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PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF MICROSYMBIONTS ASSOCIATED WITH *CROTALARIA MEDICAGENIA* : A NATIVE LEGUME OF THE INDIAN THAR DESERT

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Abstract

In present investigation, the phenotypic as well as genetic diversity of twenty root nodule bacterial strains isolated from root nodules of *Crotalaria medicagenia* growing in Thar Desert of India, were investigated. All bacterial isolates were fast growing and highly diverged in their phenotypic characteristics such as salt and pH tolerance. All isolates distributed into three groups based on ARDRA (amplified r-DNA restriction analysis) pattern while formed eleven groups on the basis RAPD (random amplified polymorphic DNA) patterns, which indicates high genetic diversity among them. The 16S rDNA sequencing and Blastn result of seven isolates suggested that microsymbionts of *C. medicagenia* belong to the only *Ensifer* genus. On the basis of phylogenetic analysis of 16S r-RNA gene sequences, these *Ensifer* strains closely related to old world rhizobia (*E. kostiensis, E. saheli, E. terangae*) as well as new world rhizobia (*E. mexicanus, E. americanus*), but formed new lineages, thus these are novel strains of *Ensifer*. It is suggested that in the alkaline soil of the Thar Desert *C. medicagenia* is nodulated by diverse *Ensifer* species.

Key words : *C. medicagenia*; *Ensifer*; Thar Desert; 16S r-RNA gene.

Introduction

The legume plants are nodulated by a diverse group of bacteria, collectively known as rhizobia (Sprent, 2001). The legume-rhizobia symbiosis is one of the most important plant microbe interactions on the Earth which fix the nitrogen and play an important role in the nitrogen cycle. These rhizobia are a group of gram negative soil bacteria that are genetically diverse and physiologically heterogeneous and capable of eliciting nodules on leguminous plant roots, where atmospheric nitrogen reduced to ammonia. There are currently more than 100 symbiotic root nodule bacteria species within 13 genera which are taxonomically diverse members of α and β subclasses of the Proteobacteria (Moulin *et al.*, 2001; Chen *et al.*, 2003; Sy *et al.*, 2001; Bontemps *et al.*, 2015).

The *Crotalaria* L. is an important taxon of Papilionoidecae and comprised by more than 700 species (Lewis *et al.*, 2005), which are mainly distributed in tropical and sub-tropical regions of the world (Polhil, 1982). In India, it is the largest legume taxa having 93 species of which 27 are endemic (Ansari, 2008). Many species of *Crotalaria* have great economic importance in term of for fibers, silage and green manure which are its agronomic trails (Bhagya and Sridhar, 2009; Polhil, 1982). *Crotalaria* plants have a high dry matter production potential and are able to grow on nitrogen deficient poor soil. In different part of the world the *Crotalaria* spp. has been found to be nodulated by *Bradyrhizobium, Methlylobacterium, Rhizobium, Mesorhizobium* strains (Samba *et al.*, 1999; Sy *et al.*, 2001; Liu *et al.*, 2007; Rocha *et al.*, 2011).

The Great Indian Thar Desert which is the 18th largest desert in the world and biggest in India (Bhandari, 1990, Sprent and Gehlot, 2010) possesses several economically important native legume genera including *Crotalaria* (Lewis *et al.*, 2005). *C. medicagenia* Lam. is an erect and branched annual herb, grow in farms and loans and widely distributed in Thar Desert. It has trifoliate leaves with raceme inflorescence (5-15 yellow flowers) and used as favorable camel feed in north western India. Characterization of native rhizobial diversity is required in order to exploit the biological nitrogen fixation efficiently, for improvement of agricultural productivity. Recently,

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Gehlot *et al.* (2012; 2013) observed nodulation in several native legumes and also characterized their microsymbionts in arid and semi-arid regions of Thar Desert, but still many legume plant species are unexplored. Whole genome of one of the *Ensifer* strain associated with *Tephrosia wallichii* had also been isolated recently from Thar Desert of India (Tak *et al.*, 2013). The present study was carried out to investigate nodulation status of *C. medicagenia* in the arid region and also to characterize their associated microsymbiont at phenotypic as well as genetic level.

