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ORIGINAL PAPER

A significant proportion of indigenous rhizobia from India associated with soybean (*Glycine max* L.) distinctly belong to *Bradyrhizobium* and *Ensifer* genera

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Abstract The diversity among 269 rhizobia isolated from naturally occurring root nodules of soybean collected from two different agro-ecological regions of India, based on RFLP and sequences of the intergenic spacer (IGS) between the 16S and 23S rRNA genes, growth rate, and indole acetic acid production, revealed their significant, site-dependent genomic diversity. Among these bacteria, nine IGS genotypes were identified with two endonucleases. They were distributed into five divergent lineages by sequence analysis of each IGS representative strain, i.e., (1) comprising IGS genotypes I, II, III, and reference *Bradyrhizobium yuanmingense*; (2) with genotype IV and strains of unclassified bradyrhizobia genomic species; (3) including genotypes V, VI, and *Bradyrhizobium liaoningense*; (4) with IGS genotype VII and *Bradyrhizobium elkanii* strains; and (5) comprising IGS genotypes VIII, IX, and different *Ensifer* genus bacteria. Host-specificity test revealed that all rhizobia-nodulated soybean and cowpea and only part of them formed nodules on *Arachis hypogeae* and *Cajanus cajan*. The great diversity of soybean nodulators observed in this study emphasises that Indian soil is an important reservoir of nitrogen-fixing rhizobia.

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Introduction

Biological nitrogen fixation resulting from symbiosis between legume plants and rhizobia provides a significant boost to N_2 fertilisation and, additionally, does not cause any hazard to the environment. Soybean (*Glycine max* (L.)) forms nitrogen-fixing root nodules with diverse bacteria belonging to different genera and species. The slow-growing rhizobia that effectively nodulate soybeans are distributed among five different *Bradyrhizobium* species: *Bradyrhizobium japonicum*, *Bradyrhizobium elkanii*, *Bradyrhizobium liaoningense*, *Bradyrhizobium canariense*, and *Bradyrhizobium yuanmingense* (Vinuesa et al. 2008). Other symbionts of soybean are fast growers classified as *Sinorhizobium fredii* and *Sinorhizobium xinjiangense* and moderately slow-growing rhizobia belonging to *Mesorhizobium tianshanense* (Man et al. 2008). However, other slow- and fast-growing rhizobia genetically distinct from those mentioned above were also reported (Chen et al. 2000; Appunu et al. 2008a; Vinuesa et al. 2008).

In India, soybean cultivation started in northern, north-eastern hills, and different scattered areas of central India as soon as it was domesticated in China (Chauhan and Joshi 2005), but commercial soybean production started from the 1960s. Majority of Indian soils where soybean was planted originally were devoid of rhizobia able to nodulate effectively soybean. The yellow-seeded soybean genotypes were introduced into India in 1965 from the USA along with efficient N_2-fixing *B. japonicum* inoculum like Nitragin, Legumaid, and Dixit (Balasundaram and Subba Rao 1971). Since then, the commercial cultivation of

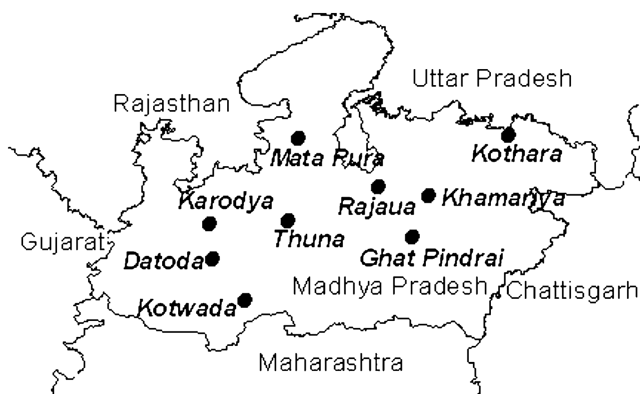

Fig. 1 Geographical location of the nine sites where nodules were collected in Madhya Pradesh, India

