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Recent Progress in the Evolution and Ecology of Actinorhizal Symbioses

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Actinorhizae are N_2-fixing symbioses between dicotyledonous plants and *Frankia*. Actinorhizal plants form microsymbiotic root nodules with *Frankia* (Frankiaceae). *Frankia* are N_2-fixing, sporulating, Gram-positive, and filamentous bacteria. These actinorhizal associations contribute significant quantities of N (1-150 kg N/ha/y) to temperate forest ecosystems (Torrey, 1978; Dawson, 1983). In addition to enhancing forest productivity by serving as a major input of N, some actinorhizal plants, such as many *Alnus* and *Casuarina* species, are commercially valuable (Diem and Dommergues, 1990; Hibbs and Cromack, 1990; Wheeler and Miller, 1990). This review gives a brief overview of actinorhizal symbioses and focuses on the significant advances in the evolutionary and ecological understanding of the symbiotic interactions between host plants and *Frankia* endosymbionts. Recent comprehensive reviews on the various aspects of actinorhizal symbioses are available.

Actinorhizal Plants

Actinorhizal plants occur in diverse environments and are early successional plants. In conventional taxonomic treatment, actinorhizal plants are diverse, belonging to 8 plant families and 24 genera (Table 1). All are perennial woody shrubs or trees except *Datisca*, which is herbaceous. The term "actinorhizal" was coined to describe their common features (Torrey and Tjepkema, 1979). In contrast to conventional taxonomy (Cronquist, 1988), recent molecular phylogenies of angiosperm plants based on *rbcL* sequences suggest a close genealogical relationship among N_2-fixing plants (Chase et al., 1993). Actinorhizal plants were found in three of the four subclades that contain N_2-fixing symbioses (Soltis et al., 1995). Soltis et al. (1995) also suggested that N_2-fixing plants, including actinorhizal plants and legumes, have a single origin in common with several non-N_2-fixing plants. This single nitrogen-fixing clade was supported by a recent angiosperm molecular systematic study that incorporated the data from the two chloroplast encoded genes, *rbcL* and *atpB*, and the nuclear gene, 18S rDNA (Soltis et al., 2000). An analysis of *rbcL* sequences combined with anatomical and morphological characters (Swensen, 1996) suggested that actinorhizal symbioses originated at least four times, not like the previously accepted idea that symbioses are chaotic (Bousquet and Lalonde, 1990; Sprent, 1994).

Frankia

The genus name *Frankia* was proposed by J. Brunchorst (1887). However, it took 100 years to isolate pure cultures. Since the first successful isolation of *Frankia* from nodule tissue (Callaham et al., 1978), much has been learned about *Frankia* taxonomy, host specificity groupings, metabolism, biochemistry, and genetics. For example, studies of the isolated strains have allowed *Frankia* strains to be grouped on the basis of host specificity, DNA-DNA re-association kinetics, restriction fragment length polymorphisms, DNA sequences, etc. (Benson and Silvester, 1993). Recent phylogenetic positioning of *Frankia* using full-length 16S rDNAs (Normand et al., 1996) suggests that *Frankia* is monophyletically distinct from close bacterial genera and is grouped into four subclades.

Ecological studies of *Frankia* in soil are in their infancy, however, primarily because of the difficulty of isolating *Frankia* from soil and of differentiating *Frankia* strains from each other and from other soil microorganisms. Although microsymbionts have been isolated from 20 of 24 actinorhizal plant genera, typical *Frankia* isolates confirmed by host re-infection and N_2-fixing ability measurement have been reported from only 10 plant genera. For example,

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Table 1. Actinorhizal plant genera and *Frankia* isolates and accession numbers of *rbcL* and 16S rDNA sequences deposited in GenBank

Family and genus	Isolates[a]	*rbcL*[b]	16S rDNA[b]
Betulaceae			
Alnus	Y, T	X56618	M88466
Casuarinaceae			
Allocasuraina	Y, T	X69527	
Casuarina	Y, T	L01893	M55343
Gymnostoma	Y, T	X69531	
Ceuthostoma	N	-	
Coriariceae			
Coriaria	Y, A	L01897	AF063641
Datiscaceae			
Datisca	Y, A	L21939	L18979[c]
Elaeagnaceae			
Elaeagnus	Y, T	U17038	L40618
Hippophaë	Y, T	U17039	L40617
Shepherdia	Y, T	-	L40619
Myricaceae			
Comptonia	Y, T	X69529	
Myrica	Y, T	L01934	L40622
Rhamnaceae			
Ceanothus	Y, A	U06795	AF050759
Colletia	Y, A	U59819	AF063641
Discaria	Y, A	U59826	
Kentrothamnus	N	-	
Retanilla	Y, ND	-	
Talguenea	N	-	
Trevoa	Y, T	U59828	AF063642
Rosaceae			
Cercocarpus	Y, A	U06796	
Chamaebatia	N	-	
Cowania	Y, ND	U59817	
Dryas	N	U06825	L40616
Purshia	Y, A	U06821	AF034776

[a] Symbols: Y, isolates reported; N, isolates not reported; T, typical strains which reinfect their original host plant species and fix N_2; A, atypical strains which do not reinfect their host plant species but usually fix N_2 in culture or associated with other host plant species; ND, infectivity and effectivity not determined.

[b] More than one sequence have been deposited from some plant genera or their *Frankia* isolates. The GenBank accession numbers provided are those of the longest or representative sequences. Only the sequences of 16rDNA from typical strains or directly amplified from uncultured microsymbiotic *Frankia* within root nodules are presented.

[c] Only partial sequence is available.

