

1 ***Frankia irregularis* sp. nov., an actinobacterium unable to nodulate its original host,**  
2 ***Casuarina equisetifolia*, but effectively nodulate members of the actinorhizal *Rhamnales***

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24 Abbreviations: A<sub>2</sub>pm, diaminopimelic acid; ANI, average nucleotide identity; dDDH, digital  
25 DNA–DNA hybridization; GGDC, Genome-to-Genome Distance Calculator; GTR, general  
26 time-reversible; ML, maximum-likelihood; MP, maximum-parsimony; MRE, maximal-  
27 relative-error; MUSCLE, Multiple Sequence Comparison by Log-Expectation; PAUP,  
28 Phylogenetic Analysis Using Parsimony; RAxML, Randomized Axelerated Maximum  
29 Likelihood; TNT, Tree analysis New Technology.

30 The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence and the draft  
31 genome sequence reported are MH145366 and FAOZ00000000, respectively.

32 **Abstract**

33 A red pigmented actinobacterium designated G2<sup>T</sup>, forming extremely branched vegetative  
34 hyphae, vesicles and multilocular sporangia, was isolated from *Casuarina equisetifolia* nodules.  
35 The strain failed to nodulate its original host plant but effectively nodulated members of  
36 actinorhizal *Rhamnales*. The taxonomic position of G2<sup>T</sup> was determined using a polyphasic  
37 approach. The peptidoglycan of the strain contained *meso*-diaminopimelic acid as diagnostic  
38 diamino acid, galactose, glucose, mannose, rhamnose, ribose and xylose. Polar lipid pattern  
39 consisted of phosphatidylinositol (PI), diphosphatidylglycerol (DPG), glycopospholipids  
40 (GPL1-2), phosphatidylglycerol (PG), aminophospholipid (APL) and unknown lipids (L). The  
41 predominant menaquinones are MK-9 (H<sub>4</sub>) and MK-9 (H<sub>6</sub>) while the major fatty acids are *iso*-  
42 C<sub>16:0</sub>, C<sub>17:1</sub> ω8c and C<sub>15:0</sub>. The size of the genome of strain G2<sup>T</sup> is 9.5 Mb and digital DNA G+C  
43 content is 70.9%. The 16S rRNA gene showed 97.4% to 99.5 % sequence identity with the type  
44 strains of the genus *Frankia*. Digital DNA:DNA hybridisation (dDDH) values between strains  
45 G2<sup>T</sup> and its nearest phylogenetic neighbor *Frankia elaeagni* and *Frankia discariae* type strains  
46 were below the threshold of 70 %. Based on these results, strain G2<sup>T</sup> (=DSM 45899<sup>T</sup> = CECT  
47 9038<sup>T</sup>) is proposed to represent the type strain of a novel species *Frankia irregularis* sp. nov.

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## 58 Introduction

59 The genus name *Frankia* was first proposed by Brunchorst (1886) [1]. It belongs to the  
60 monogeneric family *Frankiaceae* (Becking 1970 emend Zhi et al. 2009) [2-3] and the order  
61 *Frankiales* (Sen et al. 2014) [4] and encompasses soil actinobacteria best known for their  
62 facultative nitrogen-fixing symbiosis with actinorhizal plants [7]. It has been shown, based on  
63 16S rRNA [8], *gyrB* [9], *glnII* [8-9] genes, 16S-23S rRNA Internal Transcribed Spacers [10],  
64 MLSA (*atp1*, *ftsZ*, *dnaK*, *gyrA* and *secA*) [11] and core genomes [12] phylogenies, that the  
65 genus *Frankia* is structured in four clusters in concordance with the host plant specificity  
66 proposed by Baker [13]. *Frankia* of cluster 1 are found infective on host plants of *Alnus*,  
67 *Casuarina*, *Allocasuarina* and *Myricaceae*, while cluster 2 represents strains that are infective  
68 on *Coriariaceae*, *Datisceae*, *Dryadoideae*, and *Ceanothus*. Strains of cluster 3 are the most  
69 promiscuous and are infective on *Elaeagnaceae*, *Myricaceae*, *Colletieae* and *Gynmmostoma*.  
70 The fourth *Frankia* cluster consists of the atypical strains which are unable to fix nitrogen  
71 and/or to re-infect actinorhizal host plants. Recently ten species have been recognized *Frankia*  
72 *alni*, *Frankia casuarinae* [14] and *Frankia canadensis* [15] of cluster 1, *Frankia coriariae* [16],  
73 *Candidatus Frankia datiscaae* [17] and *Candidatus Frankia californiensis* [18] of cluster 2,  
74 *Frankia elaeagni* [14] and *Frankia discariae* (19) from cluster 3, *Frankia inefficax* [20],  
75 *Frankia asymbiotica* [21] and *Frankia saprophytica* [22] from cluster 4.

76 Strain G2<sup>T</sup> of phylogenetic cluster 3, was isolated from *Casuarina equisetifolia* and appears to  
77 infect members of the *Rhamnales* order but not its original host plant. Based on a polyphasic  
78 approach, G2<sup>T</sup> emerges as type strain of a new species *Frankia irregularis* sp. nov.

