

Bradyrhizobium canariense sp. nov., an acid-tolerant endosymbiont that nodulates endemic genistoid legumes (Papilionoideae: Genisteae) from the Canary Islands, along with *Bradyrhizobium japonicum* bv. *genistearum*, *Bradyrhizobium* genospecies alpha and *Bradyrhizobium* genospecies beta

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Highly diverse *Bradyrhizobium* strains nodulate genistoid legumes (brooms) in the Canary Islands, Morocco, Spain and the Americas. Phylogenetic analyses of ITS, *atpD*, *glnII* and *recA* sequences revealed that these isolates represent at least four distinct evolutionary lineages within the genus, namely *Bradyrhizobium japonicum* and three unnamed genospecies. DNA–DNA hybridization experiments confirmed that one of the latter represents a new taxonomic species for which the name *Bradyrhizobium canariense* is proposed. *B. canariense* populations experience homologous recombination at housekeeping loci, but are sexually isolated from sympatric *B. japonicum* bv. *genistearum* strains in soils of the Canary Islands. *B. canariense* strains are highly acid-tolerant, nodulate diverse legumes in the tribes Genisteae and Loteae, but not *Glycine* species, whereas acid-sensitive *B. japonicum* soybean isolates such as USDA 6^T and USDA 110 do not nodulate genistoid legumes. Based on host-range experiments and phylogenetic analyses of symbiotic *nifH* and *nodC* sequences, the biovarieties *genistearum* and *glycinearum* for the genistoid legume and soybean isolates, respectively, were proposed. *B. canariense* bv. *genistearum* strains display an overlapped host range with *B. japonicum* bv. *genistearum* isolates, both sharing monophyletic *nifH* and *nodC* alleles, possibly due to the lateral transfer of a conjugative chromosomal symbiotic island across species. *B. canariense* is the sister species of *B. japonicum*, as inferred from a maximum-likelihood *Bradyrhizobium* species phylogeny estimated from congruent *glnII*+*recA* sequence partitions, which resolves eight species clades. In addition to the currently described species, this phylogeny uncovered the novel *Bradyrhizobium* genospecies alpha and beta and the photosynthetic strains as independent evolutionary lineages. The type strain for *B. canariense* is BTA-1^T (= ATCC BAA-1002^T = LMG 22265^T = CFNE 1008^T).

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Abbreviations: ECGL, endemic Canarian genistoid legume; REP, repetitive extragenic palindromic sequence.

The GenBank/EMBL/DDBJ accession number for the *rrs* sequence of strain BC-C2 is AY577427; those for the ITS1 sequences are AY386703–AY386705, AY386707, AY386708, AY386712–AY386718, AY386721, AY386722, AY386734, AY599094 and AY599095.

Sequence accession numbers for new *Bradyrhizobium* sequences used and generated in this study, the figures discussed in the text and our final and concluding remarks are available as supplementary material in IJSEM Online.

In previous reports, we have shown that at least four *Bradyrhizobium* lineages nodulate endemic genistoid legumes such as *Adenocarpus*, *Chamaecytisus*, *Lupinus*, *Spartocytisus* and *Teline* species in soils of the Canary Islands (Fig. A, available as supplementary material in IJSEM Online). They are consistently distinguished by PCR-RFLPs of *rrs*+ITS amplicons (Jarabo-Lorenzo *et al.*, 2003; Vinuesa *et al.*, 1998, 1999), profiling of stable low-molecular-weight RNAs (Jarabo-Lorenzo *et al.*, 2000) and phylogenetic analyses of ITS (Fig. B, available as supplementary material in IJSEM Online), *atpD*, *glnII* and *recA* sequences using maximum-likelihood and Bayesian inference methods (Vinuesa *et al.*, 2005). We have shown that these lineages can be also recovered from the nodules of other genistoid legumes such as *Lupinus* spp. and from *Ornithopus* spp. (Papilionoideae: Loteae) growing in Africa, Europe and America (Jarabo-Lorenzo *et al.*, 2003; Vinuesa & Silva, 2004; Vinuesa *et al.*, 2005), using diverse complex media containing yeast-extract and mannitol such as YMA or 20E and following standard isolation procedures (León-Barrios *et al.*, 1991; Vinuesa *et al.*, 1998).

Bradyrhizobium japonicum is one of the lineages that nodulates endemic Canarian genistoid legumes (ECGLs) (Vinuesa *et al.*, 2005). The other three lineages represent unnamed genospecies that are clearly delineated in a well resolved species phylogeny based on combined *glnII*+*recA* sequences (see Fig. C, available as supplementary material in IJSEM Online).

Here we present evidence for the taxonomic distinctiveness (Stackebrandt *et al.*, 2002; Vandamme *et al.*, 1996) of one of these evolutionary lineages, for which we propose the name *Bradyrhizobium canariense*. This species can be unequivocally distinguished from the five *Bradyrhizobium* species currently described, namely *B. japonicum* (Jordan, 1982, 1984), *Bradyrhizobium elkanii* (Kuykendall *et al.*, 1992), *Bradyrhizobium liaoningense* (Xu *et al.*, 1995), *Bradyrhizobium yuanmingense* (Yao *et al.*, 2002) and *Bradyrhizobium betae* (Rivas *et al.*, 2004), by a combination of genotypic, physiological and ecological characteristics. *B. betae* was recently isolated from tumour-like root deformations of sugar beet (*Beta vulgaris*) in Northern Spain and has an unknown symbiotic status. It is possible that the four isolates used for the species description actually represent a single clone, since all presented the same ITS haplotype (Rivas *et al.*, 2004). *B. yuanmingense* was isolated from the root nodules of *Lespedeza* spp. growing in China, whereas the other three species were isolated from the nodules of soybean (*Glycine max*) in different continents (see Table A and figures provided as supplementary material in IJSEM Online).

B. canariense strains are grouped in highly supported monophyletic clusters in the gene trees inferred from a large number of ITS (Fig. B in IJSEM Online), *atpD*, *glnII* and *recA* sequences obtained from isolates of ECGLs and a diverse worldwide collection of *Bradyrhizobium* strains, including the type strains of all previously described species

in the genus (Vinuesa *et al.*, 2005). Population genetics studies of Moroccan and Canarian *B. canariense* isolates (Vinuesa *et al.*, 2005) based on repetitive extragenic palindromic sequence (REP)-PCR genomic fingerprints, multilocus enzyme electrophoresis (MLEE) and multilocus sequence (*atpD*, *glnII*, *recA*) polymorphisms revealed: i) high levels of strain diversity across sampling sites; ii) significant levels of recombination, as assessed by linkage disequilibrium analyses of MLEE data, variance- and coalescent-based estimation methods of the population recombination parameter, and the reticulated evolutionary pattern exhibited by the ITS, *atpD*, *glnII* and *recA* sequence partitions; iii) lack of genetic differentiation between continental and insular populations; and iv) significant gene flow between them. From these findings it was inferred that migration and recombination are significant evolutionary forces that provide *B. canariense* with internal cohesiveness and shape its genetic population structure (Vinuesa & Silva, 2004; Vinuesa *et al.*, 2005). Furthermore, these population genetics studies revealed that there is no significant recombination between *B. canariense* strains and the other three sympatric evolutionary lineages recovered from the nodules of ECGLs, and that the genetic differentiation between these lineages is highly significant (Vinuesa *et al.*, 2005). This finding is remarkable since the four species have overlapped ecological niches and therefore the ecological opportunity for horizontal gene transfer. In conclusion, these studies demonstrated that *B. canariense* represents a *bona fide* evolutionary, phylogenetic and cohesive species (Mayr, 1970; Templeton, 1989; Ward, 1998; Wiley, 1978).