Frankia isolates from *Ceanothus* (Lechevalier and Ruan, 1984) are unable to re-infect *Ceanothus*, although some can nodulate members of the Elaeagnaceae and Myricaceae (Baker, 1987). Ecological studies of genetic diversity involving isolation of *Frankia* strains have been limited to the microsymbiont *Frankia* of a few plant hosts. Efforts to improve the isolation methods of *Frankia* involve the investigations of resistance to antibiotics and to heavy metals of *Frankia* strains to use them as genetic markers (Richards et al., 2002; Tisa et al., 1999).

Thus far, most molecular phylogenetic studies of *Frankia* have used only 16S rDNA sequences. However, it is important to use other sequences at the same time, because conflicting molecular phylogenies could arise if lateral transfer between *Frankia* occurs. Comparison of the phylogenetic trees of 16S rDNA and *nifH* gene sequences, one of the genes involved in nitrogen fixation, suggests that both genes have evolved in a relatively tightly-linked state (Jeong et al., 1999). Thus, both of *Frankia* phylogenetic trees reconstructed using *nifH* and 16S rDNA sequences showed that sub-groupings of both trees correspond with each other in terms of plant origins of *Frankia* strains, except for a *Ceanothus* microsymbiont *Frankia*. However, it would be worthwhile to reconstruct a phylogenetic tree using more extensive *nif* genes to pinpoint co-evolutionary origins. Two recent reports dealt with *Frankia* phylogeny using the hypervariable *nifDK* intergenic spacer (Navarro et al., 1997) and *glnII* sequences (Cournoyer and Lavire, 1999). The analysis of the hypervariable *nifDK* intergenic spacer sequences suggested that *Gymnostoma* microsymbiont is phylogenetically close to *Elaeagnus*-infective *Frankia* strains. In contrast, the phylogenetic analysis of *glnII* sequences suggested that, similar to the phylogenetic trees of 16S rDNA and *nifH*, *Alnus*/*Casuarina* microsymionts are very different from *Elaeagnus* microsymbionts. However, both studies compared very short sequences.

Attributes of the Actinorhizal Symbiosis

Free-living *Frankia* differentiates into three different cell types: septate hyphae, multilocular sporangia, and thick-walled vesicles. In nodules, *Frankia* typically differentiates into vesicles and sometimes sporangia. However, actinorhizal nodules are morphologically diverse and their host plants obviously play a significant role in modifying *Frankia* morphology. For example, the mature effective form of *Frankia* strains in nodules is often but not exclusively associated with symbiotic vesicle formation, although all effective *Frankia* strains described to date form vesicles in culture (Newcomb and Wood, 1987).

Frankia enters root tissue by root hair infection (for *Alnus, Casuarina, Comptonia*, and *Myrica* species) or by intracellular penetration (for *Ceanothus, Elaeagnus*, and *Shepherdia* species) (Benson and Silvester, 1993). The host plant determines the infection route. That is, one *Frankia* strain may enter either by root hair or by intracellular space, depending on the host plant species, but a plant species

shows only one infection route (Racette and Torrey, 1989; Miller and Baker, 1986).

Evolution of the Symbiotic Interactions Between Host Plants and *Frankia* Microsymbionts

The early attempts to classify members of the genus *Frankia* related to host plants were based on infectivity groups. Using bacterial isolates, Baker (1987) grouped *Frankia* strains into the following four infectivity groups 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. (1988) used a more complex approach including diverse phenotypic characteristics. However, these studies used a limited diversity of *Frankia*, most of which were isolated from only four of eight actinorhizal plant families. The reliability of inoculation tests is also questionable because of autoregulation of nodules and because strains are not subject to competition or natural environmental conditions. Nevertheless, using the limited diversity of *Frankia*, Maggia and Bousquet (1994) suggested some degree of co-evolution between the divergence of host plants and their promiscuity toward *Frankia*. The actinorhizal taxa that diverged more recently in this group of plants were shown to be susceptible to a narrower spectrum of *Frankia*, whereas earlier diverging ancestors including some species of the genus *Myrica* were highly promiscuous, indicating that evolution has proceeded toward narrower promiscuity and greater specialization. Thus, host promiscuity is likely to be a key determinant in the establishment of an effective symbiosis.

Molecular phylogenetic analyses of both actinorhizal plants and *Frankia* (Normand et al., 1996; Swensen, 1996; Soltis et al., 1995) suggested that they are monophyletic, meaning, they have a single origin. Both trees also implied possible evolutionary relationship between host plants and their symbiotic partners, *Frankia* (Benson and Clawson, 2000). Subsequently, Jeong et al. (1999) compared the *Frankia* phylogenetic tree with the phylogenetic tree of actinorhizal plants in a rigorous way using tree matching, likelihood ratio tests, and divergence time estimations. In the comparison, they used *nifH* and 16S rDNA gene sequences from typical strains and amplified directly from uncultured microsymbiontic *Frankia* within root nodules. This selection strategy is supported by a recent study (Huguet et al., 2001) of *Frankia* diversity in sympatric plant populations that unisolated strains identified from direct PCR amplification of 16S rDNA tend to group together with typical strains, but isolates tend to group with unisolated strains and/or strains known to be atypical.

The analysis of Jeong et al. (1999) revealed that, although actinorhizal plants and *Frankia* did not strictly co-evolve, subgroupings of *Frankia* and actinorhizal plants correspond with each other in terms of symbiotic partnership. The estimated divergence time and molecular clock hypothesis test suggested that *Frankia* clades diverged more recently than plant clades, and that actinorhizal symbioses originated more than three times after the four plant clades diverged.

