The Nodular Microsymbionts of *Gymnostoma* spp. Are *Elaeagnus*-Infective *Frankia* Strains

ELISABETH NAVARRO,¹ RENAUD NALIN,² DANIEL GAUTHIER,¹ AND PHILIPPE NORMAND^{2,3*}

*Laboratoire de Microbiologie, Centre ORSTOM de Nouméa, Nouméa Cedex, New Caledonia,¹
and Laboratoire d'Ecologie Microbienne du Sol, UMR CNRS 5557,² and Centre d'Analyse
Moléculaire de la Biodiversité,³ Université Lyon I, 69622 Villeurbanne Cedex, France*

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The phylogenetic relationships of *Frankia* strains infective on *Gymnostoma* with other *Frankia* strains was analyzed. Partial sequencing of the 16S rDNA and use of specific primers showed that the *Frankia* strains present in *Gymnostoma* are phylogenetically close to *Elaeagnus*-infective strains. This finding was confirmed by using the sequences of the hypervariable *nifDK* intergenic spacer. The strains present in *Gymnostoma* nodules were close to one another, clustered with *Elaeagnus*-infective strains, and distantly related to *Casuarina* and *Alnus*-infective strains. Morphological observations of strains and cross-inoculation trials showed that *Gymnostoma*-infective strains are indistinguishable from *Elaeagnus*-infective strains. Results of both phenotypic and genotypic approaches indicate that *Gymnostoma*-infective strains are *Elaeagnus* infective and not *Casuarina* infective.

The family Casuarinaceae, which comprises woody nonleguminous plants fixing nitrogen in symbiosis with *Frankia*, includes 96 extant species belonging to four genera (*Allocasuarina*, *Casuarina*, *Ceuthostoma*, and *Gymnostoma*). They are confined to the Malaysian-Australian Melanesian region, except for the essentially littoral species *Casuarina equisetifolia* (11), which has a worldwide tropical distribution.

New Caledonia is characterized by the abundance of endemic Casuarinaceae, especially those belonging to the genus *Gymnostoma*. In fact, 8 of the 18 species of *Gymnostoma* are restricted to the Main Island flora. These species are abundant on heavy metal-rich ultramafic rock, including peridotites and serpentinites, which represent one-third of the Main Island area. Five *Gymnostoma* species are found only in soils derived from ultramafic rocks: *Gymnostoma chamaecyparis* (Poisson) L. Johnson, *G. deplancheanum* (Miquel) L. Johnson, *G. glaucescens* (Schlechter) L. Johnson, *G. leucodon* (Poisson) L. Johnson, and *G. poissonianum* (Schlechter) L. Johnson. This was also the case for the species *G. intermedium* (Poisson) L. Johnson, with the exception of one report of plants growing on acidic rocks. On the other hand, *G. webbianum* (Miquel) L. Johnson is found on both ultramafic and nonultramafic substrates. Finally, the last species, *G. nodiflorum* (Thunberg) L. Johnson, develops mainly on nonultramafic substrates and also in mixed alluvial soils (9).

So far, little is known about the microsymbionts of *Gymnostoma*. A few cross-inoculation studies and successful attempts to isolate strains have been made (24, 26, 29). Only three *Frankia* strains, isolated from *G. papuanum* (HFPGpI1), *G. sumatranum* (ISU0224887), and *G. nobilis* (G80), are available. Among these, HFPGpI1 was isolated from a *G. papuanum* seedling inoculated with crushed nodules of *Casuarina equisetifolia* (24), casting some doubts on its true origin.

It seems that strains isolated from *Gymnostoma* spp. nodulate, besides their original host, members of Elaeagnaceae and *Myrica* species but not *Casuarina* and *Allocasuarina* species (24, 26, 29). Nevertheless, conflicting results have been ob-

tained with isolated *Frankia* strains on *Gymnostoma* (29). Some *Casuarina*-infective strains were reported to nodulate *Gymnostoma* spp. at low frequency, i.e., less than 50% (29), whereas others did not nodulate at all. The relation of strains symbiotic with *Gymnostoma* to other *Frankia* strains and their phylogenetic position have not yet been clearly determined. Using sequences of 16S rDNA and the *nifDK* intergenic spacer, as well as specific amplification, we sought to relate the strains symbiotic with *Gymnostoma* to other *Frankia* strains.

Nodules and bacterial strains. Nodules from *Gymnostoma* plants were harvested from different areas of New Caledonia (Table 1). Tontouta is in the Southern part of New Caledonia. Rivière des Pirogues, Etoile Filante, Creek Pernod, and Rivière Bleue are in the Far South region. These two regions are 60 km apart. Kouaoua, Mé Aïu, and Canala are in the Central Eastern region, in a perimeter of 30 km. There is approximately 100 km between the Southern and the Central

FIG. 1. Specific amplification with primers FGPS989ac (A) and FGPS989e (B). Lanes: 1, Ccl3; 2 to 9, *Gymnostoma* nodule DNAs; 10, D11; L, 123-bp ladder. nt, nucleotides.

