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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 nifD-nifK 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 nifD-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  $\rm H_2O_2$  for 5 min, rinsed with sterile distilled water and kept at  $-20\,^{\circ}\rm C$ . One nodule lobe was crushed in 500  $\rm \mu L$  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  $\rm \mu L$  of TE buffer (pH 7.5).

# 2.3. PCR amplification of nifD-nifK intergene

For deoxyribonucleic acid (DNA) amplification of a region including the 3' end of nifD, the intergenic spacer (IGS), and the beginning of nifK, 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<sub>2</sub>, 50 mM KCl, 0.01 % [w/v] gelatine, 20 µM deoxynucleoside triphosphate [dNTP], 1 µM each of the primers and 2.5 units of TaqI 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·L<sup>-1</sup> 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.

Strain reference		Navarro et al. (1997)	This study	This study	This study	This study	This study	Navarro et al. (1997)	This study	This study	This study	This study	This study	This study	This study	Navarro et al. (1997)	This study	This study	Navarro et al. (1997)	This study	This study	This study	This study	Navarro et al. (1997)	This study	This study	This study	This study				
IGS-types		Q	ഥ	īН	Н	Q	В	О	Д	囝	Д	Ö	D	田	В	Q	Ö	В	A	В	A	В	Н	ŋ	М	О	Q	В	Ω	В	Q	D
Strain or nodule <sup>b</sup>		TC23 - TC24	TC287	KC693	PMC753	PLD170 - PLD185	RBD570	MG59	DG251	EFG308	KI55	KI72	DI247	DI249	PLL300	RPL161	RPL233	RPL526	CN61	CN721	TWN402	NHN750	KN301	KP54	KP80 - KP81	KP82	DP272	RBW161	RBW162 - RBW163	CW726	AW673	CHW746
Geographical origin <sup>a</sup>		Tontouta (NC)	Tontouta (NC)	Kouaoua (NC)	Poum (NC)	Plaine des Lacs (NC)	Rivière Bleue (NC)	Mé Aïu (NC)	Dzumac (NC)	Etoile Filante (NC)	Kouaoua (NC)	Kouaoua (NC)	Dzumac (NC)	Dzumac (NC)	Plum (NC)	Rivière des Pirogues (NC)	Rivière des Pirogues (NC)	Rivière des Pirogues (NC)	Canala (NC)	Canala (NC)	Tiwaka (NC)	Nehoue (NC)	Kouaoua (NC)	Kouaoua (NC)	Kouaoua (NC)	Kouaoua (NC)	Dzumac (NC)	Rivière Bleue (NC)	Rivière Bleue (NC)	Canala (NC)	Amieu (NC)	Chagrin (NC)
Host plant	Gumnostoma nodules	G. chamaecyparis	G. chamaecyparis	G. chamaecyparis	G. chamaecyparis		$G.\ deplancheanum$	$G.\ glaucescens$	$G.\ glaucescens$	$G.\ glaucescens$	$G.\ intermedium$	$G.\ intermedium$	$G.\ intermedium$	$G.\ intermedium$	$G.\ leucodon$	$G.\ leucodon$	$G.\ leucodon$	$G.\ leucodon$	$G.\ nodifforum$	$G.\ nodifforum$	$G.\ nodifforum$	$G.\ nodifforum$	$G.\ nodifforum$	$G.\ poissonianum$	$G.\ poissonianum$	$G.\ poissonianum$	$G.\ poissonianum$	$G.\ webbianum$	$G.\ webbianum$	$G.\ webbianum$	$G.\ webbianum$	G. webbianum

Table I. Continued.

Strain reference		1 This study	1 This study	1 Rouvier et al. (1996)	2 This study	2 Rouvier et al. (1996)	2 Rouvier et al. (1996)	3 This study	3 Rouvier et al. (1996)	3 Rouvier et al. (1996)	3 Rouvier et al. (1996)	3 Rouvier et al. (1996)	3 Rouvier et al. (1996)	3 Rouvier et al. (1996)		4 This study	4 Rouvier et al. (1996)	4 Rouvier et al. (1996)	4 Rouvier et al. (1996)	5 This study	5 Rouvier et al. (1996)	5 Rouvier et al. (1996)						
IGS-types																												
Strain or nodule b		1Cei1	19Ce1	CeCB	CeGB	CePall	CeSB	CeWg2	CeWB	14Ce2	CeWg1	CeMLB	7Cc1	CcBK	CCHRC	$C_{cJF}$	CcOCC	$C_{c}TC$	$C_cWW$		37At1	Altarri - Altarr2	AltBW	AltNLC	11Al1	AllAT	AllKur1 - AllKur2	
Geographical origin <sup>a</sup>		Alva Beach (A)	Horseshoe Bay (A)	Cow Bay (A)	Garners Beach (A)	Pallarenda (A)	Sauders Beach (A)	Wangetti Beach (A)	Wongalinda Beach (A)	Cape Hillsborough Beach (A)	Wangetti Beach (A)	Mount Low Beach (A)	Cattle Creek (A)	Bakerville Creek (A)	Herbert River Crossing (A)	Jourama Falls (A)	Old Chinaman Creek (A)	Tinaroo Creek (A)	West Watsonville (A)		Paluma Road (A)	Atherton Rifle Range (A)	Bluewater (A)	North of Lawyer Creek (A)	Eungella Road (A)	Atherton (A)	Kuranda (A)	
Host plant	Casuarina nodules	C. equisetifolia/incana	C. equisetifolia	$C.\ equisetifolia$	C. equisetifolia	$C.\ equisetifolia$	C. equisetifolia	C. equisetifolia	C. equisetifolia	$C.\ equisetifolia$	C. equisetifolia	C. equisetifolia	C. cunninghamania	C. cunninghamania	C. cunninghamania	C. cunninghamania	C. cunninghamania	C. cunninghamania	$C.\ cunninghamania$	Allocasuarina nodules	A. torulosa	A. torulosa	A. torulosa	A. torulosa	$A.\ littoralis$	A. littoralis	A. littoralis	

Table I. Continued.

