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Genetic Diversity among *Frankia* Strains Nodulating Members of the Family Casuarinaceae in Australia Revealed by PCR and Restriction Fragment Length Polymorphism Analysis with Crushed Root Nodules

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DNA extracted directly from nodules was used to assess the genetic diversity of *Frankia* strains symbiotically associated with two species of the genus *Casuarina* and two of the genus *Allocasuarina* naturally occurring in northeastern Australia. DNA from field-collected nodules or extracted from reference cultures of *Casuarina*-infective *Frankia* strains was used as the template in PCRs with primers targeting two DNA regions, one in the ribosomal operon and the other in the *nif* operon. PCR products were then analyzed by using a set of restriction endonucleases. Five distinct genetic groups were recognized on the basis of these restriction patterns. These groups were consistently associated with the host species from which the nodules originated. All isolated reference strains had similar patterns and were assigned to group 1 along with six of the eight unisolated *Frankia* strains from *Casuarina equisetifolia* in Australia. Group 2 consisted of two unisolated *Frankia* strains from *C. equisetifolia*, whereas groups 3 to 5 comprised all unisolated strains from *Casuarina cunninghamiana*, *Allocasuarina torulosa*, and *Allocasuarina littoralis*, respectively. These results demonstrate that, contrary to the results of previous molecular studies of isolated strains, there is genetic diversity among *Frankia* strains that infect members of the family Casuarinacaeae. The apparent high homogeneity of *Frankia* strains in these previous studies probably relates to the single host species from which the strains were obtained and the origin of these strains from areas outside the natural geographic range of members of the family Casuarinaceae, where genetic diversity could be lower than in Australia.

The angiosperm family Casuarinaceae originates in Australia and the islands of the western Pacific (17), where its members are found in environments ranging from tropical rain forests to arid woodlands and saline sites. The family comprises more than 80 species of trees and shrubs in four genera: *Allocasuarina*, *Casuarina*, *Gymnostoma*, and *Ceuthostoma* (48). A number of species of Casuarinaceae are noted for their rapid growth rates on infertile soils and are now grown widely in tropical and subtropical regions for windbreaks, sand dune stabilization, and production of high quality fuelwood (8). The successful establishment and growth of these species on nutrient-deficient soils often depends on the formation of effective actinorhizal root nodules in association with the soil actinomycete *Frankia* sp. These nodules are capable of nitrogen fixation at rates comparable to those found for nodulated legumes (46).

Actinorhizal species are found in at least 24 plant genera in eight angiosperm families. A number of host inoculation groups have been recognized on the basis of the *Frankia* strains that successfully nodulate them (1). In comparison with other inoculation groups, such as *Alnus* and members of the family Elaeagnaceae, few *Frankia* strains infective on their original hosts have been isolated from the Casuarinaceae, despite numerous attempts (39). The low growth rate of these strains has sometimes favored the emergence of contaminating *Frankia* strains called atypical strains (27), which are unable to nodulate their original hosts but can nodulate members of the Elaeagnaceae (9).

The genetic diversity of the infective *Frankia* isolates reported to date for the Casuarinaceae appears low. For example, a high degree of DNA-DNA homology was found between Casuarinaceae-infective *Frankia* strains isolated from geographically distant areas, including Senegal, the United States Brazil, Thailand, and Madagascar (11), and all strains were grouped into a single genomic species, genomic species 9 (11). Further, the use of symbiotic genes as hybridization probes for restriction fragment length polymorphism (RFLP) studies led to the conclusion that a high genetic homogeneity exists between the infective strains isolated from Casuarinaceae (27). Sequencing of hypervariable regions of the 16S and 23S rRNA genes (rDNAs), the IGS (intergenic spacer) between the 16S and 23S rDNAs, and the IGS between the *nifH* and *nifD* genes of the nitrogenase complex showed very high sequence conservation between these strains (14, 21, 26, 40). The apparent high genetic homogeneity of *Casuarina*-infective *Frankia* strains has not been found in other inoculation groups (11, 26). Moreover, if we consider the molecular clock hypothesis of Ochman and Wilson (30), a 1% divergence in the 16S rRNA sequence corresponds to a time elapsed of about 50 million years. Therefore, more diversity than that observed until now would be expected among *Frankia* strains nodulating Casuarinaceae, since this host plant family originated 60 millions years ago, according to the pollen record (17, 20). The full extent of

