

1 ***Vigna unguiculata* is nodulated in Spain by endosymbionts of Genisteae legumes and by
2 a new symbiovar (*vignae*) of genus *Bradyrhizobium***

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16

17 **Abstract**

18

19 *Vigna unguiculata* was introduced in Europe from Africa where it has its distribution center
20 being currently cultivated in Mediterranean regions with adequate edapho-climatic conditions
21 and where the slow growing rhizobia nodulating this legume have not yet been studied.
22 Previous studies based on *rrs* gene and ITS region analyses showed that *B. yuanmingense* and
23 *B. elkanii* nodulated *V. unguiculata* in Africa, two species that were not found in this study.
24 Using the same phylogenetic markers we showed that in Spain *V. unguiculata*, a legume from
25 Tribe Phaseolae, is nodulated by two species of group I, *B. cytisi* and *B. canariense*, which are
26 common endosymbionts of Genisteae in both Europe and Africa. These two species have not
27 been found up to date in *V. unguiculata* nodules in its African distribution centers. All strains
28 from *Bradyrhizobium* group I isolated in Spain belong to the symbiovar genistearum found at
29 present only in Genisteae legumes in both Africa and Europe. *V. unguiculata* is also nodulated
30 in Spain by a strain from the group II of *Bradyrhizobium* belonging to a novel symbiovar
31 (*vignae*). Some African *V. unguiculata*- nodulating strains also belong to this symbiovar
32 proposed here.

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34 **Key words:** *Bradyrhizobium*, *Vigna unguiculata*, symbiovar, phylogeny, Spain

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38 **Introduction**

39
40 The species *Vigna unguiculata* (cowpea) from the Tribe Phaseolae is indigenous to Africa
41 where the Transvaal region is considered its evolutionary center since the oldest varieties of
42 this legume were found in this region [20]. *V. unguiculata* takes part of human diet in African
43 countries [12] because it has high contents in protein (23%), carbohydrates (56%) and fiber
44 (4%) that can fulfil the human essential amino acid requirements complemented with cereals
45 [11]. Moreover this legume has great agronomic interest due to its resistance to acidity,
46 dryness and high temperatures [5, 8] and by the establishment of nitrogen-fixing symbiosis
47 that allow its use in intercrops with cereals, mainly maize, in the African countries [9, 14]. In
48 these African countries this legume establishes nitrogen-fixing symbioses with several slow
49 growing strains from genus *Bradyrhizobium* being *B. yuanmingense* and *B. elkanii* the main
50 species identified in cowpea nodules [16, 25, 36].

51 *V. unguiculata* was introduced from Northern Africa in the South of Europe where this
52 legume is currently cultivated in Mediterranean regions such as Extremadura (Spain), a warm
53 region with acidic soils suitable for the cropping of this legume and where it is very
54 appreciated by the consumers. Nevertheless, despite the interest of this legume for
55 intercropping or rotation with non-legumes overall in European Mediterranean countries there
56 are no data about the rhizobia establishing nitrogen-fixing symbiosis with this legume in
57 Europe .

58 This work is the first one carried out in an European country that aimed the identification of
59 slow growing strains nodulating *V. unguiculata* as well as the analysis of their phylogenetic
60 relationships with those nodulating this legume in African countries. Surprisingly, the results
61 of this work showed the nodulation of *V. unguiculata*, a legume that belongs to the Tribe
62 Phaseolae, by endosymbionts of legumes from Tribe Genisteae that have not been found in
63 African countries. In addition, we detected a phylogenetic lineage belonging to the
64 *Bradyrhizobium* group II scarcely present in Europe whose *nodC* gene correspond to a novel
65 symbiovar within this genus also present in Africa for which we propose the name *vignae*.

66

67 **Materials and methods**

68 *Strains and nodulation experiments*

69 Plants of *V. unguiculata* were used as trap plants in a soil from Extremadura in Spain. The
70 rhizobial strains were isolated from the nodules according to the method of Vincent [42]. In
71 order to confirm the nodulation capacity of the strains, infectivity tests were conducted in a

72 growth chamber with controlled conditions using sterile vermiculite as substrate. *Vigna*
73 *unguiculata* were surface disinfected and seedlings were inoculated as described by Ramírez-
74 Bahena *et al.* [27].

75

76 *RAPD fingerprinting*

77 RAPD patterns were obtained as previously described [30] using the primer M13 (5'-
78 GAGGGTGGCGGTTCT -3') and the GoTaq Flexi DNA polymerase (Promega). PCR
79 conditions were: preheating at 95 °C for 9 min; 35 cycles of denaturing at 95 °C for 1 min;
80 annealing at 45 °C for 1 min and extension at 75 °C for 2 min, and a final extension at 72 °C
81 for 7 min. 10 µl of each PCR products were electrophoresed on 1.5% (w/v) agarose gel in
82 TBE buffer (100 mM Tris, 83 mM boric acid, 1 mM EDTA, pH 8.5) at 6 V/cm, stained in a
83 solution containing 0.5 g/ml ethidium bromide, and photographed under UV light. Standard
84 VI (Roche, USA) was used as a size marker. A dendrogram was constructed based on the
85 matrix generated using UPGMA method and the Pearson coefficient with Bionumerics
86 version 4.0 software (Applied Maths, Austin, TX)

