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Phylogenetic diversity of *Bradyrhizobium* strains nodulating *Calicotome spinosa* in the Northeast of Algeria

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ABSTRACT

Fifty-two slow-growing strains were isolated from root nodules of *Calicotome spinosa* grown in the Northeast of Algeria and grouped in 24 rep-PCR clusters. One representative strain for each profile was further phylogenetically characterized. The nearly complete 16S rRNA gene sequence indicated that all strains were affiliated to *Bradyrhizobium*. Multi-Locus Sequence Analysis (MLSA) of the *atpD*, *glnII* and *recA* genes and of the 16S-23S rRNA internal transcribed spacer (ITS) showed that these strains formed four divergent clusters: one close to *Bradyrhizobium canariense* and *Bradyrhizobium lupini* and three others separate from all the described species, representing three putative new *Bradyrhizobium* species. A phylogenetic analysis based on the *nodC* gene sequence affiliated the strains to either of the two symbiovars, genistearum or retamae.

Introduction

Legumes interact symbiotically with nitrogen-fixing rhizobia, which are soil bacteria that may reside intracellularly within nodules developing on plant roots [21]. Thanks to this symbiosis, legumes can grow in arid, nitrogen-deficient soils, acting as pioneer plants for soil stabilization, colonization and fertility enhancement, thus helping to prevent soil erosion and desertification [9].

The *Genisteae* members form a large tribe with around 25 genera [8], including shrubs, small trees and herbs [15]. They mainly grow in the Mediterranean regions [50], where they are ecologically important due to their prevalence among plant communities [40].

Calicotome belongs to the *Genisteae* tribe; this genus essentially consists of four species *Calicotome infesta* (C. Presl) *Calicotome intermedia* (C. Presl) *Calicotome spinosa* (L.) and *Calicotome villosa* (Poir.) [43]. *C. spinosa*, known as thorny broom or spiny broom, is a very spiny, densely branched shrub that can reach up to three meters in height. *C. spinosa* grows in the western Mediterranean region on sunny slopes and on dry rocky ground. It is native to Spain, France, Italy, Croatia and Algeria, and has been introduced into New Zealand. From March to June *C. spinosa* produces bright yellow flowers, which are borne singly or in small clusters. The seedpods are 30 mm long and are almost hairless (Germplasm Resources Information Network-(GRIN) [Online Data- base, URL: http://www.ars-grin.gov/cgi-bin/npgs/html/genusfamfind.pl]).

Genisteae have been described as being mainly nodulated by diverse slow-growing strains affiliated to *Bradyrhizobium* [5,6,9,12,14,28,29,35,36,47,51,53,58,60]. However, some exceptions have been reported, with fast-growing rhizobia being identified among isolates from root nodules of *Retama raetam*, *Genista saharae*, *Lupinus micranthus* and *Lupinus luteus,* which were affiliated to *Ensifer*, *Rhizobium*, *Microvirga,* and *Phyllobacterium* [11,31,32,35,36].

To our knowledge, there are no available reports on symbiotic nitrogen-fixing bacteria associated with *C. spinosa* in Algeria. The primary objective of this research was therefore to identify and characterize the rhizobial symbionts within nodules of *C. spinosa* grown in the Northeast of Algeria, by studying their genetic diversity and phylogenetic relationships.

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Table 1
Origins of *Calicotome spinosa* rhizobial isolates and pH of soil, ecological, climatic characteristics of their sampling sites.

Sites	Coordinates	Mediterranean-type climate	Ecosystem and soil characteristics	pH of soil
Azzaba (Skikda)	36°43′14.6″N-7°10′00.1″E	Subhumid	Embankment soil	6.1
Sidi Aich (Bejaia)	36°35′23.2″N-4°39′51.8″E	Humid	Oued bed, sandy gravel	7.2
Azazga (Tizi Ouzou)	36°45′48.0″N-4°23′48.9″E	Semiarid	Maquis, rocky clay	6.9
Ain El Turk (Bouira)	36°23′48.1″N-3°48′′48.5″E	Semiarid	Oued bed, sandy gravel	7.5

Materials and methods

Rhizobial isolation and culturing conditions

Bacteria were isolated from root nodules collected on wild *C. spinosa* plants grown at four different geographical sites in north-eastern Algeria (see Supplementary Fig. S1 in the online version at DOI: 10.1016/j.syapm.2018.05.005). The sampling sites were chosen along a climatic gradient from humid to semi-arid. The ecological and climatic characteristics of the sampling sites are indicated in Table 1. The rhizobial strains used in this study are listed in Table 2.

Nodules were surface-sterilized by immersion in 96% ethanol for 30 s, in 3% sodium hypochloride for 3 min, then thoroughly rinsed ten times in sterile distilled water. Next, nodules were individually crushed on sterile plates and a loopful of nodule suspension streaked onto yeast extract mannitol Agar (YMA) in Petri dishes, where rhizobia were incubated at 28 °C according to the protocol described by Vincent [59]. Isolates were long-term stored in YMB adjusted to 20% glycerol (v/v) at -80 °C.

