphal matrix (19, 20).

Amplification from diverse actinorhizal nodules. Nodules from eight additional plant species belonging to six of the eight plant families reported to bear actinorhizal nodules were prepared by the protocols described above (Table 1). Each of these species gave the appropriately sized band upon amplification, although some gave a low signal, presumably because of the small amount of DNA initially present in the sample (Fig. 2). This problem was particularly evident with *P. tridentata* nodules, which yielded few hyphal clusters from the small-diameter nodule lobes used. An undiluted DNA sample resulted in more satisfactory amplification (data not shown).

Sequence analysis. To demonstrate that *Frankia* DNA was being amplified, a section embracing regions V1 and V2 of the 16S rDNA and corresponding to *E. coli* 16S rRNA coordinates 28 to 419 (between primers) was reamplified from the 1,500-bp products by using primers fD1 and rDB1. rDB1 was designed to amplify bacterial but not plastid DNA. The plants chosen for analysis were *Ceanothus griseus*, *Coriaria arborea*, *Coriaria plumosa*, *Discaria toumatou*, and *P. tridentata*. None of these plants has yielded isolates, nor have their nodule symbionts been studied by 16S rRNA analysis. The amplified products were purified and cycle sequenced in both directions. The resulting sequences and an alignment with *Frankia* 16S rDNA sequences are shown in Fig. 3.

The 378-bp sequences obtained are closely related to homologous sequences that have been reported for other *Frankia* strains. A comparison of the sequences with all of the small-subunit rRNA Ribosomal Database Project data available through the worldwide web resulted in identification of *Frankia* strains as the nearest neighbors for all of the sequences (11). Altogether, 38 variable sites were identified among the *Frankia* strains analyzed, with high levels of diversity occurring between positions 42 to 60 (V1) and positions 144 to 155 (V2). Sequences were aligned with the Pileup (6) and Clustal W (29) programs, and phylogenetic trees were constructed by the unweighted pair group with mathematical average method in the Genetics Computer Group Pileup program (6), by the maximum-likelihood method with the fastDNAml program (11), and by the parsimony method with the DNAPars program in PHYLIP, version 3.5c, with bootstrapping (Fig. 4) (7).

When the sequence from G48, an actinomycete isolated from *Podocarpus* roots, was used as an outgroup, three distinct *Frankia* lineages were revealed by all three methods. The sequences from a *Discaria* nodule and from members of the Elaeagnaceae form one group. The characteristic signature of this group includes a 2-bp deletion at positions 50 and 51 and a C at position 116 (Fig. 3). A second group is composed of sequences from *Coriaria* nodules plus sequences derived from *Purshia*, *Ceanothus*, and *Dryas* nodules. The *Purshia* sequence and two *Ceanothus griseus* sequences are identical to the sequence reported previously for *Dryas drummondii*, a member of the Rosaceae. These sequences are unique at positions 155 (A) and 226 (C). A diverse third group is formed by strains isolated from various *Alnus* spp. and *Casuarina equisetifolia* (strain ORS020606) and a strain from a *Myrica* nodule.

DISCUSSION

We used a relatively rapid method for isolating and amplifying *Frankia* DNA that minimized the amount of actinorhizal plant nodule tissue required for analysis. Sufficient DNA was obtained to yield a nearly full-length amplicon of the small-subunit rDNA from as little as 1 mg of nodule tissue. We estimated that about 90% of the initial tissue was removed by fractionation, leaving less than 100 μg (wet weight) of *Frankia* hyphal clusters and embedded plant organelles as the starting material for DNA isolation.

Amplification and sequencing of 16S rDNA from nodules have been used previously to identify *Frankia* strains in nodules (12, 14–17, 21, 22, 24). Our method differs from some previously described methods in that we used a small amount of tissue; larger amounts increased the chance of amplifying non-*Frankia* DNA or DNA from more than one resident *Frankia*