Materials and Methods

Nodule sampling, isolation of rhizobia

In order to explore rhizobial diversity associated with *Crotalaria medicagenia*, a survey was conducted at 14 sampling sites throughout Thar Desert (table 1) in the monsoon and post monsoon season (July–September) in the years 2010-2012. The plant was identified with the help of Flora of the Indian Desert (Bhandari, 1990) as well as taking help from regional office of the Botanical Survey of India (BSI). Whole plant were excavated with intact root system and then thoroughly washed with tap water. Roots were gently separated from one another and then position (attachment on roots), numbers, color, shape and size of nodules were recorded and 4-5 nodulated plants were kept moist and brought to the laboratory for preservation of nodules and isolation of rhizobia. The viable seeds of *Crotalaria medicagenia* were also collected to perform host authentication experiments. Root nodule bacterial strains were isolated and purified from sterile root nodules of *Crotalaria medicagenia* according to standard procedure as described by Vincent (1970) and Somasegaran and Hoben (1994). All purified bacterial strains were maintained on congo red-Yeast Extract Mannitol Agar (CR-YEMA) and incubated at 28ºC temperature in an incubator. YEM agar plates were checked regularly to record colony characteristics of isolates and purity. In addition rhizospheric soil from each sampling sites were also collected to determine the pH of sampling sites.

Phenotypic characteristics of isolates

All isolates were characterized for their phenotypic features such as acid or alkali production, utilization of carbon sources, resistance to antibiotics, pH and NaCl tolerance by using standards methods as described in Cappuccino and Sherman (2007) and Somasegaran and Hoben (1994). Acid or alkali production was visually determined on the YEM medium supplemented with bromothymol blue as a pH indicator. Tolerance to sodium chloride (NaCl) was assessed by inoculating each bacterial culture to YEMA plates supplemented with different concentration of salt from 0.5 to 4% (w/v). Tolerance to acidic or alkaline pH was determined by spot inoculation of fresh and activated bacterial cultures to YEMA plates. The pH was adjusted (pH4 to pH11) by using 1N HCl and 1N NaOH (Cappuccino and Sherman 2007) and media were buffered with 20mM HOMOPIPES (pH 4.5; 5.0), 40mM MES (pH 5.5; 6.0), 30mM HEPES (pH 6.8 – 8.2) and CHES (pH 9.0-10). Growth was examined after an incubation of 2 or 5 days for the fast-growing or slow-growing bacteria, respectively.

Authentication and host range of isolates

All isolates were evaluated for their ability to form nodules on their original host plant. Scarified seeds of *C. medicagenia* were surface sterilized by sequentially treatment with 95% (v/v) ethanol for 2 min and 0.1%$HgCl_2$ for 3 min followed by several washes with sterile distilled water to eliminate any trace of $HgCl_2$ and then allowed to germinate in petri-plates, containing sterilized moist filter paper. Three to four germinated seedlings were planted aseptically into each plastic pot containing washed and sterilized sand, with two replicate. Each seedling was inoculated with 1 ml (10^9cell/ml) bacterial culture at exponential growth phase. Non-inoculated seedlings, either supplied with mineral nitrogen (as 0.1% KNO_3 in nutrient solution) or grown without nitrogen, served as positive (N^+) and negative controls (N^-), respectively. Seedlings were grown under natural sunlight and temperature in green house. Nodule assessment and harvesting accomplished after 6-8 weeks of inoculation as described by Somasegaran and Hoben (1994). Rhizobia were re-isolated and purified from nodules of these harvested plants and matched with the parental strains on the basis of colony morphology and genetic fingerprints (RAPD pattern) using RPO1 primers (Richardson *et al.*, 1995).