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TABLE 1. Bacterial strains used in this study

Registry no.	Usual name	Original host plant	Geographical origin	Reference
ORS020606	CeD	*C. equisetifolia*	Senegal	7
ORS020607	CeF	*C. equisetifolia*	United States	7
ORS020608	Br	*C. equisetifolia*	Brazil	8a
ORS020609	M2	*C. equisetifolia*	Madagascar	8a
ORS021001	Cj1-82	*C. junghuniana*[a]	Thailand	10
ORS022602		*A. torulosa*[b]	Australia	6a
HFP020203	CcI3	*C. cunninghamiana*[c]	United States	50
HFP022801	AllI1	*A. lehmaniana*[c]	United States	51

[a] Hybrid of *C. equisetifolia* and *C. junghuniana*.
[b] Inoculated with crushed nodules of *C. cunninghamiana* (6b).
[c] Inoculated with crushed nodules of *C. equisetifolia*.

genetic diversity in this association may not yet have been revealed, even though isoenzymes, and especially esterases, have been found to discriminate the *Frankia* strains isolated from Casuarinaceae (12, 21, 22, 40).

The apparent lack of genetic diversity observed among *Frankia* isolates that are infective on Casuarinaceae could be explained in a number of ways. First, very few strains capable of reinfecting Casuarinaceae have been isolated from root nodules of this family. Thus, isolation procedures may have introduced a bias, leading to the selection of strains that are adapted to culture conditions but are not representative of the full genetic diversity of *Frankia* strains that nodulate Casuarinaceae. Second, most of these reference strains were isolated either directly or indirectly from only one species, *Casuarina equisetifolia*. Those being described as originating from *Casuarina cunninghamiana* and *Allocasuarina lehmaniana* were in fact obtained following artificial inoculation of these host plant species with crushed nodules from *C. equisetifolia* (50, 51). If a particular genotype is associated with this host plant species, it could explain the genetic homogeneity observed so far. Third, most strains were isolated from Casuarinaceae growing in areas where the family is not native. In this situation, the genetic diversity of *Frankia* strains capable of infecting *Casuarina* species could be low and restricted to *Frankia* strains that were introduced as inoculants to improve tree growth (8, 45). Moreover, according to Torrey and Racette (47), inoculation (with surrounding soil or crushed nodules) of introduced Casuarinaceae may have been done purposefully in the early 1900s, at the beginning of the exportation of these trees into tropical and subtropical countries. The genetic homogeneity of isolated *Frankia* strains might then be due to the small number of host plant species exported or to the selection of strains adapted to saprophytic life and thus able to survive outside Australia.

Extraction of DNA directly from nodules and amplification by PCR have previously been used to study the phylogenetic relationships between *Frankia* reference strains and unisolated *Frankia* strains in nodules of *Alnus*, *Coriaria*, and *Dryas* species (4, 26, 28). We have modified these procedures to examine the possible genetic diversity that may occur among *Frankia* strains that nodulate native populations of *Casuarina* and *Allocasuarina* species in tropical northeastern Australia. This approach avoids the bias-inducing process of isolation of *Frankia* strains from field-collected nodules. We extracted DNA directly from nodules and amplified two regions (the ribosomal operon and the *nifD* and *nifK* genes) by PCR. Diversity in amplification products was then determined by RFLP analysis. Previously isolated *Frankia* strains infective on Casuarinaceae were used as reference strains in this study.

MATERIALS AND METHODS

Reference strains and field-collected nodules. Eight *Frankia* strains previously isolated from species of Casuarinaceae (Table 1) were used as reference strains. All were cultured at 28°C in BAP medium (23) with sodium propionate at 10 mM as the sole carbon source. Nodules of two species of *Casuarina* and two of *Allocasuarina* were harvested from one or two trees at a total of 22 sites (Table 2). One nodule was collected from each tree sampled. To differentiate these *Frankia* strains (whose DNA was directly amplified from the nodules) from the isolates used as reference strains, we call them unisolated strains. The designation, host of origin, and collection location for each of these unisolated strains are presented in Table 2.

