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Use of Repetitive Sequences and the Polymerase Chain Reaction (rep-PCR) to Fingerprint the Genomes of *Frankia* Isolates

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Abstract

Oligonucleotide primers complimentary to consensus sequence motifs of repetitive elements common to prokaryotic genomes, REP (repetitive extragenic palindromic), ERIC (enterobacterial repetitive intergenic consensus), and BOX elements, were used to amplify intervening sequences of *Frankia* genomic DNA using the polymerase chain reaction (termed rep-PCR). The PCR reaction products were separated electrophoretically producing a complex banding pattern or fingerprint that was characteristic of each strain. Members of the same genomic species were found to display similar fingerprints, but the rep-PCR technique was sensitive enough to distinguish closely related strains and provides an effective means to rapidly differentiate between *Frankia* isolates at the sub-species level.

Keywords: *Frankia*, rep-PCR, genomic fingerprinting, 16S rRNA

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1. Introduction

The filamentous soil bacterium *Frankia* (Actinomycetales) infects roots of a diverse array of woody dicotyledonous plants, termed actinorhizal plants, producing nodules capable of dinitrogen-fixation. Since the first successful isolation of the endophyte in 1978 (Callaham et al., 1978), hundreds of strains have been isolated from more than half of the 24 genera of known host plants (Benson and Silvester, 1993), allowing a wide range of morphological, physiological and molecular studies of *Frankia*. Thus, the genus is now relatively well described as a group of symbiotic, vesicle-forming, sporulating, filamentous actinomycetes (Lechevalier and Lechevalier, 1990).

Studies using pure cultures of available *Frankia* isolates (Jiabin et al., 1985; Baker 1987; Torrey and Racette, 1989; Torrey 1990) indicate three overlapping host-specificity groups (HSG): 1) Strains that infect *Alnus, Comptonia* and *Myrica;* 2) Strains that infect the *Casuarinas* and *Myrica;* and 3) Strains that infect the *Elaeagnaceae* and *Myrica*. Although strains that infect these three major host groups also infect some species of the promiscuous host genera *Myrica,* members of a fourth group infect only members of the *Elaeagnaceae* (Baker 1987).

Phenotypic comparisons based on protein patterns (Benson et al., 1984; Gardes and Lalonde, 1987), isozyme patterns (Gardes et al., 1987; Girgis and Schwencke, 1993), fatty acid profiles (Lechevalier et al., 1883; Mirza et al., 1991), correlate relatively well with the proposed HSG. Genotypic analyses (for example, An et al., 1985; Dobrisita 1985; Fernandez et al., 1989; Nittayajarn et al., 1990; Nazaret et al., 1991) support these groupings as well, but indicate greater diversity than previously expected from phenotypic characterization.

More than nine genomic species of *Frankia* were delineated on the basis of DNA-DNA reassociation kinetics in an analysis of 43 strains isolated from the three major HSG. These include three species within the *Alnus* HSG, five within the *Eleagnus* infectivity group, one that includes the "typical" Casuarina-compatible strains (ie. strains which reinfect the original host plant) and several stains, including "atypical" isolates (ie. *Eleagnaceae*-compatible strains which do not reinfect their original hosts, Baker 1987), that could not be grouped (Fernandez et al., 1989). Phylogenetic analysis of partial 16S rRNA sequence comparisons correlate well with the DNA-DNA hybridization data (Nazaret et al., 1991).

Short palindromic repetitive sequence elements, typically found in intergenic transcribed but not translated regions of enteric bacteria (Lupski and Weinstock, 1992), appear to be widely distributed in the genomes of prokaryotes (Versalovic et al., 1991; 1994; de Bruijn, 1992). Sequence

comparisons of Repetitive Extragenic Palindromic (REP) (Stern et al., 1984) and Enterobacterial Repetitive Intergenic Consensus (ERIC) (Hulton et al., 1991) elements of enteric bacteria has lead to the identification of consensus sequences that appear to be present in a wide variety of eubacteria by DNA-DNA hybridization studies (Versalovic et al., 1991). Oligonucleotide primers corresponding to these highly conserved regions have been used to amplify the intervening genomic DNA located between these conserved sequences by the PCR reaction, resulting in the generation of a highly reproducible set of distinct PCR products that can be separated on simple agarose gels to obtain genomic fingerprints (refered to as rep-PCR fingerprinting; Versalovic et al., 1991; 1994; de Bruijn 1992). The more recent identification of analogous repetitive sequences in a gram-positive bacterium (BOX elements; Martin et al., 1992) has led to the development of a third set of primers (BOX primers) that can also be used to generate genomic fingerprints of both gram-positive and gram-negative bacteria (Louws et al., 1994; 1995; Versalovic et al., 1994). The distribution of repetitive elements is thought to represent an intrinsic property of the structure of bacterial genomes. Rep-PCR has been used to develop phylogenetic relationships between closely related strains that correlate with groupings discerned using other, more traditional approaches (de Bruijn 1992; Judd et al., 1993; Versalovic et al., 1994; Strain et al., 1994; Louws et al., 1995).

