



# Diversity of Nitrogen-Fixing Symbiotic Rhizobia with Special Reference to Indian Thar Desert

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## Abstract

The symbiotic nitrogen-fixing bacteria are found in diverse climatic conditions and are ecologically important. The classification of rhizobia has always been fascinating; with the advent of polyphasic approaches, it is continuously changing by addition of new genera and species and the reclassification and discovery of nontraditional rhizobia. In comparison with crop legumes, the study of symbiotic associations in wild/native legumes has led to the discovery of several genetically diverse rhizobia. In the era of global climate change, increasing desertification, and for food security, the identification and characterization of rhizobia adapted to arid and hot climatic conditions are important. With this aim desert rhizobia associated with several native legumes belonging to different tribes have been broadly studied from less-explored regions of Indian Thar Desert. The diverse legumes in alkaline soils of the Thar Desert are found to be nodulated by traditional rhizobial genera, *Ensifer* and *Bradyrhizobium*. On the basis of core gene phylogeny, the *Ensifer* strains affiliated to mimosoid, cesalpinoid, and papilionoid legumes clustered into novel clades and lineages. *Bradyrhizobium* strains phylogenetically diversified from the *B. yuanmingense* type strain are microsymbiont of species of *Tephrosia*, *Alysicarpus*, *Crotalaria*, and *Chamaecrista* in addition to strains of *Ensifer*. The tree rhizobia (isolated from *Vachellia*, *Senegalia*, *Prosopis*, *Mimosa*) have host range restricted to tree species and therefore could be used as an inoculum in forestry practices. The other native rhizobia isolated from wild legumes (*Tephrosia* and *Chamaecrista*) are compatible with crop legumes (*Vigna*, *Cyamopsis*, *Glycine max*) and can be useful in preparation of consortia for extension of agricultural practices.

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## 2.1 Introduction

The ability to fix atmospheric nitrogen to ammonia is exclusively confined to prokaryotic organisms (diazotrophs) that contain the nitrogenase enzyme complex and the phenomenon is known as biological nitrogen fixation (BNF). Diazotrophs can be broadly divided into two groups: (i) free-living/nonsymbiotic and (ii) mutualistic/symbiotic. Eukaryotic organisms are incapable to fix nitrogen, and therefore few of them establish a symbiotic relationship with diazotrophic bacteria. In these interactions, eukaryotic organisms supply nutrients and energy to the diazotrophs in exchange for fixed nitrogen. Legume-rhizobia symbiosis is one of such plant-microbe interactions that contribute fixed nitrogen to the biosphere in a cost-effective and eco-friendly manner. The symbiotic diazotrophs invade or colonize the root (and occasionally stem) of the host plant, where they multiply and induce nodule organogenesis. Rhizobia are heterotrophic, aerobic, non-sporulated, gram-negative, and rod-shaped nitrogen-fixing bacteria that have coevolved with host legumes (Sprent 2001). Rhizobia are widely distributed in diverse geographical and ecological niches of the world. Besides diazotrophy, a group of rhizobia have been discovered which show methylotrophy, for example, *Methylobacterium* strains isolated from species of *Crotalaria* (Sy et al. 2001), and stem-nodulating photosynthetic bradyrhizobia have been isolated from species of *Aeschynomene* (Molouba et al. 1999). The rhizobia are among the most extensively studied group of bacteria due to their nitrogen-fixing ability and could replace synthetic nitrogen fertilizers, in nitrogen-poor soils (Zahran 2001). Relatively little is known about the nitrogen-fixing value of most of the wild legume species found in arid conditions (Sprent 2001). Rhizobia associated with wild/native legumes establish effective symbiosis under harsh environmental conditions and are more tolerant to abiotic stresses (salinity, alkalinity, drought, elevated temperatures, etc.) than the crop rhizobia (Zahran 2001). Thus symbiotically efficient rhizobia with increased tolerance to high salt, pH, and temperature could enhance the production of food and forage legumes in semiarid and arid regions of the world. There are many potentially nodulated legumes native to arid and semiarid areas worldwide which need to be explored for their microsymbiont diversity (Sprent and Gehlot 2010; Panwar et al. 2014).

All bacteria isolated from root nodules were initially classified into the genus *Rhizobium* until the early 1980s. Later two distinct groups of rhizobia were recognized based on growth on culture medium: fast- and slow-growing. The fast-growing rhizobia are acid-producing and associated mainly with temperate legumes, while the slow-growing (bradyrhizobia) are associated with tropical and subtropical legumes. For a long time, the grouping of root nodule bacteria was based on nodulation with certain host plants called as the cross-inoculation group. The Bergey's manual has played a fundamental role in rhizobial taxonomy. Development in

molecular biology techniques and with the advent of numerical taxonomy considering wide range of characteristics led to the definition of new genera and species and also the renaming of some species (Jordan 1982; Graham et al. 1991; Young et al. 2003). Moulin et al. (2001) published in Nature the nodulation of legumes by *Burkholderia*, a genus that belongs to class beta-proteobacteria. Sawada et al. (2003) reported 44 bacterial species distributed in 12 genera, 10 of which belong to the class “alpha-proteobacteria” (*Allorhizobium*, *Azorhizobium*, *Blastobacter*, *Bradyrhizobium*, *Devosia*, *Mesorhizobium*, *Methylobacterium*, *Ochrobactrum*, *Rhizobium*, and *Ensifer*) and 2 to the class “beta-proteobacteria” (*Burkholderia* and *Cupriavidus*). Peix et al. (2015) reviewed that there are 15 rhizobial genera belonging to proteobacteria under “classical rhizobia” (*Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, and *Ensifer*) and “new rhizobia” (*Aminobacter*, *Burkholderia*, *Cupriavidus*, *Devosia*, *Herbaspirillum*, *Methylobacterium*, *Microvirga*, *Ochrobactrum*, *Phyllobacterium*, and *Shinella*) with nodulating and non-nodulating species. Shamseldin et al. (2017) mentioned about 18 genera of rhizobia with more than 250 species belonging to class alpha- and beta-proteobacteria. At the time of writing this book chapter, the following legume-nodulating genera are known: *Rhizobium*, *Neorhizobium*, *Pararhizobium*, *Ensifer*, *Shinella*, *Mesorhizobium*, *Phyllobacterium*, *Aminobacter*, *Bradyrhizobium*, *Blastobacter*, *Photrhizobium*, *Devosia*, *Azorhizobium*, *Methylobacterium*, *Microvirga*, and *Ochrobactrum* in alpha-proteobacteria and *Paraburkholderia* (Sawana et al. 2014), *Cupriavidus*, *Trinickia*, and *Herbaspirillum* in beta-proteobacteria (List of Prokaryotic Names with Standing in Nomenclature (LPSN); <http://www.bacterio.net>).

## 2.2 Diversity of Classical and Unconventional Rhizobia

Rhizobial systematics is becoming complex and rapidly changing, and recently many new species have been recognized. The term “root nodule bacteria” (RNB) is nowadays used for all groups of bacteria that have been isolated from root nodule; they may be nodulating or non-nodulating. The legume-nodulating bacterial species within class alpha-proteobacteria comprises of six families, namely, *Bradyrhizobiaceae*, *Brucellaceae*, *Hyphomicrobiaceae*, *Methylobacteriaceae*, *Phyllobacteriaceae*, and *Rhizobiaceae*, under the order *Rhizobiales* and families *Burkholderiaceae* and *Oxalobacteraceae* within the order *Burkholderiales* in class beta-proteobacteria. In addition to classical and new rhizobia, there are few reports from the class gamma-proteobacteria (Benhizia et al. 2004; Shiraishi et al. 2010). Non-rhizobial root nodule endophytes belonging to gamma-proteobacteria (*Enterobacter*, *Klebsiella*, *Pantoea*, *Pseudomonas*, *Stenotrophomonas*), Actinobacteria (*Arthrobacter*, *Brevibacterium*, *Microbacterium*, *Micromonospora*, *Mycobacterium*, *Streptomyces*), Firmicutes (*Bacillus*, *Fontibacillus*, *Paenibacillus*), and *Sphingobacteria* (from *Clitoria ternatea*) have also been isolated from many indigenous legumes (Hoque et al. 2011; Aserse et al. 2013; de Meyer et al. 2015; Boukhatem et al. 2016). Martínez-Hidalgo and Hirsch (2017) reviewed the

existence of many non-rhizobial root nodule endophytes, many of them are nitrogen fixers and few of them were able to induce the formation of nodules. Biogeography of nodulated legumes and associated nitrogen-fixing microsymbionts was reviewed by Sprent et al. (2017) in terms of both longitudinal and latitudinal trends. Our knowledge about rhizobial diversity associated with nodulated legumes is limited due to culture-based approaches used to characterize the strains. The root nodules are house for many conventional and unconventional rhizobia. In this chapter we emphasized mainly the diversity of rhizobial genera belonging to class alpha- and beta-proteobacteria and some recent changes made in classification.