Molecular fingerprinting by RAPD

All purified and actively grown strains in YEM broth were used for isolation of genomic DNA by using the standard method given by Cheng and Jiang (2006). RPO1based primer-(nif gene directed primer widely used for study of genetic diversity) was used to randomly amplify genomic DNA and pattern studied as described by Richardson *et al.* (1995). The reactions were carried out in a final volume of 20ìl containing: 1.5ìl of template DNA (90 ng/µl), 1U of Taq DNA polymerase, 3µM of PRO1 primer, 3.75mM $MgCl_2$, 150µM of each dNTP and 1X PCR buffer. The PCR cycling condition were as follow: initial denaturation at 94°C for 5 min. followed by

5 cycles at 94°C for 30 s, 50°C for 60 s, 72°C for 90 s and then 30 cycles at 94°C for 30 s, 55°C for 25 s and 72°C for 90 s and a final extension at 72°C for 5 min. The amplified DNA fragments were separated by running the PCR products on 2.0% agarose gel (containing Ethidium bromide) at 80 V for 1-2 hrs along with DNA ladder of 100bp and generated band pattern were visually identified under BIO-RAD Gel Doc System (Bio Rad SR+, USA Inc.).

Amplification of 16S rRNA gene and ARDRA pattern (Amplified r-DNA Restriction Analysis)

The universal primers 18F and 1492R (Weisburg *et al.*, 1991) were used for amplification of 16S rRNA gene of bacterial strains. PCR reaction was carried out in a final volume 20ìl containing: 1ìl of template DNA (90 ng/ µl), 0.6U of Taq DNA polymerase, 1.2µM of each of the primers, 2mM $MgCl_2$, 150µM of each dNTP and 1X PCR buffer. The PCR cycling condition as follow: initial denaturation 94°C for 5 min followed by 35 cycles of 94º C for 30 s, 53º C for 30 s, 72º C for 60 s and a final extension at 72°C for 5 min. Amplified PCR products along with 500bp marker were run on 0.89% (w/v) agarose gel, prepared in 1X TAE buffer and were visualized by staining with ethidium bromide under BIO-RAD Gel Doc System.

The amplified PCR products (16S r-DNA) were incubated with restriction endonucleases *Msp*I (10U/ µl) (Genei Bangalore) for overnight at 37º C for restriction digestion. The reactions were carried out in a total final volume of 20 µl containing 1X buffer, 2.5U of *Msp*1 enzyme, 10 µl aliquots of PCR products (amplified 16S r-DNA) and 7.75 µl of nuclease free water. The digested PCR products were separated by running on 2.0% agarose gel at 80 V for 1-2 hrs and restriction band patterns were visualized by staining with ethidium bromide under UV light using BIO-RAD Gel Doc System.

Sequencing of 16S rRNA gene

The amplified product of 16s rDNA were sequenced from outsourcing Xcleris Genomics Labs Ltd., Ahmedabad. Universal external primers 18F; 1492R (Weisburg *et al.*, 1991) and internal primers 800F; 820R (Yanagi and Yamasato, 1993) were used to obtain the almost complete (1.5kb) nucleotides sequence of 16S rRNA gene. The sequencing reactions were performed by using the Applied Biosystems (96 capillary) Big Dye version 3.1 terminator and 5X buffer. Gene Tool Lite 1.0 (2000) software (Doubletwist, Inc., Oakland, CA, USA) and Bio-Edit were used for analysis of chromatograms, sequence editing and FASTA formatting of different partial sequences.

The BLAST algorithm (Altschul *et al.*, 1990) at the National Centre for Biotechnology Information (NCBI) was used to compare a query sequence with a library or database of sequences, and identify library sequences that resemble the query sequence above a certain threshold.

Phylogenetic analysis of gene sequences

All the 16S rDNA sequences of root nodule bacterial strains isolated from *C. medicagenia* were submitted in the GenBank database, and the accession numbers are individually specified in the phylograms. The CLUSTAL W (Thompson *et al.*, 1994), a multiple sequence alignment program were used to align the acquired sequences in present study, together with related sequences of type strains retrieved from the NCBI database. The phylogenetic trees were inferred using the software MEGA 6 (Tamura *et al.*, 2013) with maximum likelihood (Felsenstein, 1981) based on a GTR+G+I mode (Tavare, 1986). The phylogenetic trees were bootstrapped with 1,000 bootstrap replications to attach confidence estimates for the tree topologies.