To determine the utility of rep-PCR in fingerprinting and grouping isolates of *Frankia*, a gram-positive actinomycete, genomic DNA from 38 isolates was amplified using REP, ERIC and BOX primers. Electrophoretic separation of the amplification products generated from each primer set revealed complex banding patterns that were highly specific and resolved strains at the sub-species level. Isolates from the same genomic species produced similar fingerprints allowing a facile means to divide isolates into groups.

2. Materials and Methods

Bacterial strains

The *Frankia* strains used in this study and their sources are listed in Table 1. Strains isolated from *Casuarina* were cultured in BAP media (Murry et al., 1984), *Alnus* isolates were cultured in either BAP or M6B media (Callaham, Torrey and del Tredici, 1979) and isolates from the Rhamnaceae were grown using S+Tween media (Lechevalier et al., 1983), as previously described (Murry et al., 1984).

Table 1. *Frankia* isolates utilized

Strain	Registry number	HSG#	GS#	Original host	Geographical origin	Reference [source]*
From *Alnus*						
ARI3	HFP013003	1	1	*Alnus rubra*	Oregon, USA	Berry and Torrey (1979) [A,F]
ACNIag	ULQ0102001007	1	1	*Alnus crispa*	Quebec, Canada	Lalonde (1979)[B]
As2-3	nd	1	1	*Alnus siberica*	Heilongjiang, China	Zhang, Z. (PC)[E]
32-85	LLR013013	1		*Alnus incana*,ssp. *rugosa*	Massachusetts, USA	Lechevalier (1986)[C]
AirI1	LLR01321	1		*Alnus incana* ssp. *rugosa*	Vermont, USA	Lechevalier et al. (1983)[C]
AirI2	LLR01322	1		*Alnus incana* ssp. *rugosa*	Massachusetts, USA	Lechevalier et al. (1983)[C]
32-83	LLR01325			*Alnus incana* ssp. *rugosa*	USA	Beyazova and Lechevalier (1992)[C]
AvsI3	DDB01360610	1		*Alnus viridis* ssp. *sinuata*	Washington, USA	Baker (1987)[C]
Ar24H3	ULF0131024083	1	1	*Alnus rubra*	Orleans, France	Fernandez et al. (1989) [D]
AcoN24d	ULF01010244	1	1	*Alnus cordata*	Orleans, France	Simonet et al. (1985) [D]
Ar2402	ULF0131024152	1	1	*Alnus rubra*	Orleans, France	Fernandez et al. (1989) [D]
Aph1	nd	1		*Alnus hirsuta*	Mt. Chanbai, China	Zhang, Z. (PC)[I]
Ahp2	nd	1		*Alnus hirsuta*	Mt. Chanbai, China	Zhang, Z. (PC)[I]
Ahp3	nd	1		*Alnus hirsuta*	Mt. Chanbai, China	Zhang, Z. (PC)[I]
At39	nd	1		*Alnus tinctoria*	Liaonong, China	Zhang, Z. (PC)[I]
At45	nd	1		*Alnus tinctoria*	Liaonong, China	Zhang, Z. (PC)[I]
At48	nd	1		*Alnus tinctoria*	Liaonong, China	Zhang, Z. (PC)[I]
At79	nd	1		*Alnus tinctoria*	Liaonong, China	Zhang, Z. (PC)[I]
At97	nd	1		*Alnus tinctoria*	Liaonong, China	Zhang, Z. (PC)[I]
AgI1		1		*Alnus glutinosa*	USA	Baker (1987)[I]
From Myricacacea						
CpI1	HFP070101	1	1	*Comptonia pergrina*	Massachusetts, USA	Callaham et al. (1978)[A,E,I]
CpI3	DDB07010310	1		*Comptonia pergrina*	New Hampshire, USA	Baker (1987) [C]