Horizontal gene transfer was detected across *B. canariense* and *B. japonicum* at the symbiotic *nifH* and *nodC* loci, which map more than 250 kb apart one from the other on the chromosomal symbiotic region of *B. japonicum* USDA 110 (Göttfert *et al.*, 2001; Kaneko *et al.*, 2002). The *nifH* and *nodC* phylogenies correlated well with the host range of the ECGL isolates (Jarabo-Lorenzo *et al.*, 2003; Vinuesa *et al.*, 2005), but were incongruent with the maximum-likelihood species phylogeny (Felsenstein, 2004; Nichols, 2001) inferred from combined and congruent *glnII* plus *recA* (compare Figs C and D, available as supplementary material in IJSEM Online) partitions (Vinuesa *et al.*, 2005). Regardless of their geographical origin and (geno)species assignment, all isolates from genistoid legumes and *Ornithopus* spp. contained *nifH* and *nodC* alleles that were recovered in highly supported clades in the corresponding gene trees (see Fig. Da and Db in IJSEM Online), highlighting the independent evolutionary histories of adaptative (accessory) and housekeeping (core) loci (Lan & Reeves, 2000; Wernegreen & Riley, 1999). The most parsimonious explanation for the observed phylogenetic incongruence between housekeeping and *sym* loci is that lateral transfer events of symbiotic islands took place across species, probably mediated by an illegitimate recombination mechanism (Kaneko *et al.*, 2002; Sullivan & Ronson, 1998). Therefore, phylogenetic analysis of these two symbiotic genes, coupled with host-range experiments (Table 1), allowed us to uncover and delineate

Table 1. Host-range experiments performed in Leonard jars using selected *Bradyrhizobium* strains and legume hosts

<i>Bradyrhizobium</i> species, biovar and strain*	Host†					
	<i>C. proliferus</i>	<i>T. stenopetala</i>	<i>L. luteus</i>	<i>G. max</i>	<i>G. soja</i>	<i>M. atropurpureum</i>
<i>B. canariense</i> bv. <i>genistearum</i> BTA-1 ^T	Fix ^{+‡}	Fix ⁺	Fix ⁺	Nod ⁻	Nod ⁻	Fix ^{+/-}
<i>B. canariense</i> bv. <i>genistearum</i> BC-C2	Fix ⁺	Fix ⁺	Fix ⁺	Nod ⁻	Nod ⁻	Fix ^{+/-}
<i>B. canariense</i> bv. <i>genistearum</i> BC-MAM1	Fix ⁺	Fix ⁺	Fix ⁺	Nod ⁻	Nod ⁻	Fix ^{+/-}
<i>B. canariense</i> bv. <i>genistearum</i> ISLU16	Fix ⁺	Fix ⁺	Fix ⁺	Nod ⁻	Nod ⁻	ND
<i>B. japonicum</i> bv. <i>genistearum</i> BGA-1	Fix ⁺	Fix ⁺	Fix ⁺	Nod ⁻	Nod ⁻	Fix ^{+/-}
<i>B. japonicum</i> bv. <i>genistearum</i> FN13	Fix ⁺	Fix ⁺	Fix ⁺	Nod ⁻	Nod ⁻	ND
<i>B. japonicum</i> bv. <i>genistearum</i> Blup-MR1	Fix ⁺	Fix ⁺	Fix ⁺	Nod ⁻	Nod ⁻	ND
<i>B. japonicum</i> bv. <i>glycinearum</i> USDA 110	Nod ⁻	Nod ⁻	Nod ⁻	Fix ⁺	Fix ⁺	Fix ⁺
<i>B. japonicum</i> bv. <i>glycinearum</i> DSM 30131 ^T	Nod ⁻	Nod ⁻	Nod ⁻	Fix ⁺	Fix ⁺	Fix ^{+/-}
<i>B. liaoningense</i> bv. <i>glycinearum</i> LMG 18230 ^T	Nod ⁻	Nod ⁻	Nod ⁻	Fix ⁺	ND	ND
<i>B. yuanmingense</i> CCAU 10071 ^T	Nod ⁻	Nod ⁻	Nod ⁻	Nod ⁻	ND	ND

*Species and biovar assignment of strains is supported by the ITS, *glnII*+*recA*, *niH* and *nodC* phylogenies presented in Figs B, C and D (available as supplementary material in IJSEM Online) and further evidence presented in Vinuesa *et al.* (2005).

†*C. proliferus*, *T. canariense*, *L. luteus*, *G. max*, *G. soja* and *M. atropurpureum* are species of the genera *Chamaecytisus*, *Teline*, *Lupinus*, *Glycine* and *Macroptilium*, respectively.

‡Fix⁺ indicates a nitrogen-fixing symbiosis, as revealed by the acetylene reduction assay; Fix^{+/-} indicates weak levels of acetylene reduction; Nod⁻ indicates a non-nodulating interaction; ND, not determined. Plant germination, inoculation and cultivation were as described previously (Vinuesa *et al.*, 1998).

for the first time *Bradyrhizobium* biovarieties (symbiotic ecotypes, Fig. D in IJSEM Online), as defined in Vinuesa *et al.* (2005), and according to the proposed minimal standards for the description of new genera and species of root- and stem-nodulating bacteria (Graham *et al.*, 1991). These biovarieties should not be confounded with new species on the basis of their symbiotic (host range) phenotypes (Graham *et al.*, 1991; Lan & Reeves, 2001). We agree with Graham *et al.* (1991) that species descriptions of symbiotic rhizobia should be accompanied by a definition of their biovariety in the form of a latin trinomial, although we disagree with the proposal of equating each ecotype with a new species (Cohan, 2002), since the ecological characters conferred by symbiotic plasmids or islands are highly prone to rapid gain and loss events and horizontal transfer, and well delineated evolutionary species, such as *B. japonicum*, have more than one biovariety (see Fig. Da and Db in IJSEM Online).

An estimate of the *Bradyrhizobium* species phylogeny (Felsenstein, 2004; Nichols, 2001) based on a maximum-likelihood analysis of congruent *glnII*+*recA* sequence partitions (Fig. C in IJSEM Online) provided strong evidence that *B. canariense* is the sister species of *B. japonicum*, which is consistent with a Bayesian phylogeny presented elsewhere (Vinuesa *et al.*, 2005). This species phylogeny is congruent with the current taxonomy of the genus. Only the position of *B. betae* remains uncertain because *rrs* and ITS sequences do not resolve its phylogenetic placement (see Figs B and E, available as supplementary material in IJSEM Online) and protein-coding gene sequences are not available for this taxon yet. Importantly, the *glnII*+*recA* species phylogeny

(see Fig. C and the final and concluding remarks in IJSEM Online) does not provide conclusive support to the hypotheses derived from numerical taxonomy and *rrs* sequence analyses that the photosynthetic bradyrhizobia and *B. elkanii* may represent new genera (Ladha & So, 1994; van Berkum & Eardly, 1998; Willems *et al.*, 2001b). Taking a more conservative classification criterion based on a consensus of the different data sources available at the moment for these bacteria, and considering the topology presented in Fig. C, favours their classification as basal lineages of the genus *Bradyrhizobium* (see the final and concluding remarks in IJSEM Online), which supports the conclusions reached by So *et al.* (1994) based on *rrs* and fatty acid analysis that they are bradyrhizobia, as well as the old hypothesis of a photosynthetic ancestor for the genus (Jarvis *et al.*, 1986; Vinuesa *et al.*, 2005).

