Chapter 2

POLYPHASIC TAXONOMY OF THE GENUS *FRANKIA*

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1. INTRODUCTION

Members of the genus *Frankia* are generally described as nitrogen-fixing actinomycetes that form root nodules in symbiosis with more than 200 species of non-leguminous woody plants in 25 genera of angiosperms (Benson and Silvester, 1993; Huss-Danell, 1997; Schwintzer and Tjepkema, 1990). Although knowledge about the interaction of woody plants and *Frankia* has a long history, pure cultures of this actinomycete have been obtained only relatively recently. Initial attempts to characterize the bacterium taxonomically referred to the microsymbiont as an obligate symbiotic bacterium and used its ability to form root nodules and morphological characteristics, such as filament and vesicle formation in cells of these root nodules, as discriminative criteria with respect to other actinomycetes (Becking, 1970). These criteria led to the emendation of the family *Frankiaceae* with the type genus *Frankia* (Becking, 1970). Ten species were proposed within the genus *Frankia* based on the formation of specific host-plant infection groups after inoculation with crushed nodules (Becking, 1970). Later investigations with pure cultures, however, demonstrated that the host-plant infection groups were quite different (Baker, 1987; Normand and Lalonde, 1986).

The first successful isolation of *Frankia* occurred less than 30 years ago (Callaham *et al.,* 1978). Since then, hundreds of *Frankia* isolates have been obtained from root nodules, a natural locale of enrichment for this organism, using different isolation techniques (Baker, 1990; Baker *et al.,* 1979a; 1979b; Baker and O'Keefe, 1984; Benson, 1982; Berry and Torrey, 1979; Burggraaf, 1984; Diem and Dommergues, 1983; Hiyoshi *et al.,* 1988; Kim *et al.*, 1993). However, no general isolation protocols have been developed and only a small percentage of isolation

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attempts succeeds (Rosbrook *et al.,* 1989; St-Laurent and Lalonde, 1987). Successful attempts to isolate frankiae from soil, its second ecological niche, have been reported only once (Baker and O'Keefe, 1984).

Problems with the isolation of frankiae have been attributed to long generation times, the requirement of special isolation factors (Quispel *et al.*, 1989), the lack of specific nutritional requirements of pure cultures obtained so far (Akkermans *et al.,* 1992), and the large variability of isolates combined in the genus *Frankia* (Akimov and Dobritsa, 1992; Fernandez *et al.,* 1989; Lechevalier, 1994). Isolation and cultivation, however, are prerequisites for the characterization of *Frankia* by traditional techniques that focus on the analysis of protein and isoenzyme patterns, susceptibility and resistance to antibiotics, sugar or fatty acid composition, or other criteria, such as pigment production (Benson and Hanna, 1983; Benson *et al.,* 1984; Dobritsa, 1998; Gardes *et al.,* 1987; Gardes and Lalonde, 1987; Girgis and Schwencke, 1993; Hafeez *et al.,* 1984; Igual *et al.*, 2001; Lalonde *et al*., 1988; Lechevalier and Ruan, 1984; Mirza *et al.,* 1991; Mort *et al.,* 1983; Simon *et al.,* 1989; St-Laurent *et al.,* 1987; Wheeler *et al.,* 1986).

Many isolates can clearly be distinguished from other bacterial genera on the basis of morphological, cytochemical, and physiological characteristics (Baker, 1990). Distinctive morphological characteristics of isolates include filamentous growth, the formation of multilocular sporangia containing non-motile spores, and the differentiation of thick-walled vesicular structures at the tips of filaments (vesicles), which are the principal locus of nitrogenase and, thus, of nitrogen fixation (Lechevalier, 1994). Cells are characterized by a type-III cell wall that contains *meso*-diaminopimelic acid, glutamic acid, alanine, glucosamine and muramic acid, type PI phospholipids with phosphatidylinositol, phosphatidylinositol mannosides and diphosphatidylglycerol, and 2-*O*-methyl-D-mannose as a diagnostic sugar (Lechevalier, 1994). The most prominent physiological characteristics are their ability to fix N_2 under nitrogen-limited conditions, even in pure culture, and the formation of root nodules with specific host plants (Lechevalier, 1994).

This combination of phenotypic characteristics has frequently proven useful for the identification of frankiae, even though single features might be shared by other organisms. In certain morphological features, such as cells dividing in more than one plane, *Frankia* strains resemble *Geodermatophilus obscurus* and *Dermatophilus congolensis*, but differ from these in the possession of sporangia. Still, morphological features and cell-wall type of both *Frankia* and *Geodermatophilus* were the basis for a classification in a taxon "multilocular sporangia" (Goodfellow, 1986). The expression and thus the presence of many of the characteristic phenotypic features of *Frankia*, however, might vary with respect to the isolate and its growth conditions. The extent of sporangia formation, for instance, varies from isolate to isolate and might be totally suppressed under certain growth conditions. Although many *Frankia* strains produce typical terminal sporangia in liquid culture, the extent to which these sporangia are formed can differ significantly between strains (Figure 1). Others do not form terminal sporangia under the same growth conditions, but display intercalary sporangia only or no sporangia formation at all

(Figure 1). The latter two might form typical terminal sporangia under different growth conditions. Spores of frankiae are also found in root nodules of several actinorhizal plants (*e.g*., *Alnus* sp.) (van Dijk, 1984; van Dijk *et al.,* 1988; Weber, 1986). However, isolates producing spores in root nodules (spore (+) type) have not yet been obtained in pure culture, thus, all *Frankia* strains are of the spore (–) type.

Vesicles, the sites of nitrogen fixation, are generally formed under nitrogen-limited growth conditions, but may be formed, though inactive, in the presence of fixed-nitrogen resources (Figure 2). In addition to the typical diazotrophic frankiae, some non-N_2-fixing (atypical) isolates have been obtained from actinorhizal nodules (Baker *et al.,* 1980; Hahn *et al.,* 1988; Mirza *et al.,* 1992). Recently, these atypical *Frankia* strains have been shown to occur quite commonly in natural habitats (Wolters *et al.,* 1997a; 1997b). Atypical isolates produce hyphae and terminal sporangia, but no vesicles in either pure culture or root nodules (Figure 2); consequently, they do not grow in the absence of fixed-nitrogen resources (Hahn *et al.*, 1988).

