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**TAXONOMIC DESCRIPTION**

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# *Bradyrhizobium cenepequi* sp. nov., *Bradyrhizobium semiaridum* sp. nov., *Bradyrhizobium hereditatis* sp. nov. and *Bradyrhizobium australafricanum* sp. nov., symbionts of different leguminous plants of Western Australia and South Africa and definition of three novel symbiovars

Milena Serenato Klepa[1,2,3]†, Luisa Caroline Ferraz Helene[1,2]†, Graham O´Hara[4] and Mariangela Hungria[1,2,3,*]

**Abstract**

*Bradyrhizobium* is a heterogeneous bacterial genus capable of establishing symbiotic associations with a broad range of legume hosts, including species of economic and environmental importance. This study was focused on the taxonomic and symbiovar definition of four strains – CNPSo 4026$^T$, WSM 1704$^T$, WSM 1738$^T$ and WSM 4400$^T$ – previously isolated from nodules of legumes in Western Australia and South Africa. The 16S rRNA gene phylogenetic tree allocated the strains to the *Bradyrhizobium elkanii* supergroup. The multilocus sequence analysis (MLSA) with partial sequences of six housekeeping genes – *atpD*, *dnaK*, *glnII*, *gyrB*, *recA* and *rpoB* – did not cluster the strains under study as conspecific to any described *Bradyrhizobium* species. Average nucleotide identity and digital DNA–DNA hybridization values were calculated for the four strains of this study and the closest species according to the MLSA phylogeny with the highest values being 95.46 and 62.20%, respectively; therefore, both being lower than the species delineation cut-off values. The *nodC* and *nifH* phylogenies included strains WSM 1738$^T$ and WSM 4400$^T$ in the symbiovars retamae and vignae respectively, and also allowed the definition of three new symbiovars, sv. cenepequi, sv. glycinis, and sv. cajani. Analysis of morphophysiological characterization reinforced the identification of four novel proposed *Bradyrhizobium* species that are accordingly named as follows: *Bradyrhizobium cenepequi* sp. nov. (CNPSo 4026$^T$=WSM 4798$^T$=LMG 31653$^T$), isolated from *Vigna unguiculata*; *Bradyrhizobium semiaridum* sp. nov. (WSM 1704$^T$=CNPSo 4028$^T$=LMG 31654$^T$), isolated from *Tephrosia gardneri*; *Bradyrhizobium hereditatis* sp. nov. (WSM 1738$^T$=CNPSo 4025$^T$=LMG 31652$^T$), isolated from *Indigofera* sp.; and *Bradyrhizobium australafricanum* sp. nov. (WSM 4400$^T$=CNPSo 4015$^T$=LMG 31648$^T$) isolated from *Glycine* sp.

## INTRODUCTION

Nitrogen is the nutrient most required by plants and is incorporated into a variety of molecules essential for plant growth, especially DNA, RNA and proteins [1]. The main natural input of N into the biosphere occurs via biological nitrogen fixation performed by prokaryotic organisms [2]. A special group of bacteria, collectively called rhizobia, is able to fix atmospheric nitrogen ($N_2$) in symbiosis with species of the Fabaceae (=Leguminosae) in specialized structures called nodules, mostly formed on roots and, occasionally, on stems [1]. The symbiosis between rhizobia and legumes is reliant upon various genes; the rhizobial *nif* and *fix* genes are key to the synthesis and regulation of nitrogenase, the enzyme responsible for the reduction of $N_2$, whereas

**Author affiliations:** [1]Embrapa Soja, C.P. 231, 86001-970, Londrina, Paraná, Brazil; [2]Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, SBN, Quadra 2, Bloco L, Lote 06, Edifício Capes, 70.040-020, Brasília, Distrito Federal, Brazil; [3]Department of Microbiology, Universidade Estadual de Londrina, C.P. 10011, 86057-970, Londrina, Paraná, Brazil; [4]Centre for Rhizobium Studies (CRS), Murdoch University 90 South St. Murdoch, WA, Australia.

***Correspondence:** Mariangela Hungria, mariangela.hungria@embrapa.br

**Keywords:** *Bradyrhizobium*; MLSA; genome of prokaryotes; ANI; dDDH; symbiovars.

**Abbreviations:** ANI, average nucleotide identity; dDDH, digital DNA–DNA hybridization; HGT, horizontal gene transfer; LB, Luria–Bertani; ML, maximum-likelihood; MLSA, multilocus sequence analysis; NI, nucleotide identity; YMA, yeast–mannitol agar.

Genome and 16S rRNA accession numbers of *B. cenepequi* CNPSo 4026$^T$ (JAGKJI000000000 and MK676055), *B. semiaridum* WSM 1704$^T$ (JAGKJJ000000000 and MK676057); *B. hereditatis* WSM 1738$^T$ (JAGKJK000000000 and MK676061); *B. australafricanum* WSM 4400$^T$ (JAGKJL000000000 and MK676054).

†These authors contributed equally to this work

Ten supplementary figures and three supplementary tables are available with the online version of this article.

the nodulation process depends on the expression of a group of genes referred to as *nod*, *noe* and *nol* genes [3]. The core *nod* genes are generally located in the *nodABC* operon, which is responsible for the synthesis of the main structure of the Nod factor, lipochitooligosaccharide molecules responsible for bacterial infection and nodule organogenesis [4, 5]; *nodD* is a regulatory gene located upstream of the *nod* gene operon responsible for starting Nod factor synthesis [3, 5]. The remaining proteins coded by *nod*, *noe* and *nol* genes are involved in the modification of the Nod factor structure in order to ensure host specificity [4, 5].

*Bradyrhizobium* is one of the largest and most intriguing genera of rhizobia and can be isolated from nodules of a broad host-range of legumes, including both ancient and more recently evolved species from the Papilionoideae and Caesalpinioideae subfamilies [6, 7]. Many *Bradyrhizobium* strains associate with crops of great agronomic importance, such as soybean (*Glycine max* (L.) Merr.) [6]. In addition to those known to have a symbiotic lifestyle, some non-symbiotic *Bradyrhizobium* ecotypes are found living freely in soils [8], while others have the ability to promote plant growth when in endophytic associations [9, 10]. The genus also has strains which are highly effective nodulators of the non-legume *Parasponia* [11], and some strains have both the ability to photosynthesize and to nodulate legumes without the Nod factor mechanism [12, 13].

In view of the broad host-range of the genus, several symbiovars have been described within the genus *Bradyrhizobium*. The term symbiovar (sv.) was coined by Rogel *et al.* [14] and refers to lineages of different or the same species that are able to establish symbiosis with distinct leguminous species, these entities are differentiated on host range and symbiotic phylogenies. Currently, there are 12 symbiovars described for *Bradyrhizobium*, based mainly on the phylogeny of the *nodC* gene, chosen due to its key role in the synthesis of the Nod factor: sv. glycinearum, sv. genistearum, sv. retamae, sv. vignae, sv. sierranevadense, sv. centrosemae, sv. phaseolarum, sv. tropici, sv. pachyrhizi, sv. sojae, sv. lupini and sv. septentrionale [15–22].

Despite the increasing number of studies reporting great genetic diversity in *Bradyrhizobium* from a great variety of ecosystems [e.g. 6, 15–22], genomic and statistical studies suggest that a far higher number of genotypes estimated at 800 species still remain to be described [23, 24]. Here we delineate and describe four novel *Bradyrhizobium* species based on a polyphasic approach, as well as three novel symbiovars based on *nodC* and *nifH* phylogenies, increasing the current knowledge of *Bradyrhizobium* diversity and the evolutionary history of the rhizobia–legume symbiosis.

## ISOLATION AND ECOLOGY

The four novel species described in this study have recently been characterized by Helene *et al.* [25] and emphasize the high diversity of *Bradyrhizobium* strains isolated from indigenous legumes of Western Australia and South Africa. Strain CNPSo 4026$^T$ was isolated from root nodules of *Vigna unguiculata* used as trapping host in Western Australian soils. Strain WSM 4400$^T$ was isolated from nodules of *Glycine* sp., a legume used for cattle forage, grown in Stutterheim, Eastern Cape, in the Amathole District, South Africa. WSM 4400$^T$ is deposited at the WSM Culture Collection and was chosen for the study due to a slower growing property *in vitro*. Strains WSM 1704$^T$ and WSM 1738$^T$ were isolated from *Tephrosia gardneri* and *Indigofera* sp., respectively, in Western Australia by Yates *et al.* [26]. Details of the origin of these strains, as well as the type strains used in this study are shown in Table 1.