It had been shown previously that ITS sequence clades correlate reasonably well with DNA-homology groups (Willems *et al.*, 2001a, 2003). Therefore, we used the topologies inferred from the ITS and *glnII*+*recA* sequence data (Figs B and C in IJSEM Online) to select a number of representative *B. canariense*, *B. japonicum* and *B. liaoningense* strains to perform DNA-DNA hybridization experiments, using a filter hybridization (dot-blot) technique. Three replicate samples of 2 µg of purified genomic DNA (genomic DNA purification kit; Roche Molecular Biochemicals) were vacuum-blotted onto nylon membranes and cross-linked with UV light. Five-hundred nanograms of purified genomic DNA from three distinct *B. canariense* strains (BC-C2, BES-1 and BTA-1^T) were randomly labelled with digoxigenin using the DIG-labelling system (Roche

Table 2. Percentage of relative DNA–DNA hybridization obtained between *Bradyrhizobium canariense* BC-C2, BES-1 and BTA-1^T, conspecific isolates and selected *B. japonicum* and *B. liaoningense* strains

<i>Bradyrhizobium</i> species, biovar and strain	DNA homology (%) with probe:			Avg.*
	BC-C2	BES-1	BTA-1 ^T	
<i>B. canariense</i> bv. <i>genistearum</i> BC-C2	100	85 ± 5	81 ± 3	82.9 ± 9.5, n = 18
<i>B. canariense</i> bv. <i>genistearum</i> BES-1	74 ± 4	100	88 ± 4	
<i>B. canariense</i> bv. <i>genistearum</i> BTA-1 ^T	70 ± 3	85 ± 3	100	
<i>B. canariense</i> bv. <i>genistearum</i> BC-P1	76 ± 3	84 ± 4	84 ± 4	
<i>B. canariense</i> bv. <i>genistearum</i> BC-P5	69 ± 1	81 ± 3	80 ± 2	
<i>B. canariense</i> bv. <i>genistearum</i> BC-MAM1	84 ± 3	73 ± 4	78 ± 5	34.9 ± 10.6, n = 12
<i>B. japonicum</i> bv. <i>genistearum</i> BGA-1	38 ± 6	41 ± 2	46 ± 4	
<i>B. japonicum</i> bv. <i>glycinearum</i> DSM 30131 ^T	24 ± 3	41 ± 4	39 ± 2	
<i>B. japonicum</i> bv. <i>glycinearum</i> USDA 110	13 ± 6	34 ± 4	30 ± 7	
<i>B. japonicum</i> bv. <i>glycinearum</i> X6-9	22 ± 4	47 ± 3	44 ± 4	
<i>B. liaoningense</i> bv. <i>glycinearum</i> LMG 18230 ^T	25 ± 5	48 ± 9	37 ± 6	33.2 ± 10.4, n = 6
<i>B. liaoningense</i> Spr3-7	19 ± 3	39 ± 8	31 ± 6	

*Avg., average percentage of DNA hybridization signal among strains for the number (*n*) of comparisons indicated.

Molecular Biochemicals) and used as probes (adjusted to 20 ng ml⁻¹ in the hybridization solution). Stringent hybridization was carried out overnight at 68 °C, followed by high-stringency washings at 68 °C in 0.5 × SSC. Hybridization signals were detected by chemiluminescence using anti DIG Fab fragments and the enhanced chemifluorescence substrate (Roche Molecular Biochemicals), and quantified using a Storm 860 phosphorimager (Molecular Dynamics) equipped with the ImageQuant software (Amersham Pharmacia Biotech). The hybridization results are shown in Table 2 and indicated that the three probes

hybridized significantly stronger (in the 69–88% range) with *B. canariense* isolates than with *B. japonicum* and *B. liaoningense* strains (13–48% range).

B. canariense strains can be further distinguished from all other described *Bradyrhizobium* species by a combination of genotypic, physiological and ecological traits, as indicated in the species description. Distinctive phenotypic features of *B. canariense* are presented in Table 3. It should be noted, however, that such phenotypic traits are highly inconsistent when large populations are studied (Xu *et al.*,

Table 3. Distinctive phenotypic features for *Bradyrhizobium canariense* and reference *Bradyrhizobium* species

Species: 1, *B. canariense*; 2, *B. japonicum*; 3, *B. elkanii*; 4, *B. liaoningense*; 5, *B. yuanmingense*; 6, *B. betae*. Data for *B. japonicum*, *B. elkanii* and *B. yuanmingense* were taken from Table 3 of Yao *et al.* (2002), data for *B. liaoningense* from Xu *et al.* (1995), and data for *B. betae* from Rivas *et al.* (2004). +, >95% of isolates were positive; –, >95% of the isolates were negative; +/-, variable; NR, not reported.

Characteristics	1	2	3	4	5	6
C sources:						
D-Fructose	+	–*	+	+/-	–	+/-
Lactose	–	+*	+/-	–	–	–
Maltose/sucrose	–	+*	+*	–	+/-	+/-
N source:						
L-Glycine	–	+*	+*	NR	–	NR
Resistance to:						
Erythromycin (100 µg ml ⁻¹)	+/-	–*	+	NR	–	+/-
Growth characteristics:						
pH 4.5	+	–	+	NR	–	NR
pH 10	–	–	+/-	–	–	NR
1.0% NaCl	–	+*	+*	–	–	+
Colony size (mm) after 7 days incubation in YMA	1.0–1.5	1.0	1.0	0.2–1.0	1.0–2.0	NR
Generation time (h) in YM broth, pH 6.8	>6	>6	>6	16–40	9.5–16	12–16

*Our own data are not consistent with those reported previously.

1995; Yao *et al.*, 2002), and it is well documented that phenotypic and genotypic clustering of *Bradyrhizobium* strains correlates poorly (Dupuy *et al.*, 1994; So *et al.*, 1994; van Rossum *et al.*, 1995; Zhang *et al.*, 1999).

The description of *Bradyrhizobium canariense* sp. nov. is therefore supported by the population genetics, phylogenetic, DNA homology, physiological and ecological evidence presented above. This is the first description of a novel root nodule microsymbiont species that is primarily based on molecular evolutionary criteria, using a large collection of strains from different hosts and geographical origins, which have been extensively characterized by a broad range of genotyping methods (REP-PCR, *rrs*+ITS PCR-RFLPs, MLEE and stable low-molecular-weight RNAs), as well as by state-of-the-art phylogenetic methods using seven gene partitions [five informational/housekeeping loci (*rrs*, ITS, *atpD*, *glnII* and *recA*) and two *sym* loci (*nifH* and *nodC*)]. Therefore, this work follows the recommendations made by the ad hoc committee for the re-evaluation of the species definition in bacteriology (Stackebrandt *et al.*, 2002). Furthermore, it augments and actualizes the proposed minimal standards for the description of new genera and species of root- and stem-nodulating bacteria (Graham *et al.*, 1991) by the use of more advanced analytical tools, a highly resolved *Bradyrhizobium* species phylogeny and an updated theoretical framework. Finally, in view of the richness of evolutionary and ecological inferences that can be made from sequence data (see the final and concluding remarks provided in IJSEM Online), we would like to encourage (brady)rhizobial taxonomists to make more extensive use of them in future works. In doing so, a large multilocus sequence database could be built up quickly and used as the primary source of characters for molecular evolutionary and systematic studies. Only sequence data are highly portable and freely exchangeable by different researchers for unambiguous comparative analyses.

Description of *Bradyrhizobium canariense* sp. nov.

Bradyrhizobium canariense [ca.na.ri.en'se. N.L. neut. adj. *canariense* pertaining to the Canary Islands (Islas Canarias), where it is the dominant species nodulating endemic shrub legumes Papilionoideae: Genisteeae].