soybean advanced to new agricultural regions in the central part of India, especially Madhya Pradesh state which is now considered as the soybean hub of India. Today, soybean is one of the most important food crops and a part of routine diet of the people in the Indian subcontinent, because its seeds are source of relatively high-quality, inexpensive oil and proteins (Chauhan and Joshi 2005). The high protein content in the seeds (40–45%) demands a heavy supply of nitrogen which can be met to a sizable extent through an effective host-*Rhizobium* symbiosis. Now, soybeans are nodulated by soil indigenous rhizobia with varied level of symbiotic effectiveness (Appunu et al. 2008b). One of the reasons for variation in symbiotic effectiveness of the indigenous rhizobia may either be ancient inoculants subjected to genetic drift in the soil or may be derivatives of rhizobia native to India that acquired in some way (e.g., horizontal gene transfer) the ability to nodulate soybean (Barcellos et al. 2007). Therefore, analysis of rhizobial diversity is essential for the successful management of symbiotic associations between various soybean cultivars and indigenous nodule bacteria.

Today, little is known on the diversity and phylogeny of rhizobial population able to symbiosis with soybean present in the soils of Madhya Pradesh except preliminary reports on a small sample (Appunu et al. 2008a). Therefore, this study evaluates the large rhizobial population trapped by soybean plants at nine sites within two different agro-eco-climatic conditions in traditional soybean cultivating regions of Madhya Pradesh, India.

Materials and methods

Bacterial strains and growth conditions A total of 259 rhizobia from root nodules of soybean-growing areas in Madhya Pradesh, India (Fig. 1; Table 1), were isolated and purified by following the method of Somasegaran and Hoben (1994). Each isolate was tested for nodulation on soybean cultivar JS335 as explained elsewhere (Appunu et al. 2008a).

Phenotypic characterization The growth rate was determined as reported by Somasegaran and Hoben (1994). Gordon and Weber (1951) colorimetric procedure was followed for the estimation of Indole acetic acid (IAA) production by the isolates grown in YM broth supplemented with L-tryptophan (100 µg/mL). The change to pink colour, after the addition of freshly prepared reagent $FeCl\text{-}HClO_4$, was an indication of IAA presence and was read at 530 nm.

Genomic DNA isolation, rDNA IGS amplification, and RFLP analysis The genomic DNAs for polymerase chain reaction (PCR) templates were prepared according to the method described by Appunu and Dhar (2008). The rDNA intergenic spacer (IGS) was amplified using FGPS1490-72 (5′-TGCGGCTGGATCACCTCCTT-3′; Navarro et al.

Table 1 Geographic origin of soybean rhizobial isolates from Madhya Pradesh, India

Origin	Designation of isolates	Number of isolates	Previous crop	Agro-eco-climatic region and its characteristics[a]
Karodiya, Ujjain	SR200-226	27	Greengram	A: Hot semi arid with medium and deep black soils
Datoda, Indore	SR227-259	33	Fallow	A
Kotwada, Khandwa	SR260-288	29	Wheat	A
Thuna, Sehore	SR289-324	36	Wheat	A
Mata Pura, Guna	SR325-348	24	Sorghum	B: Hot sub humid with red and black soils
Rajaua, Sagar	SR349-376	28	Chickpea	B
Khamariya, Damoh	SR377-406	30	Fallow	B
Ghat Pindrai, Narsinghpur	SR407-431	25	Redgram	B
Kothara, Satna	SR432-458	27	Rice	B

[a] A: Coarse to fine loamy, highly alkaline, moderate to gentle slope, mean annual rainfall of 500–1,100 mm; moisture index of (−)33.3 to (−)66.7; temperature>22°C and length of growing period (LGP) of 90–150 days. B: Deep loamy, neutral to slightly acidic, moderate to gentle slope, mean annual rainfall of 1,000–1,500 mm; moisture index of (+)20 to (−)33.3; temperature of 15–22°C and LGP of 150–180 days

1992) and FGPL132-38 (5′-CCGGGTTTCCCCATTCGG-3′; Ponsonnet and Nesme 1994) primers. PCR amplifications were performed using standard temperature profile (Laguerre et al. 1996) with a modification in the elongation time of 1 min 45 s at 72°C. Restriction fragment length polymorphism (RFLP) analysis was carried out with *Hae*III and *Cfo*I (=*Hha*I) tetrameric endonucleases as explained elsewhere (Laguerre et al. 1994).