All strains are deposited at the 'Diazotrophic and Plant Growth Promoting Bacteria Culture Collection of Embrapa Soja' (WFCC Collection no. 1213, WDCM Collection no. 1054), in Londrina, State of Parana, Brazil, as well as at the Western Australian Soil

**Table 1.** Strains used in this study

| Species/strain name | Other nomenclatures | Original host species | Geographical origin | Reference |
|---|---|---|---|---|
| ***B. cenepequi* CNPSo 4026$^T$** | WSM 4798$^T$=LMG 31653$^T$ | *Vigna unguiculata* | Salmon Gums, WA | Helene *et al.* [25] |
| ***B. semiaridum* WSM 1704$^T$** | CNPSo 4028$^T$=LMG 31654$^T$ | *Tephrosia gardneri* | Carnarvon, WA | Yates *et al.* [26] |
| ***B. hereditatis* WSM 1738$^T$** | CNPSo 4025$^T$=LMG 31652$^T$ | *Indigofera* sp. | Cape Range National Park, WA | Yates *et al.* [26] |
| ***B. australafricanum* WSM 4400$^T$** | CNPSo 4015$^T$=LMG 31648$^T$ | *Glycine* sp. | Amathole District, South Africa | Helene *et al.* [25] |
| *B. archetypum* WSM 1744$^T$ | CNPSo 4013$^T$=LMG 31646$^T$ | *Muelleranthus trifoliolatus* | Wooramel, WA | Helene *et al.* [38] |
| *B. brasilense* UFLA03-321$^T$ | CBAS645$^T$=LMG 29353$^T$ | *Vigna unguiculata* | Minas Gerais, Brazil | Costa *et al.* [66] |
| *B. elkanii* USDA 76$^T$ | CNPSo 62$^T$=LMG 6134$^T$ | *Glycine max* | USA | Kuykendall *et al.* [64] |
| *B. ivorense* CI-1B$^T$ | CCOS 1862$^T$=CCMM$^T$=B1296$^T$ | *Cajanus cajan* | Ivory Coast | Fossou *et al.* [59] |
| *B. pachyrhizi* PAC48$^T$ | CECT 7396$^T$=LMG 24246$^T$ | *Pachyrhizus erosus* | Costa Rica | Ramírez-Bahena *et al.* [67] |

Microbiology Gene Bank (WSM Culture Collection), at the Belgian Coordinated Collections of Microorganisms (BCCM/LMG), and also at the Culture Collection of the Department of Microbiology of the University of Seville, Spain.

The strains were maintained on modified-yeast extract–mannitol agar (YMA) medium [27] at 4 °C in a cold room for short-term preservation and were lyophilized and stored in modified-yeast extract–mannitol (YM) broth with 30% (v/v) glycerol at −80 °C and −150 °C by cryopreservation for long-term storage, as previously described [28].

## PHYLOGENY

The 16S rRNA gene, and four housekeeping genes (*dnaK*, *glnII*, *gyrB* and *recA*) were previously amplified and sequenced [25]. The accession numbers of the 16S rRNA sequences of the strains are: CNPSo 4026$^T$ (MK676055), WSM 1704$^T$ (MK676057), WSM 1738$^T$ (MK676061) and WSM 4400$^T$ (MK676054). Complete sequences of the housekeeping genes *atpD*, *dnaK*, *glnII*, *gyrB*, *recA* and *rpoB* were also retrieved from the genomes of strains CNPSo 4026$^T$, WSM 1704$^T$, WSM 1738$^T$, WSM 4400$^T$ and other *Bradyrhizobium* type strains with available genomes in the GenBank database of the National Center for Biotechnology Information (NCBI: www.ncbi.nlm.nih.gov). The partial and complete housekeeping gene datasets were used to reconstruct phylogenetic trees of single and concatenated genes.

The symbiotic gene, *nodC*, was amplified and sequenced as previously described [25]. Sequences of *nodC* and *nifH* were also retrieved from the available genomes of the strains used in this study. The symbiovar definition was based upon the phylogeny of the *nodC* and *nifH* symbiotic genes.

The accession numbers of all sequences used in this study are listed in Table S1 and, whenever possible, in parentheses on the phylogenetic trees. MEGA software version 7 [29] was used to obtain the multiple sequence alignments using the MUSCLE algorithm [30], and to reconstruct the maximum-likelihood (ML) phylogenies based on the evolutionary distance models inferred by the lowest Bayesian information criterion scores [31], with 1000 times bootstrap re-sampling [32, 33]. The evolutionary model used for each phylogeny is listed in the corresponding figure caption. In the multilocus sequence analysis (MLSA), the concatenation of the complete and partial housekeeping gene sequences was performed manually. Although nucleotide identity (NI) is a mathematic and not a phylogenetic parameter, the NI values of specific genes can be used for species delimitation. BioEdit version 7.0.4.1 [34] was used to calculate NI, and the values are indicated in the manuscript and in Table 2, Table S2 as well as Table S3. Since we used the same single gene alignment to reconstruct the phylogenies and matrix of identity, the results were discussed together.

Based on previous molecular evidence and on a robust phylogenetic analysis of the ribosomal region of the genus *Bradyrhizobium*, Menna *et al*. [35] highlighted that 16S rRNA analyses were able to divide the genus into two well-supported groups: the *Bradyrhizobium japonicum* and the *Bradyrhizobium elkanii* supergroups. The four strains from our study were located in the *B. elkanii* supergroup in the 16S rRNA phylogeny (1314 bp) (Fig. 1). Strain CNPSo 4026$^T$ clustered with *B. neotropicale* BR 10247$^T$ and *B. centrolobii* BR 10245$^T$ with a 99% bootstrap support and 99.6 and 99% NI, respectively. Strain WSM 1738$^T$ clustered with *B. archetypum* WSM 1744$^T$ and *B. retamae* Ro19$^T$, sharing 99.8 and 99.7% NI respectively; nevertheless, the cluster had low bootstrap. Strains WSM 1704$^T$ and WSM 4400$^T$ clustered with eight other species with 75% bootstrap support, *B. brasilense* UFLA03-321$^T$, *B. pachyrhizi* PAC48$^T$ and *B. ripae* WR4$^T$, all three with 99.9–100% NI, and with *B. elkanii* USDA 76$^T$ (99.8–99.9%), *B. macuxiense* BR 10303$^T$ (99.8–99.9%), *B. ivorense* Cl-1B$^T$ (99.7–99.8%), *B. tropiciagri* CNPSo 1112$^T$ (99.2–99.3%), and *B. ferriligni* CCBAU 51502$^T$ (97.9–98%) (Table 2). High NI values were also found among the strains CNPSo 4026$^T$, WSM 1704$^T$, WSM 1738$^T$ and WSM 4400$^T$, ranging from 98.4 ro 99.9% (Table 2). It is worth mentioning that the majority of the NI values found in the 16S rRNA analysis are above the 98.65% cut-off for species delineation defined by Kim *et al*. [36], confirming that the 16S rRNA gene is very conserved within the genus *Bradyrhizobium*, allowing only limited resolution for species delineation.

Taking into account the high conservation of the 16S rRNA sequences, phylogenetic trees were reconstructed with single and concatenated housekeeping datasets, as they provide more information due to these genes possessing a faster evolutionary rate [37]. The phylogenies of single housekeeping genes *atpD* (398 bp), *dnaK* (221 bp), *glnII* (504 bp), *gyrB* (553 bp), *recA* (360 bp) and *rpoB* (439 bp) were able to differentiate the strains from all described *Bradyrhizobium* species (Figs S1–S6, available in the online version of this article). In general, the phylogeny obtained of each housekeeping gene was congruent to each other (Figs S1–S6). In order to avoid possible discrepancies caused by events of recombination at a single locus, an MLSA was performed with the partial sequences of the housekeeping genes *atpD +dnaK+glnII +gyrB+recA +rpoB* (2475 bp). Based on the MLSA tree (Fig. 2) and on the NI matrix (Table 2), strain CNPSo 4026$^T$ occupied a basal position with 89% bootstrap support; strain WSM 1704$^T$ grouped with *B. ivorense* CI-1B$^T$ with 99% bootstrap support and 95.2% NI; strain WSM 1738$^T$ was clustered with *B. archetypum* WSM 1744$^T$ and *B. namibiense* 5-10$^T$ with 81% bootstrap support and sharing 93.2 and 93.9% NI, respectively; finally, strain WSM 4400$^T$ remained in the same group of the species *B. brasilense* UFLA03-321$^T$, *B. pachyrhizi* PAC48$^T$ and *B. elkanii* USDA 76$^T$ with 99% bootstrap support, and 98.6, 97.9 and 97.7% NI, respectively (Fig. 2, Table 2). An MLSA based on six genes belonging to the core genome was also performed using the complete sequences of the housekeeping genes *atpD +dnaK+glnII +gyrB+rec A +rpoB* (11,676 bp) (Fig. S7) including the *Bradyrhizobium* type strains with genomes available. The evolutionary pattern was maintained for the strains of this study; however, strains WSM 1738$^T$ and CNPSo 4026$^T$, presented a basal position with high