## 2.2.1 Alpha-Proteobacteria

### 2.2.1.1 Rhizobiaceae

Family *Rhizobiaceae* comprises of more than 120 sp. that are distributed into the following genera: *Agrobacterium*, *Allorhizobium*, *Ciceribacter*, *Ensifer*, *Neorhizobium*, *Rhizobium*, and *Shinella* (Mousavi et al. 2014). The largest genus *Rhizobium* is extremely heterogeneous and has more than 100 species, comprising of both nodulating and few non-nodulating strains. It has gone through major revision based on multilocus sequence phylogenetic analysis of housekeeping genes. The genus *Neorhizobium* encompasses the former species of *Rhizobium* (*R. alkalisolii*, *R. huautlense*, *R. vignae*, and *R. galegae*), and three new species combinations were described, namely, *N. galegae*, *N. huautlense*, and *N. alkalisolii* (Mousavi et al. 2014). The novel genus *Pararhizobium* recently described by Mousavi et al. (2015) comprised of four new species (*P. giardinii*, *P. capsulatum*, *P. herbae*, and *P. sphaerophysae*).

The genus *Ensifer* (formerly *Sinorhizobium*) (Chen et al. 1988) presently contains 21 species, of which 19 have been validly published based on polyphasic studies. Most of the species have been isolated from root nodules of *Glycine max* (for details of species, see the review by Shamseldin et al. 2017) and tree species from the African continent (de Lajudie et al. 1994; Nick et al. 1999). Few strains (*E. meliloti* and *E. medicae*) nodulate specifically medics, melilots, and spp. of *Trigonella* (de Lajudie et al. 1994; Rome et al. 1996; El Batanony et al. 2015; Gaur et al. 2018) in different parts of the world. From the New World, species like *E. americanus* (from *Acacia acatlensis*) (Toledo et al. 2003) and *E. mexicanus* (*Acacia angustissima*) (Lloret et al. 2007) have been reported. Two species, namely, *E. abri* and *E. indiaense*, isolated from tropical legumes, *Abrus precatorius* and *Sesbania rostrata*, respectively, have been published from India (Ogasawara et al. 2003), but they have not yet been included in the Validation List of the International Journal of Systematic and Evolutionary Microbiology, and no type strains have been submitted so far at international culture collections. Recently new species *E. aridi* (not validly published) has been reported from hot-arid regions of three continents (Asia, Africa, and America). The reported strains of *E. aridi* isolated from different legume host were genetically similar but harbored different *sym* genes (Le Queré et al. 2017).

*Ensifer sojae* has been isolated from root nodules of *G. max* grown in saline-alkaline soils of China (Li et al. 2011). Similarly, Li et al. (2016) reported genetically diverse rhizobia nodulating *Sesbania cannabina* in saline-alkaline soils. Cheng et al. (2002) observed that the nodulation response of *Medicago sativa* and *Medicago murex* differed with soil acidity.

The genus *Allorhizobium* described by de Lajudie et al. (1998) contains one species (*A. undicola*) which effectively nodulates *Neptunia natans* in Senegal. The merging of the three genera (*Agrobacterium*, *Rhizobium*, and *Allorhizobium*) was done into a single genus, *Rhizobium*. On the basis of 16S rRNA phylogenetic analyses, four species of *Agrobacterium* (*A. tumefaciens*, *A. radiobacter*, *A. rhizogenes*, and *A. vitis*) were transferred to the genus *Rhizobium* (Young et al. 2003). Bacteria related to *Agrobacterium* were identified among the root nodules of several tropical leguminous plants from Africa (de Lajudie et al. 1999; Mhamdi et al. 2005). Nodulation and nitrogen-fixing genes were not detected in these strains, and it has been confirmed that these *Agrobacterium*-like strains enter the nodules by mixed infection with a rhizobia capable of inducing nodule resulting in mixed population within the nodule (Mhamdi et al. 2005).

The genus *Shinella* has one species (*S. kummerowiae*), a symbiotic bacterium isolated from root nodules of the herbal legume *Kummerowia stipulacea* grown in Shandong province of China (Lin et al. 2008). Newly described genus, *Ciceribacter* (*C. lividus*), was isolated from rhizosphere soil of *Cicer arietinum* from Kannivadi, India (Kathiravan et al. 2013).

### 2.2.1.2 Phyllobacteriaceae

As per Shamseldin et al. (2017), family *Phyllobacteriaceae* comprises about 49 nitrogen-fixing species within three genera, namely, *Mesorhizobium* (40 species), *Phyllobacterium* (8 species), and *Aminobacter* (1 species). The genus *Mesorhizobium* was described by Jarvis et al. (1997) with the aim to reassign some species previously included in the *Rhizobium* genus. Several *Rhizobium* species were transferred to this genus (*M. loti*, *M. huakuii*, *M. ciceri*, *M. tianshanense*, *M. mediterraneum*). *Mesorhizobium* is a wide spread rhizobial genus nodulating a wide range of legumes in addition to *Cicer arietinum* growing mainly in acidic soils (Peix et al. 2015). However, Zhang et al. (2012) suggested that mesorhizobia are the preferred micro-symbionts of chickpea growing in alkaline soils of northwest China.

Genus *Phyllobacterium* was proposed to accommodate bacterial species isolated from leaf nodules of members of Rubiaceae (*Pavetta*, *Psychotria*, and *Sericanthe*) and Myrsinaceae (*Ardisia*) in tropical continental Africa and Asia (Knösel, 1984). *Chromobacterium lividum* has also been reported from the leaf-nodulated members of these families. *Phyllobacterium trifolii* was isolated from root nodules of *Trifolium pratense* growing in a Spanish soil (Valverde et al. 2005). It harbors symbiotic genes and infectivity tests experiments with this species revealed that it forms nodules on the roots of *Trifolium repens* and *Lupinus albus* (Valverde et al. 2005). Recently *P. loti* was isolated from nodules of *Lotus corniculatus* (Sánchez et al. 2014).

Maynaud et al. (2012) described *Aminobacter anthyllidis*, a metal-resistant bacteria nodulating *Anthyllis vulneraria* a legume host suitable for phytostabilization in mining areas.

