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Molecular Phylogeny of the Genus *Frankia* and Related Genera and Emendation of the Family *Frankiaceae*

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The members of the actinomycete genus *Frankia* are nitrogen-fixing symbionts of many species of woody dicotyledonous plants belonging to eight families. Several strains isolated from diverse actinorhizal plants growing in different geographical areas were used in this study. The phylogenetic relationships of these organisms and uncharacterized microsymbionts that are recalcitrant to isolation in pure culture were determined by comparing complete 16S ribosomal DNA sequences. The resulting phylogenetic tree revealed that there was greater diversity among the *Alnus*-infective strains than among the strains that infect other host plants. The four main subdivisions of the genus *Frankia* revealed by this phylogenetic analysis are (i) a very large group comprising *Frankia alni* and related organisms (including *Alnus rugosa* Sp+ microsymbionts that are seldom isolated in pure culture), to which *Casuarina*-infective strains, a *Myrica nagi* microsymbiont, and other effective *Alnus*-infective strains are related; (ii) unisolated microsymbionts of *Dryas*, *Coriaria*, and *Datisca* species; (iii) *Elaeagnus*-infective strains; and (iv) "atypical" strains (a group which includes an *Alnus*-infective, non-nitrogen-fixing strain). Taxa that are related to this well-defined, coherent *Frankia* cluster are the genera *Geodermatophilus*, "*Blastococcus*," *Sporichthya*, *Acidothermus*, and *Actinoplanes*. However, the two genera whose members have multilocular sporangia (the genera *Frankia* and *Geodermatophilus*) did not form a coherent group. For this reason, we propose that the family *Frankiaceae* should be emended so that the genera *Geodermatophilus* and "*Blastococcus*" are excluded and only the genus *Frankia* is retained.

The slowly growing members of the actinomycete genus *Frankia* are root symbionts that nodulate a wide range of perennial woody dicotyledonous plants. This nitrogen-fixing symbiosis is known to occur in more than 200 species of plants belonging to 24 genera and eight families that are called actinorhizal (6). The first *Frankia* strain was isolated in 1956 by Pommer (43), but this strain was subsequently lost. In 1978, Callaham et al. (8) isolated an infective *Frankia* strain from *Comptonia peregrina*, and since then hundreds of isolates have been obtained from a number of plant species growing in many geographical areas.

Becking (5) was unsuccessful in isolating the causative agent of actinorhizal nodules despite numerous attempts. He suggested that this organism was an "obligate symbiont" and devised a classification scheme based on cross-inoculation groups and on the morphology of the endosymbiont. This scheme was subsequently found to be erroneous when pure cultures became available (8). Nonetheless, Becking (5) correctly perceived the bacterial nature of the microsymbiont, named it *Frankia* sp., and classified it as the only member of the family *Frankiaceae* in the order *Actinomycetales*.

The members of the genus *Frankia* can now be clearly distinguished from other bacterial genera on the basis of their host specificity, their morphology (hyphae, a specialized thick-walled organelle called a diazovesicle, and multilocular sporangia containing nonmotile spores), their biochemistry (a type III cell wall that contains *meso*-diaminopimelic acid, glutamic acid, alanine, glucosamine, and muramic acid and type PI phospholipids that include phosphatidylinositol, phosphatidylinositol mannosides, and diphosphatidylglycerol), the presence of 2-*O*-methyl-D-mannose, and their physiology (29). The first attempts to classify members of the genus *Frankia* were based on infectivity groups. Baker (4) grouped *Frankia* strains into the following four infectivity groups by using pure cultures in cross-inoculation tests: strains that infect *Alnus* and *Myrica* species, strains that infect *Casuarina* and *Myrica* species, strains that infect *Elaeagnus* and *Myrica* species, and strains that infect only *Elaeagnus* species. Lalonde et al. (28) used a more complex approach, in which they relied on diverse phenotypic characteristics, and described two species, *Frankia alni* and *Frankia elaeagni*. These attempts at classification did not include microsymbionts of members of known host genera from which isolates have not been obtained.

It has always been agreed that taxonomy should be consistent with phylogeny and that polyphyletic units, for instance, should not include clearly foreign taxa even though the foreign taxa may share several physiological characteristics with the polyphyletic units. This process has resulted in the splitting of some genera, such as the genera *Pseudomonas* and *Rhizobium*, and the lumping together of other taxa, such as *Rhodococcus chlorophenolicus* and the genus *Mycobacterium* (7). The phylogeny of the actinomycetes was recently reviewed by Embley and Stackebrandt (12), and these authors did not suggest that the status of the family *Frankiaceae* as one of the eight families of the order *Actinomycetales* should be modified. 16S rRNA and 16S ribosomal DNA (rDNA) comparisons are becoming the preferred reference tools for bacterial taxonomy and phylogeny. This approach was used previously with partial (280-nucleotide) sequences of a limited set of strains and yielded a

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TABLE 1. Bacteria used in this study

Trivial designation of strain or organism	Characteristic or origin	Designation in trees	Reference or other designation	GenBank accession no.
Frankia spp. strains				
ARgP5	*Alnus* infective	FA-ARgP5	41a	L40612
ACoN24d	*Alnus* infective	FA-ACoN24d	48	L40610
AVN17s	*Alnus* infective	FA-AVN17s	14	L40613
AgB1-9	*Alnus* infective	FA-AgB1-9	19	L40611
Ea1-28	*Elaeagnus* infective	FE-Ea12	14	L40618
HR27-14	*Elaeagnus* infective	FE-Hr27-14	14	L40617
SCN10a	*Elaeagnus* infective	FE-SCN10a	41a	L40619
PtI1	*Purshia tridentata* isolate	F!-PtI1	4	L41048
Geodermatophilus obscurus				
subsp. *obscurus*	Amargosa Desert, Nev., soil	*Geodermatophilus-obs-obscurus*	ATCC 25078	L40620
subsp. *dictyosporus* G97	California soil	*Geodermatophilus-obs-dictyosporus*	17	L40621
Geodermatophilus obscurus "*Blastococcus aggregatus*"	Baltic Sea	*Blastococcus-aggregatus*	ATCC 25902	L40614
Dermatophilus congolensis	Scab from infected cattle	*Dermatophilus-congolensis*	ATCC 14637	L40615
Actinoplanes sp. strain A8232	Soil	*Actinoplanes-sp.* A8232	This study	L41047
Unisolated nodule strains				
Alnus rugosa (Sp+ nodule)	Trois-Rivières, Canada	AlnusR[a]	This study	L40956
Dryas drummondii	Gaspé, Canada	Dryas[a]	This study	L40616
Myrica nagi	Lawjinrew, Meghalaya, India	Myrica[a]	This study	L40622