87

88 *Analysis of rrs, atpD and nodC genes and 16S-23S intergenic spacer (ITS)*

89 The *rrs* was amplified and sequenced according to Rivas *et al.* [28], the ITS as described by
90 Peix *et al.* [22]. The *nodC* gene was amplified with the primers and conditions described by
91 Laguerre *et al.* [17], except for the strain VUPME 10 whose *nodC* genes were amplified and
92 sequenced with the following primers nodCBradyF (5' CGCAAGGCGCAGWTCGC 3') and
93 nodCBradyR (5' GGKGTGVAGCGMGAAGCCG 3'). PCR amplifications were performed
94 with a REDExtract-N-Amp PCR Kit (Sigma) or GoTaq Flexi DNA polymerase kit (Promega)
95 or DreamTaq Green DNA polymerase (Thermo) following the manufacturers' instructions.
96 The bands corresponding to the different genes were purified either directly from the gel by
97 room temperature centrifugation using a DNA gel extraction device (Millipore Co., USA) for
98 10 min at 5000 g or by elution of the excised band and filtration through silicagel columns
99 using the Qiaquick DNA Gel Extraction Kit (Qiagen, Germany) in all cases following the
100 manufacturers' instructions. In the case of *nodC* gene from strain VUPME10, The amplified
101 product obtained in the previous section (ca. 800 bp) was cloned into the vector pJet1.2/blunt
102 Cloning Vector using the CloneJET PCR Cloning Kit (Thermo) according to manufacturer's
103 instructions for the Sticky-End Cloning Protocol. Transformation Protocol was performed
104 using Promega instructions for competent cells JM109 (Promega). The analysis of the

105 recombinant clones was carried out following the protocol for colony screening by PCR
106 following the cloning kit manufacturer's instructions.

107 The sequence reaction was performed on an ABI PRISM 3100 sequencer using a BigDye
108 terminator v3.1 cycle sequencing kit (Applied Biosystems Inc., USA) as supplied by the
109 manufacturer. The sequences obtained were compared to those held in GenBank by using the
110 BLASTN program [1]. They were aligned by using Clustal W software [40]. Distances
111 calculated according to Kimura's two-parameter model [15] were used to infer phylogenetic
112 trees with the neighbour-joining and maximum likelihood methods [6, 33] with MEGA5
113 software [39]. Confidence values for nodes in the trees were generated by bootstrap analysis
114 using 1000 permutations of the data sets.

115

116 **Results and discussion**

117

118 *RAPD fingerprinting analysis*

119 We isolated 40 slow growing strains on YMA plates with typical morphology of genus
120 *Bradyrhizobium* that were able to nodulate this host forming effective nodules (pink red
121 colour). These isolates were analysed by RAPD fingerprinting that allows the differentiation
122 among strains of the same *Bradyrhizobium* species [7, 26]. This technique provides an
123 estimation of the genetic diversity showing that strains with about 75% identity belong to the
124 same species [26]. Our isolates were this way distributed into nine groups of RAPD with
125 similarity percentages lower than this value (Fig. 1), from which we selected representative
126 strains for gene sequence analysis.

127

128 *Analysis of rrs gene and 16S-23S ITS region*

129

130 The strains isolated from *V. unguiculata* nodules in Africa belong to genus *Bradyrhizobium*
131 and have been mainly identified on the basis of their *rrs* gene and ITS region [16, 25, 36] that
132 allowed their placement into the groups I and II of *Bradyrhizobium* proposed by Menna *et al.*
133 [19]. The results of the analysis of these phylogenetic markers showed that our strains mostly
134 belong to the group I with a single strain clustering into group II (Figs. 2 and 3, S1 and S2).
135 Within group I, the isolates cluster in different phylogenetic lineages some of them branching
136 with previously described species such as that represented by the strain VUPME29, (RAPD
137 group III) which was identified as *B. canariense* since it has *rrs* gene and ITS sequences
138 identical to those of its type strain BTA-1^T.

139 The strains VUPME04, VUPME82 and VUPME26 representing the RAPD groups VI, VII
140 and VIII, respectively, were identified as *B. cytisi* since they have *rrs* gene and ITS sequences
141 almost identical to those of its type strain CTAW11^T.

142 The strain VUPME50 representing the RAPD group VII has *rrs* gene and ITS region 100%
143 identical to that of strain BGA-1 currently classified into *B. japonicum*.

144 The strains VUPMI37, presenting the RAPD type IV and VUPMI11 representing the RAPD
145 group IX, formed a cluster related to *B. canariense* with more than 99% and 96.8% identity in
146 the *rrs* gene and ITS region, respectively.

147 The strain VUPMI33, presenting the RAPD type I, formed independent branches in the
148 phylogenetic trees of both *rrs* gene and ITS region, being close to *B. japonicum* USDA 6^T
149 with 97.5% identity in this last region.

150 Finally, within the group II we only found a strain, VUPME10 showing the RAPD type II,
151 closely related to *B. pachyrhizi* PAC48^T with identities higher than 99% in the *rrs* gene and
152 97% in the ITS region.

153 Our results contrast with those obtained in Africa, where *V. unguiculata* was distributed to
154 Europe, since we did not find in our work the species *B. yuanmingense* from group I and *B.*
155 *elkanii* from group II reported in *V. unguiculata* nodules in different African countries (Figs. 2
156 and 3, S1 and S2), including the considered distribution centers of this legume [25, 36].

157 Interestingly, the strains isolated from *V. unguiculata* in Spain were identified as *B. cytisi* and
158 *B. canariense*, species not found up to date in *V. unguiculata* nodules in Africa despite *B.*
159 *cytisi* was firstly isolated in this continent [4] and *B. canariense* was found in *Lupinus* nodules
160 in South Africa [37]. These two species are common endosymbionts of Genisteae legumes
161 being *B. canariense* the main endosymbiont of Genisteae in Europe [2, 35, 38, 41] where this
162 species is probably indigenous [37].

163 Only a common cluster was found both in Europe (reported in this work) and in Africa [36],
164 which is the cluster of strain BGA-1, branching in *Bradyrhizobium* group I (see Figs. 2 and
165 3). This strain is currently classified as *B. japonicum* although it formed a lineage within this
166 species with only 97% identity with respect to the type strain USDA 6^T in the ITS region. As
167 occurs with *B. cytisi* and *B. canariense*, this lineage is commonly found in Europe and Africa
168 in Genisteae legumes [13, 35, 37, 38, 41].