Root-nodule formation by atypical isolates has been observed, but might be suppressed by the plant (Hahn *et al.*, 1988; van Dijk and Sluimer, 1994), thus, limiting the validity of root-nodule formation as a characteristic trait of frankiae. Root-nodule formation on specific host plants has been used as a differentiating characteristic between groups of frankiae. Most isolates obtained to date can be classified into four host-infection groups; those that nodulate i) *Alnus* and *Myrica*,

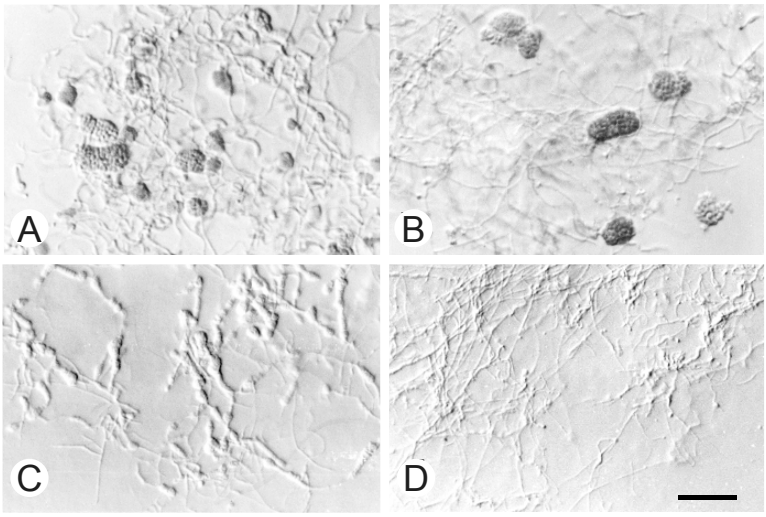

Figure 1. Frankia *strains AgGS'84/44 (A), AgGS'84/45 (B), AgKG'84/4 (C), and AgGA'84/2 (D) grown in liquid P+N medium. Although AgGS'84/44 and AgGS'84/45 form typical terminal sporangia, but to different extents, strain AgKG'84/4 forms intercalary sporangia only and AgGA'84/2 exhibits no sporangia formation on this medium. Bar represents 50 µm.*

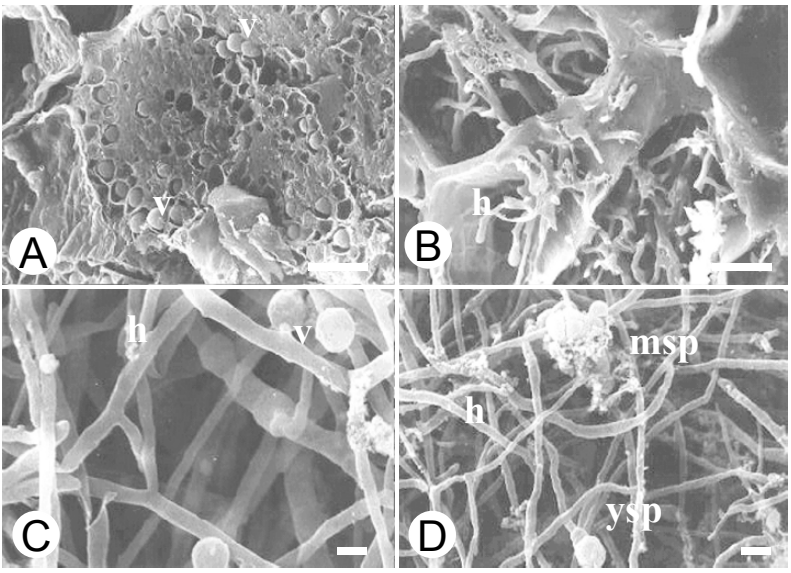

Figure 2. Root nodules formed by the N_2-fixing Frankia *strain Ag45/Mut15 (A) display typical vesicle (v) clusters in plant cells, whereas those formed by the non-N_2-fixing (atypical)* Frankia *strain AgB1.9 (B) only harbor hyphae (h) but not vesicles. The N_2-fixing strain develops vesicles in pure culture, but not sporangia (C). Sporangia (young (ysp) and mature (msp) sporangia), but no vesicles, are produced by the non-N_2-fixing strain (D). Bars represent 10 µm (A, B) and 1 µm (C, D), respectively (modified from Hahn et al., 1988).*

ii) *Casuarina* and *Myrica*, iii) *Myrica* and *Elaeagnus*, and iv) members of the *Elaeagnaceae* (*Elaeagnus*, *Hippophaë*, *Shepherdia*) (Baker, 1987). However, this classification is incomplete because isolates from several actinorhizal plant taxa (*e.g.*, *Coriaria*, *Datisca*) are still lacking. Furthermore, some isolates cross the boundaries between host-infection groups (Bosco *et al.,* 1992), whereas others show variable compatibility to plant species of the same genus (Hahn *et al.*, 1988; Huss-Darnell and Myrold, 1994; Weber, 1990). Thus, the usefulness of "unique" morphological and physiological features for the identification of frankiae is limited. Consequently, a reliable and more objective classification and identification of frankiae and *Frankia*-like bacteria requires the introduction and application of new taxonomic methods, notably chemical, numerical and molecular taxonomic procedures.

Recently, molecular taxonomic procedures have increasingly been used as alternatives to traditional techniques that are limited by successful isolation of *Frankia*. Whole plasmids (Simonet *et al*., 1988), *nif* genes (Baker and Mullin, 1994; Rouvier *et al*., 1996; Mirza *et al.*, 1994; Normand *et al*., 1992; Simonet *et al.*, 1990; 1991), intergenic spacer regions (IGS) (Bosco *et al*., 1996; Lumini and Bosco, 1996; Lumini *et al*., 1996; Navarro *et al*., 1997; Rouvier *et al.*, 1992; Simonet *et al.*, 1991), glutamine synthetase genes (*gln*) (Cournoyer and Lavire, 1999; Hosted *et al*.,

1993; Rochefort and Benson, 1990), the *rec*A gene (Marechal *et al.*, 2000) and ribosomal RNAs (Bosco *et al.*, 1992; Hahn *et al*., 1989a; Hahn *et al*., 1997; Hönerlage *et al*., 1994; Mirza *et al.*, 1992; Mirza *et al.*, 1994; Navarro *et al.*, 1997; Nazaret *et al*., 1989; 1991; Rouvier *et al.*, 1992; 1996; Simonet *et al*., 1994; Zepp *et al.*, 1997) have all been used successfully as specific targets for the characterization of isolates as well as for the identification of uncultured endophytes in root nodules. These new procedures reveal much about the genetic diversity and distribution of *Frankia*, and have refined and expanded knowledge about endophyte-host specificities. The most prominent advancement in studies on the taxonomy of *Frankia* has resulted from the use of polyphasic taxonomy approaches integrating information retrieved by a wide range of techniques on different levels of taxonomic resolution (Murray *et al.*, 1990).

