



4 Natural diversity of nodular microsymbionts of *Alnus glutinosa* 5 in the Tormes River basin

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12 **Key words:** *Alnus glutinosa*, altitude, *Frankia*, genetic diversity, glutamine synthetase gene, 16S–23S IGS
13 RFLP

14 Abstract

15 The genetic diversity of *Frankia* strains nodulating *Alnus glutinosa* along the basin of the Tormes River was
16 studied on DNA extracted directly from nodules. *Frankia* strains inhabiting root nodules at 12 different
17 locations, ranging in altitude from 409 to 1181 m, were characterized. For that, we amplified the whole IGS
18 region between 16S–23S rDNA and performed a restriction fragment length polymorphism (RFLP)
19 analysis with four restriction enzymes. Two different RFLP patterns (termed A and B) were obtained with
20 *HaeIII*, indicating the existence of two different groups of *Frankia* strains. Three different nodule extracts
21 from each of the two RFLP groups were selected for further analyses. Sequencing of the 16S–23S rDNA
22 IGS showed a 100% of intragroup homology and also confirmed the difference (98.4% level of similarity)
23 between the *Frankia* strains in the two nodule extract groups. The phylogenetic analyses based on the two
24 16S–23S rDNA IGS sequences obtained in this study and other previously published sequences indicated
25 that *Frankia* strains TFAg5 and TFAg23 (chosen as representative of *HaeIII*–RFLP group A and B,
26 respectively) are quite similar to other strains nodulating plants of *A. rhombifolia* and *A. viridis* in Cali-
27 fornia (pairwise levels of similarity including gaps ranged from 97.8% to 98.6%), together with which they
28 form a single group. To put the *Frankia* strains representative of each *HaeIII*–RFLP group in the context of
29 overall *Frankia* diversity we amplified and sequenced the 16S rDNA and *glnII* gene from nodular DNA. An
30 also remarkable fact found in this study was that *Frankia* strains belonging to the *HaeIII*–RFLP group A
31 were distributed all along the river course, from the lowest site sampled to the highest, while *Frankia* strains
32 placed into RFLP group B were restricted to the upper Tormes River, being exclusively found at altitudes
33 of 946 m or higher.
34

35 Introduction

37 Actinomycetes included in genus *Frankia* are the
38 microsymbiont of the N₂-fixing actinorhizal sym-
39 bioses. Actinorhizal plants are spread out in 24

genera belonging to 8 families of angiosperms. 40
They are pioneer species that thrive in most 41
diverse environmental conditions and geographi- 42
cal zones, improving the growth of associated 43
species by increasing the amounts of nitrogen 44
and organic matter in soils (HussDanell, 45
1997). In addition to their important ecological 46
role, some species of actinorhizal plants have 47

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48 economic use or potential as timber and fuel
49 wood and in forestry, biomass production, land
50 reclamation, and amenity planting.

51 Classical physiological testing is of little use in
52 characterizing *Frankia* strains because they are
53 difficult to isolate, or even refractory to isolation,
54 and grow slowly (doubling times of 15–48 h or
55 more). Polymerase chain reaction (PCR)-based
56 procedures have proved to be very useful to
57 characterize cultured as well nonculturable *Frankia*
58 strains (Hahn et al., 1999). In studying the
59 diversity of *Frankia*, several genes of different
60 taxonomic resolution have been targeted includ-
61 ing the glutamine synthetase gene (Cournoyer
62 and Normand, 1994), and the intergenic spacer
63 (IGS) regions between the *NifH*–*NifD*, *NifD*–
64 *NifK* (Jamann et al., 1993; Maggia et al., 1992;
65 Nalin et al., 1995; Simonet et al., 1999), and
66 16S–23S rRNA genes. In the ribosomal operon,
67 the 16S–23S IGS is the region showing the high-
68 est variability (Normand et al., 1992; Rouvier
69 et al., 1996), which allows discrimination between
70 closely related *Frankia* strains by using restriction
71 fragment length polymorphism (RFLP) analysis
72 (Maggia et al., 1992; Navarro et al., 1999; Ritchie
73 and Myrold, 1999a, b). However, in determin-
74 ing the phylogenetic relationships among the
75 *Frankia* strains, the more conserved 16S rRNA
76 gene is usually studied.

77 *Alnus* is the only actinorhizal genus within
78 the family *Betulaceae*. Among its species, black
79 alder [*Alnus glutinosa* (L.) Gaertn] is renowned
80 as a very adaptable and fast growing species.
81 It is a common tree species in riparian forests
82 and, as a deep-rooted species, plays a crucial role
83 in maintaining the soil in river banks and reduces
84 the effects of erosion. Due to its ability to fix
85 nitrogen that enriches the soil and also owing to
86 its multiple uses in silviculture and the wood
87 industry, black alder is considered a very impor-
88 tant forest species. It is found all over Europe
89 from Ireland in the west to western Siberia in the
90 East, as far South as North Africa and up to 65°
91 North. It has been introduced into the Azores
92 and the USA. Its range both in Europe and Africa
93 is markedly scattered. Black alder is mainly
94 found in the northern and western sides of the
95 Iberian Peninsula (Kajba and Gracan, 2003).