169 Within group II the lineage found in Spain has not been found up to date in African countries
170 (figures 2 and 3). It is remarkable that strains from this group have been scarcely found in
171 European soils with only some strains nodulating *Lupinus mariae-josephi* [34] and one strain
172 of the recently described species, *B. retamae* [7].

173 Therefore, the results of the analysis of core markers showed that *V. unguiculata* strains
174 isolated in Spain mostly belong to species that were not found up to date in its nodules in the
175 African countries. It is therefore an interesting finding the fact that the species *B. canariense*
176 and *B. cytisi* nodulate this legume in Spain because they are common endosymbionts of
177 Genisteae legumes and *V. unguiculata* belongs to the Tribe Phaseolae. Nevertheless, since the
178 ability to nodulate legumes is dependent of their promiscuity degree as well as to the host
179 range of rhizobia, the most interesting point is to analyze the symbiovars to which the strains
180 isolated in this study belong.

181

182 *Analysis of nodC gene*

183

184 The *nodC* gene is a phylogenetic marker related to the host range of rhizobia and promiscuity
185 level of legumes [10, 24, 26, 29, 31, 32], and the analysis of its sequences has been recently
186 proposed to be considered a minimal standard for definition of symbiovars in rhizobia [21,
187 32]. This gene has been used to delineate the symbiovars defined within genus
188 *Bradyrhizobium*, which currently are genistearum, glycinearum, retamae and sierranevadense
189 [2, 7, 43]. Although it has not been established a cutoff value to differentiate these
190 symbiovars, the available data suggest to consider it around 90% similarity since values
191 higher than this percentage are presented by strains from symbiovar glycinearum (Figs. 4 and
192 S3).

193 According to the results, all strains from *Bradyrhizobium* group I nodulating *V. unguiculata* in
194 this study belong to symbiovar genistearum (Figs. 4 and S3) that is the common
195 endosymbiont of Genisteae legumes in Europe [2, 38, 41] and Africa [3, 37]. However this
196 symbiovar was not previously found in *V. unguiculata* in Africa. In fact African *V.*
197 *unguiculata* strains were reported to cluster within a clade different from that occupied by
198 strains isolated from Genisteae legumes according to the *nodA* gene analysis [36]. The
199 available *nodC* gene sequences of African strains belonging to *Bradyrhizobium* group I placed
200 them into two clusters (Figs. 4 and S3), one of them also containing the type strain of *B.*
201 *arachidis* and other corresponding to the symbiovar glycinearum, a common endosymbiont of
202 the Phaseolae *Glycine max* (soybean), to which also belong the type strains of *B. japonicum*
203 and *B. daqingense* nodulating this legume and *B. huanghuaihaiense* that nodulated soybean
204 and *V. unguiculata* [44]. Therefore, this is the first report about the ability of the symbiovar
205 genistearum, common endosymbiont of Genisteae legumes, to nodulate the Phaseolae legume

206 *V. unguiculata* in Europe, proposed geographical origin of strains nodulating Genisteae
207 legumes [37].

208 On the other hand, the single strain belonging to the group II of *Bradyrhizobium*, VUPME 10,
209 clustered with other species of this group such as *B. pachyrhizi* and *B. elkanii* but forming a
210 divergent new lineage that presented less than 89% identity with respect to the remaining
211 symbiovars of genus *Bradyrhizobium* (Figs. 4 and S3). This new lineage is also present in *V.*
212 *unguiculata* nodules in Africa constituting a novel symbiovar of *Bradyrhizobium* able to
213 nodulate *Vigna*, for which we propose the name *vignae*.

214 All our results confirm those obtained after the *nodA* gene analysis in African strains showing
215 that *V. unguiculata* is a very promiscuous legume able to establish symbiosis with slow
216 growing rhizobia belonging to different clades [36]. This is a common finding not only in *V.*
217 *unguiculata* [18, 44], but also in other Phaseolae legumes nodulated by *Bradyrhizobium* such
218 as soybean or *Pachyrhizus* that are nodulated by several *nodC* lineages (Figs. 4 and S3). This
219 promiscuity allows the Phaseolae legume *V. unguiculata* to nodulate with the symbiovar
220 *genistearum* of *Bradyrhizobium* which is the common endosymbiont of Genisteae legumes in
221 Europe [2, 38, 41]. The presence of the new symbiovar *vignae* in both African and European
222 soils and the complete identity of the *nodC* gene from the strains isolated in these two
223 continents indicate that they have the same origin suggesting the dispersion of this symbiovar
224 with *V. unguiculata* seeds from Africa to Europe, as reported for other rhizobial
225 endosymbionts and their hosts' seeds [23]. This hypothesis should be further confirmed when
226 more isolates from this new symbiovar are available in African and European soils.

227

228 In summary, our results based on the analysis of the core phylogenetic markers showed that:
229 (i) *V. unguiculata* is nodulated by two species not previously found in this legume in Africa,
230 *B. canariense* and *B. cytisi*, only found up to date in nodules of Genisteae legumes; (ii) *B.*
231 *cytisi* is reported for the first time in this work to be present in Europe; (iii) The analysis of
232 the symbiotic *nodC* gene confirmed the high promiscuity of *V. unguiculata* able to nodulate
233 with several symbiovars including the symbiovar *genistearum*, found in this work by first
234 time in *V. unguiculata* nodules in Europe, and by a new symbiovar (*vignae*) present in both
235 Africa and Europe.

236

237 **Acknowledgments**

238 MHRB is recipient of a JAE-Doc researcher contract from CSIC cofinanced by ERDF.

239

240 **References**

241

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388 **Figure legends**

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390 Figure 1. Dendrogram obtained for the strains of *Vigna unguiculata* using Pearson's
391 coefficient and UPGMA analysis of the RAPD profiles.

