Symbiosis (2015) 67:3–10
DOI 10.1007/s13199-015-0365-8

The diversity of rhizobia nodulating the *Medicago*, *Melilotus* and *Trigonella* inoculation group in Egypt is marked by the dominance of two genetic types

Nadia H. El Batanony[1] · **Antonio Castellano-Hinojosa**[2] · **David Correa-Galeote**[2] · **Eulogio J. Bedmar**[2]

Received: 15 July 2015 / Accepted: 23 November 2015 / Published online: 30 November 2015

Abstract Twenty four rhizobial strains were isolated from root nodules of *Melilotus*, *Medicago* and *Trigonella* plants growing wild in soils throughout Egypt. The nearly complete 16S rRNA gene sequence from each strain showed that 12 strains (50 %) were closely related to the *Ensifer meliloti* LMG6133^T type strain with identity values higher than 99.0 %, that 9 (37.5 %) strains were more than 99 % identical to the *E. medicae* WSM419^T type strain, and that 3 (12.5 %) strains showed 100 % identity with the type strain of *N. huautlense* S02^T. Accordingly, the diversity of rhizobial strains nodulating wild *Melilotus*, *Medicago* and *Trigonella* species in Egypt is marked by predominance of two genetic types, *E. meliloti* and *E. medicae*, although the frequency of isolation was slightly higher in *E. meliloti*. Sequencing of the symbiotic *nodC* gene from selected *Medicago* and *Melilotus* strains revealed that they were all similar to those of the *E. meliloti* LMG6133^T and *E. medicae* WSM419^T type strains, respectively. Similarly, *nodC* sequences of strains identified as members of the genus *Neorhizobium* were more than 99 % identical to that of *N. galegae* symbiovar officinalis HAMBI 114.

Keywords *Melilotus* · *Medicago* · *Trigonella* · Egypt · Nodulation · *Ensifer* · *Neorhizobium* · *nodC*

Presented at the XV SEFIN National Meeting of the Spanish Society of Nitrogen Fixation, June 16–18, 2015, León, Spain

✉ Eulogio J. Bedmar
eulogio.bedmar@eez.csic.es

[1] Environmental Studies and Research Institute (ESRI), University of Sadat City, Sadat City, Menoufiya, Egypt

[2] Department of Soil Microbiology and Symbiotic Systems, Estación Experimental del Zaidín, CSIC, E-419 Granada, Spain

1 Introduction

Together with the actinorhizal plants, legumes are best characterized by their ability to establish dinitrogen (N_2)-fixing symbiotic associations with soil bacteria collectively referred to as rhizobia. Comprehensive reviews of the associations of bacteria with legumes and the current available knowledge on the phylogenetic diversity of both rhizobia and other endophytic bacteria inhabiting root nodules have been published recently (Rivas et al. 2009; Velázquez et al. 2010; Gyaneshwar et al. 2011; Peix et al. 2015). During the plant-rhizobial interaction process an exchange of molecular signals occurs between the two partners leading to the formation of root nodules, within which symbiotic N_2 fixation occurs (Graham 2008).

Among members of the Leguminosae (Fabaceae), the genera *Medicago*, *Melilotus* and *Trigonella* comprise a large number of species of annual herbs, herbaceous perennials and shrubs, mostly native to the Mediterranean region (Lesins and Lesins 1979). These legumes are a biological source of nitrogen that gives them economic significance in cultivation for forage or pasture, as well as environmental value in non-managed ecosystems, and makes them excellent candidates for use in sustainable agricultural systems (Graham 2008; Howieson et al. 2008). The N_2-fixing rhizobia that are currently known to nodulate *Medicago* species are from the genus *Ensifer* (syn. *Sinorhizobium*), of which the species *E. meliloti* (de Lajudie et al. 1994) and *E. medicae* (Rome et al. 1996) are well characterized microsymbionts (Garau et al. 2005; Peix et al. 2015). In addition to *Ensifer*, *Rhizobium mongolense* also nodulates *Melilotus ruthenica* (van Berkum et al. 1998). *Ensifer meliloti* has also been isolated from nodules of *Melilotus* spp. (Yan et al. 2000) and *Melilotus officinalis* and *Medicago monspelliaca* (Pandey et al. 2004; del Villar et al. 2008). Information about the

symbiosis between *Trigonella* and rhizobia is relatively scarce even though it is within the *Medicago-Melilotus* cross inoculation group. However, recent studies from China have shown that the symbionts of *T. arcuata* are *Ensifer* (He et al. 2011), and that *Rhizobium tibeticum*, which was first isolated from root nodules of *T. archiducis-nicolai* in Tibet (Hou et al. 2009), also nodulated *Medicago lupulina*, *Medicago sativa* and *Medicago officinalis* (Hou et al. 2009). On the other hand, Rajendran et al. (2012) failed to isolate rhizobial strains from nodules of *T. foenum-graecum* during a study performed to investigate the most common nodule-associated bacteria. Almost nothing is known about the symbionts of *Trigonella* spp. growing in North African and Mediterranean countries.