DNA extraction. Total genomic DNA was recovered from reference strains and purified by using previously described methods (43). DNA was extracted directly from root nodules of unisolated strains, using a modification of the method of Bousquet et al. (5). After surface sterilization of the nodule in 30% (vol/vol) H_2O_2, a single lobe was selected and the outer layers were removed. Each lobe was then crushed with a sterile plastic pestle in 300 μl of extraction buffer (100 mM Tris [pH 8], 20 mM EDTA, 1.4 M NaCl, 2% [wt/vol] cetyltrimethyl ammonium bromide, 1% [wt/vol] polyvinyl polypyrrolidone) in a 1.5-ml Eppendorf tube. The homogenate was incubated at 65°C for 60 min and centrifuged for 10 min at 7,000 × *g* to remove plant debris and polysaccharides trapped by the polyvinyl polypyrrolidone (32). The supernatant was then extracted with an equal volume of chloroform-isoamyl alcohol (24:1, vol/vol) and centrifuged at 13,000 × *g* for 15 min. DNA from the aqueous phase was precipitated with the addition of 2 volumes of ethanol, and the sample was centrifuged at 13,000 × *g* for a further 30 min. The resulting DNA pellet was washed with 70% (vol/vol) ethanol, vacuum dried, and solubilized in 10 μl of ultrapure water.

PCR amplification. Two DNA regions were amplified: one comprising the 3′ end of the 16S rDNA, the IGS, and the 5′ part of the 23S rDNA (i.e., *rrn* region), and another comprising the 3′ end of the *nifD* gene, the IGS, and the 5′ part of the *nifK* gene (i.e., *nif* region). Amplification of the *rrn* region was performed with primers FGPS989ac (5′-GGGGTCCGTAAGGGTC-3′) (3) and FGPL132′ (5′-CCGGGTTTCCCCATTCGG-3′) (33). The *nif* region was amplified with primers FGPD807 (5′-CACTGCTACCGGTCGATGAA-3′) and FGPK700′ (5′-CGAGGTAGGTCTCGAAACCGG-3′) as previously reported by Jamann et al. (16). PCR amplifications were performed under standard conditions described by Simonet et al. (44). Amplifications were in a total volume of 50 μl including 1 μl of template DNA and 0.2 μM each primer and were run for 35 cycles (95°C for 1 min, 50°C [*rrn* region] or 55°C [*nif* region] for 1 min, and 72°C for 2 min). To check the efficiency of amplification, 1/10 of the PCR products was visualized by electrophoresis on a 0.8% (wt/vol) agarose gel at 30 V cm^{-1} for 2 h and then stained with ethidium bromide (1 μg ml^{-1}).

Restriction analysis of PCR products. Five-microliter aliquots of PCR products were digested by 5 U of the respective restriction endonuclease in a 20-μl final volume for 1 h at the optimal temperature suggested by the manufacturer. The following restriction endonucleases were used: *Bso*FI, *Bst*NI, *Sau*3AI, *Scr*FI (all from Ozyme, Montigny le Bretonneux, France), *Nci*I, and *Sau*96I (both from Boehringer, Meylan, France) for the *rrn* region and *Alu*I, *Cfo*I, *Nci*I, *Rsa*I (all from Boehringer), *Bst*UI, and *Sau*3AI (both from Ozyme) for the *nif* region. Restricted fragments were separated by electrophoresis on a horizontal 4% (wt/vol) NuSieve (FMC, Rockland, Maine) agarose gel containing ethidium bromide (1 μg ml^{-1}). Gels were run at 5 V cm^{-1}, and profiles were visualized with a transilluminator at 312 nm and photographed with Ilford FP4 film. A kilobase ladder (Gibco BRL, Eragny, France) was used as a molecular size standard.