2. POLYPHASIC TAXONOMY APPROACH

Polyphasic taxonomy approaches integrate phenotypic, genotypic, and phylogenetic information retrieved by a relatively large set of techniques with different resolving power (Figure 3; see Rossello-Mora and Amann, 2001; Vandamme *et al.*, 1996 for review). Methods that retrieve phenotypic information comprise all methods not directed toward DNA or RNA, including chemotaxonomic techniques (Vandamme *et al.*, 1996). Examples are API or BIOLOG substrate-utilization screening, cell wall-structure analysis, fatty-acid methyl-ester analysis (FAME), whole-cell protein profiling, both multilocus- and iso-enzyme electrophoresis (MLEE, IEE), and serological and phage-typing techniques (Figure 3).

Genotypic information is obtained by methods that use either complete or partial features of the genome. The former include DNA base-composition analysis (%G+C), DNA-DNA relatedness or re-association studies, and fingerprinting techniques, such as ribo-typing, low-frequency restriction-fragment analysis (LFRFA), pulsed-field gel electrophoresis (PFGE), and restriction fragment length polymorphism (RFLP) analysis. Partial genomic-typing techniques use the potential of the polymerase chain reaction (PCR) that enables thermocyclic amplification of target sequences on DNA by extension of specifically annealed primers by a thermostable DNA polymerase (e.g. *Taq* polymerase) (Mullis and Faloona, 1987; Saiki *et al.*, 1988). PCR-based typing techniques include arbitrary primed PCR (AP-PCR), randomly amplified polymorphic DNA analysis (RAPD), amplified ribosomal DNA restriction analysis (ARDRA), and genomic fingerprinting that targets endogenous interspersed repetitive sequences (rep-PCR). The introduction of PCR and the shift in focus from the complete genome to specific genes has opened possibilities to characterize uncultured organisms. PCR-assisted retrieval of specific genes, e.g. ribosomal RNA genes that carry phylogenetically significant sequence information, might then be used as basis for comparative sequence or probe analyses (Ludwig *et al.*, 1998).

A reliable classification should focus on different levels of taxonomic resolution. At higher taxonomic levels, comparative 16S-rRNA sequence analysis is most appropriate because large differences in sequence between organisms can be expected. Comparative 16S-rRNA sequence analysis is reliably used for

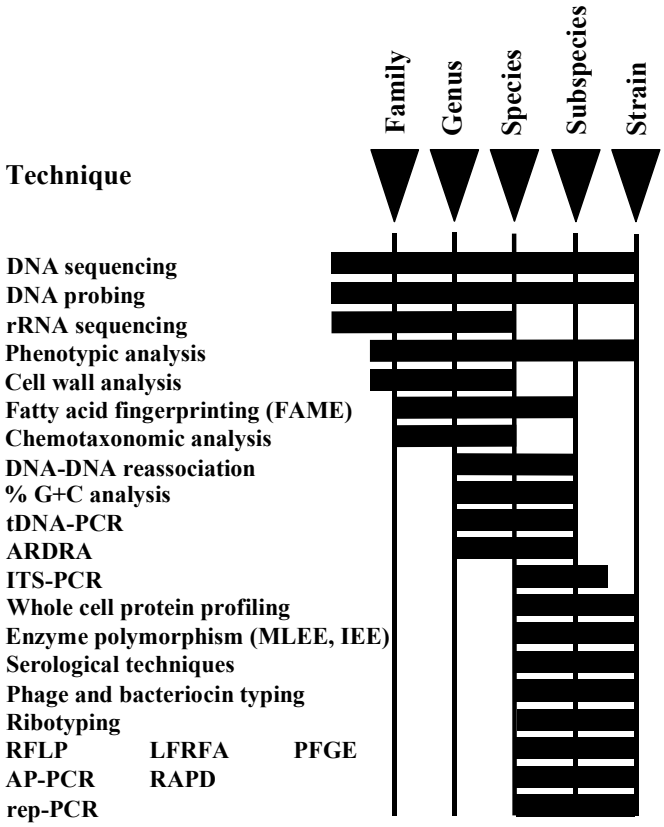

Figure 3. Taxonomic resolution of some currently used techniques (modified from Vandamme et al., 1996).

assignments from the domain level down to the genus level as characterized by a 95% sequence similarity borderline (Ludwig *et al.*, 1998). Further assignment at the species level is indicated by a 97% similarity borderline of the 16S rRNA, however, although generally confirmatory (Rossello-Mora and Amann, 2001; Stackebrandt and Goebel, 1994), there are several examples of different species with identical or nearly identical 16S-rRNA sequences (Fox, Wisotzkey, and Jurtshuk, 1992; Martinez-Murcia, Benlloch, and Collins, 1992). Furthermore, both micro-heterogeneity of the 16S-rRNA genes within a single species (Bennasar, Rossello-Mora, Lalucat, and Moore, 1996) and single organisms with two or more 16S rRNA genes with relatively high sequence divergence (Mylvaganam and Dennis, 1992; Nübel *et al.*, 1996) have been reported.

The current standard for species delineation is DNA-DNA relatedness because a high degree of correlation was found between DNA-DNA relatedness and

chemotaxonomic, genomic, serological and numerical similarity (Rossello-Mora and Amann, 2001). A species is defined as a group of strains, including the type strain, sharing 70% or greater whole genomic DNA-DNA relatedness. A further division of the species into subspecies, the lowest official taxonomic rank in nomenclature, could be based on different typing techniques, indicating minor but consistent pheno- or genotypic differences between strains of the species (Rossello-Mora and Amann, 2001). However, consistency is often not achieved and, thus, typing techniques often result in groupings of strains without formal nomenclatural standing.

For *Frankia*-like isolates or uncultured endophytes in root nodules, a classification or identification should incorporate information obtained in a polyphasic top-to-bottom approach of taxonomic resolution. Focussing on nucleic-acid analyses, an initial comparative 16S-rRNA sequence analysis provides information on their phylogenetic position and permits an assignment on the genus level. Subsequent characterization on the species level through DNA-DNA relatedness studies is followed up by sub-species level analyses provided by either comparative sequence analysis of variable regions on rRNAs or by fingerprinting techniques.

3. 16S-rRNA SEQUENCE DATA

Because of their ubiquity and genetic stability, ribosomal RNA (rRNA) sequences have been used intensively to investigate quantitative evolutionary relationships among numerous bacteria (Ludwig *et al.*, 1998; Woese, 1987; Woese, Kandler, and Wheelis, 1990). Phylogenetic characterization has been performed using sequence comparison of all three rRNAs (5S, 16S and 23S rRNA) (Fox and Stackebrandt, 1987; Ludwig *et al.*, 1992; Ludwig *et al.*, 1998; Woese *et al.*, 1990; Winker and Woese, 1991), even though only 16S and 23S rRNA molecules are of appropriate size for broad phylogenetic analyses. At the moment, the most elaborated databases of primary structures of informative macromolecules have been compiled for 16S-rRNA molecules (Maidak *et al.*, 1997).

