

Assessing the phylogeny of *Frankia*-actinorhizal plant nitrogen-fixing root nodule symbioses with *Frankia* 16S rRNA and glutamine synthetase gene sequences

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Abstract

Actinomycetes from the genus *Frankia* induce nitrogen-fixing root nodules on actinorhizal plants in the “core rosid” clade of eudicots. Reported here are nine partial *Frankia* 16S rRNA gene sequences including the first from host plants of the rosaceous genera *Cercocarpus* and *Chamaebatia*, 24 partial glutamine synthetase (GSI; *glnA*) sequences from *Frankia* in nodules of 17 of the 23 actinorhizal genera, and the partial *glnA* sequence of *Acidotherrmus cellulolyticus*. Phylogenetic analyses of combined *Frankia* 16S rDNA and *glnA* sequences indicate that infective strains belong to three major clades (I–III) and that Clade I strains consisting of unisolated symbionts from the Coriariaceae, Datisceae, Rosaceae, and *Ceanothus* of the Rhamnaceae are basal to the other clades. Clock-like mutation rates in *glnA* sequence alignments indicate that all three major *Frankia* clades diverged early during the emergence of eudicots in the Cretaceous period, and suggest that present-day symbioses are the result of an ancestral symbiosis that emerged before the divergence of extant actinorhizal plants.

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1. Introduction

Angiosperms that bear N₂-fixing root nodules occur in 10 families distributed among five of eight main lineages of the “core rosids” (Magallon et al., 1999). Two groups of bacteria participate in the symbioses. One group consists of diverse proteobacteria possessing the rhizobial nodulation genes that confer the ability to nodulate legumes of the Fabaceae and the single genus *Parasponia* of the Ulmaceae. The other group consists of high mol% G + C actinomycetes from the genus *Frankia* that nodulate plants from eight additional families shown in Table 1 (Benson and Silvester, 1993).

The restriction of nodulating ability to the core rosids has led to the hypothesis that the predisposition to form nitrogen-fixing root nodule symbioses emerged once

during angiosperm evolution (Soltis et al., 1995). The diversity of plants infected by *Frankia* has led to the suggestion that actinorhizal plants expressed this predisposition for symbiosis on multiple, perhaps three to six, occasions during angiosperm evolution (Jeong et al., 1999; Roy and Bousquet, 1996; Swensen, 1996).

From the bacterial perspective, phylogenetic studies of effective (capable of nodulating plant roots and fixing nitrogen) *Frankia* strains based on 16S rDNA or *nifH* sequences have generally revealed three major clades (Benson and Clawson, 2000; Jeong et al., 1999; Nick et al., 1992; Normand et al., 1996; Wolters et al., 1997). *Frankia* strains in these clades can be defined by behavior in culture, by morphology, and by mode of infection (Benson and Silvester, 1993). Strains of Clade I have not been isolated in culture, infect hosts by intercellular penetration through the root surface (Berry and Sunell, 1990; Kohls et al., 1994; Liu and Berry, 1991; Miller and Baker, 1985, 1986; Valverde and Wall, 1999) and form primitive vesicles in symbiosis (Newcomb et al., 1987). Strains in Clades II and III can be grown

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Table 1
Phylogenetic groups, families and genera of actinorhizal plants

Phylogenetic groups of “core rosids” (Eurosids I sensu APG) ^a	Family ^b	# Genera/# nod.	Genera
“Higher” Hamamelididae (Fagales sensu APG)	Betulaceae	6/1	<i>Alnus</i>
	Casuarinaceae	4/4	<i>Allocasuarina</i> , <i>Casuarina</i> , <i>Ceuthostoma</i> , <i>Gymnostoma</i>
	Myricaceae	3/2	<i>Comptonia</i> , <i>Myrica</i>
Urticales (included in Rosales sensu APG)	Elaeagnaceae	3/3	<i>Elaeagnus</i> , <i>Hippophae</i> , <i>Shepherdia</i>
	Rhamnaceae	55/7	<i>Ceanothus</i> , <i>Colletia</i> , <i>Discaria</i> , <i>Kentrothamnus</i> , <i>Retanilla</i> , <i>Talguenea</i> , <i>Trevoa</i>
Rosaceae (included in Rosales sensu APG)	Rosaceae	100/4	<i>Chamaebatia</i> , <i>Cercocarpus</i> , <i>Dryas</i> , <i>Purshia</i> (includes <i>Cowania</i>) ^c
Cucurbitales (Cucurbitales sensu APG)	Coriariaceae	1/1	<i>Coriaria</i>
	Datisceae	3/1	<i>Datisca</i>

^a According to Magallon et al. (1999) and Angiosperm Phylogeny Group (1998).