79 Strain G2<sup>T</sup> was isolated from nodules collected in the INRA Research Station, Saint-François,  
80 Grande Terre, Guadeloupe [23]. The type strains of *Frankia alni*, *Frankia casuarinae*, *Frankia*  
81 *elaeagni* and *Frankia discariae*, *Frankia inefficax*, *Frankia asymbiotica*, *Frankia saprophytica*,  
82 *Frankia Canadensis*, *Frankia coriaria* together with the studied strain G2<sup>T</sup> were maintained in  
83 Basic Propionate (BAP)[24] broth medium supplemented with NH<sub>4</sub>Cl at 28°C without shaking  
84 as previously described [14]. Phenotypic characterization was performed on 4 weeks old  
85 cultures. Freeze dried cells were used for chemotaxonomic analyses while a fresh wet biomass  
86 were examined for fatty acids profile and biochemical and morphological features. In this  
87 context, scanning electron microscope (FE-SEM Merlin, Zeiss, Germany) and GENIII  
88 microplates in an Omnilog device (Biolog Inc., Haywood, USA) were used as described by  
89 Nouioui *et al.* [14]. All analysed tests were carried out in duplicate.

90 Red pigmented colonies were developed after 3-4 weeks incubation of the type strain in BAP  
91 broth medium at 28°C without shaking. The colonies were formed with extremely branched  
92 vegetative hyphae, vesicles and multilocular sporangia as shown in Fig. 1 a-b, features observed  
93 as well for *F. elaeagni* DSM 46783<sup>T</sup> and *F. discariae* DSM 46785<sup>T</sup>. The ability of the type strain  
94 to fix atmospheric nitrogen and to nodulate member of the order *Rhiziales* were examined by  
95 Diem *et al.* [23]. It has been shown that strain G2<sup>T</sup> was unable to re-infect its host plant  
96 *Casuarina equisetifolia* [23]. The type strain can be distinguished from its nearest phylogenetic  
97 neighbours, *F. elaeagni* DSM 46783<sup>T</sup>, by its red pigmentation and several biochemical  
98 properties including its ability to metabolise bromo-succinic acid, guanidine hydrochloride,  
99 methyl pyruvate, potassium tellurite and 1% sodium lactate, and to grow in presence of  
100 minocycline and vancomycin. Moreover, strain G2<sup>T</sup> was unable to oxidise D-glucose-6-  
101 phosphate, D-fructose-6-phosphate and  $\beta$ -hydroxy-butyric acid unlike its phylogenetic  
102 neighbour (Table 1). Thus morphological, physiological and cultural traits of strain G2<sup>T</sup> are  
103 consistent with the genus *Frankia*.

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105 Chemotaxonomic traits of strain G2<sup>T</sup> have been determined based on thin layer chromatography  
106 procedures. Menaquinones and polar lipid profiles as well as diaminopimelic acids and sugars  
107 contents of whole cell hydrolysate were identified following the same protocols used by  
108 Nouioui *et al.* [14]. Fatty acid methyl esters (FAMES) analyses for strain G2<sup>T</sup> and the reference  
109 strains cited above were extracted and identified following the modified protocol of Miller  
110 (1982) [25] by Kuykendall *et al.* [26] and as described by Nouioui *et al.* [14]. Strain G2<sup>T</sup> was  
111 characterized by the presence of (i) *meso*-A<sub>2</sub>pm, galactose, glucose, mannose, rhamnose, ribose  
112 and xylose in its whole cell hydrolysates, (ii) isoprenologue profile consisted of MK-9(H<sub>4</sub>) and  
113 MK-9(H<sub>6</sub>) as the predominant ones (>20%) and by (iii) polar lipid pattern consisted of  
114 phosphatidylinositol (PI), diphosphatidylglycerol (DPG), glycerophospholipids (GPL1-2),  
115 phosphatidylglycerol (PG), aminophospholipid (APL) and uncharacterized lipids (L). Apart  
116 from the presence of APL, the chemotaxonomic features of strain G2<sup>T</sup> are in line with those of  
117 the type species of the genus, *Frankia alni* DSM 45986<sup>T</sup>, and with its nearest phylogenetic  
118 neighbors; *F. elaeagni* DSM 46783<sup>T</sup> and *F. discariae* DSM 46785<sup>T</sup> excepting that MK-9 (H<sub>4</sub>)  
119 was the major menaquinone and lacks rhamnose in cell wall sugars in *F. discariae* DSM  
120 46785<sup>T</sup>. In addition that the strain G2<sup>T</sup> contained APLThe major fatty acids (>15%) of the type  
121 strain are *iso*-C<sub>16:0</sub>, C<sub>17:1</sub>  $\omega$ 8c and C<sub>15:0</sub> while the type strains of *F. elaeagni* and *F. discariae* species  
122 have C<sub>16:0</sub> instead of the C<sub>15:0</sub>.

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124 The almost complete sequences of 16S rRNA gene of strain G2<sup>T</sup> extracted from the draft  
125 genome and obtained from PCR-product are 100% identical to each other. Pairwise 16S rRNA  
126 gene sequence similarities and phylogenetic trees were determined using the GGDC web server  
127 [27] and according to Meier-Kolthoff *et al.* [28]. Maximum-likelihood (ML) and maximum-  
128 parsimony (MP) trees were inferred on DSMZ phylogenomic pipeline [29] and using the  
129 GTR+GAMMA model. For ML and MP trees, the sequences were aligned using RAxML [30]  
130 and TNT [31], respectively. Rapid bootstrapping in conjunction with the autoMRE  
131 bootstopping criterion [32] was used for ML while 1000 bootstrapping replicates in  
132 conjunction with tree-bisection-and-reconnection branch swapping and ten random sequence  
133 addition replicates was used for MP. Multiple sequence alignment were determined using  
134 MUSCLE program [33] while X<sup>2</sup> test as implemented in PAUP [34] was used to check the  
135 sequence for a compositional bias.

