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Genome diversity of *Frankia* strains from different origins and host plants by AFLP analysis

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Abstract
Twenty three selected, culturable, *Frankia* strains belonging to different geographical origins and host-specificity groups were clustered on the basis of their AFLP patterns. A significant diversity was found among strains. The majority of strains studied corresponding to the three host-specificity groups *Alnus*, *Casuarina* and *Elaeagnus* (*Frankia* clusters 1 and 3) fell into the cluster group B. Within this group, two composite subgroups were found, B1 consisting of *Frankia* clusters 1 and 3. *Frankia* strains nodulating the Myricaceae family were not found closely related, although they were clustered within subgroup B1, CcI3 and Ce20 strains both *Casuarina* microsymbionts clusters in different subgroups, A and B1, respectively. The concordance between the genospecies previously described show that the grouping may reflect the taxonomic structure of the genus *Frankia*.

Keywords: *Frankia*, genome diversity, AFLP analysis

1. Introduction

The actinomycete of the genus *Frankia* is a nitrogen-fixing symbiont associated to some angiosperms denominated actinorhizal plants. These plants are able to grow in marginal soils mostly due to this interaction. Identification of *Frankia* has been historically based on molecular methods, characteristic traits such as the presence of a plasmid (Simonet et al., 1998) as well as functional targets such as nitrogenase genes have successfully been used (Baker and Mullin, 1994; Mirza et al., 1993; Prin et al., 1993; Simonet et al., 1991). A differentiation of the genus *Frankia* from other nitrogen-fixing organisms was achieved by using the size of the intergenic spacer (IGS) between the nitrogenase *nif*H and *nif*D (*nif*H-D) gene (Simonet et al., 1991) or *nif*D and *nif*K (*nif*D-K) (Jamann et al., 1993). To differentiate the genus from other actinomycetes, the glutamine synthetase II (*gln*II) gene as the target has been useful (Cournoyer and Normand, 1994).

At a low taxonomic level the identification of *Frankia* was achieved by restriction fragment length polymorphism (RFLP) analysis of total DNA and subsequent hybridization with a *nif*D-H gene probe (Baker and Mullin, 1994), and also by the *gln*II gene (Cournoyer and Normand, 1994), the IGS of the 16S-23S rRNA operon (Maggia et al., 1992), the IGS of *nif*H-D (Cournoyer and Normand, 1994), and the IGS of *nif*D-K (Jamann et al., 1993).

Phylogenetic characterization of microorganisms has been done on the basis of sequence comparison of all three rRNAs (5S, 16S and 23S rRNA) (Woese, 1987; Woese et al., 1990), though only 16S and 23S rRNA molecules are of appropriate size for broad phylogenetic analysis. Ribosomal RNA molecules or their genes are almost perfect targets for the detection of microorganisms by molecular methods. They are ubiquitously distributed, functionally and evolutionary homologous in all organisms, and extremely conserved in secondary and tertiary structure.

Remarkable variation in this region was observed within the genus *Frankia* whose sequences of strains belonging to the *Alnus* host infection group (Hönerlage et al., 1994; Roller et al., 1992) and those of strains belonging to other

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host infection groups (Hönerlage et al., 1994).

These results not only confirmed the classification of the strains into host infection groups but made it possible to differentiate among those belonging to the *Casuarina*, the *Elaeagnus* and the *Alnus* host infection groups, respectively. While *Frankia* strains belonging to the *Casuarina* and the *Elaeagnus* host infection groups were characterized by only small sequence variation, strains belonging to the *Alnus* host infection group could be separated roughly into four subgroups, three containing typical nitrogen-fixing strains and a fourth one containing only non-nitrogen-fixing strains (Hönerlage et al., 1994). Utilizing the analysis of the same genes, recently, a further subgroup containing typical nitrogen-fixing *Frankia* strains of the *Alnus* host infection group was proposed (Maunuksela et al., 1999).

A differentiation of *Frankia* at a lower taxonomic level based on RFLP analysis of the 16S rDNA permitted discrimination among different *Frankia* genomic groups (McEwan et al., 1994). At the sub-species level, arbitrary primers (Sellstedt et al., 1992), as well as intervening sequences of *Frankia* genomic DNA amplified by PCR with oligonucleotide primers targeting consensus motifs of repetitive elements common to prokaryotic genomes such as RAPD, REP or ERIC elements (rep-PCR) (Maunuksela et al., 1999; Murry et al., 1997; Murry et al., 1995; Pérez et al., 1999; Valdés et al., 2001), provided an effective means to distinguish closely related strains.