Gram-negative, aerobic, slow-growing, non-spore-forming rods, as for other species of the genus, motile by a single subpolar flagellum (León-Barrios *et al.*, 1991). Phenotypically, *B. canariense* strains are highly diverse. Colonies on YMA (pH 6.8) are white or creamy, 1–1.5 mm in diameter after 7 days incubation at 28 °C, producing an acid reaction and variable amounts of exopolysaccharides, as reflected by the diverse textures, consistencies and growth patterns they exhibit on solid media. Their lipopolysaccharide (LPS) O-antigens are also highly diverse as determined by DOC-PAGE analysis of purified LPSs and immunological cross-reactions (León-Barrios *et al.*, 1991; Santamaría *et al.*, 1997). Optimum growth temperature is 28–30 °C, but inhibited at

37 °C. No growth is observed at pH 9, or in the presence of 1 % NaCl on YMA. They use (+)-D-glucose, (+)-D-mannose, (+)-D-galactose, (–)-D-fructose, (–)-L-rhamnose, (+)-D-xylose, (–)-D-ribose, (–)-D-arabinose, glycerol, mannitol, sorbitol, citrate, fumarate and succinate, but not (–)-L-sorbose, melibiose, lactose, sucrose, (+)-D-trehalose, inulin, starch or catechol as sole carbon sources. Use L-glutamine but not L-glycine as sole N source. They are highly acid-tolerant, forming colonies of 1 mm in diameter after 6 to 7 days incubation at 30 °C on acidified 20E plates at pH 4.2 solidified with GelRite (Roth, Germany) and buffered with 25 mM Homopipes (Vinueza *et al.*, 2003). The symbiotic genes map to the chromosome, lacking plasmids as revealed by Eckhardt gel-electrophoresis (Eckhardt, 1978). Its known geographical distribution includes Spain, Morocco, the Canary Islands and the Americas. A single biovariety (bv. *genistearum*) is presently known, which nodulates different genera and species in the legume tribe Genisteeae (e.g. *Lupinus* spp., *Adenocarpus* spp., *Chamaecytisus proliferus*, *Spartocytisus supranubius* and *Teline* spp.), as well as *Ornithopus* spp. (Papilionoideae: Loteae), but does not nodulate soybeans (*Glycine max* or *Glycine soja*, Papilionoideae: Phaseoleae). At the molecular level this species can be easily distinguished from strains of its sister species *B. japonicum* and all other described *Bradyrhizobium* species by the unique 16S rRNA PCR-RFLP genotype obtained with the endonucleases *HhaI*, *DdeI* and *HinfI* (Jarabo-Lorenzo *et al.*, 2000, 2003; Vinueza *et al.*, 1998, 1999, 2005). *B. canariense* strains also display a distinct fingerprint of stable low-molecular-weight RNAs (Jarabo-Lorenzo *et al.*, 2000). It forms statistically highly supported ITS, *atpD*, *glnII* and *recA* sequence clades under the maximum-likelihood optimality criterion using best-fit models of nucleotide substitution (with bootstrap support >90 % in all cases). *B. canariense* strains are only weakly clonal, with significant recombination taking place within populations. DNA homology is greater than 69 % between *B. canariense* strains, and lower than 50 % with *B. japonicum* or *B. liaoningense* strains, its closest phylogenetic relatives.

The type strain, BTA-1^T (= ATCC BAA-1002^T = LMG 22265^T = CFNE 1008^T), was isolated from the root nodules of *Chamaecytisus proliferus* subsp. *proliferus* var. *palmensis* (Papilionoideae: Genisteeae) in La Laguna, Tenerife, Canary Islands, Spain, and has a G + C content of 63.8 mol%. This and other *B. canariense* strains have been deposited at the strain collections of the CIFN-UNAM and the Departments of Microbiology at the Universities of La Laguna and Gent, from where they are freely available.

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Table A. Sequence accession numbers for new *Bradyrhizobium* sequences used and generated (bold-face) in this study

Primers and amplification protocols were reported by Vinuesa *et al.* (2005) along with a larger dataset for all protein-coding loci, and including *atpD* sequences.

Isolate or strain*	<i>rrs</i>	ITS	<i>glnII</i>	<i>recA</i>	<i>nifH</i>	Host†	Reference or source‡
ECGL isolates							
<i>Bradyrhizobium</i> genosp. α BC-C1 #		AY386703	AY386761	AY591540	AY386781	<i>C. proliferus</i>	Vinuesa <i>et al.</i> (1998)
<i>B. canariense</i> BC-C2 #	AY577427	AY386704	AY386762	AY591541	AY386782	<i>C. proliferus</i>	Vinuesa <i>et al.</i> (1998)
<i>B. canariense</i> BC-P5 #		AY386705	AY386763	AY591542		<i>C. proliferus</i>	Vinuesa <i>et al.</i> (1998)
<i>B. japonicum</i> BC-P14 #		AY386713	AY386771	AY591544		<i>C. proliferus</i>	Vinuesa <i>et al.</i> (2005)
<i>B. canariense</i> BES-1 #		AY386707	AY386764	AY591548	AY599093	<i>C. proliferus</i>	Jarabo-Lorenzo <i>et al.</i> (2003)
<i>B. japonicum</i> BGA-1 #		AY386714	AY386772	AY591558	AY386784	<i>T. stenopetala</i>	Jarabo-Lorenzo <i>et al.</i> (2003)
<i>B. canariense</i> BTA-1 ^T #		AY386708	AY386765	AY591553	AY386783	<i>C. proliferus</i>	Jarabo-Lorenzo <i>et al.</i> (2003)
<i>B. canariense</i> BC-Mam1 #			AY386767	AY591546		<i>C. proliferus</i>	Vinuesa <i>et al.</i> (2005)
<i>Bradyrhizobium</i> genosp. β BC-MK1 #		AY386721				<i>C. proliferus</i>	Vinuesa <i>et al.</i> (2005)
<i>Bradyrhizobium</i> genosp. β BC-MK6 #		AY386722	AY386779	AY591554		<i>C. proliferus</i>	Vinuesa <i>et al.</i> (2005)
<i>Bradyrhizobium</i> genosp. β BRE-1 #		AY386715	AY599112	AY591551		<i>T. canariense</i>	Jarabo-Lorenzo <i>et al.</i> (2003)
Other genistoid legume isolates							
<i>B. japonicum</i> FN13 #		AY386716	AY386773	AY591560	AY386785	<i>L. montanus</i>	Barrera <i>et al.</i> (1997)
<i>B. japonicum</i> Blup-MR1 #		AY386717	AY386774	AY591559	AY386786	<i>L. polyphillus</i>	Vinuesa <i>et al.</i> (2005)
<i>B. japonicum</i> ISLU-16 #		AY386712	AY599108	AY591576		<i>Ornithopus</i> sp.	Jarabo-Lorenzo <i>et al.</i> (2003)
IRRI collection							
photosynthetic strains							
<i>Bradyrhizobium</i> sp. BTAi1 #			AY599123	AY591570		<i>A. indica</i>	So <i>et al.</i> (1994) / IRRI
<i>Bradyrhizobium</i> sp. IRBG127 #			AY599122	AY591571		<i>A. pratensis</i>	So <i>et al.</i> (1994) / IRRI
<i>Bradyrhizobium</i> sp. IRBG231 #			AY599124	AY591572		<i>A. denticulata</i>	So <i>et al.</i> (1994) / IRRI
non-photosynthetic							
<i>B. yuanmingense</i> TAL760 #		AY599095	AY599114	AY591565		<i>I. hirsuta</i>	So <i>et al.</i> (1994) / IRRI
Chinese soybean isolates							
<i>B. japonicum</i> X3-1 #			AY599120	AY591556		<i>G. max</i>	Vinuesa <i>et al.</i> (2005)
<i>B. japonicum</i> X6-9 #			AY599121	AY591557		<i>G. max</i>	Vinuesa <i>et al.</i> (2005)
USDA collection							
<i>B. elkanii</i> USDA 46			AY599125	AY591576	AY599091	<i>G. max</i>	van Berkum, USDA
<i>B. elkanii</i> USDA 76 ^T			AY599117	AY591568		<i>G. max</i>	van Berkum, USDA
<i>B. elkanii</i> USDA 94			AY599118	AY591569	AY599092	<i>G. max</i>	van Berkum, USDA
<i>B. japonicum</i> USDA 122			AY386777	AY591562	AY599084	<i>G. max</i>	van Berkum, USDA
Other reference strains							
<i>B. japonicum</i> DSM30131 ^T			AY599119	AY591555	AY599085	<i>G. max</i>	DSMZ
<i>B. liaoningense</i> LMG18230 ^T			AY386775	AY591564	AY599087	<i>G. max</i>	Xu <i>et al.</i> (1995)
<i>B. yuanmingense</i> CCBAU10071 ^T		AY386734	AY386780	AY591566	AY599090	<i>Le. cuneata</i>	Yao <i>et al.</i> (2002)
<i>B. japonicum</i> Nep1 #		AY386718	AY386776	AY591563		<i>G. max</i>	Vinuesa <i>et al.</i> (2005)
<i>B. liaoningense</i> Spr3-7 #			AY599116	AY591574	AY386787	<i>Ar. hypogaea</i>	Zhang <i>et al.</i> (1999)
<i>B. yuanmingense</i> LTMR28 #		AY599094	AY599115	AY591573		<i>P. lunatus</i>	Vinuesa <i>et al.</i> (2005)
<i>Bradyrhizobium</i> genosp. α CIAT3101 #			AY653774	AY591567		<i>Ce. plumieri</i>	Vinuesa <i>et al.</i> (1998)