Host plant	Geographical origin <sup>a</sup>	Strain or nodule <sup>b</sup>	IGS-types	Strain reference
Casuarina strains C. equisetifolia C. cunninghamania C. equisetifolia	Dakar (Senegal) Florida (USA) Senegal	D11 (atypical) (U) Cc13 (9) CeD (9)	ND 1	Gauthier et al. (1981) Zhang et al. (1984) Diem and Dommerones (1983)
$Elaeagnaceae strains \ Elaeagnus \ umbellata$	Illinois (USA)	EUN1f (6)	. QN	Lalonde et al. (1981)
Hippophaë rhamnoides	,	HRN18a (7)	ND	Moiroud and Faure-Reynaud (1983)
$E.\ angustifolia$		EaN1-pec (5)	ND	Lalonde et al. (1981)
$E.\ angustifolia$		Ea1-12(4)	ND	Fernandez et al. (1989)
Sherpherdia canadensis		SCN10a (U)	ND	Mort et al. (1983)
Alnus strain	(		!	
Alnus rubra	Oregon (USA)	ArI3 (1)	ND	Berry and Torrey $(1979)$

<sup>a</sup> NC: New Caledonia, A: Australia; <sup>b</sup> 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  $\mu$ L of PCR reaction mixture for each reaction. The endonucleases, NcI1, MspI, HaeIII (all from Boehringer Mannheim, Meylan, France) and ScrF1 (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  $\mu$ g mL<sup>-1</sup> ethidium bromide, using TBE electrophoresis buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA). Gels were run at 4 V cm<sup>-1</sup> 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 nifD-nifK 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*).

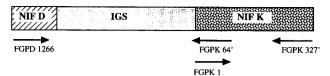


Figure 1. Sequencing strategy for the *nif*D-*nif*K intergenic spacers (IGS). The arrows show the direction of the sequencing reactions.