[a] Organism that was not isolated before molecular characterization.

preliminary *Frankia* phylogeny (39). The purpose of this study was to use complete 16S rDNA sequences from a more comprehensive set of *Frankia* strains, avoiding strains having identical partial sequences (39), to obtain a more comprehensive view of the molecular phylogeny of this group of organisms.

MATERIALS AND METHODS

Bacterial strains. In this study we used eight *Frankia* strains, as well as unisolated nodular members of the genus *Frankia* and closely related actinomycetes (Table 1). The strains were grown at 28°C in F medium (*Alnus*-infective strains) (50), F medium containing Tween 80 (*Elaeagnus*-infective strains), or BAP medium (other strains) (38).

DNA extraction. Total DNAs were extracted from pure cultures (48) and from actinorhizal nodules (49) and used for amplification and sequencing.

PCR amplification. Double-stranded amplification was performed by using the whole *rrs* gene coding for 16S rRNA and a modification of the PCR procedure of Mullis and Faloona (37). The primers used to amplify the whole 16S rRNA gene that permitted subsequent cloning in a directional manner included primer FGPS5-255 (5′-TGGAAAGCTTGATCCTGGCT-3′), which contains a *Hin*dIII restriction site, and primer FGPS1509′-153 (5′-AAGGAGGGGATCCAGCCG CA-3′), which contains a *Bam*HI restriction site (the restriction sites are underlined). Each PCR was performed in a 50-μl (final volume) reaction mixture containing template DNA, reaction buffer (10 mM Tris-HCl [pH 8.3], 1.5 mM $MgCl_2$, 50 μM KCl, 10% [wt/vol] gelatin), each deoxynucleoside triphosphate at a concentration of 200 mM, 0.5 μM oligonucleotides, and 2 U of *Taq*I DNA polymerase (Gibco-BRL, Cergy-Pontoise, France). In some cases, the amplification reaction was performed directly with cells resuspended in amplification buffer. The amplification reactions were performed for 35 cycles, each of which consisted of denaturation at 95°C for 1 min, annealing for 1 min at 55°C, and extension at 72°C for 2 min. To analyze the amplification products, 5 μl of each reaction mixture was separated by electrophoresis on a 2% (wt/vol) agarose gel (NuSieve; FMC, Rockland, Maine). The amplified material consisted of a 1,500-bp double-stranded DNA fragment. The amplified fragments were digested with *Bam*HI and *Hin*dIII (digestion with *Hin*dIII had to be carried out overnight since the *Hin*dIII site is close to the extremities), cloned into *Bam*HI-*Hin*dIII-cut pBluescript II SK- vector (Stratagene, La Jolla, Calif.), and transformed into *Escherichia coli* DH5αF′ (Bethesda Research Laboratories).

Sequencing of DNA fragments. Five or more clones were obtained, pooled, and used in the sequencing reaction to obscure possible errors due to the *Taq* polymerase or to differences between copies of the gene. Below, the designations of the primers used for sequencing refer to the "small-subunit ribosomal gene," the number in each designation is the coordinate of the 5′ end in the *Frankia* sp. strain CeD homologous sequence (41) (GenBank accession number M55343), and a prime indicates that the primer is in the direction opposite transcription. Primers FGPS310-20 (GAGACACGGCCCAGACTCCT), FGPS485-292 (CAG CAGCCGCGGTAA), FGPS747-293 (AACAGGATTAGATAC), FGPS1047-295 (ATGTTGGGTTAAGTC), FGPS1156-39 (GACGTCAAGTCACATGC CC), FGPS305′-78 (CCAGTGTGGCCGGTCGCCCTCTC), FGPS505′-313 (GTATTACCGCGGCTGCTG), FGPS659′64 (CACCGCTACACCAGGAAT TC), FGPS910′-270 (AGCCTTGCGGCCGTACTCCC), and FGPS1176′-112 (GGGGCATGATCACTTGACGTC), as well as primers T3 and T7 (Pharmacia), were necessary to determine the sequence of the whole 16S rDNA molecule for both strands. The dideoxy chain termination sequencing procedure was performed by using T7 sequencing kits (Pharmacia LKB, Uppsala, Sweden). The sequence of *Frankia* sp. strain PtI1 was determined as described previously (20).

Data analysis. GenBank release 86 was scanned for related sequences by using the algorithms FASTA of Lipman and Pearson (31) as implemented by Higgins and Gouy (21) and BLAST (3). Sequences were aligned by using CLUSTAL V (22). Indel-containing regions (regions containing an insertion in one sequence or a deletion in another sequence) were excluded from the analysis. Matrix pairwise comparisons were corrected for multiple-base substitutions by the method of Kimura (25). Phylogenetic trees were constructed by the neighbor-joining (46) and parsimony (11, 26) methods. A bootstrap confidence analysis was performed with 1,000 replicates to determine the reliability of the distance tree topologies obtained (13). The resulting tree was drawn by using the NJPlot software of M. Gouy (Laboratoire de Biométrie, U.R.A. C.N.R.S. 2055, Université Lyon I).