Initially, oligonucleotide catalogs obtained by partial sequence analysis, using oligonucleotide fragments produced by digestion with ribonuclease T_1, were used to establish distant phylogenetic relationships (Fox and Stackebrandt, 1987; Stackebrandt *et al.*, 1985). Oligonucleotide catalogs of the 16S rRNA of *Frankia* strain AirI1 and *Geodermatophilus obscurus* indicated a high degree of phylogenetic relationship between the organisms, which led to considerations of assigning both genera to the family *Frankiaceae*. This informal proposal was confirmed when a combination of oligonucleotide cataloguing and reverse-transcriptase sequencing of 16S rRNA (Embley *et al.*, 1988; Lane *et al.*, 1985; Lane *et al.*, 1988) was used to refine the phylogenetic position of two *Frankia* strains, AirI1 and Ag45/Mut15 (Hahn *et al.*, 1989b). This analysis led to the clustering of *Frankia*, "*Blastococcus*" and *Geodermatophilus* into one family *Frankiaceae*, which, however, excluded *Dermatophilus* (Hahn *et al.*, 1989b).

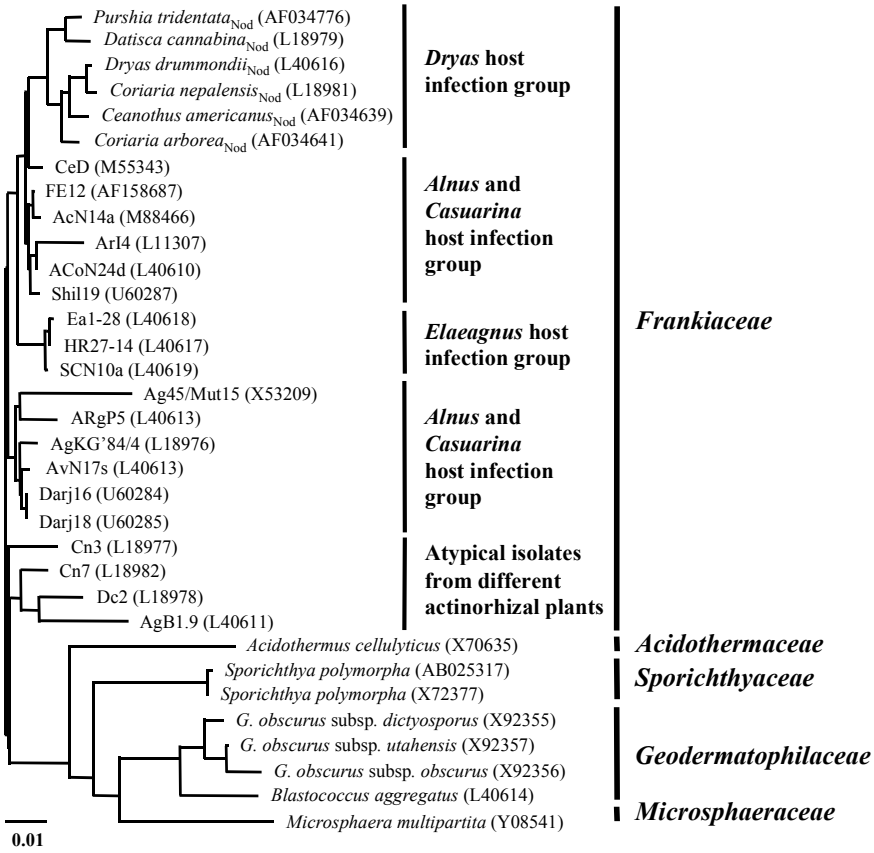

Figure 4. Neighbor-joining tree of aligned, almost complete 16S rRNA sequences of selected members of the suborder Frankineae *within the order Actinomycetales.*

The emendation of the family *Frankiaceae* was refined again when rapid sequencing protocols and large databases became available. Comparative sequence analysis of almost complete PCR-amplified 16S rDNAs of pure cultures of *Frankia*, as well as of uncultured endophytes in root nodules, led to the emendation of the family *Frankiaceae* to contain only the genus *Frankia* (Normand *et al.*, 1996). Taxa related to *Frankia* were the genera *Geodermatophilus*, "*Blastococcus*", *Sporichthya*, *Acidothermus*, and *Actinoplanes*. Similar results were obtained when a new hierarchical classification system, which was solely based on 16S rRNA and rDNA, was proposed for the taxa between the levels of genus and class for the actinomycete line of descent (Stackebrandt et al., 1997). The genus *Frankia* was the only genus within the family *Frankiaceae*. Together with the families *Acidothermaceae*, *Geodermatophilaceae*, *Microsphaeraceae* and *Sporichthyaceae*,

the family *Frankiaceae* were assigned to the suborder *Frankineae*, one of ten suborders within the order *Actinomycetales* (Stackebrandt *et al.*, 1997).

With the exception of the *Geodermatophilaceae*, each family is represented by a single genus; *Microsphaera*, *Acidothermus*, *Sporichthya*, *Geodermatophilus* and *Blastococcus*, and *Frankia*, respectively (Figure 4). Within the genus *Frankia*, four main subdivisions were proposed (Normand *et al.*, 1996). These superseded an earlier separation of closely related strains that belonged to the *Alnus/Casuarina* host-infection group from strains of the *Elaeagnus* host-infection group (Nazaret *et al.*, 1991). These new subdivisions included: (i) a large group mainly comprised of typical N_2-fixing strains belonging to the *Alnus* and the *Casuarina* host-infection groups; (ii) uncultured endophytes of *Dryas*, *Coriaria*, and *Datisca* species; (iii) strains of the *Elaeagnus* host-infection group; and (iv) atypical non-N_2-fixing strains (Figures 4 and 5) (Normand *et al.*, 1996). Although based on a relatively small number of sequences, the proposed subdivisions are still valid for sequences obtained from isolates or uncultured nodule populations, all of which cluster into the respective groups, even though many sequences are only short and partial (Figure 5).

Isolates and/or uncultured endophytes of nodules from *Gymnostoma* (Navarro *et al.*, 1997) and from *Colletia hystrix*, *Talguenea quinquenervia*, *Trevoa trinervis*, *Retanilla ephedra*, *Discaria serratifolia*, and *D. toumatou*, for example, were closely related to frankiae of the *Elaeagnus* host-infection group (Figure 5) (Benson *et al.,* 1996). Further studies suggested that frankiae that infect members of the *Elaeagnaceae* might share a common ancestor with uncultured endophytes in root nodules of *Ceanothus* spp. (Murry *et al.,* 1997). Endophytes in nodules of *C. americanus* (Clawson *et al*., 1998), *C. griseus* (Benson *et al.*, 1996) and *C. caerulens* (Ramirez-Saad *et al*., 1998) were assigned to the *Dryas* host-infection group (Figures 4 and 5). The uncultured endophytes in root nodules of *Purshia tridentata* (Benson *et al.*, 1996)*, Coriaria arborea* (Nick et al., 1992), *Coriaria plumosa* (Benson *et al.*, 1996), as well as the closely related endophytes in nodules of *Datisca cannabina* (Mirza *et al.*, 1994), also clustered with the *Dryas* group. Non-N_2-fixing and non-nodulating isolates from nodules of *C. caerulens* (Ramirez-Saad *et al.*, 1998), *D. cannabina* and *Coriaria nepalensis* (Mirza *et al.*, 1994) were not related to this group, however (Normand *et al.*, 1996), and clustered with the atypical frankiae isolates derived from *A. glutinosa* nodules (Figure 4). Uncultured non-N_2-fixing endophytes in nodules of *A. glutinosa* were found to belong to this hitherto undescribed cluster within the genus *Frankia* (Wolters *et al.*, 1997b).

