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TAXONOMIC DESCRIPTION

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Bradyrhizobium ivorense sp. nov. as a potential local bioinoculant for *Cajanus cajan* cultures in Côte d'Ivoire

Romain K. Fossou[1,2], Joël F. Pothier[3], Adolphe Zézé[2] and Xavier Perret[1,*]

Abstract

For many smallholder farmers of Sub-Saharan Africa, pigeonpea (*Cajanus cajan*) is an important crop to make ends meet. To ascertain the taxonomic status of pigeonpea isolates of Côte d'Ivoire previously identified as bradyrhizobia, a polyphasic approach was applied to strains CI-1B^T, CI-14A, CI-19D and CI-41S. Phylogeny of 16S ribosomal RNA (rRNA) genes placed these nodule isolates in a separate lineage from current species of the *B. elkanii* super clade. In phylogenetic analyses of single and concatenated partial *dnaK*, *glnII*, *gyrB*, *recA* and *rpoB* sequences, the *C. cajan* isolates again formed a separate lineage, with strain CI-1B^T sharing the highest sequence similarity (95.2%) with *B. tropiciagri* SEMIA 6148^T. Comparative genomic analyses corroborated the novel species status, with 86% ANIb and 89% ANIm as the highest average nucleotide identity (ANI) values with *B. elkanii* USDA 76^T. Although CI-1B^T, CI-14A, CI-19D and CI-41S shared similar phenotypic and metabolic properties, growth of CI-41S was slower in/on various media. Symbiotic efficacy varied significantly between isolates, with CI-1B^T and CI-41S scoring on the *C. cajan* 'Light-Brown' landrace as the most and least proficient bacteria, respectively. Also proficient on *Vigna radiata* (mung bean), *Vigna unguiculata* (cowpea, niébé) and additional *C. cajan* cultivars, CI-1B^T represents a potential bioinoculant adapted to local soil conditions and capable of fostering the growth of diverse legume crops in Côte d'Ivoire. Given the data presented here, we propose the 19 *C. cajan* isolates to belong to a novel species called *Bradyrhizobium ivorense* sp. nov., with CI-1B^T (=CCOS 1862^T=CCMM B1296^T) as a type strain.

Cultivated worldwide as a sole crop or in association with short-maturing cereals and legumes, or with long duration crops like cotton, pigeonpea (*Cajanus cajan* L.) is an important source of proteins for many smallholder farmers of developing countries [1]. In Côte d'Ivoire, diverse *C. cajan* landraces are grown for human consumption and animal feed [2], but also as plants to improve soil fertility in savannahs [3], to reduce soil erosion [4], and to serve as intercrops in upland rice cropping systems [5]. As a legume crop that forms nitrogen-fixing symbioses with soil bacteria collectively known as rhizobia, pigeonpea may overcome the low nitrogen levels often encountered in tropical and subtropical fields providing proficient rhizobia inhabit those soils. Although pigeonpea was reported to occasionally associate with fast-growing strains of the *Rhizobium* [6, 7] and *Sinorhizobium* (*Ensifer*) [8] genera, most proficient symbionts of *C. cajan* are slow-growing strains that belong to the *Bradyrhizobium* genus [9, 10] with *B. cajani* sp. nov. AMBPC1010^T as the unique type strain that was isolated from a nodule of *C. cajan* during a survey in the Dominican Republic [11].

Author affiliations: [1]Department of Botany and Plant Biology, Microbiology Unit, University of Geneva, Sciences III, 30 quai Ernest-Ansermet, CH-1211 Geneva 4, Switzerland; [2]Laboratoire de Biotechnologies Végétale et Microbienne, Unité Mixte de Recherche et d'Innovation en Sciences Agronomiques et Génie Rural, Institut National Polytechnique Felix Houphouët-Boigny, Yamoussoukro, Côte d'Ivoire; [3]Environmental Genomics and Systems Biology Research Group, Institute of Natural Resource Sciences, Zurich University of Applied Sciences (ZHAW), Einsiedlerstrasse 31, CH-8820 Wädenswil, Switzerland.

***Correspondence:** Xavier Perret, xavier.perret@unige.ch

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Abbreviations: ANI, average nucleotide identity; B&D, Broughton and Dilworth nitrogen-free plant growth solution; DDH, DNA-DNA hybridisation; ddH2O, double distilled water; ML, maximum-likelyhood; MLSA, multilocus sequence analysis; RMM, Rhizobium minimal medium; RMS, RMM medium supplemented with succinate.

All sequences determined for *Bradyrhizobium ivorense* sp. nov. isolates described in this study were deposited in GenBank/EMBL/DDBJ under the following accessions: dnaK of strain CI-1B^T (MK376326), CI-14A (MK376327), CI-19D (MK376328), CI-41S (MK376329); glnII of strain CI-1B^T (MH756157), CI-14A (MH756158), CI-19D (MH756159), CI-41S (MH756160); gyrB of strain CI-1B^T (MH756161), CI-14A (MH756162), CI-19D (MH756163), CI-41S (MH756164); recA of strain CI-1B^T (MK376330), CI-14A (MK376331), CI-19D (MK376332), CI-41S (MK376333); rpoB of strain CI-1B^T (KX388393), CI-14A (MK376334), CI-19D (MK376335), CI-41S (MK376336). Genome sequences for CI-1B^T and CI-41S strains can be accessed via GenBank Assembly accessions GCA_900696085 and GCA_900696075, respectively.

Eight supplementary figures and eight supplementary tables are available with the online version of this article.