RESULTS

Amplification from chromosomal DNA. Chromosomal DNAs of *Frankia* spp. that were obtained from pure cultures or nodules were used as sources of amplifiable material. In all cases, the amplified DNA was a single product of the expected size (approximately 1,500 bp between primers FGPS5-255 and FGPS1509′-153). Several clones (more than five) were pooled to avoid errors due to *Taq* amplification.

Sequencing and comparison of amplified 16S rDNAs. We determined the complete 16S rDNA sequences of all of the strains studied (Fig. 1) except the amplified fragment from *Actinoplanes* sp. isolate A8232, which has a *Hin*dIII site at coordinate 180. The sequence of this uncloned fragment had to be determined later by direct sequencing (39). The sequences were aligned with previously published 16S rRNA sequences of *Frankia* strains available from the GenBank database (Table 2).

In some cases amplification of nodule DNAs with the primers which we used resulted in amplification of *Frankia*-like sequences, as in the case of *Dryas drummondii*. In other cases mostly chloroplast-like sequences were obtained (for example, with *Alnus crispa*). A search of the GenBank database (by

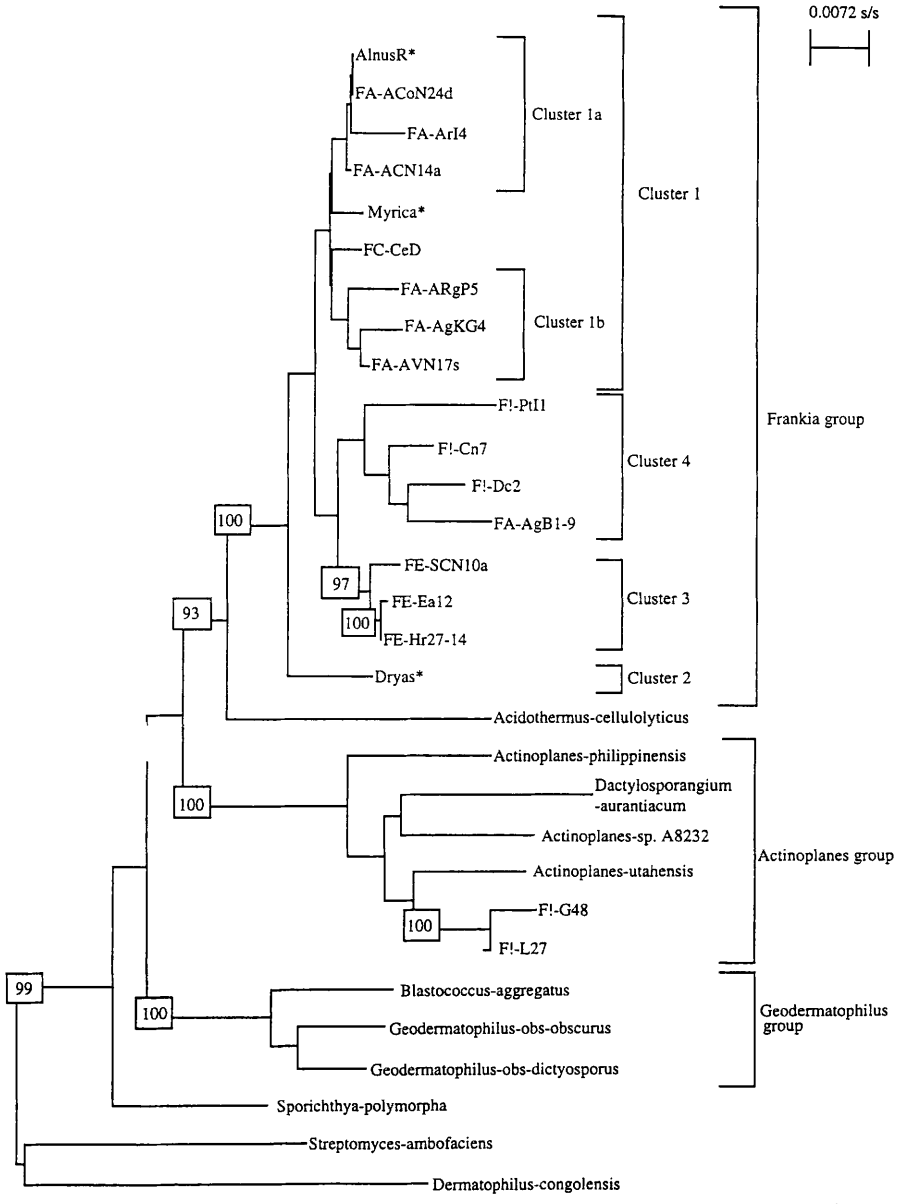

FIG. 1. Phylogenetic tree obtained by the neighbor-joining method (46). The *Streptomyces ambofaciens* and *Dermatophilus congolensis* sequences were used to define the root. The numbers in the boxes are bootstrap values; only the values that were greater than 90% are shown. A total of 1,389 sites were used for the calculations. Scale bar = 0.0072 substitution per site (s/s). An asterisk indicates that the *Frankia* strain was not isolated prior to molecular characterization.

using the BLAST or FASTA algorithms) revealed that the closest relatives of the *Alnus crispa* 16S rRNA amplicon were *Nicotiana plumbaginifolia* (locus TOBCP16SRD) and *Alnus incana* (locus AIU03555. RR1) chloroplast 16S rRNAs. In the case of *Alnus rugosa* nodules, 5 of 10 clones had chloroplast-like sequences, while the other 5 clones had typical *Frankia* sequences, which were included in this study. A comparison of the chloroplast 16S rRNA sequences with other bacterial 16S rRNA sequences revealed that a T was present in most chloroplast sequences at coordinate 30, while a C was present in all other bacteria, including, surprisingly, all cyanobacteria. This finding allowed us to synthesize primer FGP4-330 (5′-ATGGA GAGTTTGATCATGGCTCAGGAC-3′), which contained a *Bcl*I restriction site (underlined) (used for cloning) and effectively amplified nonchloroplast 16S rDNA preferentially from plant tissues. The addition to the amplification reaction mixture of restriction enzyme *Nru*I, for which a site is present in most chloroplast 16S rRNAs but absent in actinomycetes rRNAs, also helped eliminate unwanted chloroplast 16S rRNA molecules.