136 Pairwise sequence similarities for 16S rRNA gene sequence between strain G2<sup>T</sup> and the type  
137 strains of *Frankia* species varied from 97.4% to 99.5%. The highest values (above 99.0%) have  
138 been found with the type strains of *F. elaeagni* and *F. discariae* species which belong, with  
139 strain G2<sup>T</sup>, to cluster 3 of the genus *Frankia* [9-10, 35-36]. In the ML phylogenetic tree, strain  
140 G2<sup>T</sup> appeared, in highly supported clade, closely related to the type strain of *F. elaeagni* species  
141 (99.5%) forming a subclade next to the one encompasses the type strain of *F. discariae* (99.4%),  
142 *F. saprophytica* (98.1%), *F. inefficax* (97.6%) and *F. asymbiotica* (97.8%) (Fig. 2a).

143  
144 The genome sequences of the *Frankia* and representative strains from other related genera were  
145 annotated using Prokka v1.11 [37] and were compared using BPGA 1.3 pipeline [38]. The  
146 missing data and poorly aligned regions from concatenated protein sequence alignment of the  
147 core genome were removed using Gblocks [39]. A ML tree was constructed from the resulting  
148 alignment of 10,491 amino acids using LG+F+G4 substitution model by IQ-Tree with 100,000  
149 ultrafast bootstrap iterations and SH-like approximate likelihood ratio tests [40]. Another ML  
150 tree was generated using PhyloPhlAn [41] which extracts subsets of amino acid sequences from  
151 400 universal proteins and calculate phylogeny from the concatenated alignment using RAxML  
152 [42]. This approach is particularly suitable for an accurate determination of taxonomic  
153 relationships from the genomic data [41]. The phylogenetic position of strain G2<sup>T</sup> (Fig2b and  
154 Fig2.c) is in concordance with the ML 16SrRNA gene tree

155  
156 Digital DNA:DNA hybridisation (dDDH) between strain G2<sup>T</sup> and its nearest phylogenetic  
157 neighbour cited above was calculated using genome to genome distance calculator with formula

158 2 available at DSMZ server (<http://ggdc.dsmz.de/distcalc2.php>). Strain G2<sup>T</sup> and its  
159 phylogenetic relatives cited above showed dDDH values below the threshold of 70% designed  
160 by Wayne *et al.* [43] for delineation a novel prokaryotic species (Table 2). Strain G2<sup>T</sup> has a  
161 genome size of 9.5 Mb with 70.9 % of G+C content while its nearest neighbours, *F. elaeagni*  
162 DSM 46783<sup>T</sup> and *F. discariae* DSM 46785<sup>T</sup> have respectively 7.6 Mb and 7.9 Mb with 71.7 %  
163 and 72.4 % of G+C content.

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165 It can be concluded from the wealth of the present polyphasic study that strain G2<sup>T</sup> has  
166 phenotypic and genetic features consistent with those of the genus *Frankia* and distinguishable  
167 from the other *Frankia* species. Therefore, strain G2<sup>T</sup> forms a new lineage of the genus and  
168 merits to be recognised as a new species within the genus for which the name *Frankia*  
169 *irregularis* sp. nov. is proposed.

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#### 171 **Description of *Frankia irregularis* sp. nov.**

172 *Frankia irregularis* (ir.re.gu.la'ris. L. fem. adj. *irregularis* of irregular, referring to the inability  
173 of the species to infect its original host plant and to infect taxonomically disparate host plants)  
174 Nitrogen fixing Gram-positive aerobic, heterotrophic and chemoorganotrophic actinobacterium  
175 known by its red pigmentation; colonies were formed by three cell structures: substrate hyphae,  
176 multilocular sporangia and vesicles. Optimal growth was observed on BAP medium for 3-4  
177 weeks at 28°C and from pH 6.3 to 6.8. It is able to oxidise D-cellobiose, *α*-keto-butyric acid,  
178 methyl pyruvate, L-lactic acid, bromo-succinic acid, acetic acid, guanidine hydrochloride;  
179 growth in presence of 1% sodium lactate, potassium tellurite, lincomycin, minocycline and  
180 vancomycin. Whole cell hydrolysates are formed by meso diaminopimelic acid, galactose,  
181 glucose, mannose, rhamnose, ribose and xylose; polar lipid pattern consisted of  
182 phosphatidylinositol (PI), diphosphatidylglycerol (DPG), glycopospholipids (GPL1-2),  
183 phosphatidylglycerol (PG), aminophospholipid (APL) and unknown lipids (L) (Fig. S1) and  
184 predominant menaquinones (>20%) are MK-9 (H<sub>4</sub>) and MK-9 (H<sub>6</sub>). The major fatty acids  
185 (>15%) are *iso*-C<sub>16:0</sub>, C<sub>17:1 ω8c</sub> and C<sub>15:0</sub>.

186 The type strain G2<sup>T</sup> (=DSM 45899<sup>T</sup> = CECT 9038<sup>T</sup>) was isolated from *Casuarina equisetifolia*  
187 [23]. The size of the genome is 9.5 Mb and digital DNA G+C content is 70.9%.

188 The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence and draft  
189 genome sequence reported are MH145366 and FAOZ00000000, respectively.

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191 **Conflicts of interest** Authors have no conflict of interest to declare.