Although 16S rRNA sequences enabled differentiation between frankiae of different host-infection groups as well as between uncultured endophytes of the spore(+) and spore(–) types in nodules of *A. incana* (Simonet *et al.*, 1994), differences are generally relatively small. Therefore, 16S-rRNA sequence analysis is the method of choice for the classification of frankiae at the genus level, but is of limited value for further classification within the genus (Hahn *et al.*, 1989a; Nazaret *et al.*, 1991), even though some correlation between groupings based on the analysis of variable regions of 16S rRNA (Nazaret *et al.*, 1991) and DNA-DNA relatedness studies (Fernandez *et al.*, 1989) has been observed.

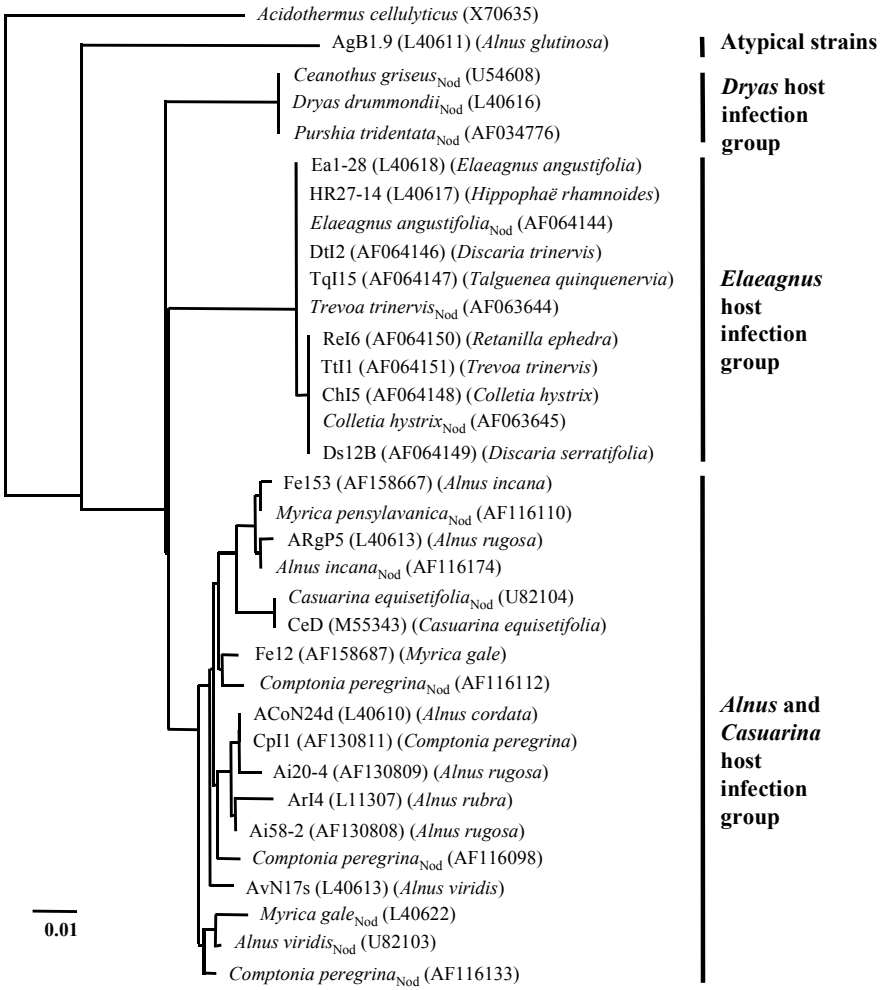

Figure 5. Neighbor-joining tree of aligned, mainly partial 16S rRNA sequences of selected Frankia *strains (with original host plant in brackets) and of uncultured nodule populations (host plant$_{Nod}$).*

4. DNA-DNA RELATEDNESS

The species in bacterial taxonomy is defined as a group of strains, including the type strain, that share 70% or greater whole genomic DNA-DNA relatedness with a ΔT_m of 5°C or less (Stackebrandt and Goebel, 1994; Wayne *et al.*, 1987). T_m is the

Strains analyzed	Genomic species (Original No.)	*Reference*	Genomic species (Proposed No.)
***Alnus* and *Casuarina* host infection group**			
ArI4, ArbN4b, ACN1, AGN1g, CpI1, $AGN1_{EXO}^{AG}$, MpI1, AirI1, AvcI1	A1	(An *et al.*, 1985)	1
ACoN24d, Ar24H3, Ar24O2, $Ag24_{251}$, ArI3, ARgN22d, ACN1, CpI1, A2J	1	(Fernandez *et al.*, 1989)	1
AvcI1, RBR162013, RBR162008, RBR162010, RBR162014	B1	(Bloom *et al.*, 1989)	1
CpI1, ArI3, ArI4, AvcI1, AvcI1.R1, AirI1, An2.1, An2.24, AgPM2.8, A7, A43, A51, A153	P1	(Akimov and Dobritsa, 1992)	1
ArI4	S2	(Shi and Ruan, 1992)	1
AV22c, ANV17o, $Ac23_{40}$, $AI43_1$	2	(Fernandez *et al.*, 1989)	2
$ARgP5^{AG}$	3	(Fernandez *et al.*, 1989)	3
Ai1c, Ai15a, Ag9b	P2	(Akimov and Dobritsa, 1992)	?
Ai6b	P3	(Akimov and Dobritsa, 1992)	?
AirI2	P5	(Akimov and Dobritsa, 1992)	?
CeD, CcI3, CeF, AllI1, ORS020608, ORS020609, CjI82, ORS022602	9	(Fernandez *et al.*, 1989)	4
C1, C2, C3, C4, C5	S1	(Shi and Ruan, 1992)	?
***Elaeagnus* host infection group**			
$Ea1.12_{,}$ $Ea1_{2,}$ $Ea2_{6}$, $Ea3_{3}$, $Eacm5_{1}$, $HR27_{14}$	4	(Fernandez *et al.*, 1989)	5
$TX31e^{HR}$, $EAN1_{pec}$, HRX401a	5	(Fernandez *et al.*, 1989)	6
EUN1f	6	(Fernandez *et al.*, 1989)	7
HRN18a	7	(Fernandez *et al.*, 1989)	8
$Ea50_1$	8	(Fernandez *et al.*, 1989)	9
EaI10, EaI11, EaI8	P6	(Akimov and Dobritsa, 1992)	?
Cc1.17	P7	(Akimov and Dobritsa, 1992)	?
S15, S14, S13, Hr5-o, H109	P8	(Akimov and Dobritsa, 1992)	?
E1, E13, E15	10	(Lumini *et al.*, 1996)	?
2.1.7, HrI1	11	(Lumini *et al.*, 1996)	?
EuI1b	P9	(Akimov and Dobritsa, 1992)	?
G2, D11**	A2	(An *et al.*, 1985)	?
Atypical isolates from different actinorhizal plants			
AgI5	P4	(Akimov and Dobritsa, 1992)	10
PtI1	S3	(Shi and Ruan, 1992)	11