Refer to next page for footnotes to table.

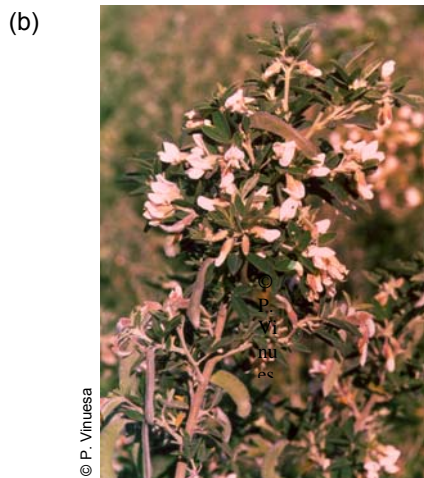
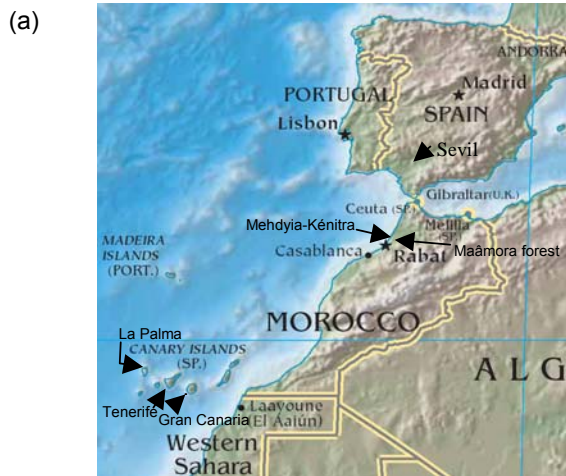
Vinuesa, P., León-Barrios, M., Silva, C., Willems, A., Jarabo-Lorenzo, A., Pérez-Galdona, R., Werner, D. & Martínez-Romero, E. (2005). *Bradyrhizobium canariense* sp. nov., an acid-tolerant endosymbiont that nodulates endemic genistoid legumes (Papilionoideae: Genisteae) from the Canary Islands, along with *Bradyrhizobium japonicum* bv. *genistearum*, *Bradyrhizobium* genospecies alpha and *Bradyrhizobium* genospecies beta. *Int J Syst Evol Microbiol* **55**, 569–575.

*ECGL stands for endemic Canarian genistoid legumes (brooms). The strains marked with # were classified in this study based on the *glnII+recA* species phylogeny presented in Fig. D and the additional phylogenetic evidence gained from *atpD*, *glnII* and *recA* sequence analyses presented by Vinuesa *et al.* (2005). Genosp. α and genosp. β correspond to genospecies alpha and beta, respectively. Notice that new *B. liaoningense* and *B. yuanmingense* strains were found nodulating diverse hosts on different continents, revealing that these taxa have a broader host range and geographical distribution than that reported in the original species descriptions. The three photosynthetic strains included in this study represent a third unnamed genospecies. The classification proposals are further supported by DNA–DNA hybridizations, chemotaxonomical and phenotypic evidence presented in this and previous studies, as discussed in the section on final and concluding remarks.

†The abbreviations for the host generic names correspond to: *A.*, *Aeschynomene*; *Ar.*, *Arachis*; *C.*, *Chamaecytisus*; *Ce.*, *Centrosema*; *G.*, *Glycine*; *I.*, *Indigofera*; *L.*, *Lupinus*; *Le.*, *Lespedeza*; *P.*, *Phaseolus*; *T.*, *Teline*.

‡The indicated references acknowledge key papers in which the corresponding strains were characterized, but do not necessarily correspond to the reference describing the original isolation of the strains. Otherwise, the source of the strains is indicated. IRRI is the International Rice Research Institute, Los Baños, Philippines; USDA is the United States Department of Agriculture, Beltsville, MA; DSMZ is the Deutsche Sammlung für Mikroorganismen und Zellkulturen, Braunschweig, Germany.

Fig. A. Location (arrows) of some of the sampling sites (a) from which *Bradyrhizobium canariense*, *B. japonicum*, *Bradyrhizobium* genospecies alpha and *Bradyrhizobium* genospecies beta isolates were obtained (more details are provided in the tree labels, Figs B–E). Examples (b) of endemic Canarian genistoid legumes (ECGLs, brooms) (Papilionoideae:Genisteae) growing in their natural habitats. Notice the contrasting growth forms of the three species shown. Some of these shrub legumes are of great ecological relevance, as they are dominant members, or key indicator species, of several plant communities of the archipelago, particularly in the laurel and pine forests, and in the subalpine scrub. For more information on the evolutionary ecology of the Canary Islands see Baldwin *et al.* (1998) and Juan *et al.* (2000). Details on the phytogeography, N₂-fixation potential and historical aspects of the uses and distribution of tagasaste, the best known of the ECGLs, have been presented elsewhere (Francisco-Ortega *et al.*, 1991, 1994; Ovalle *et al.*, 1996). A recent molecular evolutionary analysis of ECGLs was presented by Percy & Cronk (2002).



Chamaecytisus proliferus
ssp. *proliferus* var. *palmensis*
(tagasaste)
El Paso, La Palma



Spartocytisus supranubius
("retama del Teide", background)
and *Adenocarpus viscosus*
("codeso de cumbre", foreground)
Zapato de la Reina, Teide National Park,
Tenerife

Fig. B. Maximum likelihood (ML) gene phylogeny for 40 *rrs* sequences based on the model and parameter values indicated. ML-bootstrap support values $\geq 69\%$ for 100 pseudoreplicates of the dataset are shown at the relevant nodes. The analysis demonstrates that *Bradyrhizobium rrs* sequences are not divergent enough to provide adequate resolution for molecular systematic studies of the genus. Consequently, this gene is not suited to infer phylogenetic relationships between *Bradyrhizobium* strains. However, *rrs* sequences have been used in previous *Bradyrhizobium* species descriptions as the only source of molecular phylogenetic characters.

