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Typical *Frankia* infect actinorhizal plants exotic to New Zealand

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Abstract *Frankia* strains are actinomycetes that fix N_2 in root nodules of plants classified in eight families of the Angiospermae. The wide variety of plants infected has raised questions about the ecology and diversity of *Frankia* symbionts, and their co-evolution with actinorhizal plants. Partial sequencing was done of the small subunit (16S) rRNA gene from *Frankia* endophytes present in the root nodules of *Alnus cordata*, *A. glutinosa*, *A. viridis*, *Casuarina equisetifolia*, *Coriaria arborea*, *C. plumosa*, and *Elaeagnus pungens* growing in New Zealand. With the exceptions of the *Coriaria* spp., all of the plants are exotic to New Zealand. Analysis of the 16S rRNA gene indicates that the exotic species were nodulated by *Frankia* strains that cluster with strain groups previously shown to be typical for each actinorhizal host. *Frankia* in 12 native *Coriaria* spp. nodules yielded two sequences differing in only one position, suggesting a relatively low diversity compared with the exotic species.

Keywords *Frankia*; actinorhizal; root nodule; nitrogen fixation; 16S rRNA; rDNA; *Alnus*; *Coriaria*; *Elaeagnus*; *Casuarina*

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INTRODUCTION

The N_2-fixing actinomycetes that define the genus *Frankia* form root nodules on plants belonging to 24 genera of woody, dicotyledonous angiosperms in the Betulaceae, Casuarinaceae, Coriariaceae, Datiscaceae, Elaeagnaceae, Myricaceae, Rhamnaceae, and Rosaceae (Benson & Silvester 1993). Plants infected by *Frankia* are typically pioneer species that colonise soil of marginal fertility and are found on every continent except Antarctica (Baker & Schwintzer 1990). They fall within an N_2-fixing clade of the Angiospermae (Chase et al. 1993; Soltis et al. 1995) and recent studies have indicated some congruency between actinorhizal plant and *Frankia* evolution (Cournoyer et al. 1993; Maggia & Bousquet 1994; Normand et al. 1996).

Some studies have addressed the identification and diversity of unculturable or non-cultured *Frankia* endophytes through 16S rDNA analysis (Simonet et al. 1990; Nazaret et al. 1991; Mirza et al. 1992; Nick et al. 1992; Bosco et al. 1993; Mirza et al. 1994a, 1994b, 1994c; Simonet et al. 1994; Benson et al. 1996). This approach has lead to improved phylogenetic placements within the Frankiaceae (Normand et al. 1996) and has facilitated studies on the diversity of *Frankia* that cannot be obtained in culture.

Frankia are characterised by their unique morphology that includes vesicles, filamentous hyphae, and multilocular sporangia (Benson & Silvester 1993). They are known to occupy two ecological niches – the root nodule and the soil (Benson & Silvester 1993). What remains unclear is the distribution of, and ratio between, obligate symbionts, obligate saprophytes, and strains capable of growing in either environment. Their ecological significance also remains unclear. Some studies have suggested that *Frankia* strains have an active free-living existence outside the normal geographic distribution of host plants (Benecke 1969; Tyson & Silver 1979) or after the host plant is no longer present (Rodriguez-Barrueco 1968; Wollum II et al. 1968; Van Dijk 1984; Smolander & Sundman 1987). Most such studies are done by "trapping" *Frankia*

from the soil by planting hosts and observing nodule formation. Such an approach is subject to uncertainty over the identity of the infecting microorganism. Strains can be "typical" (able to re-infect and re-establish a symbiosis with the host of origin) or "atypical" (unable to re-infect and re-establish a symbiosis with the host of origin, but will often do so with another host) (Benson & Silvester 1993). Some may also primarily nodulate one host and poorly nodulate a second host, perhaps ineffectively (Bosco et al. 1992). Thus, *Frankia* strains in nodules of plants exotic to an environment cannot be assumed to be "typical" to the introduced host, unless additional information is available. Since *Frankia* are so difficult to isolate from actinorhizal root nodules, an alternate approach is used here, sequencing of the 16S rRNA genes of frankiae as they occur in root nodules.