Map locations of restriction sites in the *rrn* region. Each strain was assigned to a composite genetic group, using a combination of restriction patterns obtained with the seven and six endonucleases used on the *rrn* and *nif* regions, respectively. The map location of each restriction site was determined only for

TABLE 2. Unisolated strains used in this study

Tree species	Locality	Designation of unisolated strain	South latitude, east longitude
C. equisetifolia	Cow Bay	CeCB	16°14′, 145°28′
	Garners Beach	CeGB	17°48′, 146°06′
	Mount Low Beach	CeMLB	19°10′, 146°42′
	Pallarenda	CePall	19°12′, 146°44′
	Saunders Beach	CeSB	19°11′, 146°37′
	Wangetti Beach[a]	CeWg1, CeWg2	16°41′, 145°35′
	Wongalinga Beach	CeWB	17°56′, 146°06′
C. cunninghamiana	Bakerville Creek	CcBK	17°22′, 145°15′
	Herbert River Crossing	CcHRC	18°15′, 145°22′
	Jourama Falls[b]	CcJF	18°52′, 146°07′
	Old Chinaman Creek	CcOCC	17°26′, 145°16′
	Tinaroo Creek	CcTC	17°39′, 145°17′
	West Watsonville	CcWW	17°28′, 145°16′
A. torulosa	Atherton Rifle Range[a]	AltARR1, AltARR2	17°18′, 145°29′
	Bluewater	AltBW	19°11′, 146°35′
	North of Lawyer Creek	AltNLC	17°55′, 145°09′
A. littoralis	Atherton	AllAT	16°25′, 145°24′
	Kuranda[a]	AllKur1, AllKur2	16°50′, 145°39′
	West of Herberton	AllWH	17°23′, 145°23′

[a] Two trees were sampled.
[b] Aerial nodule.

the *rrn* region because no sequence data were available for the *nif* region. Map locations were inferred from the known sequence of *Casuarina*-nodulating *Frankia* strain ORS020606 (29 [GenBank accession number M58598]). The IBI-MacVector software (IBI, New Haven, Conn.) gave, for this sequence and for each enzyme, the locations of the restriction sites and the sizes of the restriction fragments expected. Comparison of restriction patterns obtained from unknown strains with expected patterns of the reference strain ORS020606 allowed inference, from the size of restriction fragments, of the location of restriction sites in the *rrn* region of unknown strains. Fragment sizes were calculated by using RFLP-Mac software (15) based on the algorithm of Schaeffer and Sederoff (41).

RESULTS AND DISCUSSION

PCR-RFLP analysis of DNA extracted from crushed nodules. The procedure described above allowed extraction of DNA from a single nodule lobe with sufficient purity to be amplified. As expected from the complete sequencing of the ribosomal operon of *Casuarina*-infective *Frankia* strain ORS020606, a 1,067-bp fragment was obtained in the *rrn* region of each sample tested. PCR products from the *nif* region (which has not been sequenced) yielded a fragment of 1,400 bp with all template DNAs tested.

Frankia-specific primers were needed, as the DNA obtained from nodule lobes was composed of a mixture of DNA from the host plant, the endosymbiont, and possibly contaminating bacteria that were not eliminated by the surface sterilization and peeling process. For the *rrn* region, primer FGPS989ac, developed by Bosco et al. (3), was used to obtain specific amplification of *Frankia* DNA from Casuarinaceae and *Alnus* inoculation groups. This primer did not amplify DNA from uninfective, atypical *Frankia* strains genetically related to the Elaeagnaceae inoculation group (26) which can be present in Casuarinaceae nodules. For the *nif* region, primer FGPK700′ was specific enough to avoid amplification of *Alnus*-compatible *Frankia* strains as previously reported (16, 24).

Amplification products were analyzed by RFLP, which has been found to permit rapid bacterial identification (18, 35). Moreover, this method can also be used to study the genetic structures of populations or for epidemiological purposes (31, 33). For *rrn* and *nif* regions, each enzyme gave two to four different restriction patterns, depending on its discriminating power (Table 3). For each DNA region, isolated and unisolated strains were grouped when they shared the same combination of restriction patterns. Five distinct PCR-RFLP groups were recognized on the basis of restriction patterns for the *rrn* and *nif* regions (Table 3). The nitrogenase (*nif*) and 16S rRNA genes fulfill coherent taxonomic criteria, having coevolved with the bacteria which carry them (13). Our results strengthen this hypothesis, as the groupings based on the *nif* and *rrn* DNA regions were composed of the same strains. For each DNA region, two restriction enzymes were sufficient to rapidly discriminate the five PCR-RFLP groups: *Nci*I and *Scr*FI for the *rrn* region (Fig. 1) and *Bst*UI and *Cfo*I for the *nif* region (Fig. 2).