Table 1. Continued

Strain	Registry number	HSG#	GS#	Original host	Geographical origin	Reference [source]*
From Eleagnaceae						
HRX401a	ULF140104001	3	5	*Hippophae rhamnoides*	Ornon, France	Normand et al. (1988) [D]
From Rhamnaceae						
R2	LLR03013	3		*Ceanothus americanus*	Vermont, USA	Lechevalier and Ruan(1984)[C]
CaI1	LLR03011	3		*Ceanothus americanus*	USA	Lechevalier and Ruan(1984)[C]
WgCcI.7	nd	3		*Colletia cruciata*	The Netherlands	Nittayajarn et al. (1990) [F]
From Casuarinaceae						
CcI2	HFP020202	3		*Casuarina cunninhamiana*	Florida, USA	Zhang et al. (1984) [A,E,F,I]
CcI3	HFP020203	2	9	*Casuarina cunninhamiana*	Florida, USA	Zhang et al. (1984) [A,E,F,G,I]
CeF	ORS020607	2	9	*Casuarina equisitifolia*	Florida, USA	Diem and Dommergues (1983)[[B,G,I]
M2	ORS020609	2	9	*Casuarina equisitifolia*	Madagascar	Nazaret et al. (1989) [G]
CeD	ORS020606	2	9	*Casuarina equisitifolia*	Dakar, Senegal	Diem and Dommergues (1983)[[B,G,I]
D11	ORS020602	3		*Casuarina equisitifolia*	Dakar, Senegal	Gauthier et al. (1981) [G]
G12	DDB020210			*Casuarina equisitifolia*	Florida, USA	Lechevalier (1986) [C]
CeSI5	RHB0206606	1/3		*Casuarina equisitifolia*	Florida, USA	Lechevalier (1986) [C]
CjI-82	ORS021001	2		*Casuarina junghuhniana*	Thailand	Tzean et al. (1989) [H]
CgI4	HFP020804	2		*Casuarina glauca*	Egypt	Diem et al. (1983) [G]
AllI1	HFP022801	2		*Alloc. lehmaniana*	Florida, USA	Mansour et al. (1990) [F]
–	ORS022602	2		*Allocasuarina tortulosa*	Australia	Zhang and Torrey 1985)[G]

*Source (and date of receipt) of strains used in this study: [A] J. G. Torrey, Harvard Forest, Petersham, MA (1986); [B] P. Normand, University of Laval, Quebec (1986); [C] ARS Culture Collection, USDA, Peoria, IL (1993); [D] P. Simonet, University of Lyon, France; purified DNA (1993); [E] A. Hirsch, University of California, Los Angeles, CA (1991); [F] D.D. Baker, PanLabs Inc., Bothell, WA (1991); [G] P. Normand, University of Lyon, Fance; purified DNA (1993); [H] H. Berg, Tennessee State University, Nashville, TN (1989); [I] Z. Zhang, Institute of Applied Ecology, Shenyang, China.

DNA extraction

Total DNA was isolated from 2 to 20 ml of 10 to 20-day old axenic cultures by a modification of an earlier technique (Simonet et al., 1985). Filaments were harvested by centrifugation, washed twice in TE (Tris-Cl, 10 mM; EDTA, 1 mM, pH 8.0), and resuspended in 1 ml TES buffer (Tris•Cl, 50 mM; EDTA, 100 mM and sucrose, 15% w/v, pH 8.0). Filaments were centrifuged for 5 min at 12,000 g at 4°C , and the pellet resuspended in 5 volumes of TES with lysozyme at 10 mg/ml and achromopeptidase (Wako Chemicals, Dallas, TX) at 0.5 mg/ml. The pellet was homogenized mechanically and the suspension incubated at 37°C for 30 minutes. Sodium dodecyl sulfate (SDS), 30% w/v, was added at 0.2 volumes, the mixture vortexed briefly and incubated for 15 min at 65°C to break down the cell membrane and to inactivate nucleases. The lysed cells were centrifuged for 10 min at 12,000 g at 40°C and the supernatant extracted with a phenol:chloroform (1:1) mixture saturated with TE. DNA was precipitated with ethanol, washed once in 70% ethanol, resuspended in 50 µl of TE (refered to as crude cell extracts in the text) and, in most cases, further purified using the "Prep-a-Gene" kit (Bio-Rad, Richmond, CA) according to the manufacturers recommendations. DNA was quantified using Hoechst dye 33258 and a DNA fluorometer (Model TKO 100) (Hoefer Scientific Instruments, San Francisco, CA) according to the manufacturers' instructions.