The COMPONENT program (Page, 1994), which compares topologies of two phylogenetic trees, also determined that the observed degree of fit between the *Frankia* and plant trees was significantly better ("leaves added," $P(H_o) = 0.003$; "losses," $P(H_o) = 0.004$) than the fit between the *Frankia* and 1000 randomized plant trees. These results, which are considerable in terms of the method of phylogenetic inference and the evolutionary models used, falsify the null hypothesis of chance similarity between the plant and *Frankia* trees. Their reconciled tree shows eight events of cospeciation and hypothesizes three corresponding subgroups of plants and *Frankia* (Fig. 1). However, likelihood ratio tests and molecular clock hypothesis tests (Huelsenbeck and Rannala, 1997) that estimate branching lengths as well as branching pattern, and so provide stricter co-evolution model, did not provide significant support for congruence between the two phylogenies.

Because tree mapping identified three corresponding clades of *Frankia* and plant trees, they also tested molecular clock hypothesis for each clade and estimated the approximate divergence times among the three clades of *Frankia* and among four clades of plants using average pairwise distances. The molecular clock hypothesis could not be rejected for both the subgroups of the actinorhizal plants and the *Frankia* ($P > 0.05$), and it appeared that plants and *Frankia* have interacted several times with each other to form symbiotic relationship. Divergence times were estimated using mean substitution rates obtained from comparison of 16S rDNA and *rbcL* nucleotide sequences on the basis of fossil record, geographic separation, etc. (Albert et al., 1994; Moran et al., 1993; Ochman and Wilson, 1987). The estimated divergence time among *Frankia* clades ranged from 39 to 125 million years; for plant clades, it ranged from 170 to 429 million years ago. Although the maximum approximate value of plant clade divergence times is unrealistically high considering the origin of angiosperms 415 million years ago, these calculations indicate that plant clades diverged earlier than *Frankia* clades. Taken together, the analysis of corresponding subgroups identified by tree mapping indicated that actinorhizal symbioses originated several times long after plant clades diverged. The results also indicated that once symbioses established, plants and *Frankia* were retained within certain taxonomic group with limited lateral transfer.

Consequently the direct comparison of phylogenetic trees

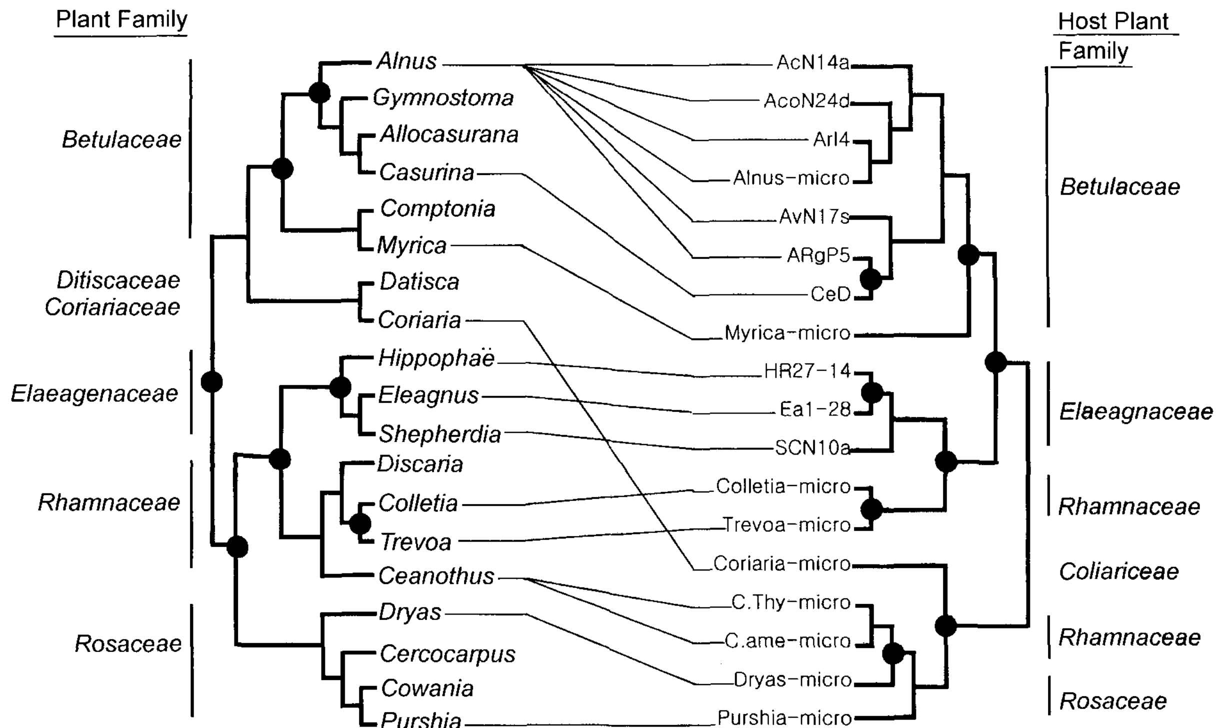

Fig. 1. Phylograms for actinorhizal plants and their microsymbionts, *Frankia*, with their host-symbiont relationships (modified from Jeong et al., 1999). The identified eight co-evolutionary (codivergent) events (●) are indicated on the nodes of this reconciled tree.