96 Up to date, only very limited research on
97 genetic diversity of *Frankia* strains nodulating
98 actinorhizal plants in the Iberian Peninsula has

99 been conducted (Huguet et al., 2004) and, to the
100 best of our knowledge, none on the diversity of
101 *Alnus*-infective *Frankia* strains. The aim of this
102 study was to assess the genetic diversity of *Frankia*
103 strains nodulating *A. glutinosa* along the Tor-
104 mes River. For that, PCR–RFLP analysis of the
105 16S–23S ribosomal spacer region was performed
106 on DNA directly extracted from nodules, and
107 16S rRNA and *glnII* gene sequences were then
108 used to establish phylogenetic relationships with
109 other *Frankia*.

110 Materials and methods

111 Nodule sampling and DNA extraction

112 The nodule samples were collected from individ-
113 ual trees of *A. glutinosa* growing along the river
114 Tormes' banks at 12 different locations (Table 1).
115 Three nodule samples per site were collected
116 accounting a total of 36 nodule samples. The river
117 Tormes is born about 2000 m in the Sierra de
118 Gredos (Spain) and flows northwest for 284 km
119 to the Douro River, at the international bound-
120 ary between Spain and Portugal (339 m altitude
121 above the sea level) (Figure 1). Nodules were
122 collected from near the root collars, frozen in dry
123 ice and then kept at –20 °C until use. One
124 nodule cluster per tree was sampled. After rins-
125 ing thoroughly with tap water, 15–20 lobe tips
126 from each nodule were detached with a scalpel,
127 soaked in 5% sodium hypochlorite for 20 min,
128 for sterilization purposes, and then rinsed 5 times
129 in sterile water to remove the bleach. After steril-
130 ization, each sample was ground in a mortar
131 with a pestle in liquid nitrogen, and DNA ex-
132 tracted by using DNeasy® Plant Mini Kit (Qia-
133 gen), according to the manufacturer's manual.

134 PCR amplification

135 PCR amplifications were performed on a What-
136 man Biometra® Tgradient thermocycler. The same
137 DNA extracts were used for 16S–23S IGS, 16S
138 rRNA and *glnII* genes amplifications. The oligo-
139 nucleotide PCR primers used to amplify 16S–23S
140 spacer regions of *Frankia* were: FG PS1493 (5'-
141 GGCTGGATCACCTCCTTTCT-3') and FGPL
142 2054' (5'-CCGGGTTTCCCCATTTCGG-3') (Sim-
143 onet et al., 1991). PCR was performed using *Taq*

Table 1. Information about sampling sites, chemical soil properties, nodule and restriction patterns of amplified 16S–23S IGS regions from nodular *Frankia* strains

Site name	Geographic position	Altitude (m)	Soil pH	Soil OM (%)	Nodule code	RFLP pattern (<i>Hae</i> III)
1. Trabanca-Fermoselle	41°16' N 6°23' W	409	5.31	1.87	TFAg1, TFAg2	A
2. Ledesma	41°05' N 6°00' W	741	7.07	5.69	TFAg3	A
3. C.I.A. "El Tormes"	41°03' N 5°49' W	764	5.28	2.79	TFAg4	A
4. Salamanca	40°54' N 5°46' W	775	4.49	7.71	TFAg9, TFAg10	A
5. Matacán	40°56' N 5°28' W	793	4.98	5.58	TFAg5, TFAg6	A
6. Alba de Tormes	40°49' N 5°31' W	818	6.85	3.24	TFAg7	A
7. Galisancho	40°44' N 5°33' W	843	6.94	9.53	TFAg8	A
8. Puente Congosto	40°29' N 5°31' W	946	4.89	3.09	TFAg11, TFAg12 TFAg13, TFAg14	A B
9. Barco de Avila	40°21' N 5°31' W	1004	6.27	1.04	TFAg15, TFAg16	B
10. Bohoyo	40°19' N 5°26' W	1083	5.12	3.88	TFAg17 TFAg18	A B
11. Bohoyo	40°19' N 5°24' W	1110	4.40	4.55	TFAg19	B
12. La Angostura	40°20' N 5°20' W	1181	6.35	1.98	TFAg20, TFAg21, TFAg22 TFAg23	A B

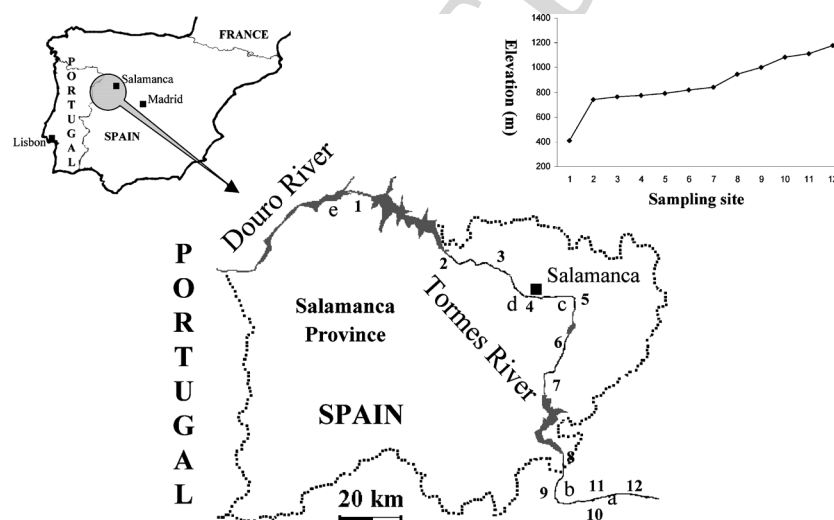


Figure 1. Geographic locations of the sampling sites (numbers) and of the meteorological stations (letters) from which climatic data were collected as listed in Tables 1 and 2, respectively. The graph on the upper right corner summarizes the altitudes of the corresponding sampling sites.