Rep-PCR reactions

The oligonucleotide PCR primers used are shown in Table 2. Primers were synthesized by the Macromolecular Structure, Sequence, and Synthesis Facility at Michigan State University using an Applied Biosystems DNA synthesizer (Model 380B, Foster City, CA). The PCR reactions were performed in Gitschier buffer (Tris-HCl, 67 mM; $MgCl_2$, 6.7 mM; ammonium acetate, 16.6 mM; EDTA, 6.7 µM; β-mercaptoethanol, 30 mM; bovine serum albumin, 170 µg/ml; pH 8.8; Kogan et al., 1987) with DMSO at 10% v:v, 50 pmol each of opposing primers (50 pmol of the one BOX primer), 50 ng of template DNA, 2 units of AmpliTaq DNA polymerase (Perkin-Elmer) and 1.25 mM of each of the 4 dNTPs in a 25 µl reaction mix. PCR amplifications were performed in an automated thermal cycler (Perkin-Elmer DNA Thermal Cycler) with an initial denaturation (95°C for 7 min) followed by 35 cycles of denaturation (94°C, 1 min), annealing (40°C for REP primers and 52°C for ERIC and BOX primers, 1 min) and extension (65°C, 8 min) and a final extension period of 16 min at 65°C. Six to ten µl of PCR products were separated electrophoretically on 1.5 % agarose gels in 0.5X TAE buffer at 4.5–5 V/cm, stained with ethidium bromide and photographed.

Table 2. Nucleotide sequences of PCR Primers

A.	REP (Repetitive Extragenic Palindromic) primers:	
	REP 1R-I	3′ CGGICTACIGCIGCIIII 5′
	REP2-1	5′ ICGICTTATCIGGCCTAC 5′
		(Versalovic et al., 1991)
B.	ERIC (Enterobacterial repetitive intergenic consensus) primers:	
	ERIC1R	3′ CACTTAGGGGTCCTCGAATGTA 5′
	ERIC2	5′ AAGTAAGTGACTGGGGTGAGCG 3′
		(Versalovic et al., 1991)
C.	BOX A1R primer:	
		5′ CTACGGCAAGGCGACGCTGACG 3′
		(Versalovic et al., 1994)
D.	16SrDNA prokaryotic primers: amplifies a 325bp fragment of 16 S rDNA	
	16S rDNA-1	5'GCC TTG GGA GTA CGG CCG CA 3'
		(na# 849, Nazaret et al., 1991)
	16SrDNA-2	5'GGG GCA TGA TGA CTT GAC GT 3'
		(na#1146, Nazaret et al., 1991)

Amplification and DNA sequence analysis of 16S rRNA gene fragments

Genomic DNA from selected Alnus isolates (ie. Ahp 1, Ahp 2, At48, As2-3 and AgI1) were amplified by PCR using primers (Table 2) complimentary to conserved sequences (from nucleic acid 849 and from nucleic acid 1176 in the *E. coli* numbering system) flanking two variable regions of the 16S rDNA gene. The parameters for the PCR reactions were essentially as described by Nazaret et al. (1991) except that PCR reactions (100 µl) were carried out using Gitschier buffer (Kogan et al., 1987) and 10% (v/v) DMSO in the reaction mixture. Sequence comparisons of these regions have been earlier utilized in phylogenetic analysis of *Frankia* spp. (Nazaret et al., 1991; Nick et al., 1992; Cournoyer et al., 1993).

The amplification products generated using the 16S rDNA primers were analyzed by electrophoresis in 2% (w/v) agarose gels to assess the homogeneity and size of the products. The PCR products were purified using the "Wizard PCR Prep" (Promega, Madison, WI) and sequenced in both directions using dye terminators with the automated sequencing apparatus (Model 373A) from Applied Biosystems (Foster City, CA). The sequencing primers were the same as the amplification primers. Sequences were aligned with homologous regions from other species. Aligned sequences were imported into Mega (Kumar

et al., 1993) and Neighbor-Joining (NJ) trees were constructed using the Jukes-Cantor distance method. Bootstrap analyses to assess the reliability of our trees were also performed with a standard of 1000 replicates.

3. Results and Discussion

Genomic fingerprints that resulted from electrophoretic separation of the PCR products of representative *Frankia* strains using BOX and ERIC primers are shown in Fig. 1. For each strain, a unique fingerprint pattern was generated by using the three primer sets shown in Table 2 (REP, ERIC and BOX). For example, compare fingerprints patterns for each strain in Fig. 1A [ERIC primers] vs. 1B [BOX primers]. While BOX-PCR gave very consistent results, amplification of *Frankia* DNA using the REP, and to a lesser extent, the ERIC, primers was sometimes erratic and low amounts of PCR product were formed. Often, genomic DNA preparations that gave characteristic fingerprints using the BOX primers, could not be fully amplified using the REP or ERIC primers (compare lanes 14 and 27 in Fig. 1A vs. 1B). It appears that the poor efficiency of the REP and ERIC-PCR reactions is due to particular impurities within different *Frankia* DNA preparations, since amplification of highly purified DNA was consistently successful using these primers. In some cases, notably when the strains were grown on defined BAP medium, crude cell extracts were successfully amplified using each primer set. However, further purification was usually required for reproducible amplification using REP and ERIC primers when cells were cultured in rich organic media.