* Corresponding author. Mailing address: Ecologie Microbienne du Sol, UMR CNRS 5557, Université Lyon I, 69622 Villeurbanne Cedex, France. Phone: (33) 4 72 43 13 77. Fax: (33) 4 72 43 12 23. E-mail: normand@biomserv.univ-lyon1.fr.

TABLE 1. Origin of DNA nodules and isolated strains used in this study

Strain or nodule ^a	GenBank accession number	Host plant	Geographical origin and soil type	Source or reference
<i>Gymnostoma</i> nodules				
RPL161	U63334	<i>G. leucodon</i>	Rivière des Pirogues (New Caledonia) Ultramafic alluvium	This study
TC24	U63692	<i>G. chamaecyparis</i>	Tontouta (New Caledonia) Hypermagnesian incepsol	This study
CN61	U63693	<i>G. nodiflorum</i>	Canala (New Caledonia) Ultramafic and acidic alluvium	This study
MG59	U63691	<i>G. glaucescens</i>	Me Aiu (New Caledonia) Oxisol weakly desaturated	This study
KP54	U63694	<i>G. poissonianum</i>	Kouaoua (New Caledonia) Oxisol weakly desaturated	This study
MI60		<i>G. intermedium</i>	Me Aiu (New Caledonia) Indurated oxisol	This study
<i>Gymnostoma</i> strains				
GD2		<i>G. deplancheanum</i>	Creek Pernod (New Caledonia) Indurated oxisol	This study
GG46		<i>G. glaucescens</i>	Etoile Filante (New Caledonia) Colluvio alluvial oxisol	This study
GW1		<i>G. webbium</i>	Rivière Bleue (New Caledonia) Ultramafic alluvium	This study
Elaeagnaceae strains				
EUN1f (6)	L37664	<i>E. umbellata</i>	Illinois	12
HRN18a (7)	U63696	<i>Hippophaë rhamnoides</i>	Alps (France)	14
EaN1-pec (5)	U63698	<i>E. angustifolia</i>	Ohio	12
Ea1-12 (4)	U63697	<i>E. angustifolia</i>	Ecully (France)	5
SCN10a (U)	U63695	<i>Shepherdia canadensis</i>	Québec (Canada)	15
<i>Casuarina</i> strains				
D11 (atypical) (U)	U63700	<i>C. equisetifolia</i>	Dakar (Senegal)	6
CcI3 (9)	U63699	<i>C. cunninghamiana</i>	Florida	31
Cj1-82 (9)		<i>C. junghuhniana</i>	Thailand	4
<i>Alnus</i> strain				
ArI3 (1)	L35557	<i>A. rubra</i>	Oregon	2

^a Genomic species numbers are in parentheses. U, undetermined species.

Eastern regions. Reference *Frankia* strains (Table 1) were grown as described previously (23).

DNA extraction from nodules. After peeling of the superficial layers, nodule lobes were disinfected with 30% (wt/vol) H₂O₂ for 5 min, rinsed with sterile distilled water, and kept at -20°C. One nodule lobe was crushed in 500 µl of TCP buffer (100 mM Tris-HCl [pH 8], 1.4 M NaCl, 20 mM EDTA [pH 8], 2% [wt/vol] cetyltrimethylammonium bromide [CTAB; Sigma, St. Louis, Mo.], 3% [wt/vol] polyvinylpyrrolidone [PVPP; Sigma]). The mixture was incubated at 65°C for 1 h and centrifuged at 3,000 × g for 5 min at 20°C. The supernatant was chloroform extracted and ethanol precipitated. The DNA pellet was dissolved in 10 µl of Tris-EDTA (TE) buffer (pH 7.5).

PCR amplification. Amplification of the 16S rRNA fragment (positions 921 to 1189 of the *Escherichia coli* sequence) with primers FGPS849 (5'-GCCTTGGGAGTACGGCCG CA-3') and FGPS1146' (5'-GGGGCATGATGACTTGACG TC-3') was performed as described by Nazaret et al. (21).

Specific amplification with primers FGPS989ac (5'-GGGG TCCGTAAGGGTC-3') or FGPS989e (5'-GGGGTCCTTAG GGGCT-3') (3) and FGPL1973' (5'-ATCGGCTCGAGGTG CCAAGGGTC-3') (19) was performed under the standard conditions described by Simonet et al. (27).