**Table 2.** Nucleotide Identity (NI) among new lineages of *Bradyrhizobium* and closely related species, based on the sequences of single and concatenated housekeeping genes (*atpD*, *dnaK*, *glnII*, *gyrB*, *recA* and *rpoB*) and 16S rRNA

| | Nucleotide identity | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Strains** | **16S rRNA (1314 bp)** | **MLSA (2475 bp)** | **MLSA (11676 bp)** | ***atpD* (398 bp)** | ***dnaK* (221 bp)** | ***glnII* (504 bp)** | ***gyrB* (553 bp)** | ***recA* (360 bp)** | ***rpoB* (439 bp)** |
| | | | | | ***Bradyrhizobium cenepequi* CNPSo 4026$^T$** | | | | |
| *B. neotropicale* BR 10247$^T$ | 99.6 | 89 | 89.9 | 90.8 | 87.3 | 88.6 | 88 | 91.6 | 87.5 |
| *B. centrolobii* BR 10245$^T$ | 99 | 89.6 | 90.5 | 92.4 | 90.4 | 89.6 | 88.2 | 90.8 | 87.5 |
| *B. elkanii* USDA 76$^T$ | 98.7 | 89.8 | 91.2 | 89.8 | 88.2 | 88.4 | 89.8 | 91.9 | 90.3 |
| *B. ivorense* CI-1B$^T$ | 98.6 | 90.4 | 91.2 | 90.6 | 89.5 | 88.4 | 89.8 | 91.1 | 93.1 |
| *B. semiaridum* WSM 1704$^T$ | 98.8 | 89.7 | 91 | 91.1 | 89.1 | 88.2 | 88.9 | 89.4 | 91.9 |
| *B. hereditatis* WSM 1738$^T$ | 98.4 | 89.9 | 90.2 | 93.1 | 91.4 | 87.8 | 87.6 | 88.6 | 92.6 |
| *B. australafricanum* WSM 4400$^T$ | 98.7 | 89.8 | 91 | 90.3 | 88.6 | 87.6 | 90 | 92.2 | 90.3 |
| | | | | | ***Bradyrhizobium semiaridum* WSM 1704$^T$** | | | | |
| *B. brasilense* UFLA03-321$^T$ | 99.9 | 94.6 | 95 | 93.6 | 94.5 | 95.6 | 94.1 | 93.8 | 95.6 |
| *B. pachyrhizi* PAC48$^T$ | 99.9 | 94.9 | 95.1 | 94.6 | 94.5 | 94.8 | 94.1 | 95.8 | 95.6 |
| *B. ripae* WR4$^T$ | 99.9 | – | – | – | 95.4 | 94.8 | 93.3 | 93.6 | 96.5 |
| *B. elkanii* USDA 76$^T$ | 99.8 | 94.4 | 95 | 94.4 | 94.5 | 95.4 | 94.2 | 91.9 | 95.6 |
| *B. macuxiense* BR 10303$^T$ | 99.8 | 94 | 92.2 | 95.1 | 94.1 | 93.6 | 92.4 | 92.7 | 96.5 |
| *B. ivorense* CI-1B$^T$ | 99.7 | 95.2 | 95.4 | 95.1 | 95.4 | 92.8 | 96.8 | 94.1 | 97 |
| *B. tropiciagri* CNPSo 1112$^T$ | 99.2 | 94 | 94.8 | 94.4 | 94.5 | 93.8 | 93.5 | 92.7 | 95.4 |
| *B. ferriligni* CCBAU 51502$^T$ | 97.9 | – | – | – | 95 | 94.4 | 90.4 | 93 | 95.4 |
| *B. cenepequi* CNPSo 4026$^T$ | 98.8 | 89.7 | 91 | 91.1 | 89.1 | 88.2 | 88.9 | 89.4 | 91.9 |
| *B. hereditatis* WSM 1738$^T$ | 98.4 | 89.9 | 90.9 | 95.4 | 91.4 | 88.6 | 89.1 | 86.6 | 89.1 |
| *B. australafricanum* WSM 4400$^T$ | 99.9 | 94.4 | 95 | 94.4 | 94.5 | 95.4 | 94.1 | 92.7 | 95.1 |
| | | | | | ***Bradyrhizobium hereditatis* WSM 1738$^T$** | | | | |
| *B. archetypum* WSM 1744$^T$ | 99.8 | 93.2 | 93.3 | 95.1 | 90 | 93.8 | 93.3 | 91.1 | 91 |
| *B. retamae* Ro19$^T$ | 99.7 | 90.6 | 92.6 | 93.6 | 92.3 | 89.6 | 90.9 | 91.6 | 86.8 |
| *Bradyrhizobium namibiense* 5-10$^T$ | 99.4 | 93.9 | 93.3 | 95.1 | 90 | 90 | 92.2 | 91.1 | 90.5 |

Continued

**Table 2.** Continued

| Strains | Nucleotide identity | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 16S rRNA (1314 bp) | MLSA (2475 bp) | MLSA (11676 bp) | *atpD* (398 bp) | *dnaK* (221 bp) | *glnII* (504 bp) | *gyrB* (553 bp) | *recA* (360 bp) | *rpoB* (439 bp) |
| *B. cenepequi* CNPSo 4026$^T$ | 98.4 | 89.9 | 90.2 | 93.1 | 91.4 | 87.8 | 87.6 | 88.6 | 92.6 |
| *B. semiaridum* WSM 1704$^T$ | 99.1 | 89.9 | 90.9 | 95.4 | 91.4 | 88.6 | 89.1 | 86.6 | 89.1 |
| *B. australafricanum* WSM 4400$^T$ | 99 | 90.7 | 91 | 93.4 | 91.8 | 90.2 | 90.2 | 89.7 | 89.6 |
| | ***Bradyrhizobium australafricanum* WSM 4400$^T$** | | | | | | | | |
| *B. brasilense* UFLA03-321$^T$ | 100 | 98.6 | 99 | 97.7 | 100 | 99.8 | 99 | 95.8 | 99.5 |
| *B. pachyrhizi* PAC48$^T$ | 100 | 97.9 | 97.9 | 95.6 | 99.5 | 99 | 98.3 | 95.2 | 99.5 |
| *B. ripae* WR4$^T$ | 100 | – | – | – | 97.2 | 96.4 | 95.2 | 96.1 | 97.7 |
| *B. elkanii* USDA 76$^T$ | 99.9 | 97.7 | 98 | 97.4 | 99.5 | 96.4 | 98.5 | 96.1 | 99 |
| *B. macuxiense* BR 10303$^T$ | 99.9 | 94.5 | 92.2 | 96.4 | 94.1 | 95 | 94.6 | 93 | 96.5 |
| *B. ivorense* Cl-1B$^T$ | 99.8 | 94.2 | 94.8 | 96.2 | 95 | 95 | 94.6 | 93 | 95.1 |
| *B. tropiciagri* CNPSo 1112$^T$ | 99.3 | 95.8 | 96.6 | 95.6 | 96.8 | 96.4 | 96.3 | 94.7 | 97.9 |
| *B. ferriligni* CCBAU 51502$^T$ | 98 | – | – | – | 99 | 98.2 | 93.5 | 99.1 | 99.3 |
| *B. cenepequi* CNPSo 4026$^T$ | 98.7 | 89.8 | 91 | 90.3 | 88.6 | 87.6 | 90 | 92.2 | 90.3 |
| *B. semiaridum* WSM 1704$^T$ | 99.9 | 94.4 | 95 | 94.4 | 94.5 | 95.4 | 94.1 | 92.7 | 95.1 |
| *B. hereditatis* WSM 1738$^T$ | 99 | 90.7 | 91 | 93.4 | 91.8 | 90.2 | 90.2 | 89.7 | 89.6 |

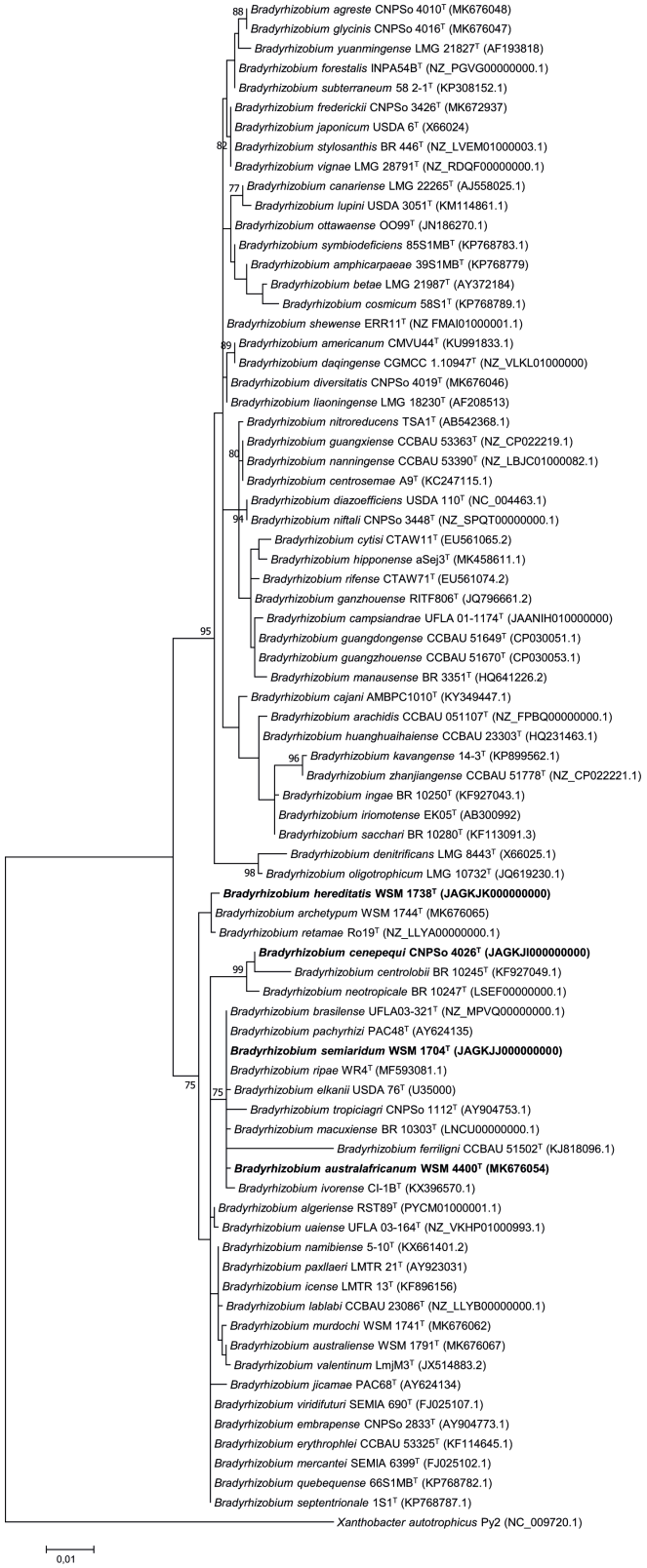


**Fig. 1.** Maximum-likelihood phylogeny based on the 16S rRNA gene alignment (1,314 bp), using the T92: Tamura three-parameter+G+I model in MEGA version 7. Accession numbers are indicated in parentheses and in Table S1. The novel species are shown in bold. Bootstrap values>70% are indicated at the nodes. *Xanthobacter autotrophicus* Py2 was used as outgroup. Bar indicates one substitution per 100 nucleotide positions.