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Initially proposed by Jordan as a separate taxon for slow-growing root nodule bacteria of legumes [12], the genus *Bradyrhizobium* now encompasses more than 50 proposed species [13]. Phylogenetic analyses based upon sequences of the 16S ribosomal RNA (rRNA) and several core protein genes, later separated bradyrhizobia species into two main lineages: the *B. japonicum* and *B. elkanii* super clades [14]. More recent phylogenetic analyses on larger sets of isolates and gene sequences showed bradyrhizobia strains clustered into more than two discreet subgroups, however [13, 15]. Nevertheless, with more than 30 species the *B. japonicum* supergroup includes plant associated bacteria as diverse as *B. arachidis* CCBAU 051107^T that was isolated from root nodules of *Arachis hypogaea* in Hebei, China [16]; *B. betae* LMG 21987^T from a tumour-like outgrowth of sugar beet (*Beta vulgaris*) grown in northern Spain [17]; *B. canariense* BTA-1^T that nodulated genistoid legumes from the Canary Islands [18]; *B. diazoefficiens* (previously *japonicum*) USDA 110^T that was isolated from a soybean nodule in Florida (US Department of Agriculture, Beltsville, MD); *B. iriomotense* strain EK05^T which infected a root-outgrowth of an *Entada koshunensis* legume in the Okinawa island in Japan [19]; *B. japonicum* USDA 6^T that was found in a soybean nodule collected in Japan and entered a strain collection as early as 1929 [20]; the extra-slow growing *B. liaoningense* USDA 3622^T (ESG 2281^T) found inside a soybean nodule collected in the Heilongjiang Province of PR China [21]; *B. ottawaense* OO99^T isolated from a soybean field near Ottawa, Canada [22] as well as the *B. cajani* AMBPC1010^T described above [11]. Together, these nine type strains of the *B. japonicum* lineage already illustrate the diversity, ubiquitous geographic distribution and relative plant promiscuity that characterise many of the bradyrhizobia strains [13].

Yet, as recently noted by Grönemeyer and Reinhold-Hurek [23], our knowledge on the diversity and biogeography of bradyrhizobia species relies primarily on data collected in the Americas, Asia and Europe. For examples, type strains of the *B. elkanii* supergroup include *B. icense* LMTR 13^T isolated from *Phaseolus lunatus* in Peru [24]; *B. macuxiense* UFLA03-321^T found inside a nodule of *Centrolobium parense* in Amazonia [25] and *B. retamae* Ro19^T identified as a symbiont of *Retama monosperma* in the Mediterranean basin [26]. A number of recent surveys identified bradyrhizobia in sub-Saharan countries, however, including in Angola and Namibia [27], Ethiopia [14], Kenya [28] and Senegal [29]. In addition, a sampling of six fields in three geographically distant regions of Côte d'Ivoire has led to the molecular characterisation of >80 pigeonpea isolates by matrix-assisted laser desorption/ionization (MALDI) time of flight (TOF) mass spectrometry (MS) and DNA sequencing [30]. That data indicated that 63 of the 85 nodule isolates (74%) were bradyrhizobia, only one of which (strain CI-41A) belonged to the *B. japonicum* super clade. Hierarchical cluster analysis of mass spectra separated the remaining 62 nodule isolates into a group of 43 strains close to *B. elkanii* USDA 76^T (cluster I strains) and a second group of 19 bradyrhizobia (cluster II), whose taxonomic status was unclear. Phylogenetic analyses reported herein show the cluster II strains to belong to a separate lineage of the *B. elkanii* super clade, with levels of similarity for partial *dnaK*, *glnII*, *gyrB*, *recA* and *rpoB* sequences and genomic average nucleotide identity (ANI) values supporting the designation of *Bradyrhizobium ivorense* sp. nov. as a novel species with CI-1B^T being proposed as the type strain.

PHYLOGENETIC CHARACTERIZATION

Although still recognized as universal genetic markers for classifying bacterial isolates, 16S rRNA sequences were occasionally found to be too conserved for accurately delineating bradyrhizobia species [24, 31]. By contrast multilocus sequence analysis (MLSA) of concatenated partial sequences for conserved protein-coding genes has been shown to provide reliable phylogenies, to reflect overall genome similarities and to match DNA–DNA hybridization (DDH) values [32]. MLSA has been repeatedly used for resolving bradyrhizobia lineages and has become one of the hallmark criteria for delimiting novel species (e.g [33, 34].) Similarly, DDH, that was long considered as another 'gold standard' for delimiting bacterial species, has been superseded by the more informative pairwise comparisons of genome sequences [35, 36]. Accordingly, a polyphasic approach was applied to determine the taxonomic status of the 19 pigeonpea isolates of Côte d'Ivoire grouped in cluster II. Isolates CI-1B^T, CI-14A, CI-19D and CI-41S were selected as representative strains because they originated from different fields and regions of Côte d'Ivoire (Table S1, available in the online version of this article) and possessed protein mass spectra that reflected the genetic diversity existing within cluster II isolates [30]. For long term preservation, 30% glycerol stocks were kept at −60 °C and bacteria were routinely grown in/on *Rhizobium* minimal medium (RMM) supplemented with 12 mM succinate (RMS) [37], or in/on RMM containing alternative carbon sources when specific metabolic analyses were needed.