144 DNA Polymerase from Amersham Pharmacia
 145 Biotech, following the manufacturer's instruction:
 146 5–10 ng of genomic DNA, 2.5 μ L of Taq poly-
 147 merase buffer 10 \times , 1 μ L of BSA 0.1%, 2.5 μ L of
 148 dNTPs mix (2 mM), 2.5 μ L of each primer (2 μ M)
 149 and 1 U of Taq DNA polymerase for 25 μ L of
 150 final reaction volume. PCR conditions were as
 151 follow: preheating at 95 $^{\circ}$ C for 5 min; 35 cycles of

denaturing at 94 $^{\circ}$ C for 1 min, annealing at 57 $^{\circ}$ C
 for 1 min and extension at 72 $^{\circ}$ C for 2 min,
 and a final extension at 72 $^{\circ}$ C for 7 min. To
 amplify the 16S rRNA gene of *Frankia*, primers
 FN1F (5'-GCTCTGGGATAACCTCG-3'; posi-
 tions 22–39 on *Escherichia coli* 16S rDNA)
 and FN1R (5'-CA GGACCCTTAAGGACCC-3';
 positions 1141–1159 on *E. coli* 16S rDNA) were

152
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160 used. These two primers were designed to amplify
161 specifically sequences corresponding to the
162 16S rRNA gene of *Frankia*. To amplify the
163 *glnII* gene of *Frankia*, primers FGgs19 (5'-TAC
164 ATCTGGATCCACGGCAC-3') and FGgs417
165 (5'-GCCGACGCCGCAGTAGTA-3') (Cournoy-
166 er and Lavire, 1999) were used. PCR conditions
167 for amplification of *Frankia* 16S rDNA and *glnII*
168 gene were as above except that the annealing tem-
169 perature was 52 °C. To check the efficiency of
170 amplification, 5 µL of the amplification reactions
171 were analysed by electrophoresis in TAE buffer on
172 a 1% (w/v) agarose gel and visualized under UV
173 light after staining with ethidium bromide.

174 *PCR product restriction analysis*

175 After PCR amplification of the 16S–23S IGS,
176 5 µL of each product was digested separately by
177 the restriction enzymes *CfoI* (Sigma), *HaeIII*,
178 *AfaI* and *MspI* (Amersham Biosciences), follow-
179 ing the manufacturer's instructions, electrophore-
180 sed on a 2% (w/v) agarose gel in 1.0×TAE
181 buffer and visualized under UV light after stain-
182 ing with ethidium bromide. The size of restricted
183 products was estimated by comparison with a
184 50 bp DNA Ladder (Amersham Pharmacia bio-
185 tech).

186 The restriction patterns with *HaeII* allowed
187 distinguishing two PCR–RFLP groups among all
188 the samples analysed in this study. Three repre-
189 sentative nodule sample from each group was
190 chosen for DNA sequencing.

191 *DNA sequencing and analysis of the sequence data*

192 The bands corresponding to the 16S–23S IGS,
193 16S rRNA and *glnII* genes were purified directly
194 from the respective gels by centrifugation using
195 the QIAquick® Gel Extraction Kit (Qiagen)
196 according to the manufacturer's instructions.
197 Sequencing reactions were performed on an
198 ABI377 sequencer (Applied Biosystems) using a
199 BigDye terminator v3.0 cycle sequencing kit as
200 supplied by the manufacturer. Those primers
201 used for amplification were also used as sequenc-
202 ing primers.

203 The sequences obtained were compared with
204 those from the GenBank using the FASTA
205 program (Pearson and Lipman, 1988). Sequences
206 were aligned using the Clustal W software

(Thompson et al., 1977) and distances were cal-
207 culated according to Kimura's two-parameter
208 method (Kimura, 1980). Phylogenetic trees were
209 inferred using the neighbour-joining method (Sai-
210 tou and Nei, 1987). Bootstrap analysis was based
211 on 1000 resamplings. The MEGA 2.1 package
212 (Kumar et al., 2001) was used for all analyses. 213

Nucleotide sequence accession numbers 214

The nucleotide sequences determined in this
215 study have been deposited in the GenBank data-
216 base under the following accession numbers:
217 DQ141195 and DQ141196 for the 16S rRNA;
218 DQ141197 and DQ141198 for the 16S–23S IGS
219 sequences; and DQ150529 and DQ150530 for the
220 *glnII* sequences. 221