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## 196 **References**

- 197 1. Brunchorst. J. Über einige Wurzelanschwellungen, besonders diejenigen von Alnus und den  
198 Elaegnaceen. *Botanische Institut Tübingen* 1886;2:151-77.
- 199 2. Becking JH. *Frankiaceae* fam. nov. (Actinomycetales) with one new combination and six new  
200 species of the genus *Frankia* Brunchorst 1886, 174. *Int J Syst Bacteriol* 1970;20:201-20.
- 201 3. Zhi XY, Li WJ, Stackebrandt E. An update of the structure and 16S rRNA gene sequence based  
202 definition of higher ranks of the class *Actinobacteria*, with the proposal of two new suborders and  
203 four new families and emended descriptions of the existing higher taxa. *Int J Syst Evol Microbiol*  
204 2009;59:589-608.
- 205 4. Sen A, Daubin V, Abrouk D, Gifford I, Berry AM, *et al.* Phylogeny of the class *Actinobacteria*  
206 revisited in the light of complete genomes. The orders 'Frankiales' and *Micrococcales* should be  
207 split into coherent entities: proposal of *Frankiales* ord. nov., *Geodermatophilales* ord. nov.,  
208 *Acidothermales* ord. nov. and *Nakamurellales* ord. nov. *Int J Syst Evol Microbiol* 2014;64(Pt  
209 11):3821-3832.
- 210 5. Benson DR, Silvester WB. Biology of *Frankia* strains, actinomycete symbionts of actinorhizal plants.  
211 *Microbiol Rev* 1993;57:293-319.
- 212 6. Schwencke J, Carú M. Advances in actinorhizal symbiosis: host plant-*Frankia* interactions, biology,  
213 and applications in arid land reclamation. *Arid Land Res Manag* 2001; 15:285-327.
- 214 7. Chaia EE, Wall L, Huss-Danell K. Life in soil by actinorhizal root nodule endophyte *Frankia*. *A review*  
215 *Symbiosis* 2010; 51:201-226.
- 216 8. Normand P, Orso S, Cournoyer B, Jeannin P, Chapelon C, *et al.* Molecular phylogeny of the genus  
217 *Frankia* and related genera and emendation of the family *Frankiaceae*. *Int J Syst Bacteriol*  
218 1996;46:1-9.
- 219 9. Nouioui I, Ghodhbane-Gtari F, Beauchemin NJ, Tisa LS, Gtari M. Phylogeny of members of the  
220 *Frankia* genus based on *gyrB*, *nifH* and *glnII* sequences. *Antonie Van Leeuwenhoek* 2011;100:579-  
221 587.
- 222 10. Ghodhbane-Gtari F, Nouioui I, Chair M, Boudabous A, Gtari M. 16S-23S rRNA intergenic spacer  
223 region variability in the genus *Frankia*. *Microb Ecol* 2010;60:487-495.
- 224 11. Gtari M, Ghodhbane-Gtari F, Nouioui I, Ktari A, Hezbri K, *et al.* Cultivating the uncultured: growing  
225 the recalcitrant cluster-2 *Frankia* strains. *Sci Rep* 2015;5:13112.
- 226 12. Louis ST, Oshone R, Sarkar I, Ktari A, Sen A, *et al.* Genomic approaches toward understanding the  
227 actinorhizal symbiosis: an update on the status of the *Frankia* genomes. *Symbiosis* 2016; 70:5-16.
- 228 13. Baker DD. Relationships among pure cultured strains of *Frankia* based on host specificity. *Physiol*  
229 *Plant* 1987;70:245-248.
- 230 14. Nouioui I, Ghodhbane-Gtari F, Montero-Calasanz MD, Göker M, Meier-Kolthoff JP, *et al.* Proposal  
231 of a type strain for *Frankia alni* (Woronin 1866) Von Tubeuf 1895, emended description of *Frankia*  
232 *alni*, and recognition of *Frankia casuarinae* sp. nov. and *Frankia elaeagni* sp. nov. *Int J Syst Evol*  
233 *Microbiol* 2016;66:5201-5210.
- 234 15. Normand N, Nouioui I, Pujic P, Fournier P, Dubost A, *et al.* *Frankia canadensis* sp. nov., isolated  
235 from root nodules of *Alnus incana* subspecies *rugosa* growing in Canada. *Int J Evol Syst Microbiol*  
236 2018;Accepted.

