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Bradyrhizobium centrosemae (symbiovar centrosemae) sp. nov., *Bradyrhizobium americanum* (symbiovar phaseolarum) sp. nov. and a new symbiovar (tropici) of *Bradyrhizobium viridifuturi* establish symbiosis with *Centrosema* species native to America

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ABSTRACT

In this work we analyze through a polyphasic approach several *Bradyrhizobium* strains isolated in Venezuela from root nodules of *Centrosema* species. The analysis of the 16S rRNA gene showed that the strains belong to three clusters within genus *Bradyrhizobium* which have 100% similarity with *Bradyrhizobium daqingense* CCBAU 15774^T *Bradyrhizobium guangxiense* CCBAU 53363^T and *Bradyrhizobium viridifuturi* SEMIA 690^T. The results of *recA* and *glnII* gene analysis confirmed the identification of the strains CMVU02 and CMVU30 as *Bradyrhizobium viridifuturi* but the *nodC* gene analysis showed that they belong to a new symbiovar for which we propose the name tropici. Nevertheless, the concatenated *recA* and *glnII* gene phylogenetic analysis, DNA–DNA hybridization and phenotypic characterization showed that the strains A9^T, CMVU44^T and CMVU04 belong to two novel *Bradyrhizobium* species. The analysis of the *nodC* gene showed that these strains also represent two new symbiovars. Based on these results we propose the classification of the strain A9^T isolated from *Centrosema molle* into the novel species *Bradyrhizobium centrosemae* (sv. centrosemae) sp. nov. (type strain A9^T = LMG 29515^T = CECT 9095^T). and the classification of the strains CMVU44^T and CMVU04 isolated from *C. macrocarpum* into the novel species *Bradyrhizobium americanum* (sv. phaseolarum) sp. nov. (type strain CMVU44^T = LMG 29514^T = CECT 9096^T).

Centrosema is a leguminous genus of tribe Phaseolae whose species are widely distributed in the savanna and forests of tropical regions establishing symbiosis with strains of genus *Bradyrhizobium* [9,13,15]. In a previous work we showed that strains nodulating *Centrosema macrocarpum* and *Centrosema molle* in Venezuela constituted three different core and symbiotic phylogenetic lineages within this genus [16].

The objective of this work was to investigate the taxonomic status of these *Centrosema* nodulating strains through a polyphasic approach. The genetic and phenotypic characteristics support the classification of the strains CMVU02 and CMVU30 into the species *Bradyrhizobium viridifuturi* within a new symbiovar for which we propose the name tropici. The strains CMVU44^T and CMVU04 should be classified as a new species and symbiovar for which we propose the name *Bradyrhizobium americanum* sp. nov. symbiovar phaseolarum, and the strain A9^T as a novel species and symbiovar for which we propose the name *Bradyrhizobium centrosemae* sp. nov. symbiovar centrosemae.

The 16S rRNA, *recA* and *nodC* gene sequences of *Centrosema* strains were previously obtained [16]. The *glnII* gene sequences were obtained in this work for all strains as described by

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Vinuesa et al. [22]. All these sequences were compared with those held in Genbank [1] and aligned with those of the *Bradyrhizobium* species using the Clustal W program [19]. The distances were calculated according to Kimura's two-parameter model [10]. The phylogenetic trees were inferred using the neighbour-joining and maximum likelihood (ML) models [7,17] that yielded similar results and then only the results of ML analysis are shown. MEGA5.0 [18] was used for all the phylogenetic analyses.

The analysis of 16S rRNA gene, including all the recently described species of *Bradyrhizobium*, confirmed the placement of strains CMVU44^T, CMVU04 and A9^T into the group I and that of strains CMVU02 and CMVU30 into the group II according to the phylogenetic division of genus *Bradyrhizobium* proposed by Menna et al. [13] (Fig. 1). Identical 16S rRNA gene sequences were found between strains CMVU44^T and CMVU04, and between CMVU02 and CMVU30. Therefore, only the 16S rRNA sequences of strains CMVU44^T and CMVU02 were included in the phylogenetic analysis (Fig. 1). The 16S rRNA gene sequences of strains CMVU44^T, A9^T and CMVU02 were identical to those of *Bradyrhizobium daqingense* CCBAU 15774^T, *Bradyrhizobium guangxiense* CCBAU 53363^T and *Bradyrhizobium viridifuturi* SEMIA 69^T, respectively. Such 100% similarity in the 16S rRNA gene does not imply the *Centrosema* strains to belong to these species since many species of genus *Bradyrhizobium* have identical or almost identical 16S rRNA gene sequence (see Fig. 1), but they can be differentiated by their housekeeping genes.

Hence, in this work we analyzed the sequences of the two housekeeping genes, *recA* and *glnII*, that have been analyzed in all *Bradyrhizobium* species. The results of the phylogenetic analysis of the concatenated sequences of these two genes (Fig. 2) showed that the strains from *Bradyrhizobium* group II, CMVU02 and CMVU30, clustered in a separate branch with the type strain of *B. viridifuturi* SEMIA 690^T. Sequence similarity values higher than 99% were found among the strains CMVU02, CMVU30 and the type strain *B. viridifuturi* SEMIA 690^T in both *recA* and *glnII* genes. Therefore strains CMVU02 and CMVU30 were classified into the species *B. viridifuturi* whose type strain SEMIA 690^T was isolated from *Centrosema pubescens* nodules in Brazil [9].

The strains CMVU44^T and CMVU04 are phylogenetically related to *B. daqingense* CCBAU 15774^T (Fig. 2) and showed 99% similarity in both *recA* and *glnII* genes between them and less than 96% and 98.5% in *recA* and *glnII* genes, respectively, with respect to the type strain of *B. daqingense* CCBAU 15774^T.

