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Original article

Evolution of *Frankia*–Casuarinaceae interactions

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Abstract – Nonisolated *Frankia* strains present in the root nodules of three of the four genera of the Casuarinaceae family (namely, *Casuarina*, *Allocasuarina* and *Gymnostoma*) have been characterised through polymerase chain reaction/restriction fragment length polymorphism (PCR/RFLP) analyses and sequencing of their *nif*D-*nif*K intergenic spacer (IGS). Analyses of the aligned sequences were used to deduce phylogenetic relations of these genes. Strains from *Casuarina* and *Allocasuarina* were found to be in the same cluster, while strains from *Gymnostoma* were closer to Elaeagnaceae strains. The relationships between IGS subgrouping and symbiotic (host spectrum) characteristics of the nonisolated strain confirmed the differences between *Casuarina/Allocasuarina* and *Gymnostoma* symbiosis. Genetic diversity among *Casuarina* and *Allocasuarina* microsymbionts seems to be host species-dependent. In contrast, no relation could be found between *Gymnostoma* microsymbionts and host species. The comparison between phylogenic analyses of the host plants and their microsymbionts suggests that the most coherent evolutionary scenario would be that an early split occurred in the evolution of Casuarinaceae, resulting into two distinct lines of descent. © Inra/Elsevier, Paris

diversity / *Frankia* / Casuarinaceae / coevolution

Résumé – Évolution des interactions *Frankia*–Casuarinaceae. En utilisant des analyses PCR/RFLP et le séquençage de l'intergène *nif*D-K, des souches non isolées de *Frankia* présentes dans les nodosités de trois des quatre genres constituant la famille des Casuarinacées (*Casuarina*, *Allocasuarina* and *Gymnostoma*) ont été caractérisées. L'analyse des séquences alignées a permis d'établir les relations phylogénétiques entre ces souches. Les souches infectives sur *Casuarina* et *Allocasuarina* appartiennent au

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même groupe phylogénétique, alors que les souches infectives sur *Gymnostoma* sont regroupées avec les souches d'Elaeagnacées. Les relations entre le groupage moléculaire et les caractéristiques symbiotiques du micro-organisme (spectre d'hôte) confirment les différences entre les symbioses impliquant *Casuarina/Allocasuarina* et *Gymnostoma*. La diversité génétique des microsymbiotes de *Casuarina* et *Allocasuarina* semble être corrélée à l'espèce de la plante hôte. Au contraire, aucune relation n'a été trouvée entre le type de microsymbiotes de *Gymnostoma* et l'espèce végétale. La comparaison des arbres phylogénétiques des plantes hôtes et de leurs microsymbiotes suggère qu'une séparation précoce soit survenue dans l'évolution des Casuarinacées, entraînant l'existence de deux lignées de descendants. © Inra/Elsevier, Paris

diversité / *Frankia* / Casuarinacées / coévolution

1. INTRODUCTION

The actinomycete *Frankia* has established a nitrogen-fixing symbiosis with a wide range of dicotyledonous plants. This symbiosis is known to occur in more than 200 species of plants belonging to eight families (Betulaceae, Casuarinaceae, Myricaceae, Elaeagnaceae, Rhamnaceae, Rosaceae, Coriariaceae and Datiscaceae) (Benson and Silvester, 1993).

The Casuarinaceae family is composed of the four genera of tropical dicotyledonous plants *Allocasuarina*, *Casuarina*, *Ceuthostoma* and *Gymnostoma*, of which *Gymnostoma* is considered the most primitive (Johnson and Wilson, 1989). These plants are naturally confined to the Malaysian-Australian Melanesian region but some species, and particularly *Casuarina equisetifolia*, have been exported extensively to other tropical areas worldwide, to be used as windbreaks, to stabilise sand dunes or as a source of fuel wood (Diem et al., 1988; Diem and Dommergues, 1990). This is due in part to the nitrogen-fixing symbiosis that most of the 96 extant species from this family have established with the actinomycete *Frankia* permitting the plants to develop on poor soils.

No study has been carried out on the evolution of *Frankia*-Casuarinaceae relationships. Most of the genetic diversity work on Casuarinaceae infective strains has been done on strains isolated from *Casuarina* spp. and *Allocasuarina* spp. growing in areas where they are not native (Nazaret et al., 1991; Rouvier et al., 1992). Little is known about *Gymnostoma* microsymbionts, the only reports in the literature dealing with three successful isolations of *Frankia* strains in pure culture (Racette and Torrey, 1989; Savouré and Lim, 1991). Therefore, the phylogenetic relationships of *Frankia* strains infective on *Casuarina*, *Allocasuarina* and *Gymnostoma* genera from native areas have not been studied.

With this in mind, we studied the diversity of Casuarinaceae microsymbionts in northeastern Australia and New Caledonia, areas in the natural geographic range of the host plants. Using sequencing and polymerase chain reaction/restriction fragment length polymorphism (PCR/RFLP) analysis, we sought to compare Casuarinaceae microsymbionts and to determine the level of diversity among these strains and their relationships with host plant species.

2. MATERIALS AND METHODS

2.1. Nodules and bacterial strains

Nodules and reference strains used are described in *table I.*

2.2. DNA extraction from nodules

After peeling off the superficial layers, nodule lobes were disinfected with 30 % w/v H_2O_2 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, 2 % w/v CTAB [Sigma, St Louis, MO, USA] and 3 % w/v PVPP [Sigma], pH 8). The mixture was incubated at 65 °C for 1 h and centrifuged at 3 000 *g* for 5 min (20 °C). The supernatant was chloroform-extracted and ethanol-precipitated. The DNA pellet was dissolved in 10 µL of TE buffer (pH 7.5).

2.3. PCR amplification of *nif*D-*nif*K intergene

For deoxyribonucleic acid (DNA) amplification of a region including the 3′ end of *nif*D, the intergenic spacer (IGS), and the beginning of *nif*K, primers FGPD807 (5′-CACTGCTACCGGTCGATGAA-3′) (Jamann et al., 1993) and FGPK333′ (5′-CCGGGCGAAGTGGCT-3′) (Nalin et al., 1995) were used. PCR amplification was performed in 0.5 mL Eppendorf tubes in a total volume of 50 µL containing: template DNA (approximately 0.1 µg), polymerase reaction buffer (10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.01 % [w/v] gelatine, 20 µM deoxynucleoside triphosphate [dNTP], 1 µM each of the primers and 2.5 units of *Taq*I DNA polymerase [Gibco BRL, Gaithersburg, MD, USA]). DNA amplification was done in a thermocycler (Perkin Elmer, Norwalk, CT, USA) using the following programme: 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 % w/v) in TBE buffer with 5 µL of PCR product. The gel was stained in an aqueous solution of 1 $mg \cdot L^{-1}$ ethidium bromide and photographed with HP5 film with a 302-nm ultraviolet source.