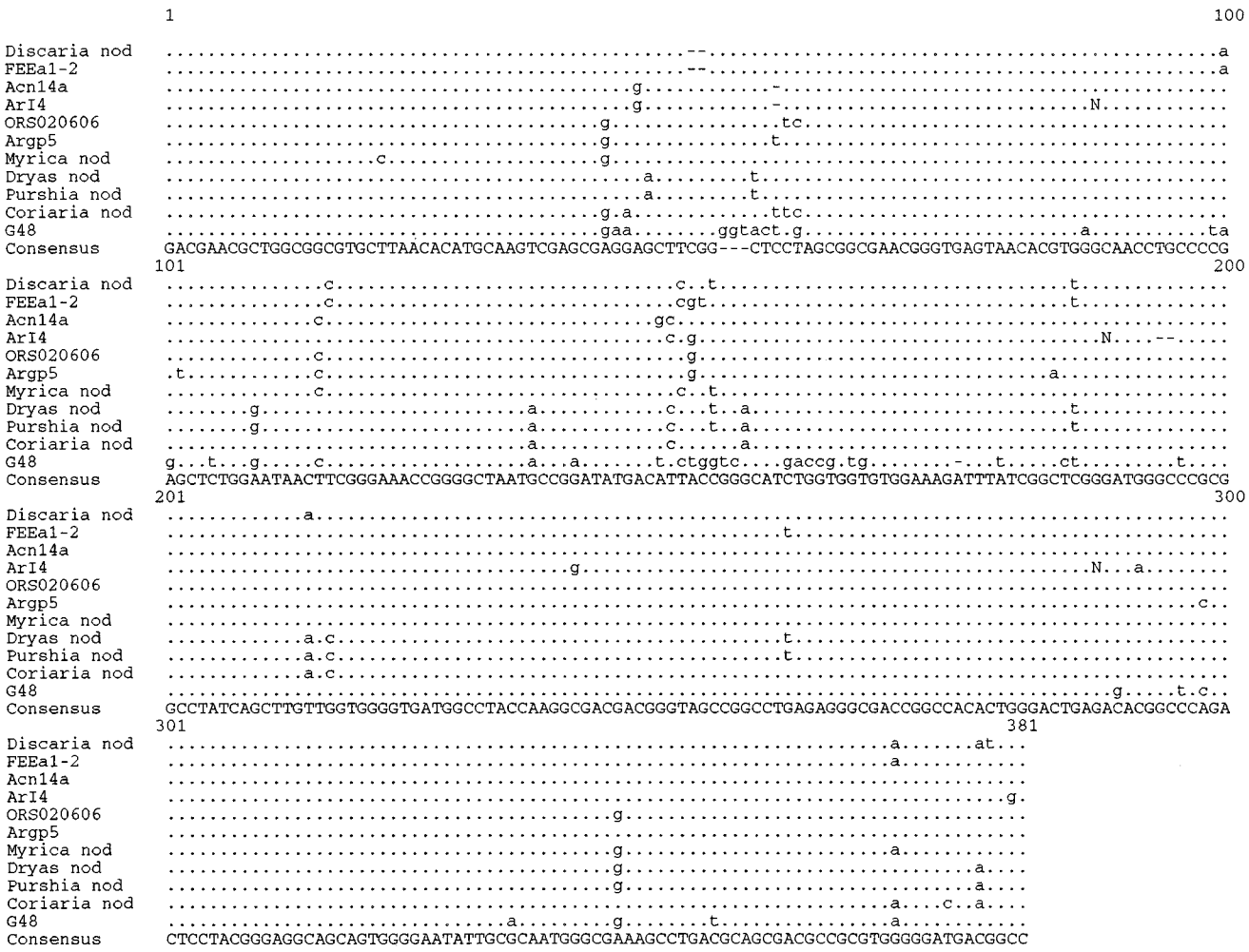

FIG. 3. Alignment of the partial 16S rRNA gene with other *Frankia* sequences. The alignment was created by using the Genetics Computer Group Pileup program. The positions correspond to *E. coli* coordinates 28 to 419. The primer fD1 and rDB1 sequences were not included in the analysis. The sequences used for alignment (and their accession numbers) were the Acn14a (M88466) (*Alnus* sp.), ArI4 (L11307) (*Alnus rubra*), Argp5 (L40612) (*Alnus* sp.), *Casuarina equisetifolia* strain ORS020606 (M55343), *Coriaria arborea* and *Coriaria plumosa* nodule (U54610), *Ceanothus griseus* nodule (U54608), *Discaria toumatou* nodule (U54609), *Dryas drummondii* nodule (L40616), FE-Ea12 (L40619) (*Elaeagnus* sp.), *Purshia tridentata* nodule (U54611), and isolate G48 (L11306) sequences. Nucleotides identical to the consensus nucleotides are indicated by dots, indeterminate nucleotides are indicated by N, and insertion-deletions are indicated by dashes. nod, nodule.

strain, as has been observed in other studies (15). We also avoided using digesting enzymes that can contribute spurious amplification signals. Adding ascorbate to maintain phenolic compounds in the reduced state was critical for isolating useful template DNA; other workers have used polyvinyl polypyrrolidone for this purpose (24). This addition shortened the extraction procedure to only a few steps. Additional time saving could be generated by directly cycle sequencing the amplified products from nodule lobes. Direct double-stranded sequencing from amplification products of isolated strains and some nodules has been done previously (17).

The amplicons chosen for sequencing were from nodules of members of plant genera that have not yielded *Frankia* isolates despite repeated attempts. The sequences clustered in three subclades, including (i) organisms that infect members of the Elaeagnaceae plus the genus *Discaria* (Rhamnaceae); (ii) symbionts obtained from members of the Rosaceae and Coriariaceae plus the genus *Ceanothus* (Rhamnaceae); and (iii) relatively more diverse symbionts obtained from *Alnus* (Betulaceae), *Casuarina* (Casuarinaceae), and *Myrica* (Myricaceae) nodules (17). Our results, like those of Nick et al. (21), place coriaria *Frankia* strains separate from the strains from the genera *Alnus* and *Elaeagnus*. The branch nearest the *Coriaria* sequence leads to endophytes of the Rosaceae and the genus *Ceanothus*. The partial sequence determined by Mirza et al. (14) for a *Coriaria* nodule endophyte falls into this group as well (data not shown).