Sequencing and phylogenetic analysis PCR product of the rDNA IGS was amplified (minicycler; MJ Research, Waltham, MA, USA), purified (Perfectprep Gel Cleanup Kit; Eppendorf, Germany), and sequenced using PCR-producing primers. The nucleotide sequences were determined using the dideoxy chain-termination method with the Big-Dye terminator kit of the ABI Prism 310 DNA sequencer (Applied Biosystems; Foster City, CA, USA).

The IGS sequences were compared with related sequences from the GenBank database by Blast program (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Multiple sequence alignment was performed with ClustalW version 1.8 package (Thompson et al. 1997). Neighbour-joining tree was constructed under the Jukes–Cantor model and bootstrapped with 1,000 replications using the program MEGA3.1 (Kumar et al. 2004).

Host range test Healthy seeds of all legumes were surface sterilised and germinated as explained previously (Appunu et al. 2005). Each seedling was then transferred to a plastic pot filled with sterilised sand and soil (50:50) mixtures. Two-days-old seedlings were inoculated with 1 ml of rhizobial suspension (approximately 10^8 cells ml^{-1}) and transferred to a plant growth chamber. Each plant treatment was in five repetitions. Plants were harvested at 6 weeks from sowing, and the nodulation ability was recorded by the procedure of Appunu and Dhar (2006).

Nucleotide sequence accession numbers The newly determined rDNA IGS sequences were deposited in the GenBank data base under accession number FN376848 through FN376851.

Results and discussion

Isolation and morphological characteristics A total of 259 nodule isolates obtained from naturally occurring root nodules of soybean cultivars grown in nine different sites of Madhya Pradesh, India, were studied (Fig. 1; Table 1). The generation time of the isolates varied from 2.3 to 32 h (Table 2). About 90% of these isolates were slow growers with generation times (GM) varying from 7 to 32 h, whereas 10% of fast growers recorded doubling time (GM) varying from 2.3 to 4.6 h. In the presence of tryptophan, nearly 9% of slow-growing and 10% of fast-growing rhizobial isolates synthesised IAA (Table 2). Production of the phytohormone IAA has been used primarily to distinguish between the two species of soybean-nodulating *Bradyrhizobium* genus, i.e., *B. japonicum* (non-IAA producers), and *B. elkanii* (IAA producers; Minamisawa and Fukai 1991). By IAA test, about 81% of studied slow-growing soybean nodulators were included into *B. japonicum* group, and only 9% were identified as *B. elkanii*. The 10% of IAA positive nodule isolates were fast-growing rhizobia, and they were included into genus *Ensifer* by rDNA IGS sequence analysis (Fig. 2). Production of IAA in the culture by fast-growing rhizobia has been reported earlier (Annapurna et al. 2007).

Analysis of 16S–23S rDNA IGS RFLP and sequence analyses The 16S–23S rDNA IGS region of all studied soybean nodule isolates were amplified by PCR with the FGPS1490 and FGPL132 primer pair. For each strain, a

Table 2 Intergenic spacer (IGS) genotypes identified by restriction fragment length polymorphism (RFLP) analysis of polymerase chain reaction-amplified 16S–23S rDNA regions and other characteristics of strains belong to respective genotypes

S. No.	IGS RFLP patterns[a]	IGS genotype	Growth rate (h)	IAA production	Percent of isolates
1	AA	I	9.3–15.2	No	18
2	AB	II	7.8–12.2	No	7
3	BA	III	10.3–16.2	No	8
4	CC	IV	12.7–18.9	No	37
5	DD	V	14.5–32	No	3
6	DE	VI	14.5–32	No	8
7	E–	VII	8.1–16.4	Yes	9
8	F–	VIII	2.5–4.3	Yes	6
9	G–	IX	2.3–4.6	Yes	4