2.4. PCR amplification of 16S-23S intergene

Amplifications of a part of the 16S gene and the IGS were performed by using the standard conditions as described previously. Primers FGPS989e (5′-GGGGTCCTTAGGGGCT-3′) (Bosco et al., 1992) and FGPL1973′ (5′-ATCGGCTCGAGGTGCCAAGGGTC-3′) (Navarro et al., 1992) were used for *Gymnostoma* DNA amplifications. Primers FGPS989ac (5′-GGGGTCCGT-AAGGGTC-3′) (Bosco et al., 1992) and FGPL132′ (5′-CCGGGTTTCCCATT-CGG-3′) (Ponsonnet and Nesme, 1994) were used for *Casuarina/Allocasuarina* DNA amplifications.

Table I. Origin of DNA nodules and isolated strains used in this study and 16S-23S IGS-types.

Host plant	Geographical origin [a]	Strain or nodule [b]	IGS-types	Strain reference
Gymnostoma nodules				
G. chamaecyparis	Tontouta (NC)	TC23 - TC24	D	Navarro et al. (1997)
G. chamaecyparis	Tontouta (NC)	TC287	F	This study
G. chamaecyparis	Kouaoua (NC)	KC693	F	This study
G. chamaecyparis	Poum (NC)	PMC753	H	This study
G. deplancheanum	Plaine des Lacs (NC)	PLD170 - PLD185	D	This study
G. deplancheanum	Rivière Bleue (NC)	RBD570	B	This study
G. glaucescens	Mé Aïu (NC)	MG59	D	Navarro et al. (1997)
G. glaucescens	Dzumac (NC)	DG251	D	This study
G. glaucescens	Etoile Filante (NC)	EFG308	E	This study
G. intermedium	Kouaoua (NC)	KI55	D	This study
G. intermedium	Kouaoua (NC)	KI72	C	This study
G. intermedium	Dzumac (NC)	DI247	D	This study
G. intermedium	Dzumac (NC)	DI249	E	This study
G. leucodon	Plum (NC)	PLL300	B	This study
G. leucodon	Rivière des Pirogues (NC)	RPL161	D	Navarro et al. (1997)
G. leucodon	Rivière des Pirogues (NC)	RPL233	C	This study
G. leucodon	Rivière des Pirogues (NC)	RPL526	B	This study
G. nodiflorum	Canala (NC)	CN61	A	Navarro et al. (1997)
G. nodiflorum	Canala (NC)	CN721	B	This study
G. nodiflorum	Tiwaka (NC)	TWN402	A	This study
G. nodiflorum	Nehoue (NC)	NHN750	B	This study
G. nodiflorum	Kouaoua (NC)	KN301	H	This study
G. poissonianum	Kouaoua (NC)	KP54	G	Navarro et al. (1997)
G. poissonianum	Kouaoua (NC)	KP80 - KP81	B	This study
G. poissonianum	Kouaoua (NC)	KP82	D	This study
G. poissonianum	Dzumac (NC)	DP272	D	This study
G. webbianum	Rivière Bleue (NC)	RBW161	B	This study
G. webbianum	Rivière Bleue (NC)	RBW162 - RBW163	D	This study
G. webbianum	Canala (NC)	CW726	B	This study
G. webbianum	Amieu (NC)	AW673	D	This study
G. webbianum	Chagrin (NC)	CHW746	D	This study

Table I. Continued.

Host plant	Geographical origin [a]	Strain or nodule [b]	IGS-types	Strain reference
Casuarina nodules				
C. equisetifolia/incana	Alva Beach (A)	1Ceil	1	This study
C. equisetifolia	Horseshoe Bay (A)	19Ce1	1	This study
C. equisetifolia	Cow Bay (A)	CeCB	1	Rouvier et al. (1996)
C. equisetifolia	Garners Beach (A)	CeGB	1	Rouvier et al. (1996)
C. equisetifolia	Pallarenda (A)	CePall	1	Rouvier et al. (1996)
C. equisetifolia	Sauders Beach (A)	CeSB	1	Rouvier et al. (1996)
C. equisetifolia	Wangetti Beach (A)	CeWg2	1	Rouvier et al. (1996)
C. equisetifolia	Wongalinda Beach (A)	CeWB	1	Rouvier et al. (1996)
C. equisetifolia	Cape Hillsborough Beach (A)	14Ce2	2	This study
C. equisetifolia	Wangetti Beach (A)	CeWg1	2	Rouvier et al. (1996)
C. equisetifolia	Mount Low Beach (A)	CeMLB	2	Rouvier et al. (1996)
C. cunninghamania	Cattle Creek (A)	7Cc1	3	This study
C. cunninghamania	Bakerville Creek (A)	CcBK	3	Rouvier et al. (1996)
C. cunninghamania	Herbert River Crossing (A)	CcHRC	3	Rouvier et al. (1996)
C. cunninghamania	Jourama Falls (A)	CcJF	3	Rouvier et al. (1996)
C. cunninghamania	Old Chinaman Creek (A)	CcOCC	3	Rouvier et al. (1996)
C. cunninghamania	Tinaroo Creek (A)	CcTC	3	Rouvier et al. (1996)
C. cunninghamania	West Watsonville (A)	CcWW	3	Rouvier et al. (1996)
Allocasuarina nodules				
A. torulosa	Paluma Road (A)	37At1	4	This study
A. torulosa	Atherton Rifle Range (A)	AltARR1 - AltARR2	4	Rouvier et al. (1996)
A. torulosa	Bluewater (A)	AltBW	4	Rouvier et al. (1996)
A. torulosa	North of Lawyer Creek (A)	AltNLC	4	Rouvier et al. (1996)
A. littoralis	Eungella Road (A)	11All	5	This study
A. littoralis	Atherton (A)	AllAT	5	Rouvier et al. (1996)
A. littoralis	Kuranda (A)	AllKur1 - AllKur2	5	Rouvier et al. (1996)
A. littoralis	West of Herberton (A)	AllWH	5	Rouvier et al. (1996)

Table I. Continued.