222 **Results and discussion**

Genetic diversity of PCR–RFLP patterns 223

Satisfactory PCR amplifications of the 16S–23S
224 IGS regions of *Frankia* were obtained from 23 of
225 the 36 nodular DNAs. PCR reactions yielded an
226 expected-size 510 bp amplicon. RFLP analysis of
227 amplicons was then performed with *CfoI*, *HaeIII*,
228 *AfaI* and *MspI*. Digests with *HaeIII* produced
229 two clear and different RFLP patterns which
230 were assigned the letters A and B. None of the
231 other three restriction enzymes (*CfoI*, *AfaI* and
232 *MspI*) generated distinguishing RFLP patterns
233 for the *Frankia* strains symbiotic with *A. glutin-*
234 *osa* here studied (Figure 2). 235

The whole 16S–23S IGS region and the first
236 76 bp of the 23S rRNA gene of three representa-
237 tive nodular symbiont from each of the two
238 RFLP groups obtained with *HaeIII* were then se-
239 quenced. Sequencing of the 16S–23S rDNA IGS
240 showed a 100% of intragroup homology and,
241 therefore, we chose TFAg5 and TFAg23 as rep-
242 resentative nodular symbionts of the *HaeIII*–
243 RFLP group A and B, respectively, for further
244 analyses. The sequences of TFAg5 (Group A)
245 and TFAg23 (Group B) (Table 1) differed by 7
246 mismatches (2 transitions and 5 transversion)
247 and one indel (level of similarity, 98.4% includ-
248 ing gaps). Therefore, the sequencing results veri-
249 fied the PCR–RFLP analysis results. 250

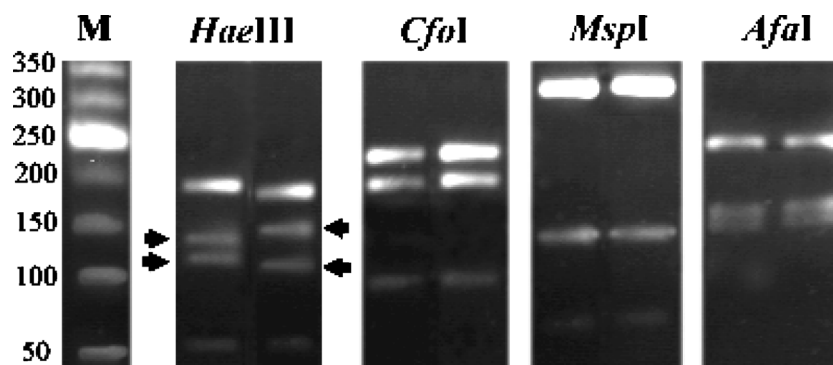


Figure 2. Restriction patterns of 16S–23S rDNA IGS region of the nodular *Frankia* strains TFAg5 (left lanes) and TFAg23 (right lanes) after digestion with *Hae*III, *Cfo*I, *Msp*I and *Afa*I. The bands indicated by the arrowheads allow to differentiate between *Hae*III–RFLP patterns A (right) and B (left). M, 50 bp DNA ladder (Amersham Pharmacia).

251 When compared to previously published se- 284
 252 quences (Figure 3), it was found that the two 285
 253 strains sequenced in this study joined other *Alnus* 286
 254 (Oakley et al., 2004; Varghese et al., 2003) and 287
 255 *Casuarina* (Normand et al., 1992) infective 288
 256 strains and are more distant to *Frankia* strains 289
 257 that infect plant species from the family *Rhamna-* 290
 258 *ceae* (*Ceanothus*, for instance) (Jeong and Myr- 291
 259 old, 1999; Ritchie and Myrold, 1999a, b), in 292
 260 accordance with the established subdivisions in 293
 261 the genus *Frankia* (Normand et al., 1996). This 294
 262 phylogenetic analyses indicated that even though 295
 263 the *Frankia* strains nodulating alders along the 296
 264 Tormes River are very homogeneous, they form 297
 265 two distinct groups (Figure 3). *Frankia* strains 298
 266 TFAg5 and TFAg23 are quite similar (pairwise 299
 267 levels of similarity including gaps ranged from 300
 268 97.8% to 98.6%) to those strains nodulating 301
 269 plants of *A. rhombifolia* (strain ALRH70) and 302
 270 *A. viridis* (strain ALVI390) in California (Oakley 303
 271 et al., 2004), together with which they form a 304
 272 single group (Figure 3). However, strain TFAg23 305
 273 shows a level of similarity with the Californian 306
 274 strain ALVI390 higher than with TFAg5 (98.6% 307
 275 vs. 98.4%, including gaps).