### 2.2.1.3 Bradyrhizobiaceae

Family *Bradyrhizobiaceae* contains three nitrogen-fixing genera *Bradyrhizobium*, *Blastobacter*, and *Photobacterium*. The genus *Bradyrhizobium* was created to accommodate slow-growing bacteria capable of establishing nitrogen-fixing symbioses with a broad range of plants belonging to three subfamilies of the family Leguminosae and characterized by an alkaline reaction in culture media. The genus *Bradyrhizobium* presently includes more than 40 species isolated from the nodules of highly divergent legume tribes, including herbaceous and woody species of tropical and temperate origin (Sprent et al. 2017; Shamseldin et al. 2017). Jordan (1982) reported *B. japonicum* from root nodules of *Glycine max*, and since then many species (*B. daqingense*, *B. huanghuaihaiense*, *B. liaoningense*, and *B. ottawaense*) have been isolated which are associated with soybean. Bradyrhizobial species have also been reported as microsymbionts of *Arachis hypogaea* (*B. arachidis*, *B. guangxiense*, *B. guangdongense*, and *B. subterraneum*) and *Vigna unguiculata* (*B. kavangense*, *B. brasilense*, *B. vignae*, and *B. manausense*). Type strain *B. canariense* is an acid-tolerant endosymbiont that effectively nodulates shrubs of the tribes Genisteae and Loteae (Vinuesa et al. 2005). Strain *B. iriomotense* was isolated from a tumor-like root of the legume *Entada koschunensis* from Japan (Islam et al. 2008). Ramírez-Bahena et al. (2009) described two species (*B. pachyrhizi* and *B. jicamae*) that nodulate *Pachyrhizus erosus*. From Morocco (Africa) *B. cytisi* was isolated from nodules of *Cytisus villosus* and *B. retamae* from *Retama monosperma* (Chahboune et al. 2011).

The type strains of *Bradyrhizobium* on the basis of housekeeping genes phylogeny clusters into two mega clades (Ojha et al. 2017). *Bradyrhizobium* strains belonging to two mega clades do not show a particular geographical pattern as observed for type strains of *Ensifer* (Sankhla et al. 2017); instead they have been found intermingled in both clades representing different continents from where bradyrhizobia have been reported. Mega clade-I contains several Asian species (*B. arachidis*, *B. daqingense*, *B. ganzhouense*, *B. guangxiense*, *B. guangdongense*, *B. huanghuaihaiense*, *B. iriomotense*, *B. japonicum*, *B. liaoningense*, and *B. yuanmingense*), African species (*B. cytisi*, *B. kavangense*, *B. rifense*, *B. subterraneum*, and *B. vignae*), and few species from Europe (*B. betae* and *B. canariense*). From the New World, species such as *B. americanum*, *B. centrosemae*, *B. centrolobii*, *B. forestalis*, *B. ingae*, *B. manausense*, *B. neotropicale*, *B. ottawaense*, and *B. stylosanthi* have been reported. The mega clade-II also contains few Asian (*B. erythrophlei*, *B. ferrii*, and *B. lablabi*), African (*B. namibiense* and *B. retamae*), and European (*B. valentinum*) species. Mega clade-II is mainly represented by species isolated from the New World such as *B. brasilense*, *B. elkanii*, *B. embrapense*, *B. icense*, *B. jicamae*, *B. mercantei*, *B. macuxiense*, *B. pachyrhizi*, *B. paxllaeri*, *B. tropiciagri*, and *B. viridifuturi*.

Symbiotic *Bradyrhizobium* strains have also been isolated from the nodules of non-legume plants (*Trema aspera*) growing between rows of tea in the Pangia district of New Guinea (Trinick 1973). The discovery of photosynthetic *Bradyrhizobium* strains that can induce nitrogen-fixing nodules on stems of the legume *Aeschynomene* was made (Molouba et al. 1999). The microsymbionts of *Aeschynomene indica* are unique as they form nodules on plant stems, branches, and roots; and some of them produce the photosynthetic pigments. The genus *Photorhizobium* contains only a single photosynthetically active species *P. thompsonianum* efficiently nodulating *A. indica* (Eaglesham et al. 1990). The aquatic budding bacterium *Blastobacter denitrificans* also forms nitrogen-fixing symbioses with *A. indica* (van Berkum and Eardly 2002).

#### 2.2.1.4 Hyphomicrobiaceae

Family *Hyphomicrobiaceae* includes two nodulating and nitrogen-fixing genera *Devosia* and *Azorhizobium*. The genus *Azorhizobium* described by Dreyfus et al. (1988) includes nitrogen-fixing root and stem-nodulating microsymbiont species that can also fix nitrogen *ex planta* under micro-aerobic conditions. Genus *Azorhizobium* contains three species; two of them effectively fix nitrogen with species of *Sesbania*. *Azorhizobium caulinodans* was isolated from the stem nodules of *Sesbania rostrata* from Africa (Dreyfus et al. 1988), and *A. doebereineriae* is microsymbiont of *Sesbania virgata* in Brazil (de Souza Moreira et al. 2006). The unique feature of *A. caulinodans* is that it fixes nitrogen both in aerobic cultures and in micro-aerobic symbiosis with its legume host *S. rostrata*. Recently strain *A. oxalatifilum* was isolated from macerated petioles of *Rumex* sp. through enrichment in mineral medium.

The genus *Devosia* has single nodulating species, *D. neptuniae*, identified by Rivas et al. (2003) that efficiently nodulates aquatic legume *Neptunia natans* in India. This strain through horizontal transfer has acquired symbiotic genes from a broad host range strain, *Rhizobium tropici* (Rivas et al. 2003).

#### 2.2.1.5 Methylobacteriaceae

Family *Methylobacteriaceae* comprises of two rhizobial genera *Methylobacterium* and *Microvirga* with three and four species, respectively, that can induce nitrogen-fixing nodules on roots of legume plant. The genus *Methylobacterium* includes pink-pigmented facultative methylotrophic (PPFM) bacteria that are strictly aerobic and able to grow on one-carbon compounds such as formate, formaldehyde, and methanol as sole carbon source. Sy et al. (2001) reported facultative methylotrophic species forming nodules in the *Crotalaria* species. The type strain *M. nodulans* was isolated from *C. podocarpa* from Senegal (Jourand et al. 2004). *Methylobacterium* strains within the root nodules obtain carbon from photosynthates of host plant as well as from methylotrophy. Madhaiyan et al. (2006) isolated several nodulating and plant-growth promoting *Methylobacterium* species from tropical legumes.

Ardley et al. (2012) reported three species of *Microvirga*, namely, *M. lupini*, isolated from nitrogen-fixing nodules of *Lupinus texensis* in Texas, and *M. lotononidis* and *M. zambiensis* from *Listia angolensis* collected in Zambia.

### 2.2.1.6 Brucellaceae

The family *Brucellaceae* contains fast-growing, nitrogen-fixing strains in the genus *Ochrobactrum*, which has two species, namely, *O. lupini* and *O. cytisi*, isolated from the nodules of *Lupinus honoratus* and *Cytisus scoparius*, respectively (Trujillo et al. 2005; Zurdo-Piñeiro et al. 2007).

## 2.2.2 Beta-Proteobacteria

### 2.2.2.1 Burkholderiaceae

Family *Burkholderiaceae* comprises of the two genera *Burkholderia sensu lato* and *Cupriavidus* (former *Ralstonia*). Genus *Burkholderia sensu lato* is a big and complex group including diverse species with different physiological and ecological properties; pathogenic strains have been isolated from animals and humans; some are phytopathogenic and others are beneficially associated with plant (root nodule symbionts and nonsymbiotic strains) and have also been isolated from a very wide range of environmental habitats (soil and water) [Gyaneshwar et al. 2011]. Taxonomy of *Burkholderia sensu lato* has undergone major changes several times based on concatenated phylogenetic analysis of housekeeping gene fragments and whole genome sequences. Sawana et al. (2014) based on molecular signatures and phylogenomic analysis proposed for the division of *Burkholderia sensu lato* containing pathogenic organisms, and a separate new genus *Paraburkholderia* was proposed for harboring environmental species. Eleven species of the genus *Burkholderia* were later transferred to genus *Paraburkholderia*, and a new genus *Caballeronia* was proposed to accommodate 12 species of the genera *Burkholderia* and *Paraburkholderia* (Dobritsa and Samadpour, 2016). Presently this complex group of *Burkholderia sensu lato* is split into the following genera: *Burkholderia sensu stricto* (includes the human and animal pathogens), *Caballeronia* (includes the plant beneficial and environmental strains), and *Paraburkholderia* (includes the nitrogen-fixing legume microsymbionts) (Sawana et al. 2014; Dobritsa and Samadpour 2016). Based on comparative genomic analysis recently, Estrada-de los Santos et al. (2018) described two novel genera *Mycetohabitans* and *Trinickia* which form two distinct and unique clades, including two (*M. endofungorum* and *M. rhizoxinica*) and four (*T. caryophylli*, *T. dabaoshanensis*, *T. soli*, and *T. symbiotica*) new combinations of species.