using representative *Frankia* and actinorhizal plants revealed some degree of co-evolution between the symbionts. The phylogenetic analysis of additional 16S rDNA sequences especially from *Datiscaceae* and *Coriariceae* and other nitrogen-fixing genes will be needed in order to better understand the co-evolution. Such an analysis should not only clarify co-evolutionary relationship between *Frankia* and actinorhizal plants but also address the question of at which taxonomic levels the actinorhizal symbiosis has strictly co-evolved. Parts of these questions have been recently addressed in the investigations of diversity and specificity of *Frankia* strains in nodules of sympatric host plant species (Clawson and Benson, 1999; Huguet et al., 1999; Jeong and Myrold, 1999; Navarro et al. 1999; Ritchie and Myrold 1999; Simonet et al., 1999). All these studies utilized gene sequences amplified from root nodules as a means to estimate the diversity of *Frankia* strains. The accumulation of data have revealed that, although geographic separation and soil type play a more important role for divergence of microsymbiont *Frankia* than host plant, the diversity of *Frankia* strains that inhabit in the nodules of the same plant genus differs at the plant species level rather than at the genus or family level, and its level of the diversity correlates with the level of diversity of plant hosts. For example, Jeong and Myrold (1999) collected nodules and soil from three sites where *C. velutinus* and *C. integerrimus, C. velutinus* and *C. prostratus*, and *C. integerrimus* and *C. sanguineus* grow together. According to the traditional classification (McMinn, 1942) and molecular phylogeny (Jeong et al., 1997) of the genus *Ceanothus*, *C. prostratus* belongs to the subgenus Cerastes, *C. sanguineus* to the deciduous group of the subgenus Ceanothus, and *C. velutinus* and *C. integerrimus* to the evergreen group of the subgenus Ceanothus. The intergenic spacer region between 16S and 23S rRNA gene sequences suggested that *Ceanothus*-microsymbiont *Frankia* were almost identical and closely related at the intraspecific level. Fingerprints of nodule DNAs using rep-PCR showed that *Ceanothus*-microsymbiont *Frankia* exhibited less diversity within co-populations of host plants than among co-populations, suggesting that geographic separation plays a more important role for divergence of *Ceanothus*-microsymbiont *Frankia* than host plant. The analysis of *Frankia* strains inhabiting root nodules of nine *Ceanothus* species representing the taxonomic diversity and geographic range of the genus at the same laboratory gave essentially the same conclusion (Ritchie and Myrold, 1999).

Ecology of the Symbiotic Interactions Between Host Plants and *Frankia* Microsymbiont

Frankia can proliferate in two niches: root nodules and soil. The isolation from root nodules (Callaham et al., 1978) and soil (Baker and O'Keefe, 1984) of *Frankia* that can be

successfully cultured in simple media suggests that *Frankia* is a saprophyte and a facultative symbiont. However, evidence for the saprophytic condition comes from indirect observations and experiments, because of the difficulty in differentiating between spores and hyphae (Benson and Silvester, 1993).

Generally, high *Frankia* populations in soil are observed near soils of host plants (Zimpfer et al., 1999). However, many observations also indicate that *Frankia* is present in soils well outside the normal geographic range of host plants (Benecke, 1969), under non-host plant stands (Maunuksela et al., 1999; Smolander and Sundman, 1987), or long after host plants have disappeared from a site (Jeong and Myrold, 2001; Wollum et al., 1968). Interestingly, Gauthier et al. (2000) suggested that *Frankia* have a positive tropism towards non-host species belonging to the families having nodulated species. The nodulating capacity of the rhizosphere of *Aphitonia neocaledonica* (non-host species belonging to the host family *Rhamnaceae*) was almost similar to that of *Gymnostoma* species (symbiotic host), and *Frankia* strains from both rhizospheres were similar. In addition to host plants, *Frankia* soil populations are controlled by soil variables. The population size of *Frankia* in soils was positively correlated with pH 3 up to pH 8.0 in a study of Finnish soils (Smolander and Sundman, 1987). In contrast, Myrold and Huss-Danell (1994) observed no significant correlation between pH and population size in a study of Swedish soils. However, they found that carbon level may regulate the size of infective *Frankia* populations. There is some indication that the speed of nodulation during stand development is a function of soil calcium levels (Crannell et al., 1994; Hilger and Myrold, 1992; Scott, 1973).

Plant bioassay using a nodulation capacity (van Dijk, 1984) and a most-probable-number (MPN) method (Huss-Danell and Myrold, 1994) has been most widely used for quantitative studies of *Frankia* populations. The MPN method, which measures nodulation units (NUs), has been useful in surveying *Frankia* populations in various forest soils (Jeong and Myrold, 2001; Huss-Danell and Myrold, 1994; Smolander and Sundman, 1987; van Dijk et al., 1988) and in following the survival of *Frankia* introduced into soil under laboratory conditions (Smolander et al., 1988). These studies have given similar results: (1) numbers of *Frankia* range from zero to a few thousand NU g^{-1} soil; (2) *Frankia* populations differ according to the tree species present; and (3) native and introduced populations of *Frankia* are favored by higher soil pH or liming.

Although the MPN method provides a quantitative measure of NUs, it is unclear whether these numbers reflect *Frankia* biomass in soil. A complicating factor is that spores appear to be much more infective than hyphae (Burleigh and Torrey, 1990; van Dijk, 1984). Furthermore, calculations based on DNA content extracted from soil and assayed with *Frankia*-specific probes (Hahn et al., 1990; Simonet et al., 1991) suggest that even viable counts represent only a fraction of the *Frankia* genomes present in soil (Myrold et al., 1990). More recently, use of the polymerase chain reaction (PCR) has substantiated that the number of *Frankia* genomes (genomic units, GUs) in soil is significantly greater than the number of *Frankia* NUs (Hilger and Myrold, 1992; Myrold and Huss-Danell, 1994; Picard et al., 1992). It is now possible to expand research on *Frankia* ecology to include forms of *Frankia* that have so far eluded measurement. An interesting molecular technique to study *Frankia* population is to track down introduced *Frankia* strains by *in situ* hybridization using Cy-3-labeled oligonucleotide probes targeting the 23S rRNA insertion of *Frankia* strains. Using this method, Nickel et al. (2001) showed that inoculation or leaf litter amendment that may enhance saprophytic growth of *Frankia* help *Frankia* populations with high specific N_2-fixing capacities successfully to compete for nodule formation with other indigenous or inoculated *Frankia* populations.