For DNA amplification of a region including the 3' end of *nifD*, the intergenic spacer, and the beginning of *nifK*, two primers, FGPD807 (5'-CACTGCTACCGGTCGATGAA-3')

(10) and FGPK333' (5'-CCGGGCGAAGTGGCT-3') (17), were used. PCR amplification was performed in 0.2-ml Eppendorf tubes in a total volume of 50 µl containing template DNA (approximately 0.1 µg) and polymerase reaction buffer (10 mM Tris-HCl [pH 8.3], 1.5 mM MgCl₂, 50 mM KCl, 0.01% [wt/vol] gelatin, 20 µM deoxynucleoside triphosphate, 1 µM each of the primers, 2.5 U of *TaqI* DNA polymerase [Gibco BRL, Gaithersburg, Md.]). DNA amplification was done in a thermocycler (Perkin Elmer, Norwalk, Conn.) with the following program: initial denaturation for 3 min at 95°C; 35 cycles of denaturation (30 s at 95°C), annealing (30 s at 63°C), and extension (30 s at 72°C); and a final extension (2 min at 72°C). PCR amplification of DNA was checked by agarose gel electrophoresis (2%, wt/vol) in Tris-borate-EDTA (TBE) buffer with 5 µl of PCR product. The gel was stained in a aqueous solution of 1 mg of ethidium bromide liter⁻¹ and photographed with HP5 film with a 302-nm UV source.

Sequencing of intergenic spacer amplicons. Before sequencing, the amplification reaction mix was purified with Centricon-30 concentrators (Amicon-Grace Company, Epernon, France). The amplicons were sequenced with the Deaza G/A sequencing kit (Pharmacia Biotech SA, St-Quentin-Yvelines, France) by the direct DNA sequencing method described by Winship (30). The fragments were sequenced in both directions. In the case of the *nifDK* spacer, the two amplification primers FGPD807 and FGPK333' were used as well as FGPK1 (5'-GTGACGA