[a] The letters identify RFLP patterns obtained with *Hae*III and *Cfo*I

– not determined

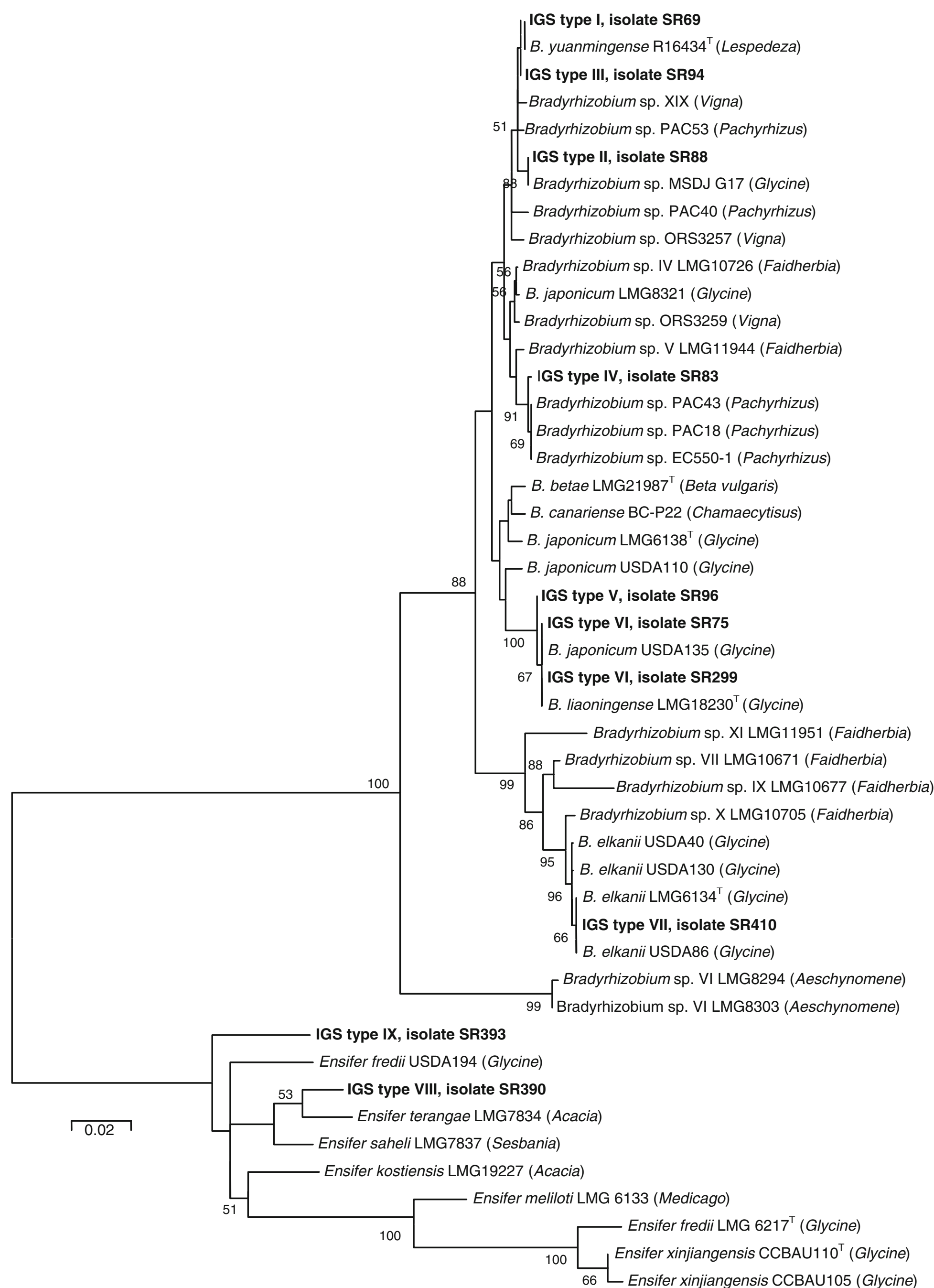

Fig. 2 Neighbour-joining tree constructed with rDNA intergenic spacer (IGS) sequences showing the phylogenetic relationships. *Values at branching points* indicate bootstrap support higher than 50% (1,000 replicates). The *scale bar* indicates 2% nucleotide substitutions. Soybean isolates of Indian origin are shown in *bold* with their IGS genotype. The sequences determined in this study are indicated with a *cross*

single DNA band size from 910 to 990 bp was obtained. Analyses of rDNA IGS by RFLP with two endonucleases, *Hae*III and *Cfo*I, revealed nine different combinations of restriction patterns corresponding to IGS genotypes I–IX (Table 2). These results revealed that studied *G. max* microsymbionts were genetically different and belong to distinct rhizobial genomic groups. Earlier, the IGS analyses have been successfully used to group genetically related rhizobia at intra-species and inter-species levels (Willems et al. 2003).