ArI3	GATICTUTCICC	COCCACATOC	ACATECOCO M	C33C3CCC	GGGTGGGG 4 GG					• 100
RPL161		CGCGACATGG								
MG59										
TC24										
CN61 KP54	CONCURRENCE	CGCGACATGG	ACATGGCCAT	CAACAGCCCG	ACCTGGGACC	TGATGGAGAC	CCCGTGGTCG	AAGTCCGGAG	AGGTCTTCTG	AGCAG-TAGT
EUN1 f	GATCTTCCC	COCCACATOG	ACATCOCCAT	CAACAGCCCG	ACCIGGGACC	TGATGGAGAC	CCCGTGGTCG	AAGTCCGGAG	AGGTCTTCTG	ACCAGTTAGT
SCN10A	GATCTTCGCC	CCCCACATGG	ACATCOCCAT	CARCAGOCCO	ACCIGGGACC	TGATGGAGAC	CCCGTGGTCG	AAGTCCGGAG	AGGTCTCCTG	ACCAGCTAGT
HRN18a	GCTCTTCGCC	CGCGACATGG	ACATCGCGAT	CAACGGCCCG	ACCTGGGACC	TGATGGAGAC	CCCCTGGTCG	AAGTCCGGAG	AGGTCTTCTG	ACCCGTTAG-
Ea1-12	GGTCTTCGCC	CGCGACATOG	ACATCGCGAT	CAACAGCCCG	ACCTGGGACC	TGATGGAGAC	CCCCTGGTCG	AAGGCCGGAG	AGGTGTGCTG	ACCCG2TC.T
EaN1-pec D11	GGTCTTCGCC	CGCGACATOG	ACATCGCGAT	CAACAGCCCG	ACCTGGGACC	TCATGGAGAC	CCCCTGGTCG	AAGGCCGGAG	AGGTGTGCTG	ACCGGATT-T
Cci3										
Ced	GGTCTTCGCC	CGTGACATGG	ACATUGUGAT ACATYOCOGAT	CAACAGCCCG	ACCTGGGACC	TCATGGAGAC	CCCCTGGTCG	AAGGCCGGAG	AGGIGITCIG	ACCGGCCGGT
1Ceil	GGTCTTCGCC	CGTGACATGG	ACATCGCGAT	CAACAGCCCG	ACCTGGGACC	TCATGGAGAC	CCCCTGGTCG	AAGGCCGGAG	AGGTGTTCTG	ACCGGCCGGT
19Ce1	GGTCTTCGCC	CGTGACATGG	ACATCGCGAT	CAACAGCCCG	ACCTGGGACC	TCATGGAGAC	CCCCIGGICG	ANGGCCGGAG	AGGTGTTCTG	ACCOGGCCGGT
14Ce2 7Cc1	GGTCTTCGCC	CGTGACATGG	ACATTGCGAT	CAACAGCCCG	ACCTGGGACC	TCATGGAGAC	CCCCTGGTCG	AAGGCCGGAG	AGGTGTTCTG	ACCOGCCGGT
37At1	GGTCTTCGCC	CGTGACATGG	ACATTGCGAT	CAACAGCCCG	ACCTGGGACC	TCCTGGAGAC	CCCCTGGTCG	AAGGCCGGAG	AGGTGTTCTG	ACCGGCCGGT
11A11	COTOTTOGCO	CGCGACATGG CGTGACATGG	ACATCGCGAT	CAACAGCCCG	ACCTGGGACC	TCCTGGAGAC	CCCCTGGTCG	AAGGCCGGAG	AGGTGTTCTG	ACCGGCCGGT
	GGICIICGCC	COTOMCATOG	ACATCGCGAT	CAACAGCCCG	ACCIGGGACC	TCCTGGAGAC	CCCCTGGTCG	AAGGCCGGAG	AGGTGTTCTG	ACCGGCCGGT
	101									200
ArI3	CTCCGGGCAC	CCGGGCGCGA	GGCGCCCAGC	AGTAGACGGC	CACTTGCCGT	-AGACGGCCA	CTCGCAGTA-	GACGGCCACT	CCCCCTAGAC	
RPL161 MG59										
TC24	CUGCACGC	GGCAACCA CCGGCAACCA CGCAACCA	CCGGCGACCA	CCGGTGATCA	CCGGTGGCTG	-ccggcggcc	ATCAGTGGG-	TCTTTTCCTT	CGCACGGTGT	ACGGCCACGA
CN61	-CCGGCACCG	CGCAACCA	CCCCCCCACCA	CCCCTCATCA	CCCGTGGCTG	-ccgccgcc	ATCAGTGGG-	TCTTTTCCTT	CGCACGGTGT	ACGGCCACGA
KP54	CGGCACCG	GCCAACCA	CCGGCGACCA	CCGGCGANCA	CCGGTGGCTG	ACCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC	ATCAGGGGG-	CCTTCTCCAT	CGCACGGGGC	ACGGCCAGGA
EUN1 f	TCCGGCTCCG	GCCAACCA CGCGCAACCA CCGGCGACCA	CCGGCGACCA	CCGGTGATCA	CCGGTGGCTG	-CCGGCCGGCC	ATCAGTGAG.	COMPONENT	crecoccocc	ACCCCCA CCA
SCN10A										
HRN18a Eal-12										
EaN1-pec	TUUGGTUTUU	GCCT	~~GGCGAT							
D11	CCG	GCC- CCTCG-CA CCGCGGTAGC	CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC	TOT	CCCCT					
Cci3	-ccccccc	CCGCGGTAGC	CGCAGT						ACCGG	CCGGCGAGCG
Ced										
1Cei1 19Cel										
14Ce2	-000000000	CCGCGGTAGC	CGCAGT							
7Cc1	-cogcoccc	CCGCGGTAGC CCGCGGTAGC	CCCAGT							
37At1	-000000000	CCGCGGTAGC	CGCAGT							
11Al1	-CCGGCCCCG	CCGCGGTAGC	CGCAGT							
ArT3	201	CACOTOCCACO	100001010	<b>#</b> 1 0mm as man						300
ArI3 RPL161	AGTAGACGGC	CACTTGCAGT	AGCTGACCAG	TAGTTCATCC	AGGCCTGGCC	GAACCAGCCG	CCAGCGACCC	CGTGACCGGA	GAAACGGTCG	300 CTGGCGGCTG