392

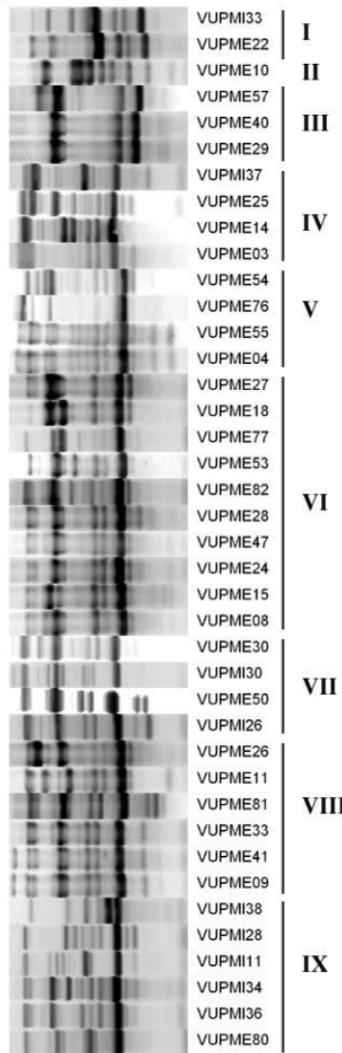
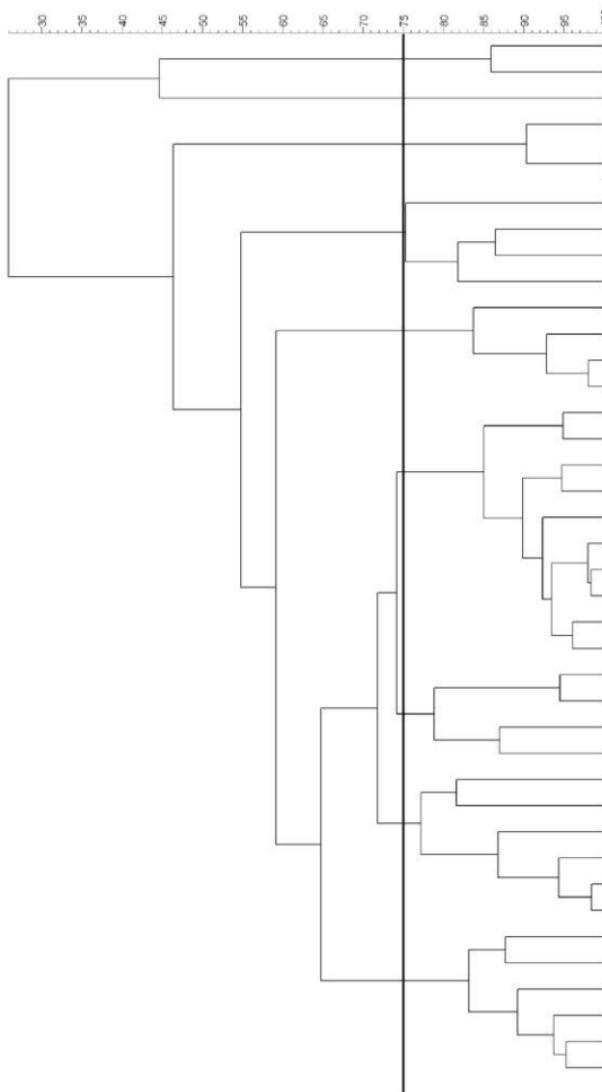
393 Figure 2. Neighbour-joining phylogenetic rooted tree based on *rrs* gene sequences (1485 nt)
394 showing the taxonomic affiliation of the strains representative of the different RAPD groups.
395 Bootstrap values calculated for 1000 replications are indicated. Bar, 1 nt substitution per 100
396 nt. Genbank Accession numbers are given in brackets.

