Systematic and Applied Microbiology 36 (2013) 392–400

Contents lists available at SciVerse ScienceDirect

Systematic and Applied Microbiology

journal homepage: www.elsevier.de/syapm

Centrosema is a promiscuous legume nodulated by several new putative species and symbiovars of *Bradyrhizobium* in various American countries

Martha-Helena Ramírez-Bahena[a,b], Rajaa Chahboune[c], Encarna Velázquez[b,d], Alicia Gómez-Moriano[a], Edith Mora[e], Alvaro Peix[a,b,*], Marcia Toro[e]

[a] *Instituto de Recursos Naturales y Agrobiología, IRNASA-CSIC, c/Cordel de Merinas 40-52, 37008 Salamanca, Spain*
[b] *Unidad Asociada Universidad de Salamanca-CSIC 'Interacción Planta-Microorganismo', Salamanca, Spain*
[c] *Department of Biology, Faculty of Sciences and Techniques, University Abdelmalek Essaadi, Tangier, Morocco*
[d] *Departamento de Microbiología y Genética, Lab. 209, Universidad de Salamanca, Edificio Departamental de Biología, Campus M. Unamuno, Salamanca, Spain*
[e] *Laboratorio de Estudios Ambientales, Instituto de Zoología Tropical, Facultad de Ciencias, Universidad Central de Venezuela, Caracas 1041-A, Apartado 47.058, Venezuela*

ARTICLE INFO

Article history:
Received 14 January 2013
Received in revised form 5 March 2013
Accepted 15 March 2013

Keywords:
Rhizobia
Bradyrhizobium
Centrosema
Biodiversity
Phylogeny

ABSTRACT

Centrosema is an American indigenous legume that can be used in agroecosystems for recovery of acidic and degraded soils. In this study, a *Centrosema*-nodulating rhizobial collection of strains isolated in a poor acid savanna soil from Venezuela was characterized, and the members of the collection were compared to other *Centrosema* strains from America. The analysis of the *rrs* gene showed that the strains nodulating *Centrosema* in American countries were closely related to different species of the genus *Bradyrhizobium*. However, the analysis of the *atpD* and *recA* genes, as well as the 16S–23S ITS region, showed that they formed several new phylogenetic lineages within this genus. The Venezuela strains formed three lineages that were divergent among themselves and with respect to those formed by *Centrosema* strains isolated in other countries, as well as to the currently described species and genospecies of *Bradyrhizobium*. In addition, the symbiotic genes *nodC* and *nifH* carried by *Centrosema*-nodulating strains were analyzed for the first time, and it was shown that they belonged to three new phylogenetic lineages within *Bradyrhizobium*. The *nodC* genes of the *Centrosema* strains were divergent among themselves and with respect to the *genistearum* and *glycinearum* symbiovars, indicating that *Centrosema* is a promiscuous legume. According to these results, the currently known *Centrosema*-nodulating strains represent several new putative species and symbiovars of the genus *Bradyrhizobium*.

Introduction

Centrosema is a leguminous genus of voluble climbing herbs or subshrubs within the tribe *Phaseolae* that includes 34 species, all of them native to America. Most species are widely distributed in the savanna and forests of tropical regions (Penteado et al., 1996). *Centrosema* is a very nutritive pasture legume occurring naturally in arid tropical ecosystems, and many species of this genus become well adapted to very diverse tropical environments including highlands, dried or seasonally flooded areas, as well as acidic soils showing low fertility (Keller-Grein et al., 2000; Schultze-Kraft et al., 1990). In addition, *Centrosema* has been studied recently in phytoremediation schemes (Nwaichi et al., 2011) and investigated as a medicinal plant for its likely antiproliferative activity in leukemia (Mani and Lakshmi, 2010). Due to its importance, several studies on the biology and agronomic performance of *Centrosema* species have been conducted in several America (Keller-Grein et al., 2000; Reátegui et al., 1985; Sousa et al., 2011), Africa (Odeyinka et al., 2008) and Asia (Humphreys et al., 1990) countries, as well as in Australia (Schultze-Kraft et al., 1997). Some species, such as *Centrosema molle* or *Centrosema macrocarpum*, are commonly cultivated as pastures in agricultural savanna ecosystems in Venezuela because of their property for increasing the nitrogen content in soils. These legumes are combined with other crops in tropical agroecosystems where acid soils with poor fertility occur, and they act as plant covers that provide protein and energy sources for animals in the drought season, which is the most critical season for the production of forage (Alguacil et al., 2010).