^b Classification according to Cronquist (1981).

^c *Cowania* has been included with *Purshia* (Henrickson, 1986).

in culture and form multicellular lipid-enveloped structures termed vesicles in both culture and symbiosis. Clade II strains prefer to grow on organic acids, whereas Clade III strains grow on both organic acids and simple sugars (Benson and Silvester, 1993). Although relatively few plants have been studied, Clade II strains appear to infect their hosts by root hair infection and Clade III strains infect by either intercellular penetration or root hair infection depending on the plant being infected (Berry et al., 1986; Bosco et al., 1992; Callaham et al., 1979; Cournoyer et al., 1993; Miller and Baker, 1985; Racette and Torrey, 1989).

While comparisons of the 16S rRNA genes are useful, the variability of the gene among frankiae is low and phylogenetic relationships are sometimes ambiguous with partial sequences or with closely related strains (Benson and Clawson, 2000). The more variable *nifH* gene allows for a more detailed analysis but relatives that lack the ability to fix atmospheric nitrogen cannot be included in analyses (Jeong et al., 1999). We chose to use the gene for glutamine synthetase I (*glnA*) since it is almost universally present in prokaryotes and has more variability than the 16S rRNA gene while still being conserved. In addition, the *glnA* gene is not directly involved in nitrogen fixation and is under different selective pressure than either *nifH* or 16S rDNA. Here, we examine the phylogeny of *Frankia* as it relates to the evolution of the actinorhizal symbioses using *Frankia* partial 16S rDNA and *glnA* sequences obtained from representatives that infect almost all known actinorhizal plant genera.

2. Materials and methods

2.1. Bacterial strains

Table 2 lists the sources of sequences used in this study. Root nodules were collected from 17 of the 23

known actinorhizal plant genera and stored in 95% ethanol at -20°C .

2.2. Amplification of bacterial 16S rDNA and *glnA* gene fragments

Frankia genomic DNA was purified from plant root nodules or cultured strains as previously described (Clawson et al., 1998). Portions of *Frankia* 16S rDNA and glutamine synthetase (*glnA*) genes were amplified from the same DNA preparations. Polymerase chain reaction (PCR) primers used for the 16S rRNA gene were fD1 (Weisburg et al., 1991) and rDB1 (Benson et al., 1996). The *glnA* PCR primers were designated DB41 (5'-TTCTTCATCCACGACCCG-3') and DB44 (5'-GG CTTCGGCATGAAGGT-3') and yielded a 477 bp fragment encompassing about one-third of *glnA*. The *glnA* gene fragment from *Acidothermus cellulolyticus* did not amplify with DB41 and DB44 so additional primers were made that overlapped the region of interest. These primers were designated DB43f (5'-GGCTA CTTCCCGGTCC-3'), AB43r (5'-GGACCGGGAAGTAGCC-3'), and AB1f (5'-GGCCATCCACGAGTC CG-3'). The PCR amplifications were done as described previously (Clawson and Benson, 1999). All amplifications were done in quadruplicate in 50 μL reactions and combined to mask PCR errors. The amplicons were cycle sequenced in both directions using an ABI cycle sequencing kit (Perkin-Elmer) and the sequencing reactions were analyzed with an Applied Biosystems (Perkin-Elmer, Norwalk, CT) Prizm sequencer.

2.3. Phylogenetic analyses of bacterial DNA sequence alignments

Alignments of bacterial 16S rDNA, *glnA*, and concatenated 16S rDNA and *glnA* sequences were generated with ClustalW (Thompson et al., 1994). Distance and

Table 2

Nodule and strain sources, locations and accession numbers of sequences used in this study