PCR has therefore been employed mainly for the phylogenetic analysis and differentiation among closely related *Frankia* species of isolates and uncultured endophytes in nodules. The assignment of isolates or uncultured nodule endophytes to the genus *Frankia* is usually based on PCR-assisted sequence retrieval, subsequent cloning and sequencing of the amplicons, and finally a comparative analysis of sequences derived from pure cultures or uncultured populations with those of existing in reference collections. Comparative sequences analysis of PCR-amplified 16S rDNA of pure cultures of *Frankia*, as well as of uncultured endophytes in root nodules led to the emmendation of the family Frankiaceae to contain only the genus *Frankia* (Normand et al., 1996).

However, several of these studies have only been focused on a small number of target groups with no inclusion of plant host groups belonging to different geographical areas. With the aim to compare the previous results of the genome characterization of *Frankia* we report here the results of Fluorescent AFLP analysis of 23 *Frankia* strains from diverse origin plant hosts.

This communication reinforces the results by Normand (2002) and by Huguet et al. (2001) by documenting high levels of similarity within homologous strains and high levels of variability within other strains considered previously not specific, respectively.

2. Materials and Methods

Frankia *strains and cultural conditions and DNA extraction*

Frankia strains used in this study are listed in Table 1. *Frankia* strains were grown in modified BAP medium (Svetlana, 1998). Genomic DNA from each isolate was extracted from a culture by using the Genomic DNA purification protocol from PROMEGA as previously described (Barnes, 1989), and modified by us. The purity and quality of the DNA were determined by UV absorption with a UV spectrophotometer (Beckman Instruments, Inc., Fullerton, CA).

AFLP analysis of Frankia *genomic DNA*

One microgram of genomic DNA was used for AFLP analysis. The procedures employed for template DNA preparation, PCR amplification, and gel electrophoresis are described elsewhere (McClenaghan et al., 1984). Restriction enzymes *Eco*RI and *Mse*I were utilized in combination to generate template DNAs for AFLP analysis. The primer and adapter sequences used for AFLP were:

*Eco*RI+A = 5'-GACTGCGTACCAATTTC/A,
*Eco*RI+C = 5'-GACTGCGTACCAATTC/C,
*Mse*I+0 = 5'-GATGAGTCCTGAGTAA,
*Mse*I+T = 5'-GATGAGTCCTGAGTAA/T.

Table 1. *Frankia* strains used and their sources.

Strain	Original host	FC	Geographical origin
ACN1	*Alnus crispa*	1	Canada
ACN14a	*Alnus cordata*	1	Canada
AirI1	*Alnus incana* spp. *rugosa*	1	Vermont, USA
AirI3	*Alnus rubra*	1	Oregon, USA
AvcI1	*Alnus viridis* spp. *crispa*	1	Ontario, Canada
B16219	*Elaeagnus angustifolia*	3	Wyoming, USA
CcI3	*Casuarina cunninghamiana*	1	Florida, USA
CeSI5	*Casuarina equisetifolia*	1	USA
ChI1	*Colletia hystrix*	3	Chile
CpI1	*Comptonia peregrina*	1	USA
DC2	*Datisca* sp.	4	New Zealand
DC12	*Datisca cannabina*	1	USA
EANIpec	*Elaeagnus angustifolia*	3	Ohio, USA
EuI1	*Elaeagnus umbellate*	3	Massachusetts, USA
IPNCe20	*Casuarina equisetifolia*	1	Miraflores, Edo. de Mexico
M3	*Myrica cerifera*	1	El Esquilón, Ver, Mexico
PtI1	*Pusrshia tridentata*	4	New Zealand
ReI6	*Retanilla ephedra*	3	Chile
S14	*Shepherdia argentea*	3	Samara, Russia
TqI15	*Talguenea quinauinervia*	3	Chile
TtI11	*Trevoa trinervis*	3	Chile
TtI12	*Trevoa trinervis*	3	Chile
TtI42	*Trevoa trinervis*	3	Chile

AFLP analysis

Frankia total DNA was digested with the restriction enzymes *Eco*RI and *Mse*I. Simultaneously, generally applicable double-stranded oligonucleotide adaptors, composed of a unique sequence and an overhang complementary to the restriction sites in the genomic digest, were ligated to the restriction fragments for 16 h at 37°C. This mixture consisted of 250 ng of *Frankia* DNA, 1 U of *Eco*RI, 1 U of *Mse*I, 2 pmol of *Eco*RI adaptor, 2 pmol of *Mse*I adaptor, 1 ml of ligase buffer (50 mM Tris-HCl [pH 7.5] containing 0.6 U of T4 DNA-ligase, and H_2O up to 25 ml.