For MLSA studies, the *dnaK*, *glnII*, *gyrB*, *recA* and *rpoB* marker genes were amplified using procedures described in Fossou *et al.* [30] and primers specifically designed for bradyrhizobia sequences (Table S2). Marker genes were sequenced on both strands using Sanger sequencing. The resulting sequences were assembled and manually curated for primers and ambiguities before being archived in GenBank, with their respective accession numbers listed in Table S2. As preliminary analyses showed the pigeonpea isolates to share identical 16S rDNA sequences and to belong to the *B. elkanii* super clade [30], subsequent phylogenetic analyses included all 21 types strains currently described for this lineage. An additional eight type strains representative of the *B. japonicum* super clade as well as the unrelated *B. oligotrophicum* LMG 10732^T strain were also included in phylogenies [13]. Together with the associated accession numbers that characterise them, the bradyrhizobia type strains of the *B. japonicum* and *B. elkanii* super clades are listed in Tables S3a and S3b, respectively. Except for type strains of the *B. erythrophlei*, *B. ferriligni*, *B. namibiense* and *B. ripae* species for which no genome data was accessible at the time of writing, all other sequence accessions

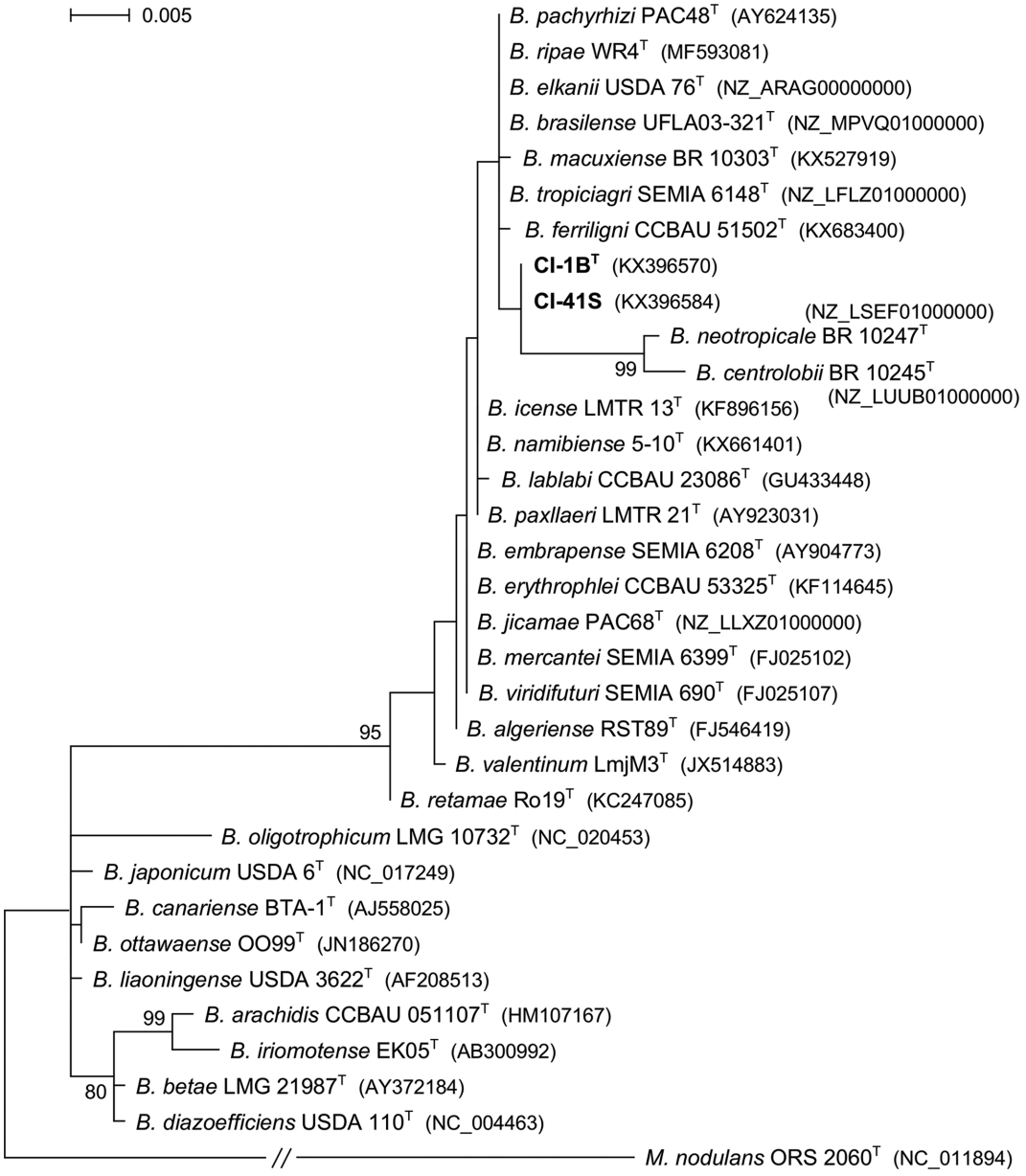

Fig. 1. Maximum-likelihood (ML) unrooted phylogeny of 16S rRNA gene sequences of type strains of the *Bradyrhizobium* genus and of *B. ivorense* sp. nov CI-1B^T and CI-41S. Sequences were aligned with MAFFT version 7 using the Q-INS-i method. Analysis used the T92 +G+I model, a total of 1182 positions, 1000 pseudoreplicates with bootstrap values>70% shown at branch nodes. Sequences for the 21 members of the *B. elkanii* super clade and *B. oligotrophicum* LMG 10732^T were 1179 bp long. Except for *B. iriomotense* (1178 bp), sequences for the *B. japonicum* super clade type strains were 1177 bp long. To improve resolution of ML tree, the branch for the *M. nodulans* strain ORS 2060^T outgroup was truncated. Accession numbers or source for 16S rRNA gene sequences are indicated within parentheses next to the species name, with strains of the novel species marked in bold. Bar represents the expected number of substitutions per site.