Initially two legume-nodulating *Burkholderia (sensu lato)* strains, *P. tuberum* STM678<sup>T</sup> and *P. phymatum* STM815<sup>T</sup>, were isolated from root nodules of *Aspalathus carnosa* and *Machaerium lunatum*, respectively (Moulin et al. 2001). Presently, the following legume-nodulating species of genus *Paraburkholderia* have been described including *P. aspalathi*, *P. caballeronis*, *P. caribensis*, *P. diazotrophica*, *P. dilworthii*, *P. kirstenboschensis*, *P. mimosarum*, *P. nodosa*, *P. phenolirup-trix*, *P. piptadeniae*, *P. phymatum*, *P. tuberum*, *P. rhynchosiae*, *P. ribeironis*, *P. sabiae*, and *P. spreintiae* (Estrada-de los Santos et al. 2018). The following species of *Paraburkholderia* are root nodule symbionts of species of *Mimosa* (Estrada-de los Santos et al. 2018): *P. caribensis* (*M. pudica* and *M. diplotricha*); *P.*



*diazotrophica* (*Mimosa* spp.); *P. mimosarum* (*M. pigra* and *M. pudica*); *P. nodosa* (*M. bimucronata* and *M. scabrella*); *P. phenoliruptrix* (*M. flocculosa*); *P. phymatum* (*M. pudica*); and *P. sabiae* (*M. caesalpiniiifolia*). Other beta rhizobia strains (*C. taiwanensis* and *T. symbiotica*) belonging to different genera are specifically symbionts of species of *Mimosa*. Two newly described species *P. piptadeniae* and *P. ribeironis* nodulates piptadenia group (*Piptadenia gonoacantha*, tribe mimoseae) in Brazil (Bournaud et al. 2013, 2017). The following species of *Paraburkholderia* nodulates papilionoids (Estrada-de los Santos et al. 2018): *P. sprentiae* (isolated from *Lebeckia ambigua* root nodules); *P. rhynchosiae* (from root nodules of *Rhynchosia ferulifolia*); *P. dilworthii* (from *Lebeckia ambigua* root nodules); *P. kirstenboschensis* (nodulates papilionoid legumes *Virgilia oroboides*, *Hypocalyptus coluteoides*, *H. oxalidifolius*, and *H. sophoroides* indigenous to South Africa); and *P. tubereum* (nodulates several *Cyclopia* species). *Paraburkholderia* type strains (*P. phymatum* and *P. nodosa*) belonging to mimosoid clade also nodulate the promiscuous papilionoid (*Phaseolus vulgaris*, *Vigna unguiculata*, and *Macroptilium atropurpureum*) legumes of tribe Phaseoleae, whereas the *T. symbiotica* strain has restricted host range (*Mimosa* spp.) and failed to nodulate these promiscuous legumes. Type strain *P. caballeronis* originally isolated from tomato has the ability to effectively nodulate *P. vulgaris* (Martínez-Aguilar et al. 2013). Type strain *P. aspalathi* was isolated from root nodules of the South African legume *Aspalathus abietina* (tribe Crotalariaeae). The legume-nodulating *Paraburkholderia* strains of class beta-proteobacteria possess nodulation genes phylogenetically related to those found in legume symbionts of the class alpha-proteobacteria suggesting that the beta rhizobia have evolved through lateral gene transfers (Chen et al. 2003).

Genus *Cupriavidus* presently contains two rhizobial species, *C. taiwanensis* nodulating *Mimosa* sp. which has been repeatedly isolated from root nodules on the pan-tropical weeds *M. pudica* and *M. diplotricha* in Taiwan (Chen et al. 2001, 2003) and also from India (Gehlot et al. 2013). Another species is *C. necator* that was isolated through trap (in soils of Brazil) experiments from root nodules of two promiscuous legume species, *P. vulgaris* and *Leucaena leucocephala* (da Silva et al. 2012). This species can effectively nodulate other promiscuous legumes such as *M. atropurpureum*, *V. unguiculata*, and *M. caesalpiniaefolia* (da Silva et al. 2012).

### 2.2.2.2 Oxalobacteraceae

The genus *Herbaspirillum* in family *Oxalobacteraceae* within the order *Burkholderiales* was first described to include bacterial strains associated with roots of several cereals. Valverde et al. (2003) reported a nitrogen-fixing bacterium *H. lusitanum* associated with root nodules of *P. vulgaris* plants grown in soil from Portugal. Genus *Herbaspirillum* and another genus *Variovorax* of the beta-proteobacteria have been isolated from root nodules of species of *Acacia* (*A. salicina* and *A. stenophylla*) from Southeastern Australia (Hoque et al. 2011). It has also been isolated from nodules of *Aspalathus linearis*, but nodulation has not been confirmed (Hassen et al. 2012).

### 2.2.3 Gamma-Proteobacteria

Besides nodulating bacteria from alpha- and beta-proteobacteria, there is a report of gamma-proteobacteria nodulating *Hedysarum* sp. (Benhizia et al. 2004). *Enterobacter cloacae* and *E. kobei* were isolated from root nodules of the three species of *Hedysarum* (*H. carnosum*, *H. spinosissimum*, and *H. pallidum*) growing in native stands in different habitats in Algeria (Benhizia et al. 2004). Muresu et al. (2008) concluded there are frequent reports of coexistence of symbiotic culturable rhizobia with non-culturable rhizobia and other endophytic bacterial taxa (of the order *Enterobacteriales* or *Pseudomonadales*) within root nodules suggesting that diversity of bacterial species nodulating legumes may be broader than expected. Shiraishi et al. (2010) noted the ability of *Pseudomonas* sp. to nodulate *Robinia pseudoacacia*. These gamma-proteobacteria strains probably acquired the symbiotic genes from symbiotic-rhizobial species in the soil and rhizosphere through lateral gene transfer.

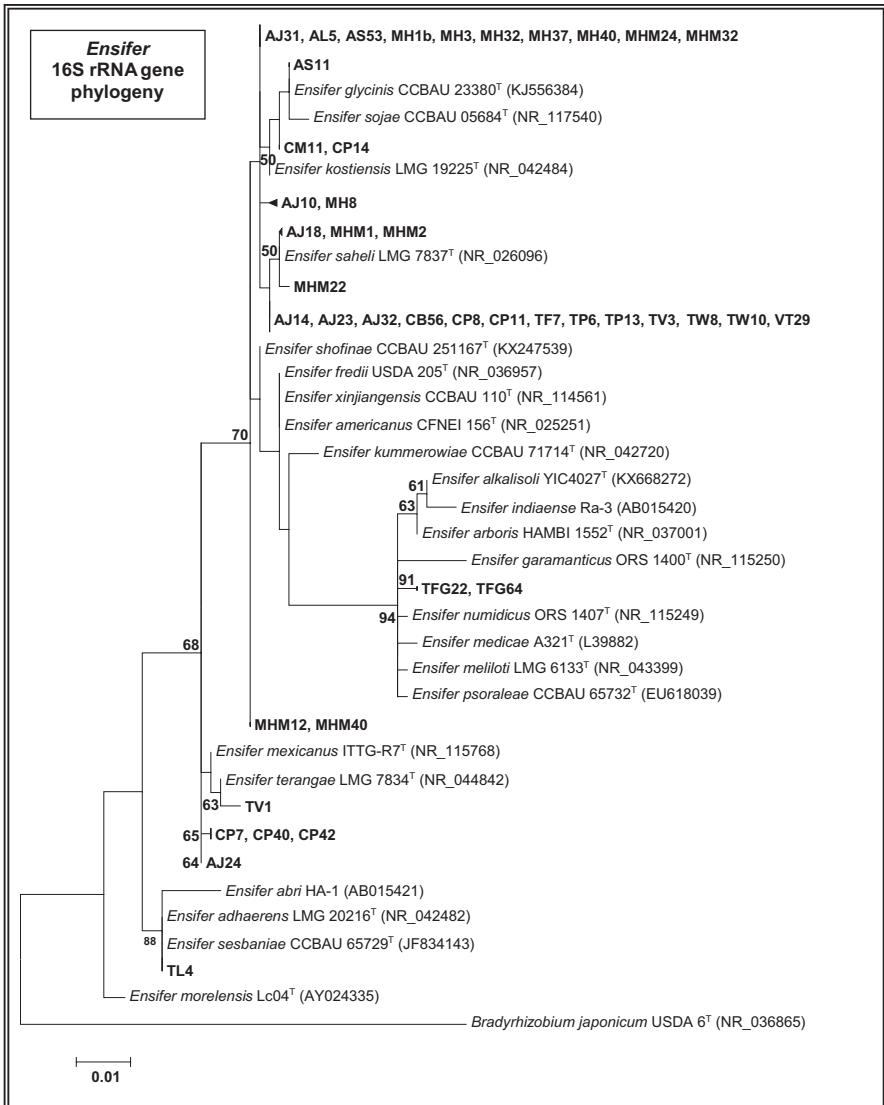
## 2.3 Genetic Diversity of Indian Thar Desert Rhizobia