After restriction and ligation, the DNA was diluted with H_2O to a final volume of 100 µl and stored at –20°C until it was further analyzed. The ligation products, now having unique sequences from the adaptors at both sites, were amplified by PCR with adaptor-specific primers.

The initial PCR was performed as follows: 5 µl of the ligation products, 40 µl of primer mix, 5 µl of 10X PCR buffer (Mg^{+2}) and 1 U of Taq DNA polymerase in a thermocycler iCycler (BioRad®) using an initial denaturation step at 94°C for 3 min, followed by 20 cycles consisting of denaturation at 94°C for 30 s, annealing at 56°C for 60 s, and extension at 72°C for 60 s. The selective PCR was performed as follows: 5 µl of the first PCR reaction, 2 µl of 10X PCR buffer (Mg^{+2}), 5 µm of 5'*Eco* and 3'*Mse* primers, and 1U of *Taq* DNA polymerase. The PCR conditions were by using an initial denaturation step at 94°C for 3 min, followed by 13 cycles consisting of denaturation at 94°C for 30 s, then, the annealing temperature was decreasing by 0.7°C per cycle (touchdown) and extension at 72°C for 60 s.

The primers had, in addition to the adaptor-specific sequence (*Eco*+A and *Mse*+T) or one (*Eco*+C and *Mse*-0). This selective nucleotide allowed the amplification of only a subset of restriction fragments when the banding pattern, obtained after PCR with primers without a selective nucleotide, was too complex. The *Eco*+C primer or the *Eco*+A primer was fluorescently labeled with IRD 800.

The PCR products were separated in a denaturing (6.5%) polyacrylamide sequencing gel with a Li-Cor 4202G automated sequencer. The running conditions were: 1500 V, 40 W, 40 mA at 45°C, laser speed 4 for 210 min.

Data analysis

Fluoroimages of the banding pattern saved by the sequencer in a computer TIFF file were analyzed with GelImage software (Kodak Digital Science™ 1D). Fluorescent amplification fragments between 50 and 400 bp were included in the cluster. Images were straightened, unwarped, and normalized by alignment to a reference strain included in each gel and/or to an RTS-Ready Label molecular weight marker (Bio-Rad Laboratories). Bands were selected by the computer program, with visual assistance for correction or addition of bands. Dendrograms were created by computing similarity values according to the position of the bands to infer a phylogenetic tree based on AFLP profiles which were obtained by the neighbor-joining algorithm (Saitou and Nei, 1987). The final phylogram was visualized with the TreeView program, version 1.30 (Page, 1996).

3. Results

Using a selection of *Frankia* strains, the most discriminatory primer combinations in the AFLP reaction was determined. The primer combinations *Eco*+A–*Mse*+T and *Eco*+C–*Mse*+0 were selected, since AFLP fingerprints of *Frankia* strains showed different banding patterns and a sufficient number of bands to be informative. These primers were used for all *Frankia* strains analysis. A total of 215 amplified fragments were considered into the range from 50 to 400 bp. A representative gel image of AFLP analysis of *Frankia* strains from different origins with the *Eco*R1+C–*Mse*I+0 combination is shown in Fig. 1.

AFLP analysis

Cluster groups were defined at the 30% of dissimilarity distance level. These levels were selected after the dendrogram analysis, as they were the lowest, which gave clear grouping. The NJ phylogenetic tree based on the AFLP data is shown in Fig. 2. The results of the AFLP analysis showed great genetic diversity among the *Frankia* isolates with three discrete groups identified. The diversity does not appear to be geographically based as almost all members are in different groups (Fig. 2).

North American *Frankia* strains used in this study were clustered in group A (CcI3 and B16219), together with PtI1 an ineffective strain from New Zealand, group B1 (Eulb, Cpl-P, CeS15, AvcI1, ArI3, AirI, ACN14a, ACN1, M3 and ChI1). Similar results were found for South American *Frankia* strains, where strains TtI42 and TgI15 formed group B2 in addition of a non-infective strain DC2 from Oceania. Strain RelI6 showed to be an independent group within group B2. Strains TtI12 and TtI11 from South America were closely located in group B1. The largest diversity of *Frankia* strains was observed in group B1 that include strains from three different regions – South, Central and North America.