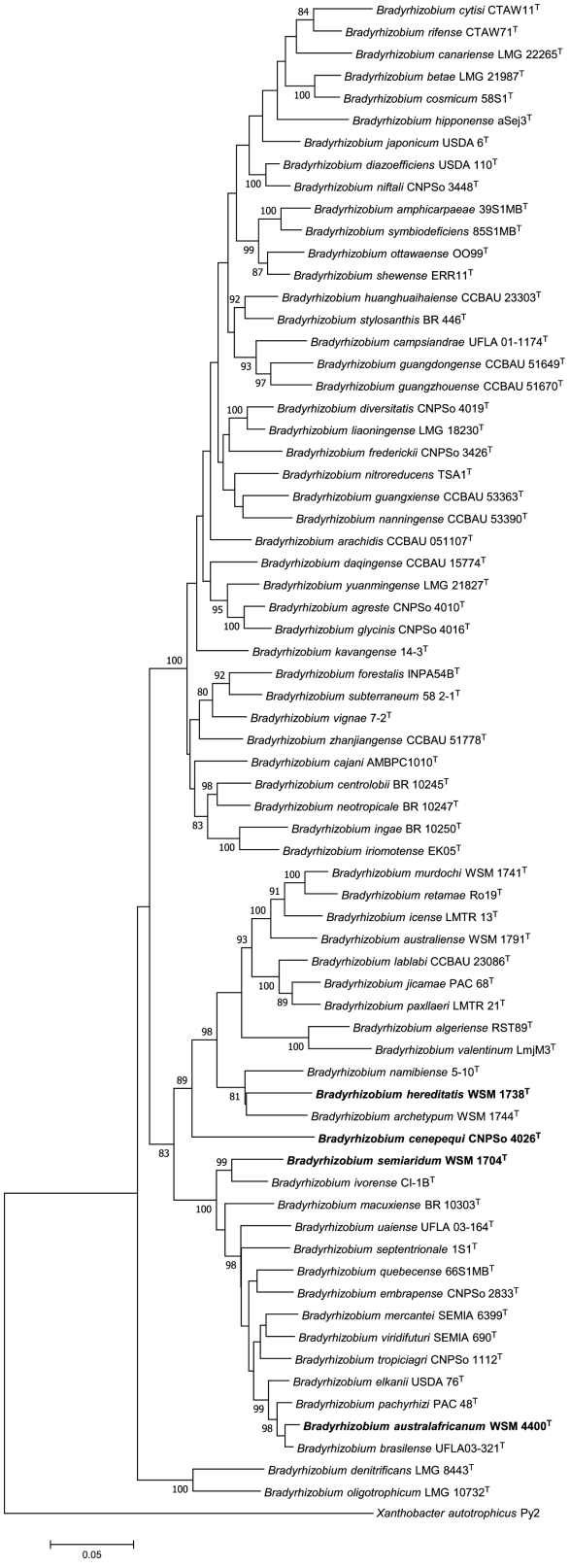


**Fig. 2.** Maximum-likelihood phylogeny based on concatenated alignment of the partial sequences of *atpD +dnaK+glnII +gyrB+recA +rpoB* genes (2475 bp), using the GTR: general time reversible +G+I model in MEGA version 7. Accession numbers are indicated in Table S1. The novel species are shown in bold. Bootstrap values>70% are indicated at the nodes. *Xanthobacter autotrophicus* Py2 was used as outgroup. Bar indicates five substitutions per 100 nucleotide positions.

bootstrap support to a cluster including *B. jicamae* PAC 68$^T$, *B. paxllaeri* LMTR 21$^T$, *B. lablabi* CCBAU 23086$^T$, *B. valentinum* LmjM3$^T$, *B. icense* LMTR 13$^T$, *B. retamae* Ro19$^T$ and *B. murdochi* WSM 1741$^T$, which were recently described as belonging to the *B. jicamae* supergroup in a phylogenomic study of *Bradyrhizobium* [23]. Since the MLSA using the complete sequences is a larger dataset, the NI values and bootstrap support were slightly higher for the analyses on this dataset versus those based on the alignment with the partial sequences (Table 2), as also observed in other studies [38, 39].

Based on the phylogeny of concatenated sequences of *recA*, *atpD*, *glnII*, *dnaK* and *gyrB*, Durán *et al.* [40] suggested the cut-off of 97% for species delineation in the genus *Bradyrhizobium*. Even though the NI values of WSM 4400$^T$ with *B. brasilense* UFLA03-321$^T$, *B. pachyrhizi* PAC48$^T$ and *B. elkanii* USDA 76$^T$ were above 97% in the MLSA with partial sequences of six housekeeping genes, that included the *rpoB* gene, strains WSM 4400$^T$, WSM 1738$^T$, WSM 1704$^T$ and CNPSo 4026$^T$ were clearly separated from all described *Bradyrhizobium* species, indicating that they are novel lineages.

The analysis of the symbiotic genes may reveal useful information about the evolutionary history of symbiosis in rhizobia [14, 41]. Here two genes, *nodC*, which encodes the main chito-oligosaccharide component of the Nod factor backbone, and *nifH*, encoding the iron subunit of the nitrogenase enzyme [3], were used to infer the diversity of symbiotic genes. Strain WSM 1704$^T$ was not included in the symbiotic analysis, as we were unable to get a successful amplification from this strain with the *nodC* primer used in our study, as well as unable to find both genes in its genome. The NI of *nodC* and *nifH* genes of strains from this study and of close strains are shown in Tables S2 and S3.

The three strains from this study that had an identifiable *nodC* were positioned in three different groups in the *nodC* tree (335 bp) (Fig. 3). Strain CNPSo 4026$^T$, isolated from *Vigna unguiculata*, occupied a position with 88% bootstrap support and the NI values were equal to or less than 91.6% with all strains used in this analysis. Strain WSM 1738$^T$, isolated from nodules of *Indigofera* sp., *B. lablab* CCBAU 23086$^T$ isolated from *Lablab purpureus* [42], *B. murdochii* WSM 1741$^T$ from *Rhynchosia minima* [38] and *B. paxllaeri* LMTR 21$^T$ isolated from *Phaseolus lunatus* [40] were grouped with 98% bootstrap support and shared 98.5, 98.5 and 97.6% NI, respectively (Table S2) inside the sv. retamae, which originally included strains isolated mainly from *Retama* species in Africa [16] and today allocates 11 strains. It is interesting to emphasize that all strains of sv. retamae including WSM 1738$^T$ are unable to nodulate soybean [38, 40, 42–44]. Strain WSM 4400$^T$, originally isolated from nodules of *Glycine* sp. in South Africa, grouped with 99% bootstrap support with *B. pachyrhizi* BR 3262 and *Bradyrhizobium* sp. VULI21 and presented a *nodC* sequence closely related to *Bradyrhizobium* strains isolated from *V. unguiculata* in Spain (VUPME10), Africa (STM3062), Greece (VULI11, VULI21 and VUCR24), and Brazil (BR 3262), composing the sv. vignae [17, 45, 46] with 100% bootstrap support. Even though WSM 4400$^T$ is the first strain isolated from *Glycine* sp. inside the sv. vignae, the nodulation ability of this strain in *V. unguiculata* was confirmed in this study (data not shown). The species *V. unguiculata*, known as cowpea, is indigenous to Africa and represents an important nutritional source around the world; the occurrence of the African strain WSM 4400$^T$ inside the sv. vignae corroborates with the hypothesis that Africa is the centre of origin of this symbiovar, from where the strains and host seeds were dispersed to other continents [17]. The NI values for WSM 4400$^T$ ranged from 95.2–99.1% among the strains of closely related species (Table S2), with the highest NI value found between WSM 4400$^T$ and *B. pachyrhizi* BR 3262, a strain successfully used in commercial inoculants for the cowpea crop in Brazil [45].