	1	15	16	30	31	45	46	60
1	ArI3	GATCTTCGCCCCGGA	CATGGACATCGCCAT	CAACAGCCCGGCTG	GGACCTCCTCGAGGC			
2	RPL161	GGTCTTCGCCCCGCA	CATGGACATGGCCAT	CAACAGCCCGACCTG	GGACCTGATGGAGAC			
3	MG59	GGTCTTCGCCCCGCA	CATGGACATGGCCAT	CAACAGCCCGACCTG	GGACCTGATGGAGAC			
4	TC24	GGTCTTCGCCCCGCA	CATGGACATGGCCAT	CAACAGCCCGACCTG	GGACCTGATGGAGAC			
5	CN61	GGTCTTCGCCCCGCA	CATGGACATGGCCAT	CAACAGCCCGACCTG	GGACCTGATGGAGAC			
6	KP54	GGTCTTCGCCCCGCA	CATGGACATGGCCAT	CAACAGCCCGACCTG	GGACCTGATGGAGAC			
7	EUN1f	GATCTTCGCCCCGCA	CATGGACATCGCCAT	CAACAGCCCGACCTG	GGACCTGATGGAGAC			
8	SCN10A	GATCTTCGCCCCGCA	CATGGACATCGCGAT	CAACAGCCCGACCTG	GGACCTCATGGAGAC			
9	HRN18a	GCTCTTCGCCCCGCA	CATGGACATCGCGAT	CAACAGCCCGACCTG	GGACCTGATGGAGAC			
10	Ea112	GGTCTTCGCCCCGCA	CATGGACATCGCGAT	CAACAGCCCGACCTG	GGACCTGATGGAGAC			
11	EaN1pec	GGTCTTCGCCCCGCA	CATGGACATCGCGAT	CAACAGCCCGACCTG	GGACCTCATGGAGAC			
12	CcI3	GGTCTTCGCCCCGTA	CATGGACATCGCGAT	CAACAGCCCGACCTG	GGACCTCATGGAGAC			
13	D11	GGTCTTCGCCCCGTA	CATGGACATCGCGAT	CAACAGCCCGGCTG	GGACCTGATGGAGAA			
		61	75	76	90*91	105	106	120
1	ArI3	CCCCTGGTCAAGGC	CGGCGAGGTCGCCCTG	<u>ATCC</u> ACC GCCCTCCG	GGCACCCGGGCGCGA			
2	RPL161	CCCCTGGTCAAGTC	CGGAGAGGTCTTCTG	<u>ACC</u> AGCTAGT-CCGG	CACCGGCC--AACCA			
3	MG59	CCCCTGGTCAAGTC	CGGAGAGGTCTTCTG	<u>ACC</u> AGCTAGT--CGG	CACCGGCC--AACCA			
4	TC24	CCCCTGGTCAAGTC	CGGAGAGGTCTTCTG	<u>ACC</u> AGCTAGT-CCGG	CACCGGCCGCAACCA			
5	CN61	CCCCTGGTCAAGTC	CGGAGAGGTCTTCTG	<u>AGC</u> AG-TAGT-CCGG	CACCGGCC--AACCA			
6	KP54	CCCCTGGTCAAGTC	CGGAGAGGTCTTCTG	<u>ACC</u> AGTTAGT--CGG	CACCGGCC--AACCA			
7	EUN1f	CCCCTGGTCAAGTC	CGGAGAGGTCTTCTG	<u>ACC</u> AGCTAGTTCCGG	CTCCGCGCGCAACCA			
8	SCN10A	CCCCTGGTCAAGTC	CGGAGAGGTCTTCTG	<u>ACC</u> CGTTAG-CCCG	CACCGCCGCGCAACCA			
9	HRN18a	CCCCTGGTCAAGGG	TGGAGAGGTCTTCTG	<u>ACCC</u> G-----AAG	ACTCG-GCG--TCCG			
10	Ea112	CCCCTGGTCAAGGC	CGGAGAGGTGTGCTG	<u>ACC</u> GGATC-TTCCGG	TCTCCG-----CCT			
11	EaN1pec	CCCCTGGTCAAGGC	CGGAGAGGTGTGCTG	<u>ACC</u> GGATT-TTCCGG	TCTCCG-----CC-			
12	CcI3	CCCCTGGTCAAGGC	CGGAGAGGTGTGCTG	<u>ACC</u> GGCCGGT-CCGG	CCCCGCGCGGCTAGC			
13	D11	CCCCTGGTCAAGGC	CTGGAGAAAGGCCCTG	<u>ACC</u> GGG-----	--CCG--CCTCG-CA			
		121	135	136	150	151	165	180
1	ArI3	GGCGCCACGACAGTAG	ACGGCCACTTGCCGT	-AGACGGCCACTCGC	AGTA-GACGGCCACT			
2	RPL161	CCGGCGACCACCGGT	GATCACCGGTGGCTG	GCCGGCGGCCATCAG	TGGGGTCTTTCTCTT			
3	MG59	CCGGCGACCACCGGT	GATCACCGGTGGCTG	-CCGGCGGCCATCAG	TGGG-TCTTTCTCTT			
4	TC24	CCGGCGACCACCGGT	GATCACCGGTGGCTG	-CCGGCGGCCATCAG	TGGG-TCTTTCTCTT			
5	CN61	CCGGCGACCACCGGT	GATCACCGGTGGCTG	-CCGGCGGCCATCAG	GGGG-CCTTCTCCAT			
6	KP54	CCGGCGACCACCGGC	GANCAACCGGTGGCCG	ACGGCGGCCATCAG	T-----			
7	EUN1f	CCGGCGACCACCGGT	GATCACCGGTGGCTG	-CCGGCGGCCATCAG	TGAG-CCTTCTCCAT			
8	SCN10A	TCGGCGACCACCGGC	GATCACCGGTGGCC-	GCCGGCGGCCATC-	-----			
9	HRN18a	C-GGTGACNCCCGT	GGT-----	-----	-----			
10	Ea112	--GGCGAT-----	-----	-----	-----			
11	EaN1pec	--GGCGAT-----	-----	-----	-----			
12	CcI3	CGCAGT-----	-----	-----	-----			
13	D11	CGCCGGG-----T	GGT--CCGGT----	-----	-----			
		181	195	196	210	211	225	240
1	ArI3	CGCCGTAGACGGCCA	CTCGCAGTAGACGGC	CACTTGCAGTAGCTG	ACCAGTAGTTCATCC			
2	RPL161	CGCACGGGTGACGGC	CACGAATGCCGTGAG	CCCGAGTCGACGACC	GAAAAGG-----			
3	MG59	CGCACGGGTGACGGC	CACGAATGCCGTGAG	CCCGAGTCGACGACC	GAAAAGG-----			
4	TC24	CGCACGGGTGACGGC	CACGAATGCCGTGAG	CCCGAGTCGACGACC	GAAA-GG-----			
5	CN61	CGCACGGGGCACGGC	CAGGAA--CGTGAG	CCCGAACTGGCGATG	GAAAAGG-----			
6	KP54	-----	--ACGCA-GAAAGGC	TGATGTTACGCGGTG	CGTCCGT-----			
7	EUN1f	CTCGCGGGCGACGGC	CAGGAATGCCGTGAG	CCCGAGTCGCGGATG	GAAAAGG-----			
8	SCN10A	-----	-----	-----	-----			
9	HRN18a	-----	-----	-----	-----			
10	Ea112	-----	-----	-----	-----			
11	EaN1pec	-----	-----	-----	-----			
12	CcI3	-----	-----	-----	-----			
13	D11	---ACCGG--CCGGC	GAGCG--CCGGT---	-----	-----			

FIG. 2. Alignment of the *nifDK* intergenic spacer of the *Frankia* symbionts of *Gymnostoma* spp., *Elaeagnus*-infective strains (EUN1f, SCN10a, HRN18a, Ea112, EaN1pec, and D11), a *Casuarina*-infective strain (CcI3), and an *Alnus*-infective strain (ArI3). The stop codon of *nifD* and the start codon of *nifK* are underlined and marked by an asterisk.