Host plant	Geographical origin [a]	Strain or nodule [b]	IGS-types	Strain reference
Casuarina strains				
C. equisetifolia	Dakar (Senegal)	D11 (atypical) (U)	ND	Gauthier et al. (1981)
C. cunninghamania	Florida (USA)	CcI3 (9)	1	Zhang et al. (1984)
C. equisetifolia	Senegal	CeD (9)	1	Diem and Dommergues (1983)
Elaeagnaceae strains				
Elaeagnus umbellata	Illinois (USA)	EUN1f (6)	ND	Lalonde et al. (1981)
Hippophaë rhamnoides	Alps (France)	HRN18a (7)	ND	Moiroud and Faure-Reynaud (1983)
E. angustifolia	Ohio (USA)	EaN1-pec (5)	ND	Lalonde et al. (1981)
E. angustifolia	Ecully (France)	Ea1-12 (4)	ND	Fernandez et al. (1989)
Sherpherdia canadensis	Quebec (Canada)	SCN10a (U)	ND	Mort et al. (1983)
Alnus strain				
Alnus rubra	Oregon (USA)	ArI3 (1)	ND	Berry and Torrey (1979)

[a] NC: New Caledonia, A: Australia; [b] genomic species numbers are in parentheses; U: undetermined species.

2.5. Sequencing of IGS amplicons

Before sequencing, the amplification reaction mix was purified by using Centricon-30 concentrators (Amicon-Grace Company, Epernon, France). The amplicons were sequenced using the Deaza G/A sequencing kit (Pharmacia Biotech SA, St-Quentin-Yvelines, France) and the direct DNA sequencing method described by Winship (1989). The fragments were sequenced in both directions. The sequences were determined for both strands.

2.6. Data analysis

The sequences were aligned with previously published sequences (Nalin et al., 1995; Navarro et al., 1997) using the multiple-alignment CLUSTA1V algorithm (Higgins and Sharp, 1988), with manual refinements in the noncoding regions. Distances were calculated according to Kimura's two-parameter model (Kimura, 1980) and phylogenetic analyses were made using neighbour-joining (N-J) (Saitou and Nei, 1987) and parsimony methods (Swofford, 1993). A bootstrap confidence analysis was performed with 1 000 replicates to determine the reliability of the distance tree topologies obtained (Felsenstein, 1985). The resulting tree was drawn by using the N-J plot sofware (Perrière and Gouy, 1996).

2.7. Amplicons restriction analysis

Restriction endonuclease digestions were done with 15 μL of PCR reaction mixture for each reaction. The endonucleases, *NcI*1, *Msp*I, *Hae*III (all from Boehringer Mannheim, Meylan, France) and *Scr*F1 (Ozyme, Montigny Le Bretonneux, France) were used as specified by the manufacturers. Electrophoresis was carried out in a horizontal slab gel on a 4 % (w/v) Nusieve (FMC, Rockland, ME, USA) agarose gel containing 0.5 μg mL^{-1} ethidium bromide, using TBE electrophoresis buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA). Gels were run at 4 V cm^{-1} for 3 h and photographed as described previously.

3. RESULTS

3.1. Amplification

The method used for extracting DNA from Casuarinaceae nodule lobes provided endophyte DNA that was pure enough to be efficiently amplified with the sets of primers tested (data not shown). A DNA fragment corresponding to the 16S-23S IGS was obtained for all the 55 templates tested, including DNA from isolates and from nonisolated strains (data not shown).

3.2. Sequencing and comparison of the *nif*D-*nif*K intergenic spacer

By using the sequencing strategy shown in *figure 1*, a sequence that covers the 3′ end of *nif*D, the IGS and the beginning of *nif*K was obtained for all 11 DNAs studied and aligned with the published sequences (Nalin et al., 1995; Navarro et al., 1997) (*figure 2*).

NIF D IGS NIF K

FGPD 1266 FGPK 64' FGPK 327'

FGPK 1

Figure 1. Sequencing strategy for the *nif*D-*nif*K intergenic spacers (IGS). The arrows show the direction of the sequencing reactions.

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          1                                                                                                   *       100
ArI3      GATCTTCGCC CGGGACATGG ACATCGCCAT CAACAGCCCG GCCTGGGACC TCCTCGAGGC CCCCTGGTCG AAGGCCGGCG AGGTCGCCTG ATCCACCGCC
RPL161    GGTCTTCGCC CGCGACATGG ACATGGCCAT CAACAGCCCG ACCTGGGACC TGATGGAGAC CCCGTGGTCG AAGTCCGGAG AGGTCTTCTG ACCAGCTAGT
MG59      GGTCTTCGCC CGCGACATGG ACATGGCCAT CAACAGCCCG ACCTGGGACC TGATGGAGAC CCCGTGGTCG AAGTCCGGAG AGGTCTTCTG ACCAGCTAGT
TC24      GGTCTTCGCC CGCGACATGG ACATGGCCAT CAACAGCCCG ACCTGGGACC TGATGGAGAC CCCGTGGTCG AAGTCCGGAG AGGTCTTCTG ACCAGCTAGT
CN61      GGTCTTCGCC CGCGACATGG ACATGGCCAT CAACAGCCCG ACCTGGGACC TGATGGAGAC CCCGTGGTCG AAGTCCGGAG AGGTCTTCTG AGCAG-TAGT
KP54      GGTCTTCGCC CGCGACATGG ACATCGCCAT CAACAGCCCG ACCTGGGACC TGATGGAGAC CCCGTGGTCG AAGTCCGGAG AGGTCTTCTG ACCAGTTAGT
EUN1f     GATCTTCGCC CGCGACATGG ACATCGCCAT CAACAGCCCG ACCTGGGACC TGATGGAGAC CCCGTGGTCG AAGTCCGGAG AGGTCTCCTG ACCAGCTAGT
SCN10A    GATCTTCGCC CGCGACATGG ACATCGCGAT CAACAGCCCG ACCTGGGACC TCATGGAGAC CCCGTGGTCG AAGTCCGGAG AGGTCTTCTG ACCCGTTAG-
HRN18a    GCTCTTCGCC CGCGACATGG ACATCGCGAT CAACGGCCCG ACCTGGGACC TGATGGAGAC CCCCTGGTCG AAGGGTGGAG AGGTCTTCTG ACCCG-----
Eal-12    GGTCTTCGCC CGCGACATGG ACATCGCGAT CAACAGCCCG ACCTGGGACC TGATGGAGAC CCCCTGGTCG AAGGCCGGAG AGGTGTGCTG ACCGGATC-T
EaN1-pec  GGTCTTCGCC CGCGACATGG ACATCGCGAT CAACAGCCCG ACCTGGGACC TCATGGAGAC CCCCTGGTCG AAGGCCGGAG AGGTGTGCTG ACCGGATT-T
D11       GGTCTTCGCC CGTGACATGG ACATCGCGAT CAACAGCCCG GCCTGGGACC TGATGGAGAA CCCCTGGTCG AAGGCCTGGA GAAAGGCCTG ACCGGG----
Cci3      GGTCTTCGCC CGTGACATGG ACATCGCGAT CAACAGCCCG ACCTGGGACC TCATGGAGAC CCCCTGGTCG AAGGCCGGAG AGGTGTTCTG ACCGGCCGGT
Ced       GGTCTTCGCC CGTGACATGG ACATCGCGAT CAACAGCCCG ACCTGGGACC TCATGGAGAC CCCCTGGTCG AAGGCCGGAG AGGTGTTCTG ACCGGCCGGT
1Ceil     GGTCTTCGCC CGTGACATGG ACATCGCGAT CAACAGCCCG ACCTGGGACC TCATGGAGAC CCCCTGGTCG AAGGCCGGAG AGGTGTTCTG ACCGGCCGGT
19Cel     GGTCTTCGCC CGTGACATGG ACATCGCGAT CAACAGCCCG ACCTGGGACC TCATGGAGAC CCCCTGGTCG AAGGCCGGAG AGGTGTTCTG ACCGGCCGGT
14Ce2     GGTCTTCGCC CGTGACATGG ACATTGCGAT CAACAGCCCG ACCTGGGACC TCATGGAGAC CCCCTGGTCG AAGGCCGGAG AGGTGTTCTG ACCGGCCGGT
7Cc1      GGTCTTCGCC CGTGACATGG ACATTGCGAT CAACAGCCCG ACCTGGGACC TCCTGGAGAC CCCCTGGTCG AAGGCCGGAG AGGTGTTCTG ACCGGCCGGT
37At1     GGTCTTCGCC CGCGACATGG ACATCGCGAT CAACAGCCCG ACCTGGGACC TCCTGGAGAC CCCCTGGTCG AAGGCCGGAG AGGTGTTCTG ACCGGCCGGT
11A11     GGTCTTCGCC CGTGACATGG ACATCGCGAT CAACAGCCCG ACCTGGGACC TCCTGGAGAC CCCCTGGTCG AAGGCCGGAG AGGTGTTCTG ACCGGCCGGT