In the *nifH* phylogeny (205 bp), the strains of this study confirmed the same clustering as observed for the *nodC* genes, with a basal position of CNPSo 4026$^T$ with 83% bootstrap support, WSM 1738$^T$ in sv. retamae with 94% bootstrap support, and WSM 4400$^T$ in sv. vignae with 99% bootstrap support (Fig. S8). The topology of the *nifH* tree is slightly different from the *nodC* tree, as some *Bradyrhizobium* strains do not have available *nifH* sequences, e.g. strain VUPME10 of sv. vignae. In the *nifH* phylogeny, the other strains from sv. vignae were closer to each other than in the *nodC* phylogeny; in addition, the strains WSM 4400$^T$, BR 3262, VULI21, VUCR24 and VULI11 shared 99.5% NI (Table S2). Interestingly, strain STM3062 from Africa, which was close to strains of the sv. vignae in the *nodC* phylogeny, presented higher similarity to *B. elkanii* SEMIA 5019 and *B. elkanii* SEMIA 587 from sv. sojae in the *nifH* phylogeny (Fig. S8), sharing 100% NI, whereas the values among STM3062 and strains from sv. vignae ranged from 94.1–94.6% NI (data not shown).

Symbiotic genes, including nodulation and nitrogen-fixation genes, are commonly located in symbiotic plasmids in the genera *Rhizobium*, *Sinorhizobium* and *Paraburkholderia*, while in *Bradyrhizobium* and *Mesorhizobium* they are usually located in the chromosome, in a region called a symbiotic island or an integrative and conjugative element [5, 47–49]. Considering the findings of a large study on the nodulation traits in *Bradyrhizobium*, Menna and Hungria [13] suggested a monophyletic origin for the symbiotic island based upon *nodA*, *nodZ*, *nodY/K* and *nifH* phylogenies, whereafter it is shared among strains ether by vertical inheritance or horizontal transfer. Therefore, the congruence between *nodC* and *nifH* phylogenies found in the strains of the current study, as well as in other studies involving *Bradyrhizobium* [20, 46, 50], support the hypothesis of simultaneous evolution of these genes in the symbiotic island. However, the incongruence presented by STM3062 may indicate a horizontal gene transfer (HGT) event, as has also been demonstrated in other studies [51].

In order to get a better understanding of the evolutionary history of the strains from this study, two novel phylogenetic trees with the 16S rRNA and *glnII +recA* housekeeping genes including the available sequences of strains used for symbiovar definition (Fig S9 and Fig S10) were reconstructed and compared with the phylogenies of *nodC* and *nifH* genes. Core and symbiotic genes of

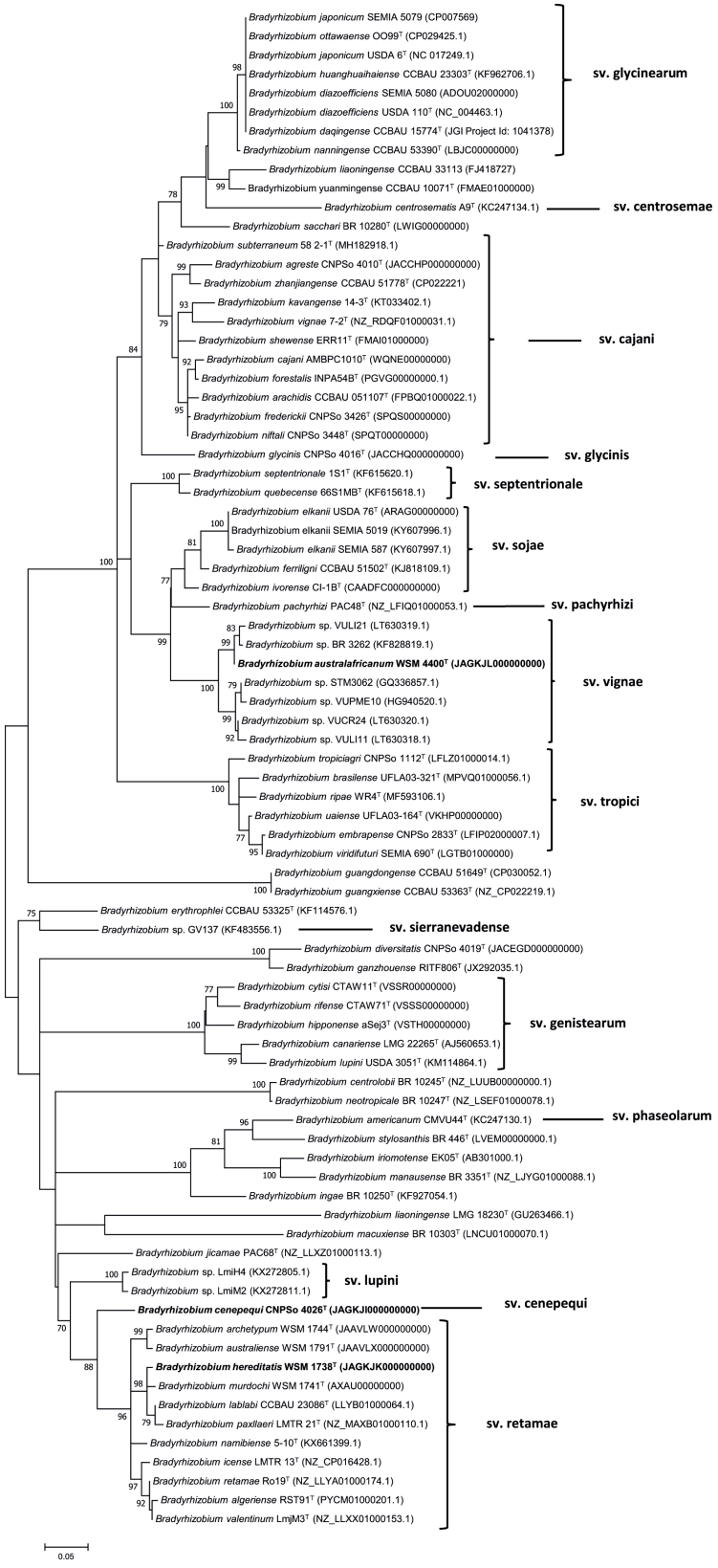


**Fig. 3.** Maximum-likelihood phylogeny based on *nodC* gene alignment (335 bp), using the T92: Tamura three-parameter+G+I model in MEGA version 7. Accession numbers are indicated in parentheses. The novel species are shown in bold. Bootstrap values >70% are indicated at the nodes. Bar indicates five substitutions per 100 nucleotide positions.

strain WSM 1738$^T$ were not congruent, with *B. archetypum* WSM 1744$^T$, *B. retamae* Ro19$^T$ and *B. namibiense* 5-10$^T$ representing the closest species in the 16S rRNA and *glnII +recA* phylogenies (Figs S9 and S10), while *B. murdochi* WSM 1741$^T$ was the closest in the symbiotic phylogenies (Fig. 3 and S8), which could indicate HGT of the symbiotic genes, a reasonably common event in rhizobia [52]. Nevertheless, strain WSM 4400$^T$ was close to *B. brasilense* UFLA03-321$^T$ and *B. pachyrhizi* PAC48$^T$ in the 16S rRNA (Fig. 1) and MLSA trees (Fig. 2), but in the novel phylogenies of core genes containing the strains used in symbiovar analysis (Figs S9 and S10), WSM 4400$^T$ was closer to the strains VULI21, VUCR24 and VULI11 from sv. vignae, congruent with *nodC* and *nifH* phylogenies (Fig. 3 and Fig S8). This pattern is generally found in *Mimosa*-nodulating *Paraburkholderia* and it seems to be related to mild HGT events [53]. Therefore, these findings reinforce that both horizontal and vertical transfer may contribute towards the evolution of this symbiosis, resulting in the great diversity of rhizobia found nowadays.

Delamuta *et al.* [20] proposed a cut-off value of approximately 92.5% in *Bradyrhizobium nodC* sequence similarity to define new symbiovars. We will suggest names for the symbiovars according to the strain occupying a central position in the cluster. Based on this cut-off, we confirm that strains WSM 1738$^T$ and WSM 4400$^T$ are included in sv. retamae and sv. vignae, respectively, and we propose the description of three novel symbiovars. More details about the symbiovars described here are shown in Table S3. Strains CNPSo 4026$^T$ described in this study and *B. glycinis* CNPSo 4016$^T$ recently described as a novel species by our research group [39] did not cluster with any *Bradyrhizobium* strain in the *nodC* phylogeny and presented NI values equal or lower than 91.6 and 92.5%, respectively; therefore, we propose two novel symbiovars named cenepequi and glycinis, respectively, named as the first and only species described so far in these symbiovars. The evolutionary history, as well as the host range of these symbiovars should be further investigated as more isolates belonging to these symbiovars become available. We also suggest a novel sv. named 'cajani' for a *nodC* lineage with 79% bootstrap support that contains *B. cajani* and another nine species (Fig. 3). The nucleotide identity of strains *B. agreste* CNPSo 4010$^T$, *B. arachidis* CCBAU 051107$^T$, *B. cajani* AMBPC1010$^T$, *B. forestalis* INPA54B$^T$, *B. frederickii* CNPSo 3426$^T$, *B. kavangense* 14-3$^T$, *B. niftali* CNPSo 3448$^T$, *B. shewense* ERR11$^T$, *B. vignae* 7-2$^T$ and *B. zhanjiangense* CCBAU 51778$^T$ ranged from 91.3 to 99.7% similarity among each other (Table S3). Even though strain *B. subterraneum* 58 2-1$^T$ was not included in the branch with 79% bootstrap support, it shared a NI from 93.7–96.4% with the other strains of the sv. cajani, and, therefore, it possibly belongs to the same symbiovar. The sv. cajani contains strains isolated from several hosts, including *Glycine clandestina*, *Arachis hypogaea*, *Cajanus cajan*, *Inga* sp., *Chamaecrista fasciculata*, *Vigna unguiculata* and *Erythrina brucei* isolated in Africa, China, USA, Australia, Brazil and the Dominican Republic (Table S3). Interestingly, all strains from sv. cajani tested for nodulation ability with soybean were unable to nodulate this legume [39, 50, 54–58].