276 *Sequencing and phylogenetic analyses of the 16S* 277 *rRNA and glnII genes*

278 To put the *Frankia* strains TFAg5 and TFAg23
 279 in the context of overall *Frankia* diversity we
 280 amplified and sequenced, in addition to the 16S–
 281 23S IGS region, the 16S rDNA and *glnII* gene
 282 from nodular DNA. The sequenced 16S rDNA
 283 region was 708 bp in length, and it is included

between positions 209 and 917 of the 16S rRNA
 gene of the *F. alni* strain ACN14a (Normand
 et al., 1996). Therefore, a considerable segment
 of the 5' 708-bp fragment sequenced in this study
 forms part of the hypervariable region of the 16S
 rRNA gene (Clawson et al., 1998; Heuvel et al.,
 2004; Varghese et al., 2003) being, thus, appro-
 priate for distinguishing diversity among strains.
 It has been shown that *Frankia* strains having
 identical sequences in the hypervariable region of
 the 16S rDNA are the same, or very closely re-
 lated, strains (Clawson et al., 1999). On the other
 hand, the genes for glutamine synthetase I and
 II, *glnI* and *glnII*, respectively, are known to
 have more variability than the 16S rRNA gene,
 and both have been used to study the phylogeny
 of *Frankia* strains (Clawson et al., 2004; Cour-
 noyer and Lavire, 1999; Gtari et al., 2004; Heu-
 vel et al., 2004). We chose to use the *glnII* gene
 since it seems to present higher variability than
 the *glnI* gene (Cournoyer and Lavire, 1999), from
 which we sequenced a region of 313 bp in length.

The partial 16S rDNA sequences of the *Frankia*
 strains TFAg5 and TFAg23 differed by 1
 mismatch (level of similarity, 99.8%). The tree
 topology obtained from 16S rDNA sequences of
Frankia strains TFAg5 and TFAg23 and se-
 quences in database shows the grouping of these
 two strains group with other *Alnus–Myrica* infec-
 tive strains (Figure 4). This grouping is coherent
 with that obtained by Normand et al. (1996)
 using complete sequences of the 16S rRNA gene,
 and also with others obtained with different
 molecular techniques (Igual et al., 2003; Veláz-
 quez et al., 1998).

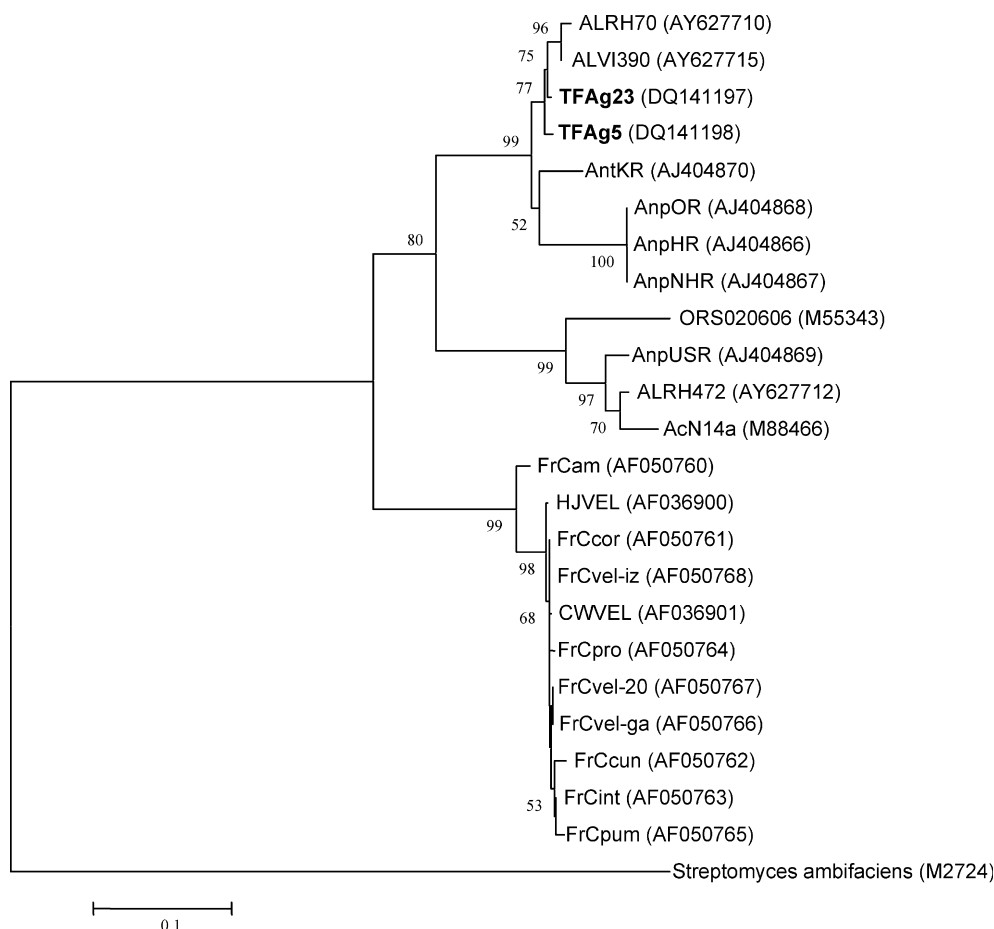


Figure 3. Comparative sequence analysis of the 16S–23S rDNA IGS regions from nodular *Frankia* strains TFAg5 and TFAg23 (in bold types) and representative *Frankia* strains from GenBank. The significance of each branch is indicated by bootstrap percentage calculated for 1000 subsets (only values greater than 50% are shown). The analysis was based on 490 nt. *Streptomyces ambifaciens* (M2724) was used as outgroup. Bar, 0.1 substitutions per site.