          101                                                                                                         200
ArI3      CTCCGGGCAC CCGGGCGCGA GGCGCCCAGC AGTAGACGGC CACTTGCCGT -AGACGGCCA CTCGCAGTA- GACGGCCACT CGCCGTAGAC GGCCACTCGC
RPL161    -CCGGCACCG GCC--AACCA CCGGCGACCA CCGGTGATCA CCGGTGGCTG GCCGGCGGCC ATCAGTGGGG TCTTTTCCTT CGCACGGTGT ACGGCCACGA
MG59      --CGGCACGC GGC--AACCA CCGGCGACCA CCGGTGATCA CCGGTGGCTG -CCGGCGGCC ATCAGTGGG- TCTTTTCCTT CGCACGGTGT ACGGCCACGA
TC24      -CCGGCACCG CCGGCAACCA CCGGCGACCA CCGGTGATCA CCGGTGGCTG -CCGGCGGCC ATCAGTGGG- TCTTTTCCTT CGCACGGTGT ACGGCCACGA
CN61      -CCGGCACCG CGC--AACCA CCGGCGACCA CCGGTGATCA CCGGTGGCTG -CCGGCGGCC ATCAGGGGG- CCTTCTCCAT CGCACGGGGC ACGGCCAGGA
KP54      --CGGCACCG GCC--AACCA CCGGCGACCA CCGGCGANCA CCGGTGGCCG ACGGCCGGCC ATCAGT---- ---------- ---------- -------ACG
EUN1f     TCCGGCTCCG CGCGCAACCA CCGGCGACCA CCGGTGATCA CCGGTGGCTG -CCGGCGGCC ATCAGTGAG- CCTTCTCCAT CTCGCGGCGC ACGGCCAGGA
SCN10A    CCCGGCACCG CCGGCGACCA TCGGCGACCA CCGGCGATCA CCGGTGGCC- GCCGGCGGCC ATC------- ---------- ---------- ----------
HRN18a    --AAGACTCG -GCG--TCCG C-GGTGACCN CCCGTGGT-- ---------- ---------- ---------- ---------- ---------- ----------
Eal-12    TCCGGTCTCC G------CCT --GGCGAT-- ---------- ---------- ---------- ---------- ---------- ---------- ----------
EaN1-pec  TCCGGTCTCC G------CC- --GGCGAT-- ---------- ---------- ---------- ---------- ---------- ---------- ----------
D11       -------CCG --CCTCG-CA CGCCGGG--- ----TGGT-- CCGGT----- ---------- ---------- ---------- ---ACCGG-- CCGGCGAGCG
Cci3      -CCGGCCCCG CCGCGGTAGC CGCAGT---- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Ced       -CCGGCCCCG CCGCGGTAGC CGCAGT---- ---------- ---------- ---------- ---------- ---------- ---------- ----------
1Ceil     -CCGGCCCCG CCGCGGTAGC CGCAGT---- ---------- ---------- ---------- ---------- ---------- ---------- ----------
19Cel     -CCGGCCCCG CCGCGGTAGC CGCAGT---- ---------- ---------- ---------- ---------- ---------- ---------- ----------
14Ce2     -CCGGCCCCG CCGCGGCAGC CGCAGT---- ---------- ---------- ---------- ---------- ---------- ---------- ----------
7Cc1      -CCGGCCCCG CCGCGGTAGC CGCAGT---- ---------- ---------- ---------- ---------- ---------- ---------- ----------
37At1     -CCGGCCCCG CCGCGGTAGC CGCAGT---- ---------- ---------- ---------- ---------- ---------- ---------- ----------
11A11     -CCGGCCCCG CCGCGGTAGC CGCAGT---- ---------- ---------- ---------- ---------- ---------- ---------- ----------

          201                                                                                                         300
ArI3      AGTAGACGGC CACTTGCAGT AGCTGACCAG TAGTTCATCC AGGCCTGGCC GAACCAGCCG CCAGCGACCC CGTGACCGGA GAAACGGTCG CTGGCGGCTG
RPL161    ATGCCGTGAG CCCGAGTCGA CGACCGAAAA GG-------- ---------- ---------- ---------- ---------- ---------- ----------
MG59      ATGCCGTGAG CCCGAGTCGA CGACCGAAAA GG-------- ---------- ---------- ---------- ---------- ---------- ----------
TC24      ATGCCGTGAG CCCGAGTCGA CGACCGAAA- GG-------- ---------- ---------- ---------- ---------- ---------- ----------
CN61      A---CGTGAG CCCGAACTGG CGATGGAAAA GG-------- ---------- ---------- ---------- ---------- ---------- ----------
KP54      CA-GAAAGGC TGATGTTACG CGGTGCGTCG GT-------- ---------- ---------- ---------- ---------- ---------- ----------
EUN1f     ATGCCGTCAG CCCGAGTCGG CGATGGAAAA GG-------- ---------- ---------- ---------- ---------- ---------- ----------
SCN10A    ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
HRN18a    ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Eal-12    ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
EaN1-pec  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
D11       --CCGGT--- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Cci3      ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Ced       ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
1Ceil     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
19Cel     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
14Ce2     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
7Cc1      ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
37At1     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
11A11     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