Egyptian *Medicago*, *Melilotus* and *Trigonella* species have been studied for years, but they are not well characterized in terms of their symbionts, although there have been several reports from neighbouring North African and Mediterranean countries, such as Tunisia (Zakhia et al. 2004) and Algeria (Sebbane et al. 2006; Arbi et al. 2015). In Egypt, research on their plant-associated microsymbionts has mainly focused on cultivated species, and those of wild legumes have been generally ignored. *Medicago*, *Melilotus* and *Trigonella* grow wild throughout Egypt and are part of the natural vegetation. Because very scarce information about their symbiosis is available, the objective of this study was to explore the diversity of the rhizobia that infect species of the three legume genera via sequencing of their core (16S rRNA) and symbiotic (*nodC*) genes, and to compare the results obtained with isolates from other legumes within the same inoculation group.

2 Materials and methods

2.1 Isolation of bacteria from nodules and culture conditions

Root fragments (5–10 cm long) containing nodules (5–10/plant) were collected from healthy *Melilotus*, *Medicago*, and *Trigonella* plants growing wild in different locations in Egypt, ranging from the North West Mediterranean coastal region to the Nile Valley (Table 1). Nodules were kept on ice until they were taken to the laboratory. They were surface-sterilized by sequential washing with 96 % ethanol for 10 s, 3 % hydrogen peroxide for 3 min and, finally, rinsed thoroughly in sterile distilled water. Tests to validate surface sterilization of plant tissues were performed by touching the disinfected nodules several times on the surface of solid yeast extract-mannitol (YEM) medium (Vincent 1970) prior to isolation of the interior microbiota. Nodules from the same plant species and location were pooled. Twelve nodules from each pool were placed independently in Petri dishes and crushed in a drop of sterile water with a sterile glass rod. The resulting suspension was streaked onto Petri dishes containing YEM supplemented with 0.025 g L^{-1} Congo Red and incubated at 30 °C for 10 d. Single colonies were picked and checked for purity by repeated streaking on YEM medium.

2.2 DNA extraction and PCR amplifications

Genomic DNA was isolated from bacterial cells using the RealPure Genomic DNA Extraction kit (Durviz, Spain), according to the manufacturer's instructions. The quantity of DNA was determined by using a Nanodrop spectrophotometer (NanoDrop ND1000). PCR amplifications of 16S rRNA gene fragments were carried out using the two opposing primers 41f and 1488r as previously reported (Herrera-Cervera et al. 1999). Three forward primers, nodCF, nodCF2 and nodCFn, and the reverse primer nodCI were used for amplification and sequencing of the *nodC* gene as indicated earlier (Laguerre et al. 2001). Amplification products were purified using the Qiagen PCR product purification system and subjected to cycle sequencing using the same primers as for PCR amplification, with ABI Prism dye chemistry and analyzed with a 3130 xl automatic sequencer at the sequencing facilities of the Estación Experimental del Zaidín, CSIC, Granada, Spain. The 16S rRNA gene sequences were compared to those deposited in EzTaxon-e (Kim et al. 2012) and those of the *nodC* gene sequences with homologous sequences in GenBank using the Phydit software (Chun 2001). Phylogenetic analyses were performed with the Geneious software package version 7.1.7 (Kearse et al. 2012), inferred using the neighbor-joining algorithm (Saitou and Nei 1987) and visualized with MEGA5 (Tamura et al. 2011).

2.3 Plant nodulation tests

Seeds of *Melilotus*, *Medicago* and *Trigonella* species were surface-sterilized with 2.5 % $HgCl_2$ for 7 min, followed by thorough washing in sterile distilled water. The seeds were then placed in Petri dishes containing 1 % water agar and allowed to germinate at 30 °C in the dark. Seedlings (3 per pot) were planted in autoclaved 1 L Leonard jar assemblies containing sand and vermiculite (1:1, v:v) and inoculated at sowing with 1 mL of a single bacterial strain (~ 10^8 cells mL^{-1}). The plants were fed with an N-free nutrient solution (Fahraeus 1957), grown in a greenhouse under natural daylight conditions, and harvested at 10 % flowering to check for nodule formation.

2.4 Accession numbers

Accession numbers of the nucleotide sequences of the strains used in this study are shown in the phylogenetic trees.

Table 1 Rhizobial strains isolated in this study from root nodules of *Melilotus*, *Medicago* and *Trigonella* plants