Based on various criteria such as annual precipitation, temperature, soil, geography, and types of plant community, the deserts can be classified to hyper-arid, arid, and semiarid (Sprent and Gehlot, 2010). The Great Indian Desert or the Indian Thar Desert comes under low altitude arid and semiarid region with an area of about 200,000 km<sup>2</sup>. It is a subtropical desert (<https://www.britannica.com/place/Thar-Desert>), and most of its part (61%) is present in Western Rajasthan which experiences an arid climatic regime (hot desert climate found under the subtropical ridge). The precipitation is low and varies from hyper-arid areas to semiarid areas, some areas have saline tracts, and soils are alkaline with low fertility. The native legumes of this region are subject to extremely stressful and harsh environmental conditions. In arid regions, heat stress affects both the free-living and symbiotic life of rhizobia, while desertification causes a negative impact on legume-rhizobia symbiosis. Still, a large number of legumes of Thar Desert belonging to different subfamilies were reported to be nodulated (Panwar et al. 2014, Gehlot et al. 2012). As questioned by Sprent and Gehlot (2010) “Nodulated legumes in arid and semiarid environments: are they important?” the answer would be yes, these legumes that have coevolved with their rhizobial partners in the Thar Desert have immense potential for fixing atmospheric nitrogen, reforestation, in controlling soil erosion, and increasing the soil fertility. Sprent and Gehlot (2010) mentioned about some nodulating native, perennial drought-tolerant, and drought-escaping annual legumes from the Thar Desert. The rhizobia of wild legumes have novel genotype and better phenotype/traits than the homologous crop rhizobia. Therefore, isolation of effective and promiscuous rhizobia from wild legumes to inoculate other legume crops is a better strategy to improve the efficiency of the rhizobium-legume symbiosis.

In the era of global climate change and for food security, studies such as exploration, identification, and characterization of indigenous nitrogen-fixing microsymbiont associated with native medicinal and food crop legumes are needed. Such studies are more relevant when working on the microsymbionts specific to legumes growing in a particular soil type and climatic conditions. Earlier research on rhizobia-legume symbiosis was restricted to few agriculturally important food legume crops like soybean, common bean, cowpea, species of *Vigna*, and associated species. In the last one decade, several studies have been conducted on molecular characterization and analysis of phylogenetic diversity of root nodule bacteria associated with native/wild legumes (such as species of *Vachellia*, *Senegalia*, *Prosopis*, *Mimosa*, *Chamaecrista*, *Crotalaria*, *Alysicarpus*, *Rhynchosia*, *Tephrosia*, *Indigofera*, *Trigonella*, and *Vigna*) of Indian Thar Desert (Gehlot et al. 2012, 2013, 2014, 2016; Tak et al. 2013, 2016a, b; Panwar et al. 2014; Ojha et al. 2015; Sankhla et al. 2015, 2017, 2018; Choudhary et al. 2017, 2018; Rathi et al. 2017, 2018; Gaur et al. 2018). Panwar et al. (2014) reviewed the status of nodulation of more than 30 native legume species belonging to 3 subfamilies and more than 10 legume genera. Gehlot et al. (2012) reported that in the hot-dry and alkaline soils of Indian Thar Desert (Western Rajasthan), native legumes are nodulated by genetically diverse nitrogen-fixing *Ensifer*, *Bradyrhizobium*, and *Rhizobium* strains. The dominant microsymbionts of most legumes were species of *Ensifer* (Gehlot et al. 2012, 2013; Ojha et al. 2015; Sankhla et al. 2015, 2017, 2018; Tak et al. 2016a, b; Ardley 2017; Choudhary et al. 2017, 2018; Rathi et al. 2017, 2018; Gaur et al. 2018) and few novel *Ensifer* strains have been characterized at genomic level (Tak et al. 2013; Gehlot et al. 2016; Le Queré et al. 2017). Some native legumes including species of *Tephrosia*, *Chamaecrista*, and *Alysicarpus* in the Thar Desert are effectively nodulated by both *Ensifer* and *Bradyrhizobium* strains (Gehlot et al. 2012; Ojha et al. 2015; Tak et al. 2016b; Rathi et al. 2017, 2018).

### 2.3.1 Phylogenetic Diversity of Thar Desert *Ensifer* Strains

Genetically distinct groups of old-world *Ensifer* strains have evolved in the Thar Desert and are more adapted to stressed environment and dominating in the alkaline soils. The Thar-*Ensifer* strains are promiscuous and nodulating papilionoid, mimosoid, and caesalpinoid legumes. In the maximum likelihood phylogenetic tree of the Thar Desert *Ensifer* strains isolated from root nodules of various wild legumes *Vachellia jacquemontii* (AJ), *Vachellia leucophloea* (AL), *Senegalia senegal* (AS), *Crotalaria burhia* (CB), *Chamaecrista pumila* (CP), *Mimosa hamata* (MH), *Mimosa himalayana* (MHM), *Trigonella foenum-graecum* (TFG), *Tephrosia falci-formis* (TF), *Tephrosia leptostachya* (TL), *Tephrosia purpurea* (TP), *Tephrosia villosa* (TV), *Tephrosia wallichii* (TW), and *Vigna trilobata* (VT) of Western Rajasthan based on 16S rRNA gene sequences (Fig. 2.1), the strains clustered into novel clades and lineages. Most of the strain isolated from mimosoid trees and shrubs (MH,



**Fig. 2.1** Maximum likelihood phylogenetic tree of **Thar Desert *Ensifer*** strains isolated from root nodules of various wild legumes of Western Rajasthan based on **16S rRNA** gene sequences. Bootstrap values more than 50% calculated for 1000 replications are indicated at internodes. The scale bar indicates 1% nucleotide substitution per site. (Abbreviation: superscript T stands for type strain)