- 237 16. Nouioui I, Ghodhbane-Gtari F, Rohde M, Klenk HP, Gtari M. *Frankia coriariae* sp. nov., an infective  
238 and effective microsymbiont isolated from *Coriaria japonica*. *Int J Syst Evol Microbiol*  
239 2017;67:1266–1270
- 240 17. Persson T, Benson DR, Normand P, Vanden Heuvel B, Pujic P, *et al.* Genome sequence of  
241 "Candidatus *Frankia datiscaae*" Dg1, the uncultured microsymbiont from nitrogen-fixing root  
242 nodules of the dicot *Datisca glomerata*. *J Bacteriol* 2011;193:7017-7018.
- 243 18. Normand P, Nguyen TV, Battenberg K, Berry AM, Heuvel BV, *et al.* Proposal of 'Candidatus *Frankia*  
244 *californiensis*', the uncultured symbiont in nitrogen-fixing root nodules of a phylogenetically broad  
245 group of hosts endemic to western North America. *Int J Syst Evol Microbiol* 2017;67:3706-3715.
- 246 19. Nouioui I, Montero-Calasanz MDC, Ghodhbane-Gtari F, Rohde M, Tisa LS, *et al.* *Frankia discariae*  
247 sp. nov.: an infective and effective microsymbiont isolated from the root nodule of *Discaria*  
248 *trinervis*. *Arch Microbiol* 2017;199: 641–647.
- 249 20. Nouioui I, Ghodhbane-Gtari F, Montero-Calasanz MDC, Rohde M, Tisa LS, *et al.* *Frankia inefficax*  
250 sp. nov., an actinobacterial endophyte inducing ineffective, non nitrogen-fixing, root nodules on  
251 its actinorhizal host plants. *Antonie Van Leeuwenhoek* 2017;110:313-320.
- 252 21. Nouioui I, Gueddou A, Ghodhbane-Gtari F, Rhode M, Gtari M, *et al.* *Frankia asymbiotica* sp. nov.,  
253 a non infective actinobacterium isolated from *Morella californica* root nodule. *Int J Syst Evol*  
254 *Microbiol* 2017; 67:4897-4901.
- 255 22. Nouioui I, Ghodhbane-Gtari F, Klenk HP, Gtari M. *Frankia saprophytica* sp. nov. an atypical non-  
256 infective (Nod–) and non-nitrogen fixing (Fix–) actinobacterium isolated from *Coriaria nepalensis*  
257 root nodules. *Int J Syst Evol Microbiol* 2018;68:1090–1095.
- 258 23. Diem HG, Gauthier D, Dommergues YR. Isolation of *Frankia* from nodules of *Casuarina*  
259 *equisetifolia*. *Can J Microbiol* 1982;28:526–530.
- 260 24. Murry MA, Fontaine MS, Torrey JG. Growth kinetics and nitrogenase induction in *Frankia* sp. HFP  
261 Arl3 grown in batch culture. *Plant Soil* 1984;78:61-78.
- 262 25. Miller LT. Single derivatization method for routine analysis of bacterial whole-cell fatty acid methyl  
263 esters, including hydroxy acids. *J Clin Microbiol* 1982;16:584-586.
- 264 26. Kuykendall LD, Roy MA, O'Neill JJ, Devine TE. Fatty Acids, Antibiotic Resistance, and  
265 Deoxyribonucleic Acid Homology Groups of *Bradyrhizobium japonicum*. *Int J Syst Evol Microbiol*  
266 1988;38:358-361.
- 267 27. Meier-Kolthoff JP, Auch AF, Klenk HP, Göker M. Genome sequence-based species delimitation  
268 with confidence intervals and improved distance functions. *BMC Bioinformatics*. 2013;14:60.
- 269 28. Meier-Kolthoff JP, Göker M, Spröer C, Klenk HP. When should a DDH experiment be mandatory in  
270 microbial taxonomy? *Arch Microbiol* 2013;195:413-418.
- 271 29. Meier-Kolthoff JP, Hahnke RL, Petersen J, Scheuner C, Michael V, *et al.* Complete genome  
272 sequence of DSM 30083(T), the type strain (U5/41(T)) of *Escherichia coli*, and a proposal for  
273 delineating subspecies in microbial taxonomy. *Stand Genomic Sci* 2014;9:2.
- 274 30. Stamatakis A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large  
275 phylogenies. *Bioinformatics* 2014;30(9):1312-1313.
- 276 31. Goloboff PA, Farris JS, Nixon KC. TNT, a free program for phylogenetic analysis. *Cladistics*  
277 2008;24:774-786.
- 278 32. Pattengale ND, Alipour M, Bininda-Emonds OR, Moret BM, Stamatakis A. How many bootstrap  
279 replicates are necessary? *J Comput Biol* 2010;17:337-354.
- 280 33. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic*  
281 *Acids Res* 2004;32:1792-1797.
- 282 34. Swofford DL. PAUP\*: Phylogenetic Analysis Using Parsimony (\*and Other Methods), Version 4.0.  
283 Sunderland: Sinauer Associates; 2002.
- 284 35. Gtari M, Brusetti L, Skander G, Mora D, Boudabous A, *et al.* Isolation of *Elaeagnus*-compatible  
285 *Frankia* from soils collected in Tunisia. *FEMS Microbiol Lett* 2004;234:349-355.
- 286 36. Gtari M, Daffonchio D, Boudabous A. Assessment of the genetic diversity of *Frankia*  
287 microsymbionts of *Elaeagnus angustifolia* L. plants growing in a Tunisian date-palm oasis by  
288 analysis of PCR amplified *nifD*-K intergenic spacer. *Can J Microbiol* 2007;53:440-445.



- 289 37. Seemann T. Prokka: rapid prokaryotic genome annotation. *Bioinformatics* 2014;15:30:2068-2069.  
290 38. Chaudhari NM, Gupta VK, Dutta C. BPGA- an ultra-fast pan-genome analysis pipeline. *Sci Rep* 2016;  
291 6: 24373.  
292 39. Castresana J. Selection of conserved blocks from multiple alignments for their use in phylogenetic  
293 analysis. *Mol Biol Evol* 2000;17:540-52.  
294 40. Minh BQ, Nguyen MA, von Haeseler A. Ultrafast approximation for phylogenetic bootstrap. *Mol*  
295 *Biol Evol* 2013;30:1188-1195.  
296 41. Segata N, Börnigen D, Morgan XC, Huttenhower C. PhyloPhlAn is a new method for improved  
297 phylogenetic and taxonomic placement of microbes. *Nat Commun* 2013;4:2304.  
298 42. Stamatakis A. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of  
299 taxa and mixed models. *Bioinformatics* 2006;22:2688-2690.  
300 34. Wayne LG, Brenner BD, Colwell RR, Grimont PAD, Kandler O, *et al.* Report of the ad hoc committee  
301 on reconciliation of approaches to bacterial systematics. *Int J Syst bacteriol* 1987; 37:463–464.  
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### 305 **Figure Legends**

306 **Figure 1.** Scanning electron (a) and light microscopy micrograph (b) of strain G2<sup>T</sup> grew on BAP  
307 media for 4 weeks at 28°C. (h), hyphae; (v), vesicles and (s), sporangia.