Strain source	Accession Nos. <i>glnA</i> /16S	Reference <i>glnA</i> /16S
<i>Acidothermus cellulolyticus</i>	AY027649/AJ007290	This study/Marechal et al. (2000)
<i>Alnus incana</i> subsp. <i>rugosa</i> nodule	AF156755/AF131893	This study/Clawson and Benson (1999)
<i>Casuarina cunninghamiana</i> strain CcI3	AF156756/AF149268	This study
<i>Ceanothus americanus</i> R2	AY027647/AY027645	This study/Lechevalier and Ruan (1984)
<i>C. americanus</i> nodule	AF156762/AF063639	This study/Clawson et al. (1998)
<i>Cercocarpus ledifolius</i> nodule	AF156763/AF149275	This study
<i>Chamaebatia foliolosa</i> nodule	AF156758/AF149272	This study
<i>Colletia hystrix</i> nodule	AF156741/AF063640	This study/Clawson et al. (1998)
<i>Comptonia peregrina</i> nodule	AF156747/AF116103	This study/Clawson and Benson (1999)
<i>Comptonia peregrina</i> strain CpI1	L10631/AF130811	Hosted et al. (1993)/This study
<i>Coriaria arborea</i> nodule	AF156764/AF063631	This study/Benson et al. (1996)
<i>Datisca cannabina</i> nodule	AF156761/AF158686	This study
<i>Discaria serratifolia</i> strain DsI2B	AF156743/AF158686	This study/Clawson et al. (1998)
<i>Elaeagnus angustifolia</i> nodule	AF156742/AF064144	This study/Clawson et al. (1998)
<i>Hippophae rhamnoides</i> nodule	AF156745/AF149269	This study
<i>Myrica gale</i> nodule 2	AF156748/AF158684	This study/Clawson and Benson (1999)
<i>Myrica gale</i> nodule 1	AF156757/AF116162	This study/Clawson and Benson (1999)
<i>Myrica pensylvanica</i> nodule	AF156754/AF116076	This study/Clawson and Benson (1999)
<i>Purshia stansburiana</i> nodule	AF156760/AF149276	This study
<i>P. glandulosa</i> nodule	AF156759/AF149273	This study
<i>P. tridentata</i> strain PtI6	AY027648/AY027646	This study
<i>Retanilla ephedra</i> strain ReI6	AF156740/AF064150	This study/Clawson et al. (1998)
<i>Talguenea quinquinervia</i> nodule	AF156746/AF063643	This study/Clawson et al. (1998)
<i>Trevoa trinervis</i> nodule	AF156744/AF063642	This study/Clawson et al. (1998)
<i>Talguenea quinquinervia</i> strain TqI5	AF156739/AF064147	This study/Clawson et al. (1998)

parsimony phylogenetic trees with bootstrap values were generated from the alignments in PHYLIP (Felsenstein, 1993). Five hundred pseudo-alignments were generated with Seqboot. Distance matrices were constructed in DNAdist with the Kimura two-parameter model (transition/transversion ratio of two). Neighbor-Joining trees were constructed with the program Neighbor (Saitou and Nei, 1987). A consensus tree was generated with Consense and viewed as a cladogram in TREEVIEW (Page, 1996). Parsimony trees were generated in PHYLIP with Dnapars from the pseudo-alignments described above. Maximum likelihood trees were produced from *glnA* alignments with either the second codon position or full-length sequence, 16S rDNA alignments, and concatenated alignments of *glnA* and 16S rDNA sequences in TREE-PUZZLE (Strimmer and Haeseler, 1996) with the HKY model of substitution (Hasegawa et al., 1985). The alignments were tested for clock-like rates of mutation with the molecular clock option in TREE-PUZZLE.

3. Results

3.1. Bacterial DNA sequence

Reported here are nine partial *Frankia* 16S rDNA sequences including the first from the rosaceous genera *Cercocarpus* and *Chamaebatia*, 24 partial *glnA* sequences, and the partial *glnA* sequence of *A. cellulolyticus*.

We used *A. cellulolyticus* as an outgroup in this study because previous phylogenetic work has shown it to be the closest known relative to *Frankia* (Marechal et al., 2000; Normand et al., 1996). The partial sequences of *Frankia* 16S rRNA genes used in this study correspond to positions 28–419 on *Escherichia coli* 16S rDNA; alignments contain 44 variable sites in 375 bp (11.7% variability in the region), or about 45% of the total number of variable sites of the full-length *Frankia* gene (Clawson et al., 1998). The portion of the *glnA* gene used spans 477 nucleotides from a total of about 1500 with 162 informative sites among the *Frankia* sequences (33.9%); this region contains a conserved 13 amino acid region involved in active site formation (Hosted et al., 1993). An alignment of GS amino acid residues revealed 30 variable positions from a total of 159 (data not shown).