4. Discussion

The genetic diversity of *Frankia* has been studied extensively in recent years due to the availability of advanced molecular techniques (Calia et al., 1998; Cameron et al., 1994; Colombo et al., 1997). Isolates have been studied focusing only on the context of their relationship to

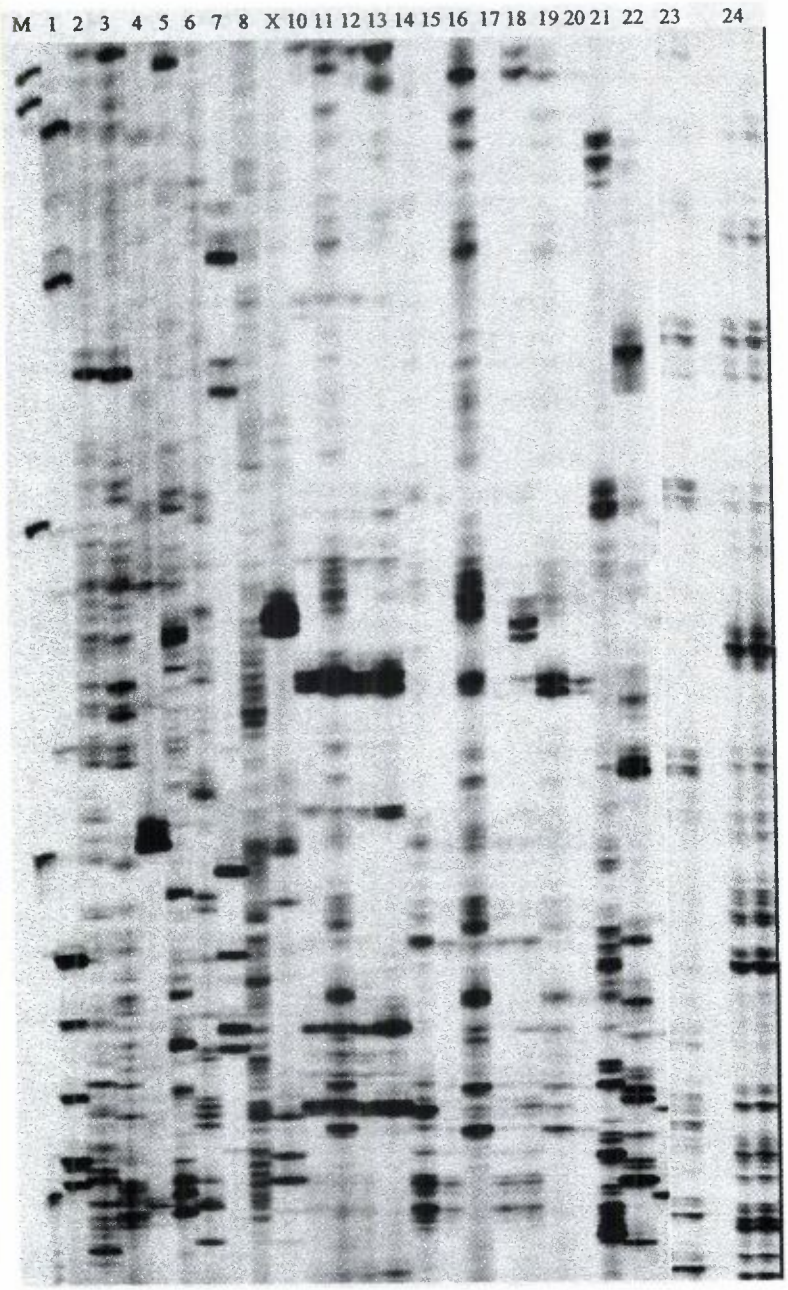

Figure 1. Representative gel image of AFLP analysis of *Frankia* sp. from different *Frankia* clusters and geographical origins with the *Eco*RI-*Mse*I combination. Lanes: M, size std; 1-DC2; 2-TtI12; 3-TtI11; 4-AirI1; 5-AvcI1; 6-CesI5; 7-S14; 8-M3; 10-DC12; 11-EAN1pec; 12-Eu1; 13-CpIP; 14-PtI1; 15-B16219; 16-CcI3; 17-AirI3; 18-TtI42; 19-TqI15; 20-IPNCe20; 21-ChI1; 22-ReI6; 23-ACN1; 24-ACN14.

host specificity groups (HSG). The results of the study reported here provide a contribution to the genetic diversity of *Frankia* coming from different origins and from different *Frankia* clusters (FC) according to Normand (2002) by AFLP analysis.

AFLP fingerprinting of DNA was first described in 1995 as a technique to detect genomic restriction fragments by PCR amplification and as being useful for DNAs of any origin or complexity (Vos et al., 1995). Fingerprints are produced without prior sequence knowledge, by using a limited set of generic primers.