308 **Figure 2.** Maximum-likelihood phylogenetic tree based on almost complete 16S rRNA gene  
309 sequences constructed using the GTR+GAMMA model. The numbers above the branches are  
310 bootstrap support values greater than 60% for ML (left) and MP (right) (a). Maximum-  
311 likelihood phylogenomic tree based on core genome sequences (b). Maximum-likelihood  
312 phylogenomic tree based on concatenated amino acid sequences from 400 universal proteins  
313 (c).

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Sodium lactate	+	+	-	+	+	+	-	+	+	+
<b>Grow in presence of</b>										
Fusidic acid	w	+	-	+	+	-	-	+	+	+
Lithium chloride	w	+	-	+	-	-	-	+	+	+
Potassium tellurite	+	+	+	+	+	-	-	+	+	+
Sodium bromate	w	+	+	+	-	-	-	-	+	+
<b>Nitrogen sources</b>										
Guanidine hydrochloride	+	+	-	+	-	-	-	+	+	-
D-serine	w	+	+	+	-	-	-	-	+	+
<b>Antibiotic resistance to<sup>#</sup></b>										
Lincomycin	R	R	S	R	S	S	R	R	R	S
Nalidixic acid	w	R	R	R	R	S	S	R	R	R
Minocycline and vancomycin	R	R	S	R	R	S	S	R	R	R
<b>Major fatty acids (&gt;15%)</b>	<i>iso</i> -C <sub>16:0</sub> , C <sub>17:1 ω</sub> 8c, C <sub>15:0</sub>	<i>iso</i> -C <sub>16:0</sub> , C <sub>17:1 ω</sub> 8c	<i>iso</i> -C <sub>16:0</sub> , C <sub>17:1 ω</sub> 8c	<i>iso</i> -C <sub>16:0</sub> , C <sub>17:1 ω</sub> 8c	<i>iso</i> -C <sub>16:0</sub> , C <sub>17:1 ω</sub> 8c	C <sub>18:1 ω</sub> 9c, C <sub>16:0</sub>	<i>iso</i> -C <sub>16:0</sub> , C <sub>17:1 ω</sub> 8c	C <sub>17:1 ω</sub> 8c, <i>iso</i> -C <sub>16:0</sub> , C <sub>16:0</sub>	<i>iso</i> -C <sub>16:0</sub> , C <sub>17:1 ω</sub> 8c, C <sub>17:0</sub> , C <sub>15:0</sub>	<i>iso</i> -C <sub>16:0</sub> , C <sub>17:1 ω</sub> 8c, C <sub>15:0</sub>
<b>Predominant menaquinones (&gt;20%)</b>	MK-9(H <sub>4</sub> ); MK-9(H <sub>6</sub> )	MK-9(H <sub>8</sub> ), MK-9(H <sub>4</sub> ) <sup>[14]</sup>	MK-9(H <sub>4</sub> ), MK-9(H <sub>6</sub> ) <sup>[21]</sup>	MK-9(H <sub>8</sub> ) <sup>[15]</sup>	MK-9(H <sub>6</sub> ), MK-9(H <sub>8</sub> ) <sup>[14]</sup>	MK9(H <sub>6</sub> ), MK9(H <sub>4</sub> ) <sup>[16]</sup>	MK-9(H <sub>4</sub> ), MK-9(H <sub>6</sub> ) <sup>[14]</sup>	MK-9(H <sub>4</sub> ) <sup>[19]</sup>	MK-9(H <sub>6</sub> ), MK-9(H <sub>4</sub> ) <sup>[20]</sup>	MK-9(H <sub>6</sub> ) <sup>[22]</sup>
<b>Polar lipids</b>	PI, DPG, GPL <sub>1-2</sub> , PG, APL, UL	PI, DPG, GPL <sub>1-3</sub> , PG, UL <sup>[14]</sup>	PI, DPG, PG, PL <sup>[21]</sup>	PI, DPG, GPL <sub>1-2</sub> , PG, PL <sub>1-3</sub> , UL <sup>[15]</sup>	PI, DPG, GPL <sub>1-3</sub> , PG, UL <sup>[14]</sup>	PI, PG, DPG, GPL <sub>1-2</sub> , UL <sup>[16]</sup>	PI, DPG, GPL <sub>1-3</sub> , PG, UL <sup>[14]</sup>	PI, DPG, GPL <sub>1-3</sub> , PG, UL <sup>[19]</sup>	PI, DPG, GPL <sub>1-2</sub> , PG, UL <sup>[20]</sup>	PI, DPG, GPL <sub>1-2</sub> , GL <sub>1-6</sub> , PG, PL, UL <sup>[22]</sup>

<b>Host plant origin</b>	<i>Casuarina equisetifolia</i>	<i>Alnus viridis ssp.crispa</i>	<i>Morella californica</i>	<i>Alnus incana ssp. rugosa</i>	<i>Casuarina cunninghamiana</i>	<i>Coriaria japonica</i>	<i>Elaeagnus angustifolia</i>	<i>Discaria trinervis</i>	<i>Elaeagnus umbellata</i>	<i>Coriaria nepalensis</i>
<b>Host plant range</b>	Rhamnales	<i>Alnus</i> , <i>Comptonia</i> , <i>Myrica</i>	-	<i>Alnus</i>	Casuarinaceae (excluding <i>Gymnostom</i> ), <i>Myricaceae</i>	<i>Coriariaceae</i> , <i>Datisceae</i>	<i>Elaeagnaceae</i> , <i>Colletieae</i> , <i>Morella</i>	<i>Colletieae</i> , <i>Elaeagnaceae</i> <i>eMorella</i>	<i>Elaeagnaceae</i> , <i>Morella</i>	-
<b>Genomic G+C content (%)</b>	70.9	72.8	72.0	72.4	70.1	71.0	71.7	72.3	72.3	71.8