Clustering. Table 3 shows the numbers of substitutions for *Frankia* strains and related strains when regions of ambiguous alignment were not considered. The following clusters were identified by using the distance matrix and the resulting phylogenetic tree (Fig. 1).

Cluster 1 is a very broad group that contains the strains that infect *Alnus* species. Strain ACoN24d, which belongs to the genomic species *F. alni* (subcluster 1a), is very closely related to the unisolated strain found in *Alnus* Sp+ nodules; we identified only a few substitutions in indel-containing regions, which were not considered in the analysis. These organisms form a tight cluster with strains ArI4 and ACN14a. Strain ArI4

TABLE 2. 16S rRNA sequences obtained from GenBank

Organism	Source	GenBank accession no.	Designation in trees
Frankia sp. strains			
ACN14a	*Alnus crispa*, Canada	M88466	FA-ACN14a
ArI4	*Alnus rugosa*, United States	L11307	FA-ArI4
AgKG4-84	*Alnus glutinosa*, The Netherlands	L18976	FA-AgKG4
CeD	*Casuarina equisetifolia*, Senegal	M55343	FC-CeD
L27	*Podocarpus* sp., People's Republic of China	L11306	F!-L27
G48	*Podocarpus* sp., People's Republic of China	M59075	F!-G48
Cn7	*Coriaria nepalensis*, Pakistan	L18982	F!-Cn7
Dc2	*Datisca cannabina*, Pakistan	L18978	F!-Dc2
Acidothermus cellulolyticus		X70635	*Acidothermus-cellulolyticus*
Actinoplanes philippinensis		X72864	*Actinoplanes-philippinensis*
Actinoplanes utahensis		X80823	*Actinoplanes-utahensis*
Dactylosporangium aurantiacum		X72779	*Dactylosporangium-aurantiacum*
Sporichthya polymorpha		X72377	*Sporichthya-polymorpha*
Streptomyces ambofaciens		M27245	*Streptomyces-ambofaciens*

is the most distantly related member of this group; it also has the sequence that contains the highest number of undetermined positions and presumably also the highest number of errors. For this reason, strain ArI4 was always only distantly related to the other subcluster 1a organisms. Closely related to this subcluster are *Casuarina*-infective *Frankia* sp. strain CeD and the unisolated *Frankia* microsymbiont present in *Myrica nagi*. The other *Alnus*-infective strains, which form subcluster 1b, may or may not cluster with the members of subcluster 1a depending on which regions are considered. A parsimony analysis also revealed subcluster 1a containing *F. alni* (as well as the *Alnus* Sp+ nodule strain and strains ArI4, ACN14a, and ACoN24d), but not subcluster 1b or cluster 1 (data not shown).

In cluster 2 the closest neighbors of the *Dryas* microsymbiont are the two unisolated microsymbionts present in the root nodules of *Coriaria nepalensis* and *Datisca cannabina*. These three organisms form a tight and very coherent group (data not shown). The *Dryas* microsymbiont sequence was the only sequence used for further analysis since the other sequences were not complete and were very similar to the sequence of the *Dryas* microsymbiont. The *Dryas* microsymbiont had no close neighbors on the neighbor-joining tree, while it was closely related to the *Casuarina*-infective strain on the parsimony tree.

The three *Elaeagnus*-infective strains form a coherent cluster 3 (97% of the bootstrap replicates). In this cluster the region where the level of sequence divergence is highest is the region between coordinates 900 and 1200, which has been described for 10 strains (39). Because there is less variability at coordinates 1 to 300 (unpublished data), we decided not to determine complete sequences for all of the *Elaeagnus*-infective strains. This cluster was also detected by parsimony analysis (data not shown). The sequences of the three cluster 3 strains exhibited only nine mismatches, one in the first, fifth (coordinate 180) and eight between coordinates 974 and 1267.

The three atypical (noninfective and non-nitrogen-fixing) strains obtained from *Coriaria*, *Datisca*, and *Purshia* species form a broad group designated cluster 4 (bootstrap value, 77%) together with *Alnus*-infective, non-nitrogen-fixing strain AgB1-9. This group was not detected by parsimony analysis.

The four clusters described above form a highly coherent *Frankia* group (with a bootstrap value of 100%). This group was also identified by parsimony analysis, but the parsimony analysis group differed in that it included the genus *Acidothermus*.

In the *Actinoplanes* group, which is farther away from the *Frankia* group, are the so-called *Frankia* strains isolated from *Podocarpus macrophylla* (strain L27) and *Podocarpus nagi* (strain G48) in Fujian Province, People's Republic of China, by D. C. Yang, Y. Shi, and J. S. Ruan (29a), and these organisms form a relatively tight cluster together with *Actinoplanes* sp. strain A8232, which was identified on the basis of its morphology (multilobed sporangia, motile spores arranged in coils) and biochemistry (type II cell wall containing *meso*-diaminopimelic acid, arabinose, xylose, and glycine and type PII phospholipids, including phosphatidylethanolamine). This group was well supported by a bootstrap value of 100% and by the results of the parsimony analysis.

In the *Geodermatophilus* group the *Geodermatophilus* and "*Blastococcus*" sequences form a tight and coherent group (100% of the bootstrap replicates), and this group was also identified by parsimony analysis.

We found that two other sequences, the sequences of *Sporichthya polymorpha* and *Acidothermus cellulolyticus*, clustered with the *Frankia* and *Geodermatophilus* sequences. *Acidothermus cellulolyticus* clustered with the *Frankia* group in 93% of the bootstrap replicates, and in the parsimony analysis, this organism also fell in the *Frankia* group.

The *Frankia*, *Actinoplanes*, and *Geodermatophilus* groups, together with *Acidothermus cellulolyticus* and *S. polymorpha*, form a coherent (99% of bootstrap replicates) subline of descent within the *Actinomycetales*.