Figure 6. Data on DNA-DNA relatedness support the differentiation into host-infection groups and indicate a high diversity of the genus Frankia *with at least eleven genomic species reliably separated and several others to be confirmed (as indicated by the question marks).*

melting temperature of the hybrid determined by stepwise denaturation, and ΔT_m is the difference in T_m in degrees Celsius between the homologous and the heterologous hybrids formed under standard conditions (Vandamme *et al.*, 1996). Attempts to define species within the genus *Frankia* were initially based on host-infection groups as the major criterion. This criterion resulted in the proposal of two species, *F. elaeagni* and *F. alni*, with the latter composed of two subspecies, *vandijkii* and *pommerii*, based on the ability or not to form spores in root nodules (Lalonde *et al.*, 1988). DNA-DNA relatedness was used to differentiate strains from different host-infection groups with ratios of homology between 67% and 94% within one host-infection group or levels lower than 50% between different host-infection groups (Akimov and Dobritsa, 1992; An *et al.,* 1985; Bloom *et al.,* 1989; Fernandez *et al.*, 1989; Shi and Ruan, 1992).

These results have led to the emendation of *F. alni* (Fernandez *et al.*, 1989), but have also indicated a much higher diversity of the genus *Frankia* than initially suggested (Lalonde *et al.*, 1988). At least four genomic species were proposed in the *Alnus* and *Casuarina* host-infection group and five genomic species in the *Elaeagnus* host-infection group (Fernandez *et al.*, 1989). The large diversity in DNA-DNA relatedness of *Frankia* strains was confirmed by other studies that distinguished five genomic species in the *Alnus* and *Casuarina* host-infection group and four within the *Elaeagnus* host-infection group (Akimov and Dobritsa, 1992). To date, data on DNA-DNA relatedness are available for about 80 strains (Figure 6). Unfortunately, virtually all data sets were obtained with different strains so making comparative analysis and delineation of distinct genomic species highly speculative. Although the assignment of 19 genomic species has been indicated among *Frankia* strains (Dobritsa, 1998), eleven genomic species might be reliably separated with several others to be confirmed (Figure 6). This confirmation and further delineation of distinct genomic species, however, requires comprehensive studies on DNA-DNA relatedness that include the respective reference strains and additional phenotypic characterization (Wayne *et al.*, 1987).

5. 23S-rRNA SEQUENCE DATA

In contrast to 16S rRNA, 23S rRNA generally shows a greater variation of both sequence and length due to the presence or absence of additional sequence stretches compared to the 23S-rRNA gene sequence of *E. coli* (Höpfl *et al.,* 1989). Domain III of the 23S rRNA of gram-positive bacteria with a high DNA G+C content contains a large insertion specific for these organisms (Roller et al., 1992). This region exhibits considerable sequence differences between organisms of different genera (Roller *et al.*, 1992). A sequence comparison of the whole ribosomal operon for two *Frankia* strains, one belonging to the *Alnus* and the other to the *Casuarina* host-infection group, showed that the large variability of this region was only exceeded by that of the intergenic spacer regions (Hahn *et al.*, 1997).

Remarkable variation in this region was observed within the genus *Frankia*, when sequences of *Frankia* strains belonging to the *Alnus* host-infection group (Hönerlage *et al.*, 1994; Maunuksela *et al.*, 1999) were compared to those of *Frankia* belonging to other host-infection groups (Hönerlage *et al.*, 1994). The

results confirmed the classification of the strains into host-infection groups (Hönerlage *et al.*, 1994) as proposed by 16S-rRNA sequence analysis (Normand *et al.*, 1996). In addition, strains belonging to the *Alnus* host-infection group could be separated roughly into four subgroups, three containing typical N_2-fixing strains and a fourth exclusively of non-N_2-fixing strains (Hönerlage *et al.*, 1994). Recently, a further subgroup containing typical N_2-fixing *Frankia* strains of the *Alnus* host-infection group was proposed (Maunuksela *et al.*, 1999).

Using sequence differences in the entire insertion as well as in target sites for existing probes as criteria for the design of new subgroups, a further differentiation could be made. Based on sequence divergence between *Frankia* strains of subgroup IIIb, for example, three subgroups represented by the original reference strain AgB32 (IIIb), as well as by strain ARgP5 (IIId) and by strain AgKG'84/4 (IIIe) might be designed within this *Alnus* host-infection subgroup (Figure 7).