+, positive reaction; -, w, weak reaction; negative reaction; R, resistant; S, sensitive; DPG: diphosphatidylglycerol; UL: unidentified lipids; PG: phosphatidylglycerol; GPL: Unknown glycopospholipid; PI: phosphatidylinositol; PL: phospholipids

**Table 2.** 16S rRNA gene sequence identities and dDDH values between type strain G2<sup>T</sup> and the type strains of the nearest phylogenetic *Frankia* species. dDDH values are in % (upper right) and 16S rRNA gene sequence similarities are in % (lower left)

	<b>G2<sup>T</sup></b>	<i>F. inefficax</i> DSM 45817 <sup>T</sup>	<i>F. elaeagni</i> DSM 46783 <sup>T</sup>	<i>F. discariae</i> DSM 46785 <sup>T</sup>	<i>F. asymbiotica</i> DSM 100626 <sup>T</sup>	<i>F. saprophytica</i> DSM 105290 <sup>T</sup>
<b>G2<sup>T</sup></b>	-	22.1 [19.8 -24.6%]	25.9 [23.6 - 28.4%]	24.9 [22.6 - 27.4%]	22.5 [20.3 - 25%]	22.8 [20.5 - 25.2%]
<i>F. inefficax</i> DSM 45817 <sup>T</sup>	97.6	-	22.2 [20 - 24.7%]	22.6 [20.3 - 25%]	25.8 [23.5 - 28.3%]	25.7 [23.4 - 28.2%]
<i>F. elaeagni</i> DSM 46783 <sup>T</sup>	99.5	97.8	-	25.6 [23.3 - 28.1%]	22.5 [20.3 - 25%]	23.0 [20.7 - 25.4%]
<i>F. discariae</i> DSM 46785 <sup>T</sup>	99.4	97.8	98.9	-	23.1 [20.8 - 25.5%]	23.3 [21 - 25.7%]

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<i>F. asymbiotica</i> DSM 100626 <sup>T</sup>	97.8	98.1	98.0	97.8	-	34.6 [32.2 - 37.1%]
<i>F. saprophytica</i> DSM 105290 <sup>T</sup>	98.1	98.5	98.2	98.0	99.4	-

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[...] confidence interval

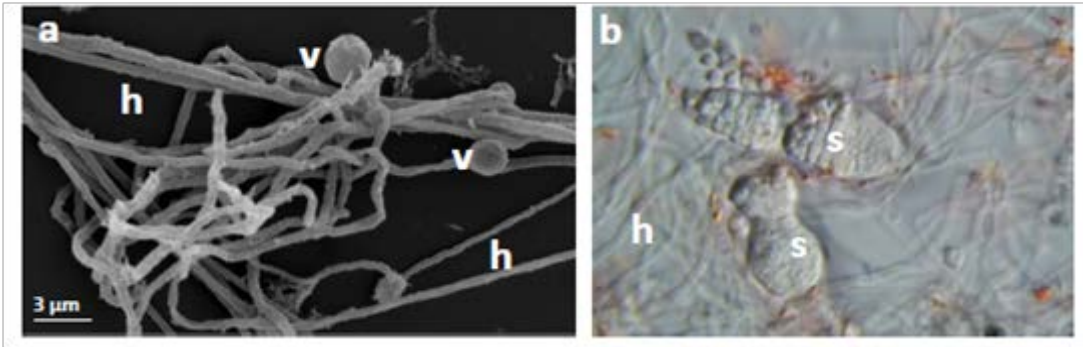
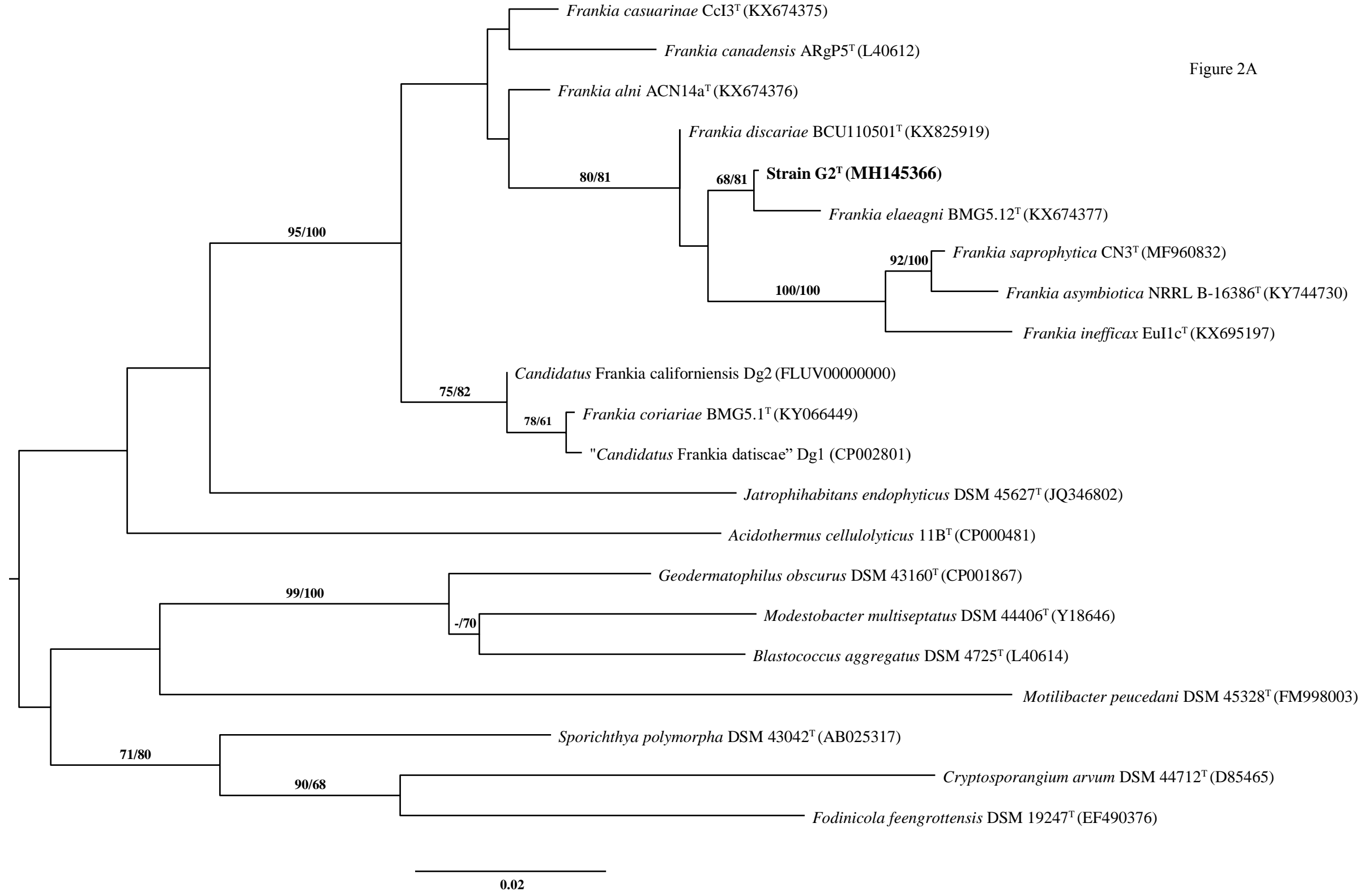