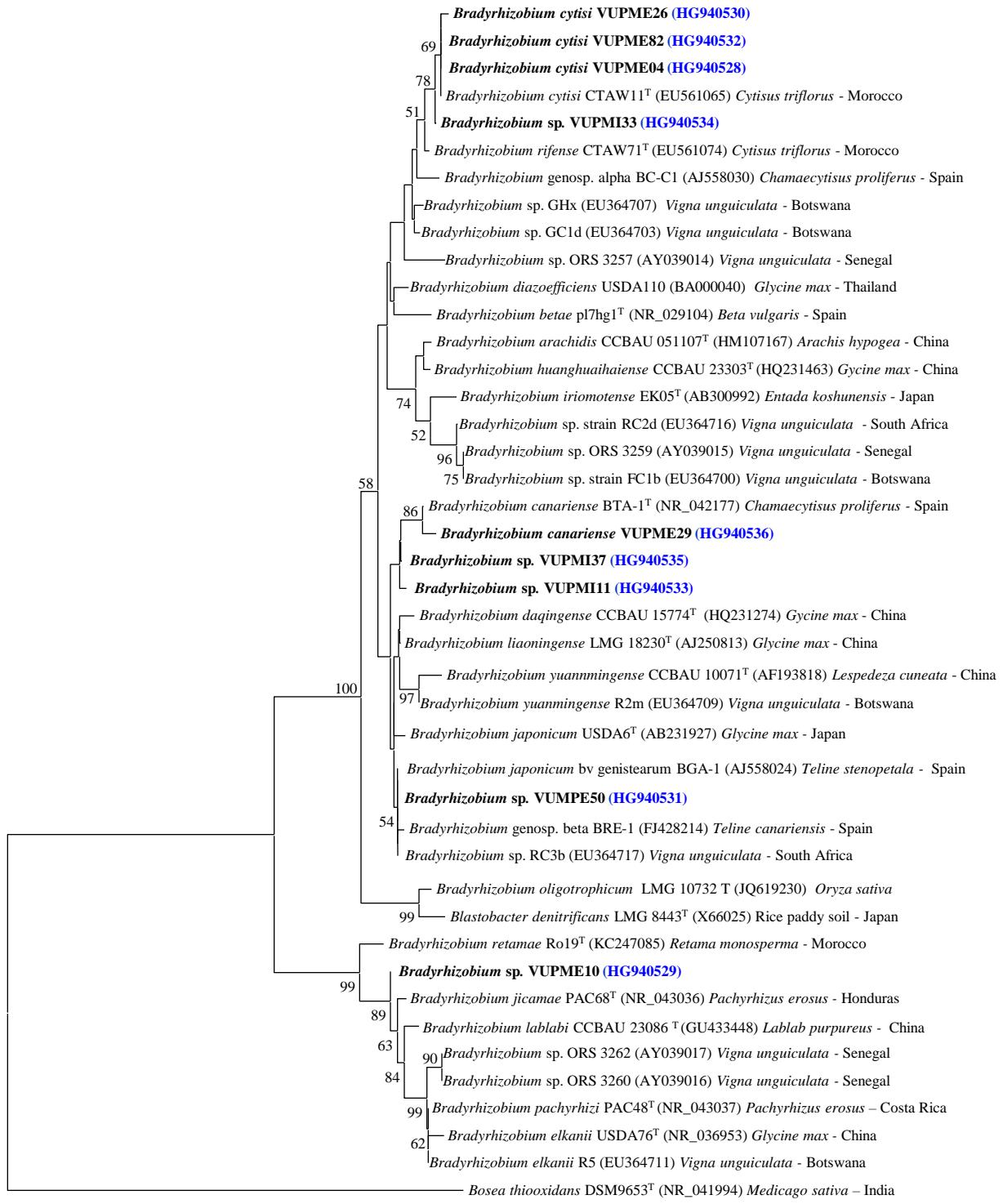
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398 Figure 3. Neighbour-joining phylogenetic rooted tree based on 16S-23S rRNA internal
399 transcribed spacer (ITS) sequences (810 nt) showing the taxonomic affiliation of the strains
400 representative of the different RAPD groups. Bootstrap values calculated for 1000
401 replications are indicated. Bar, 1 nt substitution per 100 nt. Genbank Accession numbers are
402 given in brackets.

403 Figure 4. Neighbour-joining phylogenetic tree based on *nodC* gene sequences (400 nt)
404 showing the position of representative strains from different RAPD groups. Bootstrap values
405 calculated for 1000 replications are indicated. Bar, 5 nt substitution per 100 nt. Genbank
406 Accession numbers are given in brackets.