Even though strains *B. nanningense* CCBAU 53390$^T$ and *B. ivorense* CI-1B$^T$ were not described as belonging to any symbiovar, the *nodC* phylogeny (Fig. 3) indicates that these strains belong to pre-existing symbiovars. *Bradyrhizobium nanningense* CCBAU 53390$^T$ was isolated from *A. hypogaea* in China [58] and had 98.8%similarity to strains of sv. glycinearum able to nodulate soybean [15], whereas *B. ivorense* CI-1B$^T$ isolated from *Cajanus cajan* in Africa [59] presented 93.1–95.8% similarity to strains of sv. sojae, commonly associated with *Glycine max* [20].

## GENOME FEATURES

The genomes of strains CNPSo 4026$^T$, WSM 1704$^T$, WSM 1738$^T$ and WSM 4400$^T$ were sequenced by the MiSeq platform (Illumina) at Embrapa Soja (Londrina, Brazil) using sequence libraries constructed with the Nextera XT kit (Illumina). The reads were assembled *de novo* with the A5-MiSeq pipeline version 20140604 and the genomes were annotated with Rapid Annotation using Subsystems Technology (RAST) version 2.0 [60]. The draft genomes were deposited in the GenBank database (NCBI), and received the accession numbers JAGKJI000000000 for CNPSo 4026$^T$, JAGKJJ000000000 for WSM 1704$^T$, JAGKJK000000000 for WSM 1738$^T$, and JAGKJL000000000 for WSM 4400$^T$. The final genome assemblies used the recommended statistical parameters for taxonomic purposes [61] and the detailed genomic data are shown in Table 3.

Average nucleotide identity (ANI) and digital DNA–DNA hybridization (dDDH), the state-of-the-art methods for inferring overall genome relatedness, were applied to the genomic sequences of the four strains of this study and the available genomic sequences of the closest species identified in the MLSA, *B. archetypum* WSM 1744$^T$, *B. brasilense* UFLA03-321$^T$, *B. elkanii* USDA 76$^T$, *B. ivorense* CI-1B$^T$ and *B. pachyrhizi* PAC48$^T$. The genomic comparisons were calculated using an ANI calculator [62] with

**Table 3.** Statistical parameters of final genomes assemblies of the new lineages of *Bradyrhizobium* for taxonomic purposes

| Strain | Size | No. of Contigs | N50 | Coverage | G+C content (mol%) |
|---|---|---|---|---|---|
| ***Bradyrhizobium cenepequi* CNPSo 4026$^T$** | 8472857 | 188 | 149882 | 74× | 62.3 |
| ***Bradyrhizobium semiaridum* WSM 1704$^T$** | 6712655 | 120 | 194653 | 119× | 65.1 |
| ***Bradyrhizobium hereditatis* WSM 1738$^T$** | 7871253 | 61 | 418770 | 75× | 62.0 |
| ***Bradyrhizobium australafricanum* WSM 4400$^T$** | 9684669 | 221 | 125914 | 75× | 63.1 |

default parameters and Genome-to-Genome Distance Calculator version 2.1 [63], with the recommended 'formula 2' (identities/high-scoring pairs length), and the values are indicated in Table 4. The genome of CNPSo 4026$^T$ revealed low relatedness with the genomes of the closer *Bradyrhizobium* species, with values equal or lower than 82.65% of ANI and 25.50% of dDDH. WSM 1704$^T$ showed higher genomic similarity (88.27% of ANI and 35.40% of dDDH) to *B. ivorense* CI-1B$^T$ isolated from *Cajanus cajan* in West Africa [59]. Strain WSM 1738$^T$ shared 87.59% of ANI and 34.30% of dDDH with *B. archetypum* WSM 1744$^T$. Finally, WSM 4400$^T$ shared 94.88% of ANI and 58.80% of dDDH with *B. elkanii* USDA 76$^T$, a soybean nodulating strain used as inoculant [64, 65], 89.96% of ANI and 36.70% of dDDH with *B. brasilense* UFLA03-321$^T$, isolated from *V. unguiculata* in Brazilian soils [66], and 95.46% of ANI and 62.20% of dDDH with *B. pachyrhizi* PAC48$^T$, isolated from *Pachyrhizus erosus* in Costa Rica [67]. Among the strains described in this study, the values ranged from 81.95 to 87.18% for ANI and from 24.40 to 33.0% for dDDH (Table 4). Considering that the four strains of this study showed values below the cut-off values for species delineation of 95–96% for ANI and 70% for dDDH [61, 63], the genomic analyses confirmed that CNPSo 4026$^T$, WSM 1704$^T$ and WSM 1738$^T$ from Western Australia and WSM 4400$^T$ from South Africa represent novel *Bradyrhizobium* species.

The automatic annotation from RAST showed that strain CNPso 4026$^T$ possesses the nodulation genes possibly organized in a symbiotic island starting with a *nodD*, putative *fixJ*, *nodABCSUIJ*, a pseudogene of sulfotransferase and *nolNO*, while the *nodZ* was not found. *nodZ* is an important gene for Nod factor synthesis since it is related to the fucosylation of the core lipochitooligosaccharide [68] and it has been pointed out as a host-specific nodulation gene [69]. The absence of the *nodZ* gene in strain CNPSo 4026$^T$ may indicate that the strain uses different strategies to modify the Nod factor and establish the nodulation, which should be carefully investigated in further studies. The nodulation region of WSM 1738$^T$ presented two copies of *nodD*, putative *fixJ*, followed by *nodABCSUIJ*, *nolNO* and a putative *nodZ*. StrainWSM 4400$^T$ showed *nodD2D1ABCSUIJ*, *nolNO* and a putative *nodZ*. As commented before, we did not find *nod* genes in the genome of WSM 1704$^T$, which can be related to the smaller size of the genome of this strain.

The SEED platform [60] was used to estimate the G+C genome content, defined as 62.3, 65.1, 62.0 and 63.1 mol% for CNPSo 4026$^T$, WSM 1704$^T$, WSM 1738$^T$ and WSM 4400$^T$, respectively.

## PHENOTYPIC CHARACTERIZATION

Morphophysiological analyses were carried out and compared among strains CNPSo 4026$^T$, WSM 1704$^T$, WSM 1738$^T$, WSM 4400$^T$, *B. archetypum* WSM 1744$^T$ and *B. elkanii* USDA 76$^T$. The tests were performed using modified-YMA medium at 28 °C [27], and when the strains were cultivated in different conditions this is described. Congo red was used in the modified-YMA medium to verify colony morphology after 7–10 days of growth. The physiological features were given according to adaptations from Hungria *et al.* [70] by growth in medium containing bromothymol blue as an indicator for, indicating acid, neutral or alkaline reaction; with 1% (w/v) NaCl; at 37 °C, at pH 4.0 and pH 8.0; growth on Luria–Bertani (LB) medium. Urease activity was evaluated using 2% (w/v) urea and the pH indicator phenol red. The API 50CH kit platform (bioMérieux) was used to determine carbohydrate metabolism, with bacteria grown in modified-YM-minus-mannitol with bromothymol blue. Tolerance of antibiotics was analysed by the disc-diffusion technique [71] using ampicillin (10 µg), bacitracin (10 U), cefuroxime (30 µg), chloramphenicol (30 µg), nalidixic acid (30 µg), neomycin (30 µg), penicillin (10 U), streptomycin (10 µg), tetracycline (30 µg) and erythromycin (15 µg). All tests were conducted in duplicate and the differential phenotypical features among the strains from this study and closest species are described in Table 5.

The most contrasting morphophysiological features observed were that strains WSM 1704$^T$ and WSM 1738$^T$ were able to grow on modified-YMA at 37 °C in 4 and 10 days, respectively, indicating tolerance to high temperature, especially WSM 1704$^T$. Another interesting feature is that strain CNPSo 4026$^T$ was able to grow weakly on modified-YMA with 1% (w/v) NaCl, a trait not commonly found in *Bradyrhizobium*. The strains of this study presented the ability to grow well on modified-YMA with pH 4.0 and 8.0, except for strain WSM 1738$^T$, which grew weakly at pH 4. Concerning carbohydrate metabolism, strain CNPSo 4026$^T$ was unable to use glycerol and D-mannitol, whereas WSM 1738$^T$ was only unable to use D-mannitol, while other strains were able to weakly use both C sources. Even though glycerol and D-mannitol are commonly used as C sources in YMA culture medium, it is worth mentioning that the growth conditions are different between the API 50CH platform and agar culture medium in Petri plates; therefore, it is common to find some incongruences in patterns of C-source utilization, and this also raises doubts about the usefulness of using platforms such as the API system to describe physiological features.