RPL161 MG59	AGTAGACGGC ATGCCGTGAG ATGCCGTGAG	CCCCACTCCA	CCACCCCAAAA	CC						CTGGCGGCTG
RPL161 MG59 TC24	AGTAGACGGC ATGCCGTGAG ATGCCGTGAG ATGCCGTGAG	CCCGAGTCGA CCCGAGTCGA	CGACCGAAAA CGACCGAAA	GG						CTGGCGGCTG
RPL161 MG59 TC24 CN61	AGTAGACGGC ATGCCGTGAG ATGCCGTGAG ATGCCGTGAG ACGTGAG	CCCGAGTCGA CCCGAGTCGA CCCGAACTGG	CGACCGAAAA CGACCGAAA- CGATGGAAAA	GG						CTGGCGGCTG
RPL161 MG59 TC24 CN61 KP54	AGTAGACGGC ATGCCGTGAG ATGCCGTGAG ATGCCGTGAG ACGTGAG CA-GAAAGGC	CCCGAGTCGA CCCGAGTCGA CCCGAACTGG TGATGTTACG	CGACCGAAAA CGACCGAAA- CGATGGAAAA CGGTGCGTCG	GG GG GT						CTGGCGGCTG
RPL161 MG59 TC24 CN61 KP54 EUN1f	AGTAGACGGC ATGCCGTGAG ATGCCGTGAG ATGCCGTGAG ACGTGAG CA-GAAAGGC ATGCCGTCAG	CCCGAGTCGA CCCGAGTCGA CCCGAACTGG TGATGTTACG CCCGAGTCGG	CGACCGAAAA CGACCGAAA- CGATGGAAAA CGGTGCGTCG CGATGGAAAA	GG						CTGGCGGCTG
RPL161 MG59 TC24 CN61 KP54	AGTAGACGGC ATGCCGTGAG ATGCCGTGAG ATGCCGTGAG ACGTGAG CA-GAAAGGC ATGCCGTCAG	CCCGAGTCGA CCCGAGTCGA CCCGAACTGG TGATGTTACG CCCGAGTCGG	CGACCGAAA CGACCGAAA- CGATGGAAA CGGTGCGTCG CGATGGAAAA	GG GG GG GG						CTGGCGGCTG
RPL161 MG59 TC24 CN61 KP54 EUN1f SCN10A HRN18a Ea1-12	AGTAGACGGC ATGCCGTGAG ATGCCGTGAG ATGCCGTGAG ACGTGAG CA-GAAAGGC ATGCCGTCAG	CCCGAGTCGA CCCGAGTCGA CCCGAACTGG TGATGTTACG CCCGAGTCGG	CGACCGAAAA CGACCGAAA- CGATGGAAAA CGGTGCGTCG CGATGGAAAA	GG						CTGGCGGCTG
RPL161 MG59 TC24 CN61 KP54 EUN1f SCN10A HRN18a Ea1-12 EaN1-pec	AGTAGACGGC ATGCCGTGAG ATGCCGTGAG ATGCCGTGAG ACGTGAG CA-GAAAGGC ATGCCGTCAG	CCCGAGTCGA CCCGAGTCGA CCCGAACTGG TGATGTTACG CCCGAGTCGG	CGACCGAAAA CGACCGAAA- CGATGGAAAA CGGTGCGTCG CGATGGAAAA	GG GG GT GG						CTGGCGGCTG
RPL161 MG59 TC24 CN61 KP54 EUN1f SCN10A HRN18a Ea1-12 EaN1-pec D11	AGTAGACGGC ATGCCGTGAG ATGCCGTGAG ATGCCGTGAG CA-GAAAGGC ATGCCGTCAG	CCCGAGTCGA CCCGAGTCGA CCCGAACTGG TGATGTTACG CCCGAGTCGG	CGACCGAAAA CGACCGAAA- CGATGGAAAA CGGTGCGTCG CGATGGAAAA	GG						CTGGCGGCTG
RPL161 MG59 TC24 CN61 KP54 EUN1f SCN10A HRN18a Ea1-12 EaN1-pec D11 Cci3	AGTAGACGGC ATGCCGTGAG ATGCCGTGAG ATGCCGTGAG CA-GAAAGGC ATGCCGTCAG 	CCCGAGTCGA CCCGAGTCGA CCCGAACTGG TGATGTTACG CCCGAGTCGG	CGACCGAAAA CGACCGAAAA CGATGGAAAA CGGTGGGTCG CGATGGAAAA	GG						CTGGCGGCTG
RPL161 MG59 TC24 CN61 KP54 EUN1f SCN10A HRN18a Ea1-12 EaN1-pec D11 Cci3 Ced1	AGTAGACGGC ATGCCGTGAG ATGCCGTGAG ATGCCGTGAG ACGTGAG CA-GAAAGGC ATGCCGTCAG 	CCCGAGTCGA CCCGAGTCGA CCCGAACTGG TGATGTTACG CCCGAGTCGG	CGACCGAAAA CGACCGAAAA CGATGGAAAA CGGTGCGTCG CGATGGAAAA	GG						CTGCCGCTG
RPL161 MG59 TC24 CN61 KP54 EUN1f SCN10A HRN18a Ea1-12 EaN1-pec D11 Cci3 Ced 1Cei1 19Ce1	AGTAGACGGC ATGCCGTGAG ATGCCGTGAG ATGCCGTGAG ACGTGAG CA-GAAAGGC ATGCCGTCAG 	CCCGAGTCGA CCCGAGTCGA CCCGAGTCGA TGATGTTACG CCCGAGTCGG	CGACCGAAAA CGACCGAAAA CGATGGAAAA CGGTGCGTCG CGATGGAAAA	GG						CTOGCOGGETG
RPL161 MG59 TC24 CM61 KP54 EUN1f SCN10A HRN18a Eal-12 EaN1-pec D11 Cci3 Ced 1Cei1 19Cel 14Ce2	AGTAGACGC ATGCCGTGAG ATGCCGTGAG ATGCCGTGAG ATGCCGTGAG CA-GAAAGGC ATGCCGTCAGCCGGT	CCCGAGTCGA CCCGAGTCGA CCCGAACTGG TGATGTTACG CCCGAGTCGG	CGACCGAAA CGACCGAAA- CGATGGAAA- CGATGGAAAA CGGTGCGTCG CGATGGAAAA	GG						CTOGCOGCTG