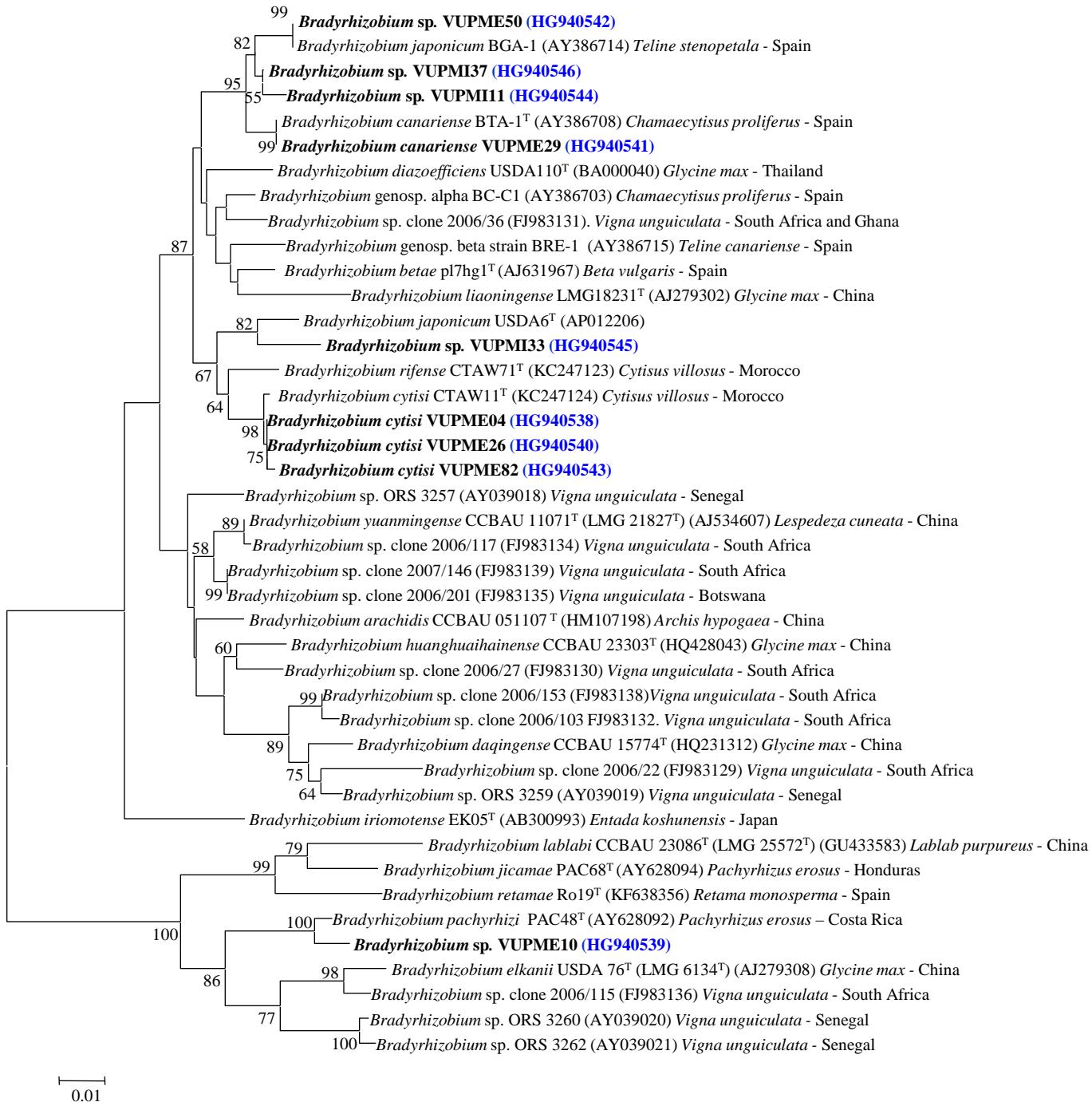
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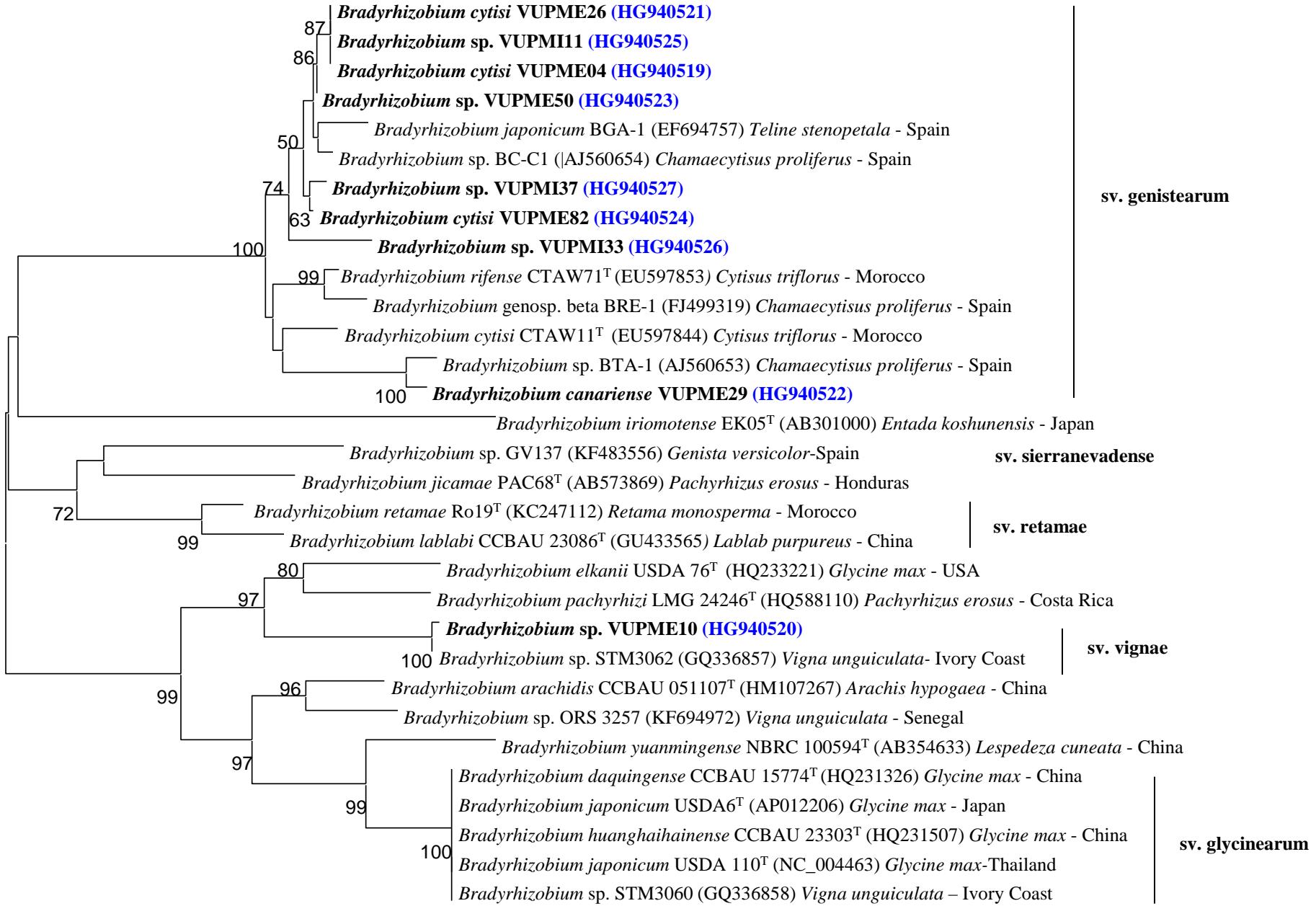