The AFLP technique is also highly reproducible because astringent reaction conditions are used for primer annealing. Since 1995, this technique has been widely used and has been demonstrated to have a high-resolution power for bacterial classification, molecular typing, and molecular epidemiology (Dijkshoorn et al., 1996; Huys et al., 1996; Janssen and Dijkshoorn, 1996; Keim et al., 1997). The genetic resolution of the AFLP method largely relies on the selection of the appropriate restriction enzymes. Janssen and Dijkshoorn (1996) suggested that the restriction enzyme *Hind*III, combined with *Taq*I, gives an adequate number of

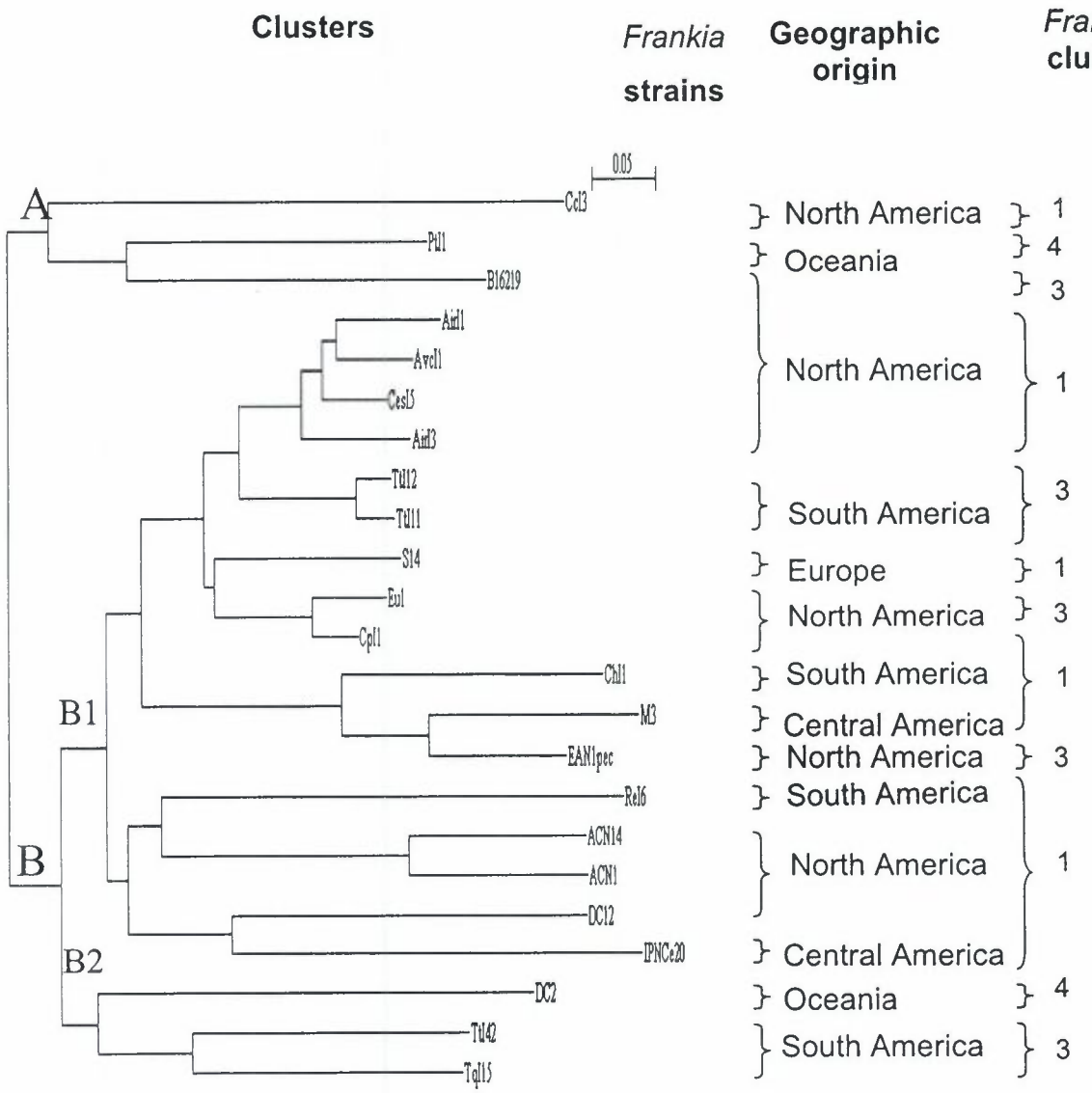

Figure 2. Neighbor-joining dendrogram showing the relationships among the studied *Frankia* strains, derived from the AFLP fingerprint data. Computing the similarity values according to the position of the bands created dendrogram.