          301                                                   *                                                     400
ArI3      CCGCGGCAGG CCTCAAGCCC CACCTGGGAC AAGCCCACAG GAGAGGAGGG ACGTCGGTGA CGACGACCCC CGAGCACACC AGCGCGGTCC CGCTGCGGGT
RPL161    ---------- ---------- ------CTCA AGTCACGGCA GAAAGGAGGA GGTCCGGTGA CGACGACTCC CGAGACGAAC AGTCACATCC CGCTCAAGGT
MG59      ---------- ---------- ------CTCA AGTCACGGCA GAAAGGAGGA GGTCCGGTGA CGACGACTCC CGAGACGAAC AGTCACATCC CGCTCAAGGT
TC24      ---------- ---------- ------CTCA AGTCACGGCA GAAAGGAGGA GGTCCGGTGA CGACGACTCC CGAGACGAAC AGTCACATCC CGCTCAAGGT
CN61      ---------- ---------- ------CTCA AGTCACGGCA GAAAGGAGGA GGTCCGGTGA CGACGACTCC CGAGACGAAC AGTCACATCC CGCTCAAGGT
KP54      ---------- ---------- ------ATCA -GTCACTGCA GAAAGGAGGA GGTCCGGTGA CGACGACTCC CGAGACGAAC AGTCACATCC CGCTGAAGGT
EUN1f     ---------- ---------- ------CTCA AGTCACGGCA GAAAGGAGGA GGTCCGGTGA CGACGACTCC CGAGACGAAC AGTCACATCC CGCTTAAGGT
SCN10A    ---------- ---------- ---------- AGTCACTGCA GAAAGGAGGA TGTCCGGTGA CGACGACTCC CGAGACGAAC AGTCACATCC CGCTGAAGGT
HRN18a    ---------- ---------- ------CATC AGTCACTGTA GAAAGGAGGA TGTCCGGTGA CGACAACTCC CGAGACAGGT AGTCACATAC CGCTGAAGGT
Eal-12    ---------- -----CGCCG GCGGCTCATC AGTCACGGTA GAAAGGAGGA TGTCCGGTGA CGACGACTCC CGAGACAGGC AGTTCCATTC CGTTGCGGGT
EaN1-pec  ---------- -----CGCCG GCGGC-CATC AGTCACGGTA GAAAGGAGGA TGTCCGGTGA CGACGACTCC GTAGACAGGC AGTTCCATTC CGTTGCGGGT
D11       ---------- ---------- --GGCCATCT AGTCACGGTA GAAAGGAGGA TGTC-GGTGA CGACGACTCC CGAGACAGGC AGTCACATCC CGCTCAAGGT
Cci3      ---------- ---------- ------CATC AGCCACGGTA GAAGGGAGGA TGTCCGGTGA CGACGACTCC GGAAACAAAC AGTTCCGTCC CGCTGCGGGT
Ced       ---------- ---------- ------CATC AGCCACGGTA GAAGGGAGGA TGTCCGGTGA CGACGACTCC GGAAACAAAC AGTTCCGTCC CGCTGCGGGT
1Ceil     ---------- ---------- ------CATC AGCCACGGTA GAAGGGAGGA TGTCCGGTGA CGACGACTCC GGAAACAAAC AGTTCCGTCC CGCTGCGGGT
19Cel     ---------- ---------- ------CATC AGCCACGGTA GAAGGGAGGA TGTCCGGTGA CGACGACTCC GGAAACAAAC AGTTCCGTCC CGCTGCGGGT
14Ce2     ---------- ---------- ------CATC AGCCACGGTA GAAGGGAGGA TGTCCGGTGA CGACGACTCC GGAAACAAAC AGTTCCGTCC CGCTGCGGGT
7Cc1      ---------- ---------- ------CATC AGCCACGGTA GAAGGGAGGA TGTCCGGTGA CGACGACTCC GGAAACAAAC AGTTCCGTCC CGCTGCGGAT
37At1     ---------- ---------- ------CATC AGCCACGGTA GAAGGGAGGA TGTCCGATGA CGACGACTCC GGAAACAAAC AGTTCCGTCC CGCTGCGGGT
11A11     ---------- ---------- ------CATC AGCCACGGTA GAAGGGAGGA TGTCCGATGA CGACGACTCC GGAAACAAAC AGTTCCGTCC CGCTGCGGGT
```

Figure 2. Alignment of the *nif*D-*nif*K intergenic spacer (IGS). The stop codon of *nif*D and the start codon of *nif*K are marked by an asterisk.

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          401                                                                                                      500
ArI3      CCTTGACCAC AACGACATCT TCCGCGACGA GGTCTACCAG AAGCAGTTCG AGGGCAAGCG CGAGTTCGAG AACGCGGCCC CGAAGGAAGA GGTCCAGCGG
RPL161    CCTCAACCAC ACCGAGCTGT TCAAGGACGA GGTCTACACC AAGCAGTTCG AGACGAAGCG CGAGTTCGAG AACGGTGCGA TCGACGCCGA GGTCAACCGG
MG59      CCTCNACCAC ACCGAGCTGT TCAAGGACGA GGTCTACACC AAGCAGTTCG AGACCAAGCG CGAGTTCGAG AACGGTGCGG ATCACGCCGA GGTCAACCGG