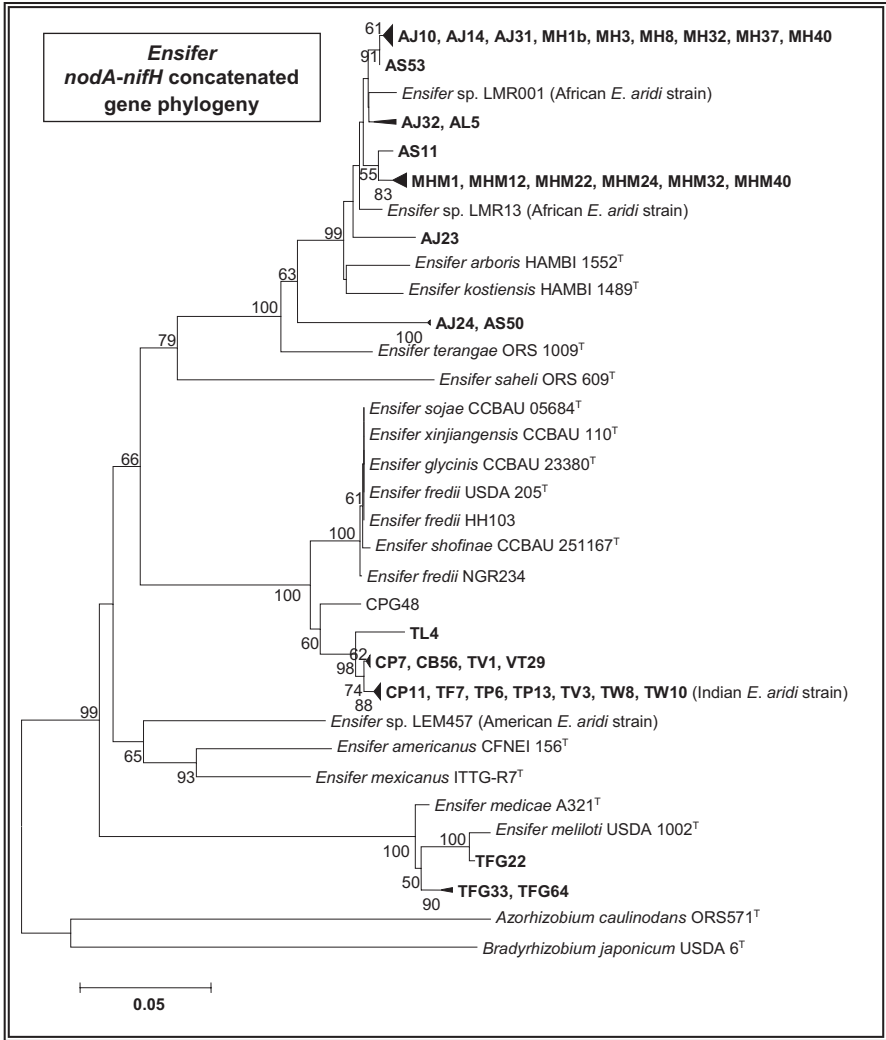
MHM, AJ, AS, AL), papilionoid shrubs/herbs (CB, TF, TP, TV, TW, VT), and caesalpinoid herb (CP) shared close similarity with *E. saheli* LMG 7837<sup>T</sup> (*Sesbania cannabina*, Senegal) (de Lajudie et al. 1994). Few strains (TV1, CP7, CP40, CP42) shared close similarity with *E. terangae* LMG 7834<sup>T</sup> (*Senegalia laeta*, Senegal) (de Lajudie et al. 1994). Strains CM11 and CP14 shared close affinity with another old-world *Ensifer*, *E. kostiensis* HAMBI 1489<sup>T</sup> (*Senegalia senegal*, Sudan) (Nick et al. 1999). Strain AS11 clustered with recently reported type strain *E. glycinis* CCBAU from root nodules of *G. max* in China. A single strain TL4 clustered with *E. adhaerens* LMG 20216<sup>T</sup> and *E. sesbaniae* CCBAU 65729<sup>T</sup> (isolated from *Sesbania cannabina*, China). Microsymbionts of legume, *Trigonella foenum-graecum* (TFG22 and TFG64) as expected, shared close similarity with type strain *E. medicae* A321<sup>T</sup> (*Medicago truncatula*, France). Few mimosoid strains formed distinct lineages within genus *Ensifer* (Fig. 2.1).

The genera in the subfamily Caesalpinioideae found in arid and semiarid regions of India are non-nodulating including species of *Cassia*, *Senna*, and *Parkinsonia* except the genus *Chamaecrista*. *Chamaecrista pumila* is a basal legume in which microsymbionts remain trapped in infection thread and are not dropped out, and these infection threads with symbiosomes are called as fixation threads. Novel findings were made related to microsymbionts of *C. pumila* in the Thar Desert. The genus *Chamaecrista* is known to be nodulated by slow-growing nitrogen-fixing *Bradyrhizobium* strains all over the world (Beukes et al. 2016; dos Santos et al. 2017). However, *C. pumila* growing in arid and semiarid regions of the Thar Desert of India is nodulated by fast-growing *Ensifer* in addition to slow-growing *Bradyrhizobium*. This is probably the first report of *Ensifer* nodulating *C. pumila*. This suggests that the host legume and naturalized fast-growing *Ensifer* of Indian Thar Desert have coevolved and both gained the ability to interact and form a symbiotic association (Rathi et al. 2018).

The limitation of 16S rRNA gene phylogeny is that closely related species cannot always be distinguished due to high level of sequence conservation. Therefore to determine the exact taxonomic position of these *Ensifer* strains, multilocus sequence analysis (MLSA) was performed for few selective strains. The MLSA based on conserved protein-coding housekeeping genes (*glnII*, *atpD*, *recA*, and *dnaK*) suggests that the Thar Desert *Ensifer* strains associated with root nodules of members of the papilionoid, mimosoid, and caesalpinoid legumes are novel species of *Ensifer* as they are significantly divergent from existing type strains of *Ensifer* (Tak et al. 2016b; Sankhla et al. 2017; Rathi et al. 2018). The *Tephrosia-Ensifer* (TP6 and TW10) strains were characterized at genomic level and designated as novel species of *E. aridi* (Le Queré et al. 2017). These Thar Desert *Ensifer* strains were found to be genetically identical to strains isolated from other parts of the world such as *Ensifer* strains from root nodules of *Acacia raddiana* and *Acacia*

*gummifera* from Merzouga Desert in Morocco (Africa) and from wild species of *Phaseolus* (*Phaseolus filiformis*) in the hot desert of Baja California in Mexico. The core genomes of six *Ensifer* strains from three different continents (Asia, Africa, and America) were compared with *Ensifer* type strains. Genome-based species delineation tools such as average nucleotide identity (ANI) and in silico-based DNA-DNA hybridization (DDH) demonstrated that they belong to a new species of *Ensifer*; however these strains were symbiotically distinct (*sym* genes continent specific). The genomic data suggested several conserved genes specific to genus *Ensifer* (Le Queré et al. 2017). Previously published work and the finding of predominance of *Ensifer* in hot-arid alkaline soils of Thar Desert suggest that *Ensifer* species have been mostly isolated from legumes growing in the arid and alkaline soils of Old World and New World (Tak et al. 2016b; Shamseldin et al. 2017; Sankhla et al. 2017; Rathi et al. 2018). Exception to this is *E. medicae* that nodulates species of *Medicago* in acidic soils (Garau et al. 2005). These findings are supported by the comparative genomic studies on *Ensifer* microsymbionts nodulating soybean growing in alkaline soil of China, suggesting that the strains of *Ensifer* have specific genes for adaptation to alkalinity, low water potential, salt stress, and high temperature (Tian et al. 2012).