CGACTCCC-3') and FGPK64' (5'-CCTCGTCCCTGAACA-3'), which were derived from a sequence deposited in GenBank under accession number L41344. For the 16S rDNA sequencing of nodule MI60, primers FGPS849 and FGPS1176' were used. The sequences were determined for both strands. The sequences were aligned and compared with alignments of *Frankia* strains and nodule DNAs (21, 22) by using the multi-

ple-alignment ClustalV algorithm (8), with manual refinements in the noncoding regions. Two additional sequences, from EUN1f (L37664; GenBank) and ArI3 (L35557; GenBank), were included (17). The distances were calculated according to Kimura's two-parameter model (12), and phylogenetic analyses were performed by the neighbor-joining methods (25) and parsimony (28).

	241	255	256	270	271	285	286	300
1 ArI3	AGGCCTGGCCGAACC	AGCCGCCAGCGACCC	CGTGACCGGAGAAAC	GGTTCGCTGGCGGCTG				
2 RPL161	-----	-----	-----	-----	-----	-----	-----	-----
3 MG59	-----	-----	-----	-----	-----	-----	-----	-----
4 TC24	-----	-----	-----	-----	-----	-----	-----	-----
5 CN61	-----	-----	-----	-----	-----	-----	-----	-----
6 KP54	-----	-----	-----	-----	-----	-----	-----	-----
7 EUN1f	-----	-----	-----	-----	-----	-----	-----	-----
8 SCN10A	-----	-----	-----	-----	-----	-----	-----	-----
9 HRN18a	-----	-----	-----	-----	-----	-----	-----	-----
10 Ea112	-----	-----	-----	-----	-----	-----	-----	-----
11 EaN1pec	-----	-----	-----	-----	-----	-----	-----	-----
12 CeI3	-----	-----	-----	-----	-----	-----	-----	-----
13 D11	-----	-----	-----	-----	-----	-----	-----	-----
	301	315	316	330	331	345	346	*360
1 ArI3	CCGCGGCAGGCCTCA	AGCCCCACCTGGGAC	AAGCCACAGGAGAG	GAGGGACGTCGGTGA				
2 RPL161	-----	-----	-----CTCA	AGTCACGGCAGAAAG	GAGGAGGTCCGGTGA			
3 MG59	-----	-----	-----CTCA	AGTCACGGCAGAAAG	GAGGAGGTCCGGTGA			
4 TC24	-----	-----	-----CTCA	AGTCACGGCAGAAAG	GAGGAGGTCCGGTGA			
5 CN61	-----	-----	-----CTCA	AGTCACGGCAGAAAG	GAGGAGGTCCGGTGA			
6 KP54	-----	-----	-----ATCA	-GTCACGTCAGAAAG	GAGGAGGTCCGGTGA			
7 EUN1f	-----	-----	-----CTCA	AGTCACGGCAGAAAG	GAGGAGGTCCGGTGA			
8 SCN10A	-----	-----	-----	AGTCACGTCAGAAAG	GAGGATGTCCGGTGA			
9 HRN18a	-----	-----	-----CATC	AGTCACGTAGAAAG	GAGGATGTCCGGTGA			
10 Ea112	-----	CGCCGGCGGCTCATC	AGTCACGGTAGAAAG	GAGGATGTCCGGTGA				
11 EaN1pec	-----	CGCCGGCGGC-CATC	AGTCACGGTAGAAAG	GAGGATGTCCGGTGA				
12 CeI3	-----	-----	AGCCACGGTAGAAGG	GAGGATGTCCGGTGA				
13 D11	-----	-----GGCCATCT	AGTCACGGTAGAAAG	GAGGATGTCCGGTGA				
	361	375	376	390	391	405	406	420
1 ArI3	CGACGACCCCGAGC	ACACCAGCGCGGTCC	CGTGC GGTCCTTG	ACCACAACGACATCT				
2 RPL161	CGACGACTCCCGAGA	CGAACAGTCACATCC	CGCTCAAGGTTCCTCA	ACCACACCGAGCTGT				
3 MG59	CGACGACTCCCGAGA	CGAACAGTCACATCC	CGCTCAAGGTTCCTCN	ACCACACCGAGCTGT				
4 TC24	CGACGACTCCCGAGA	CGAACAGTCACATCC	CGCTCAAGGTTCCTCG	ACCACACCGAGCTGT				
5 CN61	CGACGACTCCCGAGA	CGAACAGTCACATCC	CGCTCAAGGTTCCTCG	ACCACACCGAGCTGT				
6 KP54	CGACGACTCCCGAGA	CGAACAGTCACATCC	CGCTGAAGGTTCCTCG	ACCACACCGAACTGT				
7 EUN1f	CGACGACTCCCGAGA	CGAACAGTCACATCC	CGCTTAAGGTTCCTCG	ACCACACCGAGCTGT				
8 SCN10A	CGACGACTCCCGAGA	CGAACAGTCACATCC	CGCTGAAGGTTCCTCG	ACCACAACGAGCTGT				
9 HRN18a	CGACAACCTCCCGAGA	CAGGTAGTCACATAC	CGCTGAAGGTTCCTCG	ACCACAGCGAGCTGT				
10 Ea112	CGACGACTCCCGAGA	CAGGCAGTTCCATTC	CGTTGCGGGTCCTCG	ACCACAGCGAGCTGT				
11 EaN1pec	CGACGACTCCGTAGA	CAGGCAGTTCCATTC	CGTTGCGGGTCCTCG	ACCACAGCGAGCTGT				
12 CeI3	CGACGACTCCGAAA	CAAACAGTTCCGTCC	CGCTGCGGGTCCTCG	ACCACAGCGAGCTGT				
13 D11	CGACGACTCCCGAGA	CAGGCAGTCACATCC	CGCTCAAGGTGCTCG	ACCACAACGAGCTGT				
	421	435	436	450	451	465	466	480
1 ArI3	TCCGCGACGAGGTCT	ACCAGAAGCAGTTTCG	AGGGCAAGCGCGAGT	TCGAGAACGCGGCC				
2 RPL161	TCAAGGACGAGGTCT	ACACCAAGCAGTTTCG	AGACGAAGCGCGAGT	TCGAGAACGGTGCGA				
3 MG59	TCAAGGACGAGGTCT	ACACCAAGCAGTTTCG	AGACCAAGCGCGAGT	TCGAGAACGGTGCGG				
4 TC24	TCAAGGACGAGGTCT	ACACCAAGCAGTTTCG	AGACCAAGCGCGAGT	TCGAGAACGGTGCGG				
5 CN61	TCAAGGACGAGGTCT	ACACCAAGCAGTTTCG	AGACCAAGCGCGAGT	TCGAGAACGGTGCGG				
6 KP54	TCAAGGACGAGGTCT	ACACCAAGCAGTTTCG	AGACCAAGCGCGAGT	TCGAGAACGGTGCGG				
7 EUN1f	TCAAGGACGAGGTCT	ACACCAAGCAGTTTCG	AGACCAAGCGCGAGT	TCGAGAACGGTGCGG				
8 SCN10A	TCAAGGACGAGGTCT	ACACCAAGCAGTTTCG	AGACCAAGCGCGAGT	TCGAGAACGGTGCGG				
9 HRN18a	TCAAGGACGAGGTCT	ACAGGAAGCAGTTTCG	AGGGCAAGGGCGAGT	TCGAGAACGGTGCGG				
10 Ea112	TCAAGGACGAGGTCT	ACCAGAAGCAGTTTCG	AGGGCAAGGGCGAGT	TCGAGAACGGTGCGG				
11 EaN1pec	TCAAGGACGAGGTCT	ACCAGAAGCAGTTTCG	AGGGCAAGGGCGAGT	TCGAGAACGGTGCGG				
12 CeI3	TCAAGGACGAGGTCT	ACCAGAAGCAGTTTCG	AGGGCAAGGGCGAGT	TCGAGAACGGTGCGG				
13 D11	TCAAGGGCGAGGCTT	ACCACAAGCAGTTTCG	AGGGCAAGGGCGAGT	TCGAGAACGGTGCGG				