An ecological question that we addressed is whether *Frankia* strains populate soil in the absence of host plants. New Zealand is an intriguing environment in which to address this question since it is known that some exotic plants nodulate wherever planted in New Zealand soil (*Alnus* spp., *Elaeagnus* spp.) whereas others do not (*Purshia* spp., *Ceanothus* spp., *Casuarina* spp.) (Benecke 1969; Silvester 1977; Benson & Silvester 1993). Of the 24 genera of angiosperms infected by *Frankia* only two are represented in the native flora of New Zealand – *Discaria* (Rhamnaceae) and *Coriaria* (Coriariaceae) (Silvester 1977). Micro-organisms found in the exotic *Alnus* and *Elaeagnus* nodules are of interest since they are likely to be present as part of the normal soil microbiota and help to answer the question of the independent existence of *Frankia* in soil.

METHODS

Nodule preparation

The sources of the root nodules used in this study are listed in Table 1. In all cases nodules were washed in running water then frozen at –25°C upon collection or receipt at The University of Waikato.

PCR protocol

The protocols used for *Frankia* hyphae isolation and DNA preparation from actinorhizal root nodules and 16S rRNA gene amplification by the polymerase chain reaction (PCR) have been previously described (Benson 1982; Benson et al. 1996). In brief, excised nodule lobes were submerged in TEA buffer (10 m*M* Tris·HCI, 1 m*M* Na_4·EDTA, 20 m*M* ascorbic acid, pH 7.6), the nodule periderm was removed, and the *Frankia* hyphal clusters were dissected from the nodule tissue and collected on nylon screens. These manipulations removed essentially all contaminating soil bacteria and most of the plant tissue. Template DNA was prepared by lysing the isolated hyphas cells with 0.2 N NaOH/1% sodium dodecylsulfate (SDS) and precipitating the DNA as described previously (Benson et al. 1996).

Frankia 16S rDNA corresponding to *E. coli* positions 28 to 419 was amplified using primers fD1 (5′-CCGAATTCGTCGACAACAGAGTTTGA TCCTGGCTCAG-3′) (Weisburg et al. 1991) and rDB1 (5′-CCAAGCTTGAGGTTTACAACCCGAA-3′) (Benson et al. 1996). The PCR amplicons were purified on Wizard Prep columns (Fisher Sci. Co., Springfield, MA) and quantitated for cycle sequencing.

Cycle sequencing was carried out for both directions using an Amplicycle kit (Perkin Elmer, Norwalk, Connecticut). Fluorescently tagged fD1 and rDB1 primers were used for sequencing along with two internal primers; DB25 (5′-GCCTATCAGCTTGTTGGTGG-3′), and DB26 (5′-CCACCAACAAGCTGATAGGC-3′), to ensure that the entire amplicon was sequenced in both directions. Cycle sequencing was carried out in a Perkin-Elmer Model 2400 thermal cycler using about 75 fmols of template DNA. Thermal cycling was done with an initial hold at 95° for 1 min, followed by 30 cycles at 95° for 30 sec., 50° for 30 sec., and 72° for 60 sec., with a final extension at 72° for 4 minutes. Sequencing reactions were analysed using a Pharmacia LKB DNA sequencer. The Wisconsin Computer Group GCG programs run on a VAX system were used to align and edit the sequences (Devereux et al. 1984).

Table 1 Sources of nodules used in this study. (*i* = indigenous to New Zealand)