The *Dermatophilus congolensis* sequence was found to be only distantly related to the other sequences in all analyses.

DISCUSSION

The phylogeny of the genus *Frankia* and related taxa was analyzed by examining a distance matrix and performing a parsimony analysis with the 16S rDNA sequences. The distance matrix (Table 3) and a phylogenetic tree (Fig. 1) were constructed by transforming variations in the 16S rDNA sequences into evolutionary distance values. Our findings illustrate the relationships among *Frankia* spp. strains, as well as the relationships between these strains and unclassified strains. The following four main clusters were revealed by this phylogenetic analysis: (i) *F. alni*, the closely related *Casuarina*-infective *Frankia* strain, and a second subcluster of *Alnus*-infective strains; (ii) unisolated microsymbionts of *Dryas*, *Coriaria*, and *Datisca* species; (iii) the *Elaeagnus*-infective *Frankia* strains; and (iv) the atypical *Frankia* isolates (including infective but

TABLE 3. Distance matrix for taxonomic units

Organism	Distance or no. of differences																												
	FA-ACoN24d	FA-ACN14a	FA-ArI4	FA-AlnusR	FA-AVN17s	FA-ARgP5	FE-Hr27-14	FA-AgB1-9	Myrica	FA-AgKG4	F!-Dc2	F!-Cn7	FC-CeD	FE-Ea12	FE-SCN10a	Dryas	F!-PtI1	*Geodermatophilus- obs-dictyosporus*	*Geodermatophilus- obs-obscurus*	*Blastococcus-aggregatus*	F!-L27	F!-G48	*Actinoplanes-philippinensis*	*Actinoplanes-sp.* A8232	*Actinoplanes-utahensis*	*Dactylosporangium-aurantiacum*	*Streptomyces-ambofaciens*	*Sporichthya-polymorpha*	*Acidothermus-cellulolyticus*	*Dermatophilus-congolensis*
FA-ACoN24d		2	9	0	11	17	16	36	9	11	21	17	10	17	21	25	36	68	72	74	80	89	69	84	89	92	104	67	58	98
FA-ACN14a	1		11	2	9	15	18	39	9	10	22	18	8	19	21	25	38	68	74	77	78	87	67	82	87	90	104	69	56	97
FA-ArI4	7	8		9	20	26	25	46	18	15	26	22	19	26	30	33	33	75	80	83	85	85	73	90	95	98	111	74	66	104
FA-AlnusR[b]	0	1	7		11	17	16	37	9	11	21	17	10	17	21	25	36	68	72	75	80	89	69	84	89	92	104	67	58	98
FA-AVN17s	8	7	15	8		11	18	38	14	6	18	14	14	19	21	31	39	72	78	74	77	88	68	83	88	87	104	69	57	97
FA-ARgP5	12	11	19	12	8		25	42	19	12	23	20	13	26	28	34	43	75	82	79	82	92	71	88	91	93	108	76	62	101
FE-Hr27-14	12	13	19	12	13	18		33	20	15	18	14	22	1	7	29	34	75	77	78	76	86	76	85	89	86	98	65	61	96
FA-AgB1-9	27	29	35	28	28	31	24		39	19	15	18	43	34	38	54	40	80	82	84	75	87	72	89	87	90	102	73	69	96
Myrica[b]	7	7	13	7	10	14	15	29		12	22	20	10	21	25	25	40	69	72	76	77	86	66	79	84	89	102	64	60	97
FA-AgKG4	13	11	17	13	7	14	17	22	14		20	16	13	16	15	25	24	52	54	53	55	67	58	61	66	67	74	49	42	115
F!-Dc2	24	25	30	24	21	27	21	17	25	23		10	25	19	18	33	23	55	57	56	58	68	64	64	69	69	79	50	49	114
F!-Cn7	20	21	26	20	16	23	16	21	23	18	11		21	15	14	31	23	51	55	52	58	67	62	62	69	69	78	46	42	113
FC-CeD	7	6	14	7	10	9	16	32	7	15	29	24		23	25	21	43	70	73	78	78	87	69	79	86	87	107	72	59	101
FE-Ea12	12	14	19	12	14	19	1	25	15	18	22	17	17		8	30	35	76	78	79	77	87	77	84	90	86	99	66	62	96
FE-SCN10a	15	15	22	15	15	21	5	28	18	17	21	16	18	6		32	39	74	78	79	77	87	76	86	88	86	101	66	62	95
Dryas[b]	18	18	25	18	23	25	21	41	18	29	38	36	15	22	24		54	68	71	78	81	90	73	84	85	87	104	61	61	92
F!-PtI1	27	29	25	27	29	33	26	30	30	28	27	27	33	26	29	41		88	91	96	84	86	82	95	97	103	107	82	73	108
Geodermatophilus-obs-dictyosporus	53	53	59	53	56	58	58	63	54	62	65	60	54	59	58	53	70		26	38	88	96	75	94	92	102	103	69	81	92
Geodermatophilus-obs-obscurus	56	57	63	56	60	64	60	64	56	64	68	65	56	60	60	55	72	20		37	90	100	87	97	98	105	108	67	80	89
Blastococcus-aggregatus	56	59	64	57	56	60	59	64	58	63	66	61	60	60	60	59	75	29	28		101	112	91	103	108	112	115	73	83	89
F!-L27	63	61	67	63	60	64	59	59	60	68	71	71	61	60	60	64	67	71	72	81		6	45	35	30	45	112	84	86	94
F!-G48	68	67	66	68	67	71	66	67	66	81	82	81	67	67	67	69	67	76	79	87	5		52	44	40	56	126	95	97	103
Actinoplanes-philippenensis	60	58	64	59	58	61	66	62	57	71	78	76	59	67	66	63	72	66	77	79	39	44		53	38	55	101	84	79	91
Actinoplanes-sp. A8232	67	65	73	67	66	70	68	71	63	73	77	74	63	67	69	67	78	76	78	83	28	34	46		48	49	121	92	87	110
Actinoplanes-utahensis	68	66	74	68	67	70	68	66	64	79	83	83	66	69	67	65	76	72	77	84	23	30	32	38		55	121	92	92	89
Dactylosporangium-aurantiacum	77	75	83	76	72	77	71	75	74	82	85	85	72	71	71	72	87	86	89	94	37	45	48	40	45		125	101	91	105
Streptomyces-ambofaciens	80	80	87	80	80	83	75	79	79	89	96	95	83	76	78	80	84	81	85	90	90	99	89	99	94	106		94	112	85
Sporichthya-polymorpha	51	52	57	51	52	58	49	56	48	58	59	54	55	50	50	46	64	54	52	56	66	73	73	74	71	84	72		73	78
Acidothermus-cellulolyticus	50	48	58	50	49	54	53	60	52	54	63	54	51	54	54	53	65	71	70	73	78	86	79	79	81	87	100	64		104
Dermatophilus-congolensis	125	124	131	126	124	129	123	123	125	94	93	92	129	124	122	119	136	116	113	114	118	131	104	133	115	124	110	101	116	