Molecular techniques that circumvent *Frankia* isolation has innovated population studies especially for *Frankia* strains associated with plant host recalcitrant to isolation (Hahn et al., 1999). The first study was the use of strain specific *nifH* probes to assay nodule DNA extracts (Simonet et al., 1990). Consensus sequences of interspersed repetitive DNA sequence elements found in a variety of eubacteria have been used for genomic fingerprinting coupled with the PCR technique, the well known of which are REP-, ERIC, and BOX-PCR, collectively referred to as rep-PCR (Sadowsky et al., 1996; Versalovic et al., 1991; Versalovic et al., 1994). It was proposed that rep-PCR may constitute a useful method to fingerprint bacterial genomes at strain level. Recently, the utility of these techniques was also demonstrated to generate characteristic banding patterns with genomic DNA of *Frankia* isolates and nodules (Jeong and Myrold, 1999; Murry et al., 1997, 1995; Pérez et al., 1999). In particular, Jeong and Myrold (1999) showed that additional repetitive sequences, direct repeats (DR), found in high GC *Myrobacterium bovis* (Doran, 1993), are useful in fingerprinting *Frankia* strains, high GC bacteria. In a comparison of population size and diversity of *Frankia* in soils under *C. velutinus* and under old-growth Douglas-fir stands, rep-PCR was used to analyze nodule fingerprints that, most likely, represent fingerprints of *Frankia* genomes inhabiting nodules (Jeong and Myrold, 2001). The *Frankia* population size in soil under *C. velutinus* was about 10 times higher than that under Douglas-fir, suggesting that host plants have a positive influence on *Frankia* population size. Nodulation capacities among three *Ceanothus* species

used as trap plants showed no significant difference. Most nodules could fix N_2 according to the acetylene reduction activity assay. Fingerprints of nodule DNA suggested that there was no host specificity among *Ceanothus*-infective *Frankia* in the two soils. Results also suggest that the two soils shared a large, common group of *Frankia* but there were also *Frankia* strains unique to the Douglas-fir soil.

Frankia is easily isolated from some plant hosts, although it is still difficult enough to isolate relative to the fast growing bacteria such as *Rhizobium* species, but not from others. *Frankia* associated with host plants recalcitrant to isolation may be more obligated symbiont than isolated *Frankia*. Interestingly, trials to isolate *Frankia* from host plants recalcitrant to isolation have frequently resulted into the isolation of atypical strains. A recent molecular analysis suggested that those atypical strains are most likely not the real symbiont of the actinorhizal plants but were present in the rhizosphere including the nodule surface and were selected by the culture method (Ramírez-Saad et al., 1998). This proposal was supported by an inoculation study (Simonet et al., 1999) of *Myrica cerifera* seedlings, a promiscuous host, with nonsterile crushed nodules harvested in Australia and representing the six groups of unisolated strains characterized previously by Rouvier et al. (1996). Surprisingly, none of the PCR/RFLP patterns obtained from the newly developed nodules corresponded to any of the seven Australian groups described by Rouvier et al. (1996). These observations confirm that the nodules formed initially by a compatible strain can harbor other infective rhizosphere or rhizoplane *Frankia* strains. Direct evidences about how different *Frankia* strains live together and interact with each other within such a small environment still need to come.

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References

Albert, V. A., Backlund, A., Bremer, K., Chase, M. W., Manhart, J. R., Mishler, B. D. and Nixon, K. C. 1994. Functional constraints and *rbc*L evidence for land plant phylogeny. *Ann. Mo. Bot. Gard.* 81:534-567.

Baker, D. D. 1987. Relationships among pure cultured strains of *Frankia* based on host specificity. *Physiol. Plant Pathol.* 70:245-248.

Baker, D. D. and O'Keefe, D. 1984. A modified sucrose fractionation procedure for the isolation of frankiae from actinorhizal root nodules and soil samples. *Plant Soil* 78:23-28.

Benecke, U. 1969. Symbiosis of alder nodules in New Zealand. *Plant Soil* 30: 145-149.

Benson, D. R. and Clawson, M. L. 2000. Evolution of the actinorhizal plant symbiosis. In: Prokaryotic nitrogen fixation: A model system for the analysis of biological process, ed. by E.W Triplett, 207-224 pp. Horizon Scientific Press, Wymondham, UK.

Benson, D. R. and Silvester, W. B. 1993. Biology of *Frankia* strains, actinomycete symbionts of actinorhizal plants. *Microbiol. Rev.* 57:293-319.

Bousquet, J. and Lalonde, M. 1990. The Genetics of Actinorhizal Betulaceae. In: The Biology of Frankia and Actinorhizal Plants, ed. by C. R. Schwintzer and J. D. Tjepkema, 239-261 pp. Academic Press, Inc., San Diego.

Brunchorst, J. 1887. Die Struktur der Inhaltskürper in den Zellen einiger Wurzelanschwellungen. Bergens Mus. Aarsb. 235pp.

Burleigh, S. and Torrey, J. G. 1990. Effectiveness of different *Frankia* cell types as inocula for the actinorhizal plant *Casuarina*. *Appl. Environ. Microbiol.* 56:2565-2576.

Callaham, D., Del Tredici, P. and Torrey, J. G. 1978. Isolation and cultivation *in vitro* of the actinomycete causing root nodulation in *Comptonia*. *Science* 199:899-902.