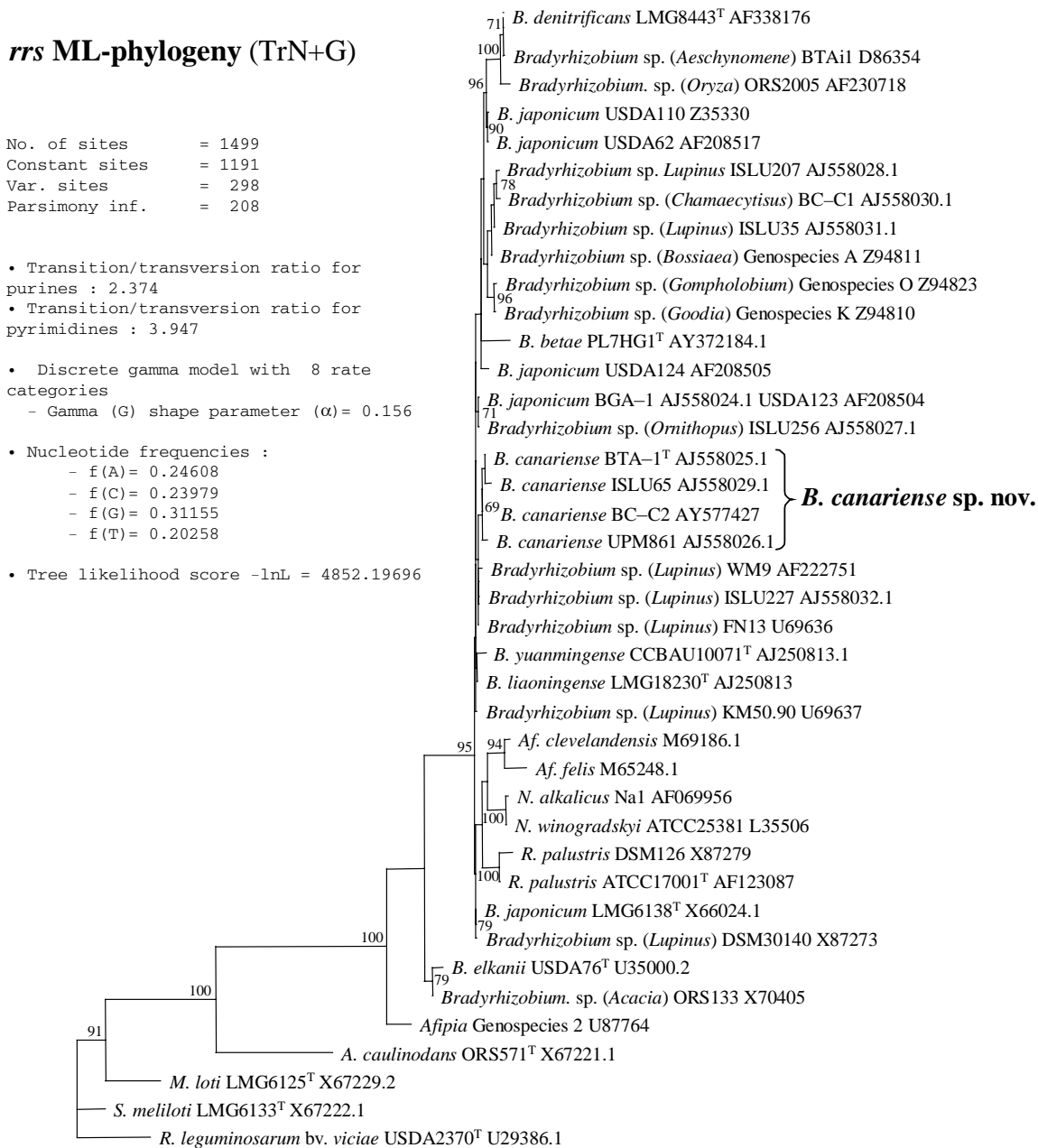


Fig. C. Maximum parsimony (MP) analysis of full-length *rrs* to *rrl* internally transcribed spacer (ITS) sequences. We show one of the 11 most parsimonious trees found in a single island with tree length 818, using a heuristic search strategy with 100 random sequence additions, TBR branch swapping and gaps coded as fifth character state. Bootstrap proportions (%) for 1000 pseudoreplicates are indicated at the relevant nodes. All analyses were performed with PAUP*4vb10 (Swofford, 2002). Notice the significant improvement in tree resolution obtained when this region is used for phylogeny reconstructions, as compared to that achieved with the *rrs* region (Fig. B). This evolutionary hypothesis (Fig. C) is based on all the phylogenetic information present in the multiple alignment, since gaps (260 sites) were also treated as character states in the analysis. The branches supported by MP analysis (heuristic search strategy as described above) of gapped sites are indicated by arrows. These branches are recovered with a 100 % frequency in a majority-rule consensus topology computed from the 96 most parsimonious trees of 539 steps. The tree was rooted with the *B. elkanii* sequences, based on the phylogenetic evidence presented in Figs B and D.