FIG. 2—Continued.

Frankia strain isolation. Nodules were directly harvested from the roots of the *Gymnostoma* plants. Each lobe was individually rinsed under tap water, disinfected for 10 to 20 min in 30% (wt/vol) H_2O_2 (13), and rinsed with sterile distilled water before being cut into small fragments. Each fragment was incubated at 30°C for 1 to 3 months in 5 ml of nitrogen-free medium (7) or complex medium (CM) containing (per liter): K_2HPO_4 , 1.67 g; KH_2PO_4 , 0.87 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 0.01; yeast extract,

0.5 g; Bacto-Peptone, 0.5 g; glucose, 2 g; sodium propionate, 1.2 g; ferric iron sodium EDTA, 0.01 g; $NaMoO_4 \cdot 2H_2O$, 0.005 g; $MnSO_4 \cdot H_2O$, 0.025 g; $ZnSO_4 \cdot 7H_2O$, 0.007 g; $CuSO_4 \cdot 5H_2O$, 0.00125 g; $CoSO_4 \cdot 7H_2O$, 0.0014 g; and H_3BO_3 , 0.0003 g (pH 6.8).

The isolated strains, identified microscopically by the presence of *Frankia*-characteristic morphological structures, such as hyphae, vesicles, sporangia, and spores, were then subsequently cultured in CM.