Strain	Locality of isolation	Host of isolation	Closest relative species on basis of 16S rRNA gene	Similarity (%)	
				EzTazon-e	Phydit
NHBM3B	Desouk	*Melilotus indicus*	*E. medicae* WSM419^T	99.24	99.36
NHBM16	Izbat Al Maaddiyyah	*Melilotus messanensis*	*E. medicae* WSM419^T	99.36	98.94
NHBM22B	Kafr El-Sheikh	*Medicago intertexta*	*E. meliloti* LMG 6133^T	99.46	98.93
NHBM5	Kom Awshim	*Melilotus indicus*	*E. medicae* WSM419^T	99.61	99.31
NHBM13	Kom Awshim	*Melilotus indicus*	*E. medicae* WSM419^T	100	99.60
NHBM14	Kom Awshim	*Melilotus indicus*	*E. meliloti* LMG 6133^T	98.52	98.52
NHBM18	Kom Awshim	*Melilotus siculus*	*E. medicae* WSM419^T	99.92	99.60
NHBM19	Kom Awshim	*Melilotus siculus*	*E. meliloti* LMG 6133^T	97.71	97.71
NHBM15	Kom Awshim	*Melilotus indicus*	*Variovorax*	not applicable	
NHBM23	Fayoum	*Medicago intertexta*	*E. medicae* WSM419^T	95.83	95.42
NHBM25	Fayoum	*Medicago polymorpha*	*N. huautlense* S02^T	100	100
NHBM9	40 km west of Rosetta sea coast	*Melilotus indicus*	*E. meliloti* LMG 6133^T	100	100
NHBM12	Janaklis	*Melilotus indicus*	*E. meliloti* LMG 6133^T	91.67	90.56
NHBM17	40 km west of Rosetta sea coast	*Melilotus messanensis*	*E. meliloti* LMG 6133^T	99.56	99.56
NHBM21	40 km west of Rosetta sea coast	*Medicago sativa*	*N. huautlense* S02^T	100	100
NHBM10A	El-Amerya	*Melilotus indicus*	*E. medicae* WSM419^T	100	100
NHBM10B	El-Amerya	*Melilotus indicus*	*E. medicae* WSM419^T	100	99.69
NHBM24	Alexandria	*Medicago polymorpha*	*E. medicae* WSM419^T	99.72	99.43
NHBTR69	Alexandria	*Trigonella maritima*	*E. meliloti* LMG 6133^T	95.49	94.66
NHBTR70	Alexandria	*Trigonella maritima*	*E. meliloti* LMG 6133^T	100	99.75
NHBTR72	Western coastal region	*Trigonella marítima*	*E. meliloti* LMG 6133^T	100	100
NHBTR74	Western coastal region	*Trigonella maritima*	*E. meliloti* LMG 6133^T	95.17	94.65
NHBM6	Al Dakhilah	*Melilotus indicus*	*Paenibacillus*	not applicable	
NHBM7	Burj Al Arab	*Melilotus indicus*	*Brevibacillus*	not applicable	
N**H**BM26	Suez	*Medicago laciniata*	*E. meliloti* LMG 6133^T	92.42	91.45
NHBM27	Western coastal region	*Medicago laciniata*	*E. meliloti* LMG 6133^T	91.78	90.81
NHBM29	Western coastal region	*Medicago laciniata*	*N. huautlense* S02^T	100	100
NHBM4	Tamalay	*Melilotus indicus*	*Paenibacillus*	not applicable	

3 Results

3.1 Phylogenetic analysis of 16S rRNA and *nodC* genes

Twenty eight bacterial strains, 12 from *Melilotus*, 8 from *Medicago* and 8 from *Trigonella* species, were isolated from extracts of nodules taken from healthy plants growing wild in different locations in Egypt (Table 1). Partial sequences of the 16S rRNA gene from each strain revealed that 24 strains were members of the family Rhizobiaceae of the Alphaproteobacteria and that the remaining 4 strains belonged to the genera *Paenibacillus* (2 strains), *Variovorax* and *Brevibacillus*, respectively (Table 1). Among the Rhizobiaceae, 21 strains were classified as belonging to the genus *Ensifer* and 3 belonged to the genus *Neorhizobium* (Table 1). A neighbor-joining tree (Fig. 1) and EzTaxon-e analysis (Table 1) constructed from the 16S rRNA gene sequences indicated that strains NHBM3B, NHBM5, NHBM10A, NHBM10B, NHBM12 and NHBM13 from *Melilotus indicus*, NHBM16 from *Melilotus messanensis*, NHBM18 from *Melilotus siculus*, NHBM23 from *Medicago intertexta* and NHBM24 from *Medicago polymorpha* clustered with *E. medicae* WSM419^T with identity values higher than 99 %. Also, that strains NHBM9, NHBM12 and NHBM14 from *Melilotus indicus*, strain NHBM17 from *Melilotus messanensis*, strain NHBM19 from *Melilotus siculus*, strain NHBM22B from *Medicago intertexta*, and strains NHBM26 and NHBM27 from *Medicago laciniata* grouped with *E. meliloti* LMG6133^T with identity values higher than 99.0 %. Strains NHBTR69, NHBTR70, NHBTR72 and NHBTR74 isolated from *T. maritima* also clustered with *E. meliloti* LMG6133^T with identity values higher than 99.0 %. Together with strains in genus *Ensifer*, strains NHBM21, NHBM25 and NHBM29 that were isolated from nodules of *Medicago sativa*, *Medicago polymorpha* and *Medicago laciniata*, respectively, showed 100 % identity with