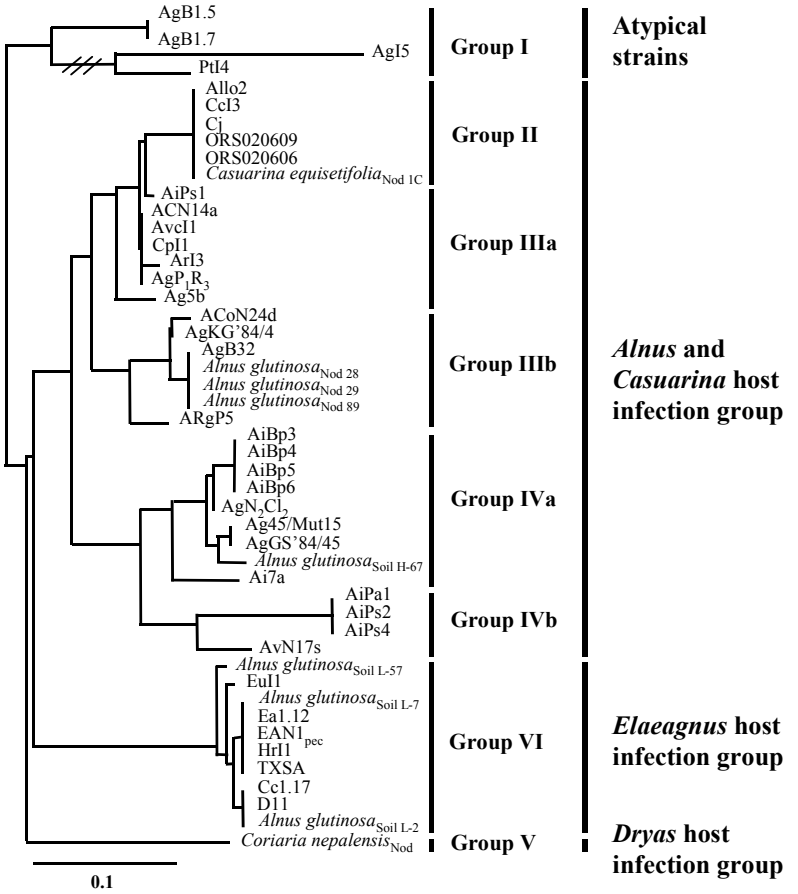

Figure 7. Neighbor-joining tree based on comparative sequence analysis of an actinomycetes-specific insertion in Domain III of the 23S rRNA of different Frankia *strains and uncultured populations in root nodules.*

Similar considerations could be made for subgroup I which comprises non-N_2-fixing *Frankia* strains. However, because members of the currently established subgroups I and IIIb were rarely encountered in root nodules of different origin (Maunuksela *et al.,* 1999; 2000; Nickel *et al.*, 1999; 2001; Zepp *et al.*, 1997), it is questionable whether the designation of further subgroups within these groups will

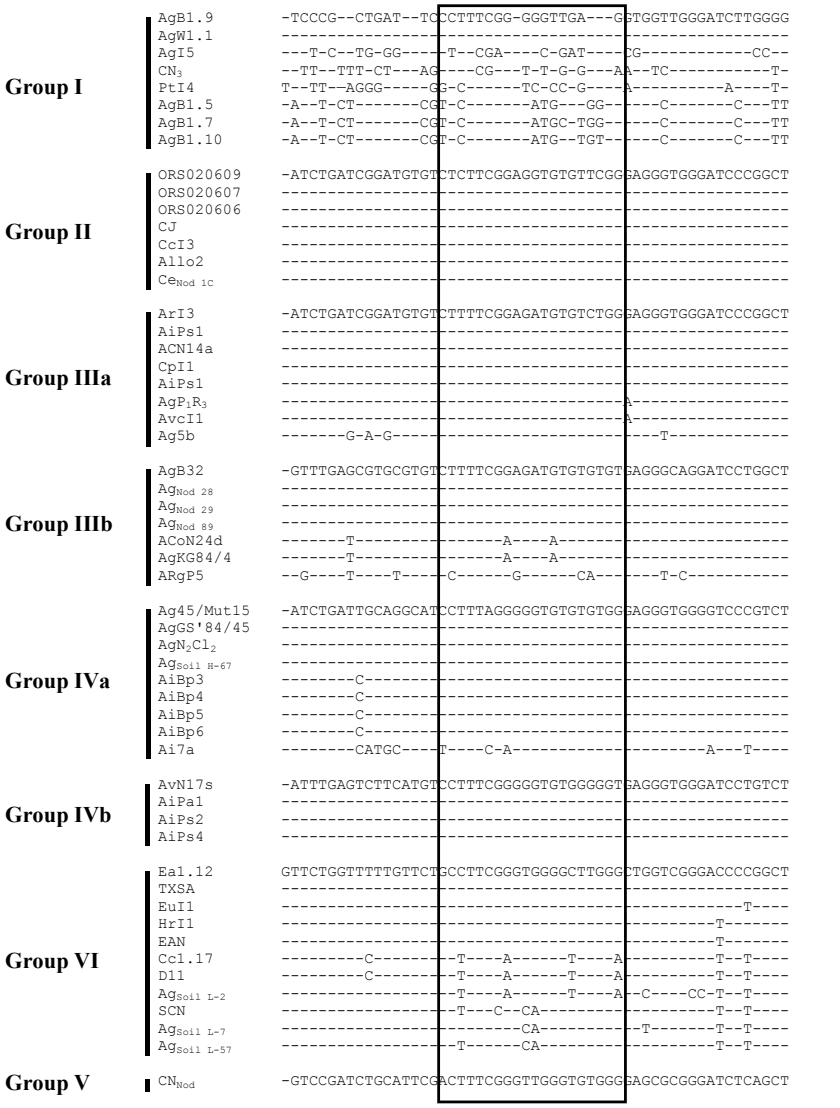

Figure 8. Potential signature sequences on the insertion in Domain III of the 23S rRNA that characterize pure cultures of Frankia *or uncultured endophytes in root nodules or soil that belong to specific host-infection groups and sub-groups.*

have any future implications on ecological studies. This might be different for the *Elaeagnus* host-infection group, where large sequence differences in the insertion of strains Cc1.17, Ea1.12 and SCN10a (up to 9 differences) might be the basis for the design of ecologically significant subgroups of frankiae.

The correlation of sequences of the highly variable insertion in Domain III of the 23S rRNA with host infection groups is surprising (Figure 8). Although this region is not supposed to have any phylogenetic significance (Roller *et al.*, 1992), these results confirm comparable data from the variable regions of 16S rRNA (Nazaret *et al.*, 1991b). The use of sequences from this region as targets for the detection and identification of frankiae is, therefore, limited to sequences retrieved from pure cultures and uncultured populations in root nodules, which allows a clear assignment of sequence information to members of the genus *Frankia* from a certain host-infection group. Based on this sequence information, attempts to detect frankiae in more complex environments, such as soils, become feasible. However, sequence information retrieved from such environments need to have its origin confirmed, even though high similarity values between the sequence retrieved and existing sequences that originated from confirmed frankiae might exist. An application of this approach for the analysis of diversity of *Frankia* populations in soils is, therefore, limited.

6. GENOMIC FINGERPRINTING

Fingerprinting or typing techniques can be used to discriminate frankiae at lower taxonomic levels. Restriction fragment length polymorphism (RFLP) analysis on DNA, either extracted from individual nodule lobes (Baker and Mullin, 1994) or on PCR-amplified nucleic acids, such as the 16S rDNA (Huguet *et al.*, 2001; McEwan *et al.,* 1994), the *gln*II gene (Cournoyer and Normand, 1994), the IGS of the 16S-23S rRNA operon (Gauthier et al., 1999; Maggia *et al.,* 1992; Navarro *et al.*, 1999; Ritchie and Myrold, 1999), the IGS of *nif*H-D (Cournoyer and Normand, 1994), or the IGS of *nif*D-K (Jamann *et al.,* 1993), have permitted discrimination among different *Frankia* genomic groups (Lumini *et al.*, 1996; McEwan *et al.*, 1994; Nalin *et al.,* 1997). However, the discriminative ability of this technique might be affected by the target nucleic acid and the restriction enzyme (or the combination of restriction enzymes) used (Normand and Chapelon, 1997).