Our studies suggest that the *Ensifer* strains associated with mimosoid members (*Vachellia*, *Senegalia*, and *Mimosa*) of the Thar Desert on the basis of their symbiotic genes are closer to type strain *E. arboris* (isolated from *Prosopis chilensis*, Sudan) [Fig. 2.2]. In species phylogeny these strains shared close similarity with *E. saheli*; this incongruence with symbiotic gene phylogeny is due to horizontal transfer of the *sym* genes (Gehlot et al. 2013; Sankhla et al. 2017; Choudhary et al. 2017, 2018). *Ensifer* strains affiliated with papilionoid and caesalpinoid legumes in the Thar desert showed maximum similarity to symbiotic genes (*nodA* and *nifH*) of closely related type strains (*E. fredii*, *E. glycinis*, *E. shofinae*, *E. sojae*, and *E. xinjiangensis*) isolated from root nodules of *G. max* and with broad host range strain (*Ensifer* sp. NGR234) (Tak et al. 2016a, b; Sankhla et al. 2018; Rathi et al. 2018) (Fig. 2.2). This phylogenetic incongruence observed in species and symbiotic gene phylogeny of Thar Desert *Ensifer* strains may be attributed to soil alkalinity and hot-arid conditions that play a major role in the evolution of these rhizobial strains. In contrast to this, the symbiotic essential genes of TFG strains had intermediate sequences diversified from closely related *E. meliloti* and *E. medicae*, as observed in their species phylogeny based on 16S rRNA gene (Gaur et al. 2018) [Fig. 2.2]. It was surprising to note that no *E. aridi* type of strains was isolated from root nodules of various mimosoids (*Vachellia*, *Senegalia*, and *Mimosa*) studied from the Thar Desert, although *E. aridi* (African strains LMR001 and LMR13) have been recovered from the species of *Acacia* (*Vachellia*) from Morocco, Africa (Fig. 2.2). The *sym* gene phylogenies of different *E. aridi* strains from three continents (American strain LEM457) clearly indicate that these strains are genetically identical but harbor different symbiotic genes specific to local environmental conditions (Fig. 2.2). The Indian *E. aridi* strains harbor novel *sym* genes diversified from *E. fredii* and cross-nodulating papilionoid, mimosoid, and caesalpinoid wild legumes as well as crops (Tak et al. 2016b; Le Queré et al. 2017).



**Fig. 2.2** Maximum likelihood concatenated phylogenetic tree of **Thar Desert *Ensifer*** strains isolated from root nodules of various wild legumes of Western Rajasthan based on *nodA-nifH* concatenated gene sequences. Bootstrap values more than 50% calculated for 1000 replications are indicated at internodes. The scale bar indicates 5% nucleotide substitution per site. (Abbreviation: superscript T stands for type strain)

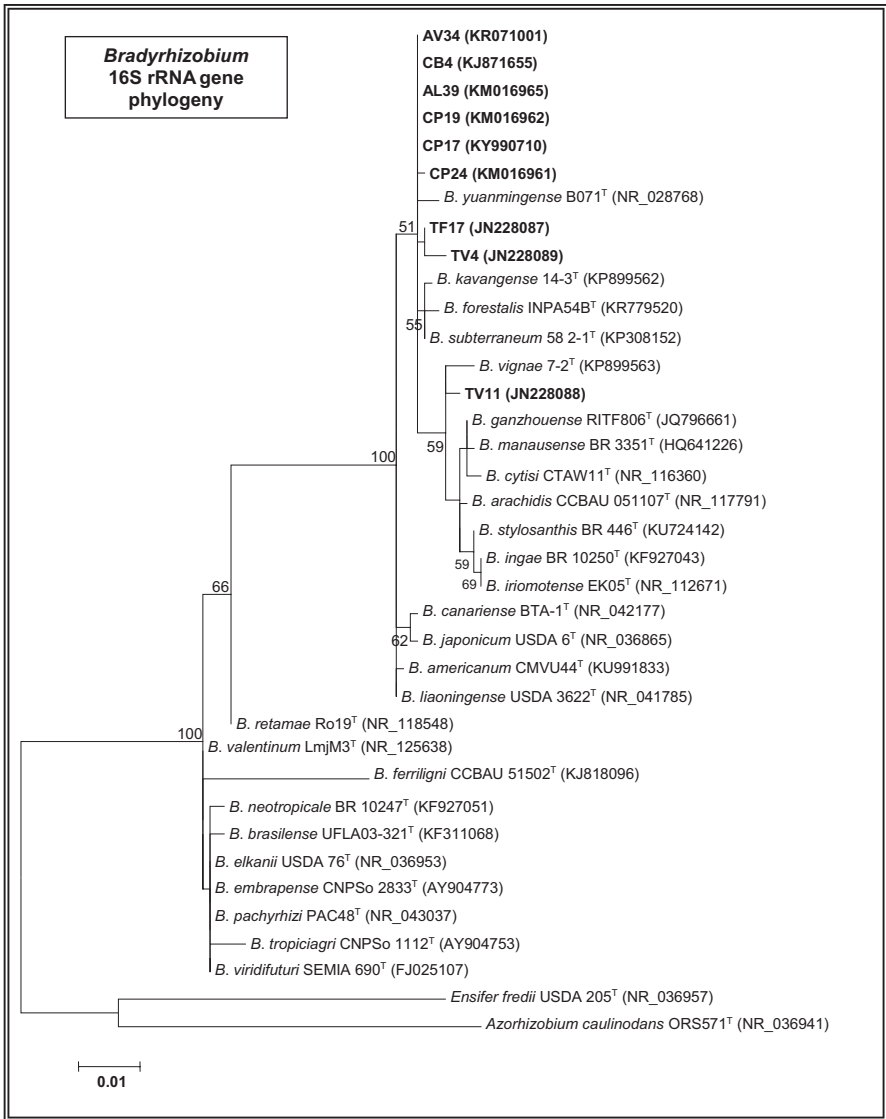
**2.3.2 Phylogenetic Diversity of Thar Desert *Bradyrhizobium* Strains**

Among the various wild legumes of the Thar Desert studied, the slow-growing bradyrhizobial strains were also recovered from root nodules of species of *Tephrosia* (tribe Millettieae, Papilionoideae) (Gehlot et al. 2012; Ojha et al. 2015), *Crotalaria*