TC24      CCTCGACCAC ACCGAGCTGT TCAAGGACGA GGTCTACACC AAGCAGTTCG AGACCAAGCG CGAGTTCGAG AACGGTGCGG ATCACGCCGA GGTCAACCGG
CN61      CCTCGACCAC ACCGAGCTGT TCAAGGACGA GGTCTACACC AAGCAGTTCG AGACCAAGCG CGAGTTCGAG AACGGTGCGG ATGACGCCGA GGTCAACCGG
KP54      CCTCGACCAC ACCGAACTGT TCAAGGACGA GGTCTACACC AAGCAGTTCG AGACCAAGCG CGAGTTCGAG AACGGTGCGG ATGACGCCGA GGTCAACCGG
EUN1f     CCTCGACCAC ACCGAGCTGT TCAAGGACGA GGTCTACACC AAGCAGTTCG AGACCAAGCG CGAGTTCGAG AACGGCGCCG ATGACGCCGA GGTCAACCGG
SCN10A    CCTCGACCAC AACGAGCTGT TCAAGGACGA GGTCTACACC AAGCAGTTCG AGACCAAGCG CGAGTTCGAG AACGGTGCGG ACGACGCCGA GGTCAACCGG
HRN18a    CCTCGACCAC AGCGAGCTGT TCAAGGACGA GGTCTACAGG AAGCAGTTCG AGACCAAGCG CGAGTTCGAG AACGGCGCCG ATGACGCCGA GGTCAACCGG
Eal-12    CCTCGACCAC AGCGAGCTGT TCAAGGACGA GGTCTACCAG AAGCAGTTCG AGGGCAAGGG CGAGTTCGAG AACGGCACGG ACGCCGCCGA GGTCAACCGG
EaN1-pec  CCTCGACCAC AGCGAGCTGT TCAAGGACGA GGTCTACCAG AAGCAGTTCG AGGGCAAGGC CGAGTTCGAG AACGGCAGTG AATCCGCCGA GGTTTCCCGA
D11       GCTCGACCAC AACGAGCTGT TCAAGGGCGA GGCCTACCAC AAGCAGTTCG AGGGAAAGAC CGAGTTCGAG AACGGCAGTG ACTCCGCCGA GGTTGCCCGC
Cci3      CCTCGACCAC AGCGAGCTGT TCAAGGACGA GGTCTACCAG AAGCAGTTCG AGGGCAAGAG CGAGTTCGAG AACGGCAGCG ACGCCGCCGA GGTGCAGCGG
Ced       CCTCGACCAC AGCGAGCTGT TCAAGGACGA GGTCTACCAG AAGCAGTTCG AGGGCAAGCG CGAGTTCGAG AACGGCAGCG ACGCCGCCGA GGTCGCCCGG
1Ceil     CCTCGACCAC AGCGAGCTGT TCAAGGACGA GGTCTACCAG AAGCAGTTCG AGGGCAAGCG CGAGTTCGAG AACGGCAGCG ACGCCGCCGA GGTCGCCCGG
19Cel     CCTCGACCAC AGCGAGCTGT TCAAGGACGA GGTCTACCAG AAGCAGTTCG AGGGCAAGCG CGAGTTCGAG AACGGCAGCG ACGCCGCCGA GGTCGCCCGG
14Ce2     CCTCGACCAC AGCGAGCTGT TCAAGGACGA GGTCTACCAG GAGCAGTTCG AGGGCAAGCG CGAGTTCGAG AACGGCAGCG ACGCCGCCGA GGTCGCCCGG
7Cc1      CCTCGACCAC AGCGAGCTGT TCAAGGACGA GGTCTACCAG GAGCAGTTCG AGGGCAAGCG CGAGTTCGAG AACGGCAGCG ACGCCGCCGA GGTAGCCCGG
37At1     CCTCGACCAC AGCGAGCTGT TCAAGGACGA GGTCTACCAG AAGCAGTTCG AGGGCAAGCG CGAGTTCGAG AACGGCAGCG ACGCCGCCGA GGTCGCCCGG
11All     CCTCGACCAC AGCGAGCTGT TCAAGGACGA GGTCTACCAG AAGCAGTTCG AGGGCAAGCG CGAGTTCGAG AACGGCAGCG ACGCCGCCGA GGTCGCCCGG

          501                                    540  544
ArI3      GTCCTCGACT GGACCCGCGG GTGGGAGTAC CGGGAGAAGA ACTT
MG59      GTCCTGGAGT GGACCCGTAC CTGGGAGTAC CGCGAGAAGA ACTT
RPL161    GTCCTGGAGT GGACCCGTAC CTGGGAGTAC CGCGAGAAGA ACTT
TC24      GTCCTGGAGT GGACCCGTAC CTGGGAGTAC CGCGAGAAGA ACTT
CN61      GTCCTGGAGT GGACCCGTAC CTCGGAGTAC CGCGAGAAGA ACTT
KP54      GTTCTGGAGT GGACCCGTAC CTGGGAGTAC CGCGAGAAGA ACTT
EUN1f     GTCCTGGAGT GGACCCGCAC CTGGGAGTAC CGCGAGAAGA ACTT
SCN10A    GTCCTGGAGT GGACCCGCAC CTGGGAGTAC CGGGAGAAGA ACTT
HRN18a    GTTCTGGAGT GGACCCGCGG CTGGGAGTAC CGCGAGAAGA ACTT
Eal-12    GTCCTCGAGT GGACCCGCGG CTGGGAGTAC CGGGAGAAGA ACTT
EaN1-pec  GTCCTCGAGT GGACCCGCGG CTGGGAGTAC CGGGAGAAGA ACTT
D11       GTTCTCGAAT GGACCCGCGG CTGGGAGTAC CGGGAGAAGA ACTT
Cci3      GTCCTGGAGT GGACCCGCGG CTGGGAGTAC CGGGAGAAGA ACTT
Ced       GTCCTGGAGT GGACCCGCGG CTGGGAGTAC CGGGAGAAGA ACTT
1Ceil     GTCCTGGAGT GGACCCGCGG CTGGGAGTAC CGGGAGAAGA ACTT
19Cel     GTCCTGGAGT GGACCCGCGG CTGGGAGTAC CGGGAGAAGA ACTT
14Ce2     GTCCTGGAGT GGACCCGCGG CTGGGAGTAC CGGGAGAAGA ACTT
7Cc1      GTCCTGGAGT GGACCCGCGG CTGGGAGTAC CGGGAGAAGA ACTT
37At1     GTCCTGGAGT GGACCCGCGG CTGGGAGTAC CGGGAGAAGA ACTT
11All     GTCCTGGAGT GGACCCGCGG CTGGGAGTAC CGGGAGAAGA ACTT
```