were manually curated using as reference the corresponding genomic data, and inaccuracies were highlighted in Table S3b. In the case of *B. ferriligni* type strain CCBAU 51502^T, the 16S rRNA gene sequence archived under GenBank accession KX683400 (deposited by Li Y. in 2016) was used instead of the KJ818096 sequence (deposited by Yao and Sui in 2014), which diverged too much from other bradyrhizobia sequences to be considered as reliable. DNA sequences were aligned with MUSCLE as implemented in MEGA software version 7 [38], and partial housekeeping gene sequences concatenated with Seaview ver. 4 [39]. Phylogenies were inferred with MEGA 7, with evolutionary trees reconstructed using the maximum likelihood (ML) method [40], a statistical support of 1000 bootstrap replicates and sequences of *Methylobacterium nodulans* strain ORS 2060^T [41] selected as outgroups. Best-fit nucleotide substitution model was selected according

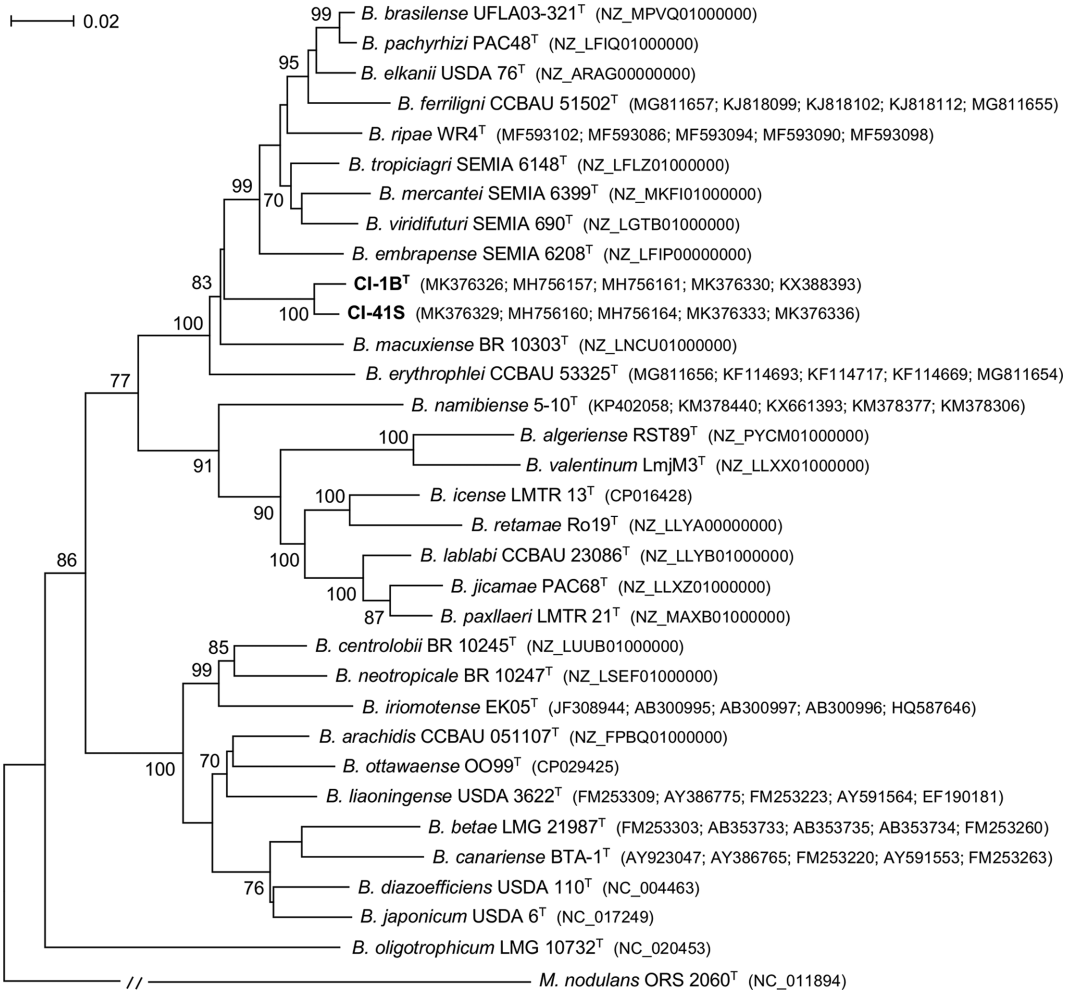

Fig. 2. Maximum-likelihood (ML) unrooted phylogram inferred from concatenated partial *dnaK* (279 bp), *glnII* (540 bp), *gyrB* (588 bp), *recA* (439 bp) and *rpoB* (714 bp) gene sequences of type strains of the *Bradyrhizobium* genus and of *B. ivorense* sp. nov CI-1B^T and CI-41S. The GTR+G+I model was used and included as parameters a total of 2566 positions and 1000 pseudoreplicates, with only bootstrap support values>70% shown at branch nodes. To improve resolution of the tree, the branch for the *M. nodulans* strain ORS 2060^T outgroup was truncated. Accession numbers or source for sequences are indicated within parentheses next to the species name. Bar represents the expected number of substitutions per site.

to the Bayesian information criterion [42], and similarity levels (uncorrected genetic distances) between concatenated sequences of selected *Bradyrhizobium* species calculated as in Rashid *et al.* [43] and reported in Table S4.