Results and Discussion

Nodulation status and morphology of nodules

C. medicagenia found in irrigated farm land and in an open land (Fig. 1A) with availability of water throughout all sampling sites in Thar Desert which indicates its sensitivity towards low soil moisture and xerophytic condition. Soil samples and nodules of *C. medicagenia* were collected from all the sampling sites in five districts of Western Rajasthan (table 1). The pH of soils throughout all sampling sites in Thar Desert was alkaline (ranged from pH 7.9 to 9) as described elsewhere (Gehlot *et al.*, 2012). Occurrence of nodules was mostly seen on the secondary and tertiary roots as compared to the nodules on the main root and collar region (Fig. 1B). Nodules collected from the excavated roots system of *Crotalaria medicagenia* in present investigation showed crotalarioid morphology as observed by Corby (1988) and were indeterminate, elongated, branched and pinkish brown in colour (Fig. 1C). Thus *C. medicagenia* is following the tribe character (Crotalarieae) as reported by Sprent (2001). The highest average number of nodules per plant was thirteen recorded in Deh (semi-arid sites from Nagaur) while lowest five in Chohtan (arid sites from Barmer) site where soil was more sandy, poor in texture and climatic condition was more harsh (high temperature and low rain fall) as compared to other sampling sites (semiarid areas). The reduction in nodule number in drought conditions may be due to reduced

Table 1: Nodulation status of *Crotalaria medicagenia* growing at different sampling site throughout the Thar Desert and isolated strains.

District	Site of soil sample	pH of soil	Average no. of nodules /plant	*C. medicagenia*
Jodhpur	JNVU Campus	8.2	9	CM4, CM7
	Kailana	8.2	8	CM8, CM9, CM10
	Pratapnagar	8.8	11	CM11*, CM12
	Shergarh	8.7	10	CM14
Nagaur	Deh	8.5	13	CM3
	Inana	8.1	8	CM5*
Barmer	Bhuka	8.8	6	CM6*
	Chohtan	8.1	5	CM19
	Nimdee	8.6	7	CM20
Jaisalmer	Kuldhera	8.6	9	CM13*, CM16*
	Pokaran	8.7	8	CM18
	Sum	8.1	8	CM1*, CM2
Bikaner	Bikaner	7.9	12	CM17
	Nokha	9.0	11	CM15*

* Strain identified as *Ensifer* sp.

colonization of root surfaces, less infection events and limited multiplication of rhizobia as assumed in several earlier reports (Zahran *et al.*, 1999).

Phenotypic characteristics of isolates and authentication to host

A total of 20 fast growing bacterial strains were isolated and purified from root nodules of *C. medicagenia*. All isolates (except CM9 and 10) showed rhizobia like colony characteristics such as white, translucent or opaque, raised, entire margin, shining with smooth edge, highly mucilaginous or non- mucilaginous colonies (Somasegaran and Hoben, 1994)). Two isolates CM9 and 10 showed non rhizobial colony characteristics as mentioned by Somasegaran and Hoben (1994). Phenotypic features of isolates related to BTB reaction, salt and pH tolerance are given in Table 2. All isolates were either acid producer or neutral, those are common to fast growing bacteria strains as reported in previous studies (Saeki *et al.*, 2005; Sharma *et al.*, 2010).

The strains were highly variable in tolerance to NaCl and pH. Most of rhizobial isolates were able to tolerate NaCl up to 2% NaCl while two non rhizobial isolates CM10 and CM9 were able to grow up to 4% and 5% NaCl, respectively. In case of pH tolerance the isolates CM1 and CM6 (*Ensifer* species) tolerated the pH4-11 while *Ensifer* sp. CM5 and CM11 tolerated the pH4-10. Other *Ensifer* sp. CM13 and CM15 tolerated the pH5-11; *Ensifer* sp. CM6 tolerated the pH5-10. The variation in phenotypic characteristics within *Ensifer* strains may be due to plasmid based characters, frequently affected by horizontal gene transfer (HGT) among variety of soil bacteria and stress condition may favour such frequent HGT (Amina and Amin, 2010). Horizontal gene transfer (HGT) has played an important role in bacterial evolution at least since the origins of the bacterial divisions, and it is still powerful driving force for creating phenotypic, biochemical and genetic diversity in bacteria (Amina and Amin, 2010; Davies 1996). Based on our present and previously reported results (Gehlot *et al.*, 2012; 2013) we speculate that harsh conditions in the Thar Desert such as high temperature, variation in diurnal temperature, soil alkalinity, variation in diurnal temperature, water stress conditions are major driving force behinds frequency of HGT among soil bacteria reflecting diversity in phenotypic traits.