3.2. *Frankia* phylogeny

The phylogeny of *Frankia* based on a data set of concatenated 16S rDNA and *glnA* sequences is shown in Fig. 1. The topology of the tree was strongly supported by Neighbor-Joining, Maximum Likelihood and Maximum Parsimony and is in agreement with previous studies by placing the strains in three major clades. In all trees, Clade II clusters with Clade III with high bootstrap support (92, 100, 81 for Neighbor-Joining, Maximum Likelihood, and Parsimony, respectively).

Sequences from unisolated *Frankia* strains of the Rosaceae genera *Cercocarpus*, *Chamaebatia* and *Purshia*

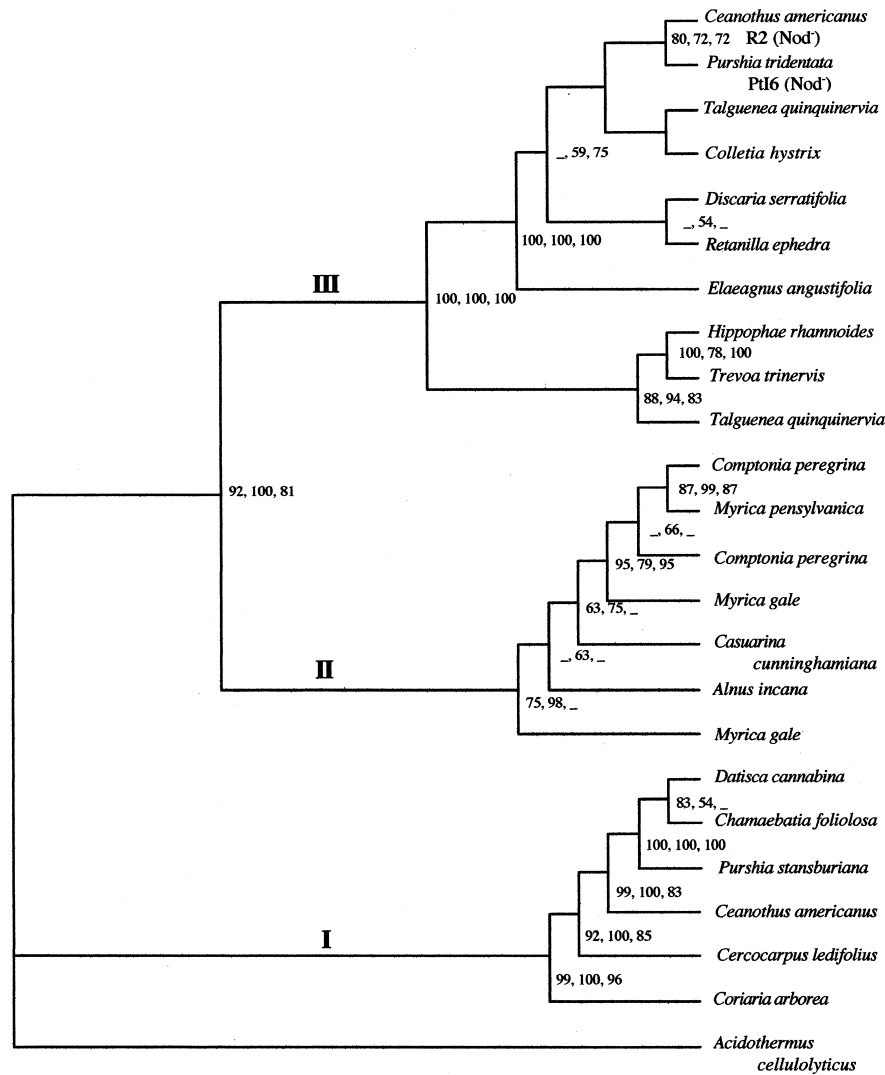


Fig. 1. The phylogeny of *Frankia* shown in a Neighbor-Joining tree of concatenated *glnA* and 16S rDNA sequences. Bootstrap values are based on 500 samplings; those above 50% are shown and represent the percentage of times the strains to the right of the node appear together. The first number corresponds to percent bootstrap support in the NJ tree shown. The second number corresponds to quartet puzzling support values from the maximum likelihood estimates of TREE-PUZZLE using 10,000 puzzling steps. The third number corresponds to percent bootstrap support in the DNAPars program of PHYLIP. A dash indicates that the support value was less than 50%. A similar phylogeny of *Frankia* strains was observed from an alignment of *glnA* amino acid sequences (data not shown).