Distribution of PCR-RFLP groups. All reference *Frankia* strains had similar patterns and were grouped together (group 1), whereas the unisolated strains from Australia were distributed across PCR-RFLP groups 1 to 5 (Table 3). Of particular interest is the consistent relationship between PCR-RFLP groups and the species of Casuarinaceae from which the *Frankia* strains were derived in the field. Two groups (groups 1 and 2) contained *Frankia* strains from *C. equisetifolia* only. The majority of the unisolated strains from *C. equisetifolia* were in group 1, with only two strains in group 2. There was one PCR-RFLP group for *Frankia* strains from each of the other three species of Casuarinaceae. Group 3 comprised all of the unisolated *Frankia* strains from *C. cunninghamiana*, including one (CcJF) derived from an aerial nodule from which no successful isolation has been achieved to date (34). The remaining two groups consisted of *Frankia* derived from the nodules of the two *Allocasuarina* species. Group 4 included all unisolated strains from *Allocasuarina torulosa*, whereas group 5 included the unisolated strains present in *Allocasuarina littoralis* nodules.

These results demonstrate that there is genetic diversity among *Frankia* strains infective on Casuarinaceae and that in native populations, the *Frankia* strain occurring in nodules seems to be host species dependent. Such host species dependence was not found when a similar PCR-RFLP method was used on the *nif* region of *Frankia* strains infective on Elaeagnaceae (16). However, recent research on the taxonomic relationships of strains in the genus *Frankia* has indicated that

TABLE 3. PCR-RFLP groups revealed by combination of restriction patterns of *rrn* and *nif* regions[a]

Strain	Restriction pattern of *rrn* region digested with:							Restriction pattern of *nif* region digested with:						PCR-RFLP group
	*Bso*FI	*Bst*NI	*Msp*I	*Nci*I	*Sau*3AI	*Sau*96I	*Scr*FI	*Alu*I	*Bst*UI	*Cfo*I	*Nci*I	*Rsa*I	*Sau*3AI	
Reference														
ORS020606	BF1	BT1	M1	N1	SA1	S1	SF1	A1	BU1	C1	N1	R1	SA1	1
ORS020607	BF1	BT1	M1	N1	SA1	S1	SF1	A1	BU1	C1	N1	R1	SA1	1
ORS020608	BF1	BT1	M1	N1	SA1	S1	SF1	A1	BU1	C1	N1	R1	SA1	1
ORS020609	BF1	BT1	M1	N1	SA1	S1	SF1	A1	BU1	C1	N1	R1	SA1	1
ORS021001	BF1	BT1	M1	N1	SA1	S1	SF1	A1	BU1	C1	N1	R1	SA1	1
ORS022602	BF1	BT1	M1	N1	SA1	S1	SF1	A1	BU1	C1	N1	R1	SA1	1
HFP020203	BF1	BT1	M1	N1	SA1	S1	SF1	A1	BU1	C1	N1	R1	SA1	1
HFP022801	BF1	BT1	M1	N1	SA1	S1	SF1	A1	BU1	C1	N1	R1	SA1	1
Unisolated														
CeCB	BF1	BT1	M1	N1	SA1	S1	SF1	A1	BU1	C1	N1	R1	SA1	1
CeGB	BF1	BT1	M1	N1	SA1	S1	SF1	A1	BU1	C1	N1	R1	SA1	1
CePall	BF1	BT1	M1	N1	SA1	S1	SF1	A1	BU1	C1	N1	R1	SA1	1
CeSB	BF1	BT1	M1	N1	SA1	S1	SF1	A1	BU1	C1	N1	R1	SA1	1
CeWg2	BF1	BT1	M1	N1	SA1	S1	SF1	A1	BU1	C1	N1	R1	SA1	1
CeWB	BF1	BT1	M1	N1	SA1	S1	SF1	A1	BU1	C1	N1	R1	SA1	1
CeMLB	BF1	BT2	M2	N2	SA2	S2	SF2	A2	BU2	C1	N1	R2	SA1	2
CeWg1	BF1	BT2	M2	N2	SA2	S2	SF2	A2	BU2	C1	N1	R2	SA1	2
CcBK	BF1	BT2	M2	N3	SA2	S2	SF3	A2	BU2	C2	N2	R2	SA2	3
CcHRC	BF1	BT2	M2	N3	SA2	S2	SF3	A2	BU2	C2	N2	R2	SA2	3
CcJF	BF1	BT2	M2	N3	SA2	S2	SF3	A2	BU2	C2	N2	R2	SA2	3
CoOCC	BF1	BT2	M2	N3	SA2	S2	SF3	A2	BU2	C2	N2	R2	SA2	3
CcTC	BF1	BT2	M2	N3	SA2	S2	SF3	A2	BU2	C2	N2	R2	SA2	3
CcWW	BF1	BT2	M2	N3	SA2	S2	SF3	A2	BU2	C2	N2	R2	SA2	3
AltARR1	BF2	BT2	M1	N1	SA3	S3	SF4	A3	BU3	C3	N3	R3	SA1	4
AltARR2	BF2	BT2	M1	N1	SA3	S3	SF4	A3	BU3	C3	N3	R3	SA1	4
AltBW	BF2	BT2	M1	N1	SA3	S3	SF4	A3	BU3	C3	N3	R3	SA1	4
AltNLC	BF2	BT2	M1	N1	SA3	S3	SF4	A3	BU3	C3	N3	R3	SA1	4
AllAT	BF1	BT3	M3	N4	SA4	S4	SF4	A4	BU4	C4	N4	R4	SA3	5
AllKur1	BF1	BT3	M3	N4	SA4	S4	SF4	A4	BU4	C4	N4	R4	SA3	5
AllKur2	BF1	BT3	M3	N4	SA4	S4	SF4	A4	BU4	C4	N4	R4	SA3	5
AllWH	BF1	BT3	M3	N4	SA4	S4	SC4	A4	BU4	C4	N4	R4	SA3	5