Nodulation and nitrogen fixation abilities were evaluated 30 days after inoculation of the strains on *Glycine max* (commercial cultivar 'BRASMAX Potência RR) and *Macroptilium atropurpureum* (commonly known as 'siratro') that were grown under controlled glasshouse conditions in Leonard jars with sterilized sand, vermiculite (2:1, v/v), and N-free nutrient solution [72]. Strains CNPSo 4026$^T$, WSM 1738$^T$ and WSM 4400$^T$ formed effective nodules, presenting red or pink colour, on siratro, whereas only WSM 4400$^T$ was able to nodulate soybean, but nodules were not as effective as in siratro, verified by the pale green colour of the leaves. Previous studies have confirmed that WSM 4400$^T$ forms effective nodules in *Vigna unguiculata* (data not shown). Even though strain WSM 1704$^T$ was isolated from nodules of *T. gardneri* [26], the strain was unable to nodulate siratro and soybean. We were also unable to amplify or find the main nodulation and nitrogen-fixation genes in the genome WSM 1704$^T$. However,

**Table 4.** Average nucleotide identity (ANI) and digital DNA–DNA hybridization (dDDH) values among new lineages of *Bradyrhizobium* and closely related *Bradyrhizobium* species

| | **ANI (%)** | **dDDH (%)** | **ANI (%)** | **dDDH (%)** | **ANI (%)** | **dDDH (%)** | **ANI (%)** | **dDDH (%)** |
|---|---|---|---|---|---|---|---|---|
| **Strains** | ***Bradyrhizobium cenepequi* CNPSo 4026$^T$** | | ***Bradyrhizobium semiaridum* WSM 1704$^T$** | | ***Bradyrhizobium hereditatis* WSM 1738$^T$** | | ***Bradyrhizobium australafricanum* WSM 4400$^T$** | |
| ***B. cenepequi* CNPSo 4026$^T$ (JAGKJI000000000)** | – | – | 82.65 | 25.20 | 82.10 | 24.60 | 82.64 | 25.40 |
| ***B. semiaridum* WSM 1704$^T$ (JAGKJJ000000000)** | 82.65 | 25.20 | – | – | 81.96 | 24.40 | 87.18 | 33.00 |
| ***B. hereditatis* WSM 1738$^T$ (JAGKJK000000000)** | 82.10 | 24.60 | 81.95 | 24.40 | – | – | 81.95 | 24.50 |
| ***B. australafricanum* WSM 4400$^T$ (JAGKJL000000000)** | 82.64 | 25.40 | 87.18 | 33.00 | 81.95 | 24.50 | – | – |
| *B. archetypum* WSM 1744$^T$ (JAAVLW000000000) | 82.18 | 24.60 | 82.00 | 24.40 | 87.59 | 34.30 | 82.10 | 24.60 |
| *B. brasilense* UFLA03-321$^T$ | 82.58 | 25.40 | 87.21 | 33.10 | 82.06 | 24.60 | 89.96 | 36.70 |
| *B. elkanii* USDA 76$^T$ (ARAG01000000) | 82.63 | 25.50 | 87.18 | 33.20 | 81.99 | 24.50 | 94.88 | 58.80 |
| *B. ivorense* CI-1B$^T$ (CAADFC000000000) | 82.65 | 25.40 | 88.27 | 35.40 | 82.13 | 24.50 | 87.32 | 33.20 |
| *B. pachyrhizi* PAC 48$^T$ (LFIQ00000000) | 82.61 | 25.30 | 87.11 | 32.90 | 82.03 | 24.30 | 95.46 | 62.20 |

**Table 5.** Distinctive phenotypical properties of new lineages of *Bradyrhizobium* and closely related strains

Strains: 1, *Bradyrhizobium cenepequi* CNPSo 4026$^T$; 2, *Bradyrhizobium semiaridum* WSM 1704$^T$; 3, *Bradyrhizobium hereditatis* WSM 1738$^T$; 4, *Bradyrhizobium australafricanum* WSM 4400$^T$; 5, *Bradyrhizobium archetypum* WSM 1744$^T$; 6, *Bradyrhizobium elkanii* USDA 6$^T$. +, Positive growth; w, weak growth; –, no growth.

| Charactheristic | 1 | 2 | 3 | 4 | 5* | 6† |
|---|---|---|---|---|---|---|
| Carbon source utilization: | | | | | | |
| Glycerol | – | w | w | w | w | w |
| Erythritol | – | – | w | w | w | – |
| L-Arabinose | – | + | + | + | w | + |
| D-Ribose | w | + | + | + | w | + |
| D-Xylose | w | + | w | + | w | + |
| D-Adonitol | – | + | w | + | + | w |
| Methyl β-D-xylopyranoside | – | – | w | – | + | – |
| D-Galactose | – | w | w | + | w | + |
| D-Glucose | – | w | w | w | w | w |
| D-Fructose | – | w | w | w | w | w |
| D-Mannose | w | w | w | + | w | + |
| L-Sorbose | – | w | w | w | + | – |
| Dulcitol | – | w | + | – | w | w |
| Inositol | – | w | – | + | w | – |
| D-Mannitol | – | w | – | w | w | w |
| D-Sorbitol | – | w | + | w | w | w |
| Methyl α-D-mannopyranoside | – | – | – | w | w | – |
| Methyl α-D-glusopyranoside | – | + | – | w | w | – |
| *N*-Acetylglicosamine | – | – | + | w | w | – |
| Amygdalin | – | w | – | – | w | – |
| Arbutin | – | + | – | – | w | – |
| Aesculin ferric citrate | + | + | + | + | + | w |
| Salicin | – | + | – | – | w | – |
| Cellobiose | – | + | w | – | w | – |
| Maltose | – | – | + | + | w | – |
| Lactose | – | – | + | + | w | – |
| Melibiose | – | – | – | + | + | – |
| Trehalose | w | – | w | – | w | – |
| Inulin | – | – | w | – | w | – |
| Melezitose | – | w | – | – | w | – |
| Raffinose | – | – | – | w | w | – |
| Glycogen | + | + | – | + | + | – |
| Xylitol | – | + | w | – | + | w |
| Gentiobiose | – | w | w | + | + | – |

Continued

**Table 5.** Continued

| Charactheristic | 1 | 2 | 3 | 4 | 5* | 6† |
|---|---|---|---|---|---|---|
| Turanose | – | – | – | W | W | – |
| D-Lyxose | + | + | W | + | W | + |
| D-Tagatose | – | – | – | W | W | – |
| D-Fucose | W | + | W | + | W | + |
| L-Fucose | + | + | W | + | + | + |
| D-Arabitol | – | + | – | + | + | W |
| L-Arabitol | – | + | – | W | + | W |
| Potassium gluconate | + | + | + | + | + | – |
| Potassium 2-keto-gluconate | + | + | + | + | + | – |
| Potassium 5-keto-gluconate | + | + | + | + | + | – |
| Growth at/in: | | | | | | |
| pH 4 | + | + | W | + | W | ND |
| 37 °C | – | + | + | – | + | – |
| 1% NaCl | W | – | – | – | – | – |
| Tolerance to antibiotics (μg disc$^{-1}$): | | | | | | |
| Ampicillin (10) | – | + | + | + | + | ND |
| Neomycin (30) | + | + | + | W | + | – |
| Penicillin G (10 U) | + | + | + | + | + | ND |
| Tetracycline (30) | + | + | – | + | + | + |
| Streptomycin (10) | – | – | + | – | – | + |
| Cefuroxima (30) | – | + | – | + | + | + |

*Data obtained from Helene *et al.* [42].
†Data obtained from Helene *et al.* [37].

in the genome WSM 1704$^T$, we did find the *nfeD* gene related to nodulation efficiency and competitiveness according to the host plant, *nifU* gene involved in the mobilization of Fe-S cluster synthesis and repair and, *fixA* which is normally part of the *fixABCX* operon, and it is required for nitrogenase activity. Therefore, further studies are needed to investigate the mechanisms involving the nodulation with the original host, or the possible loss of the symbiotic ability of this strain during the evolution process.

Based on the extensive polyphasic study presented here, we propose the description of four novel *Bradyrhizobium* species, for which we suggest the following names: *Bradyrhizobium cenepequi* sp. nov. (type strain CNPSo 4026$^T$), *Bradyrhizobium semiaridum* sp. nov. (type strain WSM 1704$^T$), *Bradyrhizobium hereditatis* sp. nov. (type strain WSM 1738$^T$) and *Bradyrhizobium australafricanum* sp. nov. (type strain WSM 4400$^T$), isolated from different regions of Western Australia and South Africa. In addition, the symbiotic gene phylogenies allow the description of three new symbiovars: cenepequi, glycinis and cajani, contributing to knowledge about the evolutionary history of symbiotic relationships.

## DESCRIPTION OF *BRADYRHIZOBIUM CENEPEQUI* SP. NOV.

*Bradyrhizobium cenepequi* [ce.ne.pe'qui. N.L. gen. n. *cenepequi*, arbitrarily formed from the acronym CNPq (Conselho Nacional de Pesquisa, Brazilian National Council for Scientific and Technological Development); in honour of the 60 years this public institution that finances research in Brazil, including international projects of collaboration].