Figure 1

Figure 2A



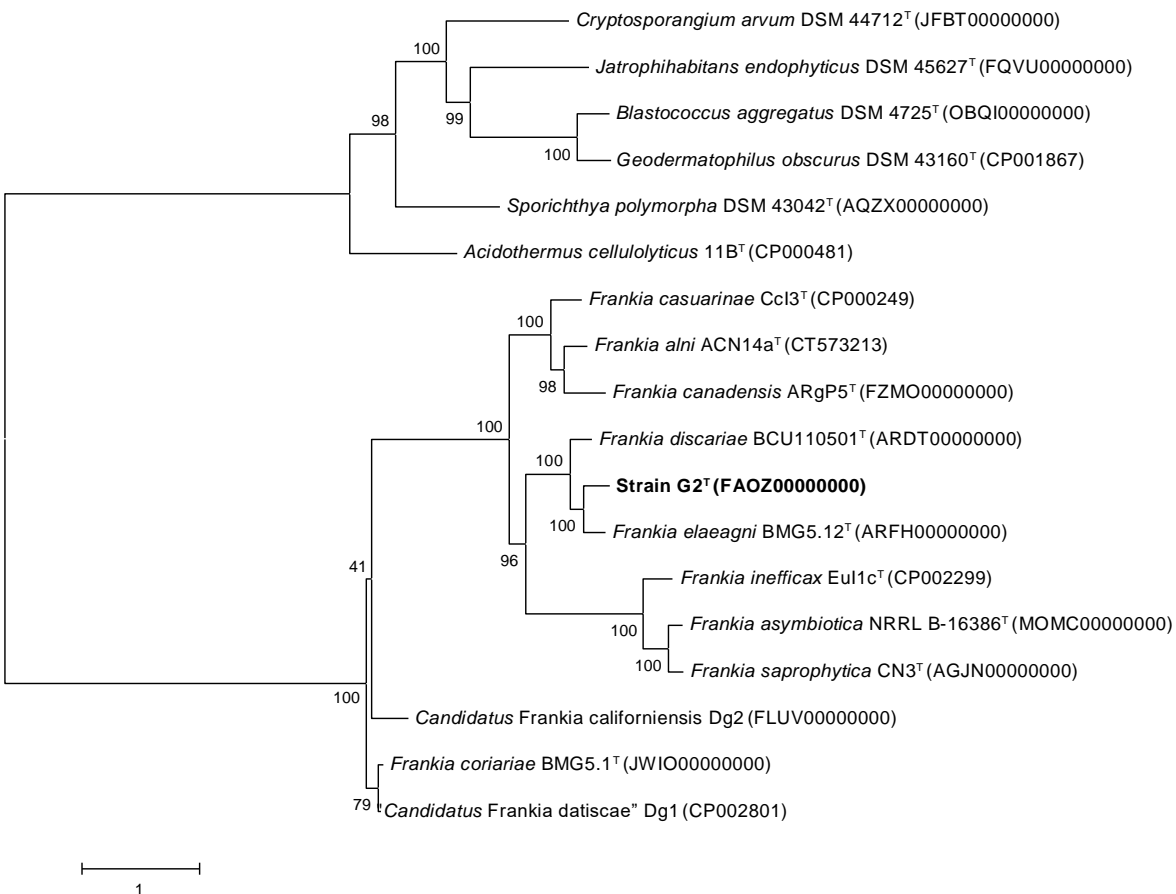


Figure 2B