Because pigeonpea isolates CI-1B^T, CI-14A, CI-19D and CI-41S share identical 16S rRNA gene sequences [30], only CI-1B^T and CI-41S are displayed in the ML phylogenetic trees for that marker. As expected, in the phylogeny based upon 1182 positions of nearly complete 16S rRNA gene sequences that are shown in Figures 1 and S1, the taxonomic position of many of the 21 species of the *B. elkanii* supergroup (e.g. *B. elkanii* USDA 76^T, *B. pachyrhizi* PAC48^T, *B. ripae* WR4^T and *B. tropiciagri* SEMIA 6148^T) remained unresolved. Yet, strains CI-1B^T and CI-41S always formed a distinct branch rooted in the *B. elkanii* clade of 16S rRNA gene trees, whether a subset of eight representative or 34 species of the *B. japonicum* supergroup were included in the analyses (see Figs 1 and S1, respectively). Phylogenetic relationships between the *C. cajan* isolates and bradyrhizobia type strains was better resolved by applying MLSA using the following partial *dnaK* (MK376326 to MK376329), *glnII* (MH756157 to MH756160), *gyrB* (MH756161 to MH756164), *recA* (MK376330 to MK376333) and *rpoB* (MK376334 to MK376336) gene sequences for the *C. cajan* isolates (see also Table S2). In order to include the same set of 30 type strains used for the 16S rRNA gene phylogeny shown in Fig. 1, individual alignment lengths were trimmed to 279 (*dnaK*), 540 (*glnII*), 588 (*gyrB*), 439 (*recA*) and 714 (*rpoB*) nucleotides. As preliminary phylogenetic analyses confirmed that trees for each of the five selected markers were largely congruent (see Fig. S2–S6), individual sequences were combined into a dataset of 2560 bp long concatenated sequences which phylogeny showed CI-1B^T

Table 1. Average nucleotide identity (ANI) values between Bradyrhizobium *ivorense* sp. nov. CI-1B^T, CI-41S and type strains of the *B. elkanii* super clade with available genome data

	CI-1B^T	
Strains	**ANIb (%)**	**ANIm (%)**
***B. ivorense* sp. nov. CI-41S**	**96.6**	**97.5**
B. elkanii USDA 76^T	86.0	89.0
B. pachyrhizi PAC48^T	85.4	88.6
B. macuxiense UFLA03-321^T	85.3	88.7
B. mercantei SEMIA 6399^T	85.2	88.6
B. tropiciagri SEMIA 6148^T	85.2	88.4
B. embrapense SEMIA 6208^T	85.0	88.3
B. viridifuturi SEMIA 690^T	85.0	88.3
B. brasilense BR 10303^T	84.1	87.9
B. lablabi CCBAU 23086^T	79.7	85.2
B. jicamae PAC68^T	79.4	85.4
B. paxllaeri LMTR 21^T	79.4	85.4
B. algeriense RST89^T	79.2	85.2
B. icense LMTR 13^T	78.9	85.3
B. retamae Ro19^T	78.9	85.2
B. valentinum LmjM3^T	78.9	85.2

and CI-41S to form a separate, highly supported lineage in a basal position of the *B. elkanii* super clade (Fig. 2). While concatenated sequences of CI-1B^T and CI-41S shared 98.4% similarity, the highest levels of sequence similarity between CI-1B^T and bradyrhizobia type strains was with *B. tropiciagri* SEMIA 6148^T (95.2%), followed by *B. elkanii* USDA 76^T, *B. pachyrhizi* PAC48^T and *B. ripae* WR4^T (95.1%) (see Table S4). Surprisingly, in this study, several type strains shared sequence similarities above the 97% threshold recently proposed to separate bradyrhizobia species, when a similar set of marker genes (*atpD*, *dnaK*, *glnII*, *gyrB*, *recA*) was used [24]. For example, concatenated sequences of *B. pachyrhizi* PAC48^T and *B. brasilense* UFLA03-321^T shared as much as 99.1% similarity, while *B. elkanii* USDA 76^T and *B. pachyrhizi* PAC48^T genes were 97.8% similar (Table S4), therefore the use of 97% sequence similarity threshold for delimiting potential *Bradyrhizobium* species requires further investigation. That *B. ivorense* isolates form a separate lineage within the *B. elkanii* clade was further confirmed by a phylogenetic analysis of concatenated partial *glnII* (522 bp) and *recA* (411 bp) gene sequences of CI-1B^T, CI-41S and 57 *Bradyrhizobium* type strains (see Fig. S7).

GENOMIC CHARACTERIZATION

To further characterize the *C. cajan* isolates, draft genomes were obtained for CI-1B^T and CI-41S using paired-end sequencing (2×300 bp) with an Illumina MiSeq instrument at the Zürich University of Applied Sciences (ZHAW). Once isolated via a NucleoSpin tissue kit (Macherey-Nagel, Düren, Germany),

Table 2. Differential characteristics of Bradyrhizobium *ivorense* sp. nov. isolates, using as reference strain *B. elkanii* USDA 76^T that was grown in the same conditions. For comparison, corresponding phenotypes of *B. pachyrhizi* PAC48^T and *B. tropiciagri* SEMIA 6148^T were taken from Ramírez-Bahena *et al.* (2009) [52] and Delamuta *et al.* (2015) [53], respectively. Bacterial response was qualified as: +, positive; ±, weak or variable; –, negative; or ND, not determined

	Strains						
Differential characteristics	**CI-1B^T**	**CI-14A**	**CI-19D**	**CI-41S**	**USDA 76^T**	**PAC48^T**	**SEMIA 6148^T**
Growth at 35 °C	–	–	±	±	+	+	+
Aesculin hydrolysis	±	–	–	ND	–	±	±
Reduction of nitrate to nitrite	–	–	–	–	+	+	ND
Assimilation as carbon source:							
Arabinose	+	±	+	ND	±	+	+
Glucose	+	±	+	±	±	+	–
Maltose	+	+	+	±	±	±	–
N-acetylglucosamine	+	+	+	ND	±	–	–
Resistant to (µg per disc):							
Ampicillin (100)	+	+	±	–	–	±	ND
Kanamycin (100)	+	+	+	+	–	–	ND
Growth in YM with 0.5% NaCl	+	+	–	+	+	+	+
0.75% NaCl	–	–	–	–	+	+	+