Cells are Gram-stain-negative, aerobic, and non-spore-forming. Culture on modified-YMA medium with pH 6.8–7.0 and Congo red resulted in slightly pink colonies, with less than 1 mm diameter, circular shape, translucent, and with low mucus production and gummy consistency after 7 days of growth at 28 °C. The strain shows alkaline reaction on modified-YMA with bromothymol

blue and positive urease activity. CNPSo 4026$^T$ grows well both at pH 4.0 and pH 8.0 after 7 days. The strain is unable to grow on solid LB medium and when incubated at 37 °C, and presented weak growth on modified-YMA containing 1% (w/v) NaCl. CNPSo 4026$^T$ is able to use D-arabinose, L-xylose, aesculin ferric citrate, starch, glycogen, D-lyxose, L-fucose, potassium gluconate, potassium 2-ketogluconate and potassium 5-ketogluconate; weakly uses D-ribose, D-xylose, D-mannose, L-rhamnose, trehalose and D-fucose; but is unable to use glycerol, erythritol, L-arabinose, D-adonitol, methyl β-D-xylopyranoside, D-galactose, D-glucose, D-fructose, L-sorbose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, *N*-acetylglucosamine, arbutin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, inulin, melezitose, raffinose, xylitol, gentiobiose, turanose, D-tagatose, D-arabitol and L-arabitol. The strain is tolerant to bacitracin (10 U), chloramphenicol (30 µg), erythromycin (15 µg), nalidixic acid (30 µg), neomycin (30 µg), penicillin G (10 U) and tetracycline (30 µg); it is sensitive to ampicillin (10 µg), cefuroxime (30 µg) and streptomycin (10 µg). The strain is able to form effective nitrogen-fixing nodules on *Macroptilium atropurpureum* and *Vigna unguiculata*, but does not nodulate *Glycine max*.

The type strain, CNPSo 4026$^T$ (=WSM 4798$^T$=LMG 31653$^T$), was isolated from root nodules of *Vigna unguiculata* used as trapping host in Western Australian soils. The DNA G+C content of strain CNPSo 4026$^T$ is 62.3 mol%.

## DESCRIPTION OF *BRADYRHIZOBIUM SEMIARIDUM* SP. NOV.

*Bradyrhizobium semiaridum* (se.mi.a'ri.dum. L. pref. *semi*, half; L. masc. adj. *aridus*, dry; N.L. neut. adj. semiaridum, half-dry).

Cells are Gram-stain-negative, aerobic and non-spore-forming. Culture on modified-YMA medium with pH 6.8–7.0 and Congo red resulted in white colonies, with less than 1 mm diameter, circular shape, opacity, and low mucus production and viscous consistency after 7 days of growth at 28 °C. The strain shows alkaline reaction on modified-YMA with bromothymol blue and positive urease activity. WSM 1704$^T$ grows well at pH 4.0 and pH 8.0 after 7 days, and also when incubated at 37 °C after 4 days. The strain is unable to grow on solid LB medium and on modified-YMA containing 1% (w/v) NaCl. WSM 1704$^T$ is able to use D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, methyl α-D-mannopyranoside, arbutin, aesculin ferric citrate, salicin, cellobiose, starch, glycogen, xylitol, D-lyxose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium 2-ketogluconate and potassium 5-ketogluconate; weakly uses glycerol, D-galactose, D-glucose, D-fructose, mannose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, amygdalin, melezitose and gentibiose; but is unable to use erythritol, methyl β-D-xylopyranoside, methyl α-D-mannopyranoside, *N*-acetylglicosamine, maltose, lactose, melibiose, sucrose, trehalose, inulin, raffinose, turanose and D-tagatose. The strain is tolerant to ampicillin (10 µg), bacitracin (10 U), cefuroxime (30 µg), chloramphenicol (30 µg), erythromycin (15 µg), nalidixic acid (30 µg), neomycin (30 µg), penicillin G (10 U) and tetracycline (30 µg); it is sensitive to streptomycin (10 µg). The strain was isolated from nodules of *Tephrosia gardneri*, and was not able to nodulate *Macroptilium atropurpureum* or *Glycine max*.

The type strain, WSM 1704$^T$ (=CNPSo 4028$^T$=LMG 31654$^T$), was isolated from nodules of *Tephrosia gardneri*, in Carnarvon, WA. The DNA G+C content of strain WSM 1704$^T$ is 65.1 mol%.

## DESCRIPTION OF *BRADYRHIZOBIUM HEREDITATIS* SP. NOV.

*Bradyrhizobium hereditatis* (he.re.di.ta'tis. L. gen. n. *hereditatis*, of heritage. To highlight the importance of preservation of World Heritage Parks, such as the Cape Range National Park, WA, a source of biodiversity hotspots).

Cells are Gram-stain-negative, aerobic and non-spore-forming. Culture on modified-YMA medium with pH 6.8–7.0 and Congo red resulted in slightly pink colonies, with less than 1 mm diameter, circular shape, translucent and low mucus production and gummy consistency after 7 days of growth at 28 °C. The strain shows alkaline reaction on modified-YMA with bromothymol blue and positive urease activity. WSM 1738$^T$ grows well at pH 8.0 after 7 days and when incubated at 37 °C after 10 days but grows weakly at pH 4.0. The strain is unable to grow on solid LB medium nor on modified-YMA containing 1% (w/v) NaCl. Strain WSM 1738$^T$ is able to use D-arabinose, L-arabinose, D-ribose, L-xylose, dulcitol, D-sorbitol, *N*-acetylglicosamine, aesculin ferric citrate, maltose, lactose, starch, potassium gluconate, potassium 2-ketogluconate and potassium 5-ketogluconate; it weakly uses glycerol, erythritol, D-xylose, D-adonitol, methyl β-D-xylopyranoside, D-galactose, D-glucose, D-fructose, D-mannose, L-sorbose, L-rhamnose, cellobiose, trehalose, inulin, xylitol, gentibiose, D-lyxose, D-fucose and L-fucose; it is unable to use inositol, D-mannitol, methyl α-D-mannopyranoside, methyl α-D-glusopyranoside, amygdalin, arbutin, salicin, melibiose, sucrose, melezitose, raffinose, glycogen, turanose, D-tagatose, D-arabitol and L-arabitol. The strain is tolerant to ampicillin (10 µg), bacitracin (10 U), chloramphenicol (30 µg), erythromycin (15 µg), nalidixic acid (30 µg), neomycin (30 µg), penicillin G (10 U) and streptomycin (10 µg); it is sensitive to cefuroxime (30 µg) and tetracycline (30 µg). The strain was isolated from effective nodules of *Indigofera* sp. and is able to form effective nitrogen-fixing nodules on *Macroptilium atropurpureum*, but not on *Glycine max*.

Strain WSM 1738$^T$ (=CNPSo 4025$^T$=LMG 31652$^T$) was isolated from nodules of *Indigofera* sp., in Cape Range National Park, WA. The DNA G+C content of strain WSM 1738$^T$ is 62.0 mol%.

## DESCRIPTION OF *BRADYRHIZOBIUM AUSTRALAFRICANUM* SP. NOV.

*Bradyrhizbium australafricanum* (aus.tral.a.fri.ca'num. L. masc. adj. *australis*, southern; L. masc. adj. *africanus*, African; N.L. neut. adj. *australafricanum*; of or pertaining to South Africa, the source of the strain).

Cells are Gram-stain-negative, aerobic and non-spore-forming. Culture on modified-YMA medium with pH 6.8–7.0 and Congo red resulted in slightly pink colonies, with less than 1 mm diameter, circular shape, opacity and low mucus production and gummy consistency after 7 days of growth at 28 °C. The strain shows alkaline reaction on modified-YMA with bromothymol blue and positive urease activity. WSM 4400$^T$ grows well at pH 4.0 and pH 8.0 after 7 days. The strain is unable to grow on solid LB medium, on modified-YMA containing 1% (w/v) NaCl and when incubated at 37 °C after ten days. WSM 4400$^T$ is able to use D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, D-galactose, D-mannose, inositol, aesculin ferric citrate, maltose, lactose, melibiose, starch, glycogen, gentiobiose, L-lyxose, D-fucose, L-fucose, D-arabitol, potassium gluconate, potassium 2-ketogluconate and potassium 5-ketogluconate; it weakly uses glycerol, erythritol, D-glucose, D-fructose, L-sorbose, L-rhamnose, D-mannitol, D-sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, *N*-acetylglicosamine, raffinose, turanose, D-tagatose and L-arabitol; it is unable to use methyl β-D-xylopyranoside, dulcitol, amygdalin, arbutin, salicin, cellobiose, sucrose, trehalose, inulin, melezitose and xylitol. The strain is tolerant to ampicillin (10 µg), bacitracin (10 U), cefuroxime (30 µg), chloramphenicol (30 µg), erythromycin (15 µg), nalidixic acid (30 µg), penicillin G (10 U) and tetracycline (30 µg); but is moderately sensitive to neomycin (30 µg); it is sensitive to streptomycin (10 µg). The strain was isolated from nodules of *Glycine* sp. and is able to form effective nitrogen-fixing nodules in *Macroptilium atropurpureum* and less-effective nodules in *Glycine max*.

The type strain, WSM 4400$^T$ (=CNPSo 4015$^T$=LMG 31648$^T$), isolated from nodules of *Glycine* sp. in the Amathole District, South Africa. The DNA G+C content of strain WSM 4400$^T$ is 63.1 mol%.

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

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