A combination of RFLP analysis of 16S-23S rDNA IGS amplicons, which were digested with the restriction enzyme *Nci*I, with hybridization to specific oligonucleotide probes of PCR amplified *nif*H-D revealed a close relationship between 60 *Frankia* isolates obtained from root nodules of *Casuarina equisetifolia* and the reference strain CeD (Maggia *et al.*, 1992), This result suggested a small genetic diversity among *Frankia* strains that infect members of the Casuarinacaeae.

In contrast, five distinct genetic groups were recognized, using a set of different restriction enzymes, when amplicons of the 16S-23S rDNA IGS and the *nif*D-K IGS of pure cultures of *Frankia* belonging to the *Casuarina* host-infection group and uncultured populations in nodules on two *Casuarina* and two *Allocasuarina* species were analyzed (Rouvier *et al.*, 1996). Again, all pure cultures had similar patterns

and were assigned to a group along with most of the uncultured endophytes in nodules from *C. equisetifolia*. A second group consisted of two uncultured endophytes from *C. equisetifolia*, whereas three further groups were comprised only of uncultured endophytes from *C. cunninghamiana*, *A. torulosa*, and *A. littoralis*, respectively (Rouvier *et al.*, 1996).

At the sub-species level, fingerprinting protocols, which are based on PCR with arbitrary primers, provide an effective means to distinguish closely related *Frankia* strains (Sellstedt *et al.,* 1992). Differentiation to the species, subspecies and strain level can also be achieved by PCR-assisted fingerprinting techniques that target consensus motifs of repetitive elements common to prokaryotic genomes (rep-PCR) (de Bruijn, 1992; Lupski and Weinstock, 1992; Nick and Lindstrom, 1994; Versalovic *et al.,* 1991; 1994) (Figure 9).

Rep-PCR genomic fingerprinting covers three PCR-based fingerprinting protocols (REP-PCR, ERIC-PCR, and BOX-PCR genomic fingerprinting, respectively), in which distinct genomic regions that are located between the

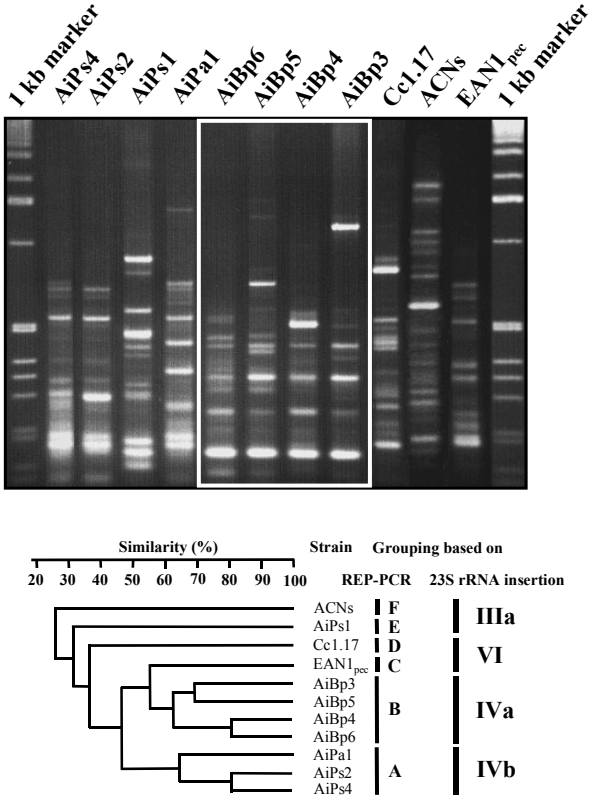

Figure 9. Rep-PCR fingerprint pattern of genomic DNA from Frankia *strains, using BOXA1R primer and the corresponding UPGMA dendrogram (modified from Maunuksela et al., 1999).*

repetitive extragenic palindromic (REP) sequence (Stern et al., 1984), the enterobacterial repetitive intergenic consensus (ERIC) sequence (Hulton *et al.,* 1991), or the BOX element (Martin *et al.*, 1992), respectively, are selectively amplified. The resulting patterns of amplification products (fingerprints) generally correlate to the classification based on other methods (Louws *et al.,* 1994; Versalovic *et al.*, 1994). For *Frankia* strains, a good correlation between degrees of relatedness has been shown by comparative 16S-rRNA sequence analysis and rep-PCR fingerprinting (Murry *et al.,* 1995). Using rep-PCR, the genomic fingerprints of DNA from root nodules of several *Ceanothus* spp. differentiated twelve distinct groups, indicating considerable genetic diversity of *Frankia* in the nodules sampled (Murry *et al.*, 1997). A similar high degree of diversity was obtained for isolates from nodules of *A. incana* (Maunuksela *et al.*, 1999; 2000) as well as for endophytes in nodule homogenates (Maunuksela *et al.*, 2000). These could not be differentiated by comparative sequence analysis of a 23S-rRNA insertion but all exhibited different rep-PCR patterns. In nodule lobe homogenates, the complexity of the fingerprint patterns was found to be similar to the patterns obtained using DNA from *Frankia* isolates (Jeong and Myrold, 1999; Maunuksela *et al.*, 1999), suggesting no significant impact of either plant or additional microbial DNA on the generation of rep-PCR fingerprint patterns.

7. CONCLUSIONS

Polyphasic taxonomy approaches that integrate information retrieved by a wide range of techniques on different levels of taxonomic resolution are revealing much about the genetic diversity and distribution of *Frankia*, and are refining and expanding knowledge of endophyte-host specificities. PCR-based approaches have been used to unravel the phylogenetic relationships of isolates, as well as of uncultured endophytes in root nodules of many actinorhizal plants from which no isolates have been obtained. Furthermore, these analyses have led to the emendation of the family *Frankiaceae* to contain a single genus *Frankia* with four main subdivisions: (i) a large group mainly comprising *Frankia alni* and other typical N_2-fixing strains belonging to the *Alnus* and the *Casuarina* host infection groups, respectively; (ii) uncultured endophytes of *Dryas*, *Coriaria*, and *Datisca* species; (iii) strains of the *Elaeagnus* host-infection group; and (iv) atypical non-N_2-fixing strains. The differentiation into host-infection groups is generally supported by data on DNA-DNA relatedness of pure cultures of *Frankia* and has led to the emendation of *F. alni* and indicated a large diversity within the genus *Frankia*. Considerable diversity among both cultured *Frankia* strains and uncultured endophytes in nodules has been confirmed by fingerprinting techniques. The growing databases of discriminative target sequences open the door to more sophisticated studies of the distribution and fate of specific *Frankia* populations in the environment and, thus, may lead to advancements in the management of actinorhizal plants and *Frankia* for human benefit.

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