Chase M. W., Soltis, D. E., Olmstead, R. G.,. Morgan, D., Les, D. H., Mishler, B. D., Duvall, M. R., Price, R. A., Hills, H.G., Qui, Y.-L., Kron, K. A., Rettig, J. H., Conti, E., Palmer, J. D., Manhart, J. R., Sytsma, K. J., Michaels, H. J., Kress, W. J., Karol, K. G., Clark, W. D., Hedrén, M., Gaut, B. S., Jansen, R. K., Kim, K.-J., Wimpee, C. F., Smith, J. F., Furnier, G. R., Strauss, S. H., Xiang, G.-Y., Plunkett, G. M., Soltis, P. S., Swensen, S. M., Williams, S. E., Gadek, P. A., Quinn, C. J., Eguiarte, L. E., Golenberg, E., Learn, Jr., G. H., Graham, S. W., Barrett, S. C. H., Dayanandan, S. and Albert, V. A. 1993. Phylogenetics of seed plants: An analysis of nucleotide sequences from .the plastid gene *rbcL*. *Ann. Mo. Bot. Gard.* 80:528-580.

Clawson, M. L. and Benson, D. R. 1999. Natural diversity of *Frankia* strains in actinorhizal root nodules from promiscuous hosts in the family Myricaceae. *Appl. Environ. Microbiol.* 65:4521-7.

Cournoyer, B. and Lavire, C. 1999. Analysis of *Frankia* evolutionary radiation using *glnII* sequences. *FEMS Microbiol. Lett.* 177:29-34.

Crannell, W. K., Tanaka, Y. and Myrold, D. D. 1994. Calcium and pH interaction on root nodulation of nursery grown red alder (*Alnus rubra* Bong.) seedlings by *Frankia*. *Soil Biol. Biochem.* 26:607-614.

Cronquist, A. 1988. The Evolution and Classification of Flowering Plants, 2nd ed. Allen, Lawrence, Kansas.

Dawson, J. O. 1983. Dinitrogen fixation in forest ecosystems. *Can. J. Microbiol.* 29:979-992.

Diem, H. G. and Dommergues, Y. R. 1990. Current and potential uses and management of Casuarinaceae in the tropics and subtropics. In: The Biology of *Frankia* and Actinorhizal Plants, ed. by C.R. Schwintzer and J.D. Tjepkema, 317-342 pp. Academic Press, Inc., San Diego.

Doran, T. J., Hodgson, A. L. M., Davies, J. K. and Radford, A. J. 1993. Characterisation of a highly repeated DNA sequence from *Mycrobacterium bovis*. *FEMS Microbiol. Lett.* 111:147-152

Gauthier, D., Jaffre, T. and Prin, Y. 2000. Abundance of *Frankia* from *Gymnostoma* spp. in the rhizosphere of *Alphitonia neocaledonica*, a non-nodulated Rhamnaceae endemic to New Caledonia. *Eur. J. Soil Biol.* 36:169-175.

Hahn, D., Kester, R., Starrenburg, M. J. C. and Akkermans, A. D. L 1990. Extraction of ribosomal RNA from soil for detection of *Frankia* with oligonucleotide probes. *Arch. Microbiol.* 154:329-335.

Hahn, D., Nickel, A. and Dawson, J. 1999. Assessing *Frankia* populations in plants and soil using molecular methods. *FEMS Microbiol. Ecol.* 29:215-227.

Hibbs, D. E. and Cromack, Jr., K. 1990. Actinorhizal plants in Pacific Northwest forests. In: The Biology of *Frankia* and Actinorhizal Plants, ed. by C.R. Schwintzer and J.D. Tjepkema, 343-363pp. Academic Press, Inc., San Diego.

Hilger, A. B. and Myrold, D. D. 1992. Quantitation of soil *Frankia* by bioassay and gene probe methods: response to host and non-host rhizospheres and liming. *Acta Ecol.* 13:505-506.

Huelsenbeck, J.P. and Rannala, B. 1997. Phylogenetic methods come of age: Testing hypotheses in an evolutionary context. *Science* 276:227-232.

Huguet, V., Batzli, J. M., Zimpfer, J. F., Normand, P., Dawson, J. O. and Fernandez, M. P. 2001. Diversity and specificity of *Frankia* strains in nodules of sympatric *Myrica gale, Alnus incana*, and *Shepherdia canadensis* determined by *rrs* gene polymorphism. *Appl. Environ. Microbiol.* 67:2116-2122.

Huss-Danell, K. and Myrold, D. D. 1994. Intrageneric variation in nodulation of *Alnus*: Consequences for quantifying *Frankia* infective units in soil. *Soil Biol. Biochem.* 26:525-531.

Jeong, S. C., Liston, A. and Myrold, D. D. 1997. Molecular phylogeny of the genus *Ceanothus* (Rhamnaceae) using *rbc*L and *ndh*F sequences. *Theo. Appl. Genet.* 94:852-857.

Jeong, S. C. and Myrold, D. D. 1999. Genomic fingerprinting of *Frankia* microsymbionts from *Ceanothus* copopulations using repetitive sequences and polymerase chain reactions. *Can. J. Bot.* 77:1220-1230.

Jeong, S. C., Ritchie, N. J. and Myrold, D. D. 1999. Molecular phylogenies of plants and *Frankia* support multiple origins of actinorhizal symbioses. *Mol. Phylogen. Evol.* 13:493-503.

Jeong, S. C. and Myrold, D. D. 2001. Population size and diversity of *Frankia* in soils under *Ceanothus velutinus* and Douglas-fir stands. *Soil Biol. Biochem.* 33:931-941.

Lechevalier, M. P. and Ruan, J.-S. 1984. Physiological and chemical diversity of *Frankia* spp. isolated from nodules of *Compotonia peregrina* (L.) Coult. and *Ceanothus americanus* L. *Plant Soil* 78:15-22.