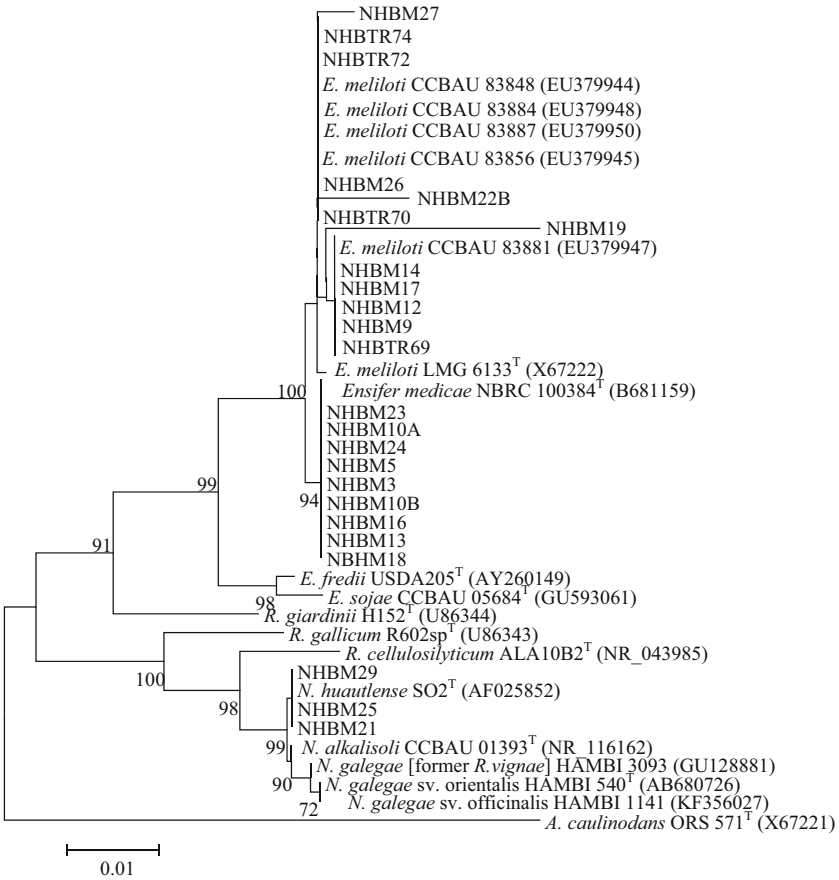

Fig. 1 Neighbor-joining phylogenetic tree based on partial 16S rRNA sequences of strains from nodules of *Melilotus*, *Medicago* and *Trigonella* and phylogenetically related species within members of the Rhizobiaceae. Bootstrap values are indicated as percentages derived from 1000 replications. Values lower than 70 are not shown. Bar, 1 nucleotide substitution per 100 nucleotides. The tree is rooted by *Azospirillum caulinodans* OTS751^T

the type strain *N. huautlense* S02^T. The 4 non-rhizobial strains from nodules of *Melilotus indicus* i.e. *Paenibacillus* strains NHBM4 and NHBM6, *Brevibacillus* strain NHBM7 and *Variovorax* strain NHBM15 (Table 1) were not further characterized in this study.

Utilization of different primer combinations resulted in amplification of the *nodC* gene of strains NHBM3B, NHBM16, NHBM18, NHBM23 and NHBM24 that were chosen as representatives of those previously identified as *E. medicae* and isolated from different host plants (Table 1). Similar results were obtained when strains NHBM9, NHBM17, NHBM19, NHBM22B and NHBM26, representing those showing identity with *E. meliloti* (Table 1), were used for PCR amplification of their *nodC* genes. The primer pair nodCF/nodCI was also useful to amplify the *nodC* gene of the strains NHBM21, NHBM25 and NHBM29 identified as *N. huautlense* that were isolated from *Medicago sativa*, *Medicago polymorpha* and *Medicago laciniata*, respectively. Pairwise alignments between globally aligned sequences of *nodC* from the strains isolated in this study with those of the corresponding *Ensifer* and *Neorhizobium* species showed that strains NHBM3B, NHBM16, NHBM18, NHBM23 and NHBM24 varied between 99.42 % and 99.74 % identity to those of the *nodC* from *E. medicae* 1037^T, that the *nodC* sequences of strains NHBM9, NHBM17, NHBM19, NHBM22B and NHBM26 were between 99.34 % and 99.74 % identical to those of *E. meliloti* ATCC 9930^T, and that the similarities of the *nodC* sequences from strains NHBM21, NHBM25 and NHBM29 to those of *N. galegae* symbiovar (sv.) officinalis HAMBI114 ranged from 98.73 % to 99.45 %. A phylogenetic tree showing the relationship between the *nodC* genes from the strains isolated from the nodules of *Melilotus*/*Medicago* showed that they belonged to the species *E. meliloti* and *E. medicae* and were affiliated to the symbiovar meliloti of *E. meliloti* (Fig. 2), whereas the strains that were related to the species *N. huautlense* were affiliated with the symbiovar officinalis of *N. huautlense* (Fig. 3).

3.2 Plant nodulation tests

Plant nodulation tests under greenhouse conditions showed that the representative strains formed effective nodules on the roots of their corresponding host plants. Indirect indications of effectiveness of the nodules for nitrogen fixation were obtained by visual inspection of the presence of the red leghemoglobin protein in nodule cross-sections and by the dark green intensity of the leaves compared to uninoculated control plants. No nodulation was detected when strains in the genera *Paenibacillus*, *Brevibacillus* and *Variovorax* were used for inoculation.