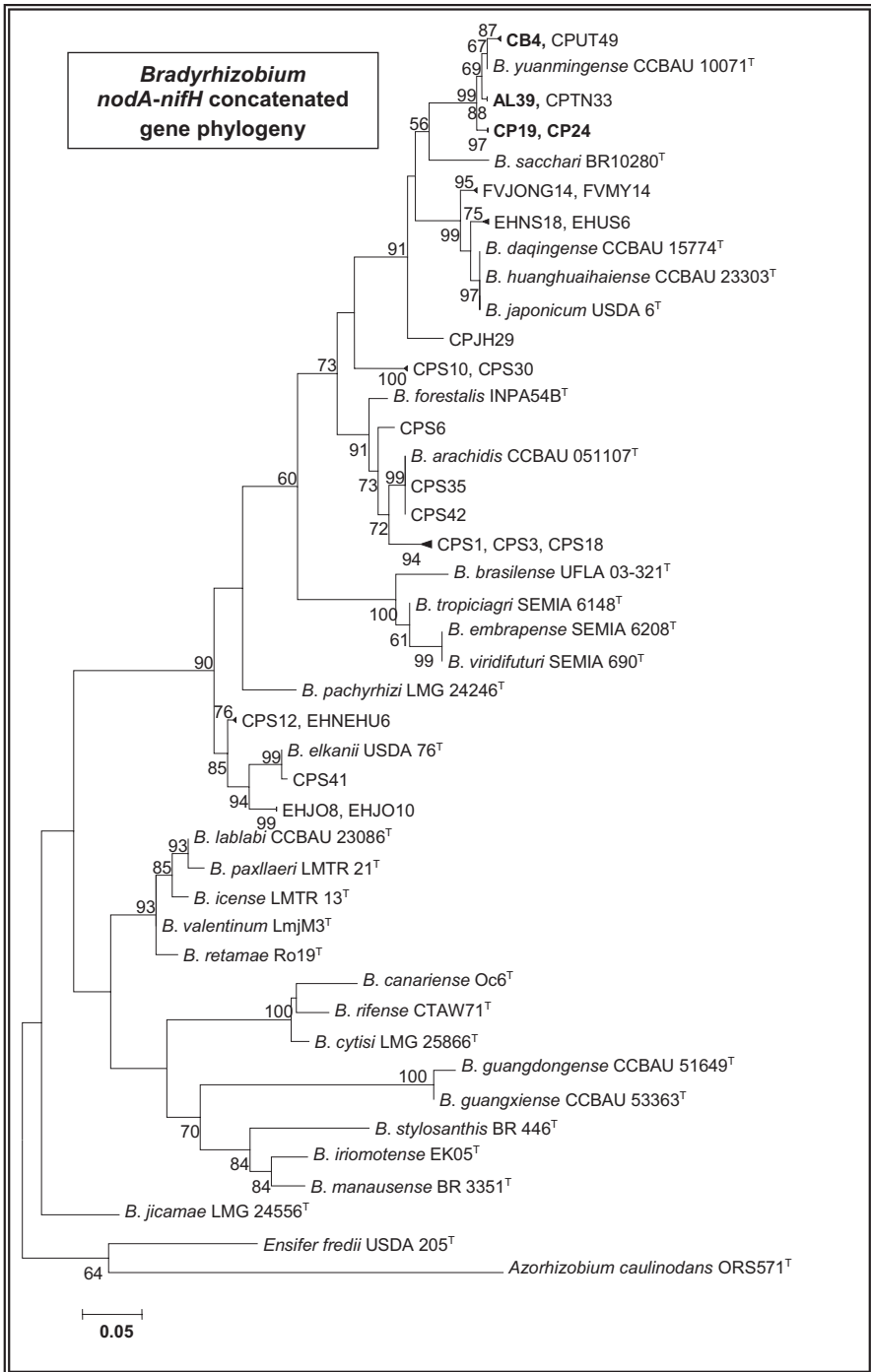
*burhia* (tribe Crotalariaeae, Papilionoideae) (Ojha et al. 2015; Sankhla et al. 2018), *Alysicarpus vaginalis* (tribe Desmodieae, Papilionoideae) (Rathi et al. 2017), and *Chamaecrista pumila* (tribe Cassieae, Caesalpinioideae) (Ojha et al. 2015; Rathi et al. 2018). The slow-growing *Bradyrhizobium* strains isolated from root nodules of these wild herbs and under shrubs on the basis of 16S rRNA and *sym* (*nodA* and *nifH*) gene phylogeny shared close affinity with the type strain *B. yuanmingense* which was initially isolated from wild legume (*Lespedeza cuneata*) of tribe Desmodieae in China (Yao et al. 2002) (Figs. 2.3 and 2.4). From India, strains sharing similarities with type strains *B. yuanmingense*, *B. liaoningense*, *B. elkanii*, and *B. japonicum* have been isolated from *G. max* growing in Madhya Pradesh, Uttar Pradesh, and Tamil Nadu having hot-arid climate and being mostly neutral to alkaline soils (Appunu et al. 2008, 2009b; Vinuesa et al. 2008). The *B. yuanmingense* has also been reported to nodulate *V. mungo*, *V. radiata*, and *V. unguiculata* growing in regions of Uttar Pradesh, Andhra Pradesh, and Tamil Nadu (Appunu et al. 2009a) and in another crop *Macrotyloma uniflorum* growing in two agro-eco-climatic regions of South India (Appunu et al. 2011). As per previous studies from various parts of India, *B. yuanmingense* is often the preferred symbionts of crops (*G. max* and *Vigna* spp.) in the hot-dry tropical climate and alkaline soils. The existing geographical factors and alkaline soil in the Thar Desert are playing a role in the dominance of *Ensifer* and the sporadic occurrence of *B. yuanmingense*-like strains in root nodules of species of *Tephrosia*, *Alysicarpus*, and *Chamaecrista* in addition to strains of *Ensifer* which might be the preferred/primary rhizobia compared to other rhizobial genera.

In contrast to the Thar Desert bradyrhizobial strains (from mega clade-I), novel species of *Bradyrhizobium* belonging to both mega clade-I and clade-II have been recently isolated from little-studied legumes *Eriosema chinense* (tribe Phaseoleae, Papilionoideae) and *Flemingia vestita* (tribe Phaseoleae, Papilionoideae) growing in acidic soil of the sub-Himalayan region of the Indian state of Meghalaya (Ojha et al. 2017). These strains isolated from acidic soils of Shillong harbor novel *nodA* and *nifH* genes (Fig. 2.4). This suggests that although selection of rhizobia by the host plants depends upon molecular signaling between the two partners, that too is influenced by ecological factors such as soil alkalinity, soil acidity, precipitation, and soil nutrients' availability in the region (Rathi et al. 2018). Caesalpinoid legume *C. pumila* in Thar Desert was nodulated by novel strains of *Ensifer* and strains divergent from *B. yuanmingense*, whereas in acidic soils and wet soils of northeastern state Meghalaya, it was found to be exclusively nodulated by diverse species of *Bradyrhizobium* belonging to both mega clade-I and clade-II. Significant symbiotic (*nodA* and *nifH* gene) diversity was observed in *C. pumila* strains isolated from acidic soils of Shillong (CPS) (Fig. 2.4). In the mimosoid legumes of Thar Desert such as *V. leucophloea* (Choudhary et al. 2017), *P. cineraria*, and *Dichrostachys cinerea* (Unpublished data, HS Gehlot), the primary dominant root nodule micro-symbionts were fast-growing species of *Ensifer*, and occasionally slow-growing *Bradyrhizobium* strains close to *B. yuanmingense* were also reported.





**Fig. 2.3** Maximum likelihood phylogenetic tree of **Thar Desert *Bradyrhizobium*** strains isolated from root nodules of various wild legumes of Western Rajasthan based on **16S rRNA** gene sequences. Bootstrap values more than 50% calculated for 1000 replications are indicated at inter-nodes. The scale bar indicates 1% nucleotide substitution per site. (Abbreviation *B.* stands for *Bradyrhizobium* and superscript T stands for type strain)



**Fig. 2.4** Maximum likelihood concatenated phylogenetic tree of Thar Desert *Bradyrhizobium* strains isolated from root nodules of various wild legumes of Western Rajasthan compared

## 2.4 Conclusions and Future Perspectives

Since the beginning of exploration of native and wild legumes from various geographical regions all over the world, the number of rhizobial genera and species has increased tremendously due to which rhizobial classification is changing. The soil alkalinity, low precipitation, and high temperature in the arid and semiarid regions of the Thar Desert are responsible for evolution and diversification of novel *Ensifer* strains when compared to slow-growing *Bradyrhizobium* from tropical and subtropical acidic soil all over the world including temperate regions. Novel *Ensifer* strains are dominant microsymbionts associated with legumes in the region. Majority of the *Ensifer* strains adapted to arid conditions of the Thar Desert are promiscuous in nature, showing geographical clustering and mosaic pattern in nucleotide sequences of housekeeping as well as symbiotic genes. Hostile environmental conditions are an important factor underlying horizontal gene transfer among related and diverse genera and species of bacteria. Molecular phylogeny of Thar Desert rhizobia provided information about their evolution and diversification and helped in better understanding the biogeography of rhizobial strains in India and in global context. Genomic characterization of diverse nitrogen-fixing novel strains from different climatic conditions will enrich our knowledge about the unique features of these strains which may open new fields for biotechnologists to improve existing microsymbiont associated with legume crops. Such studies are essential for strengthening the microbial resources in terms of agriculturally important microbes and can reduce the use of chemical fertilizers that are causing environmental pollution and adversely affecting the economy of farmers.

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**Fig. 2.4** (continued) with *Bradyrhizobium* strains isolated from other sampling sites in India having neutral [Tamil Nadu (TN), Jharkhand (JH), and Uttarakhand (UT)] and acidic [Shillong, Meghalaya] soils, based on *nodA-nifH* concatenated gene sequences. Bootstrap values more than 50% calculated for 1000 replications are indicated at internodes. The scale bar indicates 5% nucleotide substitution per site. (Abbreviation *B.* stands for *Bradyrhizobium* and superscript T stands for type strain)

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