The rDNA IGS PCR product of one representative of each IGS type (I–IX) was sequenced and compared to IGS sequences of slow-growing bradyrhizobia from India (Appunu et al. 2008a), six bradyrhizobia unclassified to species studied by Willems et al. (2003), and to rDNA IGS sequences of other rhizobia. Phylogenetic analysis revealed that the IGS sequences of soybean isolates of Indian origin were dispersed among five distinct lineages that were supported by bootstrap value higher than 50% (Fig. 2). On IGS phylogram, soybean microsymbionts representing genotypes I, II, and III are on common branch with *B. yuanmingense*. Isolate SR83 representative for genotype IV is in common group with unclassified bradyrhizobia

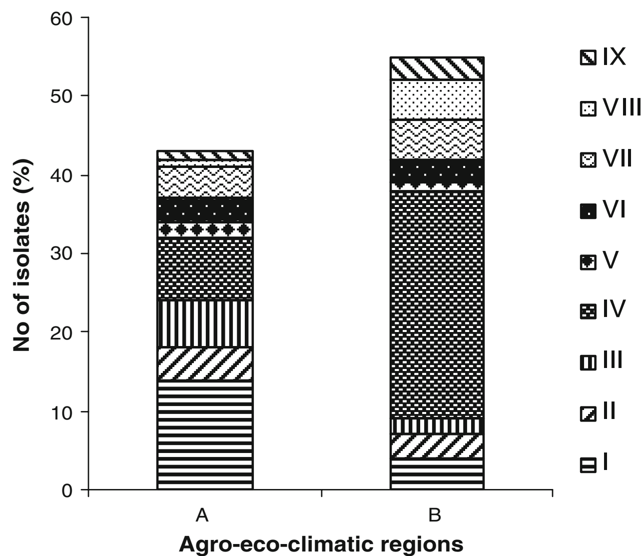

Fig. 3 Distribution of soybean rhizobial intergenic spacer (IGS) genotypes (I–IX) defined by the polymerase chain reaction restriction fragment length polymorphism analysis of 16S–23S rDNA IGS among agro-eco-climatic regions of Madhya Pradesh, India

genomic species. Strains SR96, SR75, and SR299 clustered with *B. liaoningense* LMG18230^T and *B. japonicum* USDA135 at 100% bootstrap support. In the studies of van Berkum and Fuhrmann (2000), Appunu et al. (2008a), and Saeki et al. (2005), *B. liaoningense* USDA3622^T and *B. japonicum* USDA135 shared similar rDNA IGS sequences except for one nucleotide. The representative isolate of IGS genotype VII showed 100% similarity with the corresponding sequence of *B. elkanii* USDA86 which was isolated from soybean in the USA. The soybean-nodulating strains of *B. elkanii* have never been introduced to Indian soil by inoculation, but available reports showed their existence in Asiatic continent and effective nodulation of soybean (Saeki et al. 2005; Man et al. 2008). The rhizobia representing IGS genotypes VIII and IX, together with *Ensifer* species-nodulating *G. max*, *Sesbania* sp., *Acacia* sp., and *Medicago* sp., formed out-group clearly distinct from other ones. The IGS sequences of SR393 and SR390 showed 90–93% similarities to those from *Ensifer terangae* (isolated from *Acacia* sp.) and *Ensifer fredii* (isolated from *Glycine* sp.), respectively. These isolates SR393 and SR390 showed sequence similarity of only 85% to 93% to other *Ensifer* species (*Ensifer meliloti*, *Ensifer kostiensis*, *Ensifer xinjiangensis*, and *Ensifer saheli*) included in the phylogeny analysis.

Based on the inability to produce IAA, slow-growing soybean rhizobia collected from different places in India were classified as *B. japonicum*. It was earlier suggested that this species dominate in the native bradyrhizobia population as soybean symbiont (Annapurna et al. 2007). Our study indicated that most soybean bradyrhizobia unable to synthesis IAA was related to *B. yuanmingense* which could establish symbiotic relation with soybean and unclassified bradyrhizobia genomic group (Table 2). Only 19% of *G. max* nodule isolates from India produced IAA on medium with tryptophan. About half of them formed on rDNA IGS tree common group with *B. elkanii*. The rest of IAA strains clustered with *Ensifer* species (Fig. 2).