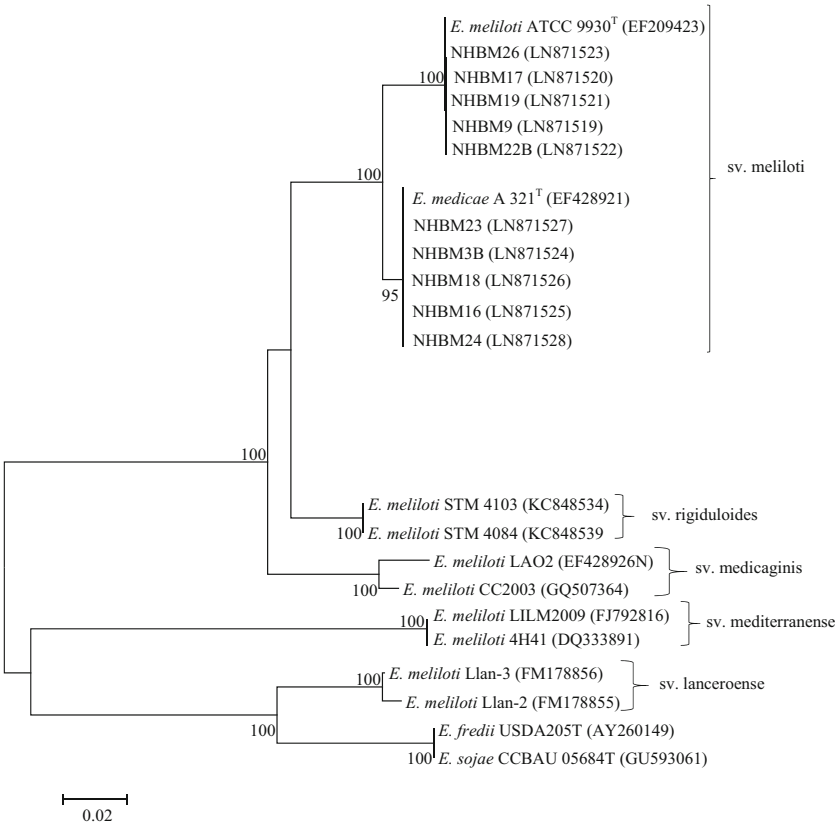

Fig. 2 Neighbor-joining phylogenetic tree based on partial *nodC* sequences of strains from nodules of *Melilotus* and *Medicago* species and of *E. meliloti* symbiovars. Bootstrap values are indicated as percentages derived from 1000 replications. Values lower than 70 are not shown. Bar, 2 nucleotide substitution per 100 nucleotides

4 Discussion

In this study, the isolation and identification of rhizobial bacteria from root nodules of *Melilotus*, *Medicago* and *Trigonella* plants growing in the wild is reported. Collectively, out of the 24 strains isolated, 12 were identified as *E. meliloti*, 9 as *E. medicae* and 3 as *N. huautlense*. These results suggest that the diversity of *Ensifer* strains nodulating species in the *Melilotus-Medicago-Trigonella* inoculation group in Egypt is marked by the predominance of two genetic types, *E. meliloti* and *E. medicae*, although the frequency with which *E. meliloti* was isolated was slightly higher (50 %) than that of *E. medicae* (37.5 %). Since the nodules were sampled from wild plants growing in very different locations throughout Egypt, the data suggest that there are no biogeographical differences among the symbionts. A much higher predominance of *E. meliloti* over *E. medicae* was reported after isolation of rhizobia from nodules of *Melilotus*, *Medicago* and *Trigonella* growing in a Central Asian soil (Roumiantseva et al. 2002) and from nodules of *Medicago sativa*, *Medicago lupulina* and

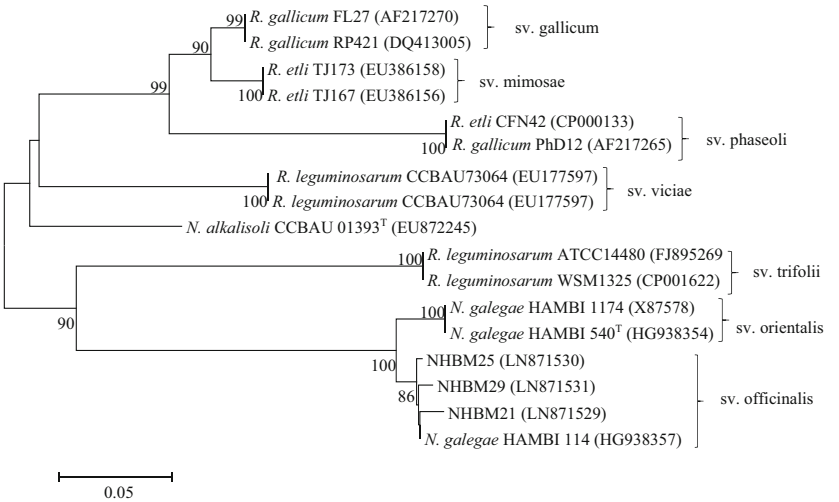

Fig. 3 Neighbor-joining phylogenetic tree based on partial *nodC* sequences of strains from nodules of *Medicago* species and of *Rhizobium* symbiovars. Bootstrap values are indicated as percentages derived from 1000 replications. Values lower than 70 are not shown. Bar, 5 nucleotide substitution per 100 nucleotides