[a] The different restriction patterns are noted with different numbers following the first letter of the enzyme. If enzymes shared the same first letter, the last letter was added.

a relationship may exist between the original host plant and the grouping of isolated strains (2, 17). Some cross-inoculation studies of the Casuarinaceae, using both crushed nodule inocula and cultured strains, have suggested that host specificity may exist between *Frankia* strains and the genera *Casuarina* and *Allocasuarina* (36, 38, 42). Our results tend to support these observations of the existence of ecological host specificity.

Map locations of discriminating restriction sites in the *rrn* region. Almost all of the differences in restriction patterns among the five PCR-RFLP groups were due to divergence of restriction sites in the IGS between 16S and 23S rDNAs (Fig. 3). The expected map of discriminating restriction sites is presented in Fig. 3 for the most discriminative endonucleases, *Nci*I and *Scr*FI. The 16S part of the *rrn* region was of little use in discriminating PCR-RFLP groups. Only *Sau*96I provided a discriminating difference in restriction sites. This finding confirms the discriminating power of the 16S-23S rDNA IGS on strains which are not differentiated by 16S rDNA sequencing (25). If isolation of strains from groups 2 to 5 is possible, it would be interesting to study their DNA-DNA homology with Casuarinaceae-infective *Frankia* strains belonging to previ-

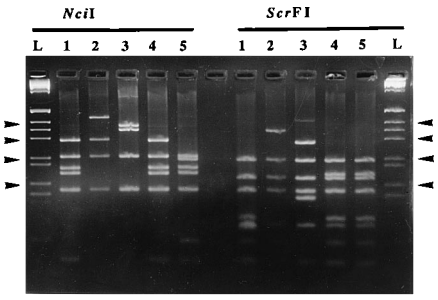

FIG. 1. Restriction patterns of the *rrn* region after digestion with *Nci*I or *Scr*FI. Lanes 1 to 5, PCR-RFLP groups 1 to 5; lane L, 1-kb ladder. Arrowheads indicate bands of 394, 298, 220, and 154 bp.