		481	495	496	510	511	525	526	540
1	ArI3	CGAAGGAAGAGGTCC	AGCGGGTCCTCGACT	GGACCCGCGGGTGGG	AGTACCCGGGAGAAGA				
2	RPL161	TCGACGCCGAGGTCA	ACCGGGTCCTGGAGT	GGACCCGTACCTGGG	AGTACCCGCGAGAAGA				
3	MG59	ATCAGCCCGAGGTCA	ACCGGGTCCTGGAGT	GGACCCGTACCTGGG	AGTACCCGCGAGAAGA				
4	TC24	ATGACGCCGAGGTCA	ACCGGGTCCTGGAGT	GGACCCGTACCTGGG	AGTACCCGCGAGAAGA				
5	CN61	ATGACGCCGAGGTCA	ACCGGGTCCTGGAGT	GGACCCGTACCTGGG	AGTACCCGCGAGAAGA				
6	KP54	ATGACGCCGAGGTCA	ACCGGGTCCTGGAGT	GGACCCGTACCTGGG	AGTACCCGCGAGAAGA				
7	EUN1f	ACGACGCCGAGGTCA	ACCGGGTCCTGGAGT	GGACCCGCACCTGGG	AGTACCCGCGAGAAGA				
8	SCN10a	ATGACGCCGAGGTCA	ACCGGGTCCTGGAGT	GGACCCGCACCTGGG	AGTACCCGGGAGAAGA				
9	HRN18a	ACGCCGCCGAGGTCA	ACCGGGTCTCTGGAGT	GGACCCGCGGCTGGG	AGTACCCGCGAGAAGA				
10	Ea112	AATCCGCCGAGGTTT	CCCGAGTCCTCGAGT	GGACCCGCGGCTGGG	AGTACCCGGGAGAAGA				
11	EaN1pec	ACTCCGCCGAGGTTG	CCCGCGTCCTCGAGT	GGACCCGCGGCTGGG	AGTACCCGGGAGAAGA				
12	CcI3	ACGCCGCCGAGGTCT	CCCGGGTCCTGGAGT	GGACCCGCGGCTGGG	AGTACCCGGGAGAAGA				
13	D11	ACGCCGCCGAGGTGC	AGCGGGTCTCTGAAT	GGACCCGCGGCTGGG	AGTACCCGGGAGAAGA				
		541	544						
1	ArI3	ACTT							
2	RPL161	ACTT							
3	MG59	ACTT							
4	TC24	ACTT							
5	CN61	ACTT							
6	KP54	ACTT							
7	EUN1f	ACTT							
8	SCN10a	ACTT							
9	HRN18a	ACTT							
10	Ea112	ACTT							
11	EaN1pec	ACTT							
12	CcI3	ACTT							
13	D11	ACTT							

FIG. 2—Continued.

Cross-inoculation trials. *Gymnostoma* spp. and *Casuarina colina* seeds were sterilized by incubation in concentrated H₂SO₄ for 2 min. *Elaeagnus* seeds were rinsed with sterile distilled water and germinated in sterile sand in a shade house. After 1 month, the seedlings were transferred into pots containing sterile sand and watered every 12 h. Plants that were 2 and 4 months old were fertilized with 1 g of standard Welgro nutrient solution (Ulvir Ltd., Barcelona, Spain) liter⁻¹. *Frankia* strains were grown in CM for 1 month. Controls were not inoculated. Plants that were 6 months old were inoculated with a cultured *Frankia* isolate (10 mg [fresh weight] per plant). After 15 (*Gymnostoma*) or 10 (*Casuarina* and *E. angustifolia*) months, the plants were uprooted and the number of nodules was scored. Uninoculated controls did not nodulate. The effectivity of nodulation was determined by an acetylene reduction assay (ARA) with a GC 6000 gas chromatograph (Farmitalia Carlo ERBA, Milan, Italy). For each trial, four plants were analyzed.

Comparative studies of the phylogenies of host plant and symbionts are a promising approach to understanding the evolution of the actinorhizal symbiosis. Of the 21 dicotyledonous genera described as actinorhizal (1), strains capable of fulfilling Koch's postulates or present as microsymbionts in 11 of these have had their 16S ribosomal DNA (rDNA) sequences determined and compared (23). This analysis has shown that the genus *Frankia* is coherent and that strains infective on *Casuarina* are phylogenetically close to those infective on *Alnus*. Study of the host plant phylogeny, on the other hand, has shown that *Casuarina* and the other Casuarinaceae genera *Allocasuarina* and *Gymnostoma* form a phylogenetically coherent family in the Hamamelidaceae (14).

It was thus expected that the microsymbionts present in the nodules of *Gymnostoma* would be phylogenetically close to those present in *Casuarina*, *Allocasuarina*, and *Alnus*. However, the present work on the 16S rDNA sequences has shown that the *Frankia* strains present in *Gymnostoma* are close to *Elaeagnus*-

infective strains. The 16S rDNA region from positions 921 to 1189 of a nodule from *Gymnostoma intermedium* (MI60) was sequenced and aligned with 35 rDNA sequences of *Frankia* strains (21, 22). This sequence was identical to the sequence of strain D11 and was very close (one mismatch) to that of strain EUN1f. A large number of *Gymnostoma* provenances were screened to assess the diversity of *Gymnostoma*-infective *Frankia* strains (Fig. 1) with the specific primers described by Bosco et al. (3), confirming these results in all cases (20). All the DNA nodules tested amplified with primer FGPS989e, which is specific to the Elaeagnaceae group, and no amplification was obtained with primer FGPS989ac (3), which is *Alnus-Casuarina* group specific.