Figure 2. (Continued).

In the group of *Casuarina/Allocasuarina*-infective strains, up to six differences were observed (corresponding to 0.018 substitutions/site). In the group of Elaeagnaceae-infective strains, between 6 and 71 differences were observed (corresponding to 0.014–0.232 substitutions/site). The *Alnus*-infective strains exhibited between 84 and 175 differences with *Casuarina*-infective strain CcI3 and *Gymnostoma* microsymbiont MG59, respectively.

Two clusters were identified by using the distance matrix (*table II*) and the resulting phylogenetic tree (*figure 3*).

Cluster 1 is a very tight group that contains *Casuarina* and *Allocasuarina* microsymbionts. Two *C. equisetifolia* microsymbionts, 1Ceil and 19Cel, have identical sequence with that of the reference *Casuarina* infective strains (*table II*). The remaining *C. equisetifolia* microsymbiont, 14Ce2, is very closely related to the *C. cunninghamiana* microsymbiont. This grouping was also detected by parsimony analysis. *A. torulosa* and *A. littoralis* microsymbionts form a group not confirmed by parsimony analysis. Nevertheless, 37At1 and 11Al1 sequences had a very low level of divergence (*table II*).

The Elaeagnaceae-infective strains form a broad group designated cluster 2. This coherent cluster (100 % of the bootstrap replicates) was also detected by parsimony analysis. The level of sequence divergences was higher in this cluster than in cluster 1. *Gymnostoma* microsymbionts belonged to cluster 2. They form a coherent group (100 % of the bootstrap replicates, parsimony analysis) with EUN1f and SCN10a.

3.3. PCR/RFLP on 16S-23S IGS

Using two restriction enzymes, IGS-types were determined (*table III*; *figure 4*). All reference *Casuarina* and *Allocasuarina* infective strains have

Table II. Distance matrix. Calculated distances (expressed as substitutions/100 sites, below diagonal) as described by Kimura (1980) and observed number of differences between pairs of sequences (above diagonal).

	ArI3	RPL16	MG59	TC24	CN61	KP54	EUN1f	SCN10	HRN18	Ea1	EaN1	D11	CcI3	CeD	1Cei	19Ce1	14Ce2	7Cc1	37At1	11Al1
ArI3	0	178	175	174	171	147	174	111	105	99	99	119	84	84	84	84	86	87	84	84
RPL161d	573	0	8	7	22	48	25	25	50	58	59	71	60	60	60	60	61	64	61	62
MG59	563	18	0	6	19	46	24	24	49	54	57	71	61	61	61	61	62	65	62	63
TC24	552	16	14	0	17	43	22	18	48	55	58	71	57	57	57	57	58	61	58	59
CN61	548	52	45	40	0	42	18	21	48	56	59	66	59	59	59	59	60	63	60	61
KP54	487	127	122	113	111	0	46	17	46	55	58	63	58	58	58	58	60	63	59	60
EUN1f	551	59	56	51	42	121	0	21	44	54	56	68	58	58	58	58	60	63	59	60
SCN10A	379	70	68	50	59	48	58	0	42	49	50	58	48	48	48	48	50	53	49	50
HRN18a	392	162	159	155	155	149	141	135	0	37	40	51	46	46	46	46	49	51	47	48
Ea1-12	358	196	182	185	189	186	180	164	121	0	9	49	35	35	35	35	37	39	36	37
EaN1-pec	360	201	194	197	201	198	189	169	133	26	0	49	32	32	32	32	34	36	33	34
D11	431	231	232	230	215	212	219	197	173	168	169	0	50	50	50	50	52	54	53	52
CcI3	297	201	206	188	197	194	192	158	153	113	103	170	0	0	0	0	3	5	3	2
CeD	297	201	206	188	197	194	192	158	153	113	103	170	0	0	0	0	3	5	3	2
1Cei1	297	201	206	188	197	194	192	158	153	113	103	170	0	0	0	0	3	5	3	2
19Ce1	297	201	206	188	197	194	192	158	153	113	103	170	0	0	0	0	3	5	3	2
14Ce2	305	205	210	192	201	202	199	165	164	120	110	178	9	9	9	9	0	4	6	5
7Cc1	310	216	222	203	213	214	211	176	172	127	117	186	15	15	15	15	12	0	6	5
37At1	296	204	210	192	201	198	196	161	156	117	106	182	9	9	9	9	18	18	0	1
11Al1	296	208	214	196	205	202	199	165	160	120	110	178	6	6	6	6	15	15	3	0

Figure 3. Phylogenetic neighbour-joining tree based on *nif*D-*nif*K intergenic spacer sequences. The bars represent 0.028 substitutions/site. The numbers are the percentages of bootstrap replicates in which the cluster was found. An asterisk indicates that the group was found by parsimony analysis.

Table III. IGS-type determination.

IGS-types	Restriction patterns of the 16S-23S IGS			
	*Msp*I	*Hae*III	*Ncl*1	*Scr*F1
A	M1	H1		
B	M1	H2		
C	M2	H1		
D	M2	H3		
E	M3	H3		
F	M3	H4		
G	M4	H1		
H	M5	H5		
1			N1	SF1
2			N2	SF2
3			N3	SF3
4			N1	SF4
5			N4	SF4

similar patterns and were grouped together (IGS-type 1), whereas the *Casuarina/Allocasuarina* microsymbionts from Australia are distributed in five IGS-types (*table I*). *C. equisetifolia* microsymbionts belong to two IGS-types: 1 and 2, the majority being in group 1. IGS-type 3 contains *C. cunninghamiana* microsymbionts. *Allocasuarina* spp. microsymbionts belong to the remaining IGS-types. IGS-type 4 includes *A. torulosa* microsymbionts, whereas IGS-type

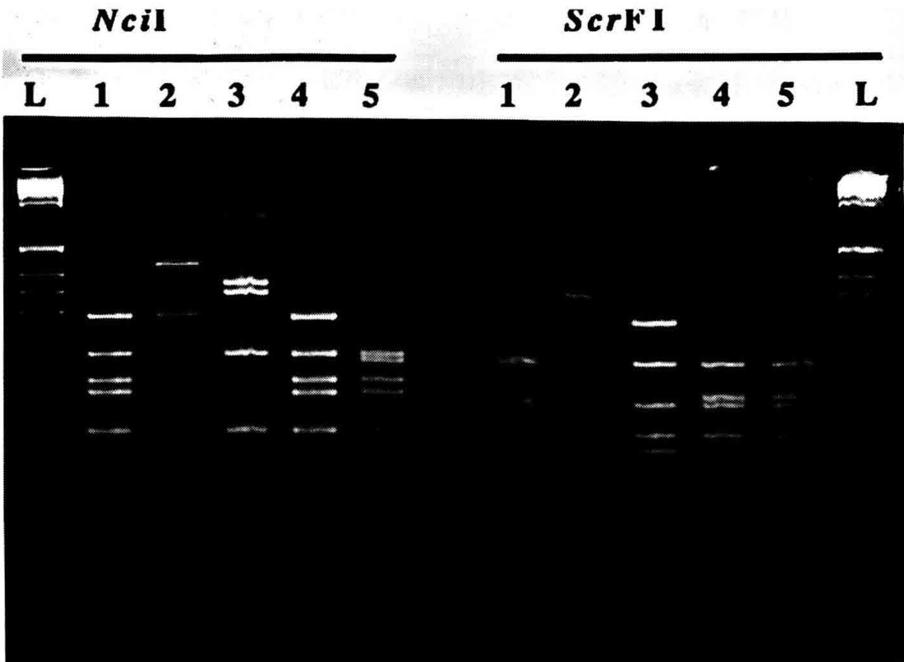

Figure 4. PCR/RFLP patterns of the 16S-23S intergenic spacer (IGS) after digestion with *NciI*, *Scr*F1. Lanes 1 to 5, IGS-types 1 to 5; lane L, 1-kb ladder.