All RNB strains (except CM9, CM10 and CM16) were found nodulating their original host *C. medicagenia*. Phenotypically, nodulated plants appeared dark-green and healthier in comparison to control and non-nodulated plants. After recording the nodulation status of inoculated plants, 2-3 isolates were successfully re-isolated and purified from nodules of each host plant. The colony characteristics and RAPD patterns (using RPO1 primer) of re-isolates were exactly similar to corresponding inoculated parental strains.

Molecular characterization

As a rapid method, molecular technique ARDRA and RPO1-PCR has been used extensively in rhizobial ecology for grouping and identifying rhizobia (Richardson *et al.*, 1995; Thies *et al.*, 2001). In present investigation the 20 isolates of *C. medicagenia* formed three groups on the basis of ARDRA patterns (table 3). Group I was the largest and comprised by 18 isolates (including seven sequenced *Ensifer* strains CM1, CM5, CM6, CM11, CM13, CM15 and CM16). Group II and III were contained single isolates CM9 and CM10, respectively and identified as non-rhizobia on the basis of colony characteristics. The 18 isolates of ARDRA group I were resolved into nine groups on the basis of RAPD pattern (table 3), thus RPO1 based fingerprinting gave better resolution of genetic diversity as compared to ARDRA (PCR-RFLP). Similar result also obtained by Sessitsch *et al.* (1997) for the rhizobial isolates of *Phaseolus vulgaris* from Australia.

The solely occupancy of *Ensifer* strains in root

Table 2: Phenotypic characteristics of root nodule bacterial strain isolated from *C. medicagenia*.

Isolates	NaCl tolerance [% of salt (w/v)]	Range of pH tolerance	BTB reaction
CM1	1	4-11	Neutral
CM2	2	4-11	Acidic
CM3	2	4-11	Neutral
CM4	2	5-10	Neutral
CM5	3	4-10	Neutral
CM6	1	5-10	Acidic
CM7	2	6-11	Neutral
CM8	2	4-11	Neutral
CM9	5	4-11	Neutral
CM10	4	5-10	Neutral
CM11	2	4-10	Acidic
CM12	2	5-11	Neutral
CM13	3	5-11	Neutral
CM14	2	6-11	Acidic
CM15	2	5-11	Acidic
CM16	2	4-11	Neutral
CM17	2	5-10	Neutral
CM18	2	5-11	Acidic
CM19	2	6-11	Acidic
CM20	2	4-11	Neutral

Table 3: Grouping of root nodule bacterial isolates of *Crotalaria medicagenia* on the basis of RAPD (by using RPO I primer) and ARDRA patterns.

RPO Group	Strains	ARDRA Group
1	CM6*, CM11*, CM12, CM13*, CM14,CM17	I (15 isolates)
2	CM4, CM8	
3	CM1*, CM18	
4	CM2, CM19	
5	CM3, CM20	
6	CM5*	
7	CM7	
8	CM15*	
9	CM16*	
10	CM10	II
11	CM9	III

* Strain identified as *Ensifer* sp on the basis of 16S rRNA gene sequences.

2009; Li *et al.*, 2011). These observations are also supported by genomic studies which suggest that core genomes of the genus *Ensifer* possess several gene clusters involved in osmoregulation and adaptation to alkaline pH (Tian *et al.*, 2012).

Phylogenetic analysis of 16S rRNA gene sequences

On the basis of ARDRA and RAPD (RPO1-PCR) profiles, the 16S rRNA gene of seven 7 isolates (CM1, CM3, CM5, CM6, CM11, CM13, CM15 and CM16) were sequenced and all identified as *Ensifer* sp. on the basis of Blastn sequence similarity search result. The closest 16S rRNA gene BLASTN match of the isolates and the percentage similarity are given in table 4. In phylogenetic analyses, these seven *Ensifer* strains formed four

Table 4: BLASTN sequence similarity percentage of 16S rRNA gene of rhizobial strains isolated from root nodules of *Crotalaria medicagenia,* with closet type strains.