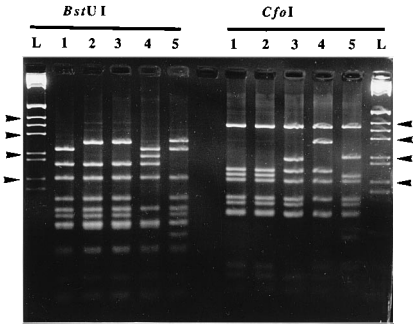

FIG. 2. Restriction patterns of the *nif* region after digestion with *Bst*UI or *Cfo*I. Lanes 1 to 5, PCR-RFLP groups 1 to 5; lane L, 1-kb ladder. Arrowheads indicate bands of 394, 298, 220, and 154 bp.

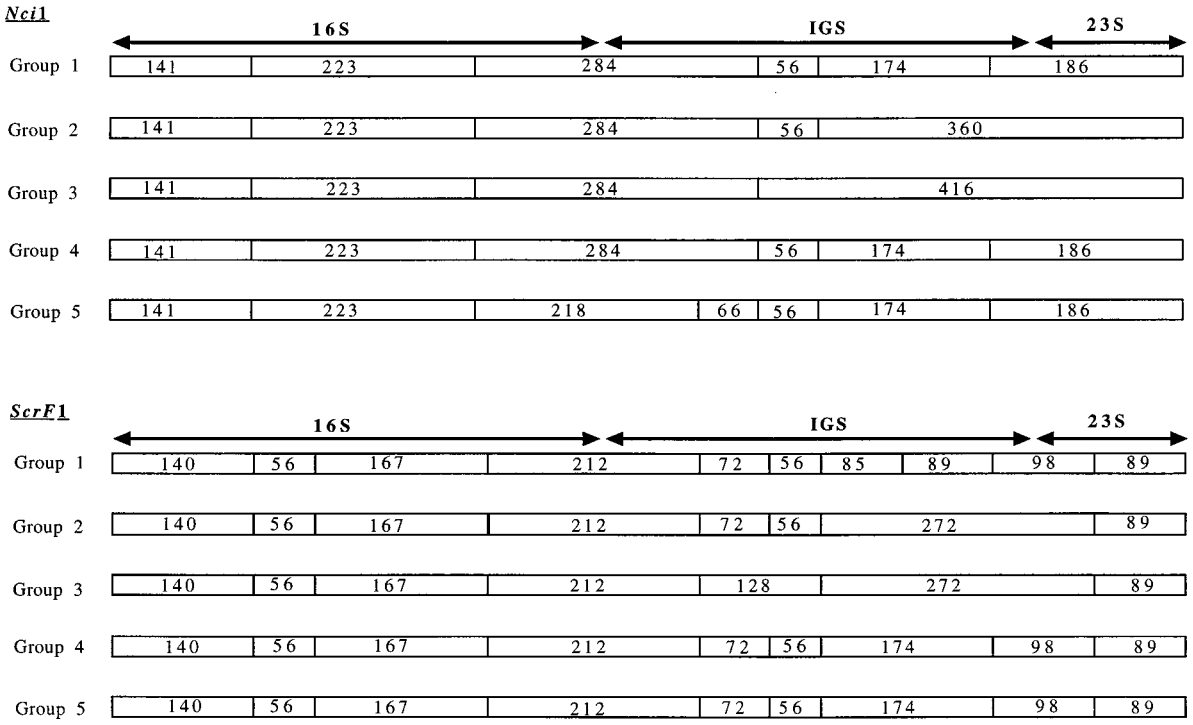

FIG. 3. Mapping of discriminative restriction sites in the *rrn* region for *Nci*I and *Scr*FI. 16S, 23S, and IGS regions are indicated by arrows. Numbers indicate the sizes of restriction fragments.

ously reported genospecies 9 (11), which were found to be part of group 1 during our study. According to Nazaret et al. (26), strains belonging to the same genomic species had the same partial 16S rRNA sequences in the hypervariable region at coordinates 900 to 1200. Our data rely only on PCR-RFLP analysis, which is less accurate than sequencing. The fact that all strains used in this study presented the same PCR-RFLP patterns in the 16S part of the *rrn* region presumably indicates that they belong to genomic species 9.