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RFL161 MG59 TC24 CN61 KF54 KF54 KF510A HEN188 Eal-1-2 EaN1-pec D11 SC01 16Ce1 19Ce1 14Ce2 TC24 CN61 KF54 EUN16 KF54 EUN16 KF54 EUN16 KF54 EUN16 KF54 EUN16 KF54 EUN16 EU	AGTINAACGC ATOCCOTTAG CCGCGCGCACG ATOCCOTTAG CCGCGCGCACG ATOCCOTTAG CCGCGCGCACG ATOCCOTTAG CCGCGCGCACG ATOCCOTTAG CCGCGCGCACG CCGCGCACG CCGCGCGCACG CCGCGCACG CCGCGCACG CCGCGCACG CCGCGCACG CCGCCGCACG CCGCCACG CCCCACG CCCCACC CCCCACG CCCCACC CCCCACC CCCCACC CCCCACC CCCCACC CCCCACC CCCCACC CCCCACC CCCCCACC CCCCCACC CCCCCACC CCCCCACC CCCCCC	CCCGAGTCGA CCCGAGTCGA CCCGAGTCGG CCCCAGTCGG CCCCGCGCG CCCCCGCGCG CCCCCGCGCG CCCCGCGCG CCCCGCGCG	CACCTGGGAAAA CGGGCGAAAA  CACCTGGGGCCGAAAA  CACCTGGGGCCGAAAA  CACCTGGGGAAAA  CACCTGGGAAAA  CACCTGGGAAAA  CACCTGGGAAAA  CACCTGGGAAAA  CACCTGGGAAAA  CACCTGGGAAAA  CACCTGGGAAAAA  CACCTGGAAAAA  CACCTGGAAAAA  CACCTGGAAAAA  CACCTGGAAAAA  CACCTGAAAAAA  CACCTGAAAAAAAAAA	AAGCCCACAG GG GG GG GG GG	GAGAGGAGG GAAAGGAGGA GAAAGGAGGA GAAAGGAGG	ACOTICGOTGA GOTICGOTGA GOTICGOTGA GOTICGOTGA GOTICGOTGA GOTICGOTGA TOTICGOTGA TOTICGOTGA TOTICGOTGA TOTICGOTGA TOTICCGOTGA TOTICCGOTGA TOTICCGOTGA TOTICCGOTGA TOTICCGOTGA TOTICCGOTGA TOTICCGOTGA TOTICCGOTGA TOTICCGOTGA	CGACGACCCC CGACGACTC CGACGACTC CGACTC CGACTC CGACTC CGACTC CGACTC CGACTC CGACTC CGACT	CGAGCACACC CGAGACGAAC CGAGACGAAC CGAGACGAAC CGAGACGAAC CGAGACGAAC CGAGACGAAC CGAGACGAAC CGAGACGAAC CGAGACCAAC CGACAC CGAGACCAAC CGAGACCAAC CGAGACCAAC CGAGACCAAC CGAGACCAAC CGAGACCAAC CGAGACCAAC CGAGACCAAC CGAGACCAAC CGAGACCAAC CGAGACCAAC CGACACCAAC CGAGACCAAC CGACACCAAC CCACACCAAC CCACACAAC CCACACCAC	AGCOCGGTCC AGTCACATCC AGTTCCGTCC	400 COCTOCOGOT COCTOCAGOT COCTOCA
RFL161 MG59 TC24 CM61 KB54 KB161 KB1	AGTINAACGC ATOCCOTTAG CCGCGCGCACG ATOCCOTTAG CCGCGCGCACG ATOCCOTTAG CCGCGCGCACG ATOCCOTTAG CCGCGCGCACG ATOCCOTTAG CCGCGCGCACG CCGCGCACG CCGCGCGCACG CCGCGCACG CCGCGCACG CCGCGCACG CCGCGCACG CCGCCGCACG CCGCCACG CCCCACG CCCCACC CCCCACG CCCCACC CCCCACC CCCCACC CCCCACC CCCCACC CCCCACC CCCCACC CCCCACC CCCCCACC CCCCCACC CCCCCACC CCCCCACC CCCCCC	CCCGAGTCGA CCCGAGTCGA CCCGAGTCGG CCCGAGTCGG CCCGAGTCGG CCCGAGTCGG CCCCAGTCGG CCCCAGTCGG CCCCGAGTCGG	CACCTGGGAAAA CGGGCGAAAA  CACCTGGGGCCGAAAA  CACCTGGGGCCGAAAA  CACCTGGGGAAAA  CACCTGGGAAAA  CACCTGGGAAAA  CACCTGGGAAAA  CACCTGGGAAAA  CACCTGGGAAAA  CACCTGGGAAAA  CACCTGGGAAAAA  CACCTGGAAAAA  CACCTGGAAAAA  CACCTGGAAAAA  CACCTGGAAAAA  CACCTGAAAAAA  CACCTGAAAAAAAAAA	AAGCCCACAG GG GG GG GG GG	GAGAGGAGG GAAAGGAGGA GAAAGGAGGA GAAAGGAGG	ACOTICGOTGA GOTICGOTGA GOTICGOTGA GOTICGOTGA GOTICGOTGA GOTICGOTGA TOTICGOTGA TOTICGOTGA TOTICGOTGA TOTICGOTGA TOTICCGOTGA TOTICCGOTGA TOTICCGOTGA TOTICCGOTGA TOTICCGOTGA TOTICCGOTGA TOTICCGOTGA TOTICCGOTGA TOTICCGOTGA	CGACGACCCC CGACGACTC CGACGACTC CGACTC CGACTC CGACTC CGACTC CGACTC CGACTC CGACTC CGACT	CGAGCACACC CGAGACGAAC CGAGACGAAC CGAGACGAAC CGAGACGAAC CGAGACGAAC CGAGACGAAC CGAGACGAAC CGAGACGAAC CGAGACCAAC CGACAC CGAGACCAAC CGAGACCAAC CGAGACCAAC CGAGACCAAC CGAGACCAAC CGAGACCAAC CGAGACCAAC CGAGACCAAC CGAGACCAAC CGAGACCAAC CGAGACCAAC CGACACCAAC CGAGACCAAC CGACACCAAC CCACACCAAC CCACACAAC CCACACCAC	AGCOCGGTCC AGTCACATCC AGTTCCGTCC	400 COCTOCOGOT COCTOCAGOT COCTOCA