suitably sized restriction fragments for bacteria with a G+C ratio of 40 to 50 mol%, and *Eco*RI-*Mse*I and *Apa*I-*Taq*I were most suited for bacterial genomes with low and high G+C contents, respectively. The genome of *Frankia* has been reported to have high G+C content, however, Huys et al. (1996) emphasized the importance of evenly distributed' bands along the length of the gel lane, because a good band distribution was critical for optimal normalization and a high level of discrimination in cluster analysis.

As is shown in Fig. 2, the genetic diversity does not seem to be geographically based as almost all members were included in diverse groups. However, according to the geographical distribution of the host plant families, some related strains were found being more affected by environmental conditions. Other studies on the 16S rDNA showed that infective *Myrica gale* strains from different geographical origin present a low diversity for this host plant considered before as promiscuous (Huguet et al., 2001). This AFLP analysis showed that the two *Frankia* strains (CpI-P and M3) from the FC1, nodulating the Myricaceae family, were not closely related as was mentioned before by Huguet et al. (2001). Our results showed that these strains are clustered in the B1 group although distant.

However, our results could be in correspondence to the *Frankia* clusters proposed by Normand (2002) who grouped together strains from FC 1 and FC 3. Isolates nodulating *Myrica* usually have been known as promiscuous according to the host assays (Baker, 1987) until the analysis 16S-rDNA sequence that showed a great diversity and specificity of *Frankia* from *Myrica* nodules.

Table 2. AMOVA of the AFLP strains according to its *Frankia* cluster.

Source of variation	d.f	Sum of squares	Variance components	Percentage of variation
Among populations	2	87.817	1.49344 Va	4.11
Within populations	20	696.357	34.81786 Vb	95.89
Total	22	784.174	36.31130	

Fixation index, F_{ST}: 0.04113
Significance tests: 1023 permutations
VA and F_{ST}:
P (rand. value > obs. value) = 0.13624
P (rand. value = obs. value) = 0.00040
P (rand. value ≥ obs. value) = 0.13663 + –0.00329

Table 3. AMOVA of the AFLP strains according to its geographical origin.

Source of variation	d.f	Sum of squares	Variance components	Percentage of variation
Among populations	4	164.174	1.78562 Va	4.93
Within populations	18	620.000	34.4444 Vb	92.07
Total	22	784.174	36.23007	

Fixation index, F_{ST}: 0.04929
Significance tests: 1023 permutations
VA and F_{ST}:
P (rand. value > obs. value) = 0.13020
P (rand. value = obs. value) = 0.00119
P (rand. value ≥ obs. value) = 0.13139 + –0.00309

The Analysis of Molecular Variance (AMOVA) shows no significant differences between *Frankia* populations (geographical origin or *Frankia* cluster). However, in both cases, there are significant differences among the *Frankia* strains. Furthermore, the variation between populations was higher when the strains were analyzed according to their geographical origin, rather than between *Frankia* strains when clusters were taken into consideration (Tables 2 and 3). At the same time the F_{ST} values were near to zero, which indicates that there is no divergence between the *Frankia* populations among the *Frankia* strains, suggesting genetic homogeneity into the *Frankia* genus.

The application of the AFLP technique in this study clearly demonstrated a genetic diversity of *Frankia* strains according to their *Frankia* cluster. These characteristics may be used for descriptions of corresponding species. However, strains CcI3 and IPNCe20 both nodulating *Casuarina* showed not to be closely related, they clustered in group A and group B1, respectively. Similar results were observed when the *nif*H genes from different *Frankia* strains were previously analyzed (Valdés, 2005). Description of one species represented by a single strain is considered to be inadequate (Vandamme et al., 1996). For this reason additional ALFP studies are needed, the results of this study represent a report of a short number of *Frankia* strains. The genome diversity of *Frankia* on a global scale is under way, the results of which will be critical to our understanding of the ecology and evolution of this actinomycte and provide further insight into the epidemiology of *Frankia*.

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REFERENCES

Baker, D. 1987. Relationships among pure cultured strains of *Frankia* based on host specificity. *Physiologia Plantarum* **70**: 245–248.

Baker, D.D. and Mullin, B.C. 1994. Diversity of *Frankia* nodule endophytes of the actinorhizal shrub *Ceanothus* as assessed by RFLP patterns from single nodule lobes. *Soil Biological Biochemistry* **26**: 547–552.