The complexity of the fingerprints of these *Frankia* strains generated by rep-PCR, varied with the specific primer used. The BOX primers produced the least complex fingerprint patterns. The average number of bands produced from BOX-PCR amplification of genomic DNA was 10.7 (+/– 2.4 sd, n = 154). The REP and ERIC primers, although derived from a consensus sequence of these elements in the enteric bacteria (Versalovic et al., 1991), produced fingerprints of greater complexity. An average of 13.95 bands (+/– 2.35 sd, n = 71 reactions) were generated using REP primers, while the ERIC primers produced on average 13.8 (+/– 2.9 sd, n = 101) bands.

The range of template DNA concentration that allowed full amplification using the REP, ERIC and BOX primers was found to be quite wide. For each primer set, a fingerprint was generated using purified genomic DNA from CcI3 at concentrations as low as 0.2 ng per µl of reaction mix and up to 10.0 ng per µl (Fig. 2). With each primer set there were very minor variations in the banding pattern associated with template concentration. To avoid problems involving

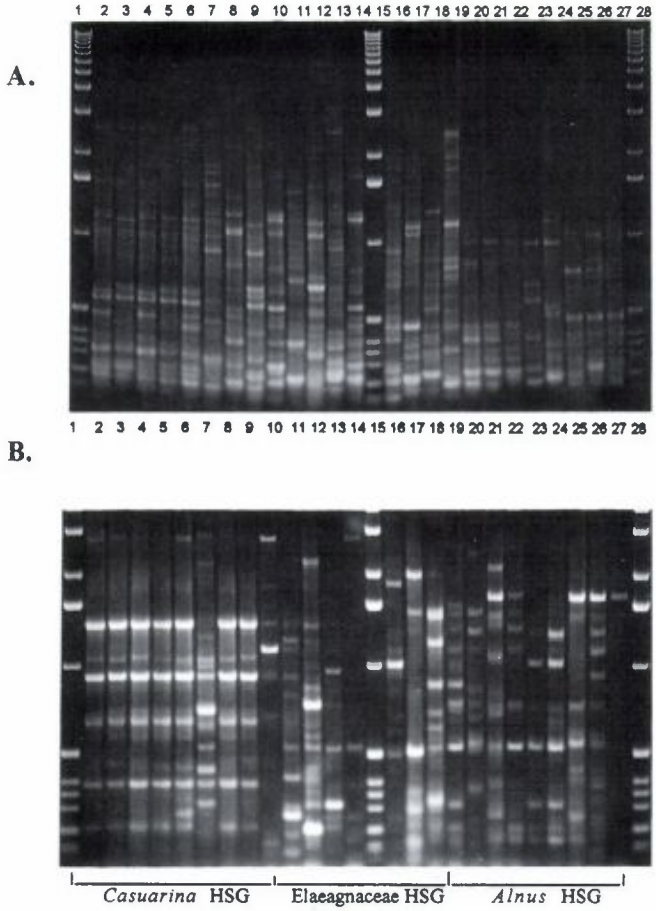

Figure 1. Rep-PCR fingerprint patterns of genomic DNA from *Frankia* sp. strains. The PCR product patterns generated using the BOX primers (A), and using the ERIC primers (B). Lanes: 1 ,15 and 30, 1Kb marker; 2, CcI3; 3, CeD; 4, CeF; 5,CjI-82; 6, Br; 7, M2; 8, CgI4 (A) and ORS0202206 (B); 9, AllI1; 10, CeSI5; 11, CcI2 ;12, G12; 13,LLR020601; 14, D11; 16, HRX401a; 17, CaI1; 18, R2; 19, WgCcI17; 20, 32-85; 21, 32-83; 22, AirI1; 23, AvsI3; 24, CpI3; 25, ACON24; 26, Ar24H3; 27, ArI3.

concentration dependent effects on banding patterns, we routinely use template DNA at a concentration of 2 ng per µl of reaction mix.