Although *Centrosema* has been widely studied for its properties as a forage legume (Navas et al., 2011; Sousa et al., 2011) and studies on *Centrosema*-rhizobia-fungi interactions have been conducted recently in Brazil (Matias et al., 2009) and Venezuela (Navas et al., 2011), little attention has been given to date to the

* Corresponding author at: Instituto de Recursos Naturales y Agrobiología, IRNASA-CSIC, c/Cordel de Merinas 40-52, 37008 Salamanca, Spain.
Tel.: +34 923219606.
E-mail address: alvaro.peix@csic.es (A. Peix).

0723-2020/$ – see front matter © 2013 Elsevier GmbH. All rights reserved.
http://dx.doi.org/10.1016/j.syapm.2013.03.007

rhizobial endosymbionts. There are no studies concerning the strains nodulating this legume in Venezuela where *Centrosema* is commonly cultivated, and only seven strains isolated from different *Centrosema* species have been reported in some studies together with strains nodulating different legumes (Menna et al., 2009; Parker, 2003; Vinuesa et al., 2005b). These strains isolated from *Centrosema pubescens*, *Centrosema plumbieri* and *Centrosema* sp. in Brazil, Colombia and Panama were classified within the genus *Bradyrhizobium* (Menna et al., 2009; Parker, 2003; Vinuesa et al., 2005b).

The genus *Bradyrhizobium* currently contains 17 species (Euzéby, J.P. LPSN – List of Prokaryotic Names with Standing in Nomenclature, http://www.bacterio.cict.fr/index.html) and they seem to be the predominant organisms establishing symbiosis with American legumes (Menna et al., 2009; Ormeño-Orrillo et al., 2011; Parker, 2003). Also, this genus includes several strains not isolated from nodules, such as *Bradyrhizobium betae* and *Bradyrhizobium iriomotense* isolated from tumors of the non-legume *Beta vulgaris* and the legume *Entada koshunensis*, respectively (Islam et al., 2008; Rivas et al., 2004), *Bradyrhizobium denitrificans* that was previously named *Blastobacter denitrificans* isolated from water (Van Berkum et al., 2006), and *Bradyrhizobium oligotrophicum* (formerly named *Agromonas oligotrophica*) isolated from a rice paddy soil (Ramírez-Bahena et al., 2013). Moreover, this genus contains photosynthetic strains, such as BTAi1, ORS278 and ORS285 that nodulate the tropical legume *Aeschynomene* whose genomes have been completely sequenced (Nzoué et al., 2009). From the strains that have not been isolated from nodules, as well as the photosynthetic bradyrhizobia mentioned, the nodulation genes have been sequenced to date only from strains *B. iriomotense* EK05^T and ORS285 (Chaintreuil et al., 2001; Islam et al., 2008).

Bradyrhizobium has been proposed to be divided into two groups according to the *rrs* gene and ITS region analyses designated I and II by Menna et al. (2009). However, the analysis of the ITS region performed in this study showed that *B. denitrificans* and the *Bradyrhizobium* photosynthetic strains clustered into a different group which we have named group III following the same nomenclature. The *Centrosema* strains included in the work of Menna et al. (2009) mostly belong to group II regardless the species of this legume and the American country from which they were isolated. However, the number of *Centrosema* strains analyzed to date is still too low, and further studies are required in order to identify the biodiversity of strains nodulating this legume in America.

Therefore, the objective of this study was to carry out a biodiversity analysis of strains nodulating *Centrosema* in Venezuela, for which their core and symbiotic genes were studied. Several putatively novel species and symbiovars of *Bradyrhizobium* were detected and this is discussed.