319 With regards to the *glnII* sequences, they dif- 333
 320 ferred by 2 mismatches (level of similarity, 99.4%) 334
 321 and, in concordance with other published studies 335
 322 (Cournoyer and Lavire, 1999; Gtari et al., 2004) 336
 323 grouped together with other *Alnus*-infective *Frankia* 337
 324 strains (Figure 5). Therefore, the sequence 338
 325 analyses of both 16S rRNA and *glnII* genes 339
 326 support the assignment of the *Frankia* strains 340
 327 nodulating *A. glutinosa* along the Tormes River 341
 328 to two different groups, confirming the results 342
 329 obtained by 16S–23S IGS–RFLP analyses. 343

330 Distribution of restriction patterns

331 *Frankia* strains belonging to the *HaeIII*–RFLP 344
 332 group A were distributed all along the river 345

course, from the lowest site sampled (Trabanca- 333
 Fermoselle, 409 m) to the highest (La Angostura, 334
 1181 m) (Table 1). However, the distribution of 335
Frankia strains placed into RFLP group B is 336
 restricted to the upper Tormes River, being exclu- 337
 sively found at altitudes of 946 m or higher 338
 (Table 1). Elevation, with its associated climatic 339
 conditions, has been proposed among the factors 340
 correlating with intra-host group *Frankia* hetero- 341
 geneity by other authors (Dai et al., 2004; Ritchie 342
 and Myrold, 1999a, b). 343

The main part of the Tormes River basin has 344
 a continental Mediterranean climate, character- 345
 ized by prolonged summer drought and contrast- 346
 ing diurnal and seasonal temperatures. However, 347
 some different climatic conditions are found at 348

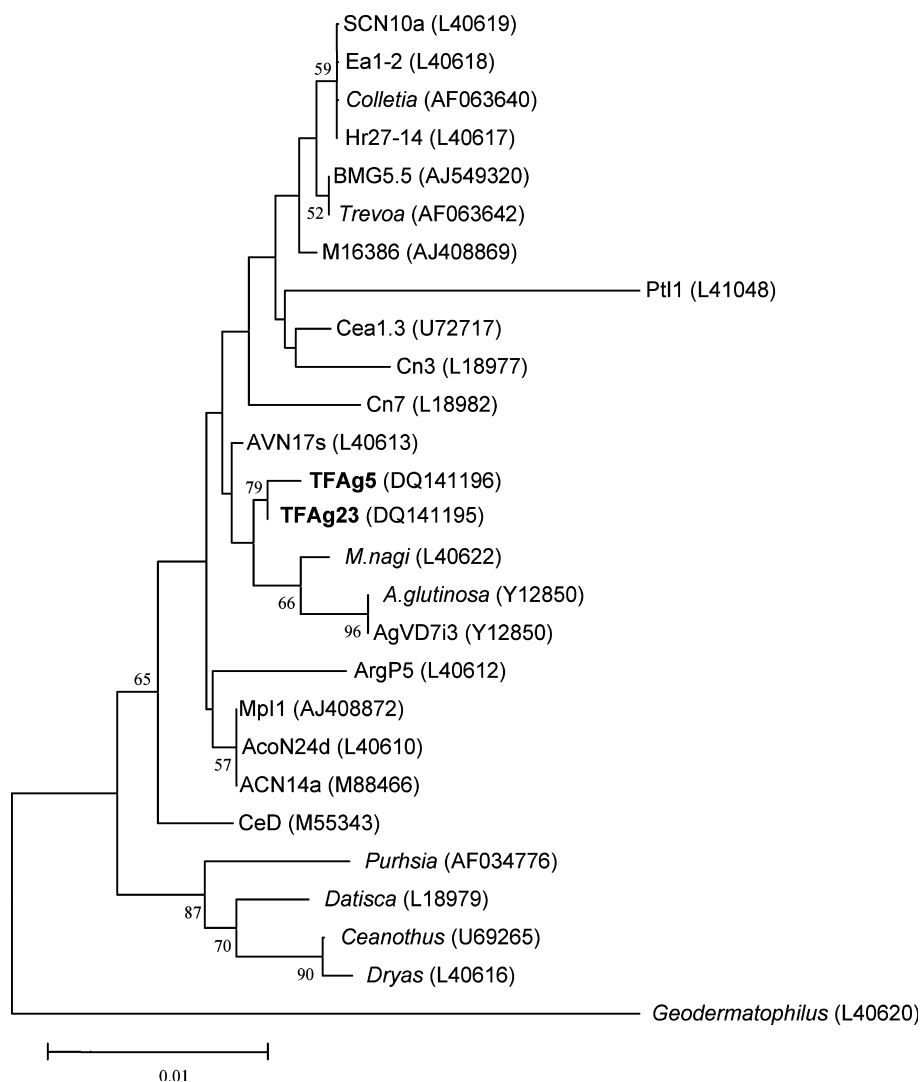


Figure 4. Comparative sequence analysis of the 16S rRNA gene from nodular *Frankia* strains TFAg5 and TFAg23 (in bold types) and representative *Frankia* strains from GenBank. The significance of each branch is indicated by bootstrap percentage calculated for 1000 subsets (only values greater than 50% are shown). The analysis was based on 708 nt. *Geodermatophilus obscurus* subsp. *obscurus* (L40620) was used as outgroup. Bar, 0.01 substitutions per site.