cluster together in Clade I along with unisolated strains from *Datisca* (Datisceae), *Coriaria* (Coriariaceae) and *Ceanothus* (Rhamnaceae). Clade II contains only *Frankia* symbionts and strains from the “higher” hamamelid lineage. Clade III contains *Frankia* strains from the Urticales lineage plus strains R2, isolated from *Ceanothus americanus* and Pt16, isolated from *Purshia tridentata*. The latter two strains represent frankiae that fail to reinfect (Nod⁻) the host of origin (Benson and Silvester, 1993). One of the strains (R2) has previously been shown to effectively infect *Elaeagnus* sp. in greenhouse trials (Baker, 1987). The ability of the other strain used in this study (Pt16) to infect *Elaeagnus* has not been reported but we may predict, on the basis of this study, that it has a high probability of doing so.

The presence of clock-like mutation rates in DNA or protein sequence alignments is helpful for correlating time within evolutionary trees. The maximum likelihood ratio test (lrt) can be used to test for molecular clocks in sequence alignments (Huelsenbeck and Rannala, 1997). The null hypothesis of the lrt is clock-like evolution (Huelsenbeck and Rannala, 1997). Thus, *p* scores less than 0.05 reject a molecular clock. In agreement with a previous study (Jeong and Myrold, 1999), the lrt rejected clock-like evolution in our alignment of *Frankia* 16S rDNA sequences (*p* = 0.0094). To test for a molecular clock that would be useful in calibrating our *glnA* phylogenetic trees, we first combined our *glnA* alignment with the following sequences: *Rhizobium leguminosarum* bv. *viciae* (AF169566), *Sinorhizobium*

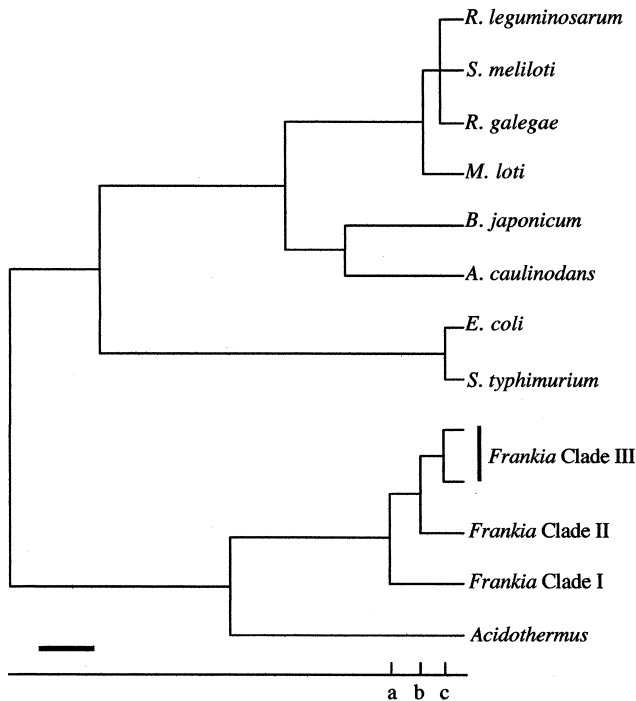


Fig. 2. The emergence of *Frankia* shown with Maximum likelihood phylogeny (TREE-PUZZLE) with a molecular clock assumption using the second codon position of *glnA*. Clades I and II are each defined by the sequence from a single *Frankia* representative phylotype, Clade III is defined by the sequence from two *Frankia* representative phylotypes. The letters on the time line indicate the relative times of divergence for: (A) Clade I *Frankia* from the progenitor of Clades II and III (263–285 Myr bp); (B) Clade II from Clade III (165–179 Myr bp); and (C) *Escherichia coli* from *Salmonella typhimurium* (69–75 Myr bp (Turner and Young, 2000)). The scale bar represents approximately 200 Myr.

meliloti (AF169573), *R. galegae* (AF169575), *Bradyrhizobium japonicum* (AF169576) *Azorhizobium caulinodans* (Y10213), *Mesorhizobium loti* (AF169563) *E. coli* (X05173), and *Salmonella typhimurium* (M14536). The molecular clock hypothesis could not be rejected when the conserved second codon position of the alignment was analyzed ($p = 0.0536$). The resulting tree from that analysis is shown in Fig. 2.