Lalonde, M., Simon, L., Bousquet, J. and Seguin A. 1988. Advances in the taxonomy of *Frankia*: recognition of species *alni* and *eleagni* and novel subspecies *pommerii* and *vandijkii*. In: Nitrogen Fixation: Hundred Years After, ed. by H. Bothe H, F.J. de Bruijn, and W.E. Newton WE, 671-680 pp. Gustav Fisher Verlag, Stutgart.

Maggia, L. and Bousquet, J. 1994. Molecular phylogeny of the actinorhizal Hamamelidae and relationship with host promiscuity towards *Frankia*. *Mol. Ecol.* 3:459-467.

Maunuksela, L., Zepp, K., Koivula, Y., Zeyer, J., Haahtela, K. and Hahn, D. 1999. Analysis of *Frankia* populations in three soils devoid of actinorhizal plants. *FEMS Microbiol. Ecol.* 28:11-21.

McMinn, H. E. 1942. *Ceanothus*: Part II; a systematic study of the genus *Ceanothus*. 129-308 pp. Santa Barbara Botanic Garden, Santa Barbara.

Miller, I. M. and Baker, D. D. 1986. Nodulation of actinorhizal plants by *Frankia* strains capable of both root hair infection and intercellular penetration. *Protoplasma* 131:82-91.

Moran, N. A., Munson, M. A., Bauman, P. and Ishikawa, H. 1993. A molecular clock in endosymbiotic bacteria is calibrated using the insect hosts. *Proc. R. Soc. Lond. B Biol. Sci.* 253:167-171.

Murry, M. A., Konopka, A. S., Pratt, S. D. and Vandergon, T. L. 1997. The use of PCR-based typing methods to assess the diversity of *Frankia* nodule endophytes of the actinorhizal shrub *Ceanothus*. *Physiol. Plant* 99:714-721.

Murry, M. A., Zhang, D., Schneider, M. and de Bruijn, F. J. 1995. Use of repetitive sequences and the polymerase chain reaction (rep-PCR) to fingerprint the genomes of *Frankia* isolates. *Symbiosis* 19:223-240.

Myrold, D. D., Hilger, A. B. and Strauss, S. H. 1990. Detecting *Frankia* in soil using PCR. 429 p. In: Nitrogen Fixation: Achievements and Objectives, ed by P.M. Gresshoff, L.E. Roth, G. Stacey, and W.E. Newton. 429 pp. Chapman and Hall, New York.

Myrold, D. D. and Huss-Danell, K. 1994. Population dynamics of *Alnus*-infective *Frankia* in a forest soil with and without host trees. *Soil Biol. Biochem.* 26:533-540.

Navarro, E., Nalin, R., Gauthier, D. and Normand, P. 1997. The nodular microsymbionts of *Gymnostoma* spp. are *Elaeagnus*-infective *Frankia* strains. *Appl. Environ. Microbiol.* 63:1610-1616.

Navarro, E., Jaffre, T., Gauthier, D., Gourbiere, F., Rinaudo, G., Simonet, P. and Normand, P. 1999. Distribution of *Gymnostoma* spp. microsymbiotic *Frankia* strains in New Caledonia is related to soil type and to host-plant species. *Mol. Ecol.* 8:1781-1788.

Newcomb, W. and Wood, S. M. 1987. Morphogenesis and fine structure of *Frankia* (Actinomycetales): the microsymbiont of nitrogen-fixing actinorhizal root nodules. *Int. Rev. Cytol.* 109:1-88.

Nickel, A., Pelz, O., Hahn, D., Saurer, M., Siegwolf, R. and Zeyer, J. 2001. Effect of inoculation and leaf litter amendment on establishment of nodule-forming *Frankia* populations in soil. *Appl. Environ. Microbiol.* 67:2603-2609.

Normand, P., Orso, S., Cournoyer, B., Jeannin, P., Chapelon, C., Dawson, J., Evtushenko, L. and Misra, A. K. 1996. Molecular phylogeny of the genus *Frankia* and related genera and emendation of the family Frankiaceae. *Int. J. System. Bacteriol.* 46:1-9.

Ochman, H. and Wilson, A. C. 1987. Evolution in bacteria: evi-

dence for a universal substitution rate in cellular genomes. *J. Mol. Evol.* 26:74-86.

Page, R. D. M. 1994. Parallel phylogenies: Reconstructing the history of host-parasite assemblages. *Clastistics* 10:155-173.

Pérez, N.-O., Vásquez, L. and Valdés, M. 1999. Genetic characterization of Mexican *Frankia* strains nodulating *Casuarina equisetfolia*. *Can. J. Bot.* 77:1214-1219.

Picard, C., Ponsonnt, C., Paget, E., Nesme, X. and Simonet, P. 1992. Detection and enumeration of bacteria in soil by direct DNA extraction and polymerase chain reaction. *Appl. Environ. Microbiol.* 58:2717-2711.

Racette, S. and Torrey, J. G. 1989. Root nodule iniation in *Gymnostoma* (*Casuarinaceae*) and *Shepherdia* (*Elaeagnaceae*) induced by *Frankia* strain HFPGpI1. *Can. J. Bot.* 67:2873-2879.

Ramírez-Saad, H., Janse, J. D. and Akkermans, A. D. L. 1998. Root nodules of Ceanothus caeruleus contain both the N_2-fixing *Frankia* endophyte and a phylogenetically related Nod^-/Fix^- actinomycete. *Can. J. Microbiol.* 44:140-148.

Richards, J. W., Krumholz, G. D., Chval, M. S. and Tisa, L. S. 2002. Heavy metal resistance patterns of *Frankia* strains. *Appl. Environ. Microbiol.* 68:923-927.