Isolates	NCBI Gen Bank accession no.	Closest Type strain in Blastn (GenBank accession no.)	Sequence similarity (%)	Biologicaland geographical origin of closest type strain
CM1	KJ871659	*Ensifer mexicanus* ITTG-R7^T(DQ411930)	99.8	*Acacia angustissima* Mexico
CM5	KJ871660	*Ensifer terangae* LMG 7834^T (X68388)	99.8	*Acacia laeta,* Senegal
CM6	KF437392	*Ensifer mexicanus* ITTG-R7^T(DQ411930)	99.8	*Acacia angustissima* Mexico
CM11	KJ871661	*Ensifer kostiensis* HAMBI 1489^T (Z78203)	99.7	*Acacia senegal* Sudan
CM13	KJ871662	*Ensifer terangae* LMG 7834^T (X68388)	99.8	*Acacia laeta,* Senegal
CM15	KJ871663	*Ensifer kostiensis* HAMBI 1489^T (Z78203)	99.9	*Acacia senegal* Sudan
CM16	KF437393	*Ensifer adhaerens* LMG 20216^T	99.9	Soil, central PA, USA

nodules of *C. medicagenia* suggested that *Ensifer* strains are well adapted to alkaline soil of Thar Desert and are dominant microsymbiont of many native legumes (Gehlot *et al.*, 2012; 2013). Our present studies again support this contention that alkaline soil and arid conditions favors more occurrences of *Ensifer* strains in root nodules of native legumes rather than other rhizobia (Han *et al.*,

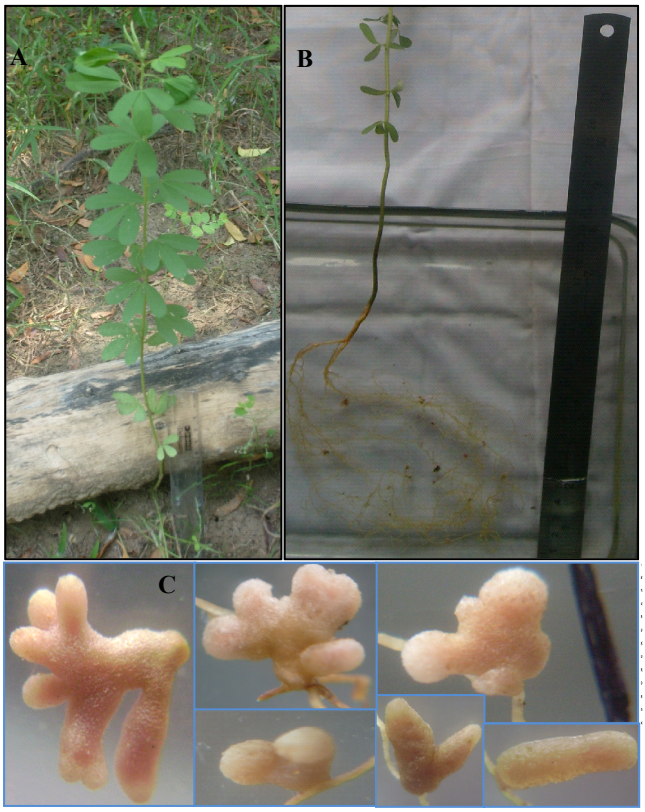

Fig. 1 : *Crotalaria medicagenia*: (A) Field view in open land in rainy season, (B) excavated root nodule system and (C) morphological appearance of nodules (scale bar 5mm).