Since all taxa in Fig. 3 adhere to clock-like behavior, nodes of the entire tree can be calibrated to the divergence of *E. coli* and *S. typhimurium*. The same two strains of these bacteria used in this study were previously estimated to diverge 69–75 Myr bp (Turner and Young, 2000). Within the calibrated tree, Clade II diverged from Clade III about 165–179 Myr bp and Clade I diverged from the common ancestor of Clades II and III around 263–285 Myr bp. Estimates of the divergence of *E. coli* and *Salmonella* vary considerably in the literature (Doolittle et al., 1996; Ochman and Wilson, 1987; Pesole et al., 1991). However, this analysis suggests that *Frankia* clades diverged early during the emergence of eudicots in the Cretaceous period, before extant actinorhizal plant families appear in the fossil record.

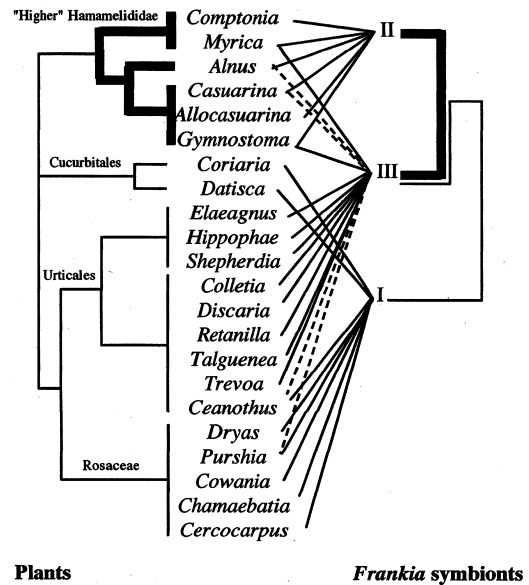


Fig. 3. Relationships between *Frankia* clades and actinorhizal plant genera from the “core Rosids.” Phylogenetic groups of the plants are shown on the left, *Frankia* clades are shown on the right. Solid lines indicate the presence of typical *Frankia* strains in each plant genus. Broken lines indicate that a *Frankia* clade is only occasionally detected in the plant genus. Thick lines denote root infection via root hairs; thin lines denote infection via intercellular penetration of the root surface.

4. Discussion

The combined data sets for 16S rRNA and *glnA* genes provide a well-supported phylogeny for effective *Frankia* strains. The basal position of Clade I in the tree presented in Fig. 1 is found in each method of analysis. The tree is the most comprehensive to date and includes data from representative strains of all known actinorhizal plant genera except for *Allocasuarina*, *Ceanothus*, *Dryas*, *Gymnostoma*, *Kentrothamnus*, and *Shepherdia*. However, the phylogenetic positions of *Frankia* sp. strains inhabiting nodules of the majority of plants from these genera are known (Bosco et al., 1994; Navarro et al., 1997; Nazaret et al., 1991; Simonet et al., 1999). The information provided allows for a more complete picture of plant–microbe relationships than previously available and provides the basis for hypotheses concerning the evolution of the symbiosis.

Fig. 3 summarizes the relationships between *Frankia* clades and actinorhizal plant clades. The latter have been established by analyses of chloroplast *rbcl* gene sequences (Jeong et al., 1999; Magallon et al., 1999; Roy and Bousquet, 1996; Soltis et al., 1995; Swensen, 1996). The basal Clade I *Frankia* strains have been found in nodules on plants from three of the four major actinorhizal lineages, Cucurbitales, Rosaceae, and Urticales (Clawson et al., 1998; Nick et al., 1992; Ritchie and Myrold, 1999). Strains from Clade II specialize in and may have coevolved with the “higher” Hamamelididae as they have been detected only in plants of this lineage.

Additional specialization, or coevolution, has been reported at lower taxonomic levels within the hamamelids and may be linked to geographical separation (Caizzi et al., 1990; Simonet et al., 1999).