[a] The values on the lower left are distances, expressed as number of substitutions per site (×1,000), as described by Kimura (25). The values on the upper right are the observed numbers of differences in the nonhypervariable regions.

[b] Organism that was not isolated before molecular characterization.

non-nitrogen-fixing strain AgB1-9). The four clusters were coherent and together formed the *Frankia* group. As determined in this analysis, the *Alnus*-infective strains (cluster 1) are by far the most diverse strains (largest difference not including indel-containing regions, 1.9%). The *Elaeagnus*-infective group (cluster 3) exhibited far less diversity (nine mismatches overall or 0.6% difference between strain SCN10a and strain Ea1-12). These organisms were predicted by the results of a PCR restriction fragment length polymorphism analysis of the *nifDK* intergene sequence (23) to be among the most distant isolates in that group of strains. Authors who arrived at different conclusions (16, 28) used a set of closely related *Alnus*-infective strains that were characteristic of the strains consistently isolated in America as well as in Europe.

(i) *F. alni* and the Sp+ phenotype (subcluster 1a). Our study included an unisolated *Frankia* strain obtained from a *Alnus rugosa* nodule in Canada. Using the PCR technique, we were able to specifically amplify the 16S rDNA of this strain by using total DNA extracted from the *Alnus* nodule. This strain corresponds to a phenotype designated Sp+ (52), which is rarely isolated in pure culture, and grouped very closely with *F. alni*, as did another *Alnus crispa* Sp+ nodule strain obtained from the French Alps (39). Thus, we concluded that a major subdivision of the genus *Frankia* should not be created for the Sp+ phenotype (28).

(ii) Subcluster 1b: the second *Alnus*-infective group of strains. The second group of *Alnus*-infective strains (which does not include *Alnus*-infective, non-nitrogen-fixing strain AgB1-9) (19) is made up of at least three strains, two of which belong to different genomic species (14). This group is not well defined, as indicated by the differences in topology observed with the method which we used, and the data probably reflect the large distances that exist between the strains. The fossil record suggests that the genera *Alnus* and *Myrica* are the oldest actinorhizal genera (33, 51). Cross-infectivity studies (4) have shown that the genus *Myrica* is a promiscuous host and thus has the largest potential range of infective strains. In the case of the genus *Alnus*, studies have revealed that the actual diversity of strains that are isolated from and infect *Alnus* species is great. The deep branching of strains that infect *Myrica* and *Alnus* species and the ancient appearance of *Alnus* and *Myrica* species in the fossil record suggest that the other groups of *Frankia* strains may have evolved from the cluster 1 strains as other host plants for *Frankia* strains evolved.

Organisms related to subclusters 1a and 1b include *Casuarina*-infective strain CeD and the *Myrica nagi* microsymbiont. The relative positions of the four subclusters are impossible to locate precisely with the data available.

(iii) *Dryas* microsymbionts. The unisolated microsymbiont present in *Dryas* species in Canada (where neither *Coriaria* nor *Datisca* species grow) was found to be very closely related to the other unisolated microsymbionts present in *Coriaria* and *Datisca* species in Pakistan (34), Mexico, New Zealand, and France (40). No strains in this well-defined taxon (100% of bootstrap replicates) have been induced to grow in the media used for pure-culture isolation of *Frankia* strains. On the other hand, morphological features (host specificity of crushed nodules, diameter of hyphae, size and shape of nitrogen-fixing vesicles) of the *Frankia* strains in nodules led Becking (5) to consider two of these three microsymbionts members of different species. The third microsymbiont infected *Datisca* plants that were not then known to be actinorhizal; these plants were later discovered to be nodulated by *Frankia* strains (9) and to have a unique morphology inside their nodules (round diazovesicles oriented toward the center of the host cell, with an excentric stele). The level of similarity of the sequences of these three organisms is high, and two of these organisms (the *Coriaria* and *Datisca* microsymbionts) can be cross-inoculated (34). On the other hand, it has been determined by *rbcL* sequence analysis that the Rosaceae (which includes the genus *Dryas*) is quite distant from the Datiscaceae and the Coriariaceae (2, 10). It is likely that *Frankia* morphology within nodules is determined by the host plant, as has been shown to be the case in *Alnus* and *Myrica* species (27). In spite of their different host plants the three microsymbionts form a well-defined and statistically significant cluster.

(iv) *Elaeagnus*-infective strains. The three *Elaeagnus*-infective *Frankia* strains, which form well-defined cluster 2, have most of their mismatches between coordinates 974 and 1267. This is in contrast to most other pairs of sequences (for example, the ACoN24d and ACN14a sequences), in which most mismatches are between coordinates 83 and 182; this conforms to the general pattern of the 16S rRNA molecule (54). For this reason the complete 16S rRNA sequences of other *Elaeagnus*-infective strains were not determined, as partial sequencing of coordinates 904 to 1188 (39) and coordinates 1 to 260 (47) revealed that different *Elaeagnus*-infective genomic species had at most nine mismatches.