5 includes *A. littoralis* microsymbionts. The genotypic grouping of the *Casuarina* and *Allocasuarina* infective strains was consistently associated with the host plant species (*table IVa*).

The 35 *Gymnostoma* microsymbionts were distributed into eight IGS-types (*table I*). For each *Gymnostoma* species, microsymbionts belong to two or three IGS-types. Conversely, microsymbionts belonging to six IGS-types were associated with several *Gymnostoma* species. IGS-type D is the most promiscuous group, being found with seven of the eight *Gymnostoma* species tested.

The grouping of the strains according to molecular criteria was not related to the grouping based on the host plant species, since each species was found to have established naturally a symbiosis with microsymbionts classified in several IGS- types (*table IVb*).

4. DISCUSSION

Coevolution has been found in several host–pathogen systems (Futuyama, 1986) and in highly specific obligate mutualism such as endosymbiosis (Futuyama, 1986; Moran et al., 1993). Coevolution can be either loose or strict, but this must be confirmed by evidence for the congruence of the two partners' phylogenetic trees.

Comparative studies of the phylogenies of host plant and symbionts constitute a promising approach for the elucidation of the evolution of actinorhizal

Table IV. Distribution of *Frankia* 16S-23S IGS-types as a function of Casuarinaceae species.
IVa. *Casuarina* and *Allocasuarina* species.

	IGS-types				
Host plant	1	2	3	4	5
C. equisetifolia	+	+			
C. cunninghamania			+		
A. torulosa				+	
A. littoralis					+

IVb. *Gymnostoma* species.

	IGS-types							
Host plant	A	B	C	D	E	F	G	H
G. chamaecyparis				+		+		+
G. deplancheanum		+		+				
G. glaucescens				+	+			
G. intermedium			+	+	+			
G. leucodon		+	+	+				
G. nodiflorum	+	+						+
G. poissonianum		+		+			+	
G. webbianum		+	+	+				

symbiosis. Of the 21 dicotyledonous genera described as actinorhizal (Benson and Silvester, 1993), strains capable of fulfilling Koch's postulates or present as microsymbionts in 11 of these have had their 16S determined and compared (Nick et al., 1992). This analysis has shown that the genus *Frankia* is coherent and that isolated strains infective on *Casuarina* are phyletically close to those infective on *Alnus*. Study of the plant phylogeny, on the other hand, has shown that *Casuarina* and the other Casuarinaceae genera *Allocasuarina* and *Gymnostoma* formed a phyletically coherent family in the Hammamelidae (Maggia and Bousquet, 1994). It was thus expected that the microsymbionts present in the nodules of these three genera would be phyletically close.

The present work on the *nif*D-*nif*K intergenic spacer has shown that, on the contrary, the nonisolated strains present in *Gymnostoma* nodules and *Casuarina/Allocasuarina* nodules belonged to different clusters. *Casuarina/Allocasuarina* microsymbionts form a tight group with a very low level of sequence divergence (*figure 3*; *table II*). The phylogenetic tree outlined in this cluster is similar to the trees obtained by PCR/RFLP analysis (*figure 5*). *Gymnostoma* microsymbionts were in the cluster of Elaeagnaceae-infective strains. Cross-inoculation studies have confirmed that *Gymnostoma*-infective strains are *Elaeagnus*-infective and not *Casuarina*-infective (Navarro et al., 1997).

Differences between *Casuarina/Allocasuarina* and *Gymnostoma* microsymbionts were confirmed by PCR/RFLP analysis of the 16S-23S intergenic spacer (*table IV*). Genetic diversity among *Casuarina* and *Allocasuarina* microsym-

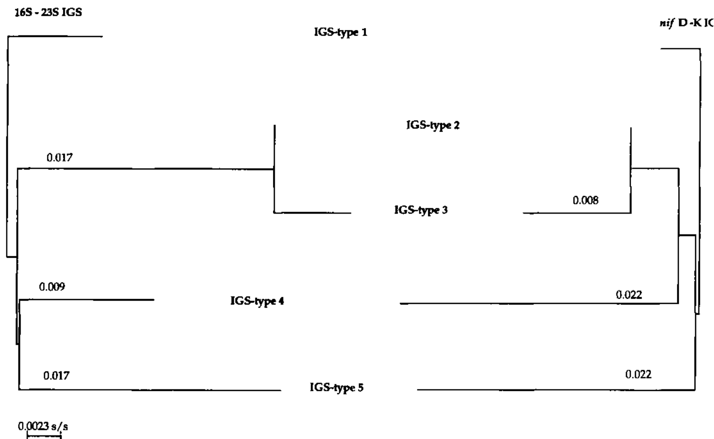

Figure 5. Dendrogram of genotypic relationship of intergenic spacer (IGS) sequences derived by PCR/RFLP analysis according to Rouvier (1995).

bionts seems to be host-species dependent. Cross-inoculation studies support this finding (Reddell and Bowen, 1985; Sellstedt, 1995). This differentiates this group from *Gymnostoma* microsymbionts for which no relation between genetic diversity and host species was observed. Identical results were obtained by cross-inoculation studies (Gauthier, personal communication).

These results mean that in the Casuarinaceae line of descent the host plants did not evolve gradually as proposed by Maggia and Bousquet (1994). These authors suggested that evolution of the symbiosis had been from a promiscuous ancestor identified as comparable to present-day *Gymnostoma* to the very restrictive descendant (*Allocasuarina*) with *Casuarina* in between. Instead, the most coherent scenario would be that an early split occurred in the evolution of Casuarinaceae, resulting in two distinct lines of descent. In each of them evolution of the symbiosis has occurred in two different ways. Evolution of the *Casuarina/Allocasuarina* symbiosis has proceeded towards a greater specificity and specialisation, and is presumably an example of coevolution. These host plants are in symbiosis with hard-to-isolate and slow-growing *Frankia* strains, suggesting that this symbiosis is becoming obligate. More saprophytic *Frankia* strains (Nalin et al., 1997), faster growing and easier to isolate, have established a nonspecific symbiotic association with *Gymnostoma*. No evolutionary relationships could be evidenced in this interaction. These hypotheses could be confirmed by comparing the phylogeny of the two symbiotic partners, using sequencing of host plant and microorganism DNA from the same nodule, from a larger sample of Casuarinaceae species.

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