Medicago polymorpha growing in Mexican soils (Silva et al. 2007). However, a predominance of *E. medicae* has been observed in the nodules of *M. lupulina* and *T. foenum-graecum* grown in Spanish soils (Iglesias et al. 2007). With regard to *Melilotus*, *Medicago* and *Trigonella* symbionts from the Mediterranean basin, Zakhia et al. (2004) have shown that, in contrast with our data, *Medicago sativa* is nodulated by *E. meliloti* in infra-arid soils from Tunisia, and Sebbane et al. (2006) found that *Medicago polymorpha* grown in Algeria is not nodulated by *E. medicae* but by taxonomically unidentified rhizobial strains. In addition, Arbi et al. (2015) have reported that *Melilotus indicus* growing wild in the Algerian Sahara forms nodules with *E. meliloti*, which agrees with the present study that both *E. meliloti* and *E. medicae* nodulate this legume species. Similar to the Egyptian species, *Medicago polymorpha* when introduced into Australia is nodulated by *E. medicae*, and it is particularly associated with annual *M. polymorpha* that are well adapted to moderately acid soils (Garau et al. 2005). The pH of the soils in which the Egyptian legumes were grown was not determined, so a relationship between pH and the predominant *Ensifer* species found in the nodules from the present study cannot be determined.

Regardless of the legume species, *E. meliloti* and *E. medicae* were isolated from nodules of both *Melilotus* and *Medicago*, but *E. medicae* was not detected within nodules from *T. maritima* (Table 1). In this study *Trigonella* were nodulated by *E. meliloti*, a result coincident with that of He et al. (2011) who showed that *T. arcuata* is also nodulated by *E. meliloti*. Indeed, *E. meliloti* strains CCBAU 83848, CCBAU 83856, CCBAU 83881, CCBAU 83884, and CCBAU 83887 isolated from *T. arcuata* clustered together with those from *T. maritima* that were isolated during the present study (Fig. 1). On the other hand, as only four strains were isolated from *T. maritima*, additional surveys are required to understand the consistency of the association between it and *E. meliloti*.

With a frequency of 12.5 %, the species *N. huautlense* was isolated exclusively from nodules of the genus *Medicago*, including *Medicago sativa*, *Medicago polymorpha* and *Medicago laciniata* (Table 1). To our knowledge this is the first report showing nodulation of *Medicago* by this rhizobial species. *Rhizobium huautlense* was first isolated from *Sesbania herbacea* (Wang et al. 1998) and, together with *R. galegae*, *R. alkalisoli* and *R. vignae*, has been shown to form a separate clade from *Rhizobium* which represents a new genus, and for which the name *Neorhizobium* was proposed (Mousavi et al. 2014).

Analysis of the *nodC* sequences revealed that strains isolated from *Melilotus* belonged to symbiovar meliloti of *E. meliloti* (Fig. 2), and that those from nodules of *Medicago* were affiliated with either *E. meliloti* symbiovar meliloti or with *N. huautlense* symbiovar officinalis (Fig. 3). A high degree of similarity (higher than 99 %) was found between the *nodC* genes of *E. medicae* and *E. meliloti*. These results agree with those of Ramírez-Bahena et al. (2015) who suggested that symbiovar meliloti should be recognised within the species *E. medicae*. Because of the very few divergences found among the sequences within each species, diversity is scarce among the strains isolated in this study. The 12 *E. meliloti*, 9 *E. medicae* and the 3 *N. huautlense* strains identified in this study are true symbionts of their corresponding host legumes as, after nodule isolation, they were able to establish effective symbioses with the *Medicago sativa*, *Medicago polymorpha* and *T. maritima* seedlings that were used for the plant inoculation tests. Cross-inoculation tests were not carried out in this study.

Nodules from *Melilotus indicus* also harbored species in the genera *Paenibacillus*, *Variovorax* and *Brevibacillus* (Table 1), but the isolation of these endophytes does not necessarily mean that they are restricted to *Melilotus indicus*. Together with rhizobia, legume nodules are occupied by a variable microbiome composed of very phylogenetically diverse bacteria, mainly species, genera, families and classes within the phyla Proteobacteria, Firmicutes and Actinobacteria (reviewed in Velázquez et al. 2013; Peix et al. 2015). Nodule endophytes have been shown to produce indole acetic acid (IAA) and siderophores, to express both N_2-fixation and 1-amino-cyclopropane 1-carboxylate (ACC) deaminase activities, and are also involved in antifungal biocontrol. However, although all the aforementioned traits are related to plant growth promotion ability (Pérez-Montaño et al. 2014), the isolation of endophytic bacteria from nodules is also considered to be a source of potential confusion when trying to identify the actual nodulating symbiont (Gyaneshwar et al. 2011). Moreover, recent work has shown that some endophytes have the capacity to accompany rhizobial cells during the infection process to enter inside the root nodules using it as a niche without any obvious benefit to the plant (Zgadzaj et al. 2015).