This study further confirmed the low presence of fast-growing soybean-nodulating rhizobia in Indian soils as reported earlier (Annapurna et al. 2007) and that could be one of the reasons why they occupy soybean nodules less frequently compared to bradyrhizobia. *Bradyrhizobium* and *Ensifer* genus bacteria-nodulating soybeans have been found in China, which is the primary centre of soybean origin. Similar diversity of microsymbionts was also noted in our studies. *G. max* cultivated in India (secondary centre of this legume origin) entered symbiosis with bacteria of

Table 3 Host range test of soybean rhizobial isolates on several host legumes

Rhizobial strain	Host plant						
	Glycine max	*Vigna mungo*	*Vigna radiata*	*Vigna unguiculata*	*Macrotyloma uniflorum*	*Arachis hypogeae*	*Cajanus cajan*
SR212 (IGS I)	+	+	+	+	+	−	−
SR256(IGS II)	+	+/−	+/−	+	+/−	−	−
SR257(IGS III)	+	+/−	+/−	+	+/−	−	−
SR415(IGS IV)	+	−	−	+/−	−	−	−
SR288(IGS V)	+	+	+	+	+	+	+
SR299(IGS VI)	+	+	+	+	+	+	+
SR410(IGS VII)	+	+	+	+	+	−	−
SR390 (IGS VIII)	+	−	−	+	−	−	−
SR393 (IGS IX)	+	−	−	+	−	−	−

+ indicates a nitrogen-fixing symbiosis, as revealed by the acetylene reduction assay; +/− indicates weak levels of acetylene reduction; − indicates a non-nodulating interaction

both genera, i.e., *Bradyrhizobium* and *Ensifer*. Although, the IGS sequence analysis of *G. max* symbionts from India clearly suggests their relationship with bacteria of the genera *Bradyrhizobium* and *Ensifer*, multilocus sequence analysis of housekeeping genes and DNA–DNA hybridization are necessary to support this conclusion.

Geographic distribution of G. max rhizobial isolates Rhizobial occurrence is considered to be independent on host origin (Martinez-Romero and Caballero-Mellado 1996). All sampled sites in this study were grouped into two different agro-ecological zones. All slow-growing *Bradyrhizobium* genotypes were present in both sampled zones. Rhizobial strains phylogenetically closely related to *B. yuanmingense* dominated ecoregion A, whereas rhizobial isolates phylogenetically closely related to *B. japonicum*, *B. laiaoningense*, and some other unnamed bradyrhizobia dominated ecoregion B (Fig. 3). More than 90% of fast-growing soybean nodulators were derived from the ecoregion B. Only 10% of fast-growing *G. max* rhizobia were isolated in ecoregion A. The soil properties, where *G. max* was planted, and geographical locations of this legume influenced the rhizobial population, as it was reported earlier (Chen et al. 2000; Yang et al. 2006; Appunu et al. 2008a; Man et al. 2008).

Host range test To study symbiotic properties of soybean nodulators deriving from India, the representatives for slow- and fast-growing nodule bacteria were chosen and checked for nodulation of legumes, which were reported to form symbiosis with *Bradyrhizobium* genus strains (Table 3; Rodriguez-Navarro et al. 2004). All tested slow- and fast-growing *G. max* rhizobia-nodulated soybean and cowpea; however, the strains representing the IGS genotype IV produced only few effective nitrogen fixation nodules on cowpea. The strain identified by rDNA IGS sequences as *B. elkanii* does not nodulate *Arachis hypogeae* and *Cajanus cajan*, whereas it is known that strains classified as *B. elkanii* have been isolated from root nodules of both these legumes (Yang et al. 2005; Yang and Zhou 2008). It was interesting that studied *G. max* rhizobia belonging to *B. elkanii* and *B. yuanmingense* phylogenetic groups (IGS genotypes I, II, III, and VII) have the same host range, and they nodulated soybean, different *Vigna* species, but not *A. hypogeae* and *C. cajan*. These results support that evolution of core and symbiotic genes of rhizobia proceeded different ways (Moulin et al. 2004).

Conclusion

The present study proves that most of soybean microsymbionts from India are slow-growing bradyrhizobia related to *B. yuanmingense* and unclassified bradyrhizobia genomic species. Less effectiveness in symbiosis with *G. max* are fast-growing rhizobia, distantly related to *E. terangae and E. fredii*, which are known as soybean nodulators.

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