Genetic homogeneity of reference strains. All reference strains clustered in group 1, along with the majority of unisolated strains from *C. equisetifolia* collected in Australia. This result shows that the discriminating power of the PCR-RFLP method that we used was comparable to those of DNA-DNA hybridization, RFLP, and sequencing methods used in previous studies of these closely related *Frankia* strains (11, 26, 27, 40). It also explains the reported genetic homogeneity of infective *Frankia* strains isolated from Casuarinaceae, as most of these originated either directly or indirectly from a single species, *C. equisetifolia* (Table 1). This result confirms that Australian nodules could have been the origin for the dissemination of this group of strains when seeds (and surrounding soil) of the host plants (mainly *C. equisetifolia*) were exported at the end of the 19th century (47).

Reference strain ORS022602 was reported to have originated from *C. cunninghamiana* nodules collected in Australia (Table 1). Curiously, this strain clustered in group 1, not in group 3 with all the unisolated *C. cunninghamiana* strains from Australia. There are a number of explanations for this apparent anomaly. Perhaps the species of origin was misidentified in the field or was from a planted, unnatural population. It is also possible that the cultivation medium had selected a strain of group 1 which coexisted in the nodule with unisolated strains of *Frankia* from group 3. In this respect, Reddell and Bowen (37) have shown that field-collected nodules from Casuarinaceae occasionally contain more than one *Frankia* strain. It should be noted that in our study, none of the restriction patterns contained a detectable mixture of bands, indicating that different PCR-RFLP groups may have been present in the nodule.

The fact that none of the reference strains from *C. equisetifolia* was clustered in group 2 is also of interest. This could be due to the small number of isolates available for testing, or it may reflect a low genetic diversity in populations of infective *Frankia* strains in areas in which *Casuarina* species have been introduced. If the latter applies, there may be significant opportunities for selecting and introducing more effective nitrogen-fixing strains of *Frankia* into areas where *C. equisetifolia* is grown. Lower diversity of endosymbionts in soils in areas outside their natural range has been shown for the leguminosae (49).

Conclusion. This study provides the first clear demonstration of genetic diversity among *Frankia* strains nodulating Casuarinaceae. Of particular interest is the apparent ecological host specificity that was found in the unisolated nodules collected from native *Casuarina* and *Allocasuarina* species in Australia. It would be interesting to determine how representative these groups are with additional sampling. This finding has implications both for inoculation programs (to ensure that a diverse range of *Frankia* strains suited to particular hosts is available for use in areas in which Casuarinaceae are introduced) and for our basic understanding of the recognition processes in this plant-microbe symbiosis. For example, some Casuarinaceae species, such as *A. torulosa* and *A. littoralis*, often grow in close proximity in native forests in Australia. Do these host species select preferred *Frankia* strains from a background soil community that contains representatives of a number of the PCR-RFLP groups? Alternatively, it could be just a

matter of which strain is growing in the vicinity of the plant in question. For the former possibility, the mechanisms that underlie this preference remain to be determined. Understanding the nature of such a process may provide new insights that will be useful for managing the symbiosis to increase the effectiveness of nitrogen fixation in plantations of Casuarinaceae. Of course, isolation of strains from various host plant species, which was recently performed (39), will be necessary for studying such plant-microorganism interactions.

Our findings also suggest an explanation for the lack of diversity observed among *Frankia* strains in previous studies. Two factors besides the isolation step are probably involved. First, most available isolated *Frankia* strains that are infective on Casuarinaceae originated from one host species, *C. equisetifolia*. Our study has shown that in Australia, nonisolated strains associated with this species were mainly in group 1, together with reference strains. Sampling from only one host plant species could have biased the sample, resulting in genetically similar strains. Second, these strains were generally isolated from nodules collected in areas outside the natural geographic range of the Casuarinaceae, mainly Australia. While two groups were defined among *Frankia* strains nodulating *C. equisetifolia* in Australia, isolated strains from outside Australia fell in only one group. As a consequence, these strains would represent an unintentionally biased *Frankia* population, which explains the lack of diversity found. This hypothesis could be confirmed by using the PCR-RFLP technique that we have developed for use on nodules collected outside Australia from several host plant species.

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