Plant	Number analysed	Location
Coriaria arborea [i]	7	Mt. Tarawera soil
	1	Raukokore
	1	Opotiki
	1	Opape
Coriaria plumosa [i]	2	Mt. Hikurangi, East Cape
Casuarina equisetifolia	2	Univ. Waikato, Hamilton
Elaeagnus pungens	2	Ruakura, Hamilton
Alnus glutinosa	2	Heritage Horticulture, Hamilton
Alnus cordata	2	Waikato River, Hamilton
Alnus viridis	1	Christchurch

```
              1                                                                                                  100
Coriaria II   -----------------------------------------g-a--------...--ttc----------------------------------------
Coriaria I    NNNNNN-----------------------------------g-a--------...--ttc----------------------------------------
Purshia       ---------------------------------------------a------...t--------------------------------------------
FE-Eal2       -------------------------------------------------..-...--------------------------------------------a
E. pungens    -------------------------------------------------..-...--------------------------------------------a
Argp5         -----------------------------------------g----------...--t------------------------------------------
C. equiset.   -----------------------------------------g----------...---tc----------------------------------------
Avn17s        NNNNNNNNN-----------------------------------g-------...---------------------------------------------
A. viridis    --------------------------------------------g-------...---------------------------------------------
A. glutinosa  --------------------------------------------g-------...---------------------------------------------
AcoN24d       NNNNNNNNNNNN--------------------------------g-------...---------------------------------------------
M. nagi nod   NNNNNNNNNNNNNNNNNNNNNNNNNNNNN------------g----------...---------------------------------------------
A. cordata    --------------------------------------------ga------...t-------------------------------------------a
G48           -----------------------------------------gaa--------ggtact-g---------------------------a----------ta
Consensus     GACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAGCGAGGAGCTTCGG---CTCCTAGCGGCGAACGGGTGAGTAACACGTGGGCAACCTGCCCCG

              101                                                                                                200
Coriaria II   --------------t-------------------a------------ct-----a---------------------------------------------
Coriaria I    --------------t-------------------a------------ct-----a---------------------------------------------
Purshia       --------g-----t-------------------a------------ct--t--a------------------------------t--------------
FE-Eal2       --------------tc--------------------------------cgt----------------------------------t--------------
E. pungens    --------------tc--------------------------------c-t----------------------------------t--------------
Argp5         -t----------------------------------------------tg---------------------------------a----------------
C. equiset.   ------------------------------------------------tg--------------------------------------------------
Avn17s        -----------------------------------------------cct--------------------------------------------------
A. viridis    ------------------------------------------------c--t------------------------------------------------
A. glutinosa  ------------------------------------------------tg--------------------------------------------------
AcoN24d       --------------t--------------------------------ctg-t------------------------------------------------
M. nagi nod   ------------------------------------------------c--t------------------------------------------------
A. cordata    ------------------------------------------------c--t---------------------------------t--------------
G48           g---t---g------.------------------a---a-------t-ctggtc----gaccg-tg--------.---t-----ct---------t----
Consensus     AGCTCTGGAATAACCTCGGGAAACCGGGGCTAATGCCGGATATGACAT-ACCGGGCATCTGGTGGTGTGGAAAGATTTATCGGCTCGGGATGGCCCGCG

              201                                                                                                300
Coriaria II   -----------------------a-c------------------------------------------t-------------------------------
Coriaria I    -----------------------a-c--------------------------------------------------------------------------
Purshia       -----------------------a-c------------------------------------------t-------------------------------
FE-Eal2       --------------------------------------------------------------------t-------------------------------
E. pungens    --------------------------------------------------------------------t-------------------------------
Argp5         ----------------------------------------------------------------------------------------------------
C. equiset.   ----------------------------------------------------------------------------------------------------