(v) Atypical strains. Unclassified strain PtI1, which was isolated from *Purshia tridentata*, was the only isolate obtained from a rosaceous plant. The family Rosaceae comprises several nonactinorhizal taxa of economic importance, such as raspberry and strawberry plants. Our phylogenetic tree (Fig. 1) revealed that strain PtI1 is separated from the other *Frankia* strains and genomic species, which is consistent with the specific characteristics of this strain. Strain PtI1 was reported to be noninfective on its original host plant and not to hybridize with *nif* probes (39). Its closest relatives are atypical strains Cn7 and Dc2, which were isolated from *Coriaria* and *Datisca* species, respectively (35), and which share with PtI1 the inability to reinfect their original hosts and also lack detectable *nif* genes. These strains group with strain AgB1-9, an *Alnus*-infective strain, with which they share an inability to fix nitrogen and a lack of *nif* hybridizing fragments (19). This broad cluster was not significant as determined by a bootstrap analysis and was not found in the parsimony study.

Family *Frankiaceae*. Grouping the *Geodermatophilus* strains with the genus *Frankia*, as proposed by Fox and Stackebrandt (15) and Hahn et al. (18), is problematic. Both the distance matrix analysis and the parsimony analysis results group the *Actinoplanes* cluster with the genus *Frankia* but not with the coherent *Geodermatophilus*-"*Blastococcus*" group. These clustering results can change according to the number of sequences, the strains included in the study, and the regions considered (because of the presence of indel-containing regions). The findings to date suggest that the three groups are equidistant from each other.

The results described above indicate that the phylogenetic tree derived from an analysis of complete 16S rDNA sequences is different from the tree based on 16S rRNA oligonucleotide catalogs and partial sequences (18); the *Actinoplanes* group is at least as close to the genus *Frankia* as the *Geodermatophilus*-"*Blastococcus*" group is.

Even if the average catalog (S_{AB}) values for *Actinoplanes* and *Frankia* strains could be considered high enough (S_{AB}, 0.51 to 0.55) (12), the relationships found when 16S rRNA oligonucleotide catalogs were used were not confirmed by the results of a study of the entire 16S rDNA molecule. Because of the imprecise relationship between S_{AB} and sequence similarity (54), catalog S_{AB} values are used only for preliminary clustering, while the use of these values in cladistic analyses can lead to erroneous conclusions, as shown by the results of this

TABLE 4. Differential characteristics of actinomycetous genera in the *Frankiaceae-Geodermatophilaceae-Actinoplanes* subline of descent

Characteristic	*Frankia*	*Geodermatophilus*	"*Blastococcus*"[a]	*Sporichthya*[b]	*Acidothermus*[c]	Actinoplanes[d]
Hyphae	Extensive	Rudimentary	Rudimentary[e]	Short, holdfasts	Slender, floccules	Extensive
Branching	++	+/−	+/−	Sparse	−	++
Sporangia	Multilocular	Multilocular	Multilocular	Fragmented hyphae	−	Oligosporous, multisporous, none
Motility	−	+/−	+/−	+/−	−	+/−
Flagellum	−	Polar	Lateral	Polar	−	Polar, lateral
Vesicles	+	−	−	−	−	−
Oxygen requirement[g]	A, MA	A	A, MA	A, AN	A	A, AN
Temp requirement	Mesophile	Mesophile	Mesophile	Mesophile	Thermophile[h]	Mesophile
Growth rate[i]	Slow (10–60 days)	Rapid	Rapid (2 h)	Rapid	Rapid (6 h)	Rapid, slow
Nitrogen fixation	+	−	NT[j]	NT	NT	NT
Cellulase activity	+	NT	−	NT	++	+/−
Gram stain reaction	+, Variable	+, Variable	+	+, Variable	−, Variable	Variable
Cell wall type(s)[k]	III	III		I	(Serine)	I, II, III, IV
Whole-cell sugar pattern[l]	D, E, or B	C		C		D
Phospholipid type[m]	PI	PII				PII
Nucleotides at positions 955 to 1225[n]	+	+	+	+	+	−
G+C content (mol%)	66–71	72.9–74.6		70	60.7	71–73
Habitat	Soil, plant roots	Soil, sea	Sea	Soil, compost	Hot spring	Soil, water
Type species (type strain)	*Frankia alni* (CpI1 [= ACoN24d = ATCC 33029])[o]	*Geodermatophilus obscurus* (ATCC 25078)	*Blastococcus aggregatus* (B15 [= ATCC 25902])	*Sporichthya polymorpha* IMRU 3913 [= ATCC 23823])	*Acidothermus cellulolyticus* (ATCC 43068)	*Actinoplanes philippinensis* (ATCC 12247)

[a] Data from reference 1, unless indicated otherwise.
[b] Data from reference 30.
[c] Data from reference 44.
[d] Data from reference 53.
[e] Data from reference 32.
[f] ++, strongly positive; +, positive; −, negative; +/−, positive or negative.
[g] A, aerobic; MA, microaerophilic; NA, anaerobic.
[h] The optimum temperature is 55°C.
[i] The values in parentheses are doubling times.
[j] NT, not tested.
[k] Type I cell walls contain LL-diaminopimelic acid, type II cell walls contain *meso*-diaminopimelic acid and glycine, and type III cell walls contain *meso*-diaminopimelic acid; all of the cell walls contain glutamic acid, alanine, glucosamine, and muramic acid.
[l] In whole-cell sugar pattern B madurose is present; in whole-cell sugar pattern C no characteristic sugar is present; in whole-cell sugar pattern D xylose and arabinose are present; and in whole-cell sugar pattern E fucose is present.
[m] In phospholipid type PII phosphatidylethanolamine and/or methylethanolamine is present; in phospholipid type PI no nitrogenous phospholipid is present.
[n] Nucleotides CATGTGGC at positions 955 to 1225 (*E. coli* numbering) are considered diagnostic for some actinomycetous sublines of descent (44).
[o] The type strain deposited in the American Type Culture Collection, strain CpI1 (= ATCC 33029), belongs to genomic species 1 (designated *F. alni*), as does strain ACoN24d. Unfortunately, type strain CpI1 appears to be difficult to grow from lyophilisates (3a). The two strains have identical partial sequences (39).

study based on 16S rDNA sequence comparisons, a more precise technique.