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Figure 3





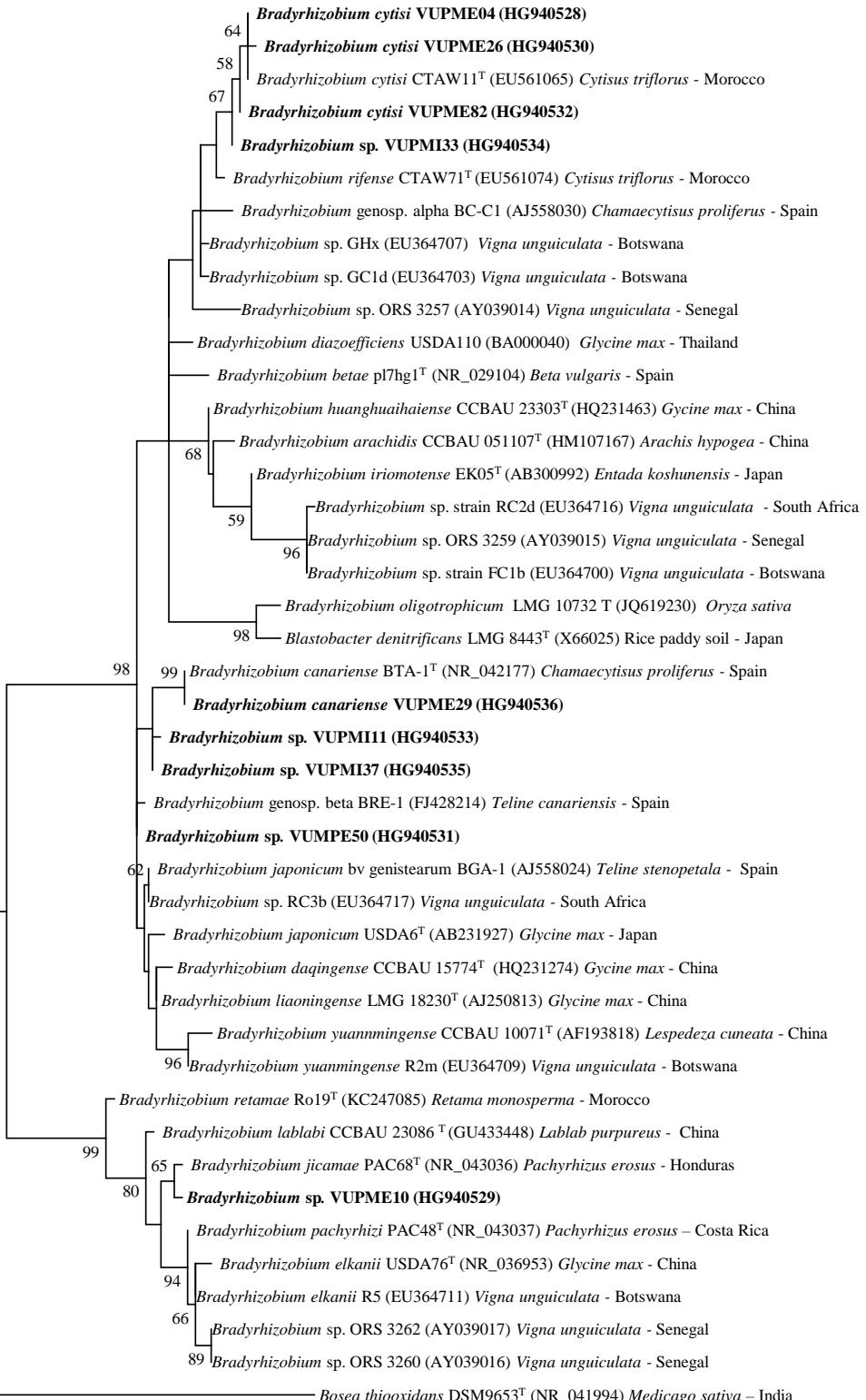


Figure S1. Maximum likelihood phylogenetic rooted tree based on *rrs* gene sequences (1485 nt) showing the taxonomic affiliation of the strains representative of the different RAPD groups. Bootstrap values calculated for 1000 replications are indicated. Bar, 1 nt substitution per 100 nt. Genbank Accession numbers are given in brackets.