Avn17s        ----------------------------------------------------------------------------------------------------
A. viridis    ----------------------------------------------------------------------------------------------------
A. glutinosa  -------------------------c--------------------------------------------------------------------------
AcoN24d       ----------------------------------------------------------------------------------------------------
M. nagi nod   ----------------------------------------------------------------------------------------------------
A. cordata    ----------------------------------------------------------------------------------------------------
G48           -------------------------------------------------------------------------------------------------g-g
Consensus     GCCTATCAGCTTGTTGGTGGGGTGATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGA

              301                                                                            381
Coriaria II   ---------------------------------------------------------------------------------
Coriaria I    ---------------------------------------------------------------------------------
Purshia       ------------------------------------------g--------------------------------------
FE-Eal2       ---------------------------------------------------------------------------------
E. pungens    -------------------------------------------------------NNNNNNNNNNNNNNNNNNNNNNNNNN
Argp5         -------c-------------------------------------------------------------------------
C. equiset.   ------------------------------------------g--------------------------------------
Avn17s        ---------------------------------------------------------------------------------
A. viridis    ---------------------------------------------------------------------------------
A. glutinosa  ---------------------------------------------------------------------------------
AcoN24d       ---------------------------------------------------------------------------------
M. nagi nod   ------------------------------------------g--------------------------------------
A. cordata    ---------------------------------------------------------------------------------
G48           -----t-c------------------------a---------g--------t-----------------------------
Consensus     CTCCTACGGGAGGCAGCAGTGGGGAATATTGCGCAATGGGCGAAAGCCTGACGCAGCGACGCCGCGTGAGGGATGACGGCC
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Fig. 1 Alignment of partial 16S rDNA sequences of *Frankia* endophytes. *E. coli* positions 28 to 419 are shown. Capital letters indicate a consensus in the strains. Differences in the consensus are represented by lower case letters, deletions are represented by (.), an absence of overall consensus is represented by (-) in the consensus line only, otherwise dashes indicate homology to the consensus character. An absence of sequence data is represented by "N". Sequences used in the alignment and their accession numbers are given in Table 2.

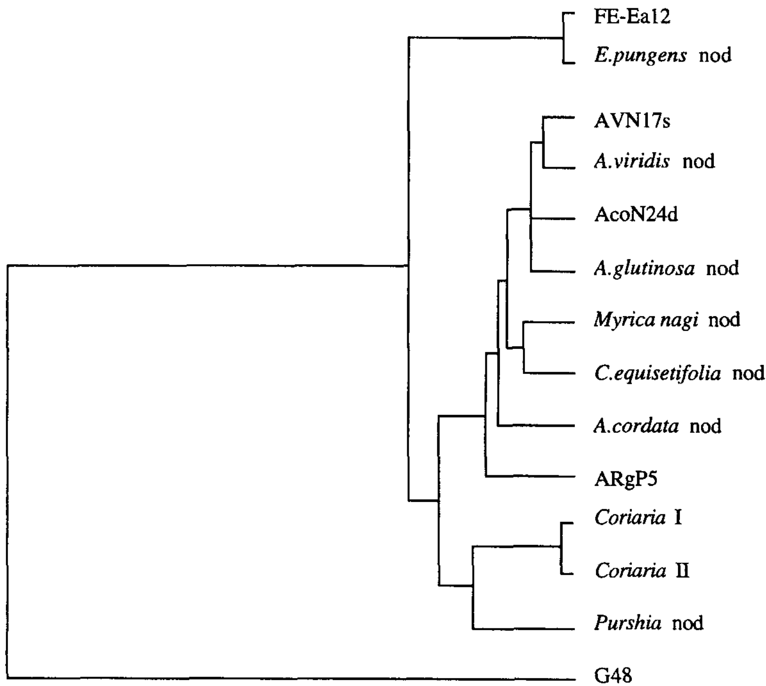

Fig. 2 Dendrogram of the aligned *Frankia* 16S rDNA using the GCG Pileup Program. The dendrogram was generated with base sequences from positions 30–355 of the alignment in Fig. 1.

RESULTS AND DISCUSSION

Endophyte 16S rRNA genes from 10 *Coriaria arborea* nodules and two *C. plumosa* nodules were partially sequenced to gain a perspective on the diversity of strains found in the nodules of exotic versus native plants. Seven of the 10 *C. arborea* plants were grown in the greenhouse, in soil obtained from seven different sites on the volcanic Mt. Tarawera, and the two *C. plumosa* nodules were collected from the slopes of Mt. Hikurangi. These nodules all yielded identical *Frankia* 16S rDNA sequences designated as *Coriaria* Type I (Fig. 1). Mt. Hikurangi and Mt. Tarawera are located about 140 km from each other on the North Island and share a common environment of elevated, mountainous terrain with

Table 2 Sequences used in the analysis of *Frankia* 16S rRNA genes.

Frankia strain or nodule source	GenBank Accession no.	Reference
AcoN24d from *Alnus cordata*	L40610	Normand et al. 1996
Argp5 from *Alnus* sp.	L40612	Normand et al. 1996
AVN17s from *Alnus* sp.	L40613	Normand et al. 1996
FE-Ea12 from *Elaeagnus* sp.	L40619	Normand et al. 1996
Myrica nagi nodule	L40622	Normand et al. 1996
Alnus cordata nodule	U82102	This study
Alnus glutinosa nodule	U82101	This study
Alnus viridis nodule	U82103	This study
Casuarina equisetifolia nodule	U82104	This study
Coriaria arborea nodule I	U54610	Benson et al. 1996
Coriaria arborea nodule II	U82100	This study
Elaeagnus pungens	U82099	This study

loose, gravelly soil, and low flora diversity, but are markedly different in that Tarawera last erupted in 1886 while Hikurangi is part of an uplifted mountain chain.