One possible result is to place the *Geodermatophilus* group in the family *Frankiaceae*, in which case the genus *Actinoplanes* would also belong to this family together with the thermophilic cellulolytic nonsporulating rod-shaped organism *Acidothermus cellulolyticus* (44) and maybe also the compost-inhabiting motile spore-forming organism *S. polymorpha* (45), thus ending their "search for a family" (30). Another possible result is to consider the family *Actinoplanaceae* and the *Geodermatophilus-"Blastococcus"* group two distinct families that are closely related to the *Frankiaceae*. When we considered phenotypic data, which must be consistent with phylogenetic results, the genera *Geodermatophilus* and *Frankia* had few distinctive characteristics compared with other actinomycetes other than multilocular sporangia. The cell wall type (type III) cannot be considered a reliable criterion as many morphologically, physiologically, and ecologically different actinomycetes have this cell wall type. On the other hand, several phenotypic features are shared by the genera *Geodermatophilus* and *Actinoplanes*, including spore motility, phospholipid type PII, peptidoglycan type A1γ, and major menaquinone MK-9(H_4).

Since the first possibility would result in lumping together taxa that have clearly different taxonomic properties (Table 4), we propose that the *Geodermatophilus-"Blastococcus"* group should be placed in a family of its own (the family *Geodermatophilaceae*) and that the paraphyletic family *Frankiaceae* as defined by Hahn et al. (18) should be emended.

Time scale of *Frankia* evolution. According to Ochman and Wilson (42) and Moran et al. (36), the evolutionary rate for the whole 16S rRNA molecule between pairs of operational taxonomic units was 1 to 2% per 50×10^6 to 55×10^6 years during the last 500×10^6 years. The maximum distance for the large coherent group containing the four *Frankia* lineages is about 4%, which corresponds to about 100×10^6 to 200×10^6 million years; this value is consistent with the time of appearance of most major groups of angiosperms, including the Myricaceae and Betulaceae, which appeared 90×10^6 to 100×10^6 years ago (33). The other *Frankia* lineages are more similar to one another; for instance, the distance between the *Elaeagnus*-infective *Frankia* strains and the *F. alni* lineage is 1%, which corresponds to about 25×10^6 to 50×10^6 years. This value is consistent with the time of appearance of the Elaeagnaceae, estimated to be about 30×10^6 years ago on the basis of the fossil record. On the other hand, the *Casuarina*-infective strains and the *F. alni* lineage differ by 0.4%, which corresponds to a time of appearance of 10×10^6 to 20×10^6 years ago, well after the first occurrence of *Casuarina* pollen (about 80×10^6 years ago) (24). It is noteworthy that these estimates of evolutionary timing concur, but imprecise dating methods preclude firm conclusions.

In this study we found that we could characterize and classify strains in pure culture and that we could also characterize at the molecular level new strains that are present in nodule tissue without the difficult and time-consuming task of isolating the symbionts from nodules. By obtaining genetic information in this manner for undescribed *Frankia* strains, such as the symbionts of actinorhizal hosts belonging to the Coriariaceae, Datiscaceae, Rhamnaceae, or Rosaceae, it should be possible to determine the relationships of these organisms with well-known strains isolated from *Alnus*, *Elaeagnus*, or *Casuarina* root nodules.

Emendation of the family *Frankiaceae* Hahn et al. 1989. Hyphae are extensive. Aerial mycelium is absent. The hyphal diameter varies from 0.5 to 2.0 μm. Branching is limited to extensive. The mycelium may bear terminal or lateral thick-walled diazovesicles. A part of the thallus is composed of irregularly shaped cuboid to oval cells that divide in more than one plane. These cells are nonmotile, possess an outer membrane, and may germinate to give filaments. Gram positive to gram variable. Aerobic to microaerophilic. The nitrogen sources used are amino acids and ammonia; atmospheric nitrogen is fixed in vivo and in vitro by most strains. The carbon sources used include carbohydrates, organic acids, and fatty acids. Found in soil and as symbionts of higher plants. The cell wall type is type III (*meso*-diaminopimelic acid, glutamic acid, alanine, glucosamine, and muramic acid). The whole-cell sugar patterns include patterns B (3-*O*-methyl-D-galactose, madurose), D (xylose), and E (fucose). 2-*O*-Methyl-D-mannose is a diagnostic sugar. Phospholipid pattern PI. The major menaquinone is MK-9(H_4). Fatty acid pattern 1. All strains tested contain hopanoid lipids (25a). The family comprises the single genus *Frankia*.

Emendation of the family *Geodermatophilaceae*. Hyphae are rudimentary. Aerial mycelium is absent. The hyphal diameter varies from 0.5 to 2.0 μm. The thallus is composed of irregularly shaped cuboid to oval cells that divide in more than one plane. These cells serve as propagules, may be motile or nonmotile, and may germinate to produce filaments or buds, which develop into motile cells. Gram positive. Aerobic. Members utilize sources of nitrogen such as amino acids and ammonia and do not fix nitrogen. The carbon sources used include carbohydrates, organic acids, and fatty acids. Found in soil and in the sea. The cell wall type is type III. The whole-cell sugar pattern is pattern C (no characteristic sugars). Phospholipid pattern PII. The major menaquinone is MK-9(H_4). Fatty acid pattern 2b. Peptidoglycan type A1γ. This family comprises the genus *"Blastococcus"* and the genus *Geodermatophilus*.

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