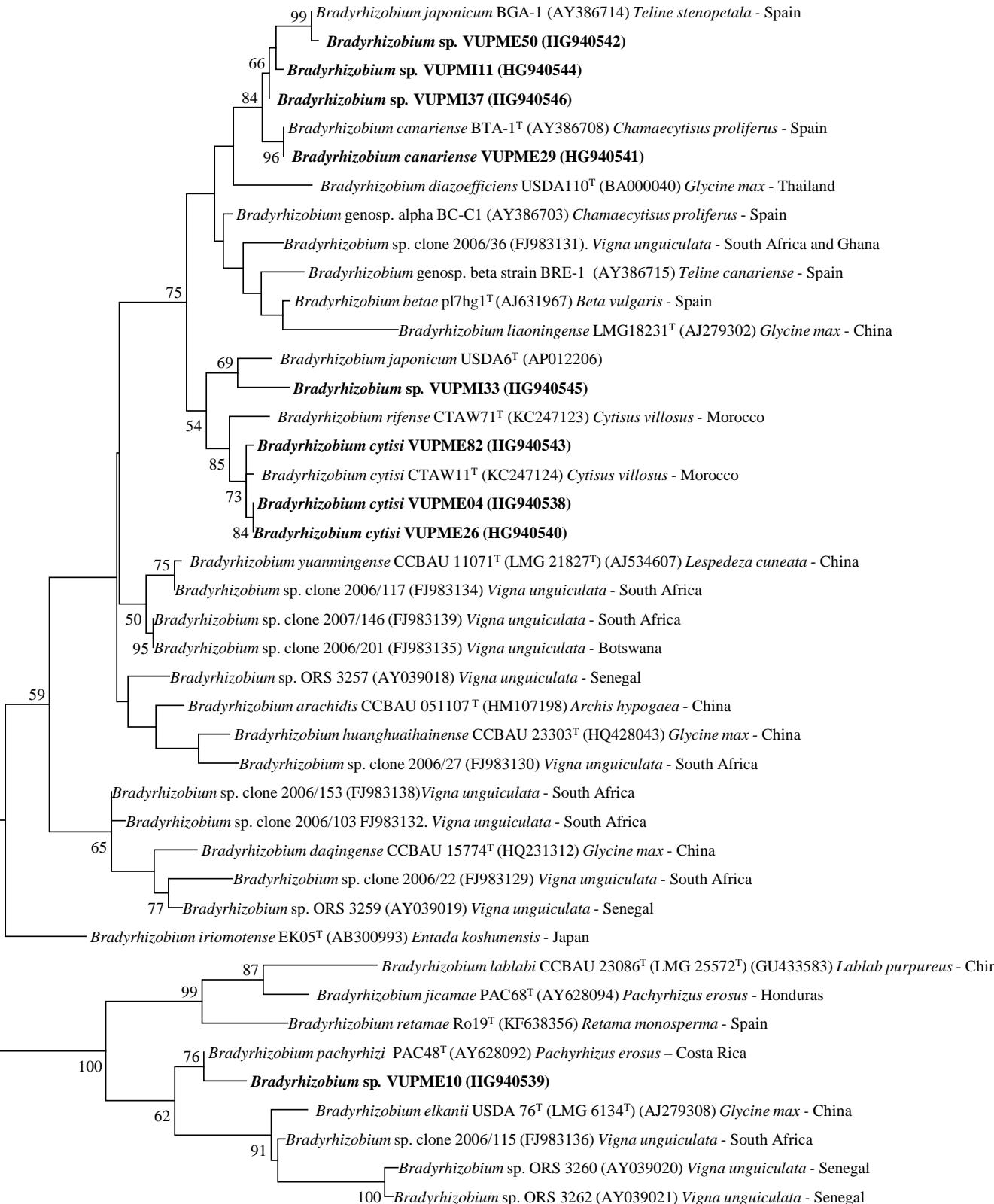


Figure S2. Maximum likelihood phylogenetic rooted tree based on 16S-23S rRNA internal transcribed spacer (ITS) sequences (810 nt) showing the taxonomic affiliation of the strains representative of the different RAPD groups. Bootstrap values calculated for 1000 replications are indicated. Bar, 2 nt substitution per 100 nt. Genbank Accession numbers are given in brackets.

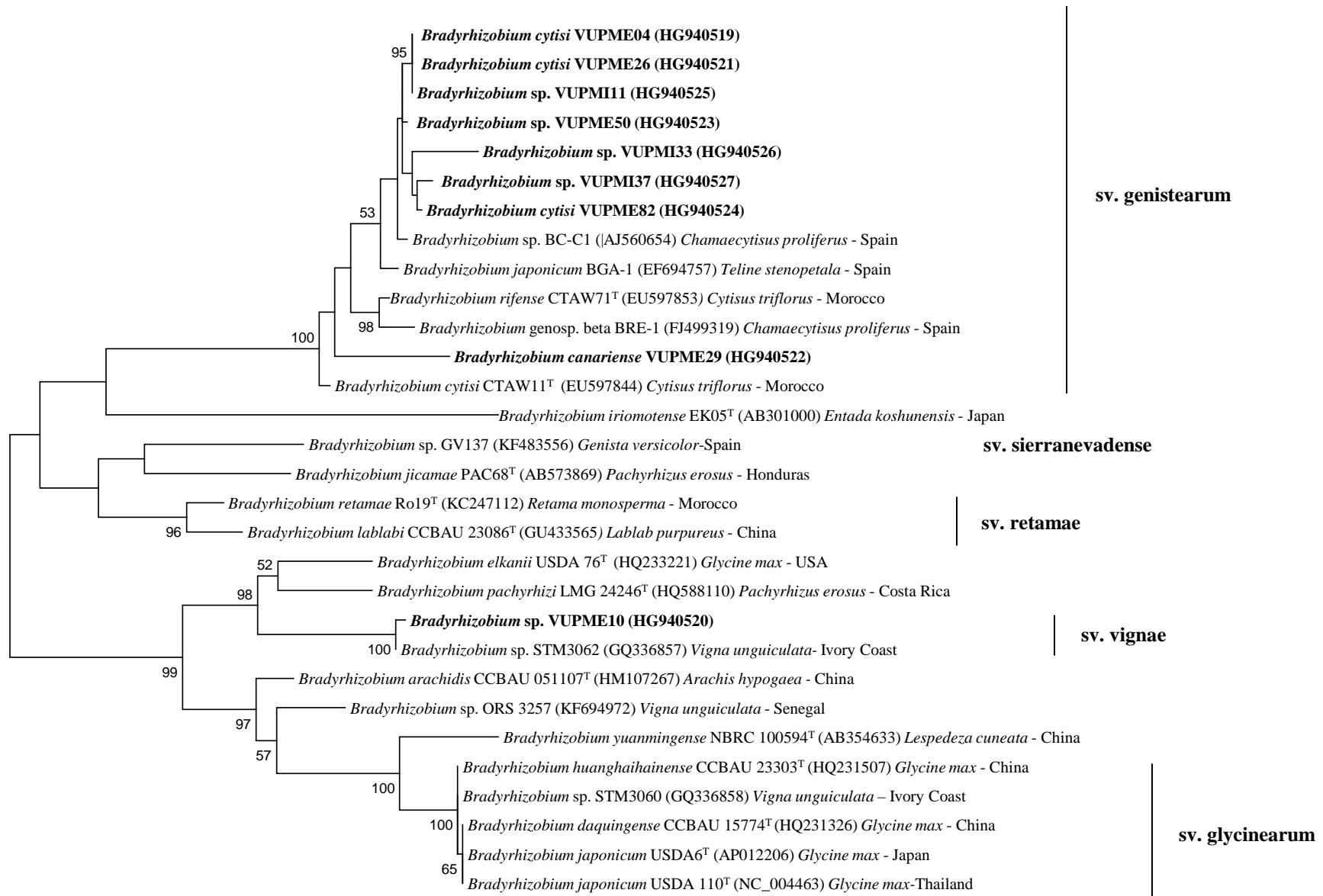


Figure S3. Maximum likelihood phylogenetic tree based on *nodC* gene sequences (400 nt) showing the position of representative strains from different RAPD groups. Bootstrap values calculated for 1000 replications are indicated. Bar, 5 nt substitution per 100 nt. Genbank Accession numbers are given in brackets.