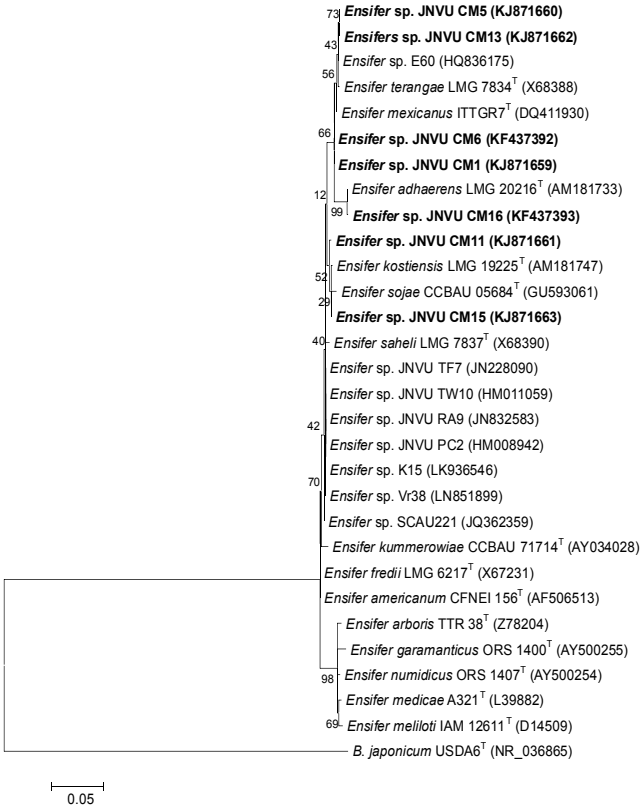

Fig. 2 : Phylogenetic tree constructed using 16S rRNA gene sequences of *Ensifer* strains isolated from the Indian native legume *C. medicagenia* together with those of type strains. The tree was built using a Maximum Likelihood (ML) method and bootstrap values calculated for 1,000 replications are indicated at internodes. The scale bar indicates 5% substitutions per site. Accession numbers from GenBank are in parenthesis. (Abbreviations: CM, *Crotalaria medicagenia*; JNVU, Jai Narain Vyas University and ^{T} indicates type strain).

different clades (fig. 2). Two isolates CM5 and CM13 showed 100 sequence similarities to each other and were clustered together along with *Ensifer* sp. E60 (isolated from acacias in Algeria) and formed I clade in this study that was very close to *Ensifer terangae* LMG 7834^{T} (de Lajudie *et al.*, 1994) with 99.8% sequence similarities (fig. 2, table 4). The strain CM1 and CM6 showed 100% similarities to each other and form II clade which was very close to new world rhizobial type strain *Ensifer mexicanus* ITTG-R7^{T} (99.8% sequence identity) that was isolated from *Acaciella angustissima* in Mexico (Lloret *et al.*, 2007). The III clade had single isolate CM16 and was very close to *Ensifer adhaerens* LMG 20216^{T} (99.9% sequence identity) that was originally isolated from soil in USA. Strain CM11 and CM15 formed the IV clade and showed 99.7% and 99.9% sequence similarities to type strain *Ensifer kostiensis* HAMBI 1489^{T} (Nick *et al.*, 1999), respectively. These two CM11 and CM15 showed 99.8% sequence similarities to each other and also showed 99.6% to *Ensifer* sp. TF7, TW10, RA9 and PC2 isolated from native legumes species of *Tephrosia*, *Rhynchosia*, *Indigofera* and *Prosopis* of the Thar Desert (Gehlot *et al.*, 2012) as well as to *Ensifer* sp. K15 and Vr38 isolated from chickpea and mung bean nodules, respectively, from Pakistan thus these isolates may be originate from the Indian subcontinent.

It can be concluded that microsymbionts of *C. medicagenia* are very diverse in phenotypic traits and also genetically different from the existing type strains of *Ensifer* species. The phylogenetic study confirms that microsymbionts of *C. medicagenia* belong to the *Ensifer* genus and are closely related to old world rhizobia (*E. kostiensis, E. saheli, E. terangae*) as well as new world rhizobia (*E. mexicanus, E. americanus*), but formed new lineages, thus these are novel strains of *Ensifer*. Furthermore polyphasic approach such as DNA–DNA hybridization, Fatty Acid Methyl Ester (FAME) of strains and multilocus sequence analysis (MLSA) of conserved housekeeping genes required for further characterization of these strains and also for description of a formal new

species of *Ensifer* nodulating native legumes of arid regions from the Thar Desert of India.

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