The reproduciblity of the PCR reaction was examined by comparing the banding pattern of the PCR products in reactions repeated at least twice using

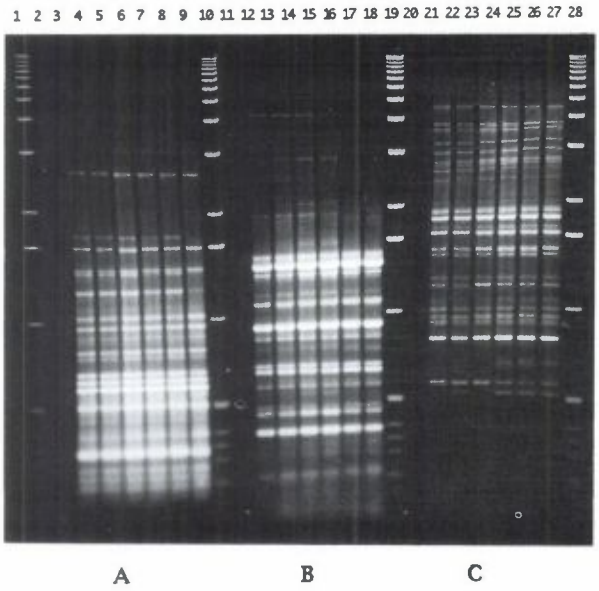

Figure 2. Effect of template concentration on amplification of genomic DNA from *Frankia* strain CcI3 using BOX (A), ERIC (B) and REP (C) primer sets. Lanes 1, 10, 19, 28: 2 µg of 1Kb marker. Lanes 2, 11, 20: no template. Lanes 3, 12, 21: 0.2 ng of template DNA per µl of reaction mix; Lanes 4,13,22: 0.5 ng per µl of reaction mix; Lanes 5,14,23: 1.0 ng per µl; Lanes 6,15,24: 2.0 ng per µl ; Lanes 8,15,26: 5.0 ng per µl ; Lanes 9,18,27: 10 ng per µl.

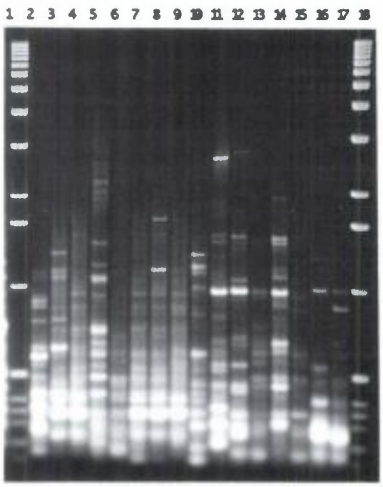

Figure 3. BOX-PCR fingerprints of *Frankia* strains isolated from Alnus spp. Lanes 1 and 18: 1Kb size marker; 2, AgI1; 3, At97; 4, At39; 5, At48; 6, At45; 7, Ahp1; 8, Ahp2; 9, Ahp3; 10, As2-3; 11, CpI3; 12, AvSI3; 13, 32-85; 14, 32-83; 15, ACN1AG; 16, ACoN24d; 17, Ar24O2.

either the same DNA preparation or, in many cases, DNA from separate extracts of the same strain. Fingerprints from individual reactions amplified using the same primer and template DNA were virtually identical. In addition, DNA from stock cultures obtained from different sources and maintained in different labs for more than a decade (strains CeF, CcI3, CpI1 and ArI3; see Table 1) gave reproducibly similar fingerprints.

Visual comparisons of fingerprint patterns generated with each of the three primer sets showed, in general, great diversity among the *Frankia* strains studied here. However, each primer set generated fingerprint patterns of the "typical" *Casuarina* isolates, ie., CcI3, CeD, CeF, CjI-82, Br, AlII1, ORS 0220602, that were remarkably similar; few bands were unique to the fingerprint characteristics of each strain (Fig. 1). Fingerprint patterns of the *Casuarina*-compatible strains M2, CgI4 and CeSI5 (Fig. 1), however, indicated genomic differences from the aforementioned *Casuarina*-infective strains. Earlier, workers observed that strain M2 shows minor 16 S rDNA sequence divergence (Nazaret et al., 1991) and polymorphisms in the *nif* structural genes (Nazaret et al., 1989) from other members of GS9. Fingerprints of CeSI5 and CgI4, *Casuarina*-infective strains which sporulate profusely in culture (Tzean and Torrey, 1989), were similar to each other but were also distinct from those of other typical *Casuarina* isolates (Fig. 1A). The genomic fingerprints of the atypical *Casuarina* isolates (ie., CcI2, G12, LLR020601 and D11) were visibly distinct from each other and bore little resemblance to those of the typical *Casuarina* isolates (Fig. 1).