**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.

	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
EUN1 f	CCTCGACCAC	ACCGAGCTGT	TCAAGGACGA	GGTCTACACC	AAGCAGTTCG	AGACCAAGCG	CGAGTTCGAG	AACGCCCCC	ATGACGCCGA	GGTCAACCGG
SCN10A	CCTCGACCAC	AACGAGCTGT	TCAAGGACGA	GGTCTACACC	AAGCAGTTCG	AGACCAAGCG	CGAGTTCGAG	AACGGTGCGG	ACGACGCCGA	GGTCAACCGG
HRN18a	CCTCGACCAC	AGCGAGCTGT	TCAAGGACGA	GGTCTACAGG	AAGCAGTTCG	AGACCAAGCG	CGAGTTCGAG	AACGGCGCCG	ATGACGCCGA	GGTCAACCGG
Ea1-12	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
11Al1	CCTCGACCAC	AGCGAGCTGT	TCAAGGACGA	GGTCTACCAG	AAGCAGTTCG	AGGGCAAGCG	CGAGTTCGAG	AACGGCAGCG	ACGCCGCCGA	GGTCGCCCGG
	501			540	544					
ArI3	501 GTCCTCGACT	GGACCCGCGG	GTGGGAGTAC	CGGGAGAAGA	544 ACTT					
MG59	501 GTCCTCGACT GTCCTGGAGT	GGACCCGCGG GGACCCGTAC	GTGGGAGTAC CTGGGAGTAC	CGGGAGAAGA CGCGAGAAGA	544 ACTT ACTT					
MG59 RPL161	501 GTCCTCGACT GTCCTGGAGT GTCCTGGAGT	GGACCCGCGG GGACCCGTAC GGACCCGTAC	GTGGGAGTAC CTGGGAGTAC CTGGGAGTAC	CGGGAGAAGA CGCGAGAAGA	544 ACTT ACTT ACTT					
MG59 RPL161 TC24	501 GTCCTCGACT GTCCTGGAGT GTCCTGGAGT GTCCTGGAGT	GGACCCGCGG GGACCCGTAC GGACCCGTAC GGACCCGTAC	GTGGGAGTAC CTGGGAGTAC CTGGGAGTAC CTGGGAGTAC	CGGGAGAAGA CGCGAGAAGA CGCGAGAAGA CGCGAGAAGA	544 ACTT ACTT ACTT ACTT					
MG59 RPL161 TC24 CN61	501 GTCCTCGACT GTCCTGGAGT GTCCTGGAGT GTCCTGGAGT	GGACCCGCGG GGACCCGTAC GGACCCGTAC GGACCCGTAC GGACCCGTAC	GTGGGAGTAC CTGGGAGTAC CTGGGAGTAC CTGGGAGTAC CTCGGAGTAC	CGGGAGAAGA CGCGAGAAGA CGCGAGAAGA CGCGAGAAGA	544 ACTT ACTT ACTT ACTT ACTT					
MG59 RPL161 TC24 CN61 KP54	501 GTCCTCGACT GTCCTGGAGT GTCCTGGAGT GTCCTGGAGT GTCCTGGAGT GTCCTGGAGT	GGACCCGCGG GGACCCGTAC GGACCCGTAC GGACCCGTAC GGACCCGTAC	GTGGGAGTAC CTGGGAGTAC CTGGGAGTAC CTGGGAGTAC CTCGGAGTAC CTGGGAGTAC	CGGGAGAAGA CGCGAGAAGA CGCGAGAAGA CGCGAGAAGA CGCGAGAAGA	544 ACTT ACTT ACTT ACTT ACTT ACTT					
MG59 RPL161 TC24 CN61 KP54 EUN1f	501 GTCCTCGACT GTCCTGGAGT GTCCTGGAGT GTCCTGGAGT GTCTGGAGT GTCTGGAGT	GGACCCGCG GGACCCGTAC GGACCCGTAC GGACCCGTAC GGACCCGTAC GGACCCGTAC	GTGGGAGTAC CTGGGAGTAC CTGGGAGTAC CTGGGAGTAC CTGGGAGTAC CTGGGAGTAC	CGGGAGAAGA CGCGAGAAGA CGCGAGAAGA CGCGAGAAGA CGCGAGAAGA CGCGAGAAGA CGCGAGAAGA	544 ACTT ACTT ACTT ACTT ACTT ACTT ACTT					
MG59 RPL161 TC24 CN61 KP54 EUN1f SCN10A	501 GTCCTCGACT GTCCTGGAGT GTCCTGGAGT GTCCTGGAGT GTCCTGGAGT GTCCTGGAGT GTCCTGGAGT	GGACCCGCG GGACCCGTAC GGACCCGTAC GGACCCGTAC GGACCCGTAC GGACCCGTAC GGACCCGCAC	GTGGGAGTAC CTGGGAGTAC CTGGGAGTAC CTGGGAGTAC CTGGGAGTAC CTGGGAGTAC CTGGGAGTAC	CGGGAGAAGA CGCGAGAAGA CGCGAGAAGA CGCGAGAAGA CGCGAGAAGA CGCGAGAAGA CGCGAGAAGA CGCGAGAAGA	544 ACTT ACTT ACTT ACTT ACTT ACTT ACTT AC					
MG59 RPL161 TC24 CN61 KP54 EUN1f SCN10A HRN18a	501 GTCCTCGACT GTCCTGGAGT GTCCTGGAGT GTCCTGGAGT GTCCTGGAGT GTCCTGGAGT GTCCTGGAGT GTCCTGGAGT	GGACCCGCG GGACCCGTAC GGACCCGTAC GGACCCGTAC GGACCCGTAC GGACCCGCAC GGACCCGCAC GGACCCGCAC	GTGGGAGTAC CTGGGAGTAC CTGGGAGTAC CTGGGAGTAC CTGGGAGTAC CTGGGAGTAC CTGGGAGTAC CTGGGAGTAC	CGGGAGAAGA CGCGAGAAGA CGCGAGAAGA CGCGAGAAGA CGCGAGAAGA CGCGAGAAGA CGCGAGAAGA CGGGAGAAGA CGCGAGAAGA CGCGAGAAGA	544 ACTT ACTT ACTT ACTT ACTT ACTT ACTT AC					
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MG59 RPL161 TC24 CN61 KP54 EUN1f SCN10A HRN18a Ea1-12 EaN1-pec D11	501 GTCCTCGACT GTCCTGGAGT GTCCTCGAGT GTCCTCGAGT GTCCTCGAGT	GGACCCGCG GGACCCGTAC GGACCCGTAC GGACCCGTAC GGACCCGTAC GGACCCGCAC GGACCCGCAC GGACCCGCAC GGACCCGCAG GGACCCGCAG GGACCCGCGG GGACCCGCGG GGACCCGCGG	GTGGGAGTAC CTGGGAGTAC CTGGGAGTAC CTGGGAGTAC CTGGGAGTAC CTGGGAGTAC CTGGGAGTAC CTGGGAGTAC CTGGGAGTAC CTGGGAGTAC CTGGGAGTAC CTGGGAGTAC	CGGGAGAAGA CGCGAGAAGA CGCGAGAAGA CGCGAGAAGA CGCGAGAAGA CGCGAGAAGA CGCGAGAAGA CGCGAGAAGA CGGGAGAAGA CGGGAGAAGA CGGGAGAAGA CGGGAGAAGA CGGGAGAAGA CGGGAGAAGA	544 ACTT ACTT ACTT ACTT ACTT ACTT ACTT AC					