Barnes, R.C. 1989. Laboratory diagnosis of human chlamydial infections. *Clinical Microbiology Review* **2**: 119–136.

Calia, K.E., Waldor, M.K., and Calderwood, S.B. 1998. Use of representational difference analysis to identify genomic differences between pathogenic strains of *Vibrio cholerae*. *Infection and Immunity* **66**: 849–852.

Cameron, D.N., Khambaty, F.M., Wachsmuth, I.K., Tauxe, R.V., and Barrett, T.J. 1994. Molecular characterization of *Vibrio cholerae* O1 strains by pulsed-field gel electrophoresis. *Journal of Clinical Microbiology* **32**: 1685–1690.

Colombo, M.M., Mastrandrea, S., Leite, F., Santona, A., Uzzau, S., Rappeli, P., Pisano, M., Rubino, S., and Cappuccinelli, P. 1997. Tracking of clinical and environmental *Vibrio cholerae* O1 strains by combined analysis of the presence of the toxin cassette, plasmid content and ERIC PCR. *FEMS Immunology and Medical Microbiology* **19**: 33–54.

Cournoyer, B. and Normand, P. 1994. Characterization of a spontaneous thiostrepton resistant *Frankia alni* infective isolate using PCR-RFLP of *nif* and *gln*II genes. *Soil Biological Biochemistry* **15**: 553–559.

Dijkshoorn, L., Aucken, H., Gerner-Smidt, P., Janssen, P., Kaufmann, M.E., Garaizar, J., Ursing, J., and Pitt, T.L. 1996. Comparison of outbreak and non outbreak *Acinetobacter baumannii* strains by genotypic and phenotypic methods. *Journal of Clinical Microbiology* **34**: 1519–1525.

Hönerlage, W., Hahn, D., Zepp, K., Zeyer, J., and Normand, P. 1994. A hypervariable 23S rRNA region provides a

discriminating target for specific characterization of uncultured and cultured *Frankia*. *Systematic and Applied Microbiology* **17**: 433–443.

Huguet, V., Mergeay, M., Cervantes, E., and Fernandez, M.P. 2001. Diversity of *Frankia* strains associated to *Myrica gale* in Western Europe: impact of host plant (*Myrica* vs. *Alnus*) and of edaphic factors. *Environmental Microbiology* **10**: 1032–1041.

Huys, G., Coopman, R., Janssen, P., and Kersters, K. 1996. High-resolution genotypic analysis of the genus *Aeromonas* by AFLP-fingerprinting. *International Journal of Systematic Bacteriology* **46**: 572–580.

Jamann, S., Fernandez, M.P., and Normand, P. 1993. Typing method for N_2-fixing bacteria based on PCR-RFLP-application to the characterization of *Frankia* strains. *Molecular Ecology* **2**: 17–26.

Janssen, P. and Dijkshoorn, L. 1996. High resolution DNA fingerprinting of *Acinetobacter* outbreak strains. *FEMS Microbiology Letter* **142**: 191–194.

Keim, P., Kalif, A., Schupp, J., Hill, K., Travis, S.E., Richmond, K., Adair, D.M., Hugh-Jones, M., Kuske, C.R., and Jackson, P. 1997. Molecular evolution and diversity in *Bacillus anthracis* as detected by amplified fragment length polymorphism markers. *Journal of Bacteriology* **179**: 818–824.

Maggia, L., Nazaret, S., and Simonet, P. 1992. Molecular characterization of *Frankia* isolates from *Casuarina equisetifolia* root nodules harvested in West Africa (Senegal and Gambia). *Acta Ecologica* **13**: 453–461.

Maunuksela, L., Zeep, K., Koivula, T., Zeyer, J., Haahtela, K., and Hahn, D. 1999. Analysis of *Frankia* populations in soils from brich-pine- and spruce-stands. *FEMS Microbiology Ecology* **28**: 11–21.

McClenaghan, M., Herring, A.J., and Aitken, I.D. 1984. Comparison of *Chlamydia psittaci* isolates by DNA restriction endonuclease analysis. *Infection and Immunity* **45**: 384–389.

McEwan, N.R., Wheeler, C.T., and Milner, J.J. 1994. Strain discrimination of cultured and symbiotic *Frankia* by RFLP-PCR. *Soil Biological Biochemistry* **26**: 541–545.

Mirza, M.S., Rademaker, J.L.W., Janse, J.D., and Akkermans, A.D.L. 1993. Specific 16S ribosomal RNA targeted oligonucleotide probe against *Clavibacter michiganensis* subsp. *Sepedonicus*. *Canadian Journal of Microbiology* **39**: 1029–1034.