The atypical *Casuarina* isolates although visibly quite diverse (Fig. 1), tended to cluster with isolates from the Rhamnaceae (ie., CaI1, R2 and WgCcI.17) and *Hippophae* (ie., HrX401a, a representative of GS5; Fernandez et al. 1989), all of which are *Elaeagnus*-compatible (Baker 1987). The great heterogeneity of fingerprint patterns within this group of strains is consistent with earlier studies that indicate *Frankia* from the *Elaeagus* HSG are genetically diverse (An et al., 1985; Jamann et al., 1993), comprising 5 of the 9 *Frankia* genomic species defined by DNA-DNA hybridization (Fernandez et al., 1989). The high similarity values for fingerprint patterns of the typical *Casuarina* isolates also conform to earlier observations that this is a remarkably homogeneous group within the genus (Fernandez et al., 1989; Nazaret et al., 1989; Girgis and Schwenke, 1993) and that the "atypical" isolates are unrelated to "typical" isolates and display greater heterogeneity (Fernandez et al., 1989; Nazaret et al., 1991; Dobritsa 1985; Nittayajarn et al., 1990; Beyazova and Lechevalier, 1992).

Fig. 3 shows BOX-PCR fingerprints of 3 *Alnus* isolates (ACNIag, ACON24d and Ar2402) which are known to be members of the GS1 group (Fernandez et al., 1989) and 12 other *Alnus*-infective strains that have not been assigned to

genomic species. With the exception of one prominent band about 1 kb in size, there were few bands in common amongst members of GS1. The Chinese isolates Aph1, Aph2 and Ahp3 were very similar to each other and to strain At39. Isolate At45 was also found to share many bands with this cluster of four. These five strains were isolated from 2 different *Alnus* species in 2 distinct localities in China, but are never the less highly related in terms of their genomic structure. Strains AgI1, At48, and As2-3 have unique fingerprint patterns.

In order to compare the patterns of relatedness generated by BOX-PCR to those derived from comparisons of partial 16S ribosomal DNA sequences, we determined the nucleotide sequence of a region of the 16S ribosomal gene (from coordinates 849 to 1179, based on the *E. coli* numbering system, Brosius et al., 1981) from five *Alnus* isolates (Fig. 4). This 325 bp region of the 16S ribosomal gene contains two hypervariable domains with 24 variable positions, and has been used in several recent studies to deduce phylogenetic relationships within the genus *Frankia* (Nazaret et al., 1991; Cournoyer et al., 1993; Nick et al., 1992). Nazaret and co-workers (1991) have demonstrated a good correlation between groupings based on partial 16S rRNA and those based on DNA-DNA hybridization (Fernandez et al., 1989). In general, strains belonging to the same genomic species have identical nucleotide sequences in this region of the 16S rRNA gene. Fig. 4 shows the partial 16S rDNA sequences from 5 *Alnus* isolates aligned with the corresponding region from GS 1, 2 and 3, reported by Nazaret et al. (1991). The nucleotide sequence from strain 32–85, which has a BOX-generated fingerprint similar to isolates from GS1, was identical to that of GS1. The BOX-generated fingerprint of strain AgI1 was distinct from those of GS1 strains, yet differed by only one nucleotide in this region of the 16S rRNA gene. BOX-PCR Fingerprints from strains Ahp1 and Ahp2 were nearly identical and these strains had the same nucleotide sequence in this region. Strains As2-3 and At48 each had distinct fingerprints and showed relatively large nucleotide differences compared to other *Alnus* isolates (Fig. 5). These data suggest a good correlation between degrees of relatedness determined by BOX-PCR finger-printing and 16S ribosomal sequence comparisons.

The rep-PCR fingerprinting of *Frankia* isolates described here is a facile and high-resolution method for discriminating strains at the sub-species level. The BOX-primers, derived from repetitive sequences in low G+C, gram-positive eubacteria, provided the most reliable system, allowing reproducible amplification of *Frankia* genomic DNA. Strains shown earlier to be members of the same genomic species by DNA-DNA hybridization (Fernandez et al., 1989) and by 16S rDNA analysis (Nazaret et al., 1991) produced visibly similar rep-PCR-fingerprints. The *Myrica* and "atypical" isolates which are known from other studies to be heterogeneous were also found here to be genetically diverse.