Clade III *Frankia* strains are effective symbionts of two of the four lineages including the actinorhizal Urticales and the “higher” Hamamelidae (Carú, 1993; Clawson and Benson, 1999; Clawson et al., 1998; Navarro et al., 1997). They are the dominant strains in the Elaeagnaceae and, except for *Ceanothus*, in the nodulated Rhamnaceae. However, they have been isolated from *Purshia* sp. (Rosaceae, strain PtI6 in Fig. 1) and *Ceanothus* sp. (Rhamnaceae, strain R2 in Fig. 1) (Lechevalier and Ruan, 1984), and their 16S rDNA has been detected in nodules of *Ceanothus* sp. (Murry et al., 1997). Some of these strains (such as R2) nodulate *Elaeagnus* sp. in greenhouse trials (Baker, 1987). Among the “higher” hamamelids, Clade III strains are common in *Myrica* and *Gymnostoma* nodules and can induce ineffective (non-nitrogen-fixing) nodules on alders (Bosco et al., 1992; Clawson and Benson, 1999; Margheri et al., 1985; Navarro et al., 1997). Some have also been isolated from *Casuarina* sp. (Diem et al., 1982; Gauthier et al., 1999; Navarro et al., 1997). Strains from Clade III therefore inhabit nodules of actinorhizal plants from three major clades, although their roles in the Rosaceae, in *Ceanothus* of the Rhamnaceae, and in some genera of the “higher” hamamelids are unclear.

The divergence of *Frankia* clades estimated in this study precedes prior estimates based on either nitrogenase (*nifH*) or 16S rRNA sequence analyses. The divergence of Clade I from the ancestor of II and III has been estimated at 62–130 Myr bp (Jeong et al., 1999) and 100–200 Myr bp (Normand et al., 1996), and the divergence of Clade II from Clade III at 46–91 Myr (Jeong et al., 1999), 25–50 Myr (Normand et al., 1996), 30 Myr (Normand and Bousquet, 1989), and 131 ± 56 Myr (Cournoyer et al., 1993). Our analysis with *glnA* sequences indicates that all three *Frankia* clades emerged well before pollen similar to present day members of the higher hamamelids appears in the fossil record around 95 Myr bp (Sims et al., 1999) and even before angiosperm fossils appear in the Cretaceous period about 125 Myr bp (Crane et al., 1995).

The dating of *Frankia* clades before the appearance of angiosperms in the fossil record obscures the history of coevolution between actinorhizal plants and their symbionts. Current associations of actinorhizal plants and *Frankia* strains indicate non-congruent phylogenies (Fig. 3) that may be the result of either multiple gains or losses of the symbiosis. Some families of actinorhizal plants have a relatively shallow fossil history. Some of the lineages have no fossil predecessors before a few tens of millions years ago despite molecular evidence that suggests that all lineages were established at about the same time in the late Cretaceous (Magallon et al., 1999).

Clearly, the lack of identifiable fossils from an actinorhizal lineage does not mean that the symbiosis did not exist prior to the deposition of fossils. Likewise, the absence of any recognizable actinorhizal lineage in the mid-Cretaceous does not mean that the ancestors of *Frankia* were not participating in plant symbioses before the lineages became established. In effect, it is difficult if not impossible to draw strong conclusions about the early evolution of the actinorhizal symbiosis.

A comparison of actinorhizal plant and *Frankia* clades indicates that intercellular penetration is the likely ancestral mode of plant infection since Clades I and III *Frankia* strains share that ability and infect a broader range of plants than Clade II strains (Fig. 3). When ancestors of Clade II specialized in infecting ancestral hamamelids via root hair infection they apparently lost the ability for intercellular penetration and subsequently coevolved with the “higher” hamamelids. This scenario differs from one proposed previously where the divergence of Clades II and III was correlated with the evolution of the intercellular penetration mode of infection when the Elaeagnaceae lineage appeared in the fossil record (Cournoyer and Lavire, 1999; Cournoyer et al., 1993; Normand et al., 1996).

In sum, the present work helps clarify the phylogeny of *Frankia* by utilizing a large collection of strains and an informative region of genomic DNA, the *glnA* gene. The evolution of three distinct *Frankia* phylotypes (Clades I–III) is predicted to predate the fossil history of extant plants. Thus, the early evolution of the actinorhizal symbiosis is obscured by an incomplete fossil record. While 16S rDNA sequences are still useful for placing strains in broad phylogenetic groups, the inclusion of *glnA* sequences allows the grouping to be done with a higher degree of confidence.

Acknowledgments

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