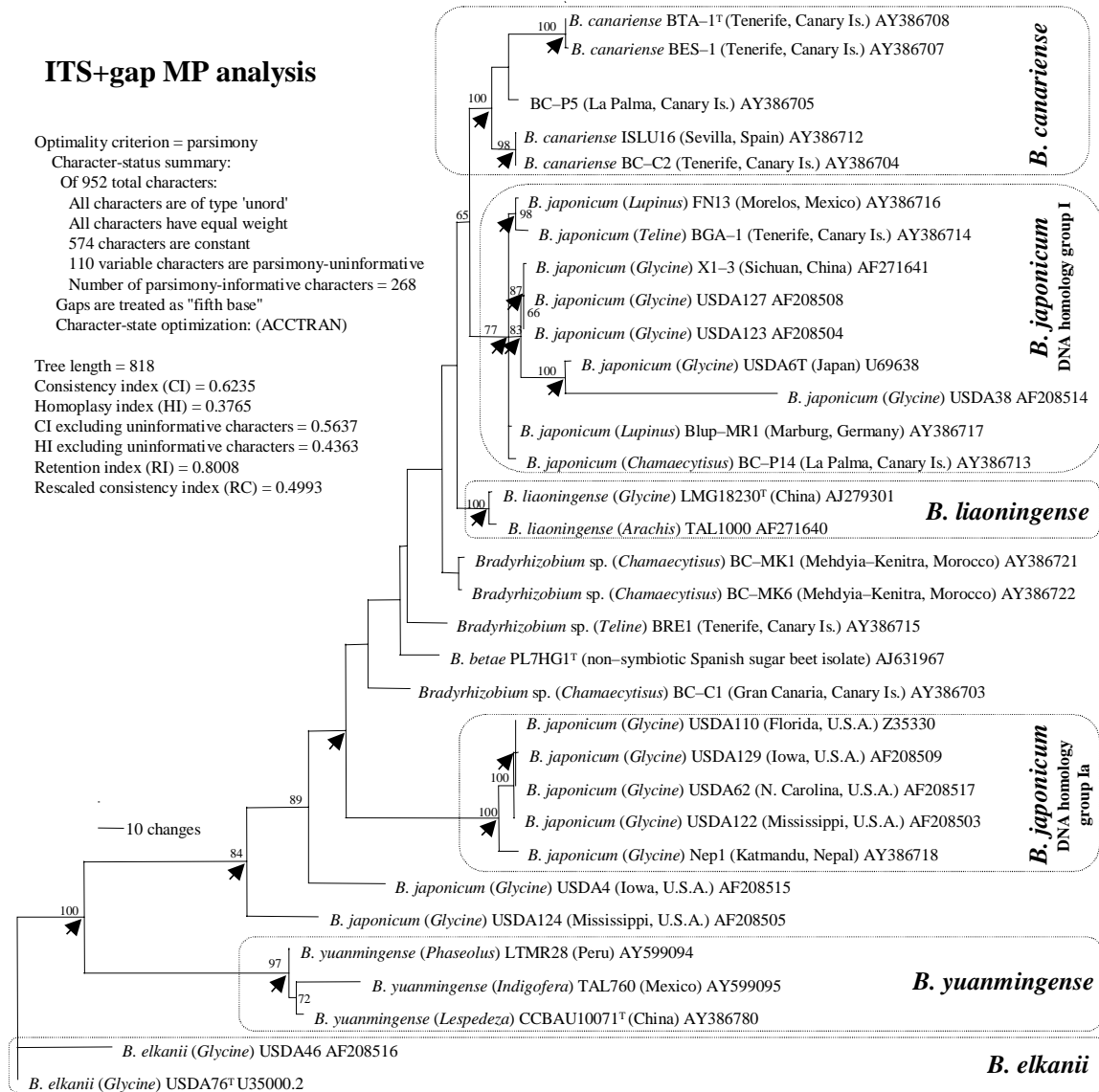
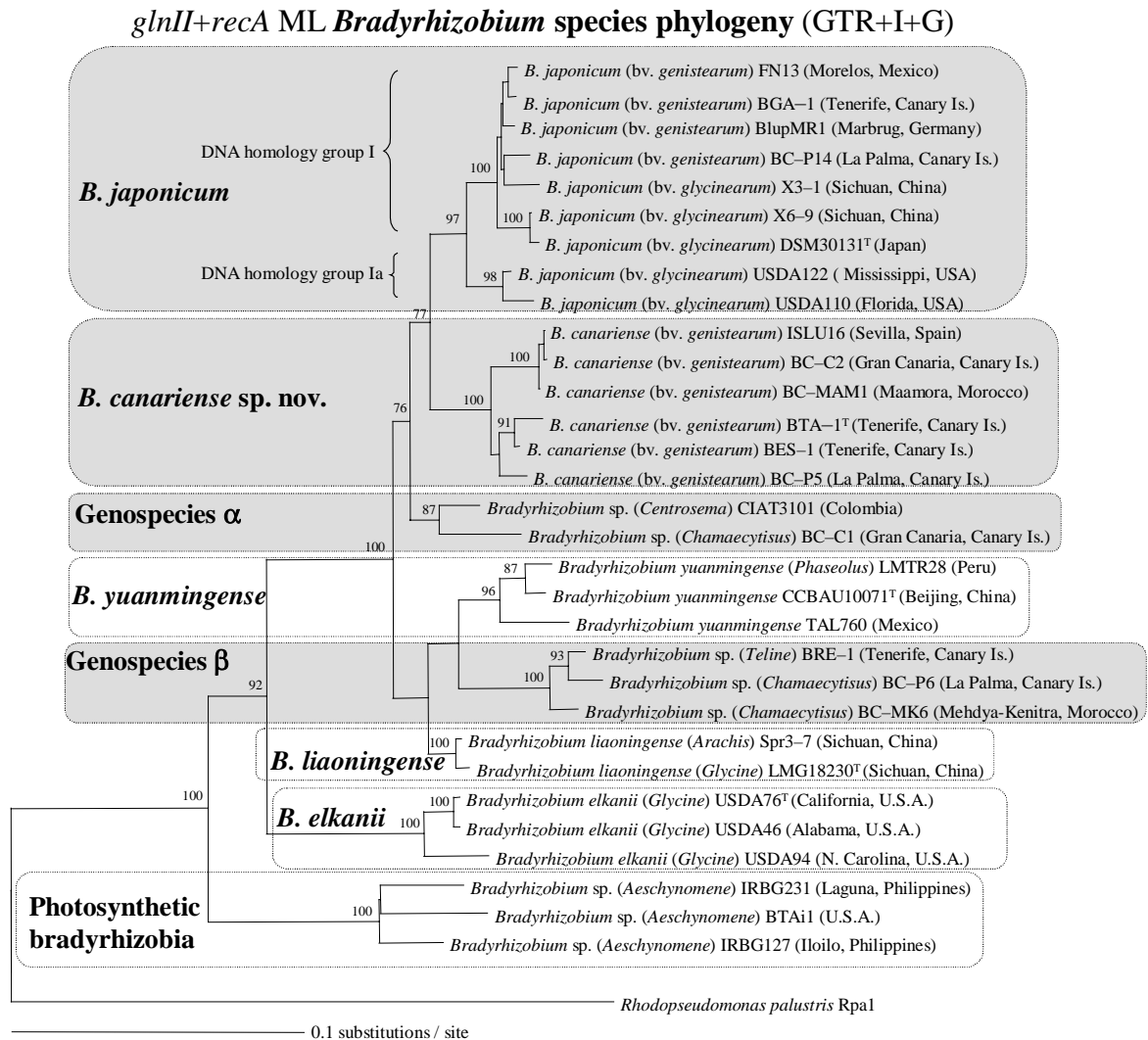


Fig. D. Maximum likelihood *Bradyrhizobium* species phylogeny (GTR+I+G) inferred from partially congruent *glnII+recA* sequence partitions (see final remarks). ML-bootstrap support values $\geq 70\%$ for 100 pseudoreplicates are indicated at the relevant nodes. Congruence among data partitions was assessed by the incongruence length difference (ILD) and Shimodaira–Hasegawa tests, as described in Vinuesa *et al.* (2005). The phylogeny shows eight highly resolved sequence clades which correspond to evolutionary species (Vinuesa & Silva, 2004; Vinuesa *et al.*, 2005), three of which (genospecies alpha, beta and photosynthetic bradyrhizobia) are not formally described as taxonomic species yet. Notice that this species phylogeny is highly congruent with current *Bradyrhizobium* taxonomy, and that it resolves speciation events for most of the taxa. Only the bipartitions connecting the *B. liaoningense*, *B. yuanmingense* and *Bradyrhizobium* genospecies beta clades are unresolved. The four evolutionary lineages that nodulate genistoid legumes in soils from the Canary Islands and in other parts of the world are highlighted.



No. of sites (594 *glnII* + 511 *recA*) = 1105; Constant sites = 718; Var. sites = 387; Parsimony inf. = 259

- Gamma (G) shape parameter (α) = 0.511; Proportion of invariant (I) = 0.318; approximated by 8 discrete rate categories.
- Nucleotide frequencies : f(A)= 0.18935; f(C)= 0.32261; f(G)= 0.31992; f(T)= 0.16812
- GTR rate parameters : A-C = 0.53140; A-G = 2.01268; A-T = 1.00190; C-G = 1.82431; C-T = 5.74493; G-T = 1.0
- Tree likelihood score: -lnL = 6075.80903

Final and concluding remarks

Figs B–D show increasingly resolved topologies. The *rrs* gene phylogeny (Fig. B) is of very limited use to delineate sequence clades. Some of the previously published *rrs*-based phylogenies of bradyrhizobia apparently have greater resolution (Chaintreuil *et al.*, 2000; Jarabo-Lorenzo *et al.*, 2003; Lafay & Burdon, 1998; van Berkum & Fuhrmann, 2000; Willems *et al.*, 2001b). This is due to the use in these studies of the simplest models of nucleotide substitution (generally neighbour-joining trees using Jukes–Cantor or Kimura two-parameter distances), which do not take into account at least two of the following characteristics of the nucleotide substitution process in rhizobial *rrs* (and other) sequences: the heterogeneity in base frequencies, transitional bias, and among-site rate variation. Consequently, such models fit the data poorly, as demonstrated by Vinuesa *et al.* (2005) using likelihood ratio tests (Huelsenbeck & Rannala, 1997). These simple models produce distance estimates with a much lower variance than those estimated by more realistic (parameter richer) models, resulting in an overestimation of nodal bootstrap support values (Bruno & Halpern, 1999; Buckley, 2002; Buckley *et al.*, 2001). Finally, great care must be taken when interpreting the results of *rrs* phylogenies of closely related taxa, as homologous recombination at this highly conserved locus may lead to gene mosaicism and distorted phylogenies (Parker, 2001; van Berkum *et al.*, 2003; Wang & Zhang, 2000). Therefore, it seems not appropriate that new *Bradyrhizobium* genospecies are named on the sole basis of *rrs* sequence polymorphisms, as has been done in the past (Lafay & Burdon, 1998).