349 the upper and lower parts of the Tormes River.
 350 Close to its mouth in the river Douro, the influ-
 351 ence of the Atlantic Ocean produces warmer and
 352 wetter conditions. At the upper part, the climate
 353 is influenced by the Sierra de Gredos mountains
 354 which produce colder temperatures and higher
 355 precipitations. We have gathered meteorological
 356 data from stations located in the vicinity of five
 357 sampling sites along the course of the Tormes
 358 River (Table 2): Bohoyo (1083 m) and Barco de
 359 Avila (1004 m), at the upper course; Matacán

(793 m) and Salamanca (775 m), at the middle
 360 course; and Trabanca-Fermoselle (409 m, data
 361 from the nearby station of Aldeadávila), at the
 362 lower course. As it can be seen (Table 2), the
 363 mean annual and maximal mean temperatures at
 364 Barco de Avila (the nearest station to Puente
 365 Congosto, that is the lowest site where *Frankia*
 366 strains belonging to the RFLP group B appear)
 367 and Bohoyo are colder than at the other three
 368 lower sites. However, there is no statistically sig-
 369 nificant differences in minimal mean temperatures
 370

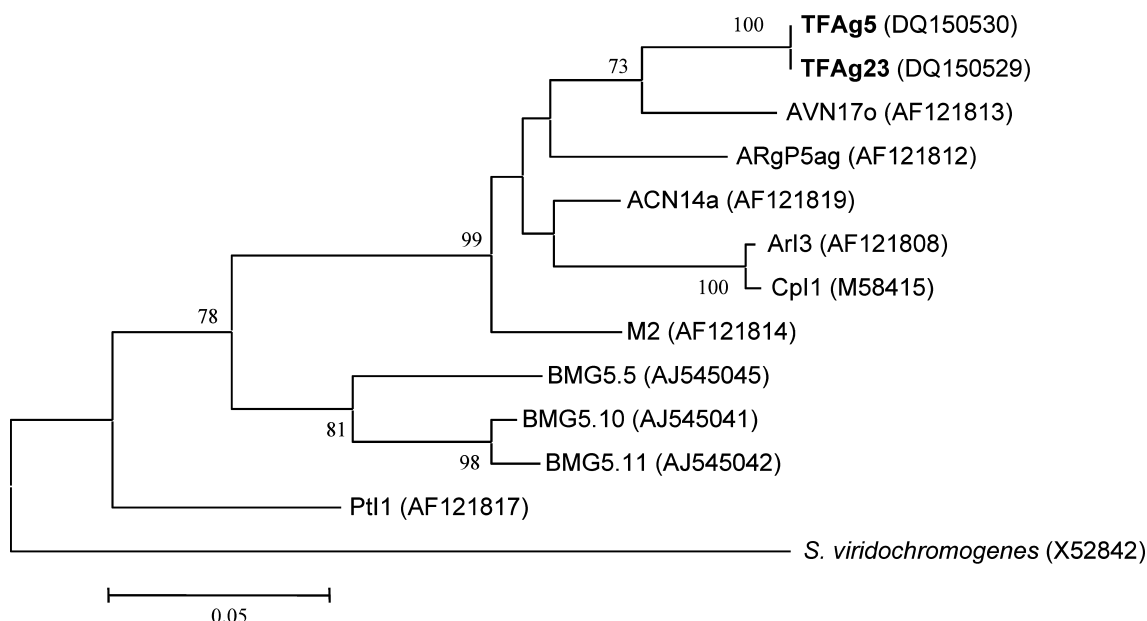


Figure 5. Comparative sequence analysis of the *glnII* gene from nodular *Frankia* strains TFAg5 and TFAg23 (in bold types) and representative *Frankia* strains from GenBank. The significance of each branch is indicated by bootstrap percentage calculated for 1000 subsets (only values greater than 50% are shown). The analysis was based on 313 nt. *Streptomyces viridochromogenes* (X52842) was used as outgroup. Bar, 0.05 substitutions per site.

Table 2. Information about precipitation (mm) and temperatures (°C) obtained from 5 stations located along the Tormes River

Station	Altitude (m)	Annual				July Maximal mean <i>T</i>	January Minimal mean <i>T</i>
		Precipitation	Maximal mean <i>T</i>	Minimal mean <i>T</i>	Mean <i>T</i>		
a. Bohoyo	1083	880 a	16.4 a	4.5 a	10.4 a	27.1 a	-1.1 a
b. Barco de Avila	1004	542 b	16.2 a	6.4 b	11.3 b	27.4 a	0.4 a
c. Matacán	793	395 c	18.3 b	5.7 b	12.0 c	29.8 b	-0.9 a
d. Salamanca	775	390 c	18.3 b	5.8 b	12.1 c	29.4 b	-0.9 a
e. Trabanca-Fermoselle*	409	809 a	21.5 c	10.5 c	16.0 d	34.7 c	3.4 b

*Data from the nearby station of Aldeadávila.