Table 3. Profiles of cellular fatty acids detected in Bradyrhizobium *ivorense* sp. nov. isolates CI-1B^T and CI-41S and in *B. elkanii* USDA 76^T as a reference strain. Reported values correspond to percentages of the total amount of fatty acid compounds found in each strain. Not detected, ND

	Strain		
Fatty acid compound detected	CI-1B^T	CI-41S	USDA 76^T
$C_{12:1}$ 3-OH	0.42	ND	ND
$C_{14:0}$	ND	0.50	ND
$C_{16:0}$	9.68	12.31	7.23
$C_{16:1}\omega5cis$	0.31	ND	0.16
$C_{16:1}\omega7cis$, $C_{16:1}\omega6cis$	1.83	1.18	0.55
$C_{17:0}$	ND	ND	0.36
$C_{17:0}$ cyclo	ND	ND	1.13
$C_{17:1}\omega8cis$	ND	ND	0.31
$C_{18:0}$	0.40	0.58	0.57
$C_{18:1}\omega5cis$	0.47	0.35	0.68
$C_{18:1}\omega7cis$	86.03	81.30	67.88
$C_{19:0}$ cyclo $\omega8cis$	0.86	3.78	21.01
$C_{20:1}\omega7cis$	ND	ND	0.11

genomic DNAs of CI-1B^T and CI-41S were fragmented with a Covaris E220 ultrasonicator, aiming for an average target size of 550 bp. Preparation of sequencing libraries followed the Illumina NeoPrep library system protocol. A total of 5090206 and 1570172 reads were obtained that provided a sequencing coverage of 115- and 36-folds for CI-1B^T and CI-41S, respectively. *De novo* sequence assemblies were obtained with MIRA version 4.0 [44] using standard settings in accurate mode. This was followed by contig reassembly using SeqMan Pro from the Lasergene genomics package version 12.1.0 (DNAStar, Madison, WI) with Pro assembler parameters and read mapping using SeqMan NGen with standard settings to check for mapping inconsistencies. Sequence contigs were ordered using Mauve 20150226 version 10 [45] and the genome of *B. elkanii* USDA 76^T as reference. Automatic genome annotation was performed using the Prokka software tool version 1.12 [46]. The main characteristics of the CI-1B^T and CI-41S genomes are reported in Table S5, with individual sequence contigs archived under the GenBank Nucleotide accessions CAADFC020000001 to CAADFC020000044 for strain CI-1B^T and CAADFB020000001 to CAADFB020000059 for strain CI-41S. ANI values between *B. ivorense* sp. nov. CI-1B^T and CI-41S, and the type strains *B. algeriense* RST89^T (PYCM01000000), *B. brasilense* BR 10303^T (MPVQ01000000), *B. elkanii* USDA 76^T (ARAG00000000), *B. embrapense* SEMIA 6208^T (LFIP00000000), *B. icense* LMTR 13^T (CP016428), *B. jicamae* PAC68^T (LLXZ01000000), *B. lablabi* CCBAU 23086^T (LLYB01000000), *B. macuxiense* UFLA03-321^T (LNCU01000000), *B. mercantei* SEMIA 6399 (MKFI01000000), *B. pachyrhizi* PAC 48^T (LFIQ01000000), *B. paxllaeri* LMTR 21^T (MAXB01000000), *B. retamae* Ro19^T (LLYA00000000), *B. tropiciagri* SEMIA 6148^T (LFLZ01000000), *B. valentinum* LmjM3^T (LLXX01000000) and *B. viridifuturi* SEMIA 690^T (LGTB01000000) were calculated with JSpeciesWS [47] using BLAST (ANIb) and Mummer (ANIm) for sequence alignments [48]. Calculations showed that CI-1B^T and closely related bradyrhizobia type strains shared as highest ANIb and ANIm, values of 86 and 89%, respectively (Table 1). Since both calculated ANIb and ANIm scores are well below the 95–96% threshold proposed for delineating species [48], genomic data also supported the proposal that cluster II *C. cajan* isolates belong to a novel *Bradyrhizobium* species.