Ritchie, N. J. and Myrold, D. D. 1999. Geographic distribution and genetic diversity of *Ceanothus*-infective *Frankia* strains. *Appl. Environ. Microbiol.* 65:1378-1383.

Rouvier, C., Prin, Y., Redell, P., Normand, P. and Simonet, P. 1996. Genetic diversity among *Frankia* strains nodulating members of the family Casuarinaceae in Australia revealed by PCR and restriction fragment length polymorphism analysis with crushed root nodules. *Appl. Environ. Microbiol.* 62:979-985.

Sadowsky, M. J., Kinkel, L. L., Bowers, J. H. and Schottel, J. L. 1996. Use of repetitive intergenic DNA sequences to classify pathogenic and disease-supressive *Streptomyces* strains. *Appl. Environ. Microbiol.* 62:3489-3493.

Scott, W. 1973. Some soil factors affecting snowbrush nodulation. Ph.D. Thesis. Oregon State Univ., Corvallis, OR. (Diss. Abstr. 33:4074).

Simonet, P., Grosjean, M.-C., Misra, A. K., Nazaret, S., Cournoyer, B. and Normand, P. 1991. *Frankia* genus-specific characterization by polymerase chain reaction. *Appl. Environ. Microbiol.* 57:3278-3286.

Simonet, P., Navarro, E., Rouvier, C., Reddell, P., Zimpfer, J., Dommergues, Y., Bardin, R., Combarro, P., Hamelin, J., Domenach, A.-M., Gourbiere, F., Prin, Y., Dawson, J. O. and Normand, P. 1999. Co-evolution between *Frankia* populations and host plants in the family Casuarinaceae and consequent patterns of global dispersal. *Environ. Microbiol.* 1:525-533.

Simonet, P., Normand, P., Moiroud, A. and Bardin, R. 1990. Identification of *Frankia* strains in nodules by hybridization of polymerase chain reaction products with strain-specific oligonucleotide probes. *Arch. Microbiol.* 153:235-240.

Smolander, A. and Sundman, V. 1987. *Frankia* in acid soils of forests devoid of actinorhizal plants. *Physiol. Plant* 70:297-303.

Smolander, A., van Dijk, C. and Sundman, V. 1988. Survival of *Frankia* strains introduced into soil. *Plant Soil* 106:65-72.

Soltis, D. E., Soltis, P. S., Morgan, D. R., Swensen, S. M., Mullin, B. C., Dowd, J. M. and Martin, P. G. 1995. Chloroplast gene sequence data suggest a single origin of the predisposition for symbiotic nitrogen fixation in angiosperms. *Proc. Natl. Acad. Sci. USA* 92:2647-2651.

Soltis, D. E., Soltis, P. S., Chase, M. W., Mort, M. E., Albach, D. C., Zanis, M., Savolainen, V., Hahn, W. H., Hoot, S. B., Fay, M. F., Axtell, M., Swensen, S. M., Prince, Li. M., Kress, W. J., Nixon, K. C. and Farris, J. S. 2000. Angiosperm phylogeny inferred from 18S rDNA, *rbcL*, and *atpB* sequences. *Bot. J. Linnean Soc.* 133:381-461.

Sprent, J. I. 1994. Evolution and diversity in the legume-rhizobium symbiosis: chaos theory? *Plant Soil* 161:1-10.

Swensen, S. M. 1996. The evolution of actinorhizal symbiosis: Evidence for multiple origins of the symbiotic association. *Am. J. Bot.* 83:1503-1512.

Tisa, L. S., Chval, M. S., Krumholz, G. D. and Richards, J. W. 1999. Antibiotic resistance patterns of *Frankia* strains. *Can. J. Bot.* 77:1257-1260.

Torrey, J. G. 1978. Nitrogen fixation by actinomycete-nodulated angiosperms. *BioSci.* 28:586-591.

Torrey, J. G. and Tjepkema, J. D. 1979. Preface. *Bot. Gaz.* 140 (Suppl): i-ii.

van Dijk, C. 1984. Ecological aspects of spore formation in the *Frankia-Alnus* symbiosis. Ph.D. Thesis. State University, Leiden, the Netherlands. 154 p.

van Dijk, C., Sluimer, A. and Weber, A. 1988. Host range differentiation of spore-positive and spore-negative strain types of *Frankia* in stands of *Alnus glutinosa* and *Alnus incana* in Finland. *Physiol. Plant* 72:349-358.

Versalovic, J., Koeuth, T. and Lupski, J. R. 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Res.* 24:6823-6831.

Versalovic, J., Schneider, M., de Bruijn, F. J. and Lupski, J. R. 1994. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods Mol. Cell. Biol.* 5:25-40.

Wall, L. G. 2000. The Actinorhizal Symbiosis. *J. Plant Growth Reg.* 19:167-182.

Wheeler, C. T. and Miller, I. M. 1990. Current and potential uses of actinorhizal plants in Europe. In: The Biology of *Frankia* and Actinorhizal Plants, ed. by C.R. Schwintzer and J.D. Tjepkema, 365-389 pp. Academic Press, Inc., San Diego.

Wollum, A. G. II, Youngberg, C. T. and Chichester, F. W. 1968. Relation of previous timber stand age to nodulation of *Ceanothus velutinus*. *For. Sci.* 14:114-118.

Zimpfer, J. F., Kennedy, G. J., Smyth, C. A., Hamelin, J., Navarro, E. and Dawson, J. O. 1999. Localization of *Casuarina*-infective *Frankia* near *Casuarina cunninghamiana* trees in Jamaica. *Can. J. Bot.* 77:1248-1256.