The ITS MP-phylogeny (Fig. C) shows a significantly higher resolution than the *rrs*-based topology. Several major sequence clades are resolved with bootstrap values >75%. A maximum parsimony analysis was used in this case since it allows to take into account the significant phylogenetic information from the insertion/deletion (indel) events (260 gapped sites in the corresponding alignment). Consequently, future studies dealing with ITS sequences should specify how gapped sites are treated for phylogeny estimation, which has not been the case in several previous works dealing with these sequences (Tan *et al.*, 2001; van Berkum & Fuhrmann, 2000). The boxed clades resolved in the ITS-based phylogenetic hypothesis presented in Fig. C correlate well with those in the *Bradyrhizobium* species phylogeny shown in Fig. D. However, the relationships between these ITS-sequence clades are not resolved. The photosynthetic bradyrhizobia were excluded from the analysis in order to improve alignment quality, which is difficult to perform unambiguously due to the different types of long insertions that these strains contain (Vinuesa *et al.*, 2005; Willems *et al.*, 2001a, 2003). It should be noted that coding gaps as fifth character state is only reasonable if the number and length of gapped sites is similar across the sequences to be compared. If the photosynthetic strains would have been included in such an analysis, the resulting trees would display very contrasting branch lengths, leading to the classical long-branch attraction problems (Felsenstein zone) and associated topological errors (Felsenstein, 2004). Notice that the long branch of the USDA 38 sequence is due to a 75 bp insertion present in the ITS of this strain. Finally, it should be noted that none of the ITS sequences from new (geno)species reported herein fell into any of the ITS-sequence clusters that correspond to the *Bradyrhizobium* genospecies (III–XI) proposed by Willems *et al.* (2003) based on ITS sequence clades and DNA-DNA hybridizations (Vinuesa, unpublished).

The protein-coding *atpD*, *glnII* and *recA* sequences have been used previously in key molecular systematic and evolutionary studies of rhizobia (Gaunt *et al.*, 2001; Turner & Young, 2000; Turner *et al.*, 2002; Wernegreen & Riley, 1999). Sequences for only one *Bradyrhizobium* strain (USDA 6^T) could be obtained in those studies due to PCR amplification problems. The primers and amplification protocols developed by Vinuesa *et al.* (2005) yielded high-quality PCR products and sequences from all bradyrhizobia tested. The *glnII+recA* ML-phylogeny presented in Fig. D is the best estimate of a *Bradyrhizobium* species phylogeny that could be made from the ITS, *atpD*, *glnII* and *recA* sequences generated and analysed by Vinuesa *et al.*, (2005). The only uncertainty present in that topology

concerns the relationships between the *B. liaoningense*, *B. yuanmingense* and *Bradyrhizobium* genospecies beta clades. Notice that these taxa correspond also with the unresolved nodes in the ITS phylogeny (Fig. C). The lack of resolution at the corresponding nodes in Fig. D is due to character incongruence between some of the sequences in the corresponding partitions, as demonstrated by statistical testing of competing hypothesis under maximum-parsimony and ML optimality criteria (Vinuesa *et al.*, 2005). These authors presented evidence from a Bayesian phylogenetic analysis that supports the following split sequence for *Bradyrhizobium* speciation events (in Newick format): (((((((((*B. japonicum*, *B. canariense*), *Bradyrhizobium* genospecies alpha), *B. liaoningense*) *Bradyrhizobium* genospecies beta), *B. yuanmingense*), *B. elkanii*), photosynthetic bradyrhizobia), *Rhodopseudomonas palustris*=outgroup). However, this hypothesis needs to be validated by further analyses (sequencing of more loci), as it is not congruent with the topologies inferred from ITS (Fig. C) or *atpD* sequences (Vinuesa *et al.*, 2005), which favour a paraphyletic status for *B. japonicum* (*sensu lato*) DNA homology groups I and Ia (Hollis *et al.*, 1981), as first noted by van Berkun & Fuhrmann (2000). The position of genospecies alpha and beta is also unstable in those gene trees. However, the hypothesis that *B. canariense* is the sister species of *B. japonicum* strains related to USDA 6^T (DNA-homology group I) is not contradicted by any of the sequence partitions analysed (Vinuesa *et al.*, 2005).

Based on the phylogenetic analyses presented herein and in Vinuesa *et al.* (2005), we conclude that the current *Bradyrhizobium* taxonomy is consistent with the species phylogeny presented in Fig. D. The phylogenetic position of *B. betae* in that species tree remains to be determined. No sound conclusions about its phylogenetic affinities can be inferred from the *rrs* or ITS sequences available for that taxon (Figs B, C), except that it does not fit within any of the resolved ITS sequence clades. The phylogenetic hypothesis presented in Fig. D provides strong evidence that the evolutionary lineages labelled as genospecies alpha, genospecies beta, and photosynthetic bradyrhizobia represent unnamed species. We refrained from making additional nomenclatural proposals at this moment for these lineages before a greater number of isolates of genospecies alpha and beta are studied, and population genetics/phylogenetic methods are applied to validate the two genospecies (VI and VIII) of photosynthetic isolates that have been proposed by Willems and colleagues based on ITS sequence analyses and DNA–DNA hybridizations (Willems *et al.*, 2001a, 2003). Particularly the taxonomic position of the photosynthetic bradyrhizobia with respect to *Blastobacter denitrificans* and *Agromonas oligotrophica* needs to be revised by a thorough phylogenetic analysis of protein-coding genes. The former species was recently shown in an elegant work to form N₂-fixing nodules on *Aeschynomene indica* plants (van Berkun & Eardly, 2002). Based on the evolutionary hypothesis presented in Fig. D, and using a conservative classification criterion, the best solution at the moment is to consider both *B. elkanii* and the photosynthetic strains as distinct basal evolutionary lineages (species) of the genus *Bradyrhizobium* (*sensu lato*), which is in line with the conclusions reached by So *et al.* (1994) from fatty acid and rRNA analyses. Therefore, previous suggestions that they may represent new genera (Eaglesham *et al.*, 1990; Ladha & So, 1994; van Berkun & Eardly, 1998; Willems *et al.*, 2001b) should be considered with caution, as they form a perfectly supported clade (100 bootstrap support) with the *bona fide* bradyrhizobia in Fig. D. Interestingly, this topology suggests that photosynthesis might represent an ancestral physiologic trait in the genus *Bradyrhizobium* (*sensu lato*), which is consistent with the evolutionary hypothesis of Jarvis *et al.* (1986) that bradyrhizobia descend from a photosynthetic ancestor related to *Rhodopseudomonas*. Therefore, rooting of the *Bradyrhizobium* species clade with the *R. palustris* sequence is consistent with this hypothesis. In this respect, the placement of *R. palustris* in the *rrs* phylogeny (Fig. B) is misleading, again illustrating the great care that has to be exercised when this gene is used for phylogenetic analysis of closely related taxa. This common practice is the major cause for the uncertainty and controversy encountered in the current molecular systematics of the genus (Lafay & Burdon, 1998; Sawada *et al.*, 2003; van Berkun & Eardly, 1998; Willems *et al.*, 2001b), not only because of the confounding effect that

homologous recombination has on traditional phylogeny estimation (Schierup & Hein, 2000; Vinuesa *et al.*, 2005), which is known to affect the *rrn* operons of rhizobia (Parker, 2001; van Berkum *et al.*, 2003), but also mainly because of the systematic error of equating a gene (*rrs* or ITS) tree with a species tree (Felsenstein, 2004; Nichols, 2001; Rosenberg, 2002; Vinuesa *et al.*, 2005), which is found throughout the systematic/taxonomic literature of rhizobia. In this regard, the relationships of *Afipia* species, *Agromonas* species, *Blastobacter* species and *Nitrobacter* species with *Bradyrhizobium* species need to be critically revised in the light of proper phylogenetic analyses based on multiple loci before a revision at a higher taxonomic level is made for these genera. Finally, the current classification of bradyrhizobia in a separate family '*Bradyrhizobiaceae*' (Sawada *et al.*, 2003) is consistent with their monophyletic grouping in the ML species phylogeny shown in Fig. D.

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