These results were confirmed by using the sequences of the hypervariable *nifDK* intergenic spacer. The length of these sequences varied from 293 to 447 bp depending on the size of the intergenic sequences. All the DNAs sequenced have different *nifDK* intergenic spacers (Fig. 2). Several indels are present in the alignment, but these were not included in the analyses. The number of pairwise differences between *Gymnostoma* sequences (not shown) varied from 6 to 48. The two closest sequences to these strains were EUN1f and SCN10a (distances of 4.2 to 7 substitutions per 100 sites). Four of the five *Gymnostoma* sequences were found to form a coherent cluster (bootstrap 95%) together with strain EUN1f isolated from an *E. umbellata* specimen in the United States, a cluster also found by parsimony analysis. Another significant cluster was made up of the same five sequences together with SCN10a, isolated from *Shepherdia* (Elaeagnaceae) (16), and the remaining *Gymnostoma* sequence (KP54). ArI3 and CcI3 formed a coherent group (bootstrap 100%) (Fig. 3). This showed that the strains present in five *Gymnostoma* nodules were close to one another, in the cluster of *Elaeagnus*-infective strains and far from *Casuarina*- and *Alnus*-infective strains.

On the basis of morphological observations, the strains isolated from *G. deplancheanum*, *G. glaucescens*, *G. webbiana*,

FIG. 3. Phylogenetic neighbor-joining tree based on *nifDK* intergenic spacer sequences. The bar represents 0.034 substitution per site. The numbers in the ovals are the percentages of bootstrap replicates in which the clusters were found. The *Gymnostoma* DNAs are in boldface type, and the genomic species described by Fernandez et al. (5) are in parentheses.

G. leucodon, and *G. chamaecyparis* were able to fulfill Koch's postulates and were comparable to *Elaeagnus*-infective strains; i.e., they had contorted hyphae, produced vesicles both in nitrogen-free and in rich media, and synthesized reddish to orange pigments. These strains nodulated *Elaeagnus* but not *Casuarina* (Table 2). They yielded effective nodules on *Elaeagnus* with ARA values on harvested nodules between 27 and 46 μmol of $\text{C}_2\text{H}_4 \text{ h}^{-1} \text{ g}$ of dry nodule $^{-1}$. No significant difference was noted between *Gymnostoma* strains on their original host and *Gymnostoma* strains on *E. angustifolia* (Table 2). These findings are in contrast to the findings by Torrey and Racette (29) that *Gymnostoma* was a promiscuous host, since we found that it was not nodulated by *Casuarina*-infective strains. This may be related to the isolation technique used by most investigators working with *Frankia*; most of the time, this did not include a single cell isolation step, which may have resulted in cocultures of *Frankia* strains with different host specificities.

These results would mean that in the Casuarinaceae line of descent, the host plants did not evolve gradually as suggested by Maggia and Bousquet (14). These authors suggested that the evolution of the symbiosis had been from a promiscuous ancestor identified as comparable to present-day *Gymnostoma*

TABLE 2. Cross-inoculation trials involving strains isolated from Casuarinaceae plants

Origin of isolates	Host plant species	Infectivity ^a	Effectivity (ARA) ^b
<i>G. deplancheanum</i> GD2	<i>G. deplancheanum</i>	4/4	27 $\pm$ 8
<i>G. glaucescens</i> GG46	<i>G. chamaecyparis</i>	4/4	27 $\pm$ 19
<i>G. webbianum</i> GW1	<i>G. deplancheanum</i>	4/4	39 $\pm$ 34
<i>G. deplancheanum</i> GD2	<i>E. angustifolia</i>	3/4	43 $\pm$ 18
<i>G. glaucescens</i> GG46	<i>E. angustifolia</i>	4/4	41 $\pm$ 20
<i>G. webbianum</i> GW1	<i>E. angustifolia</i>	3/4	46 $\pm$ 29
<i>C. junghuhniana</i> Cj1-82	<i>Gymnostoma</i> spp.	0/4	
<i>G. deplancheanum</i> GD2	<i>C. colina</i>	0/4	
<i>G. glaucescens</i> GG46	<i>C. colina</i>	0/4	
<i>G. webbianum</i> GW1	<i>C. colina</i>	0/4	

^a Number of nodulated plants/number of tested plants.

^b Micromoles of C_2H_4 per hour per gram of dry nodule $\pm$ standard error.

to the very restrictive descendant (*Allocasuarina*) with *Casuarina* as an intermediate. Instead, the most coherent scenario would be that an early split occurred in the evolution of the Casuarinaceae, resulting into two distinct lines of descent, one (*Casuarina* and *Allocasuarina*) in symbiosis with a slow-growing and hard-to-isolate *Frankia* strain phylogenically close to those infective on *Alnus* and the other in symbiosis with faster-growing *Frankia* strains that are more saprophytic and thus easier to isolate and found even in soils devoid of *Elaeagnus* (18).

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