Acknowledgments This study was supported by ERDF-cofinanced grant PE12-AGR1968 from Consejería de Economía, Innovación y Ciencia (Junta de Andalucía, Spain).

References

Arbi SB, Chekireb D, Quatrini P, Catania V, Cheriet D, Ouartsi A (2015) Phenotypic and genotypic characterization of root nodules rhizobia of *Medicago littoralis* Rhode and *Melilotus indicus* (L.) All. growing in the Oasis of Touggourt, Oued Righ Valley, in the Algerian Sahara. Symbiosis 66:75–87

Chun J (2001) PHYDIT version 3.1 (available at http://plaza.snu.ac.kr/~jchun/phydit/)

de Lajudie P, Willems A, Pot B, Dewettinck D, Maestrojuan G, Neyra M, Collins MD, Dreyfus B, Kersters K, Gillis M (1994) Polyphasic taxonomy of Rhizobia: emendation of the genus *Sinorhizobium*

and description of *Sinorhizobium meliloti* comb. nov., *Sinorhizobium saheli* sp. nov., and *Sinorhizobium teranga* sp. nov. Int J Syst Bacteriol 44:715–733

del Villar M, Rivas R, Peix A, Mateos PF, Martínez-Molina E, van Berkum P, Willems A, Veláquez E (2008) Stable molecular weight RNA profiling showed variations within *Sinorhizobium meliloti* and *Sinorhizobium medicae* nodulating different legumes from the alfalfa cross-inoculation group. FEMS Microbiol Lett 282:273–281

Fahraeus A (1957) The infection of clover root hairs by nodule bacteria studied by a simple glass slide technique. J Gen Microbiol 16:374–381

Garau G, Reeve WG, Brau L, Deiana P, Yates RJ, James D, Tiwari R, O'Hara GW, Howieson JG (2005) The symbiotic requirements of different *Medicago* spp. suggest the evolution of *Sinorhizobium meliloti* and *S. medicae* with hosts differentially adapted to soil pH. Plant Soil 276:263–277

Graham PH (2008) Ecology of the root-nodule bacteria of legumes. In: Dilworth MJ, James EK, Sprent JI, Newton WE (eds) Nitrogen-fixing leguminous symbioses. Springer, Dordrecht, pp. 23–43

Gyaneshwar P, Hirsch AM, Moulin L, Chen WM, Elliott GN, Bontemps C, Estrada-de Los Santos P, Gross E, Dos Reis FB, Sprent JI, Young JP, James EK (2011) Legume-nodulating betaproteobacteria: diversity, host range and future prospects. Mol Plant-Microbe Interact 24: 1276–1288

He YR, Wang JY, Wang ET, Feng G, Chang YL, Sui XH, Chen WX (2011) *Trigonella arcuata*-associated rhizobia—an *Ensifer* (*Sinorhizobium*) *meliloti* population adapted to a desert environment. Plant Soil 345:89–102

Herrera-Cervera JA, Cabello-Mellado J, Laguerre G, Tichy HV, Requena N, Amarger N, Martínez-Romero E, Olivares J, Sanjuan J (1999) At least five rhizobial species nodulate *Phaseolus vulgaris* in a Spanish soils. FEMS Microbiol Ecol 30:87–97

Hou BC, Wang ET, Ying LJ, Jia RZ, Chen WF, Gao Y, Don RJ, Chen WX (2009) *Rhizobium tibeticum* sp. nov., a symbiotic bacterium isolated from *Trigonella archiductis-nicolai* Vassilez. Int J Syst Evol Microbiol 59:3051–3057

Howieson JG, Yates RJ, Foster JKJ, Real D, Besier RD (2008) Prospects for the future use of legumes. In: Dilworth MJ, James EK, Sprent JI, Newton WE (eds) Nitrogen-fixing leguminous symbioses. Springer, Dordrecht, pp. 363–387

Iglesias O, Rivas R, García-Fraile P, Abril A, Mateos PF, Martinez-Molina E, Velázquez E (2007) Genetic characterization of fast-growing rhizobia able to nodulate *Prosopis alba* in North Spain. FEMS Microbiol Lett 277:210–216

Kearse M, Moir R, Wilson M, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Meintjes P, Drummond A (2012) Geneious basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28:1647–1649

Kim OS, Cho YJ, Lee K, Yoon SH, Kim M, Na H, Park SC, Jeon YS, Lee JH, Yi H, Won S, Chun J (2012) Introducing EzTaxon: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. Int J Syst Evol Microbiol 62:716–721

Laguerre G, Nour SM, Macheret V, Sanjuan J, Drouin P, Amarger N (2001) Classification of rhizobia based on *nodC* and *nifH* gene analysis reveals a close phylogenetic relationship among *Phaseolus vulgaris* symbionts. Microbiology 147:981–993

Lesins KA, Lesins I (1979) Genus *Medicago* (Leguminosae), a taxonomic study. Junk, The Hague

Mousavi SA, Österman J, Wahlbergb N, Nesmec X, Lavirec C, Vial C, Paulind L, de Lajudie P, Lindström K (2014) Phylogeny of the *Rhizobium-Allorhizobium-Agrobacterium* clade supports the delineation of *Neorhizobium* gen. nov. Syst Appl Microbiol 37:208–215