Besides, the strain A9^T showed 97.8% and 96.4% in *recA* and *glnII* genes, respectively, with respect to its closest relative *B. guangxiense* CCBAU 53363^T, grouping in the same phylogenetic cluster (Fig. 2). These results support the description of two novel species within genus *Bradyrhizobium* since several species from this genus, some of them recently described, presented similar distances between them, such as *B. canariense* and *B. lupini*, *B. huanghaihainense* and *B. arachidis*, *B. ferriligni* and *B. pachyrhizi*, *B. paxllaeri* and *B. jicamae* or *B. embrapense* and *B. tropiciagri* (Fig. 2).

The results of the housekeeping gene analysis were confirmed after DNA–DNA hybridization which was performed as indicated earlier [6,24] and showed an average of 84.5% (±12.5) between the strains CMVU44^T and CMVU04, as correspond to strains belonging to the same species, and an average of 46% (±6) between the strains CMVU44^T and *Bradyrhizobium daqingense* CCBAU 15774^T. The strain A9^T showed an average of 51% (±7) with respect to *B. guangxiense* CCBAU 53363^T. Since these values are below the 70% threshold value of DNA–DNA similarity considered for definition of bacterial species [23], the *Centrosema* strains studied in this work represent two novel species of genus *Bradyrhizobium*.

DNA for analysis of base composition was carried out as previously reported [3]. The mol% G+C content of DNA was determined using the thermal denaturation method [12]. The G+C content of strains A9^T and CMVU44^T were 65.1% and 62.7%, respectively, which is within the range reported for *Bradyrhizobium* species [11].

The phenotypic characterization was performed as was previously described for *Bradyrhizobium* [14]. API 20NE galleries and Biolog GN2 MicroPlates were inoculated according to the manufacturer's instructions. The galleries were incubated for 7 days at 28 °C. Growth temperature range was determined by incubating cultures in YMA [20] at 4, 15, 28, 37 and 45 °C. Growth pH range was determined in the same medium with final pH 4.5, 6, 7, 8, 9 and 10. PCA buffer (Na_2HPO_4 0.4 M and citric acid 0.2 M) was used to adjust the pH 4 and 6, phosphate buffer (Na_2HPO_4 0.2 M and NaH_2PO_4 0.2 M) was used for pH 7 and TE buffer 0.2 M was used for pH 8, 9 and 10. Salt tolerance was tested in the same medium containing 0.5, 1, 1.5, 2 and 2.5% (w/v) NaCl. For testing the natural antibiotic resistance the disc diffusion method on YMA was used. The discs contained the following antibiotics: ampicillin (2 μg), erythromycin (2 μg), ciprofloxacin (5 μg), penicillin (10 IU), polymyxin (300 IU), cloxacillin (1 μg), oxytetracycline (30 μg), gentamycin (10 μg), cefuroxime (30 μg), or neomycin (5 μg) (Becton Dikinson, BBL). The type strains of *B. daqingense* and *B. guangxiense* were included in the phenotypic study as reference. Phenotypic characteristics of the novel species are reported below in the species description and the differential characteristics with respect to the closest species of *Bradyrhizobium* are shown in Table 1.

The genus *Bradyrhizobium* currently contains several symbiovars described on the basis of their *nodC* gene analysis (Fig. 3). According to this analysis the strains CMVU02 and CMVU30 formed a cluster with the type strains of *B. viridifuturi* SEMIA 690^T, *B. tropiciagri* CNPSo 1112^T and *B. embrapense* CNPSo 2833^T, CNPSo 1112^T, isolated from *Centrosema pubescens, Neonotonia wightii* and *Desmodium heterocarpon* in Brazil [5,9]. This cluster is phylogenetically divergent to that formed by the *Bradyrhizobium* symbiovars defined to date, genistearum [21], glycinearum [21], retamae [8], sierranevadense [4] and vignae [2]. Therefore it constitutes a new symbiovar within genus *Bradyrhizobium* for which we propose the name tropici.

The strains CMVU44^T and CMVU04 formed an independent cluster being their closest relatives the type strains from the species *B. iriomotense* EK05^T and *B. manausense* BR 3351^T but with less than 87% similarity in the *nodC* gene. The strain A9^T also formed a divergent branch with respect to the defined simbiovars being its closest relative the species *B. yuanmingense* NBRC 100594^T and a group of species from symbiovar glycinearum nodulating soybean with similarities also lower than 87% in the *nodC* gene. Therefore the *Centrosema* strains CMVU44^T and CMVU04 and A9^T belong to two novel symbiovars within genus *Bradyrhizobium*. The strains CMVU44^T and CMVU04 with identical *nodC* genes belong to the same symbiovar for which the name phaseolarum is proposed, in reference to the tribe Phaseolae in which the legume genus *Centrosema* is included, and the strain A9^T belongs to a different symbiovar for which the name centrosemae is proposed.

Based on their phenotypic, genotypic and symbiotic characteristics we propose that the strains isolated from *Centrosema* nodules in Venezuela belong to a new symbiovar named tropici within the species *B. viridifuturi* and to two novel species and symbiovars with the names *Bradyrhizobium centrosemae* sp. nov. (sv centrosemae) and *Bradyrhizobium americanum* sp. nov. (sv. phaseolarum).

Description of *Bradyrhizobium centrosemae* sp. nov.

Bradyrhizobium centrosemae *(cen.tro.se'ma.e. N.L. gen. n. centrosemae, of* Centrosema*, isolated from* Centrosema *nodules)*

Cells are Gram negative rods as for the other species of the genus. Colonies are small, pearl white, less than 1 mm in diameter after 7