PHENOTYPIC CHARACTERIZATION

Metabolic properties of *C. cajan* isolates were characterised using a number of assays and *B. elkanii* USDA 76^T as the reference type strain. At first, API 20 NE strips (BioMérieux SA, Marcy-l'Etoile, France) were used, with slower growth of bradyrhizobia implying modifications to the manufacturer's protocol as described below. Cells were precultured at 27 °C in RMS [37] to an OD_{600} of 0.6 then washed in sterile 0.5% NaCl solution. Slots for enzymatic reactions were inoculated with 10^8 cells resuspended in saline solution, whereas ca. 10^7 cells in RMM agar were inoculated onto the various carbon sources included in the assay. Duplicated API galleries were incubated at 27 °C, in a sterile and sealed container with a moist atmosphere, with reactions assessed every second day for at least 2 weeks. When needed, sterile ddH_2O was added to compensate for evaporation. Utilization of several carbon sources was validated by additional *in vitro* assays carried out in RMM liquid cultures of 5 ml supplemented with 10 mM succinate or malate, or 20 mM galactose, glucose, fructose, maltose, mannose, pyruvate or sucrose. Cultures were inoculated with 10^7 fresh cells, in duplicate for each strain, and incubated at 27 °C with shaking, with growth monitored every third day and considered as positive if OD_{600}>0.2 after 10 days. For growth at different NaCl concentrations, OD_{600} was measured every second day for a week. Catalase activity was determined by rubbing colonies onto a glass slide in presence of 3% H_2O_2 and checking for presence of bubbles. Oxidase activity was detected by rubbing colonies on a Whatman paper soaked with 0.1% *N,N*-dimethyl-1,4-phenylenediamine oxalate solution. Sensitivity to antibiotics was determined with large (∅145 mm) RMS agar plates pre-inoculated with 10^8 cells from early log phase (0.2<OD_{600}<0.4) RMS cultures onto which antibiotic discs were placed at equidistance. Discs contained the following amounts of antibiotics: ampicillin 100 µg, chloramphenicol 500 µg, erythromycin 50 µg, gentamycin 50 µg, kanamycin 100 µg, penicillin 10 µg, streptomycin 250 µg or tetracycline 50 µg. For each strain, tests were duplicated with plates incubated at 27 °C for a maximum of 10 days, with cells considered as sensitive to the antibiotic if a growth halo of ∅>2 mm was observed from the disc edge [49]. Differential growth and metabolic properties of *C. cajan* isolates and of USDA 76^T strain are reported in Table 2, with all phenotypic characteristics listed in Table S6.

Table 4. Symbiotic efficacy of selected Bradyrhizobium *ivorense* isolates on the *Cajanus cajan* 'Light Brown' landrace. Strain proficiency is reported as the mean nodule number (mNN), nodule fresh weight (mNFW) and shoot dry weight (mSDW) per plant, with standard deviations shown in parentheses. SDW values marked with an asterisk were found to be statistically different from the corresponding values measured for CI-1B^T at the level α=5%

Strain	Test	Plants	mNN	mNFW (mg)	mSDW (mg)
No inoculum	1	6	0.0	0.0	73.7 (±7.9)*
	2	5	0.0	0.0	83.4 (±33.2)*
Bradyrhizobium elkanii USDA 76^T	1	8	32.0 (±5.8)	898.0 (±227.5)	2176.1 (±471.7)
	2	7	46.0 (±9.4)	1160.6 (±183.0)	2700.4 (±342.7)
CI-1B^T	1	8	75.1 (±25.3)	1581.1 (±477.2)	2655.0 (±777.7)
	2	8	75.9 (±12.5)	1624.1 (±213.1)	3041.6 (±483.7)
CI-4A2	1	8	59.1 (±18.6)	1050.5 (±377.8)	1876.3 (±449.8)*
CI-4A3	2	8	80.4 (±19.6)	1140.3 (±148.9)	2386.8 (±412.3)*
CI-4C	1	8	75.6 (±17.0)	1534.4 (±357.6)	2718.3 (±600.2)
CI-4D	2	8	100.0 (±35.2)	1208.1 (±286.1)	2230.3 (±404.5)*
CI-14A	1	8	58.6 (±11.4)	925.3 (±210.2)	2112.6 (±527.5)
CI-15A	2	8	35.9 (±8.0)	1184.4 (±140.8)	3027.1 (±574.0)
CI-18C	1	7	46.9 (±17.0)	943.0 (±292.2)	1833.7 (±482.9)*
CI-19D	1	8	36.5 (±7.3)	896.1 (±133.9)	1933.3 (±337.3)*
CI-33F	2	8	62.4 (±18.7)	1033.8 (±245.2)	1637.3 (±355.5)*
CI-35B	1	8	75.4 (±18.8)	910.5 (±203.5)	1765.9 (±426.1)*
CI-41S	1	8	39.4 (±7.7)	492.5 (±113.3)	691.0 (±156.3)*
	2	8	36.5 (±7.3)	896.1 (±133.9)	1155.1 (±403.8)*

The composition in cellular fatty acids of strains CI-1B^T and CI-41S was analysed at the Culture Collection of Switzerland (CCOS) in Wädenswil (Switzerland) using standard procedures and gas-chromatography-based analyses. As a reference control, the fatty acid composition of the *B. elkanii* USDA 76^T type strain was determined in parallel. The fatty acid profile obtained for USDA 76^T matched well the mean fatty acid concentrations determined for group I *B. elkanii* strains by Tighe and co-workers [50], with predominant fatty acids being of the $C_{18:1}$ω7*cis* [67.88% vs. 67.05 (±5.04)% in Tighe *et al*. 2000], $C_{19:0}$ cyclo ω8*cis* [21.01% vs. 19.99 (±3.47)%] and $C_{16:0}$ [7.23% vs. 10.49 (±2.35)%] forms. Although CI-1B^T and CI-41S strains contained lower concentrations of $C_{19:0}$ cyclo ω8*cis* (0.86% in CI-1B^T and 3.78% in CI-41S), the most abundant fatty acids remained of the $C_{18:1}$ω7*cis* and (86.03 and 81.3%, respectively) and $C_{16:0}$ (9.68 and 12.31%, respectively) types. Other differences between the CI-1B^T, CI-41S and USDA 76^T strains are the absence in the pigeonpea isolates of the $C_{17:0}$ cyclo compound found in USDA 76^T at 1.13%, and the presence in CI-41S of $C_{14:0}$ that was not detected in the other two strains. The respective concentrations of cellular fatty acids reliably detected in extracts are reported in Table 3.