Pandey P, Sahgal M, Maheswari DK, Johri BN (2004) Genetic diversity of rhizobia isolated from medicinal legumes growing in the sub-Himalayanm region of Uttaranchai. Curr Sci 86:202–207

Peix A, Ramírez-Bahena MH, Velázquez E, Bedmar EJ (2015) Bacterial associations with legumes. Crit Rev Plant Sci 34:17–42

Pérez-Montaño F, Alias-Villegas C, Bellogin RA, del Cerro P, Espuny MR, Jiménez-Guerrero I, López-Baena FJ, Ollero FJ, Cubo T (2014) Plant growth promotion in cereal and leguminous agricultural important plants: from microorganism capacities to crop production. Microbiol Res 169:325–336

Rajendran R, Patel MH, Joshi SJ (2012) Isolation and characterization of nodule-associated *Exiguobacterium* sp. from the root nodules of fenugreek (*Trigonella foenum-graecum*) and their possible role in plant growth promotion. Int J Microbiol 2012:693982

Ramírez-Bahena ME, Vargas M, Martín M, Tejedor C, Velázquez E, Peix A (2015) Alfalfa microsymbionts from different ITS and *nodC* lineages of *Ensifer meliloti* and *Ensifer medicae* symbiovar meliloti established efficient symbiosis with alfalfa in Spanish acid soils. Appl Microbiol Biotechnol 99:4855–4865

Rivas R, García-Fraile P, Velázquez E (2009) Taxonomy of bacteria nodulating legumes. Microbiol Insights 2:251–269

Rome S, Fernández MP, Brunel B, Normand P, Cleyet-Marel JC (1996) *Sinorhizobium medicae* sp. nov., isolated from annual *Medicago* spp. Int J Syst Bacteriol 46:972–980

Roumiantseva ML, Andronov EE, Sharypova LA, Dammann-Kalinowski T, Keller M, Young JP, Simarov BV (2002) Diversity of *Sinorhizobium meliloti* from the Central Asian alfalfa gene Center. Appl Environ Microbiol 68:4694–4697

Saitou N, Nei M (1987) The neigbour-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Sebbane N, Sahnoune M, Zakhia F, Willems A, Benallaoua S, de Lajudie P (2006) Phenotypical and genotypical characteristics of root-nodulating bacteria isolated from annual *Medicago* spp. in Soummam Valley (Algeria). Lett Appl Microbiol 42: 235–241

Silva C, Kan FL, Martinez-Romero E (2007) Population genetic structure of *Sinorhizobium meliloti* and *S. medicae* isolated from nodules of *Medicago* spp. in Mexico. FEMS Microbiol Ecol 60:477–489

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance and maximum parsimony methods. Mol Biol Evol 28:2731–2739

van Berkum P, Beyene D, Bao G, Campbell TA, Eardly BD (1998) *Rhizobium mongolense* sp. nov. is one of three rhizobial genotypes identified which nodulate and form nitrogen-fixing symbioses with *Medicago ruthenica* [(L.) Ledebour]. Int J Syst Bacteriol 48:13–22

Velázquez E, García-Fraile P, Ramírez-Bahena MH, Peix A, Rivas R (2010) Proteobacteria forming nitrogen fixing symbiosis with higher plants. In: Sezenna ML (ed) Proteobacteria: phylogeny, metabolic diversity and ecological effects. Nova Science Publishers, New York, pp. 37–56

Velázquez E, Martínez-Hidalgo P, Carro L, Alonso P, Peix A, Trujillo ME, Martínez-Molin E (2013) Nodular endophytes: an untapped diversity. In: Rodelas-González MB, González-López J (eds) Beneficial plant-microbial interactions: ecology and applications. CRC Press, Boca Raton, pp. 214–235

Vincent JM (1970) A manual for the practical study of root nodule bacteria. Blackwell Scientific Publications, Oxford

Wang ET, van Berkum P, Beyene D, Sui XH, Dorado O, Chen WX, Martínez-Romero E (1998) Rhizobium huautlense sp. nov., a symbiont of Sesbania herbacea that has a close phylogenetic relationship with Rhizobium galegae. Int J Syst Bacteriol. 3:687–699

Yan AM, Wang ET, Kan FL, Tan ZY, Sui XH, Reinhold-Hurek B, Chen WX (2000) *Sinorhizobium meliloti* associated with *Medicago sativa* and *Melilotus* spp. in arid saline soils in Xinjiang, China. Int J Syst Evol Microbiol 50:1887–1891

Zakhia F, Jeder H, Domergue O, Willems A, Cleyet-Marel JC, Gillis M, Dreyfus B, de Lajudie P (2004) Characterisation of wild legume nodulating bacteria (LNB) in the infra-arid zone of Tunisia. Syst Appl Microbiol 27:380–395

Zgadzaj R, James EK, Kelly S, Kawaharada Y, de Jonge N, Jensen DB, Madsen LH, Radutoiu S (2015) A legume genetic framework controls infection of nodules by symbiotic and endophytic bacteria. PLoS Genet 11:e1005280