The remaining sequences were from endophytes in the root nodules of *C. arborea* plants growing in Raukokore, Opotiki, and Opape. These locations cover a distance of approximately 70 km and are found on the East Cape of the North Island adjacent to the Bay of Plenty. Their environments are coastal, or riparian with relatively varied flora. The *C. arborea* endophytes from these regions yielded identical sequences that differed by a single base from the Type I sequence and are characterised here as *Coriaria* Type II (Fig. 1). Thus, there exists a minimum of two distinctive *Frankia* pools present within the North Island that may segregate according to their environments, although this hypothesis needs to be tested more thoroughly. Our data for *Coriaria* endophytes, based on three distinct environments and two species, suggest a relatively low overall diversity of *Coriaria Frankia* in New Zealand. This observation tends to confirm that of others who have suggested that *Coriaria Frankia* strains have an overall lack of diversity (Nick et al. 1992).

Endophytes from two *Casuarina equisetifolia* nodules growing in proximity to one another were found to have identical 16S rDNA sequences. *C. equisetifolia* plants are not normally nodulated in New Zealand soil (Benson & Silvester 1993). The plants used in this study were inoculated in a greenhouse in Hamilton with imported *Frankia* strain HFPCcI3 and transplanted outside in the mid 1980s. The nodules used here were taken from recently formed lateral roots and may represent continuing colonisation by HFPCcI3.

16S rDNA was sequenced from endophytes in two *Alnus cordata* nodules growing on the banks of the Waikato River in Hamilton, two *A. glutinosa* nodules from Heritage Horticulture (Hamilton), one *A. viridis* nodule from Christchurch on the South Island, and two *Elaeagnus pungens* root nodules in a drainage ditch in Hamilton. *Frankia* in each of the three *Alnus* species and *E. pungens* yielded a different sequence, with the two sequences from the duplicate nodules the same. An analysis of the sequences using the UPGMA (unweighted pair-group method using arithemetic averages; Devereux et al. 1984) yielded a dendrogram based on pair-wise alignments that placed the New Zealand *Frankia* sequences among other related *Frankia* sequences. In each case sequences were obtained that were similar to those typical for *Frankia* from each plant species and that were available in data bases (Fig. 2). Thus, the *Frankia* strains in the exotic plant root nodules can be characterised as "typical".

A comparison of the 16S rDNA sequences of *A. cordata*, *A. glutinosa*, and *A. viridis* reveals six variable sites (Fig. 1), reflecting a diversity in New Zealand soils similar to that found in other environments where *Alnus* is native (Normand et al. 1996). The level of diversity of *Frankia* endophytes found within the nodules of *Alnus* is noteworthy when compared with the lack of diversity of endophytes in the nodules of the native *Coriaria* spp. Although *Coriaria* plants have been present in New Zealand far longer than *Alnus* plants, the *Alnus Frankia* are much more diverse than those in *Coriaria*. In the absence of any known artificial inoculation of plants and the widespread presence of *Frankia* capable of infecting *Alnus* and *Elaeagnus*, the question of the origins of these endophytes needs to be addressed. One explanation is that a diverse population of these endophytes was already established in New Zealand before the comparatively recent arrival of *Alnus* plants over a century ago. This possibility would imply that i) a significant diversity of saprophytic *Alnus Frankia* exists in New Zealand soils compared with *Coriaria Frankia*, and ii) *Alnus* and *Elaeagnus* plants are not necessary for growth and survival of their respective *Frankia* in soil.

An alternate explanation is that *Alnus* and *Elaeagnus Frankia* were introduced to New Zealand at the same time as the plants and have since spread. However, the *Alnus* endophytes and *Elaeagnus* endophytes appear universally distributed in New Zealand soil as assessed by rapid nodulation wherever the species are planted (Benson & Silvester 1993). Furthermore, the introduction of exotic *Casuarina* has not led to a similar universal distribution of *Casuarina Frankia*.

Clearly a significant degree of circumspection is warranted for drawing conclusions on the ecology of *Frankia* in New Zealand from these results. We can conclude that i) *Alnus* and *Elaeagnus* are nodulated by homologous, "typical" endophytes in New Zealand soil, and ii) the diversity of endophytes in the root nodules of *Alnus* appears greater than the diversity of endophytes in the nodules of *Coriaria* plants. Questions remain as to the diversity of endophytic populations of *Frankia* in the nodules of other native and exotic actinorhizal plants, the diversity of saprophytic versus endophytic populations of *Frankia*, and the possibility of obligate saprophytic populations of *Frankia* being of the microecology of New Zealand.

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