To examine the symbiotic properties of *B. ivorense* sp. nov., strains were inoculated onto *C. cajan* cv. 'Light Brown' (see [30]), *Glycine max* cv. Davis, *Macroptilium atropurpureum* cv. Siratro, *Leucaena leucocephala*, *Tephrosia vogelii*, *Vigna radiata* cv. King (mung bean), and *V. unguiculata* cv. Red Caloona (cowpea). Nodulation assays were carried out in autoclaved Magenta jars containing vermiculite and nitrogen free B&D as a plant growth solution [51], using two plants per pot, an inoculum of 2×10^8 bacteria per seedling and controlled growth conditions of 12 h light phase at 10000 Lux and night/day temperatures of 20/27 °C, respectively. Depending on the legume species, plants were grown for 35 to 98 days post inoculation (dpi) with as many as 12 different *C. cajan* isolates being tested for assessing the diversity of symbiotic responses, for example in function of the geographic distribution of strains. As shown in Table 4, on *C. cajan* cv. 'Light Brown' symbiotic efficacy of *B. ivorense* sp. nov. isolates varied considerably, with CI-1B^T, CI-4C and CI-15A being the most proficient strains when shoot dry weight (SDW) was selected as criterion. When inoculated onto other legume species, *C. cajan* isolates formed nitrogen fixing nodules on cowpea, mungbean and siratro, pseudonodules on *L. leucocephala* and few sporadic pink nodules on soybean cv. Davis (see Table S7). Interestingly, on all plant species tested, CI-41S was always the least proficient bacterium, except on *T. vogelii* on which CI-41S was nearly as proficient as CI-1B^T and clearly more effective than the promiscuous *Sinorhizobium fredii* strain NGR234. Together, these nodulation assays confirmed

that strain CI-1B^T was highly proficient on several legume crops cultivated by smallholder farmers in West Africa, including cowpea (also known locally as 'niébé'), mungbean and pigeonpea. In respect to nitrogen fixation genes, CI-1B^T, CI-14A, CI-19D and CI-41S share nearly identical *nifH* genes [30], with corresponding loci of *B. elkanii* USDA 76^T and *B.ferriligni* CCBAU 51502^T strains being the closest in the *nifH* phylogeny shown in Fig. S8.

DESCRIPTION OF *BRADYRHIZOBIUM IVORENSE* SP. NOV.

Bradyrhizobium ivorense (i.vor.en'se. N.L. neut. adj. *ivorense* referring to Côte d'Ivoire, the country where isolates of this species were collected from *C. cajan* nodules).

Cells are Gram-stain-negative, aerobic, 0.6–0.7 µm wide by 1.1–3.4 µm long rods of size similar to other members of the genus. Generation time of the proposed *B. ivorense* sp. nov. CI-1B^T strain was estimated at 14 h (±3 h) when cells were grown in RMS liquid cultures and at 27 °C. On RMS agar, colonies are whitish, convex, circular, with 2 to 3 mm diameter after 10 days incubation at 27 °C. Optimal growth was observed at neutral pH, at temperatures comprised between 25 to 30 °C, and at NaCl concentrations<0.5%. Although some growth still occurred at 20 and 35 °C, plates incubated at 40 °C exhibited no growth. Strains CI-1B^T, CI-14A and CI-19D did not show significant inhibition zones when in presence of all antibiotics tested, at least when challenged with discs containing the following amounts: ampicillin 100 µg, chloramphenicol 500 µg, erythromycin 50 µg, gentamycin 50 µg, kanamycin 100 µg, penicillin 10 µg, streptomycin 250 µg or tetracycline 50 µg. Strain CI-41S was found to be sensitive to ampicillin 100 µg but grew in presence of other antibiotic discs. Strains CI-1B^T, CI-14A and CI-19D were all positive for oxidase, arginine dihydrolase and urease reactions, while only CI-1B^T responded weakly to aesculin hydrolysis. These same three strains were all negative for catalase, D-glucose fermentation, gelatin hydrolysis, indole production, nitrate reduction and para-nitrophenyl-βD-galactopyranosidase reactions. Assimilation tests were positive for adipic acid, D-glucose, maltose, D-mannitol, D-mannose, L-arabinose, N-acetyl-glucosamine and potassium gluconate; weak for malic acid, trisodium citrate, phenylacetic acid; and negative for capric acid. In addition, CI-1B^T and CI-41S were found to assimilate fructose, galactose, malate and pyruvic acid; but apparently could not use sucrose as sole carbon source. Effective nodules were formed on *C. cajan*, *M. atropurpureum*, *V. radiata*, *V. unguiculata* and *T. vogelii* but not on *G. max* and *L. leucocephala*.

The type strain, CI-1B^T (=CCOS 1862^T=CCMM B1296^T) was isolated from a root nodule of a *C. cajan* plant growing in Kossou-Bouafla, Côte d'Ivoire. The draft genome of the type strain is characterized by a size of 9.4 Mbp and a G+C content of 64.2 mol%. The GenBank accession number of the 16S rRNA gene sequence of strain CI-1B is KX396570, and its draft genome sequence accession number is GCA_900696085.

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The 19 *B. ivorense* sp. nov. strains described in this work were all isolated from nodules of pigeonpea collected in fields of Côte d'Ivoire (see [30]). Sampling of the *C. cajan* nodules in fields of Côte d'Ivoire was supported by a research collaboration fellowship attributed by the Centre Suisse de Recherche Scientifique (CSRS) of Abidjan to Adolphe Zézé (AZ) and Xavier Perret (XP), thanks to a donation of the United Nations Development Program. Transfer of *C. cajan* nodules to the University of Geneva and the subsequent genotyping of the rhizobia isolated from these nodules, were subject to conditions described in a Material Transfer Agreement that was signed by AZ and XP on July 12, 2012.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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