MG59 RPL161 TC24 CN61 KP54 EUN1f SCN10A HRN18a Eal-12 EaN1-pec D11 Cci3	501 GTCCTCGACT GTCCTGGAGT GTCCTGGAGT GTCCTGGAGT GTCCTGGAGT GTCCTGGAGT GTCCTGGAGT GTCCTCGAGT GTCCTCGAGT GTCCTCGAGT GTCCTCGAGT GTCCTCGAGT GTCCTCGAGT	GGACCCGCGG GGACCCGTAC GGACCCGTAC GGACCCGTAC GGACCCGTAC GGACCCGTAC GGACCCGCAC GGACCCGCAC GGACCCGCAC GGACCCGCAC GGACCCGCAG GGACCCGCAG GGACCCGCAG GGACCCGCAG GGACCCGCAG GGACCCGCAG	GTGGGAGTAC CTGGGAGTAC CTGGGAGTAC CTGGGAGTAC CTGGGAGTAC CTGGGAGTAC CTGGGAGTAC CTGGGAGTAC CTGGGAGTAC CTGGGAGTAC CTGGGAGTAC CTGGGAGTAC CTGGGAGTAC CTGGGAGTAC	CGGGAGAAGA CGCGAGAAGA CGCGAGAAGA CGCGAGAAGA CGCGAGAAGA CGCGAGAAGA CGCGAGAAGA CGCGAGAAGA CGCGAGAAGA CGGGAGAAGA CGGGAGAAGA CGGGAGAAGA CGGGAGAAGA CGGGAGAAGA	544 ACTT ACTT ACTT ACTT ACTT ACTT ACTT AC					
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MG59 RPL161 TC24 CN61 KP54 EUN1f SCN10A HRN18a Eal-12 EaN1-pec D11 Cci3 Ced	501 GTCCTCGACT GTCCTCGAGT GTCCTGGAGT GTCCTGGAGT GTCCTGGAGT GTCCTGGAGT GTCCTGGAGT GTCCTCGAGT	GGACCCGCGGGGGACCCGTACGGACCCGTACGGACCCGTACGGACCCGCGGGACCCGCGGGACCCGCGGGACCCGCGGGACCCGCGGGACCCGCGGGACCCGCGGGACCCGCGGGACCCGCGGGACCCGCGGGACCCGCGGGACCCGCGGGACCCGCGGGACCCGCGGGACCCGCGGGACCCGCGGGACCCGCGGGACCCGCGGGACCCGCGGGACCCG	GTUGGAGTAC CTUGGAGTAC CTUGGAGTAC CTUGGAGTAC CTUGGAGTAC CTUGGAGTAC CTUGGAGTAC CTUGGAGTAC CTUGGAGTAC CTUGGAGTAC CTUGGAGTAC CTUGGAGTAC CTUGGAGTAC CTUGGAGTAC CTUGGAGTAC CTUGGAGTAC CTUGGAGTAC	AGAAGAGES AGAAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA	544 ACTT ACTT ACTT ACTT ACTT ACTT ACTT AC					
MG59 RPL161 TC24 CN61 KF54 EUN1f SCN10A HRN18a Eal-12 EaN1-pec D11 Ccd1 Ccd1 19Ce1	501 GTCCTGGAGT GTCCTGGAGT GTCCTGGAGT GTCCTGGAGT GTCCTGGAGT GTCCTGGAGT GTCCTGGAGT GTCCTGGAGT GTCCTGGAGT GTCCTCGAGT GTCCTCGAGT GTCCTCGAGT GTCCTCGAGT GTCCTCGAGT GTCCTCGAGT GTCCTCGAGT GTCCTCGAGT GTCCTGGAGT GTCCTGGAGT GTCCTGGAGT GTCCTGGAGT GTCCTGGAGT GTCCTGGAGT	GGACCCGCG GGACCCGTAC GGACCCGTAC GGACCCGTAC GGACCCGTAC GGACCCGCAC GGACCCGCAC GGACCCGCAC GGACCCGCAC GGACCCGCAC GGACCCGCAC GGACCCGCAC GGACCCGCAC GGACCCGCAC GGACCCGCAC GGACCCGCAC GGACCCGCAC GGACCCGCAC GGACCCGCAC	GTGGGAGTAC CTGGGAGTAC	AGAGARAGA GEORGAGAGA GEORGAGA GEORGAGAGA GEORGAGAGA GEORGAGAGA GEORGAGAGA GEORGAGAGA GEORGAGA GEORGAGAGA GEORGAGA GEORGAG	544 ACIT ACIT ACIT ACIT ACIT ACIT ACIT ACIT					
MG59 RPL161 TC24 CN61 KP54 EUN1f SCN10A EAL-12 EAN1-pec D11 Cci3 Ced 1Cei1 19Ce1	501 GTCCTCGACT GTCCTCGAGT GTCCTGGAGT GTCCTGGAGT GTCCTGGAGT GTCCTGGAGT GTCCTGGAGT GTCCTCGAGT	GGACCCGCG GGACCCGTAC GGACCCGTAC GGACCCGTAC GGACCCGTAC GGACCCGTAC GGACCCGCAC GGACCCGCAC GGACCCGCAC GGACCCGCAC GGACCCGCAC GGACCCGCAC GGACCCGCAC GGACCCGCAC GGACCCGCAC GGACCCGCAC GGACCCGCAC GGACCCGCAC GGACCCGCAC GGACCCGCAC	GTUGGAGTAC CTUGGAGTAC	AGAGAGAGA  GAGAGAGA  GAGAGAGA  GAGAGAGA	544 ACTT ACTT ACTT ACTT ACTT ACTT ACTT AC					
MG59 RPL161 TC24 CN61 KP54 EUNIf SCM10A HRN18a Eal-12 EaN1-pec D11 Cci3 Ced 11Cei1 19Ce1 14Ce2	501 GTCCTCGAGT GTCCTGGAGT	GGACCOGGG GGACCCGTAC GGACCCGTAC GGACCCGTAC GGACCCGTAC GGACCCGCAC GGACCCGCAC GGACCCGCAC GGACCCGCAG GGACCCGCAG GGACCCGCAG GGACCCGCAG GGACCCGCAG GGACCCGCAG GGACCCGCAG GGACCCGCAG GGACCCGCAG GGACCCGCAG GGACCCGCAG GGACCCGCAG	GTOGGAGTAC CTOGGAGTAC	AGAGARAGA GAGAGAGA GAGAGAGA GAGAGAGA GAGAGAGA	544 ACTT ACTT ACTT ACTT ACTT ACTT ACTT AC					
MG59 RPL161 TC24 CN61 KF54 EUN1f SCN10A HRN18a Eal-12 EaN1-pec D11 Cci3 Ccd 1Cci1 19Cc1 14Cc2 7Cc1	501 GRICERGACT GROCERGAGT GROCERG	GGACCCGGGG GGACCCGTTAC GGACCCGTTAC GGACCCGTTAC GGACCCGTTAC GGACCCGTTAC GGACCCGTTAC GGACCCGCTAC GGACCCGCAC GGACCCGCAC GGACCCGCAC GGACCCGCAC GGACCCGCAC GGACCCGCAC GGACCCGCAC GGACCCGCAC GGACCCCGCAC GGACCCCGCAC GGACCCCGCAC GGACCCCGCAC GGACCCCGCAC GGACCCCGCAC GGACCCCGCAC GGACCCCGCAC GGACCCCACAC GGACCCACAC GGACCCACAC GGACCCACAC GCACCCACAC GGACCCACAC GGACCACAC GGACCCACAC GGACCCACAC GCACAC GGACCCACAC GGACCCACAC GGACCACAC GGACCCACAC GGACCACAC	GTIGGAGTAC CTIGGAGTAC	AGAGAGAGA  GAGAGAGA  GAGAGAGA  GAGAGAGA	544 ACTT ACTT ACTT ACTT ACTT ACTT ACTT AC					
MG59 RPL161 TC24 CN61 KP54 EUNIf SCM10A HRN18a Eal-12 EaN1-pec D11 Cci3 Ced 11Cei1 19Ce1 14Ce2	501 GRICERGACT GROCERGAGT GROCERG	GGACCOGGG GGACCCGTAC GGACCCGTAC GGACCCGTAC GGACCCGTAC GGACCCGCAC GGACCCGCAC GGACCCGCAC GGACCCGCAG GGACCCGCAG GGACCCGCAG GGACCCGCAG GGACCCGCAG GGACCCGCAG GGACCCGCAG GGACCCGCAG GGACCCGCAG GGACCCGCAG GGACCCGCAG GGACCCGCAG	GTIGGAGTAC CTIGGAGTAC	AGAGAGAGA  GAGAGAGA  GAGAGAGA  GAGAGAGA	544 ACTT ACTT ACTT ACTT ACTT ACTT ACTT AC					