Data represent the mean of 10 years.

Means in a column sharing the same letter are not significantly different ($P \leq 0.05$).

371 between Barco de Avila and the points situated
372 further down the river, with the exception of
373 Trabanca-Fermoselle, where the highest tempera-
374 ture values are always reached. Taking into
375 account July and January, the months in which
376 temperatures reach extremes and therefore may
377 potentially be biotically limiting factors, it is seen
378 that maximal mean temperatures in July at
379 Bohoyo and Barco de Avila are again the coldest.
380 However, with the exception of the lowest site
381 (Trabanca-Fermoselle), there is no statistically

382 significant differences in the January minimal
383 mean temperatures among the other four moni-
384 tored sites. With regards to the precipitations
385 (Table 2), annual rainfall rates were significantly
386 higher at the upper (Bohoyo and Barco de Avila)
387 and the lower (Trabanca-Fermoselle) course of
388 the Tormes River than at its middle course
389 (Matacán and Salamanca). According to our re-
390 sults, *Frankia* strains displaying the RFLP pat-
391 tern A could survive in more heterogeneous
392 climatic/altitudinal conditions than those strains

393 from the group B, which in turn would compete
394 better for nodulation at environments where the
395 maximal temperatures and the drought condi-
396 tions are less severe. More samples from other
397 places in the region should be studied in order to
398 further clarify this particular *Frankia* distribution.

399 Edaphic conditions, such as pH, organic mat-
400 ter content or soil type, have also been seen to
401 influence *Frankia* populations in soils (Huguet
402 et al., 2004; Jamann et al., 1992; Navarro et al.,
403 1999). Therefore, we performed these soil analy-
404 ses from all the sampling sites (Table 1) in order
405 to ascertain if any trend might exist between
406 such parameters and the observed distribution of
407 the two *Frankia* genotypes. All the soils are san-
408 dy soils with organic matter contents ranging
409 from 1.04% (Barco de Avila, site 9) to 9.53%
410 (Galisancho, site 7) and pH from 4.40 (Bohoyo,
411 site 11) to 7.35 (Ledesma, site 2). According to
412 our actual data (Table 1), no relationship be-
413 tween any of these two soil parameters and the
414 distribution of the two *Frankia* strains genotypes
415 can be established. However, a higher number of
416 nodule samples as well as soil samples would be
417 needed to reach secure conclusions.

418 It is also noteworthy that only two *Frankia*
419 strains were found nodulating *A. glutinosa* along
420 the Tormes River. Other authors have reported a
421 greater genetic diversity among *Frankia* strains
422 symbiotic with *Alnus*, or with its host compatible
423 genus *Myrica*, in several other geographical loca-
424 tions throughout the world (Clawson and Ben-
425 son, 1999; Clawson et al., 1999; Dai et al., 2004;
426 Huguet et al., 2004; Huguet et al., 2005; Oakley
427 et al., 2004). Several reasons could account for
428 the low diversity of *Frankia* strains detected in
429 our study as compared with those reported in
430 other works. The first possible explanation is
431 coming from the existence of unlikely climatic
432 conditions among the studied zones. In our re-
433 gion, the climate is characterized by prolonged
434 dry and hot periods that may constrain the
435 diversity of *Frankia* strains by acting as a selec-
436 tive force in favour of those *Frankia* genotypes
437 more adapted to severe drought and hot condi-
438 tions. Although climatic data were not always re-
439 ported, it can be inferred that such stresses in
440 some of the regions studied by other authors are
441 lower than in our region [i.e. France and
442 Belgium (Huguet et al., 2004) or the Northeast
443 (Clawson et al., 1999) and Midwest of the

United States (Huguet et al., 2004)] being there,
therefore, the climatic conditions less restrictive
to the adaptation of nodulating *Frankia* strains.
On the other hand, it has been reported that
close phylogenetically related *Myrica* species
(*M. faya* and *M. rivas-martinezii*) growing symp-
atrically are nodulated by different *Frankia*
strains, indicating that they have the capacity to
select specific *Frankia* genotypes present in soil
(Huguet et al., 2005). If the same occurs in the
genus *Alnus*, the range of *Frankia* strains suscep-
tible to be selected under natural conditions by a
determined *Alnus* species (or even genotype)
could vary broadly. In this regard, the low genet-
ic diversity of *A. glutinosa*-infective strains that
occurs along the Tormes River would be a conse-
quence of a lesser promiscuity of this host species
as compared with other of the studied *Alnus* spe-
cies (Clawson et al., 1999; Dai et al., 2004; Hug-
uet et al., 2001; Huguet et al., 2004; Oakley
et al., 2004). Obviously, these and other factors
could jointly account for the low genetic diversity
here observed and, therefore, more research is
needed to gain a better insight into this issue.

A better utilization of the actinorhizal symbi-
osis will require inoculations with selected *Frankia*
strains. Criteria other than a superior
nitrogen fixing capacity must be also considered,
such as the ability to persist in soil and to com-
pete favourably for nodulation with less efficient
Frankia populations. Studies of the influence
of soil and environmental factors on the diversity
of *Frankia* may improve the management of
actinorhizal symbioses (Hahn et al., 1999).

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