Plant nodulation test

Purified *C. spinosa* isolates were tested for their ability to nodulate their plant of origin. Seeds of *C. spinosa* were surface-sterilized and scarified in 98% sulfuric acid for 1 h, rinsed ten times with sterile distilled water, kept in sterile water overnight and placed in Petri dishes containing sterile absorbent paper at 20 °C and in the dark to germinate. The inoculation test was carried out on the seedlings grown in a 250 ml flask filled with liquid Jensen's medium; each seedling was inoculated with 1 ml of a rhizobial suspension (~10^8 cells ml^{-1}) in triplicate and placed in a growth chamber at 25 °C for six weeks; non-inoculated plants were included as a control. The nodulation test was carried out under controlled bacteriological conditions [5].

DNA extraction and PCR amplifications

Total genomic DNA was extracted from a yeast extract mannitol broth (YMB) bacterial culture in exponential phase using the alkaline lysis method described by Bourebaba et al. [6]. Strains were grouped by rep-PCR (repetitive extragenic palindromic-polymerase chain reactions), using primers REPIR-I and REP2-I [16]. Rep-PCR profiles were discriminated using InfoQuest 4.5. PCR amplifications of 16S rRNA and the 16S-23S rRNA internal transcribed spacer (ITS), from representative strains of each rep-PCR profile, were carried out using several sets of primers described by (16S rRNA) [25], (ITS 16S-23S rRNA) [37,62]. As a phylogenetic analysis based on the 16S rRNA gene sequence may not have provided sufficient resolution [44], the use of housekeeping genes has proved useful for classification and identification of several groups of rhizobia [17,20,33,34,39,44]. Fragments of the *atpD*, *glnII*, and *recA* housekeeping genes and the *nodC* symbiotic gene were amplified using several primers (*atpD*) [53] (*glnII*) [57], (*recA*) [6] and (*nodC*) [49]. The PCR conditions were the same as those previously described by Sanchez-Canizares et al. [48], except for ITS amplification, for which the conditions were as follows: initial denaturation (3 min at 96 °C), 35 cycles each consisting of denaturation (30 s at 95 °C), annealing (30 s at 55 °C), extension (40 s at 72 °C) and final extension for 3 min at 72 °C.

Table 2
Rep-PCR patterns, number of strains obtained per profile and cluster according to soil sampling sites used in this study.

Strains[a]	REP-PCR pattern	Number of strains per profile	Soil sampling sites	Cluster
CSA36	**A**	1	Azzaba	**B**
CSA37	**B**	1	Azzaba	**B**
CSA96, CSA63, CSS302, CSS391	**C**	4	Azzaba, Sidi Aich	**C**
CSA112	**D**	1	Azzaba	**C**
CSA115, CSA149, CSAZ498, CSS343	**E**	4	Azzaba, Azazga, Sidi Aich	**C**
CSA138, CSS312, CSS375	**F**	3	Azzaba, Sidi Aich	**C**
CSA147, CSA89, CSAZ523	**G**	3	Azzaba, Azazga	**B**
CSA159	**H**	1	Azzaba	**A**
CSA207	**I**	1	Azzaba	**B**
CSA208	**J**	1	Azzaba	**B**
CSA217, CSS360	**K**	2	Azzaba, Sidi Aich	**A**
CSA234, CSS334, CSA211, CSS372	**L**	4	Azzaba, Sidi Aich	**B**
CSA236	**M**	1	Azzaba	**B**
CSS303	**N**	1	Sidi Aich	**D**
CSS306, CSS351, CSAZ504, CSS348	**O**	4	Sidi Aich, Azazga	**A**
CSS347, CSAT444	**P**	2	Sidi Aich, Ain El Turk	**D**
CSS354, CSA158	**Q**	2	Sidi Aich, Azzaba	**A**
CSS381, CSS350	**R**	2	Sidi Aich	**A**
CSAT412	**S**	1	Ain El Turk	**D**
CSAZ503, CSA219, CSS379	**T**	3	Azazga, Azzaba, Sidi Aich	**A**
CSAZ522, CSAZ521	**U**	2	Azazga	**A**
CSAZ530, CSA157, CSS352, CSAT468	**V**	4	Azazga, Azzaba, Sidi Aich Ain El Turk	**A**
CSAT634	**W**	1	Ain El Turk	**D**
CSAT637, CSAZ507, CSAT647	**X**	3	Azazga, Ain El Turk	**A**
Total	24	52		

[a] For a given strain, CS represents the name of the host plant *C. spinosa*. The letters A, SS, AT, AZ stand for Azzaba, Sidi Aich, Ain El Turk and Azazga, respectively, the names of the locations from which plants were collected. Strains in bold were chosen as the representative strains of each REP-PCR group.

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