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, 1Cei1 and 19Ce1, 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).

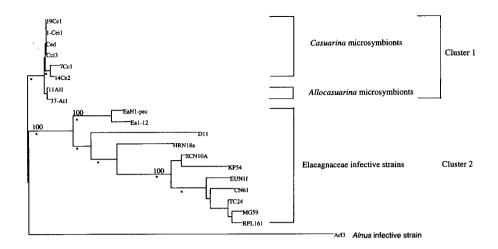
The Elaeagnaceae-infective strains form a broad group designated cluster 2. This coherent cluster (100 % of the bootstrap replicates) was also detected by parsimony analysis. The level of sequence divergences was higher in this cluster than in cluster 1. Gymnostoma microsymbionts belonged to cluster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

11All	84	62	63	59	61	09	09	20	48	37	34	52	7	7	61	7	7.0	ıcı		0
37At1	84	61	62	28	09	29	59	49	47	36	33	53	က	3	က	3	9	9	0	3
7Cc1	87	64	65	61	63	63	63	23	51	39	36	54	rΟ	52	S.	വ	4	0	18	15
14Ce2	98	61	62	28	09	09	09	50	49	37	34	52	33	က	က	က	0	12	18	15
19Cel 14Ce2	84	09	61	22	59	28	28	48	46	35	32	20	0	0	0	0	6	15	6	9
1Cei	84	90	61	22	29	28	28	48	46	35	32	20	0	0	0	0	6	15	6	9
CeD	84	09	61	22	29	28	28	48	46	35	32	20	0	0	0	0	6	15	6	9
CcI3	84	09	61	22	23	28	28	48	46	35	32	20	0	0	0	0	6	15	6	9
	1			71				28				0	170	170	170	170	178	186	182	178
EaN1	66	59	22	58	59	58	26	20	40	6	0	169	103	103	103	103	110	117	106	110
Ea1	66	28	54	55	56	55	54	49	37	0	26	168	113	113	113	113	120	127	117	120
HRN18	105	20	49	48	48	46	44	42	0	121	133	173	153	153	153	153	164	172	156	160
SCN10	111	22	24	18	21	17	21	0	135	164	169	197	158	158	158	158	165	176	161	165
EUN1f	174	25	24	22	18	46	0	28	141	180	189	219	192	192	192	192	199	211	196	199
KP54	147	48	46	43	42	0	121	48	149	186	198	212	194	194	194	194	202	214	198	202
CN61	171	22	19	17	0	1111	42	59	155	189	201	215	197	197	197	197	201	213	201	205
TC24	174	7	9	0	40	113	51	20	155	185	197	230	188	188	188	188	192	203	192	196
MG59	175	00	0	14	45	122	56	89	159	182	194	232	206	206	206	206	210	222	210	214
9	178	0								196										
ArI3	0 178	573	563	552	548	487	551	379	392	358	360	431	297	297	297	297	305	310	296	296
	ArI3																			



0.028 s/s

Figure 3. Phylogenetic neighbour-joining tree based on nifD-nifK 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	Restricti	on patterns	of the 16	S-23S IGS
	MspI	HaeIII	NcI1	ScrF1
A	M1	H1		
В	M1	H2		
$\mathbf{C}$	M2	H1		
D	M2	H3		
$\mathbf E$	M3	H3		
${f F}$	M3	H4		
$\mathbf{G}$	M4	H1		
H	M5	H5		
1			N1	SF1
$^2$			N2	${ m SF2}$
3			N3	SF3
$oldsymbol{4}$			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

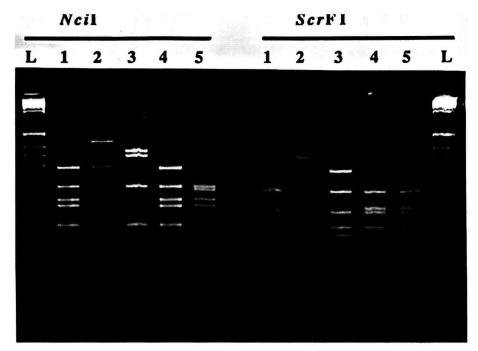


Figure 4. PCR/RFLP patterns of the 16S-23S intergenic spacer (IGS) after digestion with *Nci*1, *Scr*F1. Lanes 1 to 5, IGS-types 1 to 5; lane L, 1-kb ladder.

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.

		IG	S-type	s	
Host plant	1	2	3	4	5
C. equisetifolia	+	+			
C. cunninghamania			+		
A. torulosa				+	
$A.\ littoralis$					+

IVb. Gymnostoma species.

				IGS-t	ypes			
Host plant	A	В	С	D	E	F	G	Н
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 nifD-nifK 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-

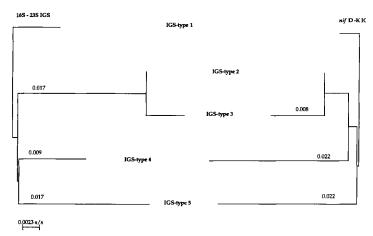


Figure 5. Dendrogram of genotypic relationship of intergenic spacer (IGS) sequences derived by PCR/RFLP analysis according to Rouvier (1995).

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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