Murry, M.A., Konopka, A.S., Pratt, S.D., and Vandergon, T.L. 1997. The use of PCR-based typing methods to assess the diversity of *Frankia* nodule endophytes of the actinorhizal shrub *Ceanothus*. *Physiologia Plantarum* **99**: 714–721.

Murry, M.A., Zhang, D., Schneider, M., and De Brujin, F.J. 1995. Use of repetitive sequences and the polymerase chain reaction (rep-PCR) to fingerprint the genomes of *Frankia* isolates. *Symbiosis* **19**: 223–240.

Normand, P., Orso, S., Cournoyer, B., Jeannin, P., Chapelon, C., Dawson, J., Evtushenko, L., and. Misra, A.K. 1996. Molecular phylogeny of the genus *Frankia* and related genera and emendation of the family Frankiaceae. *International Journal of Systematic Bacteriology* **46**: 1–9.

Normand, P. 2002. The families Frankiaceae, Geodermatophilaceae, and Sporichthyaceae. In: *The Prokaryotes: An Evolving Electronic Resource for the Microbiological Community*. 3rd edition, M. Dworkin et al., eds. Springer Verlag, New York.

Page, R.D.M. 1996. TREEVIEW: an application to display phylogenetic trees on personal computers. *Computational Applied Bioscience* **12**: 357–358.

Pérez, N.O., Olivera, H., Vásquez, L., and Valdés, M. 1999. Genetic characterization of Mexican *Frankia* strains nodulating *Casuarina equisetefolia*. *Canadian Journal of Botany* **77**: 1214–1219.

Prin, Y., Mallein-Gerin, F., and Simonet, P. 1993. Identification and localization of *Frankia* strains in *Alnus* nodules by *in situ* hybridization of *nif*H mRNA with strain specific oligonucleotide probes. *Journal of Experimental Botany* **44**: 815–820.

Roller, C., Ludwig, W., and Schleifer, H.-H. 1992. Gram-positive bacteria with a high DNA G+C content are characterized by a common insertion within their 23S rRNA genes. *Journal of General Microbiology* **138**: 1167–1175.

Saitou, N. and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **2**: 112–118.

Sellstedt, A., Wullings, B., Nyström, U., and Gustafsson, P. 1992. Identification of *Casusarina-Frankia* strains by use of polymerase chain reaction (PCR) with arbitrary primers. *FEMS Microbiology Letters* **93**: 1–6.

Simonet, P., Grosjean, M.C., Misra, A.K., Nazaret, S., Cournoyer, B., and Normand, P. 1991. *Frankia* genus-specific characterization by polymerase chain reaction. *Applied Environmental Microbiology* **57**: 3278–3286.

Simonet, P., Nguyen-Thi, L., Cross, E.T.D., and Bardin, R. 1998. Identification of *Frankia* strains by direct DNA hybridization of crushed nodules. *Applied Environmental Microbiology* **54**: 2500–2503.

Svetlana, V.D. 1998. Grouping of *Frankia* strains on the basic of susceptibility to antibiotics, pigment production and host specificity. *International Journal of Systematic Bacteriology* **48**: 1265–1275.

Valdés, M., Vásquez, L., and Pérez, N.-O. 2001. Genetic markers (RAPDs and rep-PCR) for ecological studies of the nitrogen-fixing symbiosis *Frankia-Casuarina*. *European Tropical Forestry and Research Network* **34**: 27–29.

Valdés, M., Perez, N.O., Estrada, P., Caballero-Mellado, J., Pena-Cabriales, J.J., Normand, P., and Hirsch, A.M. 2005. Non-*Frankia* actinomycetes isolated from surface-sterilized roots of *Casuarina equisetifolia* fix nitrogen. *Applied Environmental Microbiology* **71**: 460–466.

Vandamme, P., Pot, B., Gillis, M., de Vos, P., Kersters, K., and Swings, J. 1996. Polyphasic taxonomy, a consensus approach to bacterial systematics. *Microbiology Review* **60**: 407–438.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., Van De Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., and Zabeau, M. 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* **23**: 4407–4414.

Woese, C.R., Kandler, O., and Wheelis, M.L. 1990. Towards a natural system of organisms: Proposal for the domains Archaea, Bacteria and Eucarya. *Proceedings of the National Academy of Sciences USA* **87**: 4576–4579.

Woese, C.R. 1987. Bacterial evolution. *Microbiology Review* **51**: 221–271.