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      1                                                          60
GS1    TTGACGGGGG  CCCGCACAAG  CGGCGGAGCA  TGTGGCTTAA  TTCGATGCAA  CGCGAAGAAC
GS2    ..........  ..........  ..........  ..........  ..........  ..........
GS3    ..........  ..........  ..........  ..........  ..........  ..........
AgI1   ..........  ..........  ..........  ..........  ..........  ..........
32-85  ..........  ..........  ..........  ..........  ..........  ..........
Ahp1   ..........  ..........  ..........  ..........  ..........  ..........
At48   ..........  ..........  ..........  ..........  ..........  ..........
As2-3  ..........  ..........  ..........  ..........  ..........  ..........
      61                                                         120
GS1    CTTACCAGGG  CTTGACATGC  AGGGAAATCT  CGTAGAGATA  CGGGGTCCGT  AAGGGTCCTG
GS2    ..........  T.........  .........C  TCC......G  G.........  ..........
GS3    ..........  T.........  .........C  TCC......G  G.........  ..........
AgI1   ..........  ..........  ..........  ...G......  ..........  ..........
32-85  ..........  ..........  ..........  ..........  ..........  ..........
Ahp1   ..........  ..........  .T.......C  ..........  ........C.  ..........
At48   ..........  ..........  ..C.......  .C........  ........C.  .C........
As2-3  ..........  ..........  ..........  ..........  ..........  ..........
     121                                                         180
GS1    C-ACAGGTGG  TGCATGGCTG  TCGTCAGCTC  GTGTCGTGAG  ATGTTGGGTT  AAGTCCCGCA
GS2    ..........  ..........  ..........  ..........  ..........  ..........
GS3    ..........  ..........  ..........  ..........  ..........  ..........
AgI1   ..........  ..........  ..........  ..........  ..........  ..........
32-85  ..........  ..........  ..........  ..........  ..........  ..........
Ahp1   ..........  ..........  ..........  ..........  ..........  ..........
At48   ..........  ..........  ..........  ..........  ..........  ..........
As2-3  ..........  ..........  ..........  ..........  ..........  ..........
     181                                                         240
GS1    ACGAGCGCAA  CCCTCGTCCT  ATGTTGCCAG  CG--AGTTAT  GTC----GGG  GACTCATAGG
GS2    ..........  ..........  ..........  .......A..  ..........  ..........
GS3    ..........  ..........  ..........  .......CGA  ..........  ..........
AgI1   ..........  ..........  ..........  ..........  ..........  ..........
32-85  ..........  ..........  ..........  ..........  ..........  ..........
Ahp1   ..........  ..........  ..........  ......CC..  ..........  ..........
At48   ..........  ..........  ..........  ........CG.  ..........  ..........
As2-3  ..........  ..........  ..........  ......C...  ..........  ..........
     241                                 275
GS1    AGACTGCCGG  GGTCAACTCG  GAGGAAGGTG  GGGAT
GS2    ..........  ..........  ..........  .....
GS3    ..........  ..........  ..........  .....
AgI1   ..........  ..........  ..........  .....
32-85  ..........  ..........  ..........  .....
Ahp1   ..........  ..........  ..........  .....
At48   ..........  ..........  ..........  .....
As2-3  ..........  ..........  ..........  .....
```

Figure 4. Partial 16S rDNA nucleotide sequences of six *Alnus* isolates aligned with the homologous region from *Frankia* GS 1,2,3 (Nazaret et al., 1991) and from *S. ambofaciens* (Pernodet et al., 1989). Seven gaps were introduced to allow the best alignment. Nucleotide 1 corresponds to nucleotide 921 in the standard *E. coli* numbering system. The sequence data for strains AgI1, 32-85, Ahp1, At48 and As2-3 are from the present study. The nucleotide sequence of Ahp1 and Ahp2 were found to be identical.

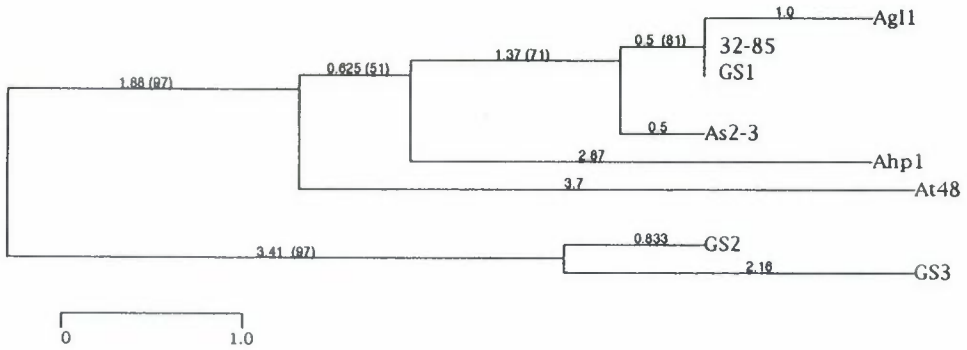

Figure 5. Neighbor joining tree showing the nucleotide differences in the partial 16S rDNA nulceotide sequences shown in Fig. 4. Numbers on branches are nucleotide differences. Numbers in parentheses are p values for boot-strap replicates (1000).

Thus, the results of this study correlate well with other (more traditional) approaches to strain classification. Because the rep-PCR fingerprinting technique is so simple and rapid, and has such high resolution, it may prove useful, as an additional method, to estimate genetic relationships between large numbers of *Frankia* isolates at the sub-species level.

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