Materials and methods

Strains and nodulation experiments

Plants of *Centrosema molle* and *C. macrocarpum* were used as trap plants in an acid soil (pH 4.99) from Guarico State in Venezuela, where savanna ecosystems are widespread. The rhizobial strains were isolated from the *Centrosema* nodules according to the method of Vincent (1970). In order to confirm the nodulation capacity of the strains, infectivity tests were conducted in a growth chamber umder controlled conditions using sterile vermiculite as substrate. The *Centrosema* seeds were surface disinfected and seedlings were inoculated as described by Ramírez-Bahena et al. (2009b).

RAPD fingerprinting

RAPD patterns were obtained as previously described (Rivas et al., 2006) using the primer M13 (5′-GAGGGTGGCGGTTCT-3′) and the GoTaq Flexi DNA polymerase (Promega) kit. PCR conditions and electrophoresis were performed as described by Faghire et al. (2012). A dendrogram was constructed based on the matrix generated using the UPGMA method and the Pearson coefficient with Bionumerics version 4.0 software (Applied Maths, Austin, TX).

Analysis of rrs, atpD, recA, nodC and nifH genes, and the 16S–23S intergenic spacer (ITS)

The *rrs* gene was amplified and sequenced according to Rivas et al. (2007a), and the ITS region as described by Peix et al. (2005). The *recA* and *atpD* genes were amplified and sequenced as described by Gaunt et al. (2001) and Vinuesa et al. (2005b). The *nodC* and *nifH* genes were amplified with the primers and conditions described by Laguerre et al. (2001) and Velázquez et al. (2010), except for strains CMVU04 and CMVU44 whose *nodC* genes were amplified and sequenced with the following primers designed in this study: nodCRB1F (5′-GGCVAASAAYGTBGGAAAGCGCAAGGCGCAGATCG-3′) and nodCRBIR (5′-AGCGNAGYTGCTGNCGHAGTATGGYC-3′). PCR amplifications were performed with a REDExtract-N-Amp PCR Kit (Sigma) or GoTaq Flexi DNA polymerase kit (Promega) following the manufacturers' instructions. The bands corresponding to the different genes were purified and sequenced as described in Faghire et al. (2012). The sequences obtained were compared to those held in GenBank by using the BLASTN program (Altschul et al., 1990). They were aligned by using Clustal W software (Thompson et al., 1997). Kimura's two-parameter model (Kimura, 1980) was used to infer phylogenetic trees with the maximum likelihood method (Felsenstein, 1981) using MEGA5 software (Tamura et al., 2011). Confidence values for nodes in the trees were generated by bootstrap analysis using 1000 permutations of the data sets.

Results and discussion

RAPD fingerprinting analysis

A total of 44 *Centrosema* nodulating strains were isolated (Table 1) and analyzed by RAPD fingerprinting that allowed differentiation between strains of the same rhizobial species, for which this technique provides an estimation of the genetic diversity (Dooley et al., 1993; Faghire et al., 2012; Iglesias et al., 2007; Moschetti et al., 2005; Rivas et al., 2006; Valverde et al., 2006). Several different RAPD profiles were obtained that were distributed into seven groups with similarity percentages lower than 85% (Fig. 1 and Table 1), from which representative strains were selected for gene sequence analysis.

Analysis of the rrs gene

The current phylogenetic classification of rhizobia is predominantly based on *rrs* gene sequences and thus the identification of nodule isolates should also be based on this gene. In *Bradyrhizobium*, as mentioned before, two well-differentiated groups, designated I and II by Menna et al. (2009), have been defined according to the 16S rRNA gene.

Centrosema strains CMVU02, CMVU20 and CMVU30 that are representative of RAPD groups V, VI and VII, respectively, belong to *Bradyrhizobium* group II and have identical *rrs* gene sequences, for which only the sequence of strain CMVU02 is shown in the phylogenetic tree (Fig. 2). The closest type strain of CMVU02 is