The ceanothus and discaria *Frankia* strain sequences are the first sequences reported from members of the Rhamnaceae. The results of the phylogenetic analysis suggest that discaria *Frankia* strains are most closely related to symbionts of members of the Elaeagnaceae. Plants belonging to the Elaeagnaceae and the Rhamnaceae are closely related in phylogenies based on *rbcL* genes (28). In contrast, the sequences obtained from two *Ceanothus griseus* root nodules are identical to each other and to the sequences obtained from nodules of the rosaceous plants *P. tridentata* and *Dryas drummondii* (23). It has been established that crushed nodule homogenates from *Dryas drummondii* produce effective root nodules on *P. tridentata* (9), so similarity between the rosaceous symbionts might

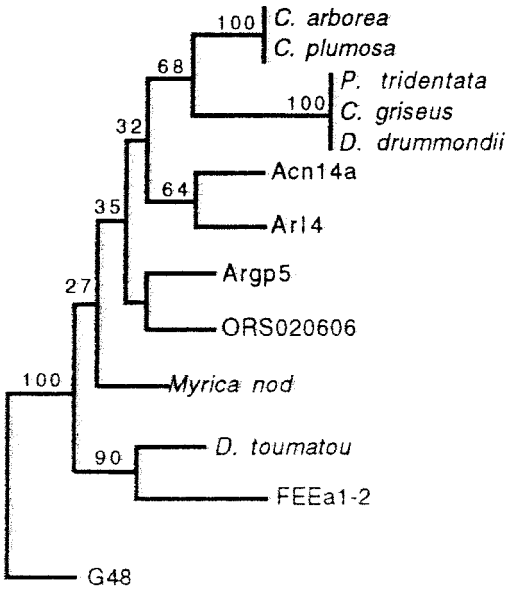

FIG. 4. Dendrogram of the aligned *Frankia* 16S rDNA sequences. After alignment with the Clustal W program (29), the strict consensus tree shown was derived from a family of trees constructed by the parsimony method, using the PHYLIP DNAPars program and a bootstrap sample of 100 replicates. The percentages of times that groups to the right of a node appeared together are shown at the branches. G48 was used as an outgroup. The GenBank accession numbers for the sequences are listed in the legend to Fig. 3.

be expected. On the other hand, the remarkable similarity of the three sequences may indicate that the diversity of *Frankia* strains that infect rosaceous plants and members of the genus *Ceanothus* is low, in contrast to what is observed in the genus *Alnus*, although a more thorough study should be done to address this issue. On the basis of the similarity of 16S rDNA sequences, one might predict that crushed nodules from *Ceanothus* sp. and crushed nodules from rosaceous species would cross nodulate.

It is surprising that the genus *Ceanothus*, in the Rhamnaceae, has a symbiont that is closely related, if not identical, to the symbionts of members of the Rosaceae. However, nodule ultrastructure is consistent with this observation. *Purshia*, *Dryas*, and *Ceanothus* symbionts all produce unusual, nonseptate vesicles in symbiosis (20). In contrast, the symbionts of members of the genus *Discaria*, another member of the Rhamnaceae, produce septate, spherical vesicles like those found in members of the Betulaceae and Elaeagnaceae (20).

Similarities and differences in vesicle morphology are generally not reliable indicators of strain identity. For example, the same *Frankia* strain can produce spherical vesicles on *Alnus* spp. and club-shaped hyphal swellings on *Myrica* spp. (10). In the case of *Purshia*, *Dryas*, and *Ceanothus* species, however, the similarity is particularly striking (18). In any event, before it is concluded that *Frankia* strains from rosaceous plants and from members of the genus *Ceanothus* are depauperate, it is necessary to conduct a more in-depth study on the inhabitants of nodules from different species and from different geographical regions.

Several partial and complete 16S rDNA sequences are now available from data banks for *Frankia* strains both in culture and within root nodules. The phylogenetic pattern that is beginning to emerge is a pattern of general congruence between the evolutionary trees of the host plants and bacterial symbionts, although the situation with the *Ceanothus* symbiont may be a very interesting exception. This pattern no doubt reflects the coevolution of actinorhizal plants and symbionts, as well as the role of symbiosis in the speciation of the microorganisms. Recent phylogenetic analyses of the large-subunit ribulose-1,5-biphosphate carboxylase/oxygenase (*rbcL*) sequence from chloroplasts has led to the identification of nitrogen-fixing lineages among plants (28). As more 16S rDNA sequences become available, it should be possible to estimate the degree to which life in the host plant has dominated the evolution of *Frankia* strains. It should also become possible to determine if members of the genus *Frankia* have entered symbiosis on more than one occasion by knowing the time of divergence of the major lineages compared with the angiosperm fossil record.

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